Sleep and circadian rhythms: the effects of ketamine, caffeine and anthracyclines

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The effects of ketamine, caffeine and anthracyclines

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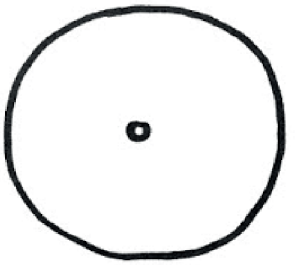
General Introduction



1. Circadian clock and sleep-wake regulation

1.1 The circadian timing system

The ancient Chinese medical book Huangdi Neijing (475-221 BC) includes circadian rhythms - physical, mental, and behavioral changes that follow a daily rhythm, a syzygial (lunar) rhythm, and a seasonal (annual) rhythm, creating a harmonious lifestyle between humans and our natural world. Ancient Chinese people thought that human rhythms are fundamental for good health, and thousands of years later this appears to be true.

The mammalian circadian clock is individually and collectively expressed by each of the ~20,000 cells of the master circadian clock, the suprachiasmatic nucleus (SCN), which is located in the hypothalamus, lateral to the third ventricle, above the optic chiasm [1]. The circadian rhythm generated is approximately 24 hours and mammals synchronize their circadian activity to the cycles of light and darkness originating from the rotation of the earth. Light information is sent directly from the retina to the SCN through the retinohypothalamic tract (RHT), which, through release of glutamate, activates neurons in the SCN [2]. Feeding and exercise can also act as zeitgebers to synchronize the central clocks [3,4]. For this, the SCN receives timing information of other brain regions via direct and indirect inputs, and sends its output, like hormonal and nervous signals, to synchronize the peripheral clocks and optimize physiology to the temporal changes in our environment [2].

Most outputs of the SCN are directed towards the hypothalamus, including the medial preoptic nucleus, dorsomedial hypothalamic nucleus, paraventricular nucleus, and dorsomedial hypothalamic nucleus [2]. The unique location of the hypothalamus allows it to directly connect to the cerebrospinal fluid, thus, the hypothalamus can convey information from the SCN in a neuroendocrine way, for example, pituitary/hypothalamus-pituitary-adrenal axis and pineal gland, and also send output via synaptic pathways to other brain areas, for example, the cortex, cerebellum or hippocampus (Figure 2, upper panel) [5,6]. The central circadian clock plays a crucial role in sleep-wake rhythm, rest-activity rhythm, thermogenesis, immunity and metabolism (Figure 1).

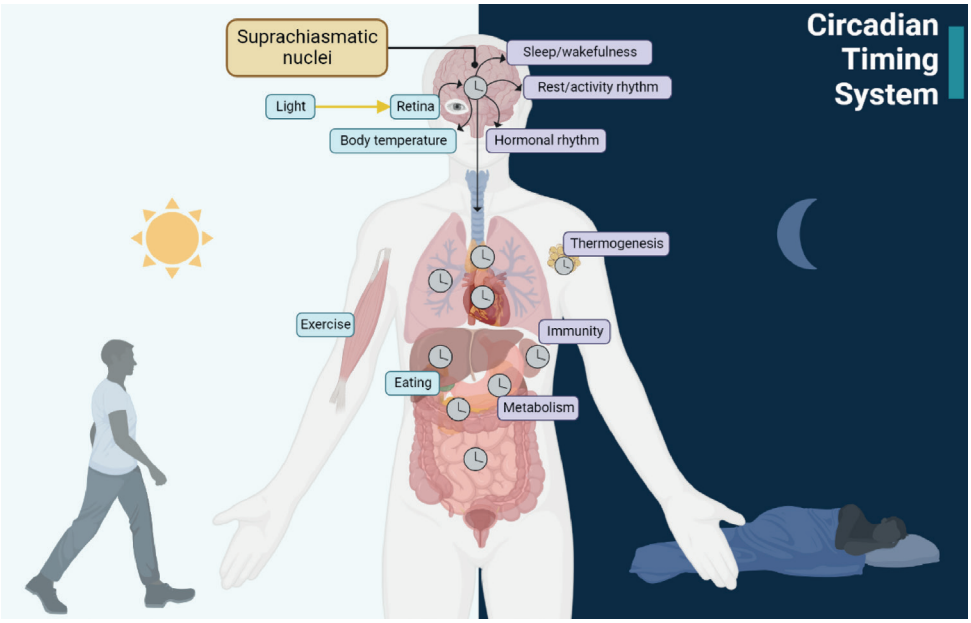


Figure 1. Schematic representation of the circadian timing system. The circadian timing system synchronizes clocks through the whole body and adapts the changes of the outside world. Created via BioRender.

The SCN is an evolutionarily conserved brain area. It generates daily electrical activity rhythms which can even be observed in brain slices, and in cultured slices (Figure 2, bottom right panel) [7]. Furthermore, SCN lesioning experiments in animals as well damage of the hypothalamus in humans show that animals and human lose their daily rest-active rhythm after such an event [8,9]. It is possible to restore circadian rhythms in SCN lesioned hamsters with SCN transplants that carry the rhythmic properties of the donor animal [10]. Thus, the SCN is considered to be the intrinsic endogenous pacemaker in the body. During the day, the neurons discharge action potential rate in the SCN is high and the resting membrane potential is more depolarized relative to the night. When SCN neurons discharge action potentials are at a lower rate and SCN neurons are most responsive to excitatory or depolarizing stimulation [11]. This kind of rhythmic firing of neurons across the 24-hour is regulated by an autoregulatory transcription-translation feedback loop (TTFL) of clock genes (Figure 2, bottom left panel) [12]. This TTFL comprises the interlocked activities of transcriptional activators (CLOCK and BMAL1) and repressors (PER and CRY). CLOCK and BMAL1 form a heterodimer that regulates the expression of clock-controlled genes as well as the repressor proteins encoded by Period and Cryptochrome. The PER/CRY heterodimer repressor complex,

in turn, inhibits CLOCK/BMAL1 activity at E-box target sequences on both clock-controlled genes and CLOCK/BMAL1 autoregulation [13]. The TTFL forms the basis for cellular rhythms in gene expression, intracellular Ca^{2+} levels, rate of action potentials, neurotransmitter release and electrical activity rhythms [13-15]. Light entrains the phase of these rhythms through release of glutamate by the RHT, which can set *Per* transcription levels and induce changes in SCN neuronal activity [2,16-18]. Multi-unit recordings reveal the synchronization of this activity across the SCN circuit [7]. In nocturnal animals, higher firing is associated with reduced locomotor activity, and decreased firing is observed during the active phase [19,20]. Diurnal rodents exhibit behavioral rhythms in the opposite phase, although the phase of clock gene and action potential rhythms are likely to be similar to that of nocturnal mammals (Figure 2, bottom right panel) [21,22].

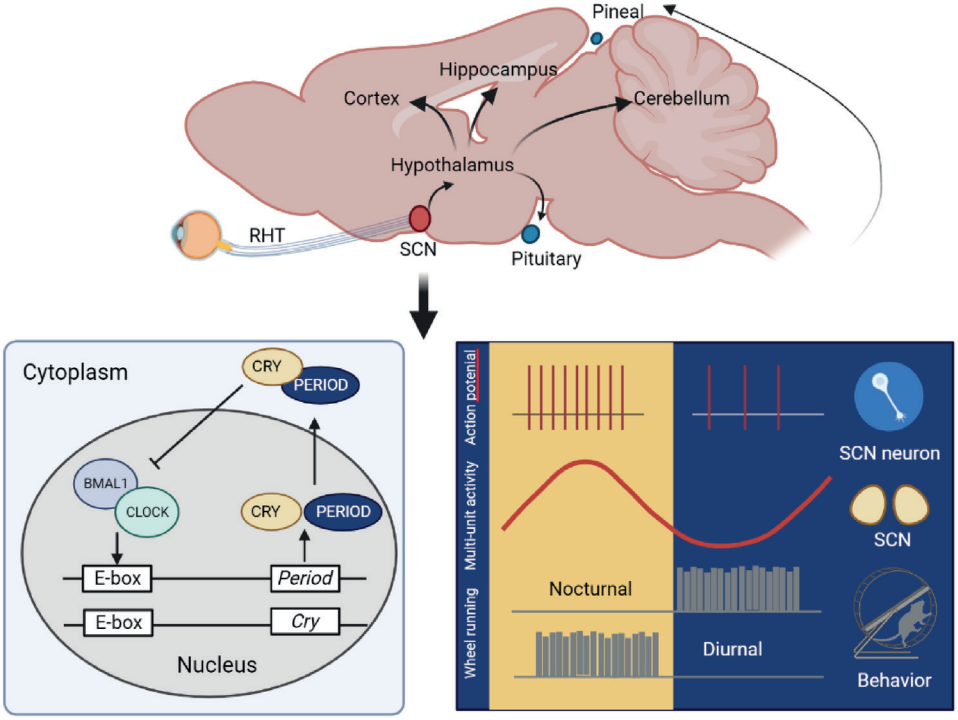


Figure 2. The suprachiasmatic nucleus is the central circadian pacemaker. Upper panel: Sagittal view of a rodent brain illustrating light input to the SCN via the RHT and output from the SCN to other brain areas. Bottom left (SCN neuron) panel is the simplified scheme of the molecular clock, the TTFL regulating the expression of clock genes and proteins that take around 24 h to complete a cycle. Bottom right panel is the representative rhythmic outputs from the SCN circuit. Adapted figure from Harvey et al., 2020 [23]. Created via BioRender.

1.2 Neurophysiology of sleep

Sleep sustains physical and cognitive performance, productivity, psychological health and well-being. Even mild sleep restriction over a few days negatively affect performance [24,25]. However, what exactly sleep is doing to our brain and body and the reason why we need sleep is still controversial in the research field. Sleep is a very complex physiological process involving the interaction of sleep-promoting and waking-promoting neural circuits. Most mammals have two different types of sleep: non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep. The changes in cortical electroencephalogram (EEG) and muscular electromyogram (EMG) are generally used to distinguish different sleep and arousal states in mammals [26]. Frequency, amplitude and morphology are critical terms to characterize the EEG and EMG for different vigilance states.

NREM sleep, is characterized by high amplitude and low frequency waves, (0.5 Hz – 4.0 Hz) also called slow-waves. Surprisingly, almost half a century after the delta activity was first demonstrated by William Grey Walter in an isolated cortex in vivo in 1966, its mechanism of generation is still not fully understood [27]. Delta waves are generated within the thalamocortical network [28] and they are thought to represent the activity of synchronized cortical neurons [29,30]. However, delta waves are consistently observed in decorticated animals [31]. Other studies show that delta waves can also be observed in the isolated thalamus [32]. Moreover, also other brain areas may be involved in generating delta waves, as is shown by lesions of the anterior hypothalamus, preoptic region, and basal forebrain, all of which can abolish delta waves [33]. Although the body weight of rats (200 – 400 grams) and mice (20 - 35 grams) can be 10 times different, their EEG power spectrums are more or less the same compared with each other (Figure. 3). However different rodent species may still show subtle differences in their EEG is also shown in Figure 3, where the peak of the NREM power spectrum (left, blue) of mice have a peak of around 3 Hz, and the peak of the NREM power spectrum (right, blue) of rats have a peak that is near 1.5 Hz.

Eugene Aserinsky and Nathaniel Kleitman first discovered REM sleep [34]. They showed that rapid eye movements occur during “active” sleep in adult human and these sleep periods with rapid eye movements may be involved in dreaming [35]. Years later, using high quality REM sleep EEG/EMG recording showed low amplitude and high frequency bands of wake-like brain activity, but with the muscle tone reaching minimum level. Because of

this low amplitude and high frequency waves, REM sleep was termed active or paradoxical sleep in early studies. In rodents, REM sleep is rich in theta activity (6 – 9 Hz), as shown in Figure 3, both mice (peak around 8 Hz) and rats (peak around 7 Hz) had a similar REM sleep power spectrum pattern and theta peak frequency, which is generated by the hippocampus [36].

The daily NREM-REM sleep cycle duration is also different across the species, for example, REM sleep occurs in the rodent approximately every 10 – 15 min. However, REM sleep occurs every 90 – 120 min in humans [37]. The overall sleep architecture is different between rodents and humans. Humans show monophasic sleep which is restricted to the night, whereas rodents display polyphasic sleep over day and night [38]. The difference is considered to be related to the size of both body and brain across the different species. The functional role of REM sleep is unclear. After decades of research and studies, it is more evident that brain areas that generate REM sleep reside in the brainstem and hypothalamus [39]. Subgroups of neurons are activated during REM sleep, called REM-on neurons, and these neurons release neurotransmitters such as γ-Aminobutyric acid (GABA), acetylcholine, and glutamate [40]. Moreover, there is also a subgroup of neurons called REM-off cells, which release neurotransmitters like norepinephrine, epinephrine, serotonin, histamine, and GABA [40].

There is a relation between the cortical EEG and theta waves in the hippocampus during REM sleep. Some studies indicated that theta activity during REM sleep is essential for motor performance, learning and memory consolidation [41,42]. REM sleep deprivation in rodents and humans resulted in impaired formation of spatial or emotional memory [40,43]. Both animal and human studies showed increased theta activity during REM sleep following a learning or memory tasks. In mice, specifically inhibiting theta activity in the hippocampus during REM sleep impaired memory consolidation, indicating that theta activity is important in memory consolidation [42]. Another study showed that increased theta activity was associated with increased cerebral blood flow during REM sleep in humans [44]. This may further indicate an increased metabolic rate and increased the energy supply from the blood to the brain areas during REM sleep.

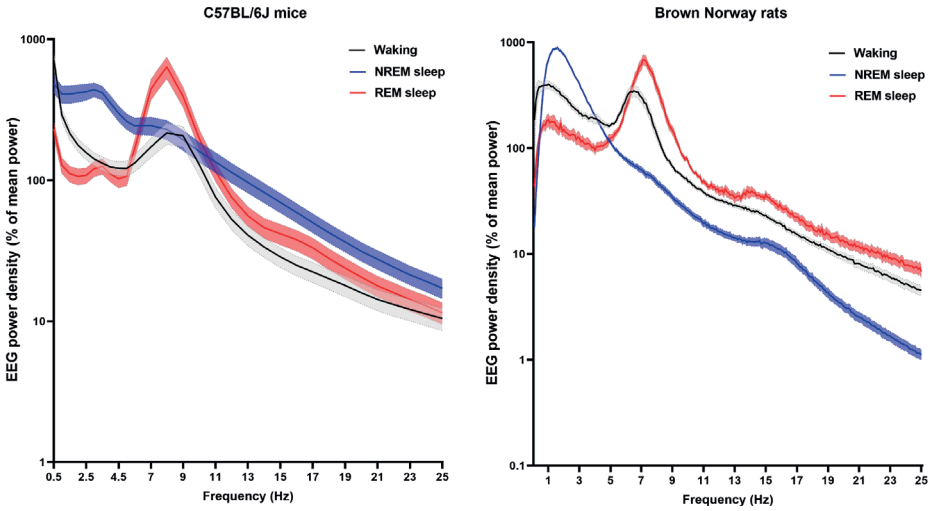


Figure.3 Spectral analysis of the EEG by a fast Fourier Transform routine. Left panel: A twenty four hours electroencephalographic power spectra in waking (black), NREM sleep (blue) and REM sleep (red) in C57BL/6J male mice under light-dark condition (n = 9). Right panel: A six hour electroencephalographic power spectra in waking (black), NREM sleep (blue) and REM sleep (red) in Brown Norway make rats under constant darkness condition (n = 8).

1.3 Homeostatic regulation of sleep

A balance of sleep and wakefulness exists, called sleep homeostasis. It means we tend to wake up when we sleep for a longer time, and when we stay awake for a longer time, the more sleepy we will feel. The sleepiness we feel is also called sleep propensity, or sleep pressure, which, in the two-process model of sleep regulation, increases during wakefulness and subsequently diminishes during sleep (Process S). This process S is thought to interact with signals received from the circadian clock (Process C) (Figure. 4). Both process C and S are influenced by external cues, like light and exercise. Slow wave activity (SWA) in the NREM sleep EEG is one of the best indicators for Process S. SWA in NREM sleep is increased with high sleep need, such as after sleep deprivation which is shown in the red line in Figure. 4 and decreased during sleep [26]. Homeostatic regulation of SWA has been demonstrated in a large number of rodents [26]. SWA is not only an indicator of Process S, but also critically important for the maintenance of sleep, brain plasticity, cognitive performance and memory [45-47].

The theories on the function of NREM sleep fall into three main categories: energy metabolism, neural plasticity, and cellular defense [48]. Sleep deprivation (SD) is often used as a mediator of sleep homeostasis and to investigate the function of sleep [49]. According to the metabolic theories on the function of sleep, the brain consumes energy during waking and restores energy in the subsequence sleep. Prolonged waking can induce an increase in adenosine. Adenosine is a ubiquitous nucleoside which serves as a building block for nucleic acids and energy storage molecules, enzyme's substrate and neuromodulator of cellular activity [50,51]. The adenosine theory states that during waking, due to neuronal activity-induced energy depletion, adenosine concentration in the brain increases, decreasing the neuronal activity of wake-active neurons and through this induces sleep [52]. Adenosine has therefore been proposed as a mediator of sleep homeostasis and a link between energy metabolism and sleep control.

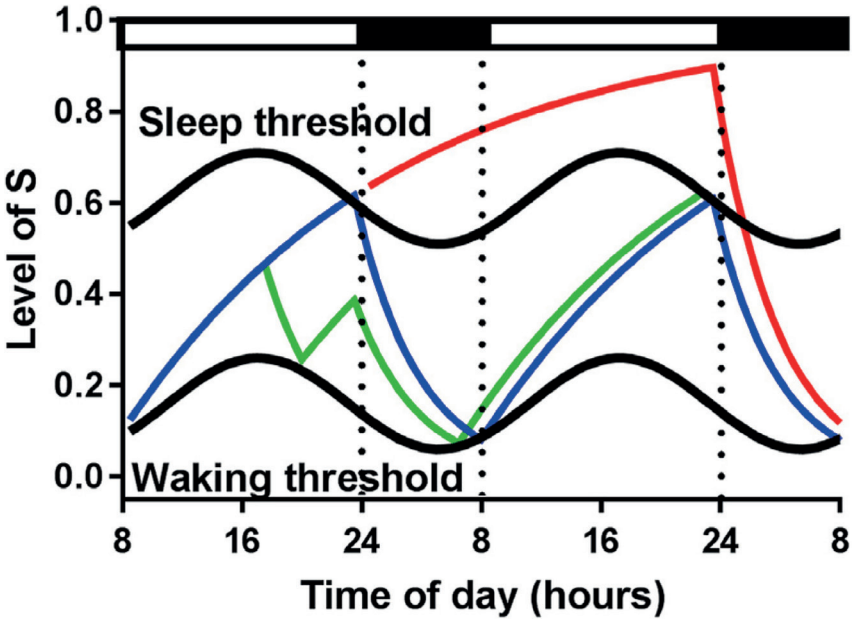


Figure 4. The two process model of sleep regulation. A simplified representation of the two process model of sleep regulation, similar to the version of the model in the initial publication (Borbely, 1982 [53]). The blue line represents the baseline condition with 8 hours of sleep and 16 hours of waking. The green line represents effects of 2-h nap and then a normal night sleep. The red line represents a 24-h sleep deprivation and followed by sleep. White and black bar represent the light and darkness. Figure adapted from Deboer 2018 [54]

2. Drugs influenced circadian clock and sleep-wake

2.1 Caffeine

As mentioned previously, adenosine may be an indicator of process S, and caffeine, which is found in coffee, tea and other types of food and beverages, is a non-selective adenosine receptor antagonist, is which widely used to induce wakefulness [55,56]. The pharmacokinetics of caffeine show a half-life of around 3 to 5 hours, and caffeine can induce waking, delay sleep onset and decrease slow-wave sleep [57]. Next to the effects on wakefulness, sleep and sleep homeostasis, caffeine is also known to improve alertness, mood, and cognitive performance, and counteract fatigue [58]. However, caffeine in doses of 5 cups of coffee or more induces anxiogenic symptoms in healthy adults and in some individuals it may induce panic disorders, and can lead to negative moods like anxiety and panic attacks [59]. Interestingly, these effects are absent when caffeine is given chronically. A recent study in mice compared the effect of acute and chronic administration of caffeine on day-night rhythm and sleep wake rhythm. Surprisingly, chronic caffeine did not induce wakefulness or disturbances of the sleep-wake cycle in contrast to acute caffeine administration. Instead, under chronic administration, it increases sleep during the rest phase and enhances sleep pressure in mice [60]. This suggests that acute and chronic caffeine influence sleep and sleep homeostasis differently.

Whether caffeine affects the circadian clock and further influences the circadian timing control of sleep is still debated. In rodent studies, increased sleep pressure by sleep deprivation can decrease the neuronal activity in the SCN, which suggests that the function of the clock can be modified by increased sleep pressure [61]. This may indicate that caffeine have an effect on the circadian clock. Acute caffeine administration can decrease homeostatic sleep pressure and increases SCN sensitivity of light in mice [62]. In humans, chronic caffeine consumption before bedtime delayed the melatonin rhythm by around 40 min, and chronic caffeine lengthened the period of circadian gene expression of human osteosarcoma U2OS cells [63].

2.2 Ketamine

Ketamine is an N-methyl-D-aspartate (NMDA) receptor antagonist, applied mainly as anesthetic, which has attracted a lot of attention in the last two

decades because of the rapid antidepressant effect in depressive patients [64]. In Berman's study, a sub-anesthetic dose in depressed patients showed a rapid antidepressant effect within 40 minutes and this effect lasted for seven days after the first infusion [64]. Ketamine may therefore have the potential to develop into a novel antidepressant; however, the mechanism of the antidepressant effect is still unknown.

Ketamine has been used as a non-barbiturate anesthetic drug for a long time, and it was described as an ideal anesthetic for its rapid onset, short duration of action, rapid recovery, and safety [65-67]. In preclinical studies, anesthetic doses had no effect or even reduced glutamate in the medial prefrontal cortex, whereas in the sub-anesthetic dose, it increased the extracellular glutamate [68]. More and more evidence from both preclinical and clinical studies have linked major depressive disorder to a dysregulated glutamatergic system, and glutamate receptors are also viewed as potential targets for antidepressant [69-71]. Thus under the sub-anesthetic does, ketamine induced an acute glutamate surge which may lead to the observed rapid-antidepressant effect. When administered in a sub-anesthetic dose, ketamine blocks NMDA receptors on GABA interneurons, thereby reducing GABA release on principal neurons and, in turn, the increasing presynaptic release of glutamate. The function of NMDARs is tightly linked to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs). AMPAR-mediated depolarization of the postsynaptic membrane is required for opening the NMDAR channel and removal of Mg^{2+} that blocks the channel pore; this is required for ketamine entry and blockade of the NMDAR channel (Figure 5, right) [72].

As referred to earlier, sleep quality is related to mood and mental health [25,73], and depressive disorders are associated with disrupted sleep and circadian rhythms [74,75]. Interestingly, SD in a subset of depressive patients is also known to induce a rapid antidepressant effect [76]. Thus, these antidepressant effects are suggested to work through sleep homeostatic mechanisms. Moreover, both SD and ketamine can increase SWA in NREM sleep and also the level of brain derived neurotrophic factor (BDNF), which are suggested to enhance the synaptic strength and plasticity [77,78]. Besides that, both SD and ketamine increase cortical excitability, and cortical excitability showed robust circadian dynamics [79]. Therefore, a rapid antidepressant effect may relate to the effects on sleep and the circadian clock. Understanding the rapid antidepressant effects of SD and ketamine from a sleep and chronobiology

perspective may contribute to exploiting the potential of these two treatments.

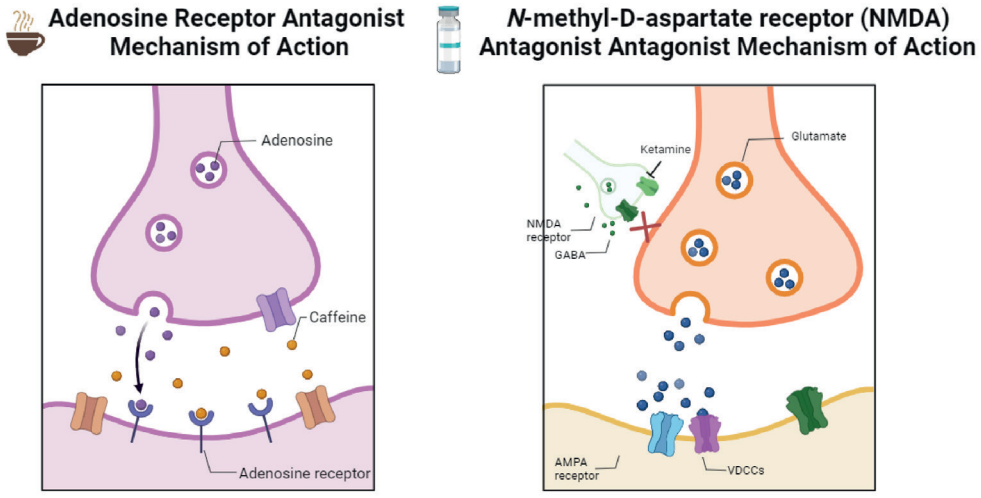


Figure 5. Action of caffeine and ketamine. *Left panel*: Proposed mechanism of caffeine's action, *Right panel*: Proposed mechanism of ketamine's antidepressant action. AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, GABA: γ-Aminobutyric acid, VDCCs: Voltage-gated calcium channels, created via BioRender.

3. Circadian clock, sleep and the relation with cancer related fatigue

3.1 Cancer related fatigue

Cancer-related fatigue (CRF) is a complex and debilitating side effect of unknown etiology, which affects more than 50% of cancer patients and cancer survivors [80,81]. CRF is defined as "a distressing, persistent, subjective sense of physical, emotional, and/or cognitive tiredness or exhaustion related to cancer or cancer treatment that is not proportional to recent activity and interferes with usual functioning." [82]. Persistent CRF has a serious effect on the quality of life of these patients since they are too tired to go to work, socialize or even perform their normal daily activities. Many cancer patients report fatigue when they get diagnosed, and this number increases in incidence and severity during and after treatment with either chemotherapy (chemotherapy related fatigue), radiotherapy, hormone therapy, surgery, or combined therapy. One third of the patients still feel fatigued five years after the end of treatment [83]. Several hypotheses describe the possible mechanism

both from a preclinical and clinical perspective. These mechanisms involve the effects of both the diseases, and the side effects of the therapies on energy metabolism, central nervous system, inflammation, immune function, hypothalamic-pituitary (HPA) axis function, antioxidant metabolism, sleep, and the circadian clock (Figure. 6) [84-92].

Many anti-cancer drugs have been approved to treat cancer. Anthracyclines are one class of chemotherapy that has been widely used for over 60 years to treat different types of cancers. The mechanisms by which anthracyclines kill tumor cells are various, including inhibition of DNA replication and RNA transcription, free radical generation leading to DNA damage or lipid peroxidation, DNA alkylation, interference with DNA unwinding or DNA strand separation and helicase activity, causing double-strand breaks (DSBs) following the poisoning of topoisomerase II, and chromatin damage, mediated through histone eviction at selected sites in the genome [93,94]. Anthracyclines have toxic side effects on nontargeted tissues, which may contribute to fatigue symptoms over a more extended period [95,96].

Recent studies document that especially the treatment of cancer is associated with both immune stimulation and immunosuppression with increased concentrations of various cytokines including tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6) and interleukin 10 (IL-10) [97,98]. Immune stimulation, increasing circulating cytokines, can reach the brain through several pathways [99]. For example, circulating cytokines can cross the blood brain barrier to enter cerebrospinal fluid and interstitial fluid spaces of the brain and spinal cord, increasing microglia activation, which can produce pro-inflammatory cytokines and chemokines [100]. Consequently, it is possible that active microglia drive neuroinflammation and further influence the function of the brain. Peripherally administered interleukin-1 (IL-1) activates the HPA axis [101], and this may further influence hormonal levels like adrenocorticotropic hormone (ACTH) and corticosterone (CORT) [102]. When the peripheral cytokines transfer into the brain, IL-1, IL-6, TNF, and their family members may mimic leptin and, hence, target hypothalamic neuropeptides that regulate food intake and energy expenditure [103]. Pro-inflammatory cytokines, cardiotrophin-like cytokine (CLC), and acute infusion of CLC into the third ventricle inhibits locomotor activity in hamsters [104]. Furthermore, a variant of cytokines is correlated with sickness behaviors and fatigue in rodents and humans, which are also the proposed mechanism for CRF [105,106]. All these pieces of evidence indicate that peripheral cytokines

may play a role in developing fatigue.

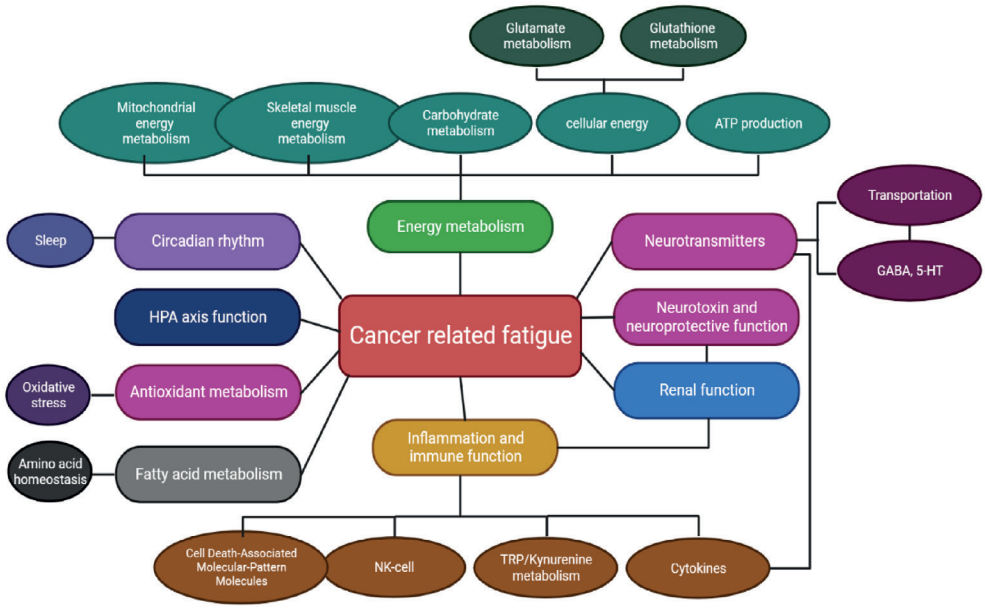


Figure.6 Possible relationships in cancer related fatigue. ATP: Adenosine triphosphate, HPA axis: hypothalamic-pituitary-adrenal axis, TRP: tryptophan, GABA: γ-Aminobutyric acid, 5- HT: serotonin. Created via BioRender.

3.2 Disruption of sleep

A common complaint of cancer patients is disrupted sleep [107]. Patients complain about poor quality of (subjective) sleep, difficulty staying asleep and insomnia during treatment and even months after the treatment [108-110]. Although we know that cancer related fatigue cannot be released after a good night of sleep or rest, a disrupted sleep-wake cycle may contribute to the fatigue experience of the patients [111]. Thus, normalizing the sleep-wake cycle of cancer patients, may help to reduce their fatigue level. However, a larger randomized trial of 219 breast cancer patients did not show any benefit of an individualized sleep therapy plan over the control intervention on fatigue [112]. Behavioral interventions aimed at improving sleep may be successful in their primary aim, but their effect on cancer-related fatigue is less obvious [113,114]. According to another study that reports sleep problems in around three thousand patients, instead of fatigue, sleep problems are more associated with pain and emotional distress [115]. The relationship between sleep and cancer related fatigue may therefore be more complicated.

3.3 Circadian disruption and cancer-related fatigue

Robust circadian rhythms are strongly associated with good health. Cancer and hospitalization can disrupt circadian rhythms [116]. One proposed underlying mechanism of CRF is disrupted circadian rhythm. Several studies used a wearable sensor to detect the subjects' activity, showing changes in daily rhythms. One study used the waist accelerometer which measured the daily activity, and further compared the daily activity before and after the first chemotherapy. This showed clear decreased activity after the chemotherapy, however, the changes in daily activity may be masked by the hospitalization [117]. Another study showed that the patients who wear the waist accelerometer show a dampened 24-h activity pattern compared with healthy subjects, and this dampened rhythm was associated with the level of fatigue, appetite and poor survival [118,119]. There is growing interest in HPA axis function and associated cortisol release in cancer survivors who have had fatigue complaints for years [120]. The daily cortisol rhythm is under strong control of the circadian clock. In healthy adults, a typical diurnal cortisol pattern is characterized by a high morning level that peaks about 30 min after awakening, followed by a decline over the course of the day with the lowest level achieved around midnight [121]. Breast cancer patients showed a dampened cortisol response compared with healthy subjects, and the decline in cortisol levels during the night is blunted in breast cancer patients [120,122]. However, it is not known whether these changes are the effects of fatigue or causal factors, for example, daily dysfunctioning.

Yet, these studies do not provide insight into the timing of the clock and the relation with fatigue. Animal models may provide more insights into the relationship among the circadian timing system, sleep-wake cycle and cancer related fatigue. Wheel running in rodent is voluntary, motivated behavior. Wheel running behavior is also a measure for accessing the circadian clock controlled behavior under continuous recording condition. Furthermore, wheel running is also viewed as a measurement of fatigue. In this case, with the animal fatigue model, it may be easier to answer the question : " Is fatigue a sleep problem, or clock problem, or both?".

Disruptions of normal circadian rhythms and sleep cycles are consequences of aging and can profoundly affect health. Accumulating evidence indicates that circadian and sleep disturbances are a risk for mood disorders and neurodegenerative conditions, and may actually drive pathogenesis early in

the course of these diseases [123-125].

In order to ensure normal functioning of the circadian clock, environmental time cues are quite important. Circadian misalignment occurs when the internal timing system runs out of synchrony with the behavioral cycle. One common example of circadian misalignment is shift work which misaligns the sleep-wake rhythm to the objective night. Shift work can result in disrupted sleep, impaired cognitive performance, fatigue, decreased alertness, impaired energy metabolism and inflammation (Figure. 7) [125-129]. Other types of misalignments include internal misalignments between the central and peripheral clocks. For example, research in animal models has demonstrated that altering the availability of food timing shifts the peripheral clock but not the central clock [130].

The rest-active rhythm is also a biomarker that reflects the robustness of the clock in patients. Interestingly, most cancer survivors benefit from daily exercise or routines [131,132]. These interventions probably help to maintain normal circadian rhythms in the body. Furthermore, cancer patients suffering from circadian disruptions have poor health outcomes compared to patients who have a robust daily activity rhythm [132,133]. Studies in rodents have added more evidence from different types of cancer, chemotherapy, radiotherapy and the consequences of circadian disruptions [134-137]. 5-fluorouracil affected rhythmicity of clock genes expression in the SCN and decreased locomotor activity during the dark phase under chronic treatment [138]. Clock mutant mice are more sensitive to the chemotherapy treatment with cyclophosphamide [139]. Recent advances identify disturbed clock gene expression and circadian rhythms to correlate with tumor development and tumor progression in mouse models [136,140,141]. These studies offer new insight into the interaction of previously unsuspected pathways with the circadian system besides cancer or treatments themselves. We can also begin to rationally develop new treatments for disorders affected by circadian disruptions.

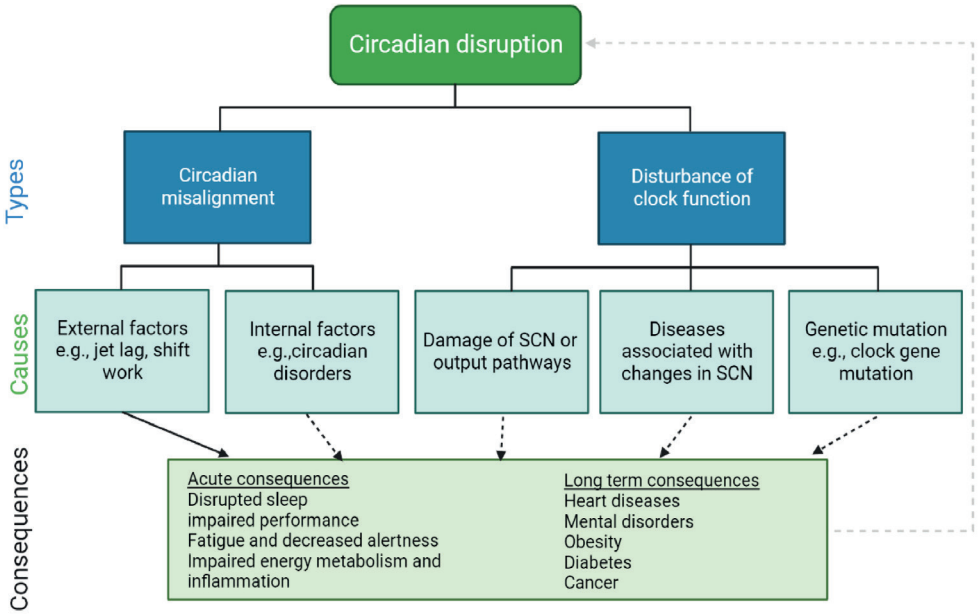


Figure 7. Schematic overview of different types of circadian disruptions, the causes and the consequences of health under acute and chronic situations. Acute and long-term consequences may send feedback and further disrupted the circadian clock. Modified from Rüger et al., 2011 [125].

Outline of this thesis

This research aims to understand how the circadian clock and sleep are influenced by different drugs, which include the most common CNS stimulant caffeine, ketamine, the newly proved anti-depressant drug, and anthracyclines, a widely used group of chemotherapeutic agents.

Caffeine is one the most widely used psychoactive stimulant across the world, it is known as a nonselective adenosine receptor antagonist, and it has been more than 60 years since adenosine has been discovered to be involved in sleep. However, most of the previous experiments have been performed under light-dark conditions and the entrainment of light has a massive effect on sleep-wake rhythm and is known to interact with caffeine. Thus, our question is how long the effect of acute caffeine lasts under constant dark conditions. **Chapter 2** describes the effect of acute administration of caffeine on sleep, sleep EEG and the circadian clock in Brown Norway rats.

As described in the introduction, mental health has a complex relationship with sleep, and the rapid-antidepressant effect of sleep deprivation is not well understood. Ketamine has a similar rapid anti-depressant effect and also changes sleep. Thus our hypothesis is that the rapid anti-depressant effect observed from both sleep deprivation and ketamine may have a similar effect on sleep and the sleep EEG. In **Chapter 3**, we investigate the relationship between the two treatments comparing the effect of sleep deprivation and low dose ketamine on sleep in Brown Norway rats.

The effect of chemotherapy on sleep and the circadian clock was investigated in **Chapter 4**. During chemotherapy, most patients complain about how tired or fatigued they feel both under the treatment and months to years after finalizing treatment. There are several hypotheses about the mechanisms of cancer-related fatigue, but there is still too much unknown about this particular type of tiredness. In this chapter, we want to investigate if CRF is a circadian problem, a sleep problem, or both. At the end of chapter 4, we conclude that it is a circadian problem, in which waking, rather than sleep is affected

Based on the conclusion in chapter 4, in **Chapter 5**, we performed a followed-up experiment that further investigated the effect on the neuronal activity of SCN and peri-SCN areas in the brain, rest-activity behavior, immune system responses and the effect on kidney and spleen, to establish where the circadian clock may be involved in CRF.

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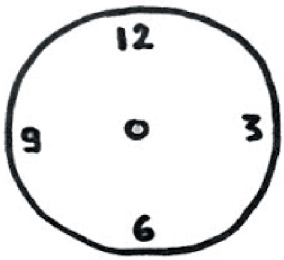
2

Long-Term Effect of a Single Dose of Caffeine on Sleep, the Sleep EEG and Neuronal Activity in the Peduncular Part of the Lateral Hypothalamus under Constant Dark Conditions



Yumeng Wang, Tom Deboer. Long-Term Effect of a Single Dose of Caffeine on Sleep, the Sleep EEG and Neuronal Activity in the Peduncular Part of the Lateral Hypothalamus under Constant Dark Conditions. Clocks & Sleep 2022, 4(2), 260-276.

Abstract

Background: Caffeine is a central nervous system stimulant that influences both the sleep–wake cycle and the circadian clock and is known to influence neuronal activity in the lateral hypothalamus, an important area involved in sleep–wake regulation. Light is a strong zeitgeber and it is known to interact with the effect of caffeine on the sleep–wake cycle. We therefore wanted to investigate the long-term effects of a single dose of caffeine under constant dark conditions. Methods: We performed long-term (2 days) electroencephalogram (EEG)/electromyogram recordings combined with multi-unit neuronal activity recordings in the peduncular part of the lateral hypothalamus (PLH) under constant darkness in Brown Norway rats, and investigated the effect of a single caffeine treatment (15 mg/kg) or saline control given 1 h after the onset of the endogenous rest phase. Results: After a reduction in sleep and an increase in waking and activity in the first hours after administration, also on the second recording day after caffeine administration, rapid eye movement (REM) sleep was still reduced. Analysis of the EEG showed that power density in the theta range during waking and REM sleep was increased for at least two days. Neuronal activity in PLH was also increased for two days after the treatment, particularly during non-rapid eye movement sleep. Conclusion: Surprisingly, the data reveal long-term effects of a single dose of caffeine on vigilance states, EEG, and neuronal activity in the PLH. The absence of a light–dark cycle may have enabled the expression of these long-term changes. It therefore may be that caffeine, or its metabolites, have a stronger and longer lasting influence, particularly on the expression of REM sleep, than acknowledged until now.

Keywords: caffeine; electroencephalogram; rapid eye movement sleep; theta activity; constant darkness; lateral hypothalamus

1. Introduction

Caffeine is the most widely used psychoactive stimulant worldwide. It has numerous pharmacological and physiological effects, including increasing arousal, cardiovascular activity, respiration, renal activity, and alertness. In addition, it has effects on mood, memory, and cognitive performance [1,2,3]. Caffeine has a noticeable effect of increasing waking and locomotor activity, and decreasing the expression of slow waves in the non-rapid eye movement (NREM) sleep electroencephalogram (EEG) [1,4], which are thought to be markers of sleep homeostasis [5]. Caffeine works via antagonism of adenosine receptor signaling across almost all brain areas and generally induces an increase in neuronal activity [6]. The levels of adenosine in the brain increase during sleep deprivation and decrease during recovery [7,8]. Therefore, adenosine is thought to be one of the substances involved in the homeostatic regulation of sleep [9].

Most of the studies investigating the effect of caffeine on sleep analyzed only the first 24 h after acute administration of caffeine. However, caffeine may have a more prolonged effect on the sleep–wake cycle. The half-life of caffeine ranges from 0.7 to 1.2 h in rats after 5–10 mg/kg [10] and from to 2.5 to 4.5 h after 4 mg/kg in adult humans (280 mg/70 kg, 2–3 cups of coffee) [11]. Moreover, 15 mg/kg caffeine (corresponding to the ingestion of 300–400 mg of caffeine in a 70 kg adult human) [6] has an effect on the vigilance states, which seems to last for approximately 3 h in albino rats [1]. However, when closely investigating the metabolism of caffeine, the effect of caffeine may last longer. The pharmacokinetic profile of caffeine shows that it remains in the bloodstream approximately 350 min after oral administration of 5 mg/kg caffeine in mice [12]. After intravenous application of 15 mg/kg of caffeine, an increased concentration of caffeine was still detectable 10 h after administration [1,13]. Next to that, metabolites of caffeine, such as paraxanthine, are also known to function on adenosine receptors [14,15]. This suggests that caffeine and its metabolites may stay in circulation and influence sleep and waking for longer than previously reported.

Light is a strong zeitgeber that entrains the endogenous circadian rest activity rhythm to the external time [16,17,18]. Adenosine may also play a role in the functioning of the circadian clock. Application of an adenosine agonist attenuates light-induced phase shifts in behavior. This attenuation can be restored by an adenosine receptor antagonist [19,20]. It has been shown that

caffeine lengthens the free-running period in mice under constant condition [21,22]. Interestingly, the effect of light on the circadian system is influenced by caffeine. It increased the light-induced phase-shift in mice and Arvicanthis, and enhanced the light-induced suprachiasmatic nucleus neural activity in mice [21,23,24,25]. Caffeine may, in that way, strengthen the rhythm of the endogenous circadian clock. These studies show that adenosine and caffeine not only affect sleep and waking, but also influence the period and light responsiveness of the circadian clock. Particularly, in a light–dark cycle, both the acute and the long-term effects on the second day may be influenced by the input of light on the circadian pacemaker. Nevertheless, there is virtually no study that investigates the sleep–wake cycle after caffeine administration under constant darkness.

Caffeine's effect on arousal is central in origin. In rat studies, 10–30 mg/kg caffeine induced large levels of c-fos expression across the brain [26]. Although most of the expression was in the cortex, it was not limited to the cortex as it was also visible in the hypothalamus, including the orexin neurons in the peduncular part of the lateral hypothalamus (PLH) [27]. When and for how long this activation in the hypothalamus lasts is unclear. Most brain areas involved in sleep–wake regulation can be found in the hypothalamus and the pons [28,29]. They consist of two ascending pathways that promote waking and wake maintenance. The first releases acetylcholine, which is produced by neurons in the pedunculopontine and laterodorsal tegmental nucleus and is active during waking and REM sleep [30]. The second releases different monoamines from the locus coeruleus (noradrenaline), raphe (serotonin), ventral periaqueductal gray matter (dopamine), and tuberomammillary neurons (dopamine). These project to the lateral hypothalamus (LH), the basal forebrain, and the cerebral cortex [31] and are most active during waking, less active during NREM sleep, and silent during REM sleep. On the opposite side, the ventrolateral preoptic area (VLPO) is thought to mainly stimulate NREM sleep. The VLPO has outputs to the acetylcholine and monoamine nuclei involved in wake maintenance and is active during sleep, when it releases the inhibitory neurotransmitters galanin and GABA [32,33,34]. Together, this forms a self-reinforcing loop, which in electrical engineering is called a "flip-flop switch", a term also coined for this system [28]. Orexin, released by specific neurons in the LH, is thought to stabilize this switch and to reinforce waking without inhibiting activity of the VLPO [28].

Based on the previous research summarized above, it is not unlikely that the effect of a single dose of caffeine is underestimated in situations where the effect of caffeine is tested under normal light–dark conditions. To further investigate these different aspects of the possible influence of caffeine, we assessed the effect of a single dose of 15 mg/kg caffeine on PLH neuronal activity, combined with vigilance state recordings in freely moving rats. Thus, in our experiment, we investigated the long-term (2 days) effect of a stimulatory concentration of caffeine on the sleep–wake cycle and EEG and locomotor activity under constant darkness. As light and caffeine interact in their effect on sleep and waking and the circadian clock, we decided to record in constant darkness to be able to distinguish the effect of a single dose of caffeine on both sleep–wake cycle and the circadian clock. In addition, to investigate the effect on neuronal activity in PLH, we recorded electrical multi-unit neuronal activity in this brain area.

2. Results

2.1. Effect of Caffeine on the Sleep–Wake Cycle and Locomotor Activity

Immediately after the caffeine injection (15 mg/kg), waking and activity were increased and the amounts of NREM sleep and REM sleep were reduced (Figure 1). This initial effect lasted for approximately 2–3 h compared with saline injection (Figure 1A–E). After that, no differences were found in the hourly values of the vigilance states or activity level. Surprisingly, a single application of caffeine on the first day still influenced the occurrence of REM sleep on the second recovery day (Figure 1C). REM sleep was decreased by 21.5% during the subjective day (the rest phase of the animal where it normally is light) relative to saline on the second recovery day (Figure 1M). In contrast, no effects on the other vigilance states or activity levels were found on the second day (Figure 1F–O; Table 1). Moreover, after caffeine, the day–night differences in waking, NREM sleep, and REM sleep were diminished on the first day (Figure 1F–H). The day–night difference after caffeine treatment was restored on the second recovery day for waking and NREM sleep (Figure 1K,L), but not for REM sleep (Figure 1M).

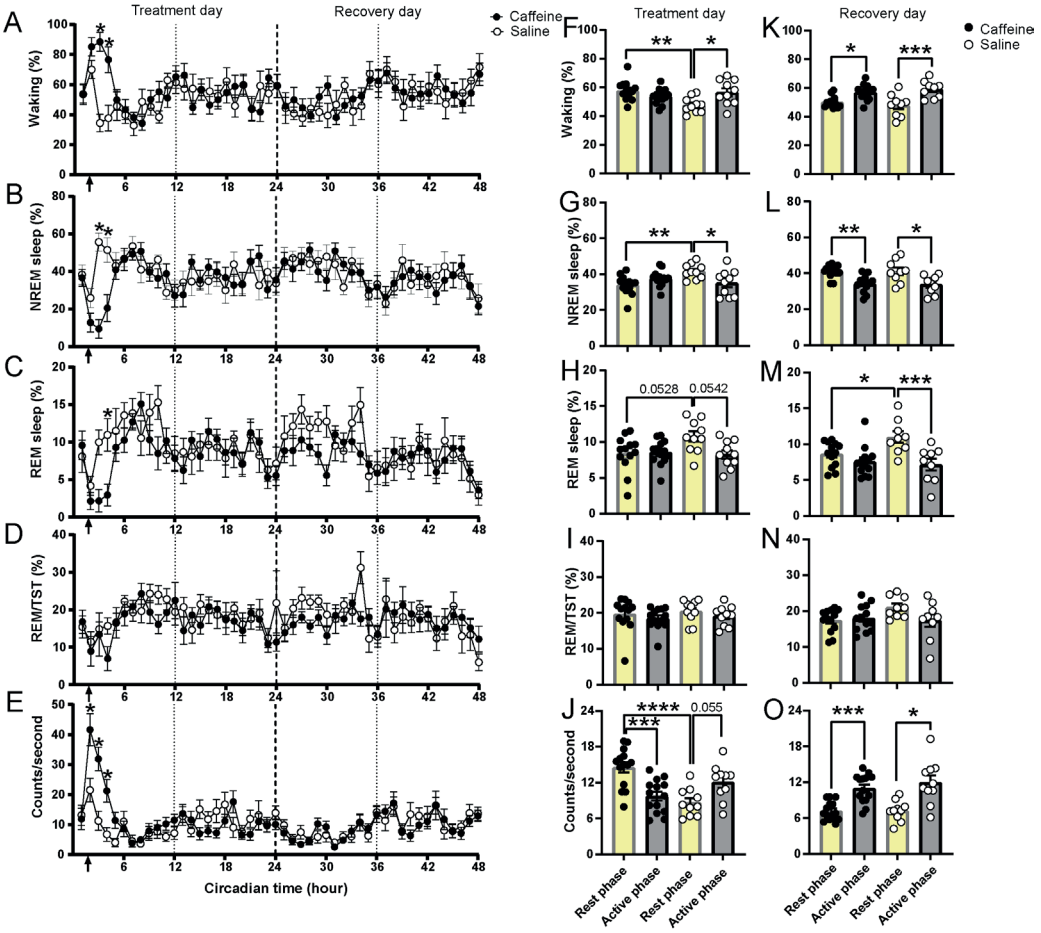


Figure 1. Vigilant states and locomotor activity of caffeine and saline treatment in 48 h recording under constant darkness. (A–E) Time course of waking, NREM sleep, REM sleep, REM sleep/total sleep time, and locomotor activity in 1 h value for caffeine (black, n = 13) and saline (white, n = 10) administration in 48 h. The first 24 h were considered as the treatment day; the second 24 h were considered as the recovery day. Arrows indicate the injection time (CT1-2). Asterisks indicate significant differences between caffeine and saline administration (* p = 0.05–0.0001, Bonferroni multiple comparisons test after significant two-way ANOVA, factors "treatment" or interaction of "circadian time" and "treatment"). (F–J) Rest and active phase values of waking, NREM sleep, REM sleep, REM sleep/total sleep time, and locomotor activity for caffeine (black, n = 13) and saline (white, n = 10) administration on treatment day. (K–O) Rest and active phase values of waking, NREM sleep, REM sleep, REM sleep/total sleep time, and locomotor activity for caffeine (black, n = 13) and saline (white, n = 10) administration on recovery day. CT0–CT12 was considered as the rest phase (yellow bar); CT12-CT24 was considered as active phase (gray bar). Asterisks indicate significant differences between caffeine and saline administration (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, Bonferroni multiple comparisons test after significant two-way ANOVA). Data are shown as mean ± SEM.

Table 1. Detailed statistics for two-way ANOVA of 48-h vigilance stages and PIR data.

Vigilance state&PIR	Time of day	Treatment	Interaction
Treating day			
Waking	P<0,0001	P=0,0359	P=0,0001
NREM sleep	P<0,0001	ns[1]	P<0,0001
REM sleep	P<0,0001	P=0,0259	P=0,0733
REM/Total sleep	P=0,0031	P=0,0420	ns
PIR	P<0,0001	P=0,0184	P<0,0001
Recovery day			
Waking	P=0,0007	ns	ns
NREM sleep	P=0,0004	ns	ns
REM sleep	P<0,0001	P=0,0259	ns
REM/Total sleep	P=0,0048	ns	ns
PIR	P<0,0001	ns	ns

[1] ns: not significant

The results obtained on the first day are in accordance with previous findings, where it was found that the effect of this dose of caffeine on waking, NREM sleep, and locomotor activity lasts for approximately 3 h following administration [1]. However, the influence on REM sleep time lasted considerably longer. Based on our findings, it seems that a single dose of 15 mg/kg caffeine can influence the occurrence of REM sleep for more than 24 h.

2.2. Effect of Caffeine on Vigilance State Episodes

The changes in the amount of waking, NREM sleep, and REM sleep were reflected in the distribution of the vigilance state episodes (Figure 2). Caffeine treatment reduced the number of short (<40 s) NREM sleep episodes during both the rest and active phase on the first day (Figure 2C,D). This change was accompanied by an increase in the number of short (<20 s) waking episodes during the subjective day (Figure 2A). However, there was an opposite effect in the subjective night (the active phase of the animal where it normally is dark) with a reduction in the number of short waking episodes (Figure 2B). Caffeine also changed the duration distribution of the REM sleep episodes, as the ANOVA indicated an overall difference in the number of episodes; however, this did not result in significant results in the post hoc *t*-test for single duration bins (Figure 2E,F). In the rest period immediately after treatment, caffeine clearly increased the number of short waking episodes and reduced the number of short NREM sleep episodes. This, however, seemed to result in the opposite response in the following active period.

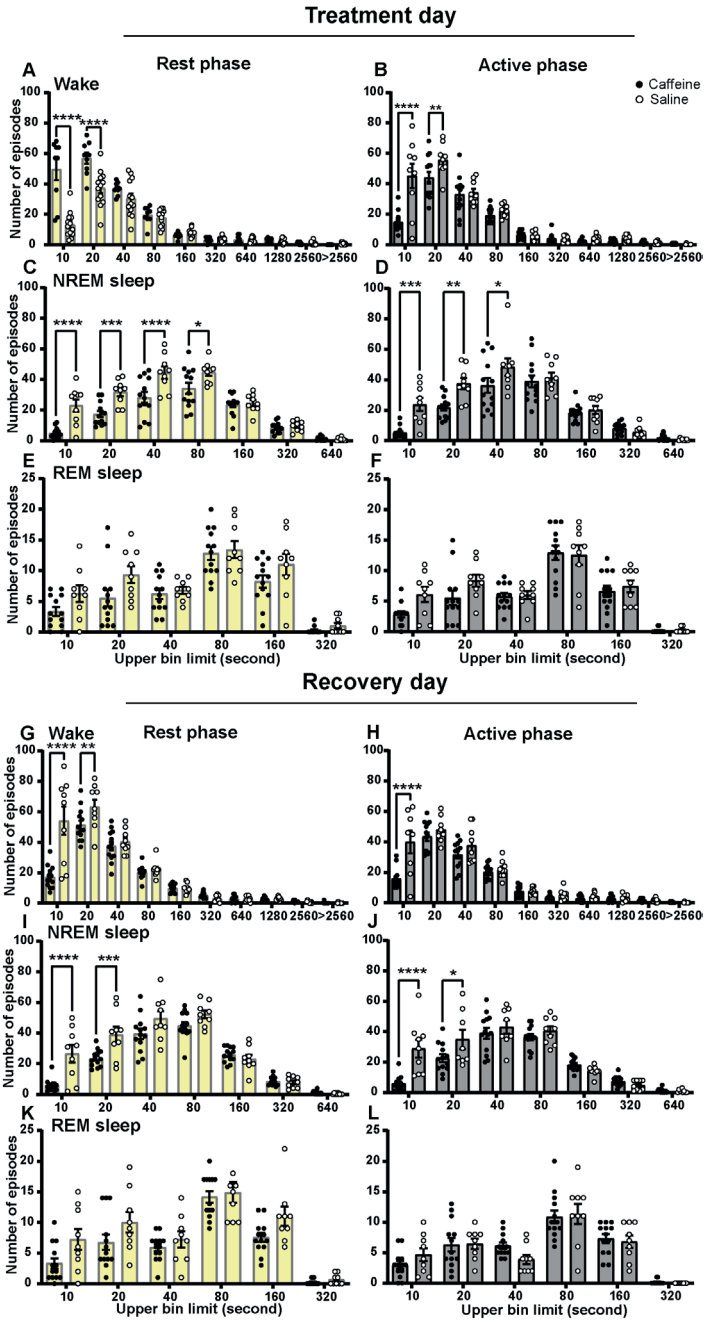


Figure 2. Episode duration histograms of rest phase and active phase. (**A**,**B**) Episode duration histograms of waking in the rest and active phase for caffeine (black, n = 13) and saline (white, n = 9) administration. (**C**,**D**) Episode duration histograms of NREM sleep in the rest phase and active phase for caffeine and saline. (**E**,**F**) Episode duration histograms of REM sleep in the rest phase and active phase for caffeine and saline. (**G**,**H**) Episode duration

histograms of waking in the rest phase and active phase for caffeine (black, $n = 13$) and saline (white, $n = 9$) administration on recovery day. (**I**,**J**) Episode duration histograms of NREM sleep in the rest phase and active phase for caffeine and saline on recovery day. (**K**,**L**) Episode duration histograms of REM sleep in the rest phase and active phase for caffeine and saline on recovery day. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ caffeine compared to saline, two-way ANOVA with Bonferroni multiple comparisons. Episodes are partitioned into ten exponentially increased duration bins from 10 to >2560 s (x-axis designates the upper limit of each bin for the following bins: 0–10, 11–20, 21–40, 41–80, 81–160, 161–320, 321–640, 641–1280, 1281–2560, >2560). Data are shown as mean ± SEM.

On the second recovery day, the reduction in short (<20 s) NREM sleep episodes under both subjective day and night was repeated (Figure 2I,J). During the subjective day, the short (<20 s) waking episodes were decreased on the second recovery day of the caffeine-treated group (Figure 2G). The number of 10 s waking episodes remained lower in the subjective night on the second day after caffeine treatment (Figure 2H). REM sleep episode distribution on the second recovery day remained different after caffeine administration compared to control treatment (Figure 2K,L). Therefore, a prolonged effect of a single dose of caffeine could also be observed here.

2.3. Effect of Caffeine on EEG Slow-Wave and Theta Activity

As we observed the long-lasting effect on REM sleep, we decided to analyze theta activity (6.0–9.0 Hz), the most prominent EEG frequency in REM sleep, in all three vigilance states (Figure 3). After caffeine treatment, theta activity was initially increased in all three vigilance states compared to saline (Figure 3A–C). In NREM sleep, values returned to baseline within a couple of hours and no differences were seen over the two days (Figure 3F,J). Increased theta activity in waking persisted for approximately 10 h, resulting in a significant increase in the average value over the rest phase (Figure 3E). On the second recovery day, this increase persisted, resulting in significantly increased theta activity values for both the rest and active phase (Figure 3I). Similarly, but more pronounced, theta activity during REM sleep remained high for the entire 47-h recording period after caffeine administration (Figure 3C,G,K; Table 2). Theta activity in both waking and NREM sleep was dependent on circadian time (Figure 3A,B, Table 2), whereas theta activity in REM sleep did not show a significant circadian rhythm (Table 2).

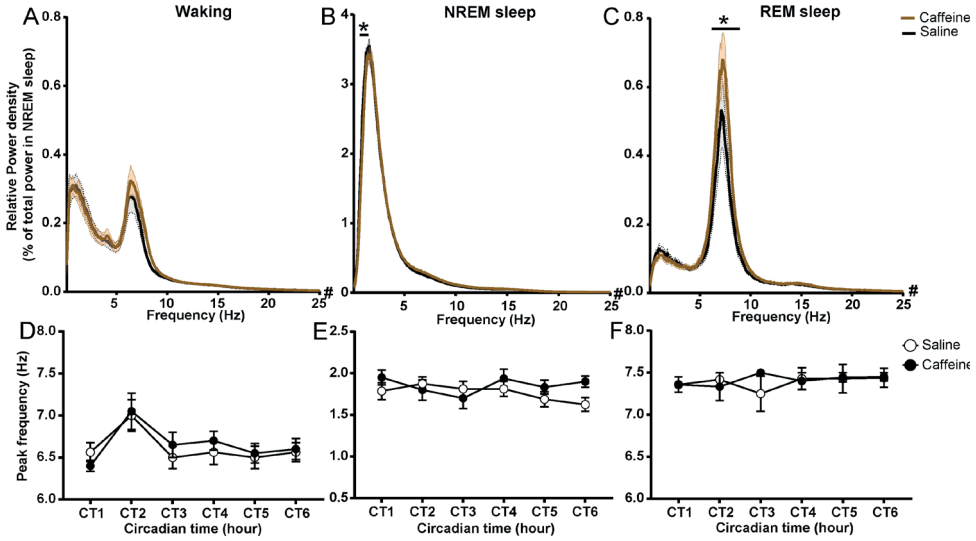


Figure 3. Theta activity in different vigilant states and slow wave activity in NREM sleep for 48 h under constant darkness. (**A**–**D**) Time course of theta activity in waking, NREM, and REM sleep (2 h interval) and slow-wave activity in NREM sleep for caffeine (black, $n = 9$) and saline (white, $n = 9$) administration in 48 h. The first 24 h were considered as the treatment day; the second 24 h were considered as the recovery day. Arrows indicate the injection time (CT1-2). Asterisks indicate significant differences between caffeine and saline administration (* $p = 0.05$–0.0001, Bonferroni multiple comparisons test after significant two-way ANOVA, factors "treatment" or interaction of "circadian time" and "treatment"). (**E**–**H**) Subjective day and night values of theta activity in waking, NREM and REM sleep, and slow-wave activity in NREM sleep for caffeine (black, $n = 9$) and saline (white, $n = 9$) administration on treatment day. (**I**–**L**) Rest and active phase values of theta activity in waking, NREM and REM sleep, and slow-wave activity in NREM sleep for caffeine (black, $n = 9$) and saline (white, $n = 9$) administration on recovery day. CT0-CT12 was considered as the subjective day (yellow bar); CT12-CT24 was considered as the subjective night (gray bar). Asterisks indicate significant differences between caffeine and saline administration or the differences between subjective day and subjective night (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, Bonferroni multiple comparisons test after significant two-way ANOVA). Data are shown as mean ± SEM.

Table 2. Detailed statistics for two-way ANOVA of 48-h theta activity and SWA.

States	Time of day	Treatment	Interaction
Treating day			
Theta in waking	P<0,0001	P<0,0001	ns[1]
Theta in NREM sleep	P=0,0278	P<0,0001	ns
Theta in REM sleep	ns	P<0,0001	ns
SWA in NREM sleep	P<0,0001	P=0,0152	ns
Recovery day			
Theta in waking	ns	P<0,0001	ns
Theta in NREM sleep	P=0,0016	P<0,0001	ns
Theta in REM sleep	ns	P<0,0001	ns
SWA in NREM sleep	P=0,0007	ns	ns

[1] ns: not significant

As slow-wave activity in NREM sleep is a marker of sleep homeostasis and has been shown in the past to be influenced by caffeine [1,4,5,9], we analyzed this variable as well. We found no significant changes in slow-wave activity in response to 15 mg/kg caffeine (Figure 3D,H,L). The slow-wave activity in NREM sleep showed a time-dependent rhythm under constant darkness in Brown Norway rats, as previously shown in other rodents (Figure 3D, Table 2) [35,36]. Maximum activity in the slow-wave range in NREM sleep seemed to be delayed on the first day after caffeine treatment compared to saline. The latter may be due to the increased waking in the first three hours after caffeine treatment.

2.4. Effect of Caffeine on the EEG Power Spectrum of Waking and REM Sleep

As we observed an overall increase in the theta activity in waking, NREM sleep, and REM sleep, we wanted to understand how caffeine influences the EEG power spectrum compared to saline administration. This is to ensure that the results we found in theta power density were not caused by changes in theta frequency over the course of the experiment. For this purpose, we analyzed the power density spectra (0–25 Hz) of waking and REM sleep after both saline and caffeine treatment, and expressed them relative to the total EEG power density of the NREM sleep EEG obtained on the first day after the saline injection. Caffeine induced an increase in EEG power density of REM sleep specifically in the theta range from 6.6 Hz to 8.1 Hz (Figure 4C), and reduced activity in the slow-wave range (0.6 Hz to 1.5 Hz) in the NREM sleep spectrum (Figure 4B). Caffeine also affected EEG activity in waking (Figure 4A, ANOVA factor "treatment" $p = 0.0023$) relative to saline administration, but no specific frequency bin was distinguished in the post hoc test. To ensure that the theta peak in the waking and REM sleep EEG remained stable after treatment, we determined its frequency for the first 6 h after treatment and found that both for waking and REM sleep, theta peak frequency after caffeine treatment was very similar to the corresponding peak frequency after saline (Figure 4D,F), indicating that caffeine did not shift the peak of theta frequency activity. The theta peak frequency of waking at CT1 was approximately 6.5 Hz, and both caffeine and saline injection increased the peak frequency to 7.0 Hz at CT2. The increased peak frequency after the injection probably reflects the change to more active waking. Additionally, the slow-wave peak in NREM sleep did not shift due to caffeine (Figure 4E). These results indicate that caffeine decreases slow-wave activity during

NREM sleep and increases theta power density in REM sleep, but does not change its frequency.

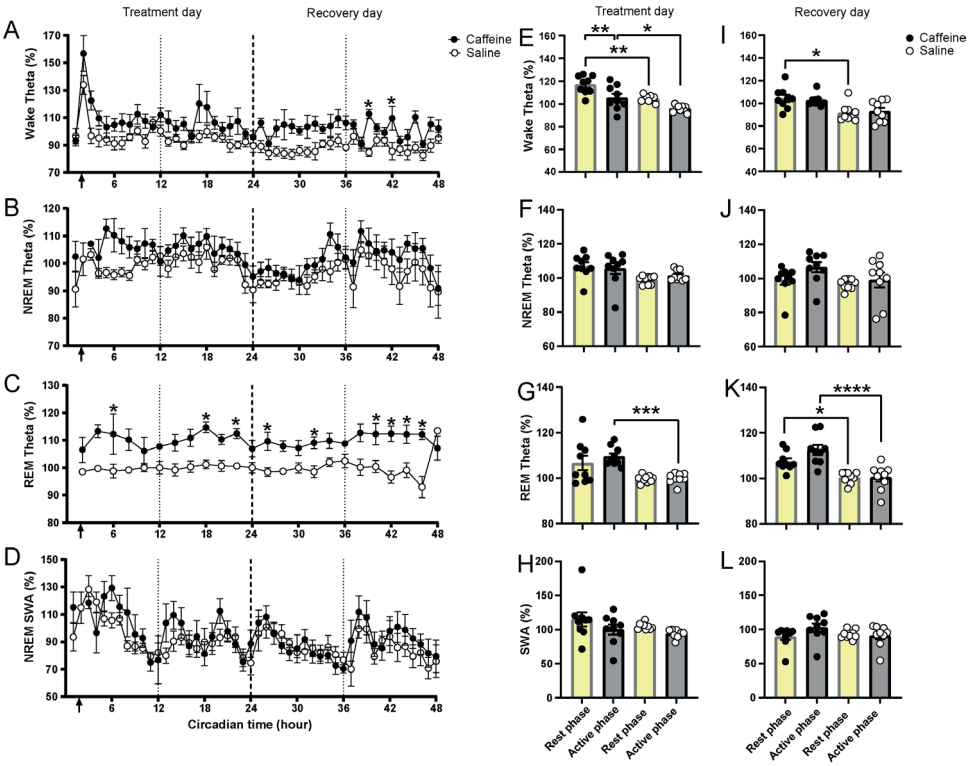


Figure 4. Effect of caffeine on power spectrum of wake, NREM sleep, and REM sleep. (**A**–**C**) Relative EEG power spectrum in waking, NREM sleep, and REM sleep during CT0–CT6 for caffeine (brown, $n = 9$) and saline (black, $n = 8$) administration. # indicates the influence of factor "treatment". Asterisk indicates significant differences between caffeine and saline administration (* $p = 0.05$–0.0001, Bonferroni multiple comparisons test after significant two-way ANOVA, factors "treatment" or interaction of "frequency" and "treatment"). (**D**–**F**) Peak frequency among 6.0–9.0 Hz in waking and REM sleep, and 0.5–2.5 Hz in NREM sleep during CT0–CT6 for caffeine (black, $n = 9$) and saline (white, $n = 8$) administration. Data are shown as mean ± SEM.

2.5. Effect of Caffeine on the Neural Activity in the Lateral Hypothalamus

Previously, it was found that an overall neuronal activation can be observed

immediately after caffeine administration, particularly in the cortex [26]. In the present data, we noticed that there was a long-term rise in the EEG theta power density, which is likely to be associated with increased synchronized activity in the hippocampus [37]. In addition, we observed changes in the amount of REM sleep over two days after treatment. It has been shown previously that 10 and 30 mg/kg caffeine can increase the activity of orexin neurons in LH [27]. This indicates that the LH might be influenced by acute caffeine. We therefore assessed the effect of acute caffeine on LH neuronal activity combined with the sleep–wake changes in our freely moving rats (Figure 5, location of the recording electrode in 5A). In these recordings, PLH electrical discharge rates were significantly higher during REM sleep compared to NREM sleep, both during subjective day and subjective night, but no day–night differences were observed (Figure 5B,C). Waking values were in between NREM and REM sleep discharge rates. After caffeine administration, PLH neural activity was increased in all three vigilance states (Figure 5D–F, Table 3). As no day–night fluctuations were observed, the neuronal activity after injection under waking, NREM, and REM sleep was averaged over the 47 h remaining for the recording. The neuronal activity during NREM sleep was significantly higher after caffeine administration (Figure 5H). A similar increase in neuronal activity during waking and REM sleep only showed a trend (Figure 5G,I). This indicates that the changes after a single application of caffeine in PLH neuronal activity also last for two days.

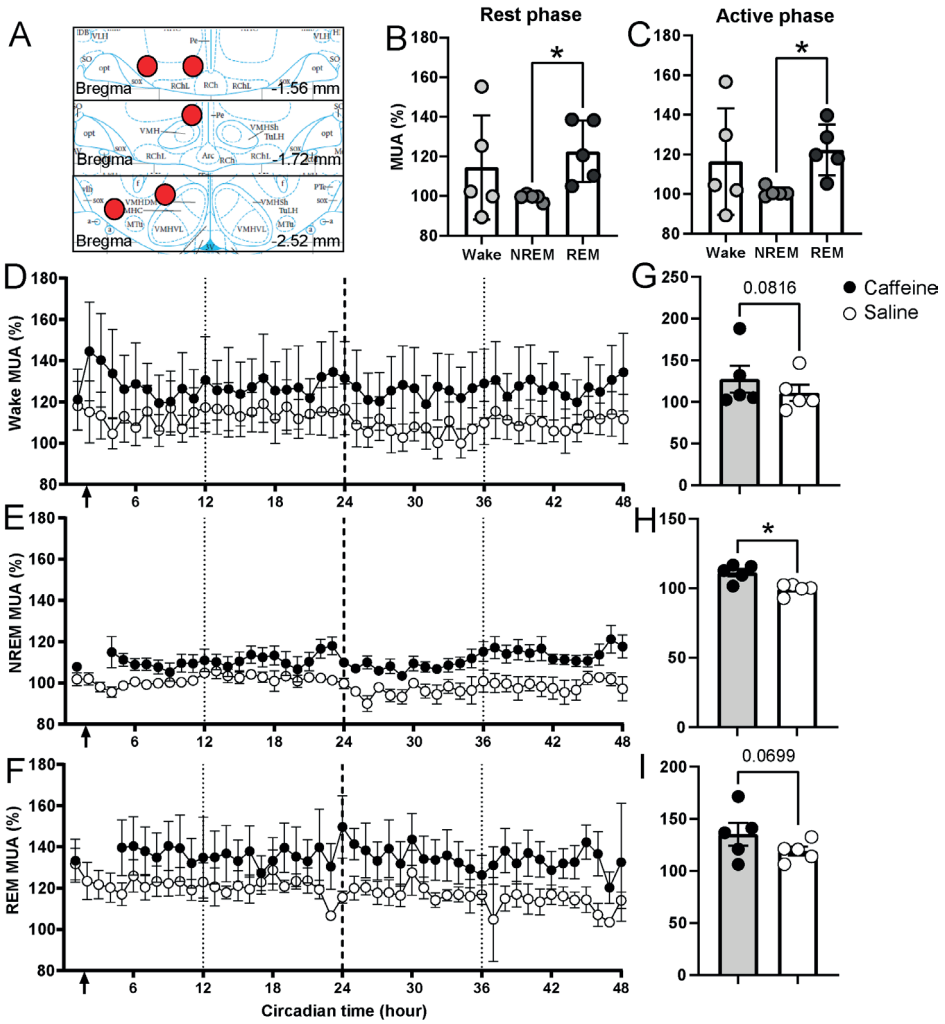


Figure 5. Electrical activity in lateral hypothalamus under caffeine and saline treatment over 48 h. The location of the electrodes in PLH is shown in (**A**). PLH activity under subjective day (**B**) and subjective night (**C**) under baseline condition (saline-treated). CT0-CT12 was considered as the subjective day, CT12-CT24 was considered as the subjective night. Asterisks indicate significant neuronal activity differences between NREM and REM states (* $p < 0.05$, paired *t*-test). (**D**–**F**) Time course of neuronal activity of PLH in waking, NREM, and REM sleep (2 h interval) for caffeine (black, $n = 5$) and saline (white, $n = 5$) administration in 48 h. The first 24 h was considered as treatment day; the second 24 h was considered as the recovery day. Arrow indicates the injection time (CT1-2). (**G**–**I**) Average value of 47 h of neuronal activity of PLH in waking, NREM, and REM sleep for caffeine (black, $n = 5$) and saline ($n = 5$). Asterisk indicates significant differences between caffeine and saline (* $p = 0.0251$, paired *t*-test). Data are shown as mean ± SEM.

Table 3. Detailed statistics for two-way ANOVA of neuronal activity under different sleep stages.

States	Time of day	Treatment	Interaction
Treating day			
Waking	ns[1]	P=0,0012	ns
NREM sleep	ns	P<0,0001	ns
REM sleep	ns	P<0,0001	ns
Recovery day			
Waking	ns	P<0,0001	ns
NREM sleep	ns	P<0,0001	ns
REM sleep	ns	P=0,0015	ns

[1] ns: not significant

3. Discussion

Caffeine is one of the most widely used stimulators, significantly promoting wakefulness. In the past decades, several researchers have documented the effects of caffeine on sleep and the circadian clock [6,38,39,40]. However, most of these studies were either short-term or did not exclude the influence of the light–dark cycle. In the present study, we tested the effects of caffeine, given 1 h after the onset of the endogenous rest phase, on vigilance states, the EEG, and neuronal activity in PLH over two days under constant dark conditions. Caffeine reduced NREM and REM sleep in the initial 3 h after the injection compared with saline, which is in accordance with previous findings in albino rats [1]. However, in addition, we found that caffeine influenced the occurrence of REM sleep for almost 36 h and REM sleep theta activity in the EEG for the whole recording duration (47 h). Correspondingly, neuronal activity in the PLH was increased during this same period. These findings confirm our idea that the effect of a single dose of caffeine may be longer than previously envisioned. We recorded the data in constant dark conditions, whereas previous experiments were all performed in a light–dark cycle. This may be why we were able to observe these long-term changes, and suggests that the influence of caffeine on sleep and circadian rhythms lasts longer in the absence of a light–dark cycle, or when light levels are low.

3.1. Acute Effect of Caffeine on Vigilance States

There are several candidate brain areas that are putative targets of caffeine to induce waking and activity. The administration of 10 mg/kg caffeine in rats leads to an acute and widespread increase in the rate of cerebral glucose utilization in the nucleus accumbens, both the shell and the core, as well as in most structures of the extrapyramidal motor system, and in many limbic

regions and cortices, which may decrease sleep. Furthermore, the basal forebrain and mesopontine tegmentum, which are arousal-promoting areas, are activated following systemic caffeine injection [41]. Caffeine may also inhibit the activation of sleep-promoting brain areas such as the ventrolateral preoptic nucleus and block the sleep-promoting adenosine receptor-mediated effects of adenosine [42,43].

In most previous studies, the effect of caffeine was examined under the influence of a light–dark cycle. As light is a strong zeitgeber for the sleep–wake cycle, the effect of caffeine on sleep will be influenced by light, or an interaction between caffeine and light may occur in these studies. Caffeine is known to significantly affect the amplitude of peripheral clocks [44]. To illuminate the effect of light, we kept our rats in constant dark conditions and administered caffeine at CT1. In our 48 h recording, caffeine treatment initially reduced sleep and increased locomotor activity, which is in line with previous sleep studies in rodents [1,45,46,47]. It is well known that the enhanced wakefulness and activity after caffeine administration is mainly caused by a blockage of adenosine receptors in the brain [6,48].

However, in addition, we observed that the amount of REM sleep was still reduced on the second day after caffeine administration. The pharmacokinetics of 15 mg/kg caffeine in rats is relatively well known. Plasma levels of caffeine are known to steadily decline, but caffeine is still elevated 8 h after application [1]. It is unlikely that levels are still high on the second day, 24 h after administration. However, on the second day after application, the amount and the circadian modulation of REM sleep were still reduced in our study. As REM sleep is under strong influence of the circadian clock [49], this may be due to a disturbance of the circadian clock by caffeine on the previous day or due to an influence of caffeine metabolites on the second day. Caffeine is known to phase delay, or slow down the circadian clock [21,22,24,40]. The circadian clock is synchronized to the normal 24 h rhythm by the external light–dark cycle. As the light–dark cycle was absent in our experiment, the effect of caffeine may be increased in our study, which may have helped to reveal the prolonged influence of caffeine or caffeine metabolites on REM sleep. These data suggest that a single dose of caffeine during the rest phase increases the voluntary movement and influences sleep architecture during the first half day and that minor but significant changes in sleep can still be present on the second day, particularly if light is absent or light levels are kept low.

3.2. Theta Activity in Different Sleep Stages

Another remarkable finding was that in the EEG of all the vigilance states, we found a specific increase in theta activity. This lasted for approximately 11–12 h in waking and NREM sleep and for more than 47 h in REM sleep. We ensured that the peak frequency did not become faster or slower after caffeine treatment and established that the increase was a genuine increase in power density in the same frequency range as the peak frequency in the control condition.

In rodents, it is well known that the theta waves in the cortical EEG during REM sleep and waking are mainly generated in the hippocampus [37]. These 6–9 Hz frequency waves have been associated with cognitive and memory performance, and voluntary movement [50,51]. Theta activity during waking was shown to be increased during sleep deprivation, which is probably due to increased activity and movement [52]. Reportedly, EEG theta oscillations during waking are an electrophysiological sign of locomotor activity and voluntary behavior in mice [53]. Due to the ability of caffeine to block the action of adenosine, caffeine has a stimulatory action on norepinephrine, dopamine, acetylcholine, serotonin, glutamate, and gamma-aminobutyric acid neurons, which can activate the motor circuit in the brain [54].

In our recordings, we found a consistent increase in theta activity in the two days after treatment. This was particularly strong in REM sleep. Both the reduction in circadian amplitude of REM sleep and the increased theta on the second day may indicate that caffeine, or its metabolites, have a particularly strong influence on REM sleep variables.

The peak frequency of the theta activity is slower in waking (~6.5 Hz) compared to REM sleep (~7.5 Hz) in our animal model, the Brown Norway rat. A recent study showed that increased theta activity in REM sleep was associated with high energy cost [55]. Caffeine, and possibly its metabolites, can regulate the metabolic rate and energy balance. Some studies show that caffeine can decrease body weight and increase energy expenditure [56,57]. This may explain the higher theta activity during REM sleep over 2 days; however, the underlying mechanism is not clear.

3.3. Activity in of the Lateral Hypothalamus

We recorded neuronal activity in different vigilance states in the PLH because previous research showed that orexin neurons in this area show increased c-fos expression after caffeine treatment [27]. We show that the activity differs between the different states, with high activity during REM sleep, intermediate activity during waking, and the lowest activity during NREM sleep. Remarkably, we did not observe a difference in activity between day and night, although in the past, it was shown that orexin (hypocretin) levels, which are dependent on neurons in the LH, show a circadian modulation [58,59]. In parallel with the increased theta in the cortex, we observed an increased neuronal firing rate in the PLH after caffeine treatment. This increase was most pronounced and significant during NREM sleep.

The hypothalamus is the central regulator of energy homeostasis in animals [60]. Caffeine administered at 10, 30, and 75 mg/kg in rats significantly increased c-fos immunoreactivity in dorsomedial and LH orexin neurons, which are thought to play an important role in arousal state control and energy expenditure [27,61,62]. An earlier study reported that caffeine promotes glutamate and histamine in the posterior hypothalamus (PH) [63]. A lower dose of caffeine may further activate the histaminergic neurons. Histamine maintains wakefulness through direct projections of PH histamine neurons to the thalamus and the cortex [63]. This suggests that the neural pathways in the hypothalamus are activated by caffeine, which may induce an increase in neural activity in the cortex, causing increases in locomotor activity, alertness, and energy expenditure. In this context, it is interesting that hypocretin receptors are also found in the hippocampus [64]. As the theta waves observed in the EEG are mainly generated in the hippocampus [37], this suggests that our finding of simultaneous increased activity in the PLH and increased theta activity in the cortical EEG may be connected to a common source.

4. Materials and Methods

4.1. Animals

Thirteen 12-week-old male Brown Norway rats (Charles River) were used in this study. Rats were group-housed under 12:12 light–dark conditions (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 (CCD, The Netherlands) and were carried out in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

4.2. Surgery

At a body weight of approximately 250 g (12 weeks of age), the animals were put under deep anesthesia with ketamine (Aescoket, Boxtel, The Netherlands; 65 mg/kg) and xylazine (Rompun, Bayer AG, Leverkusen, Germany; 13.3 mg/kg). The in vivo neuronal activity and EEG/EMG surgery techniques were used as described previously [35]. Two electrodes were aimed at the LH while the other electrode, with the insulation completely removed, was placed in the cortex for reference. For EEG, electrodes (Plastics One, Roanoke, VA, USA) were screwed through the skull on the dura over the right 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 on a plastic pedestal (Plastics One, Roanoke, VA, USA), which was fixed to the skull with dental cement and three additional support screws. The rats were allowed to recovered for at least seven days following the surgery under 12:12 LD conditions. After fully recovering from the surgery for at least seven days, the animals were connected to the recording system by a flexible cable and a counterbalanced swivel system, and they remained on the cable under constant darkness for at least one week before the start of the recording. The recording chamber was equipped with a passive infrared sensor and drinking sensor [35,36]. The animals' locomotor activity and drinking activity were recorded continuously to obtain an estimate of the circadian phase. From this, onset and offset of rest and activity were determined, and an F-periodogram analysis provided an estimate of circadian period. This enabled us to determine the time of treatment for the next recording day. All animals were free running, but with a minimal deviation from 24 h (mean 24.2 ± 0.04, range: 24.2–24.8, n = 13), which meant that we did not correct circadian phase over the two-day recording period.

4.3. Drug Treatment

Caffeine (Merck, C0750, Rahway, NJ, USA) was dissolved in 0.9% saline (LUMC Pharmacy, Leiden, The Netherlands) at a concentration of 15 mg/

mL. The concentration was used previously in several studies, and is known to significantly increase waking in rats and mice [1,65], as caffeine metabolism differs between rodents and humans; this dosage corresponds to ~5 mg/kg in humans, which is about 3 to 4 cups of coffee (each containing ~100 mg caffeine) [6]. Under constant darkness, the animals received either a caffeine (15 mg/kg) or saline (1 mL/kg) intraperitoneal injection under a randomized cross-over design at circadian time 1 (CT1). CT1 was determined by the clear on- and offset of locomotor activity (CT0) on the days before the injection. At least 3 circadian days were given between two treatments. On average, the time between treatments was 6 days (±0.6) with a range of 3–10 days.

4.4. EEG Data Acquisition

The EEG and EMG were simultaneously and continuously recorded for 48 h, as previously described [35]. The signals were amplified (amplification factor ~2000), bandpass-filtered (EEG: 0.5–30.0 Hz, −40 dB/decade; EMG 15.0–40.0 Hz, −40 dB/decade), digitized (sampling rate 100 Hz) in 10 s epochs, and automatically stored on a hard disk (Spike2, Power1401, CED, Cambridge, UK). A fast Fourier transformation routine with a 10 s window was performed offline (MATLAB, The MathWorks Inc., Natick, MA, USA) to compute EEG power density spectra within the frequency range 0.1–25.0 Hz. PLH/LA neuronal activity was recorded online (amplification factor ~50,000, bandpass-filtered between 500 and 5000 Hz, −40 dB/decade). A window discriminator converted the recorded action potentials to electronic pulses. A second window discriminator was set at a higher level to be able to exclude artifacts caused by the animal's movements. The EEG and EMG were continuously recorded and amplified (amplification factor ~2000), bandpass-filtered (between 0.5 and 30 Hz, −40 dB/decade), and subjected to analog-to-digital conversion (sampling rate 100 Hz). All data were recorded simultaneously in 10 s epochs.

4.5. 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 [35,66]. Waking, NREM, and REM sleep were determined, and artifacts were excluded for power spectral analysis. In three animals, the recording after the control injection did not last the full two days. Therefore, we had

10 animals in the control condition for the calculation of the vigilance states. Due to problems with the quality of the recordings, one animal did not contribute to the EEG spectral analysis data. Since for the spectral analysis, it was necessary to re-calculate power density values relative to the first day of the control condition, complete and clean recordings were needed from all animals for both conditions to enter the analysis. Unfortunately, this was not possible in one animal, which was therefore excluded from the analysis of the EEG power density spectra (n = 9 left). To investigate the effect of caffeine on EEG power density of different vigilance states, we analyzed the EEG power density in the slow-wave range (SWA, 1.0–4.0 Hz) in NREM sleep, and theta range (6.0–9.0 Hz) in waking, NREM, and REM sleep, as described previously [35]. The average amount of the vigilance states (waking, NREM sleep, REM sleep, and REM sleep per total sleep time) and EEG spectral data (NREM-SWA, theta) were analyzed in 1 h intervals and 12 h intervals over 48 h in both caffeine and saline treatment. The locomotor activity was collected in 10 s intervals, and was further averaged in 1 h and 12 h intervals. Spectral analysis was performed using fast Fourier transform (FFT; 0.1–25 Hz, 0.1 Hz resolution); the relative power density spectra of waking and REM sleep in caffeine and saline day (CT0-CT6) were analyzed. The peak frequency of waking (theta range) and REM sleep was determined in hourly values in the same 6 h. Sleep–wake state episodes were determined with an algorithm described previously [67]. Episodes of each vigilance state were partitioned into a maximum of ten bins with the exponentially increased duration from 10 s to >2560 s. All PLH neuronal activity and EEG power density data were standardized relative to the mean 24 h saline control injection day in NREM sleep. This enabled the calculation of mean values for the rats which had histological verification of the electrode recording site in the PLH area (n = 5).

4.6. Histology

After the recording, the mice were sacrificed in a CO_2 chamber, and a small electrolytic current (1.2 mA, 10 s) was passed through the two twisted electrodes to mark their positions. The brains were collected and kept in a 4% paraformaldehyde solution containing ferrocyanide to fix the brain tissue and to stain the recording site. After fixation, the brain was transferred into 30% sucrose and sectioned coronally and stained with cresyl violet. The marked position of the electrode was verified by microscopic inspection.

4.7. Statistics

For data analysis, Prism 8 software (Graphpad Inc., La Jolla, CA, USA). was used. Two-way ANOVA with Bonferroni multiple comparisons was used to compare the effects of treatment across time, the effects of treatment across different EEG power frequencies, and the effects of treatment across different episode durations. Detailed statistics of two-way ANOVA are shown in Table 1, Table 2 and Table 3. Paired *t*-tests were used for circadian 12 h intervals (rest or active phase) of multi-unit activity under different vigilance states. Significant differences between conditions are represented in the graphs as follow: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

5. Conclusions

In summary, we found that a single dose of caffeine not only increases waking and decreases sleep in the short term (first 3 h after administration), but also influences REM sleep and theta activity in REM sleep on the following day. In addition, neuronal activity in PLH was increased for approximately two days, particularly in NREM sleep. One of the limitations of our studies is that we only performed these recordings in male rats and therefore cannot determine whether similar changes occur in females. A limitation of the recordings of neuronal activity in the PLH is that we only recorded from a small number of animals ($n = 5$), and that we were unable to determine the type of neurons from which we recorded spiking activity. Our recordings were performed in constant darkness, which may have increased the opportunity to observe these long-term effects, as the light–dark cycle may reduce the influence of the circadian clock and reduce the effect of caffeine. The latter could also be of importance in humans. Considering the interactive effects of light and caffeine on the sleep and circadian rhythms, it may be important to consider lighting levels during the day when investigating the effects of caffeine in humans.

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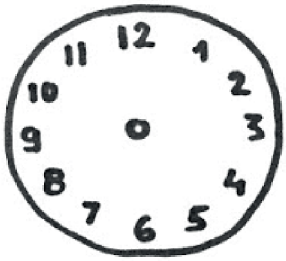
3

Comparison of sleep deprivation and a low dose of ketamine on sleep and the electroencephalogram of Brown Norway rats



Yumeng Wang, Marije Melgers, Johanna H Meijer, Tom Deboer. Comparison of sleep deprivation and a low dose of ketamine on sleep and the electroencephalogram of Brown Norway rats. *J Sleep Res. 2023 Feb 20:e13863.*

Abstract:

Ketamine is known for its antidepressive effects, but the mechanism underlying this effect remains largely unclear. In contrast to most antidepressive drugs, the action of ketamine is rapid, suggesting a different mode of action. A rapid antidepressive effect is also observed following sleep deprivation. Here we aim to evaluate the effect of a six-hour sleep deprivation (SD) and acute ketamine treatment on vigilance states, locomotor activity and electroencephalogram (EEG) power density spectra in Brown Norway rats under constant condition over two recording days. After SD and after the initial waking period induced by ketamine, both treatments induced a similar increase in non-rapid eye movement (NREM) sleep and EEG slow wave activity in NREM sleep. Rapid eye movement (REM) sleep was reduced immediately after both treatments, but was recovered later only after the sleep deprivation. The effects on the waking EEG differed between the treatments, with a faster theta peak during and after sleep deprivation, and no change in the waking spectrum after ketamine. In conclusion, sleep deprivation and ketamine both lead to an acute increment in NREM sleep slow wave activity as well as in a reduction in REM sleep. The results suggest that selective suppression of REM sleep, combined with enhancement of slow wave activity during NREM may be effective in the treatment of depression.

Key Words: sleep, sleep deprivation, ketamine, EEG, power spectrum, slow-wave

1. Introduction

Sleep quality is thought to be important in learning and memory, cognitive functioning, and mood, and disturbed sleep is a well-known risk factor in mood disorders [1,2]. Sleep abnormalities are commonly associated with psychiatric disorders such as major depressive disorder and bipolar depression disorder, and increasing evidence shows that chronic insufficient sleep may increase the risk of developing depression [2,3]. Mood disorders like depression are associated with altered sleep architecture [3]. For example, depression is related to a shorter sleep latency, increased rapid-eye movement (REM) sleep duration and REM density and also decreased non-REM (NREM) sleep [4,5]. NREM sleep and the electroencephalographic (EEG) slow-waves (EEG frequencies below 5 Hz) occurring during that sleep are considered to reflect homeostatic sleep pressure [6-8]. It has been suggested that impaired homeostatic regulation of SWA is associated with emotional dysregulation [9]. Although the mechanism of the bi-directional link between sleep and emotions is complex and not well understood, it still renders sleep a therapeutic target of antidepressant treatment.

Sleep deprivation (SD) and low dosages of the N-methyl-D-aspartate receptor (NMDAR) antagonist ketamine are two rapid-acting antidepressant treatment strategies that also influence sleep and the sleep EEG. However, little is known about the effects of ketamine on sleep regulation [10]. Ketamine has a short plasma elimination half-life but can relieve depression symptoms in humans both acutely and on the following days [11]. Total sleep deprivation is another rapid acting therapy for depression, which alleviates symptoms of depression in nearly 60% of depressed patients within hours [12]. Preclinical studies showed that ketamine induced a fast antidepressant effect in rats, 24 or 1 h prior to a forced swim test [13]. Similar to the effect of ketamine, mice and rats showed decreased immobility in the forced swim test after 12 or 24 hours of sleep deprivation [14,15]. Although the positive effect of sleep deprivation on depression is short-lasting, the rapid antidepressant effect can still be explored and compared with other rapid antidepressants to help us understand the mechanism mediating this action.

To our surprise, the effect of sleep, which showed the most pronounced changes during SD and ketamine treatment, is ignored in 80% of the papers on ketamine, SD, and mood [10]. The aim of this study was to assess to what extent ketamine and SD affect sleep, in both the acute sleep disrupted period

and in the long term (2 days), to establish similarities which may be relevant for the antidepressant effect in these two treatments. We observe similar changes in sleep and SWA in NREM sleep immediately after both treatments, and that the main differences between the two treatments lie in their effects on waking behavior and the EEG power density spectrum during waking in the initial phase of the two treatments. We also observed a long-lasting effect of the EEG power spectrum on the second recovery day in both treatments, and that REM sleep lost in the initial phase is only recovered following SD, but not after ketamine.

2. Methods

2.1 Animals

Twenty twelve-weeks old male Brown Norway rats (Charles River) were used in this study. Rats were group-housed under 12 h:12 h light-dark (LD) condition (Lights on 8:00 lights off 20:00) with food and water ad libitum in a temperature-controlled room (21–22 °C). During the experiments the animals were in constant darkness, while other conditions (food, water, temperature) remained the same. All animal experiments were approved by the Central Committee on Animals Research (CCD, 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 grams (16 weeks of age), the animals were put under deep anesthesia with ketamine (Aescoket, Boxtel, the Netherlands; 65 mg/kg) and Xylazine (Rompun, Bayer AG, Leverkusen, Germany; 13.3 mg/kg) [16]. The EEG/EMG surgery techniques were used as described previously [16-18]. 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, Roanoke, VA) 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 seven days in home cage under

12h:12h 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 on the cable under constant darkness in the recording chamber for at least one week which can let the animal familiar with the environment and used to sleep with the cable before the start of the recording. The experiments were performed under constant dark conditions because ketamine may influence the circadian clock [19], and we wanted to make sure that we would not miss this effect if it occurred. The recording chamber was equipped with a passive infrared (PIR) sensor to record locomotor activity and a sensor to record drinking behavior [16-18]. The animals' locomotor activity and drinking activity were recorded continuously to obtain an estimate of the circadian phase (Supplemental Figure S1). From this, onset and offset of rest and activity were determined and an F-periodogram analysis provided an estimate of the circadian period [20] (Supplemental Figure S1 c). This enabled us to determine the time of SD and ketamine treatment for the next recording day and to determine whether SD or ketamine influenced the circadian timing of behavior.

2.3 Experimental design

The animals were divided into two groups, one group (n = 10) received a 6-h SD, starting at the onset of the rest period, and the other group (n =10) received ketamine and saline within one hour after the start of the rest phase in a randomized cross-over design experiment. This allowed us to get rid of the confounded results from SD and ketamine/saline treatment in the same animal.

During the 6-h SD, the animals (n=10) were observed with a dim red light in addition to the online EEG recording. Whenever the animals appeared drowsy or the EEG exhibited slow-waves, they were mildly disturbed by moderate noise, by the experimenter opening the cages and putting some novel objects inside, and, if necessary, by introducing fresh food, water, or nesting material into the cage [18,21]. The animals were never touched during the SD and were not disturbed during feeding and drinking.

Ketamine (Aescoket, Boxtel, the Netherlands) was dissolved in 0.9% saline (LUMC Pharmacy) at a concentration of 25mg/ml. The concentration was used previously in several studies and is known to induce an antidepressant-like effect accompanied by a moderate increase in activity in both naive and

stressed rats [13,22]. Under constant darkness at circadian time 1 (CT1), 1 hour after the predicted offset of locomotor activity, the animals (n = 10) received either ketamine (25mg/kg) or saline at a volume of 1ml/kg body weight in an intraperitoneal injection under a randomized cross-over design. At least three days of rest were given between injections.

2.4 EEG data acquisition and EEG power spectrum analysis

The EEG and EMG were simultaneously and continuously recorded for 48 h (after saline and ketamine treatment) and 72 h (before, during and after SD) as previously described [16-18]. The EEG and EMG signals were continuously recorded and amplified (amplification factor ~ 2000) and 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) in 10-sec epochs and automatically stored on hard disk (Spike2, Power1401, CED, Cambridge, UK). A fast Fourier transformation routine with a 10-sec window was performed offline (MATLAB, The MathWorks Inc., Natick, MA) to compute EEG power density spectra within the frequency range 0.1-25.0 Hz in 0.1 Hz resolution. All data were recorded simultaneously in ten-second epochs. Since, for the spectral analysis, it was necessary to re-calculate power density values relative to the first day of the saline control or baseline (first 24-h of the 72-h recording period of the SD experiment) condition, complete and clean recordings from all animals for all days are needed to enter the analysis. Due to problems with the quality of the recordings, two animals from the SD group and one animal from ketamine group did not contribute to the SWA and EEG spectral analysis data. The waking, NREM and REM sleep power spectrum were calculated relative to either the baseline (for the SD experiment) or saline (for the ketamine experiment) NREM sleep power spectrum.

2.5 Data analysis

Three vigilance states (waking, NREM and REM sleep) were scored offline in 10-sec epochs. The manual scoring of vigilance states based on the EEG and EMG recordings was performed according to standardized criteria for rats [16-18]. Vigilance states were determined, and artifacts were excluded for power spectral analysis. 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 and 6-h intervals over 48-h and 72-h. The locomotor activity was collected in ten-second intervals and was further averaged in 1-h and

6 - h intervals. To investigate the effect of SD and ketamine administration 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 [16,17]. The 1-h and 6-h values of SWA in NREM sleep were expressed relative to the first 24-h of the saline or baseline day (=100%). The peak frequency and power density of theta in waking, REM sleep (6.0 – 9.0 Hz) and slow-waves in NREM sleep (1.0 – 4.0 Hz) was determined manually in the relative power density spectra in 0.1 Hz bins.

2.6 Statistics

For data analysis, GraphPad was used. A two-way repeated measures ANOVA was used to compare the effect of SD across time, EEG frequencies, and treatment conditions. Two-way ANOVA was used to compare the effect of drug across time, EEG frequencies, and treatment conditions. Two way ANOVA was used to compare the effect of drug and SD on EEG peak frequency and power density in this peak. In some cases, where missing values occurred in REM sleep / total sleep and SWA in NREM sleep, a mixed-effects model (REML) was used to compare the effect of SD or ketamine across circadian time. If the result was significant, we ran post hoc Bonferroni multiple comparisons corrected t-test, and the significant time points and EEG frequencies are reported in the results. The associated F-statistic is reported in all ANOVA tests. Values of $p < 0.05$ were considered statistically significant.

3 Results

3.1 Effect of sleep deprivation on vigilance states, slow-wave activity in NREM sleep, and locomotor activity.

The 6-h SD affected sleep and waking over the following 18-h. A clear decrease in waking and increases in NREM and REM sleep were observed (Fig.1 a - c, circadian time x SD interaction, $F_{71, 1278} = 4.942$, $F_{71, 1278} = 4.905$, $F_{71, 1278} = 3.623$ for waking, NREM sleep and REM sleep respectively, $p < 0.0001$). The first 5 - 6 hours after SD a clear decrease in waking and an increase in NREM sleep were seen (Fig. 1 a, $p_{CT11} = 0.0192$; Fig. 1 b, $p_{CT8} = 0.035$, $p_{CT11} = 0.0093$) and an increase in REM sleep was observed a few hours later (Fig 1 c, $p_{CT14} = 0.0481$). The decrease in waking and increase in NREM sleep were reflected in the first two 6-h values after SD (Fig 1 g, time x SD interaction, $F_{3, 54} = 207.0$, $p < 0.0001$, $p_{CT6-CT12} < 0.0001$, $p_{CT12-CT18} < 0.0001$, Fig.

1 h, $F_{3, 54} = 206.7$, $p < 0.0001$, $p_{CT6 - CT12} < 0.0001$, $p_{CT12 - CT18} < 0.0001$). The effects of SD on REM sleep started and ended later than the effects on NREM sleep and waking. The 6-h REM sleep values were increased 12-24 hours after the end of the SD (Fig.1 i, time x SD interaction, $F_{3, 54} = 109.0$, $p < 0.0001$, $p_{CT12 - CT18} < 0.0001$, $p_{CT18 - CT24} = 0.0015$). REM/total sleep in the first 6 hours after the SD was also lower compared to baseline (Fig. 1 j, SD, $F_{1, 36} = 61.38$, $p < 0.0001$, $p_{CT6 - CT12} < 0.0001$) which was due to the large increase in the amount of NREM sleep. The following active period after the SD (CT12 – CT24) showed increased REM/total sleep (Fig, 1 j, SD, $F_{1, 18} = 20.43$, $p = 0.0003$, time x SD interaction, $F_{3, 54} = 126.5$, $p < 0.0001$, $p_{CT12 - CT18} = 0.0004$, $p_{CT18 - CT24} = 0.0039$). PIR locomotor activity showed clear modulations across the day (Fig. 1 e, $F_{71, 1278} = 6.223$, $p < 0.0001$), but there was no difference in locomotor activity found after SD compared to baseline in the post hoc analysis.

SWA in NREM sleep showed a clear increase immediately after the SD, followed by a declining curve that continued for at least 6 hours (Fig. 1 f, n = 8, circadian time x SD interaction, F65, 887 = 8.324, $p < 0.0001$, $p_{CT7 - CT12} < 0.05$). Surprisingly, the animals showed an undershoot in SWA with an average of 79.83% on the second day after SD, compared with baseline condition with an average of 107.6% (Fig. 1 f, $p_{CT3} = 0.0101$ on recovery day; Figure S2, SD, $F_{3, 42} = 18,74$, $p < 0.0001$, $p_{CT0 - CT6} < 0.0001$). No effects of SD on the circadian regulation of locomotor activity, or the timing of vigilance states were found.

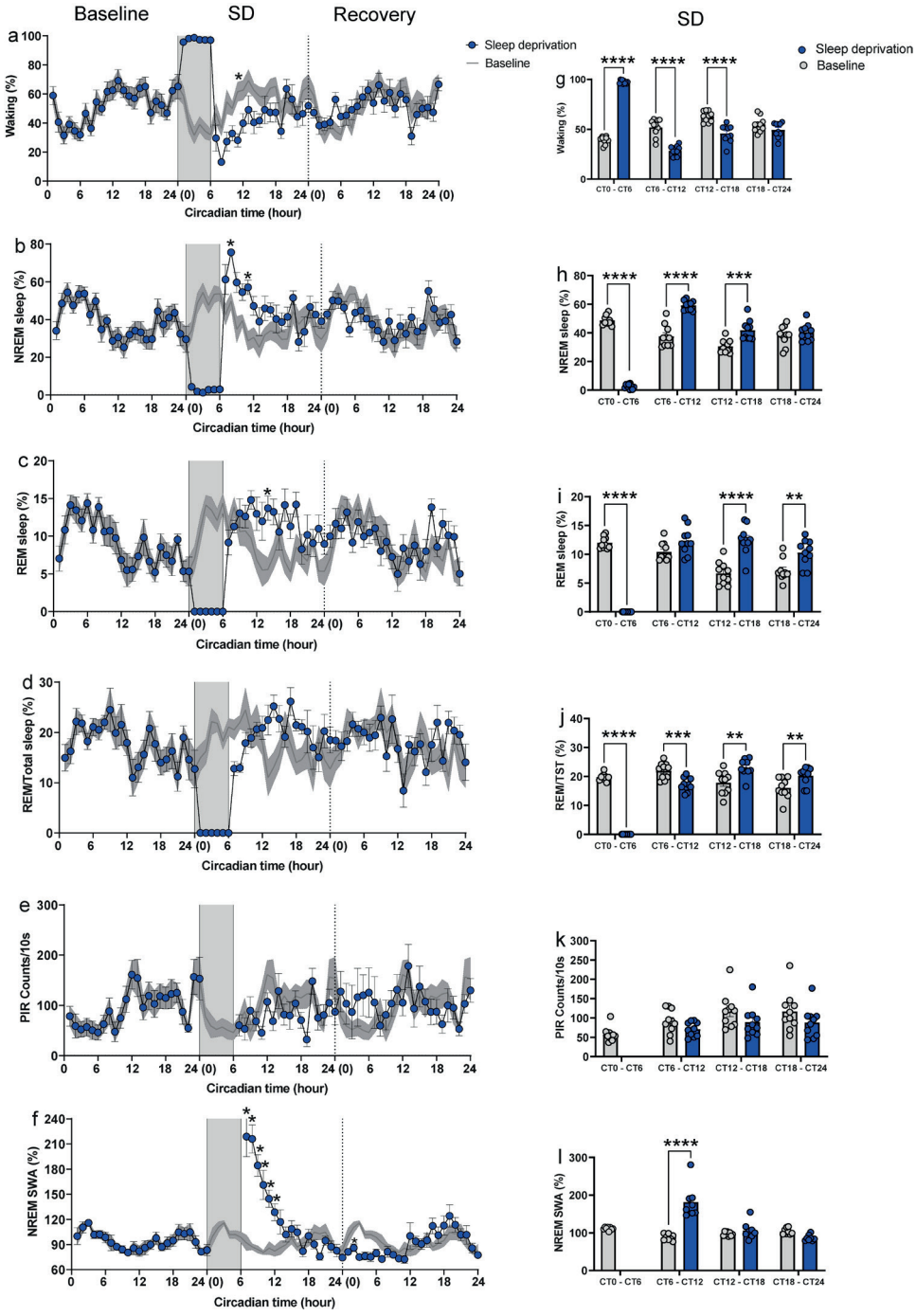


Figure 1. Vigilant stages, locomotor activity and slow-wave activity in NREM sleep of sleep deprivation over 72 hours. a – f. Time course of waking, NREM sleep, REM sleep, REM sleep/ Total sleep time, locomotor activity, and slow-wave activity (SWA) in NREM

sleep in 1-h values for sleep deprivation (n = 10 animals, n=8 for SWA) in 72 hours. The first 24-h was considered as baseline day, the second day was the sleep deprivation day, the third day was the recovery day. The baseline is repeated in the background in dark grey. The light grey area indicates the sleep deprivation period. SWA in NREM sleep is expressed relative to the average NREM sleep SWA during the first 24 hours (=100%). * $p < 0.05$. g – i. Six-hour values of waking, NREM sleep, REM sleep, REM sleep/ total sleep, locomotor activity, and SWA in NREM sleep for the sleep deprivation (blue, n = 10, n = 8 animals for SWA analysis) and baseline (grey, n = 10, n = 8 animals for SWA analysis) condition over 24 hours. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Data is shown as mean ± SEM.

3.2 Effect of acute ketamine on vigilance states, slow-wave activity in NREM sleep and locomotor activity.

Ketamine compared with vehicle, acutely increased waking and reduced NREM and REM sleep for approximately 2 hours (Fig. 2 a, drug, $F_{1, 864} = 8.780$, $p = 0.0031$; circadian time x drug interaction, $F_{47, 864} = 2.012$, $p < 0.0001$; $p_{CT2} = 0.0046$, $p_{CT3} < 0.0001$;Fig.2 b, circadian time x drug interaction, $F_{47, 864} = 2.220$, $p < 0.0001$, $p_{CT2} = 0.0014$, $p_{CT3} < 0.0001$;Fig.2 c, circadian time x drug interaction, $F_{47, 864} = 1.505$, $p = 0.0172$; $p_{CT3} = 0.0018$; Fig. 2 d, circadian time x drug interaction, $F_{47, 844} = 1.580$, $p = 0.0088$). All vigilance state 1-h values subsequently returned to baseline and no differences with vehicle were observed thereafter in any of the vigilance states (Fig 2 a-d).

The total amount of NREM sleep over the first 6 hours of the day of ketamine treatment did not differ significantly from vehicle (Fig. 2 h, time x drug interaction, $F_{3, 72} = 2.710$, $p = 0.0513$), but REM sleep was significantly reduced (Fig. 2 i, time x drug interaction, $F_{3, 72} = 4.721$, $p = 0.0046$, $p_{CT0 - CT6} = 0.0003$), and waking was enhanced (Fig. 2 g, time x drug interaction, $F_{3, 72} = 4.043$, $p = 0.0103$, $p_{CT0\text{-}CT6} = 0.0052$). As can be seen in the hourly values, the acute waking effect of ketamine lasts for two hours after which sleep is initiated. Although the first 6 hours of NREM sleep did not show notable differences between ketamine and saline, NREM sleep was significantly increased (62.24 ± 2.58 %) compared with saline administration (46.61 ± 2.12 %) during the second half of that six hours (CT4-CT6, paired t-test, $p = 0.0013$), when the main increase in sleep and SWA was found. Because of the reduction in REM sleep, REM/total sleep was also lower in the first 6-h interval after ketamine compared with vehicle (Fig.2 j, drug, F1, 72 = 4.010, p=0.0490, pCT0 – CT6 = 0.0084). In contrast to NREM sleep, REM sleep did not show big differences (9.44 ± 0.93 %) compared with saline administration (11.56 ± 0.83 %) during CT 4 – CT 6 (paired t-test, $p = 0.0551$), but due to

the increase of NREM sleep, REM/TST was decreased significantly (13.84 ± 1.4 %) compared with saline (18.66 ± 1.3 %) during CT 4 – CT 6 (paired t -test, $p = 0.0145$). REM sleep and REM/total sleep did not show a rebound in the following hours. Locomotor activity was increased for approximately one hour after ketamine administration compared with vehicle (Fig.2 e, circadian time x drug interaction, $F_{47, 694} = 1.927$, $p = 0.0003$, $p_{CT2} < 0.0001$).

SWA in NREM sleep after ketamine administration was increased considerably and significantly during CT4 and CT5 (Fig. 2 f, n = 9, drug, $F_{1, 721} = 18.96$, $p < 0.0001$, time x drug interaction, $F_{46, 721} = 1,543$, $p = 0.0135$, $p_{CT4} = 0.0006$, $p_{CT5} < 0.0001$). This was followed by a gradual decline in SWA, similar to the decline seen after SD. However, the undershoot in SWA we observed on the second day after the SD did not occur after ketamine treatment. There was no significant difference on the second day after the ketamine treatment in vigilance states, locomotor activity and SWA compared with vehicle (Fig. 2 a - f). No effects of ketamine on the circadian regulation of locomotor activity, or the timing of vigilance states were found.

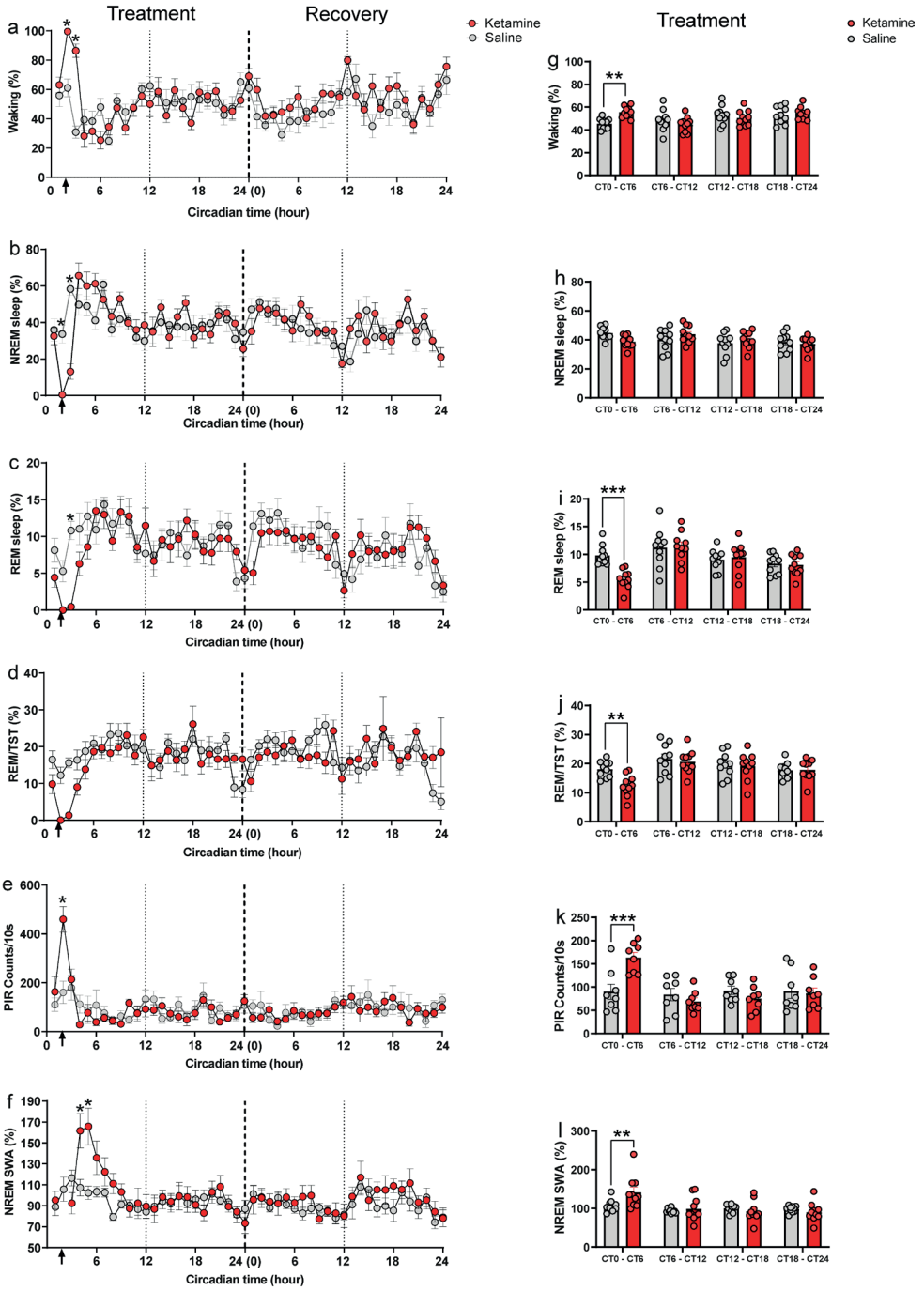


Figure 2. Vigilant stages, locomotor activity and slow-wave activity in NREM sleep after ketamine and saline treatment. a – f. Time course of waking, NREM sleep, REM sleep, REM sleep/ Total sleep time, locomotor activity and slow-wave activity (SWA) in NREM

sleep in 1-h values for ketamine and saline administration (n = 10 animals, n=9 for SWA) in 48 hours. The first 24-h was considered as treatment or control day, the second day was the recovery day. SWA in NREM sleep is expressed relative to the average NREM sleep SWA during the first 24 hours of the saline control day (=100%). * $p < 0.05$. g – i. Six-hour values of waking, NREM sleep, REM sleep, REM sleep/ total sleep, locomotor activity and SWA in NREM sleep for ketamine administration (red, n = 10, n = 9 for SWA) and saline (grey, n = 10, n = 9 for SWA) administration on the treatment day. The arrow indicates the injection time (CT1). ** $p < 0.01$, *** $p < 0.001$. Data is shown as mean ± SEM.

3.3 Effect of ketamine and sleep deprivation on the waking EEG power density spectrum

To compare the effect of ketamine and SD on the waking EEG power density spectrum, we analyzed the EEG power spectrum of the most active two hours (CT1 – CT3) after ketamine administration, which were mainly spend in waking, and the corresponding two hours during the SD. The waking power spectrum was examined to determine similarities and differences between waking induced brain activity during SD and waking induced brain activity immediately after ketamine treatment. Interestingly, the waking spectrum differed considerably between the two. Power density was increased around 7.7 Hz during the SD but not during waking after the ketamine administration (Fig.3 a, n = 8, frequency x SD interaction, $F_{249, 1750} = 1.468$, $p < 0.0001$, $p_{0.3 – 0.4\ Hz} < 0.0006$, $p_{7.4 – 7.9\ Hz} < 0.05$; Fig. 3 b, n = 9, drug, $F_{1, 3500} = 74.39$, $p < 0.0001$, frequency x drug interaction, $F_{249, 3500} = 1.253$, $p = 0.0056$). The peak frequencies differ significantly between SD baseline and saline control, which is probably due to the handling of the animals in the saline condition, which did not happen in the SD baseline condition. Concentrating on the theta peak of waking, we found that the peak frequency during SD was faster (7.5 ± 0.1 Hz) compared to baseline (6.1 ± 0.1 Hz), but that the power density, in the theta peak, when corrected for peak frequency, was not significantly different. Theta peak frequency and amplitude during waking after ketamine administration (6.9 ± 0.2 Hz), did not differ from control (6.7 ± 0.2 Hz) (Fig.3 c, treatment x group interaction, $F_{1, 30} = 21.82$, $p < 0.0001$, $p_{baseline\ vs.\ SD} < 0.0001$, $p_{baseline\ vs.\ saline} = 0.0273$, $p_{SD\ vs.\ ketamine} = 0.008$; Fig.3 d, treatment x group interaction, $F_{1, 30} = 0.9840$, ns).

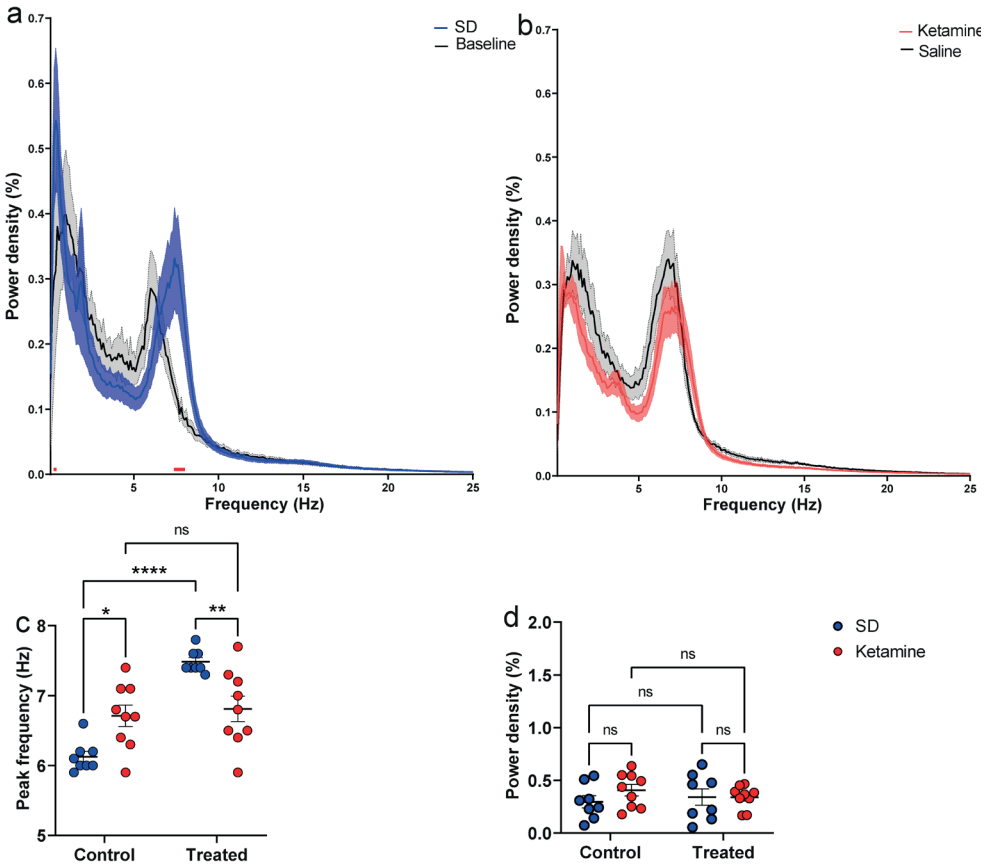


Figure 3. Acute effect of sleep deprivation and ketamine administration on the power density spectrum of the waking EEG. a – b. Relative EEG power spectrum in waking during CT 1 – CT 3 for sleep deprivation (blue, n = 8) and baseline (black, n=8) and after ketamine (red, n = 9) administration or saline (black, n = 9). The red bar indicates significant differences between sleep deprivation and baseline ($p < 0.05$). c. Peak frequency between 6.0 – 9.0 Hz in waking during CT 1 – CT 3 for sleep deprivation (blue, n = 8) and ketamine (red, n = 9) administration. d. Power density of the peak frequency from C in waking during CT 1 – CT 3 for sleep deprivation (blue, n = 8) and ketamine (red, n = 9) administration. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, ns: not significant. Data is shown as mean ± SEM.

3.4 Effect of ketamine and sleep deprivation on EEG sleep power spectrum during the early recovery period

Six hours of SD resulted in an increase in sleep and similar results were obtained after the initial 2-h waking period (CT1-CT3) induced by the ketamine administration. To further compare these two treatments, cortical EEG power density spectra were compared between CT6 – CT9 of the baseline

and SD condition, and CT3 – CT6 of vehicle and ketamine administration, which were the times where both treatments gave the highest amount of NREM sleep and the highest SWA in NREM sleep values after treatment. The power spectra in NREM sleep after SD and ketamine both showed the typical increase in EEG SWA (Fig. 4 a, frequency x SD interaction, $F_{249, 1750}$ = 212.9, p < 0,0001, SD, F1, 17500 = 6763, p < 0.0001, $p_{0.6 - 4.8\ Hz}$ < 0.05; Fig. 4 d, frequency x drug interaction, $F_{249, 4000}$ = 13.22, p < 0.0001, drug, $F_{1, 4000}$ = 455.4, p < 0.0001, $p_{0.6 - 3.2\ Hz}$ < 0.05).

Concentrating on the peak frequency and power density of the slow-wave activity peak in NREM sleep, we found that slow-wave power density was increased in the first hours after the SD (7.60 ± 0.31 %) compared with baseline (3.33 ± 0.13 %) (Fig. 4 j, treatment x group interaction, $F_{1, 30}$ = 16.37, p = 0.0003, pbaseline vs. SD < 0.0001), but also that the slow-waves were faster compared to baseline (Fig. 4 g, SD: 1.8 ± 0.1 Hz, baseline: 1.5 ± 0.1 Hz, treatment x group interaction, $F_{1, 30}$ = 5.678, p = 0.0104, , pbaseline vs. SD = 0.0237). Compared to the ketamine treatment the slow-wave peak frequency after SD was also faster and had a larger power density (Fig. 4 g, $p_{SD\ vs.\ ketamine}$ = 0.0187; Fig. 4 j, $p_{SD\ vs.\ ketamine}$ < 0.0001). As a control, we compared the spectra between the baseline day of SD and the saline control day and found no significant differences between those two (Fig. 4 g and j).

In addition, we compared the power density spectra in waking and REM sleep between the two treatments. Notably, after SD, during waking, the animals still displayed higher power density around 7.2 Hz (Fig. 4 b, SD, $F_{1, 1750}$ = 50.48, p < 0.0001, $p_{6.6 - 7.9\ Hz}$ < 0.05). The statistical analysis showed a main effect of drug (Fig. 4 e, drug, $F_{1, 4000}$ = 15.51, p < 0.0001) on the waking spectrum after ketamine administration, but the post hoc analysis did not show large differences in individual frequency bins. When comparing theta peak frequency and power density, no significant differences between ketamine and SD were found for theta peak power density during waking after the animals initiated sleep (Fig. 4 k, treatment x group interaction, F1,30 = 0.6253, ns). In the waking power spectrum, after SD, the theta peak frequency was faster compared to baseline (Fig. 4 h, Baseline: 6.3 ± 0.1 Hz, SD: 7.1 ± 0.1 Hz, treatment x group interaction, $F_{1, 30}$ = 25.16, p < 0.0001, pbaseline vs. SD < 0.0001). Ketamine treatment did not change the waking peak frequency, and the waking theta peak after SD was faster compared to that after ketamine administration (Fig. 4 h, $p_{SD\ vs.\ ketamine}$ < 0.0001).

There was a significant effect of SD on the REM sleep EEG power spectrum (Fig. 4 c, frequency x SD interaction, $F_{249,1750} = 2.031$, $p < 0.0001$, $p_{6.1-7.7\ Hz} < 0.05$), and we also found a main effect of ketamine on the REM sleep power spectrum (Fig. 4 f, drug, $F_{1,4000} = 7.660$, $p = 0.0057$), similar to waking. No significant differences between ketamine and SD were found for both theta peak frequency and theta power density in the REM sleep EEG (Fig. 4 i and l).

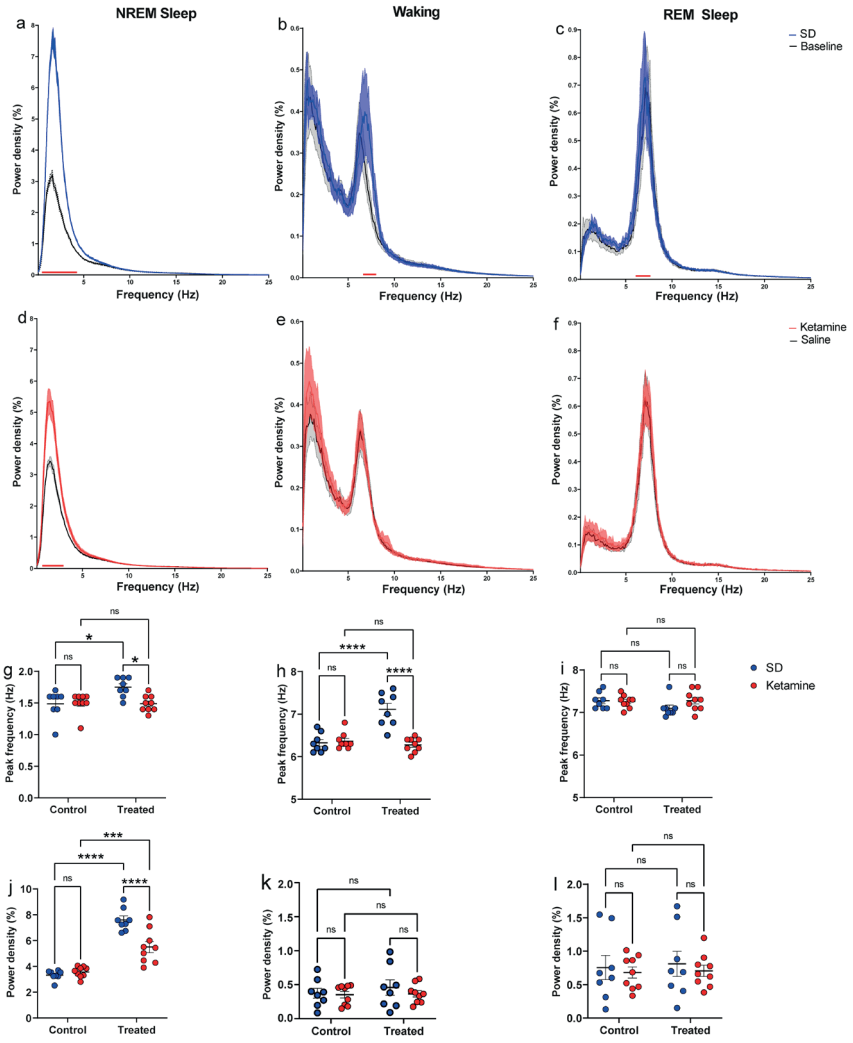


Figure 4. Effect of sleep deprivation and ketamine administration on the EEG power spectrum during the early recovery phase. a – c. Relative EEG power spectrum in NREM sleep, waking, REM sleep during CT 6 – CT 9 for sleep deprivation (blue, n = 8) and baseline (black, n = 8). Red bar indicates significant differences between sleep deprivation and baseline conditions ($p < 0.05$. d – f. Relative EEG power spectrum in NREM sleep, waking,

REM sleep during CT 3 – CT 6 for saline and ketamine administration (red, n = 9) and saline administration (black, n = 9). Red bar indicates significant differences between ketamine and saline conditions ($p < 0.05$). g – i . Peak frequency in the slow-wave range (1 - 4 Hz) for NREM sleep, and the theta range (6 - 9 Hz) for waking and REM sleep for sleep deprivation (blue, n = 8) and ketamine (red, n = 9) administration. j – l. EEG power density of the peak frequency in the slow-wave range for NREM sleep, and theta range for waking and REM sleep for sleep deprivation (blue, n = 8) and ketamine (red, n = 9) administration. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$, ns: not significant. Data is shown as mean ± SEM.

3.5 Effect of ketamine and sleep deprivation on EEG sleep power spectrum on the second recovery day

As discussed in the introduction, ketamine has an antidepressant effect that lasts longer than the acute effect it has on the vigilance states and the EEG. Therefore we analyzed the 24 hours EEG power density spectra on the second day after the SD and ketamine administration (Fig. 5). We only observed main effects on the REM sleep power density spectra after ketamine administration on the second day and no effect on the waking power density spectra (Fig. 5f, drug, REM, $F_{1, 4000} = 4.632$, $p = 0.0314$; Fig. 5d, drug, waking, $F_{1, 4000} = 1.823$, ns). Similar to ketamine, we found a main effect of SD on the REM sleep power spectrum on the second day (Fig. 5c, SD, $F_{1, 1750} = 10.30$, $p = 0.0014$, $p_{6.5\ Hz}=0.0189$, $p_{7.4\ Hz}=0.0003$, $p_{7.6\ Hz}=0.0081$). The effect of SD on the waking power spectrum was small, but still significant (Fig. 5 b, SD, $F_{1, 1750} = 4.923$, $p = 0.0266$). On the second day after the SD, we observed an undershoot in SWA (Figure S2), and we anticipated to see a similar effect in the power spectrum. Indeed, we observed between 1.2 – 2.1 Hz, a decrease in power density the second day after SD, which reflects the undershoot we observed in SWA in Supplemental Figure S21 (Fig. 5 a, SD, $F_{1, 1750} = 51.94$, $p < 0.0001$, $p_{1.2 – 2.3\ Hz} < 0.05$). For the second day of ketamine administration, we noticed a similar effect as after SD in the NREM sleep spectrum, although we did not observe the effect on the hourly values on the second day (Fig. 5 d, frequency x drug interaction, $F_{249, 4000} = 1.371$, $p = 0.0002$, $p_{1.2 – 2.5\ Hz} < 0.05$). Despite the acute sleep disturbance, ketamine administration and SD were not associated with long-term changes in sleep-wake architecture, however, both SD and ketamine were associated with long-term changes in the EEG power density spectra, especially for NREM and REM sleep.

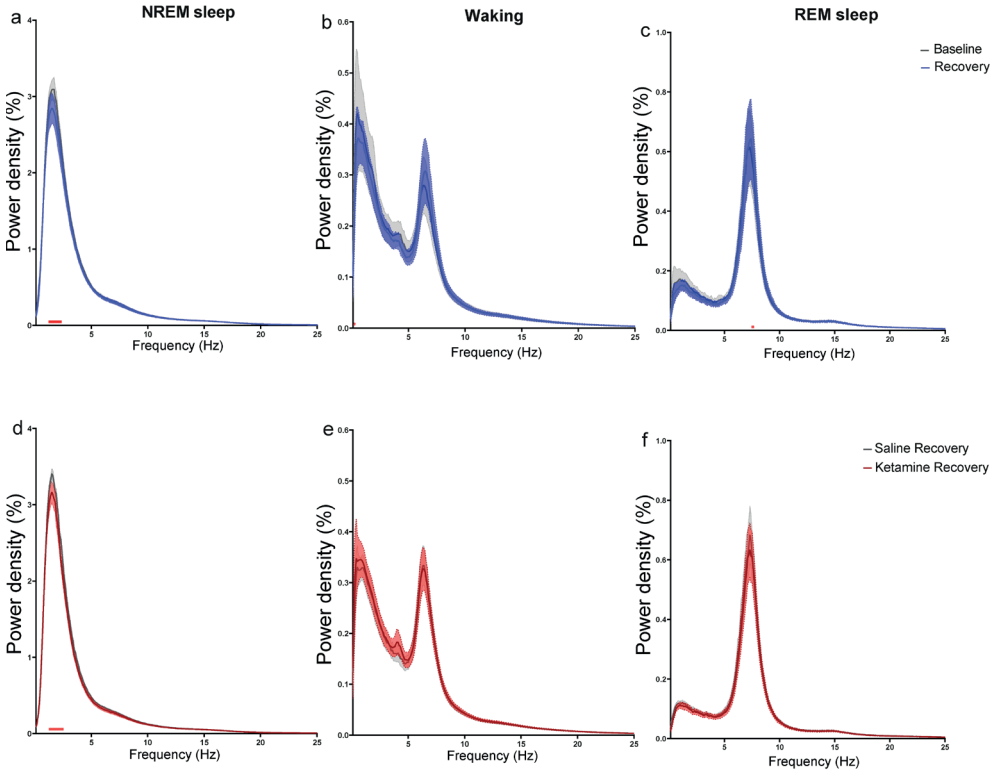


Figure 5. Effect of sleep deprivation and ketamine administration on the EEG power spectrum on the second recovery day. a - c. Relative EEG power spectrum in NREM sleep, waking, REM sleep for sleep deprivation (blue, n = 8) and baseline (black, n = 8). Red bar indicates significant differences between sleep deprivation and baseline conditions ($p < 0.05$). d - f. Relative EEG power spectrum in NREM sleep, waking, REM sleep for saline and ketamine administration (red, n = 9) and saline administration (black, n = 9). Red bar indicates significant differences between ketamine and saline conditions. Data is shown as mean ± SEM.

4. Discussion

4. 1 Effects of SD and ketamine on the sleep-wake architecture and locomotor activity

As found previously, SD induced an increase in NREM sleep and no changes in REM sleep in the first 6 hours following the SD [21,23]. The increase in NREM sleep lasted for 12 hours, and REM sleep started to increase after the

increase in NREM sleep started to diminish. It has been shown previously after SD that NREM sleep is more immediately recovered compared to REM sleep, however not after a relatively short SD of only 6 hours. Ketamine, an NMDAR antagonist, initially led to increased waking and activity which has been previously reported in rodents [24]. This increase lasted approximately 2-3 hours which corresponds well with the reported half live of 1.3 hours of ketamine in the rat [25]. Correspondingly, NMDAR antagonists are known to promote neurotransmitter release which influences brain areas that control arousal [26], but the induction of waking and suppression of sleep is still not completely understood. In this study, ketamine was expected to induce waking, however, we did not see a change in the amount of NREM sleep during the subsequent sleep period. Acute ketamine reduced REM sleep which is in line with some of the previous studies, but results tend to vary between studies [24]. This may be due to differences in light schedules, drug concentration or species. REM sleep suppression is common with many traditional antidepressants, and in some studies, there exists a relationship between the amount of REM sleep and emotional function [27,28]. Preclinical studies show that an SD lasting 24-h can increase serotonin (5 - HT) levels in the hippocampus in rats and depletion of 5 - HT actually blocks the antidepressant of ketamine in rats [13,29]. This may explain the change of sleep-wake architecture in the context of rapid changes in monoamine levels [30]. We observed a reduction in REM sleep for several hours in both treatments. REM sleep eventually recovered after SD, but not after ketamine. In view of the difference in the duration of the antidepressant effect of SD and ketamine and the difference in the effects on REM sleep between the two treatments, decreased REM sleep and the timing of REM sleep recovery may be relevant for the duration of the antidepressant therapeutic mechanism of the two treatments.

In rodents, ketamine administration can generate a behavioral syndrome characterized by hyperlocomotion, ataxia signs, and stereotypies [31]. Our recordings were obtained in a restricted condition, and the experimenters could not stay in the recording room and observe the animal after the injection. Increased locomotion, after ketamine was detected from the PIR recording, but did not allow to identify other psychotic-like behaviors. This type of behavior was also not observed in another study in rats using the same dosage, after which the animals were observed [13]. We therefore cannot exclude that this type of behavior occurred, but think that it is unlikely.

4.2 Acute effect on power spectrum and sleep homeostasis

To investigate this further, we compared the changes in EEG power density spectra during the initial phase of induced waking, the subsequent early recovery phase, and recovery on the second day, and determined similarities and differences which may elucidate the overlaps in their antidepressant action.

We show that the EEG power density spectrum of the waking period, induced in the first 2 hours after ketamine treatment, is different from that during SD, which may indicate that the fast antidepressant effect does not result from the type of brain activity during the initial waking period. Remarkably, the increase in locomotor activity after ketamine treatment was not accompanied by a change in theta EEG peak frequency as was the case during SD, where theta peak frequency became faster. Previous studies showed mixed results in rodents, with some studies showing an increase in theta activity in the first 30 min after 30 mg/kg ketamine administration, while other studies showed decreased power density within the range of 2.5–21 Hz [32,33]. The third group of studies found no effect on theta activity [24]. Recently, a study in humans showed an increase in theta activity after the ketamine administration in healthy subjects [34]. All these contradicting findings may be due to differences in recording location of the EEG, preceding sleep-wake history, circadian time of day, the drug concentration and even the species. In the present experiment we show that theta frequency became faster during SD, but when corrected for this change, there is no change in peak power density. The difference in theta frequency between SD and ketamine treatment suggests that the rapid antidepressant effect of ketamine and SD is probably not due to the quality of the initial waking period induced by the treatments.

After SD or the initial wake period induced by ketamine, both treatments showed an increase in the activity in the slow-wave range in the NREM sleep EEG. SWA is thought to reflect the build-up of sleep pressure during waking, which decreases during sleep [6]. More and more studies show that increased SWA is associated with increased BDNF, increased synaptic strength, and enhanced synaptic plasticity [35]. We performed our recording for 72- h in constant darkness, and noticed that the increase in SWA after SD was similar to that of a 24-h SD in rats under LD condition [36]. This may indicate that sleep pressure increases faster during waking under constant dark conditions. Further, we noticed an interaction between ketamine and circadian time in

SWA over 48 hours in our data. It is also possible that the effects of ketamine on sleep homeostasis depend on the circadian time. Considering the similarities in the changes in SWA after SD and ketamine treatment, it may be that SWA in NREM sleep serves as a reflection of an increased neuronal synaptic strength and plasticity, and may be related to the mechanism underlying the rapid antidepressant effects of the two treatments [37].

Ketamine did not change the REM sleep EEG power density spectrum. In contrast, we notice a change in the theta power density during REM sleep after SD. Thus, it seems that similar effects of SD and ketamine on the EEG during the early recovery period are only found in the NREM sleep EEG.

4.3 Long-term effect on power spectrum and sleep homeostasis

Analysis of the second recovery day after SD and ketamine showed a reduction of SWA in the NREM sleep EEG power spectrum and a main effect on the REM sleep power spectrum. This may indicate that both SD and ketamine influence sleep homeostasis for a longer time period under DD. This is surprising, as the plasma elimination half-life of ketamine (1.3 – 1.5 hours in rats) is short [25], and nevertheless, the changes we observed remained for at least 24 hours after administration. As we observed an undershoot in SWA on the second day which is not seen in other rat studies with such a short (6h) SD [21]. Undershoots in SWA, or negative rebounds as they are also called, have been observed before after SD in rats, but then after a 24-hour SD [36]. The strong undershoot we observed may be related to the delayed compensation in REM sleep which may suppress SWA in those hours. In summary, it seems that the effects of SD on NREM sleep SWA and REM sleep in our experiment are relatively strong when compared to other studies with a 6-h SD in rats. In contrast to the strong acute sleep disturbance, ketamine administration and SD were not associated with long-term changes in sleep-wake architecture, however, both SD and ketamine were associated with long-term changes in the EEG power density spectrum, especially in the NREM and REM sleep EEG.

There is a strong link between sleep and major depression. However, the causal relationship remains unclear. It is, for instance, unknown whether changes in depression symptoms precede or follow changes in sleep, and whether a longer or shorter sleep duration is related to improvements of depression core symptoms [6,38]. Investigating this may further explain how and when

an acute sleep deprivation exerts its rapid antidepressant effect and why it typically relapses after subsequent sleep occurs. It also needs to be researched how, with for instance light therapy, the antidepressant effect can be extended [39-42]. Other questions that remain are whether it suffices to concentrate on NREM sleep or total sleep deprivation [6], as in the past also successful treatments were achieved with REM sleep deprivation [43].

One of the limitations of this study are that we did not sample the EEG frequency over 25 Hz. It has been shown in other studies that, low dosages of ketamine increase EEG power density of gamma frequencies (30 – 100 Hz) in both human and rodents [44-46]. This enhanced gamma activity was associated with higher arousal or alertness [47]. A second limitation is that we did not use an animal model that shows behaviors characteristic of depression, but only report on healthy animals. Future studies should also include the analysis of the coherence of other cortex regions and EEG bands that have been shown to be involved in responding to ketamine or SD, such as gamma oscillations and should include animal models of depression when possible.

Taken together, the common actions of SD and ketamine on sleep consist of an acute increase in NREM sleep SWA and a reduction in REM sleep. This suggests that these two effects may be related to the rapid antidepressant effect seen after both. The finding that REM sleep eventually recovers after SD, but not after ketamine is in accordance with the assumption that reducing the amount of REM sleep and increasing REM sleep latency is antidepressant in both of these treatments, and fits with previous findings showing that the effect of SD on depressive symptoms is transient and after ketamine longer lasting [11,48]. The latter suggests that reducing REM sleep may be an important feature of a sustainable positive effect on depressive symptoms after ketamine treatment. The acute application of SD and ketamine appear to induce similar cortical changes during NREM and REM sleep and may further influence long-lasting effects in the brain. Therefore, our data suggest that a previously unappreciated targeting of NREM sleep SWA, and REM sleep may help to improve antidepressant therapies.

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Supporting information

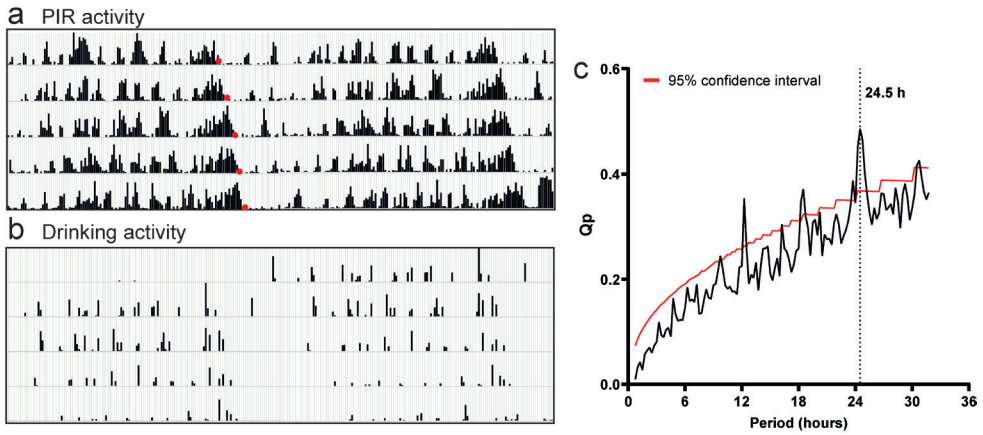


Figure. S1. Representative double plotted actogram. a. Representative double plotted actogram of PIR activity under constant darkness. Black areas indicate PIR activity. Red dot indicates the activity offset. Bin size = 10 min. b. Representative double plotted actogram of drinking activity under constant darkness. Black areas indicate drinking activity. c. The tau of the PIR activity was determined as the highest peak in the F-periodogram.

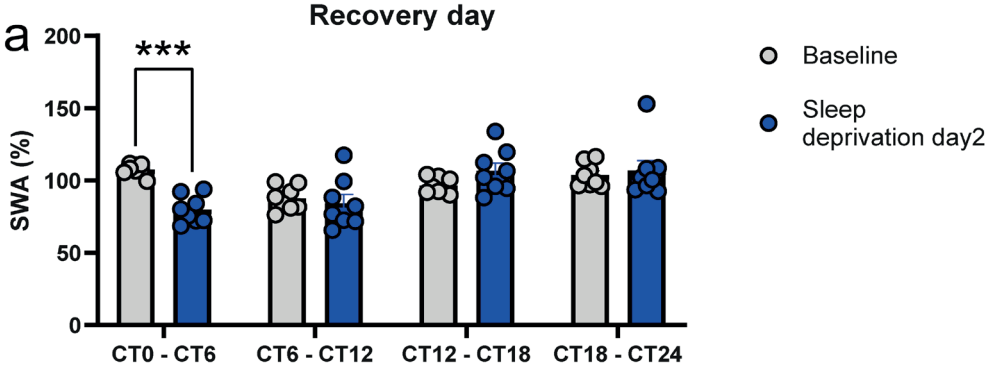
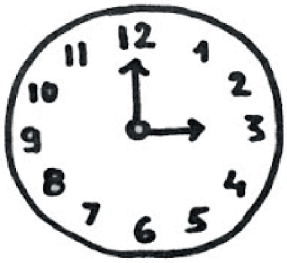


Figure. S2. Slow wave activity in NREM sleep and locomotor activity of sleep deprivation on the recovery day 2. Six-hour values of SWA in NREM sleep for the sleep deprivation (blue, n = 8) and baseline (grey, n = 8) condition on the second day after sleep deprivation. Data is shown as mean ± SEM.

4

Induction of Fatigue by Specific Anthracycline Cancer Drugs through Disruption of the Circadian Pacemaker



Yumeng Wang, Sabina Y van der Zanden, Suzanne van Leerdam, Mayke M H Tersteeg, Anneke Kastelein, Stephan Michel, Jacques Neefjes, Johanna H Meijer, Tom Deboer. Induction of Fatigue by Specific Anthracycline Cancer Drugs through Disruption of the Circadian Pacemaker. Cancers (Basel) 2022;14(10).

Abstract

Cancer-related fatigue (CRF) is the most devastating long-term side effect of many cancer survivors that confounds the quality of life for months to years after treatment. However, the cause of CRF is poorly understood. As a result, cancer survivors, at best, receive psychological support. Chemotherapy has been shown to increase the risk of CRF. Here, we study therapy-induced fatigue in a non-tumor-bearing mouse model with three different topoisomerase II-poisoning cancer drugs. These drugs either induce DNA damage and/or chromatin damage. Shortly before and several weeks after treatment, running wheel activity and electroencephalographic sleep were recorded. We show that doxorubicin, combining DNA damage with chromatin damage, unlike aclarubicin or etoposide, induces sustained CRF in this model. Surprisingly, this was not related to changes in sleep. In contrast, our data indicate that the therapy-induced CRF is associated with a disrupted circadian clock. The data suggest that CRF is probably a circadian clock disorder that influences the quality of waking and that the development of CRF depends on the type of chemotherapy provided. These findings could have implications for selecting and improving chemotherapy for the treatment of cancer in order to prevent the development of CRF.

Keywords: cancer-related fatigue; anthracyclines; circadian; sleep;quality of life

1. Introduction

Cancer-related fatigue (CRF) is a complex and debilitating side effect of cancer treatment. According to the American Cancer Society website: 'People with cancer might describe it [CRF] as feeling very weak, listless, drained, or "washed out" that may decrease for a while but then comes back. Some may feel too tired to eat, walk to the bathroom, or even use the TV remote. It can be hard to think or move. Rest might help for a short time but does not make it go away, and just a little activity can be exhausting. For some people with cancer, this kind of fatigue causes more distress than pain, nausea, vomiting, or depression' (https://www.cancer.org/treatment/treatments-and-side-effects/physical-side-effects/fatigue/what-is-cancer-related-fatigue.html (accessed on 1 April 2022)).

It affects more than 60% of cancer patients and survivors, but its etiology is unknown [1]. Persistent CRF has a serious effect on the quality of life of cancer survivors and can result in conditions where patients are too tired to go to work, socialize, or perform normal daily activities [2,3]. Many cancer patients report an increment in fatigue in the course of treatment with either chemotherapy, radiotherapy, or surgery [4]. Therapy-related CRF can persist for weeks, months, and up to many years following the finalization of treatment but varies between patients and, potentially, treatment modalities. Unfortunately, the relationship between the type of cancer treatment and CRF, as well as the molecular basis for CRF, remains unknown.

Although the cause of CRF is poorly understood, and the relative contribution of tumor type, cancer stage, and individual health status is unknown [5], converging evidence points to a major role of cytostatic drugs, including doxorubicin, in the induction of CRF [6]. Multiple mechanisms underlying the development of therapy-induced CRF have been proposed, such as inflammation, ROS formation, mitochondrial dysfunction, and central nervous system disorder [7,8,9]. Doxorubicin and its anthracycline drug family members are a cornerstone in cancer therapy, either as a single treatment or in a combination regimen, in the treatment of many solid and hematological tumors. For a long time, it was thought that the classical anti-tumor activity for these anthracycline drugs was the inhibition or poisoning of topoisomerase II [10,11]. Topoisomerase II is an enzyme critical in the relaxation of tension in the DNA, condensation of chromatin, and decatenation of DNA. To do so, topoisomerase II introduces a DNA double-strand break (DSB),

followed by DNA relaxation and re-ligation. Most anthracyclines, including doxorubicin and the structurally unrelated inhibitor etoposide, interfere with the topoisomerase-DNA interface to prevent re-ligation resulting in DNA DSBs [12]. Rapidly dividing cells, including most tumor cells, should be more sensitive to DNA DSBs than normal quiescent cells, which may explain the therapeutic window for these drugs. More recently, it became clear that anthracyclines, unlike etoposide, also induce chromatin damage as the result of histone evictions [13,14]. Studying the chemical structure of these drugs showed that chromatin damage is likely the major cause of the anticancer activity of most anthracycline drugs [15,16]. Of note, the DNA-damaging activity may conspire with chromatin damage to cause a number of serious side effects associated with anthracycline treatment, including dose-dependent cardiotoxicity and therapy-related malignant neoplasms [15].

We set out to study the putative mechanism underlying the development of therapy-induced CRF by evaluating the effect of three topoisomerase II inhibitors (doxorubicin, etoposide, and aclarubicin) on wheel-running and sleep–wake behavior in mice, which have been used previously as an overt measure of fatigue [17,18]. Wheel running in mice is voluntary, motivated behavior [19] and reflects natural activity levels of mice in the wild [20]; it is, therefore, a suitable outcome measure for a fatigue mouse model. Wheel-running activity has been recently developed as a model to measure CRF in mice, but so far, it has only been used in a limited number of studies evaluating a limited number of therapies [17,21,22,23,24].

We observed that healthy mice treated with doxorubicin showed decreased wheel-running activity, indicating fatigue-like symptoms. Surprisingly, these persistent fatigue symptoms were not associated with disrupted sleep–wake behavior. Instead, we found therapy-induced fatigue likely to be associated with disruption of the central circadian pacemaker. Our study thus suggests that CRF is not a sleep but rather a wake quality issue related to disruption of the circadian clock. This is likely caused by combined DNA- and chromatin damage activity since treatment with the topoisomerase II inhibitor family members aclarubicin (only inducing chromatin damage) or etoposide (only inducing DNA damage) failed to induce altered wheel-running behavior or affect the circadian clock. We thus provide data showing that CRF is dependent on the type of chemotherapy. CRF is then not only a psychological phenomenon but also has a physical cause, a finding that will be highly appreciated by cancer survivors. These data are important to improve the

quality of life of cancer survivors and motivate new treatment options, as fatigue is experienced as the most devastating side effect of chemotherapies.

2. Materials and Methods

2.1. Reagents

Doxorubicin and etoposide were obtained from Pharmachemie (Haarlem, The Netherlands). Aclarubicin (sc-200160) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), dissolved in dimethylsulfoxide at 5 mg/mL concentration, aliquoted, and stored at −20 °C for further use.

2.2. Cell Culture

Endogenous tagged scarlet-H2B cells [15] were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 8% FCS. MelJuSo cells stably expressing PAGFP-H2A were maintained in IMDM supplemented with 8% FCS and G-418, as described [13]. All cell lines were maintained in a humidified atmosphere of 5% CO_2 at 37 °C and regularly tested for the absence of mycoplasma.

2.3. Fractionation Assay

Cells were treated as indicated and washed and lysed directly in lysis buffer (50 mM Tris-Hcl pH 8.0, 150 mM NaCl, 5 mM $MgCl_2$, 0.5% NP40, 2.5% glycerol supplemented with protease inhibitors, 10 mM NMM, and 10 μM MG132). Subsequently, cell lysates were collected, vortexed, and incubated for 10 min on ice. To collect the cytosolic fraction, samples were centrifuged for 10 min, 15,000 g, at 4 °C. Both nuclear (pellet) and cytosolic (supernatant) fractions were washed and prepared for Western blot analysis.

2.4. Western Blot

Upon treatment, as indicated, cells were washed extensively to remove drugs. Cells were collected and lysed directly in an SDS-sample buffer (2% SDS, 10% glycerol, 5% β-mercaptoethanol, 60 mM Tris-HCl pH 6.8, and 0.01% bromophenol blue). Lysates were resolved by SDS/polyacrylamide gel electrophoresis followed by Western blotting. Primary antibodies used

for blotting: γH2AX (1:1000, 05-036, Millipore), β-actin (1:10,000, A5441, Sigma), RFP (1:2000, 6G6, Chromotek, Planegg-Martinsried, Germany), Lamin A/C (1:500, sc-20681, Santa Cruz Biotechnolgy, Santa Cruz, CA, USA), Calnexin (1:1000, C5C9, Cell signaling Technology, Danvers, MA, USA). Images were quantified with ImageJ.

2.5. Microscopy

For cytosolic H2B detection, endogenous tagged scarlet-H2B cells were seeded on coverslips. Upon treatment with 10 μM of the indicated drugs for 1 h, cells were fixed in formaldehyde (FA) 4%, permeabilized with 0.1% Triton, and stained with anti-RFP (1:100, 6G6, Chomotek, Planegg-Martinsried, Germany), goat-anti-mouse-Alexa Fluor 488 (1:400, Thermo Fisher Scientific, Waltham, MA, USA) and Alexa Fluor 647 phalloidin (1:125, A22287, Thermo fisher Scientific). Cells stably expressing PAGFP-H2A were used for histone eviction experiments. Photoactivation and time-lapse confocal imaging were performed as described [13,15], and loss of fluorescence from the photoactivated region after different treatments was quantified. Cells were analyzed by a Leica SP8 confocal microscope system with 63× lens. For live-cell imaging, a microscope equipped with a climate chamber was used. Quantification was done using ImageJ software.

2.6. Animals

Male C57BL/6J mice (10 weeks old; obtained from Envigo, Horst, The Netherlands) were group-housed in the animal facility of the Leiden University Medical Center (LUMC, Leiden, The Netherlands) under controlled conditions (12:12 h light–dark cycle; lights on at 10:00) with food and water ad libitum in a temperature-controlled room (21–24 °C). They were placed in the recording cabinets for two weeks to familiarize themselves with the new environment and entrain to the new light–dark schedule. Experiments were performed according to institutional and national guidelines and approved by the Animal Ethics Committee of the LUMC (Leiden, The Netherlands).

2.7. Behavioral Activity

Running wheel (diameter: 24 cm) activity and passive infrared (PIR) cage activity were recorded with Clocklab data collection software (Actimetrics, Wilmette, IL, USA) and were stored in 1-min bins [25]. Group housed mice (10

weeks old) were provided with a running wheel for two weeks. Subsequently, at 12 weeks of age, mice were individually housed with a PIR sensor and running wheel in a 12:12 h light–dark cycle. Baseline (BL) recordings for the running wheel and PIR activity were done for two weeks, followed by cytostatic treatment. During the treatment and recovery period, as well as during EEG/EMG recordings, only PIR activity was recorded.

2.8. EEG/EMG Surgery

Mice were anesthetized (Ketamine 100 mg/kg; Xylazine 10 mg/kg; Atropine 1 mg/kg) and implanted with EEG recording screws (placed above the somatosensory cortex and cerebellum) and electromyogram (EMG) electrodes (placed on the neck muscle) (Plastics One Roanoke, VA, USA), as described previously [25,26]. The wire branches of all electrodes were set in a plastic pedestal (Plastics One, Roanoke, VA, USA) and fixed to the skull with dental cement. Buprenorphine (0.1 mg/kg temgesic) was administered to provide post-surgery analgesia along with a heat lamp until the mice were able to move. Subsequently, the mice were allowed to recover for 7 days.

2.9. EEG/EMG Recording

The baseline recordings of RW and PIR were followed by EEG and EMG recordings (Data Sciences International). Animals (control n = 9, doxorubicin n = 8, aclarubicin n = 8, etoposide n = 7) were placed into experimental chambers, connected through a flexible cable and a counterbalanced swivel system to the recording setup. Conditions in the experimental chamber were similar to the home cage, including food and water availability. Before starting the experiment, the animals were allowed to adjust to the experimental conditions for 3 days. Subsequently, a baseline day (24 h) prior to treatment was recorded as previously described [25,26,27], starting at lights on. A post-treatment day (24 h) was recorded 21 days after the last treatment with cytostatic was finished.

2.10. Cytostatic Treatment

Mice (n = 7–9 per group) were treated as indicated (i.p.) 4 times (day 1, 2, 9 and 10) over a 10-day period with 3.75 mg/kg doxorubicin, 3.75 mg/kg aclarubicin, 11.25 mg/kg etoposide or 3.75 mL/kg saline with 4% DMSO one hour after light on. The repeated treatment with doxorubicin over 10 days

added up to a total of 15 mg/kg as applied previously in [17] in a fatigue study on doxorubicin. Aclarubicin and etoposide were given in dosages relative to doxorubicin based on our previous work in mice [15].

2.11. Data and Statistical Analysis

For the in vitro experiments, each sample was assayed in biological triplicate unless stated otherwise. All error bars denote SD. Statistical analyses were performed using Prism 8 software (Graphpad Inc. La Jolla, CA, USA). Western blot and confocal data were quantified using ImageJ software. Significance is represented on the graphs as follow: ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. No statistical methods were used to predetermine sample size. Running-wheel and PIR data were recorded by ClockLab software (Actimetrics, Wilmette, IL, USA) and further analyzed in 1-h values. The strength of the circadian clock and period of free running, wheel running, and cage activity were derived from F-periodogram analysis as previously described [25,28]. Running distance was calculated by counting the number of turns of the wheel. Activity onset was identified through ClockLab software as the activity level that exceeds exactly 20% of all non-zero counts for a 5-h period of inactivity followed by at least a 5-h period of activity. When necessary, onset points were edited manually. Vigilance states were manually scored offline using 4 s epochs and were subdivided into waking, NREM, and REM sleep. EEG power spectral analysis was done by a fast Fourier transform within the range of 0.25–25 Hz, as described previously [27]. Statistical analyses were performed using Prism 8 software (Graphpad Inc., La Jolla, CA, USA) and IBM SPSS Amos 25.0.0 (SPSS Inc., Chicago, IL, USA). Two-way ANOVA with Bonferroni multiple comparisons was used to compare the effect of different cytostatic across time. One-way ANOVA followed by an independent t-test was used to compare the strength of the circadian clock. Mann–Whitney U test was used to compare the cumulative running distance among different groups. Independent sample t-tests were used to compare Qp and tau between exercise and no-exercise groups.

3. Results

3.1. Doxorubicin-Treated Mice Develop Fatigue-Like Symptoms

Many cancer patients treated with chemotherapeutics can develop CRF [4,9]. One class of chemotherapeutics, which is commonly used as single- or combination therapy for various types of tumors, are the topoisomerase inhibitors or poisons [11,12]. Doxorubicin, one of the most used and best-known family members, has been described to induce CRF in patients [8,29]. To explore the basis of this devastating side effect, we studied the effect of three different topoisomerase drugs (doxorubicin, aclarubicin, and etoposide) on the development of fatigue-like symptoms in healthy non-tumor-bearing mice. For this purpose, 12-week-old male C57BL/6J mice were subdivided into four groups, and baseline wheel-running activity was monitored (Figure 1A,B). Subsequently, mice were treated with four intra-peritoneal injections of the indicated cytostatic drugs over a period of 16 days. Treatment was followed by monitoring body weight (which is a representative parameter of general toxicity [30]), wheel-running activity, and cage activity under a normal 12 h:12 h light–dark (LD) cycle and in constant dark (DD) conditions to evaluate the effect on the circadian clock (Figure 1A,B and Supplementary Figure S1).

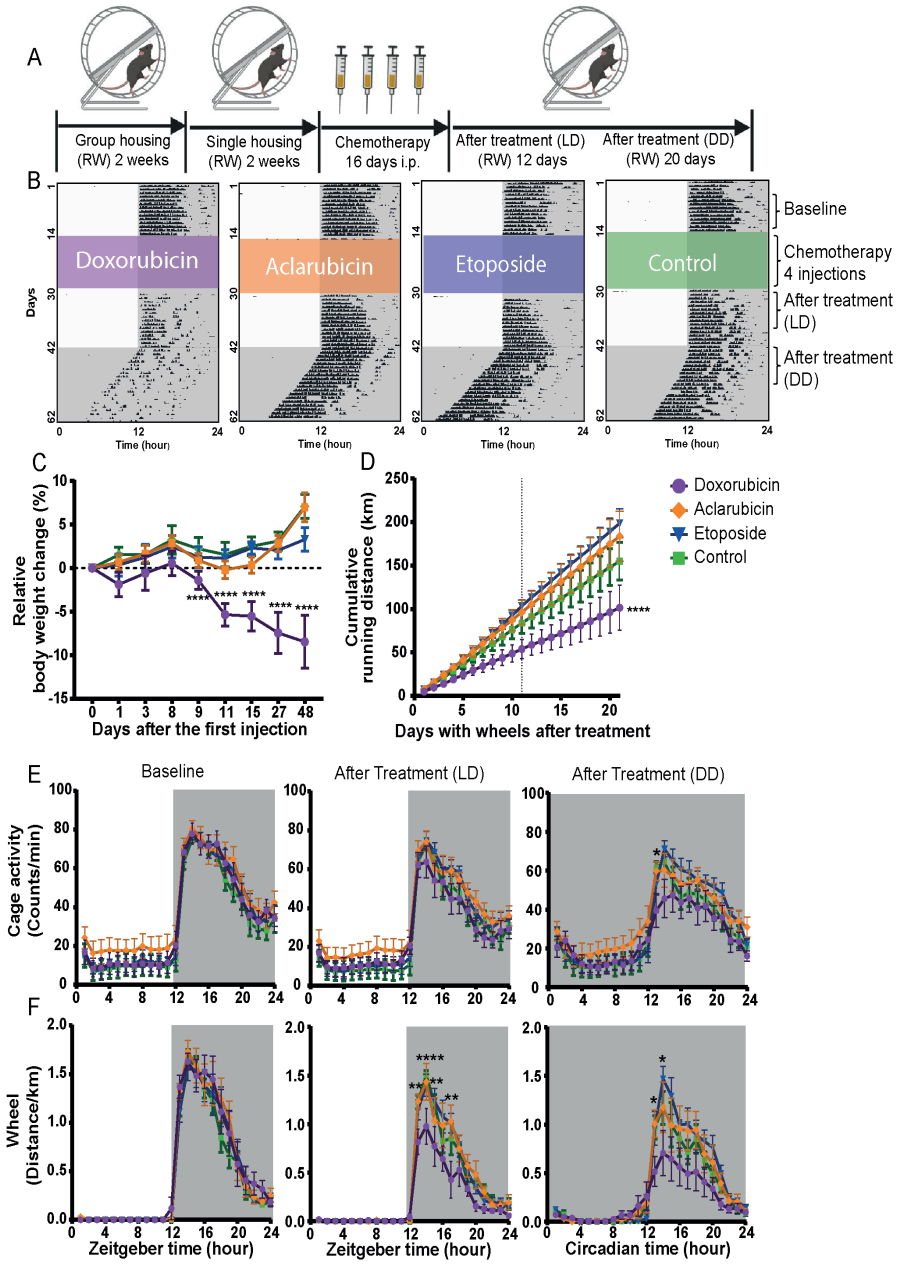


Figure 1. Doxorubicin treatment affects wheel-running activity under light-dark and constant dark conditions. (A) Experimental design of the rest-activity behavior study. RW—running wheel; LD—light-dark; DD—constant dark. (B) Representative single plotted actograms of wheel-running activity under different chemotherapy treatments during the whole experiment. Black areas indicate wheel activity. Colored areas indicate the treatment time with different cytostatic drugs. (C) Relative body weight changes after chemotherapy of

the four groups (**** $p < 0.0001$ two-way ANOVA with Bonferroni multiple comparisons, doxorubicin n = 8, all other treatments n = 9 per group). (D) Cumulative running distance during the DD phase (active phase) across 21 days, starting 7 days after the last injection. Vertical line indicated the mice were under DD condition (**** $p < 0.0001$, Mann–Whitney U test). (E) Average cage activity PIR counts/min over 10 days under baseline condition (left), treatment LD condition (middle), and treatment DD condition (right). (F) Average running distance/h over 10 days under baseline condition (left), treatment LD condition (middle), and treatment DD condition (right) (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ doxorubicin compared to control group, two-way ANOVA with Bonferroni multiple comparisons). Values are shown as mean/hour ± SEM. N = 8–9. Grey areas in B, E, and F indicate the dark periods.

Mice treated with doxorubicin showed a decreased body mass compared with etoposide-, aclarubicin-, and control-treated mice. The weight loss started 9 days after the first injection (Figure 1C). Total body mass of doxorubicin-treated mice 48 days after the last injection was still 8% lower compared to their weight pre-treatment (Figure 1C). To analyze the treatment-induced fatigue-like symptoms, we quantified the wheel-running activity profile under LD and DD conditions (Figure 1B,E,F). Particularly, doxorubicin-treated mice showed decreased running both under LD and DD conditions compared to all other treatments and relative to their own baseline activity (Figure 1D). The 21-day total average running distance of doxorubicin-treated mice (101.45 ± 25.80 km) was markedly shorter than the running distance of aclarubicin (183.61 ± 28.95 km), etoposide (198.58 ± 16.78 km), and control (170.26 ± 17.63 km) treated mice (Figure 1D). Remarkably, when we quantified the 24-h cage locomotor activity (by passive infrared, PIR recording) profile over 10 days at baseline, under LD and DD conditions (Figure 1E), no significant effect of treatment was observed under LD conditions. In constant darkness, a small but significant decrease was observed in doxorubicin-treated mice (Figure 1E). Similar to the PIR recordings, no difference was observed for aclarubicin- or etoposide-treated mice when the 24-h wheel-running activity profile was analyzed (Figure 1F). On the other hand, doxorubicin-treated mice did show a significant decrease in 24-h wheel-running activity after treatment, both under LD and DD conditions, specifically in the first half of the night (Figure 1F). Overall, the changes in wheel-running activity following the different treatments indicate that doxorubicin, unlike etoposide or aclarubicin, induces fatigue-like symptoms in non-tumor-bearing mice. This suggests that the fatigue-like symptoms measured are determined by the type of cancer treatment.

3.2. Anthracycline Variants Have Diverse Underlying Mechanism of Action

The anthracycline doxorubicin is associated with the induction of CRF [6]. This widely used chemotherapeutic is a topoisomerase II poison that functions by induction of DNA double-strand breaks following intercalation into the DNA and trapping of the enzyme [10,11]. In addition, doxorubicin also induces chromatin damage via eviction of histones (Figure 2A) [13,14]. To assess which activity (DNA damage or chromatin damage) is associated with the development of therapy-induced CRF, we tested two variant topoisomerase inhibitors: the anthracycline aclarubicin and the podophyllotoxin derivative etoposide. Similar to doxorubicin, etoposide also induces DNA double-strand breaks, as illustrated by the formation of γH2AX as detected by Western blot (Figure 2B,C) and fluorescent microscopy analysis (Supplementary Figure S2A,B). On the other hand, both doxorubicin and aclarubicin have chromatin damage activity (Figure 2D–F). To detect histone eviction, the cytosolic and nuclear fractions of cells treated with the different drugs were isolated, and endogenously tagged scarlet-H2B histones were detected by Western blot (Figure 2D,E). To confirm this observation, the localization of scarlet-H2B histones was analyzed by confocal microscopy (Supplementary Figure S2C,D). Alternatively, the fluorescent signal of photo-activated GFP-H2A histones was followed in living cells over time upon treatment with the different drugs (Figure 2F and Supplementary Figure S2E). These data confirm that both anthracyclines doxorubicin and aclarubicin, unlike etoposide, induced histone eviction. The difference in the underlying mechanism of action of these three cancer drugs inducing only DNA damage, only chromatin damage, or the combination of both, allows analyses of the contribution of these two biological effects within doxorubicin on CRF.

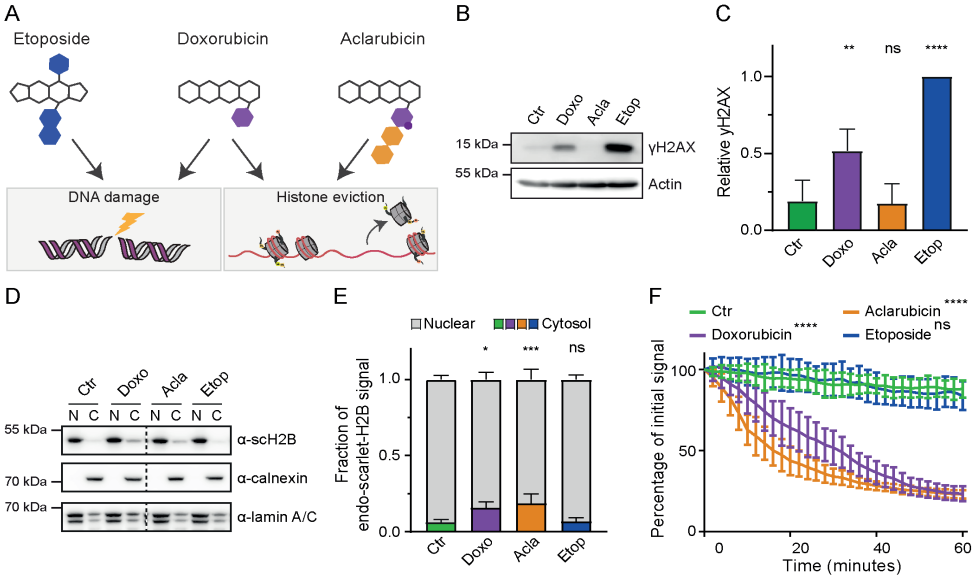


Figure 2. Mechanism of action of anthracycline drugs. (A) Schematic overview of the DNA damage (via targeting topoisomerase II) and chromatin damage (via eviction of histones) activity of the three anthracycline drugs used in this study. (B) Endogenously tagged scarlet-H2B U2Os cells were treated for 2 h with 10 μM of the indicated drugs. γH2AX levels were examined by Western blot, and actin was used as a loading control. (C) Quantification of the γH2AX levels normalized to actin. Ordinary one-way ANOVA with multiple comparisons; ns—not significant; ** $p = 0.0052$; **** $p < 0.0001$. (D) Endogenously tagged scarlet-H2B U2Os cells were treated for 1 h with 10 μM of the indicated drugs. Cells were fractionated, and the nuclear versus cytosolic fraction of H2B was examined by Western blot. Calnexin was used as cytosolic marker, and lamin A/C was used as nuclear marker. (E). Quantification of the nuclear versus cytosolic H2B signal from four independent experiments is plotted. Two-way ANOVA with multiple comparisons; ns—not significant; * $p = 0.0109$; *** $p = 0.0007$. (F). Quantification of the release of fluorescent PAGFP-H2A from the photoactivated nuclear regions upon administration of the indicated drugs. Ordinary one-way ANOVA with multiple comparisons; ns—not significant; **** $p < 0.0001$.

3.3. Sleep–Wake Patterns Were Not Affected by Chemotherapy Treatment

CRF has often been investigated in the framework of an increased need for sleep. Indeed, sleep problems are commonly reported by cancer patients undergoing chemotherapy [31,32]. These sleeping problems may, however, be independent of fatigue [33]. We, therefore, assessed the sleep–wake behavior in mice treated with either doxorubicin, etoposide, or aclarubicin. To do so, we performed EEG/electromyogram (EMG) recordings in freely moving mice with implanted stationary electrodes [25,27] before and after the completion of drug treatment (Figure 3A). Surprisingly, no significant differences in sleep and waking were observed for chemotherapeutically treated mice compared to baseline control or between treatment groups (Figure 3B–E). To further investigate the consequences of drug treatment in the different groups, we analyzed the EEG power density spectrum in the three vigilance states (Supplementary Figure S3). No alterations in the power density spectra were observed in either doxorubicin- or aclarubicin-treated mice compared to the baseline condition (Supplementary Figure S3A–F), whereas etoposide-treated mice exhibited increased EEG activity in the whole spectral range analyzed (0.5–25 Hz) in all three vigilance states (Supplementary Figure S3G–I). Since no relation to one particular vigilance state was observed, this suggests that etoposide induces a general overall change in brain activity. However, this general change in brain activity is not translated into an effect on fatigue symptoms, as no decline in wheel-running activity was observed in etoposide-treated mice (Figure 1). Taken together, the sleep and EEG data suggest that the reduction in voluntary wheel-running activity related to doxorubicin treatment is unrelated to an enhanced sleep need in these animals.

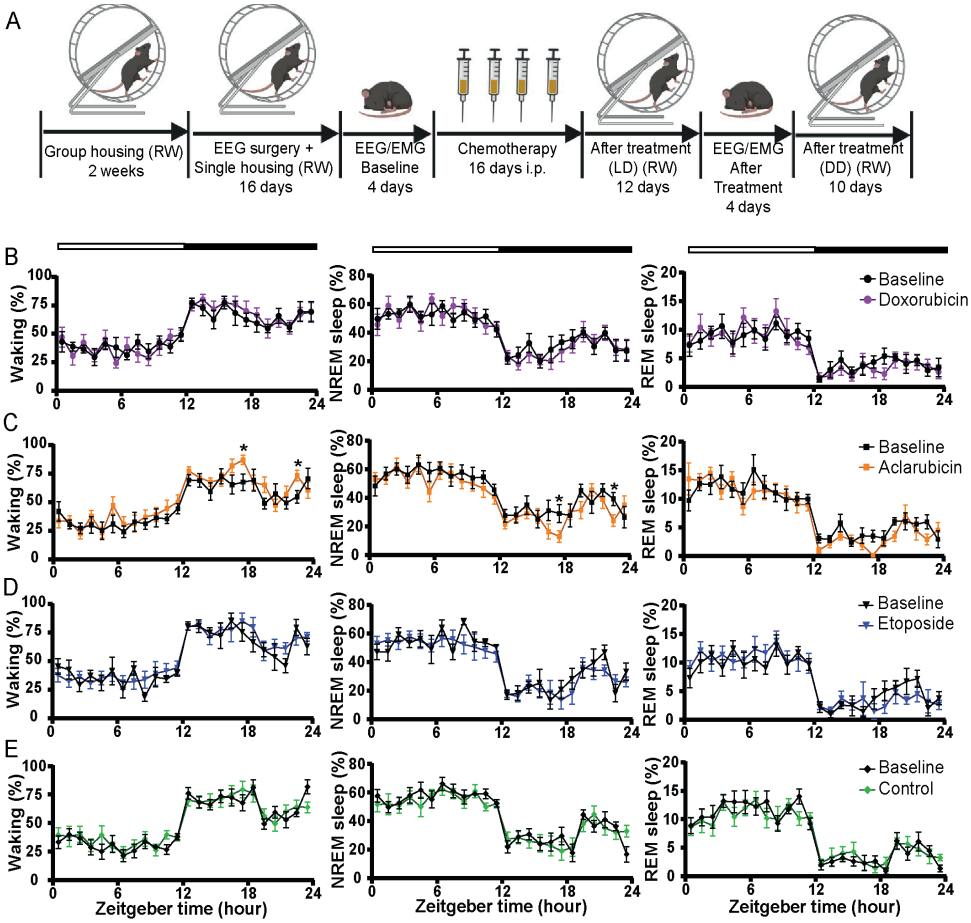


Figure 3. Chemotherapy-induced fatigue is not caused by changes in the sleep-wake rhythm. (A) Experimental design of the sleep–wake study. EEG—electroencephalogram, EMG—electromyography, RW—running wheel, LD—light-dark, DD—constant dark. (B–E) One-hour values of Waking, NREM, and REM sleep of 24 h before treatment baseline day compared with doxorubicin (B), aclarubicin (C), etoposide (D), and control (E) treatment. Values are shown as mean ± SEM. control n = 9, doxorubicin n = 8, aclarubicin n = 8, etoposide n = 7. Two-way ANOVA with Bonferroni multiple comparisons, compared with time-matched control values; * $p < 0.05$. White and black bars at the top indicate the light–dark cycle.

3.4. Doxorubicin-Treated Mice Exhibit a Disrupted Circadian Clock

If fatigue is not related to enhanced sleep and/or sleep pressure, we considered an altered waking state quality as an underlying cause rather than a change in sleep quality. In humans, wakefulness during the day is promoted by the

circadian clock of the suprachiasmatic nucleus [34]. So far, only one study has investigated the possible relation between therapy-induced CRF and a disrupted circadian clock [24]. To investigate whether doxorubicin-induced CRF might be a consequence of a disrupted circadian clock, we analyzed the precision of the behavioral rest-activity rhythm by measuring the daily onset of activity of the different treatment groups (Supplementary Figure S4), which is a routine way to test the expression of the endogenous clock [35]. Under DD conditions, doxorubicin-treated mice exhibited an unstable activity onset compared to all other groups (Figure 4A–D), while under baseline- and LD conditions following treatment, no difference between the three drugs and control groups was observed (Supplementary Figure S4B,E,H,K). To quantify this finding, we calculated the variability in activity onset over 10 days for the individual mice. Under baseline and treated LD conditions, the differences were not significant (Figure 4E,F), whereas under DD, the doxorubicin-treated mice showed vastly unstable activity onsets compared to the other treatments (Figure 4G). This suggests that treatment with doxorubicin destabilizes the circadian clock and abolishes the ability to properly time day-to-day behavior in the absence of external time cues (Figure 4A–D,G). Subsequently, we determined the period and strength of the circadian rhythm (Qp) by F-periodogram analyses [28] of mice treated with the different drugs at baseline and in LD or DD conditions (Supplementary Figure S1C). The period did not differ between the four treatment groups (doxorubicin 23.54 ± 0.14, aclarubicin 23.78 ± 0.05, etoposide 23.55 ± 0.05, saline control 23.69 ± 0.06, $p = 0.1061$, mean ± SEM, one-way ANOVA). Qp was significantly lower after doxorubicin treatment under DD conditions compared to control mice, unlike mice in the other groups (Figure 4H–J).

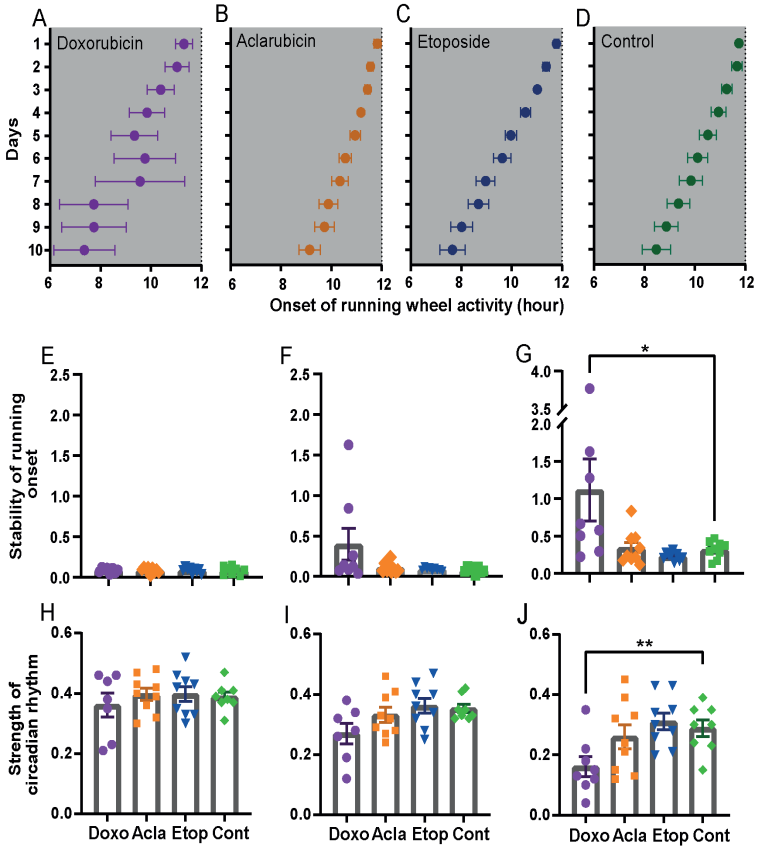


Figure 4. Doxorubicin-treated mice show a disrupted circadian clock. (A–D) Average wheel-running activity onset time of doxorubicin (A), aclarubicin (B), etoposide (C), and control (D) treated mice over a period of 10 days. Grey areas indicate the period of darkness. (E,F). Average 10 days stability of running onset time under baseline condition (E), treatment LD condition (F), and treatment DD condition (G). (H–J) Strength of the circadian rhythm (Qp) of wheel-running activity under baseline condition (H), treatment LD condition (I), and treatment DD condition (J). Data are shown as mean ± SEM. doxorubicin n = 8, all other treatments n = 9 per group. One-way ANOVA with Bonferroni multiple comparison; * $p < 0.05$, ** $p < 0.01$.

Overall, the data show that the present mouse model of CRF can discriminate between different working mechanisms of cancer treatment drugs. In addition, sleep is not affected by treatment with the drug that causes fatigue. In contrast, therapy-induced CRF is likely caused by alterations in the central circadian pacemaker as circadian rest-activity behavior shows less precise timing and has a reduced rhythm strength in mice treated with doxorubicin.

4. Discussion

Cancer-treatment-related fatigue following chemo- or radiotherapy is a devastating side effect and complaint of many cancer survivors [4]. This adverse effect accumulates during therapy and can last for a long and undefined period of time [31]. Persistent CRF has a serious and continuing effect on the quality of life of these patients since they are too tired to work, socialize, or even perform normal daily activities [1,3]. Despite the high incidence of CRF, its cause is poorly understood. Furthermore, without a clear definition of fatigue, unambiguity for measuring fatigue symptoms, and a (golden standard) therapy for CRF, the study of fatigue is complex [5]. Consequently, most studies related to the evaluation of CRF concentrate on psychological assessment and treatment [36]. To better understand the underlying molecular mechanisms of chemotherapy-induced CRF, we decided to study the effects of three topoisomerase II targeting drugs (doxorubicin, etoposide, and aclarubicin), commonly used in cancer treatment, on CRF-like symptoms in tumor-free mice [11,12]. Although these three variants belong to the same family of chemotherapeutic drugs, they function through different mechanisms. Where etoposide induces DNA double-strand breaks, aclarubicin is abstained from DNA damage activity but induces chromatin damage via eviction of histones [13]. In the case of doxorubicin, both activities (DNA- and chromatin damage) are combined in one molecule. For this reason, we wondered whether the combination of activities within doxorubicin would also be essential for the development of therapy-induced CRF in patients treated with this class of chemotherapeutic drugs.

Our current study shows that there is a clear difference in fatigue symptoms and severity between doxorubicin and its family members. Where doxorubicin treatment resulted in decreased voluntary running, both under LD and DD conditions, none of the other treatments showed an effect different from baseline activity. Furthermore, the 21-day total running distance was remarkably lower in doxorubicin-treated mice compared to other groups. These data do not prove that the combination of the two working mechanisms causes the fatigue-like symptoms, as it may also be related to other types of toxicity, but they do indicate that treatment-related CRF is therapy-specific and, in the present study, is only correlated with the topoisomerase drug that combines both DNA- and chromatin damage activity. The observed effect on fatigue symptoms by doxorubicin could be explained by attenuated DNA repair as a consequence of both DNA damage induction and eviction of

H2AX histones, which are then no longer available for phosphorylation, as is observed for other toxicities associated with doxorubicin treatment [12].

Moreover, doxorubicin caused fatigue-like behavior, with a clear influence on voluntary wheel-running activity, but it had hardly any effect on levels of other (locomotor) activity within the cage, recorded with passive infrared sensors, which is much less intense and includes foraging, eating and drinking. Therefore, the treatment with doxorubicin seems to affect specifically voluntary wheel-running behavior but leaves other, less intense behavior unaffected. This observation is in line with a previous study on CRF in doxorubicin-treated mice [17] and confirms the value of our model and wheel-running activity as a good readout for fatigue.

Given the expected interaction between sleep need and CRF, we investigated sleep quantity and quality in the different treatment groups. Sleep was recorded three weeks after completion of treatment. Surprisingly, we did not observe any significant difference in the amount or distribution of the three vigilance states, despite the obvious difference in the wheel running activity levels. We, therefore, hypothesized that therapy-related CRF might be caused by a disrupted regulation of the active period by the central circadian clock instead of being a problem related to sleep. This hypothesis is supported by our analysis of the wheel-running recordings performed under DD conditions, which show that both the precision and the strength of the circadian rhythm were reduced in doxorubicin-treated mice. The different treatments did not seem to affect the endogenous circadian period of the circadian clock but rather seemed to influence the precision of timing of onset of activity. Intriguingly, a recent study on the effect of paclitaxel, a commonly used taxane chemotherapeutic, on CRF in mice also shows disruption of the circadian clock [24]. However, paclitaxel treatment only results in a temporary decrease in wheel-running activity, which is different from our doxorubicin-treated mice with persistent CRF symptoms. This discrepancy could be explained by the difference in the molecular working mechanisms of these chemotherapeutics. Where paclitaxel is known to induce transient DNA damage by arresting cell growth by microtubule stabilization [37], doxorubicin treatment is known to attenuate DNA damage repair upon induction of DNA breaks due to the eviction of yH2AX marked histones (Figure 2) [13]. This might then affect the extent and duration of fatigue-like symptoms induced by doxorubicin. Our data showing persistent fatigue symptoms induced by doxorubicin treatment (symptoms last until the end of the experiment, several weeks after treatment) are in

line with observations of chronic CRF made in patients [3,38]. Our findings indicate that doxorubicin has a long-lasting effect on circadian-regulated rest-activity behavior, unlike etoposide or aclarubicin. Therapy-related CRF is thus strongly dependent on the type of treatment.

In the present study, we decided to include only male animals, as previous studies in male mice had provided a reasonable estimate of the dosage to use and the methods to apply [15,17]. To rule out any gender difference in the induction of therapy-induced CRF, future studies of the difference between males and females would be highly interesting to study the putative variation in response depending on sex. Furthermore, investigating where in the processing of circadian rhythms the treatments are most disturbing additional measures on circadian rhythms, for instance, body temperature, metabolism changes, and metabolomics, could improve our understanding of the mechanism of therapy-induced CRF.

In mammals, the rest–activity cycle is under the control of the central circadian pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus [39]. The SCN influences activity in brain areas such as the lateral hypothalamus, prefrontal cortex, and nucleus accumbens, which controls voluntary activity, arousal, and wakefulness, among others [40,41]. A chronic effect of chemotherapy on the central clock may therefore result in a decreased possibility to express activity during the day. Our observation of doxorubicin-induced fatigue is consistent with clinical studies showing that patients treated with chemotherapy have a dampened day-night rhythm compared to healthy volunteers [42].

5. Conclusions

Our data show that treatment with doxorubicin, which combines both DNA- and chromatin damage activity, results in increased fatigue symptoms in tumor-free mice. In contrast, treatment with aclarubicin or etoposide failed to induce fatigue, suggesting that the development of this devastating side effect is probably the result of specific cancer treatment mechanisms. These symptoms are not associated with changes in sleep but rather with a disrupted circadian clock. CRF is then dependent on the type of chemotherapy, which could be an important criterium for selecting cancer drugs for treatment.

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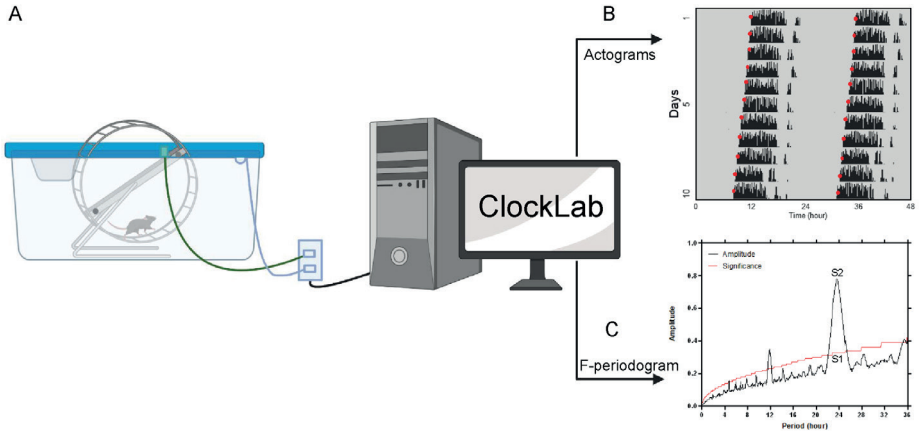
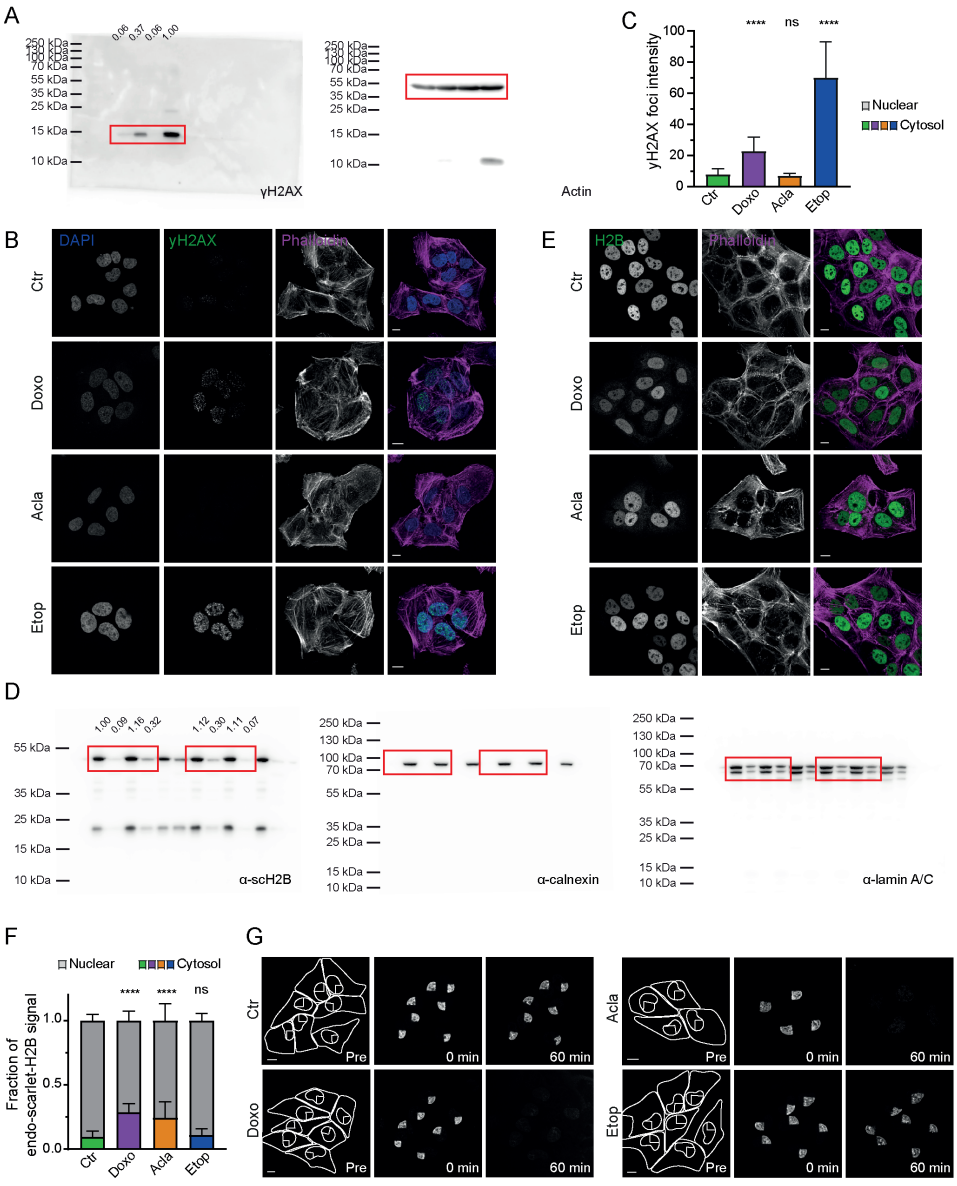
Supporting information

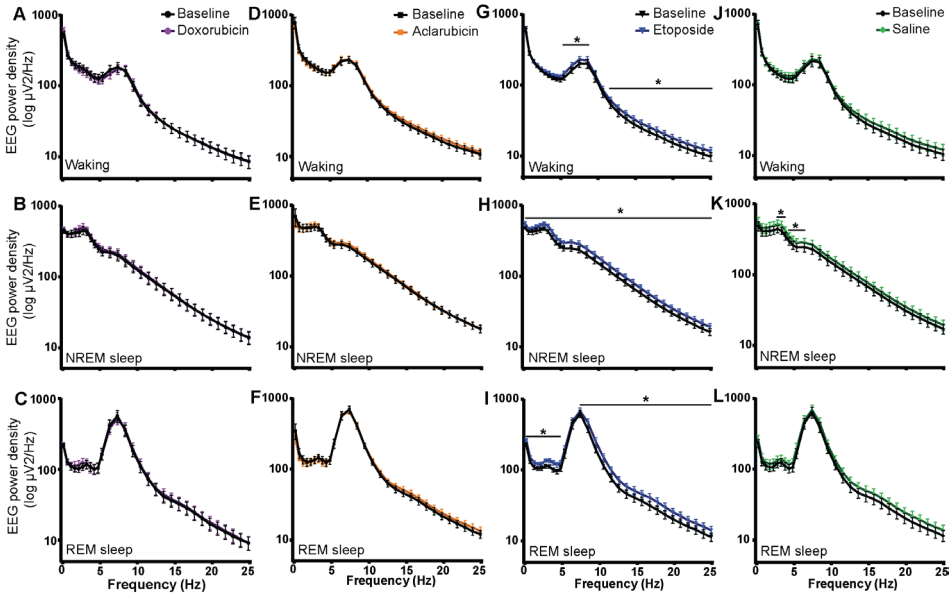


Supplementary Figure S1. Schematic representation of wheel running and passive infrared recording (PIR). Wheel running and PIR data were recorded in the Clocklab system (A). The onset of the behavioral activity was shown in the double plotted actograms with red dot (B). The period and strength of circadian clock (Qp) were determined from the F-periodogram (C). The strength of the rhythms was determined as the difference between the peak (s2) and the corresponding 95% confidence limit (s1) in F-periodogram, as previously described [30,34].

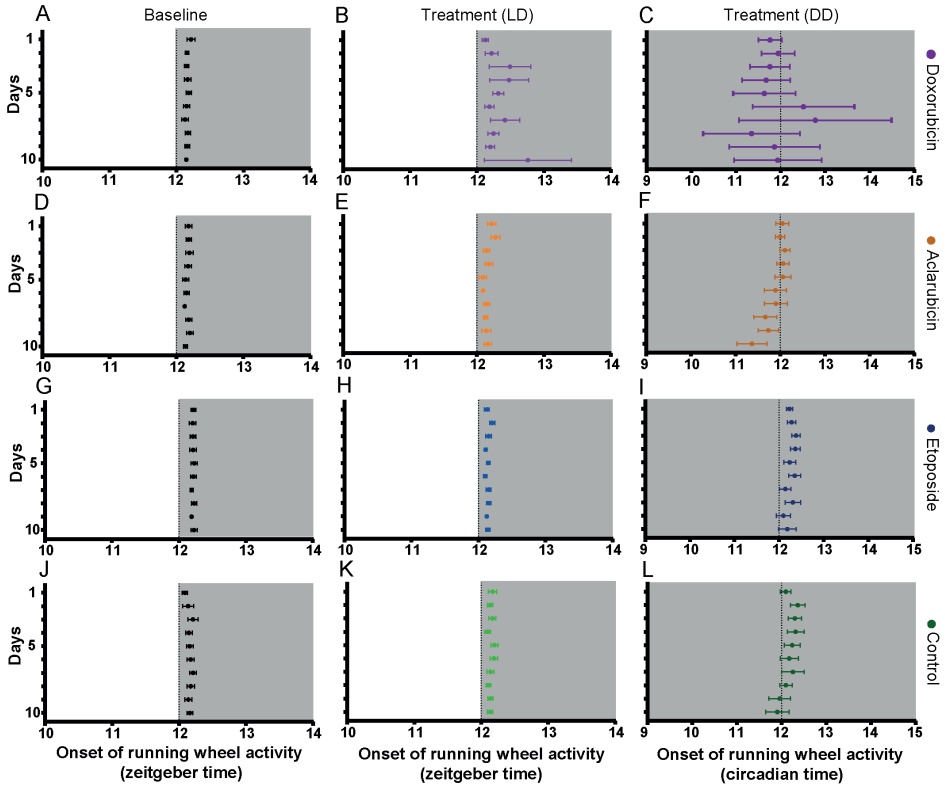


Supplementary Figure S2. Mechanism of action of anthracycline drugs. A. Whole Western blot corresponding to Fig. 2B. Cut out bands are indicated by the red box. Densitometry intensity ratio is indicated above the band. B. γH2AX (green) foci formation upon anthracycline treatment. Representative confocal image of fixed U2Os cells treated for 1 hour with the indicated drugs. DAPI (blue) and Phalloidin (magenta) are stained as nuclear and cytosol marker, respectively. Scale bar; 10 µm. C. Quantification of the γH2AX foci induced upon treatment, nCtr = 28, nDoxo = 37, nAcla = 34, nEtop = 35 analyzed from two independent experiments. Two-way ANOVA with multiple comparisons; ns, not significant, P **** < 0.0001. D. Whole Western blot corresponding to Fig. 2D. Cut out bands are indicated

by the red box. Densitometry intensity ratio is indicated above the bands. E. Nuclear versus cytosolic localization of endogenous H2B levels upon 1 hour treatment with 10μM of the indicated drugs. Representative confocal image of fixed endogenously tagged scarlet-H2B U2Os cells. Scale bar, 10 μm. F. Quantification of the nuclear versus cytosolic H2B signal, nCtr = 105, nDoxo = 102, nAcla = 106, nEtop = 105 analyzed from two independent experiments is plotted. Two-way ANOVA with multiple comparisons; ns, not significant, P **** < 0.0001. G. Part of the nucleus from MelJuSo cells stably expressing PAGFP-H2A was photo-activated. Photoactivated PAGFP-H2A was monitored by time-lapse confocal microscopy for 1 hour in the absence or presence of the indicated drug at 10 μM. Lines in the left panel define the region of cytoplasm, nucleus and activated area pre-treatment. Stills from a movie at 0 and 60 minutes after addition of the drugs are shown. Scale bar; 10 μm.



Supplementary Figure S3. Electroencephalographic (EEG) power density in Waking, NREM and REM sleep of four groups under baseline and treatment condition. Spectra are shown of Waking (A, D, G, J), NREM sleep (B, E, H, K) and REM sleep (G, F, I, L) EEG for the 24-hour before treatment baseline day and three weeks after completion of chemotherapy treatment. Power density values were calculated in 0.5-Hz bins between 0.25 and 5.0 Hz, 1-Hz bins were calculated between 5.25 and 25.0 Hz. P * = 0.05-0.0001 compared with frequency-matched baseline values, two-way ANOVA with Bonferroni multiple comparisons



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Supplementary Figure S4. Onset of wheel running activity of four groups. Average running activity onset time of doxorubicin, aclarubicin, etoposide and control treated mice over 10 days under baseline condition (A, D, G, J), treatment LD condition (B, E, H, K) and treatment DD condition (C, F, I, L). Grey areas indicate the period of darkness.

5

Misalignment of behaviour and electrical activity of SCN in cancer related fatigue animals induced by doxorubicin

Yumeng Wang, Anouk W. van Beurden, Mayke M.H. Tersteeg, Stephan Michel, Anneke Kastelein, Jacques Neefjes, Jos H.T. Rohling, Johanna H. Meijer, Tom Deboer. Internal circadian misallignment in a mouse model of chemotherapy induced fatigue. (submitted)

Abstract

Background: Cancer patients experience long lasting fatigue which correlates with poor quality of life and decreased long-term survival rates. How chemotherapy treatment contributes to this fatigue is poorly understood. Previously we have shown in a mouse model of fatigue that doxorubicin treatment induces fatigue-like symptoms related to disturbed circadian rhythms.

Methods: To investigate this further, we used the doxorubicin induced fatigue mouse model to analyze how chemotherapy affects the timing of neuronal activity in the suprachiasmatic nucleus (SCN), where master circadian pacemaker resides, in vitro and in vivo. Along with that we recorded circadian controlled behavior before and after chemotherapy treatment under both light-dark (LD) and constant darkness (DD) conditions, and we investigated inflammation related gene expression in both spleen and kidney.

Results: Doxorubicin treatment decreased both the running wheel activity and time of using the wheel. This decreased activity lasted for over five week after treatment. Surprisingly, doxorubicin treated mice still showed rhythmic SCN neuronal activity under both LD and DD conditions. However, the timing of peri-SCN areas (the brain areas surrounding SCN) activity was affected by the doxorubicin treatment, indicating an effect on the output of SCN. Furthermore, the correlation between the SCN neuronal activity and the behavior was changed after the doxorubicin treatment which indicates that the alignment between the SCN neuronal activity and behavioral activity was disturbed. Peripheral inflammation only showed small differences five weeks after the last treatment.

Conclusion: Our preclinical study demonstrates that chemotherapy induced fatigue disrupted the peripheral circadian rhythms in brain and behavior which may induce the fatigue like symptoms. Peripheral inflammation probably does not play an important role in long-lasing chemotherapy induced fatigue. Targeted circadian realignment therapies may be a novel and non-invasive way to improve patient outcomes after chemotherapy.

Keywords: Doxorubicin, SCN, circadian clock, fatigue, misalignment, peripheral clock

1. Introduction

Cancer related fatigue is a debilitating disease with grave consequences for wellbeing in cancer survivors. Yet, it is one of the most common complaints among cancer survivors months or even years after their treatment is finished. Cancer survivors have this persistent physical and mental tiredness which is neither caused by increased activity levels nor alleviated by adequate sleep or rest and decreases their quality of life, and may further contribute to increased mortality. [1] A variety of biological mechanisms of CRF have been proposed, such as cytokine dysregulation, hypothalamic-pituitary-adrenal (HPA) axis dysregulation, microglial activation, neuroinflammation and disrupted circadian clock.[1-3] Chemotherapy is one of the most common types of cancer treatment, and is associated with increases fatigue and elevations in certain pro-inflammatory cytokines. Similarly, among cancer patients undergoing chemotherapy, changes in IL-6 and TNF-α were associated with changes in fatigue over the course of treatment.[4,5] Furthermore, dampened (or flattened) daily activity and cortisol rhythms have been reported in chemotherapy-treated cancer patients and cancer survivors,[6,7] thus, chemotherapy induced fatigue may contribute to circadian misalignment. Few studies have investigated the relationship between cancer-related fatigue and the circadian clock.[8] However, interventions aimed at normalizing circadian rhythms have been shown to be able to improve fatigue in cancer patients.[9,10]

Recently we introduced a model of cancer related fatigue with the possibility to investigate differences in outcome between different treatments.[11] The results showed that doxorubicin, the treatment with the worse fatigue outcome, probably influences expression of circadian rhythms. Mammals display 24-h rhythms in behavior and physiology that are an adjustment to the 24-h period of the earth's rotation. This intrinsic rhythm system is hierarchically organized. On top of this system is the suprachiasmatic nucleus (SCN). The SCN functions as the master pacemaker and generates neuronal rhythms which are important for synchronization of the rest of the brain and the peripheral clocks in the body via neuronal and humoral signals.[12]

Doxorubicin is an anthracycline drug which is widely used to treat various types of cancer. The principal mechanism of doxorubicin cytotoxicity is the inhibition or poisoning of. Topoisomerase II, which is important in relaxation of tension in the DNA, condensation of chromatin, and decatenation of DNA

[13]. Inhibition of topoisomerase II will induce apoptosis. Thus, doxorubicin not only kills the tumor cells but also kills healthy cells and it can affect healthy organs and systems as well [14]. In rodent models of fatigue, fatigue-like behavior has been reported using different cytotoxic agents, including doxorubicin.[15,16]

In the present study we investigated the putative mechanisms of cancer treatment related fatigue by recording neuronal activity in the suprachiasmatic nucleus (SCN) of the hypothalamus together with rest-activity behavior in the same animal before and after doxorubicin treatment. The results show that in mice with chemotherapy induced fatigue SCN neuronal rhythmicity is normal. However, the mice show locomotor activity at an inappropriate time of day relative to the rhymical neuronal SCN. This suggests that doxorubicin disrupts peripheral circadian rhythms and supports the possibility that targeted circadian realignment therapies may be an intervention after chemotherapy.

2. Materials and methods

2. 1. Ethics Statement

All the experiments were performed according to institutional and national guidelines. The experiments were approved by the Central Committee on Animal Research (CCD, the Hague, 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. Animals

Male C57BL/6J mice (10 weeks old; obtained from Envigo, Horst, the Netherlands) were group housed in the animal facility of the Leiden University Medical Center (LUMC, Leiden, The Netherlands) under controlled conditions (12:12 h light:dark cycle; lights on at 9:00) with food and water ad libitum in a temperature controlled room (21–24 °C).

2. 3. Surgery

Surgery was performed when the mice were 12 weeks old. *In vivo* SCN recording techniques were as described previously.[17] Mice were anesthetized (Ketamine 100 mg/kg; Xylazine 10 mg/kg; Atropine 1 mg/kg)

and were implanted with tripolar electrodes (stainless steel, diameter 0.125 mm; Plastic One, MS333-3-BIU-SPC, Roanoke VA, United States) into the SCN. The electrodes were implanted at a 5° angle with the following coordinates: 0.61 mm lateral from Bregma and 5.38 mm ventral to the dura. The third electrode was placed in the cortex for reference. Electrodes were fixed to the skull with dental cement. Buprenorphine (0.1 mg/kg temgesic) was administered to provide post-surgery analgesia along with a heat lamp until the mice were able to move. Subsequently, the mice were allowed to recover for 7 days.

2. 4. Behavioral Assessment

Running wheel (diameter: 24cm) activity and passive infrared (PIR) cage activity were recorded with ClockLab data collection software (Actimetrics, Illinois, USA) and were stored in 1-min bins.[11] Group housed mice (10 weeks old) were provided with a running wheel for two weeks. Subsequently, after the surgery, the mice were individually housed with a PIR sensor and running wheel in a 12:12 h light: dark cycle. Baseline recordings for the running wheel and PIR activity were performed for two weeks followed by cytostatic treatment. During the treatment and recovery period, as well as, during MUA recordings only PIR activity was recorded.

2. 5. Drug Preparation

Mice were treated as indicated (i.p.) for 4 times (day 1, 2, 9 and 10) over a 10 day period with 3,75mg/kg doxorubicin (n = 26) and 0.9% saline (n = 18) one hour after light on. This concentration was found previously to induce fatigue in mice.[11]

2. 6. *In vivo* Electrophysiology

The baseline recording of running wheel and PIR were followed by *in vivo* electrophysiological recordings.[17,18] Animals were placed into a custom-designed recording chamber for measurement of SCN electrical activity and connected to a flexible cable and a counterbalanced swivel system. Conditions in the experimental chamber were similar to the home cage, including food and water availability (12:12 h light-dark cycle; lights on at 9:00). The electrical signal was amplified and bandwidth-filtered (0.5 – 5 kHz). Window discriminators were used to convert action potentials into digital pulses,

which were stored for offline analysis in 10-s epochs (CIRCAV1.9 custom-made software). During the recording, the mouse was able to move freely. The movement of the mouse was recorded by PIR detectors in 10-s epochs. All data were stored for offline analysis. Three baseline days were recorded 1 day prior to treatment. Three weeks after treatment with cytostatic or control a second recording was performed. During the second recording, the first three days were recorded under 12:12 light-dark cycle, subsequently, the mice were recorded under constant darkness condition for another three days.

2. 7. Histology

At the end of the in vivo electrophysiology experiment, the location of the electrodes was verified by histology. The mice were anesthetized with 4% - 6% isoflurane then euthanasia by the guillotine. To confirm the recording area, an electric current of 0.8 mA for 4 sec was delivered using the lesion-making device (Ugo Basile, Gemonio VA, Italy). The lesion can be confirmed by both iron staining using 0.5 % potassium ferrocyanide in 4% paraformaldehyde and Nissl staining of the brain slices (40 μm) using cresyl violet solution.

2. 8. Relative real-time quantitative PCR

RNA was extracted from spleen and kidney using Quick-RNA Miniprep Plus Kit (ZYMO research, cat#: R1058). RNA concentrations were measured using a spectrophotometer (NanoDrop) with 260/280 ratios ~1.9–2.2. One μg of cDNA per sample was reverse transcribed (RT) from extracted RNA using transcriptor first strand cDNA synthesis kit (Roche, cat#: 04896866001). Gene expression was determined using quantitative polymerase chain reaction (qPCR) reactions with a 384-well thermal cycler and the following TaqMan probes (Thermo Fisher Scientific): COX2 (assay: Mm03294838_g1), CXCL10 (assay: Mm00445235_m1), IFN-beta1 (assay: Mm00439552_s1), IFN-gamma (assay: Mm01168134_m1), IL-10 (assay: Mm01288386_m1), IL-1b (assay: Mm00434228_m1), IL-6 (assay: Mm00446190_m1), GAPDH (assay: Mm99999915_g1). Relative gene expression was ultimately calculated using the comparative CT method ($2^{-\Delta\Delta CT}$) by subtracting genes of interest (COX2, CXCL10, IFN-beta1, IFN-gamma, IL-10, IL-1b, IL-6) to the geometric mean of the housekeeping genes GADPH), and then normalizing these ΔCT values to the averageΔCT of vehicle-treated mice to determine fold change in gene expression ($2^{-\Delta\Delta CT}$).

2. 9. *Ex vivo* Electrophysiology

Ex vivo recordings were performed in a separate experiment. The experimental design is shown in supplementary Figure 3. A. *Ex vivo* SCN recording techniques were as described previously (ref).[19] Mice were under constant darkness condition for 2 weeks and then sacrificed under dim red light at circadian time (CT) 6 (± 1.0 h), which is the time with the highest neuronal activity in the SCN.[20] The brain was removed within one minute after decapitation and brain slices (450 μm thick) were prepared using a tissue chopper. The slice containing the SCN was transferred to a laminar flow chamber within five minutes after decapitation. The slices were bathed in an oxygenated artificial cerebrospinal fluid (ACSF) solution containing (in mM): NaCl 116.4, KCl 5.4, NaH2PO4 1.0, $MgSO_4$ 0.8, $CaCl_2$ 1.8, $NaHCO_3$ 23.8, glucose 15.1 and 5mg/L gentamicin (Gibco) saturated with 95% O_2 and 5% CO_2 (pH = 7.2-7.4, 290-310 mOsm). The slices were stabilized using an insulated tungsten fork and maintained for 1 hour prior to placing the electrode (50 μm; 90% platinum and 10% iridium) in the center of the SCN. Action potentials were amplified 10 times and bandpass-filtered (0.3 Hz – 3 kHz). Action potentials that exceeded a predetermined threshold set well above noise (~5 μV) were counted in 10-second bins using custom-made automated software.

2. 10. Brain-behavior correlation.

Behavioral data and MUA recordings were simultaneously collected by CIRCAV1.9 and further analyzed in MATLAB (version R2019b, MathWorks). The analysis was carried out on at least 3 days of recordings for each condition (baseline, post-treatment LD and DD). The PIR data and MUA signal were first smoothed and then a sine wave was fitted to the smoothed signals by use of FFT and nonlinear fitting. The period length (tau) and peak time were extracted from the fitted sine waves. The PIR data was smoothed with a moving average filter (movmean function, window of 1 h). The MUA signal was first low-pass filtered (lowpass function, Signal Processing Toolbox, $F_{cut\text{-}off}=2e^{-4}$ Hz) and detrended (detrend function, 4^{th} order polynomial) and then smoothed with a penalized least-squares algorithm.[21] The Pearson correlation (corr function, Statistics and Machine Learning Toolbox) was used to estimate the correlation between the behavioral data and MUA recordings. The tau of either PIR or MUA in the range of 22 – 26 hours will be include for the further analysis.

2. 11. Data Analysis

Behavioral data with wheel running was collected and further analyzed in ClockLab (version 6, Actimetrics). A rest period of at least 10 min in the wheel running or the home cage activity was used as a definition of resting time. The analysis was carried out on 10 days of wheel running and cage activity for each condition (baseline, post-treatment LD and DD). We calculated various circadian locomotor activity variables, including circadian period and strength of the circadian clock was calculated using a F-periodogram, averaged activity profile and total amount of daily activity. Analyses for non-parametric variables included interdaily stability, which quantifies the synchronization to the 24 h light–dark cycle, and intradaily variability, which quantifies rhythm fragmentation. The 24 h profiles were created by averaging the total counts from a 10-day period over the 24 h day for each mouse, and subsequently calculating group averages. In the constant darkness condition, the 24 h profiles were created similarly to those in the 12:12LD condition except that the group averages were created by aligning profiles at the onset of activity for each mouse separately. Behavioral data with MUA recordings were collected by CIRCAV1.9 and further analyzed in MATLAB (version R2019b). The analysis was carried out on at least 3 days of locomotor activity for each condition (baseline, post-treatment LD and DD).

2. 12. Statistical analysis

For data analysis, GraphPad was used. Anderson-Darling test and Shapiro-Wilk test were used for the normality test before either t-test or one-way ANOVA. Paired student's t-tests were used when distributions were normal; otherwise, the nonparametric Wilcoxon test was used to compare the difference between baseline and post-treatment conditions. Unpaired student's t-tests were used when distributions were normal; otherwise, nonparametric Mann-Whitney tests were used to determine statistically significant differences between control and doxorubicin treated groups. Two-way ANOVA was used to calculate effects of time and drug treatment. Two-way repeated measure ANOVA was used to determine the body weight change over time. If the result was significant we ran post hoc Bonferroni multiple comparisons, and the significant results are reported. One way repeated measures ANOVA with Geisser-Greenhouse correction were used to determine the effect of treatment, and Dunnett's multiple comparisons test was used to determine statistically significant differences compared with baseline. Values of $p < 0.05$

were considered statistically significant.

3. Results

3. 1. Doxorubicin treated mice showed fatigue like symptoms and disrupted circadian behavioral rhythm

We have previously shown that mice treated with doxorubicin develop fatigue like symptoms similar to cancer patients.[11] Wheel and cage activity (PIR) were recorded as it was shown in Fig.1 A. In the actograms of running wheel activity, the doxorubicin treated mice showed decreased activity under both 12h:12h light-dark (LD) and constant dark (DD) conditions, whereas the control animals did not show decreased activity after treatment (Fig. 1B). The primary measure is the wheel running distance of the mice which was also reported in our previous study [11] and in other research.[15,22] In addition, we introduce resting time as an alternative measure for chemotherapy-induced fatigue. To analyze the running distance and resting time of both doxorubicin and control treated mice, we quantified the 24 h profile of resting time under both 12h:12h LD and DD conditions over 10 days (Fig.1 C). To better visualize differences, we used the relative change compared to baseline in both doxorubicin and control groups under both LD and DD conditions (Fig.1 E and G). Treatment with doxorubicin not only decreased the running distance but also increased the resting time during darkness (Fig. 1 C and D; Suppl Figure 2 A and B). The doxorubicin treated mice showed a clear increase in resting time between ZT 14 – ZT 24 compared to control (Fig. 1 C). Over 24-h the doxorubicin treated mice (18,71 ± 0,34 hours) showed more resting time compared with baseline condition (15,85 ± 0,34 hours) (Fig.1 D). And the control mice did not showed a difference compared with baseline (15,93 ± 0,39 hours) and post-treatment condition (15,63 ± 0,32 hours) (Fig.1 D). The increased resting time can also be seen in the cage (non-wheel running) activity. Here resting time is not only increased during the dark phase or subjective night but also during the light phase or subjective day (Supplementary Fig.1A - E).

To extend these findings, we applied non-parametric circadian rhythm analysis. Reduced stability and increased variability appear to underpin circadian rhythm fragmentation and instability in doxorubicin treated mice under LD condition (Fig. 1 H and Supplementary Fig.1 F). The rhythm of control mice was more stable compared with baseline condition (Fig. 1

I and Supplementary Fig. 1G). Under DD conditions, doxorubicin treated mice exhibited a shorter circadian period in both wheel running and cage activity (Supplementary Fig.2 C, G). We further determined the strength of the circadian rhythm (Qp) by F-periodogram. We found that the Qp of wheel running of doxorubicin treated mice showed a significant decrease under DD, which indicated that treatment with doxorubicin destabilizes the circadian clock in the absence of external time cues (Supplementary Fig. 2 D). The strength of the rhythm in cage activity was decreased under LD and DD conditions (Supplementary Fig. 2 H). These latter effects were not seen in the control animals.

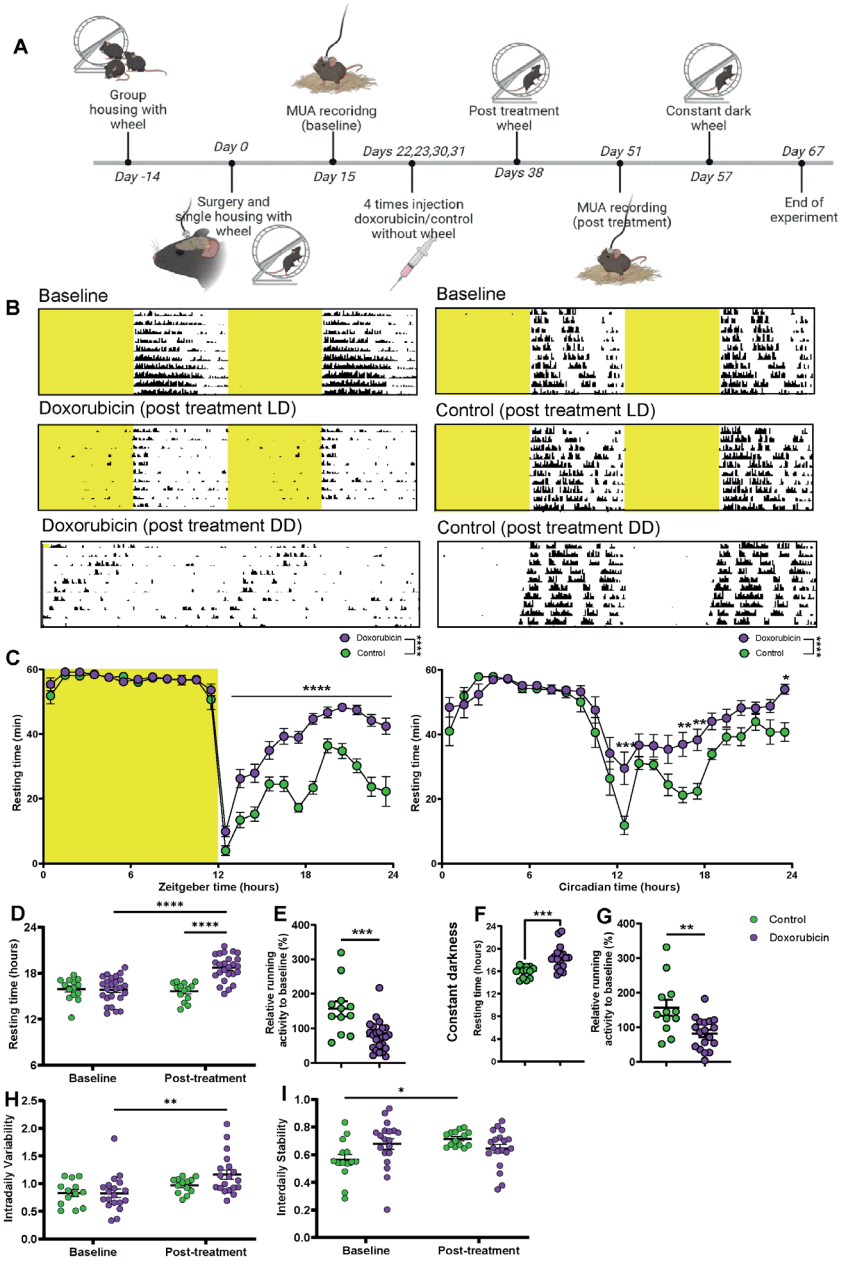

Figure 1 Doxorubicin treated mice showed fatigue like symptoms and disrupted clock. (A) Experimental design of the running wheel and multi-unit neuronal activity recording under light-dark and constant darkness conditions. (B) Representative double-plotted actograms of vehicle (right) and doxorubicin (left) treated mice under baseline, post-treatment (LD) and post treatment (DD) conditions. bin = 10 min. (C) Average resting time/h over 10 days under post-treatment LD condition (left), and post-treatment DD condition (right). (D) Average resting time/day over 10 days under baseline and post-treatment LD condition. (E) Relative running wheel activity over 10 days under LD of doxorubicin and control groups.

(F) Average resting time/day over 10 days under post-treatment DD condition. (G) Relative running wheel activity over 10 days under DD of doxorubicin and control groups (H) Intradaily variability under baseline and post-treatment LD condition. (I) Interdaily stability under baseline and post-treatment LD condition. Values are shown as mean ± SEM. Yellow indicate the light phase.

3. 2. After doxorubicin treatment the *in vitro* timing in the Suprachiasmatic Nucleus is affected

The *ex vivo* recordings from SCN slices show that the neuronal activity in both doxorubicin treated and control brain slices exhibit circadian modulations in activity (Supplementary Fig. 3B, C, E). Circadian time was calculated based on the timing of running wheel activity on the previous day. The peak time of SCN MUA activity in brain slices of doxorubicin treated mice showed considerably more variability compared with the control slices (Supplementary Fig. 2D). This may indicate that the timing of the SCN *in vitro,* in the absence of input from other brain areas, may be affected by doxorubicin. Because the rest-activity behavior is not only influenced by the output from the SCN, but can in itself also affect and feed-back on the SCN timing system. We, therefore, considered it necessary to also analyze whether the timing system of the SCN itself is also changed *in vivo*.

3. 3. After doxorubicin treatment the *in vivo* timing of neuronal activity in the Suprachiasmatic Nucleus is not affected

To investigate whether the timing system of the SCN is also affected in intact freely moving doxorubicin treated mice, we performed *in vivo* SCN neuronal activity recordings in both doxorubicin and control treated mice which were kept in 12h : 12 h LD under pre- and post-treatment conditions. The location of the electrodes is shown in Supplementary Fig. 4 for both SCN and peri-SCN area recordings. Peak time of cage activity (or locomotor activity) in the recording setup and electrical activity in SCN and peri-SCN area were extracted from the fitted sine waves (Fig. 2 A, B). The peak timing of behavior (ZT time of baseline and post treatment) was advanced compared with the baseline condition in doxorubicin treated mice, whereas there was no significant difference between these peak times in control mice (Fig. 2C). To our surprise, the timing of the MUA SCN activity peak (average ZT time) was not significantly different compared to baseline condition in either of the two treatment groups (average ZT time) (Fig. 2 D) which indicates that the SCN remains its timing of 24 hour circadian rhythmicity after doxorubicin

treatment. However, the timing system in peri-SCN area was affected by the doxorubicin treatment compared with baseline (Fig. 2 E). Here again there was no significant difference in control mice (Fig. 2 E). Under these conditions (3 days in LD), the tau of behavior and MUA activity was not significantly different in both doxorubicin and control treated mice between pre and post treatment conditions (Supplementary Fig.5).

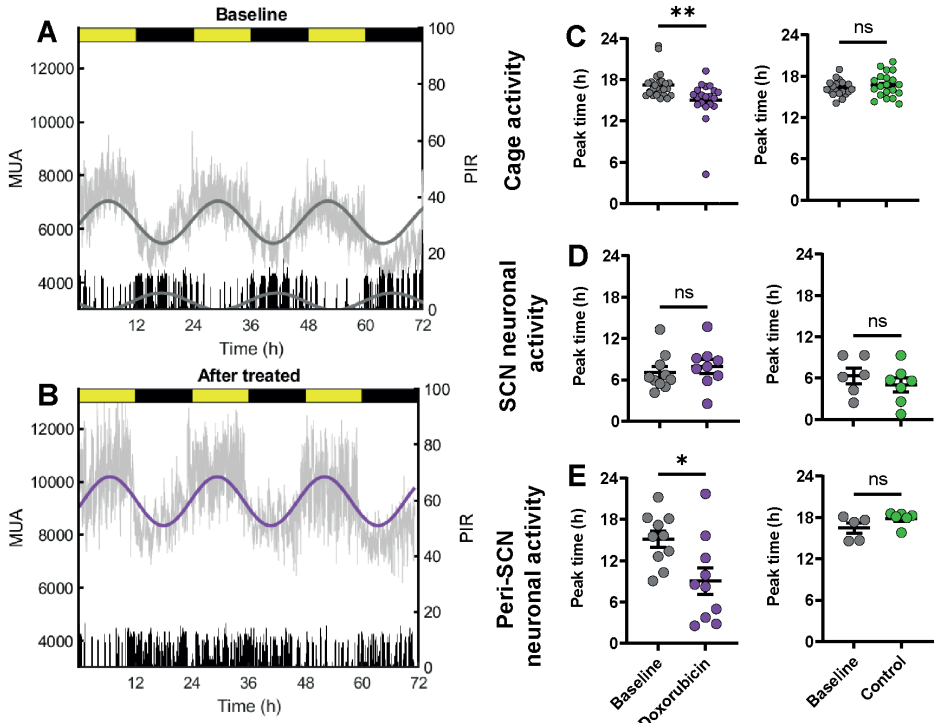

Figure 2 Timing of locomotor activity and peri-SCN are affected after doxorubicin treated in vivo. (**A**) Representative multi-unit recording and locomotor activity of doxorubicin treated mouse under baseline condition. Light grey: raw data. (**B**) Representative multi-unit recording and locomotor activity of doxorubicin treated mouse under post-treatment condition. (**C**) Peak time of cage activity of doxorubicin and vehicle treated mice under baseline and post-treatment conditions (**D**) Peak time of SCN neuronal activity of doxorubicin and vehicle treated mice under baseline and post-treatment conditions. (**E**) Peak time of peri-SCN neuronal activity of doxorubicin and vehicle treated mice under baseline and post-treatment conditions. Values are shown as mean ± SEM. Yellow indicate the light phase, black indicate the dark phase.

3. 4. Misalignment between behavioral and SCN neuronal activity in doxorubicin treated mice in light-dark condition

As we observed that the timing system of SCN does not seem to be affected

by the doxorubicin treatment under light-dark condition, but we still observed the fatigue like symptoms, we considered that the behavioral output from the SCN will show reduced precision after treatment. Cancer related fatigue may be due to a misalignment of SCN activity and behavioral activity. Under normal healthy conditions, the SCN MUA activity and behavioral activity is highly negatively correlated in nocturnal rodents.[23,24] Hence, having recorded the neural activity in the SCN, we next turned to examine the correlation between neural and behavioral activity in both doxorubicin treated and control mice. Figure 3A shows SCN neuronal activity together with behavioral activity in the recording setup for three days under 12h: 12h LD condition. It is clear that the behavioral activity in the doxorubicin treated mouse was not as rhythmic as the control. The sine-wave fit for SCN neural activity remained its robust rhythm after the doxorubicin treatment (Fig.3 A). To go one step further, we analyzed the correlation for the raw, low pass and sine-wave fit of SCN MUA with the behavioral data. In the control condition, high levels of negative correlation remained over the entire experiment (Fig. 3B, D and F). However, after the doxorubicin treatment, mice showed little or no negative correlations compared with baseline and control conditions (Fig. 3 C, E, G), which indicates that the relationship between behavior and the SCN neuronal activity was weaker compared with control mice.

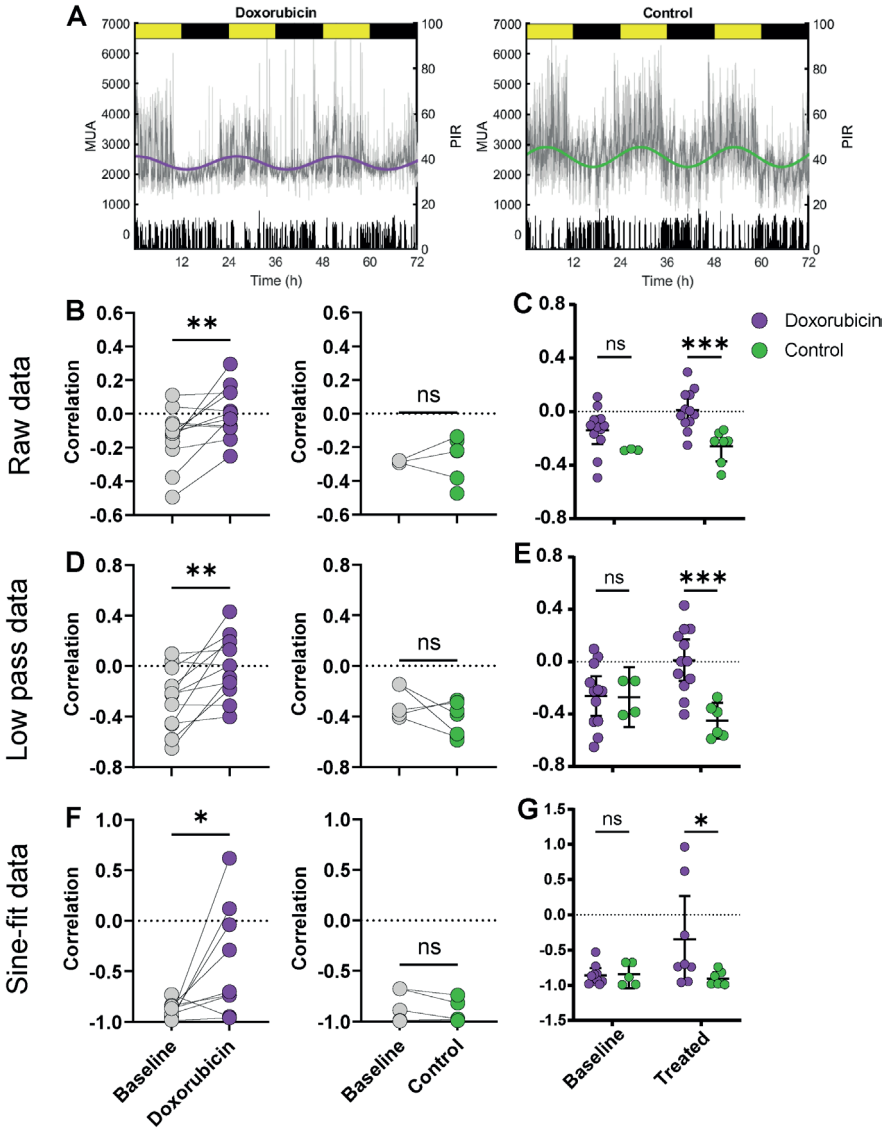

Figure 3 Doxorubicin treated mice showed misalignment of SCN electrical activity and behavioral activity (**A**) Representative sine-wave fit multi-unit activity and locomotor activity of doxorubicin (left) and vehicle (right) treated mouse under post treatment conditions. Light grey: raw data; dark grey: low-pass data; green and purple: sine-wave fit data. (**B - C**) SCN-behavior correlation of raw data of doxorubicin (left) and control (right) groups under both baseline and post-treatment (LD) conditions. (**D-E**) SCN-behavior correlation of low-pass data of doxorubicin (left) and control (right) groups under both baseline and post-treatment (LD) conditions. (**F-G**) SCN-behavior correlation of sine-wave fit data of doxorubicin (left) and control (right) groups under both baseline and post-treatment (LD) conditions. Values are shown as mean ± SEM. Yellow indicate the light phase, black indicate the dark phase.

3. 5. Misalignment of peri-SCN area neuronal and behavioral activity in doxorubicin treated mice in light-dark condition

The outflow of SCN information is only pertained to the medial hypothalamus because most of the SCN projections are directed towards target areas that contain mainly interneurons (ref).[25,26] Thus, the areas surrounding the SCN, the peri-SCN area, receive the timing information from the SCN and these areas further control the behavioral and hormonal rhythms. As is shown in the control mouse, the behavioral rhythm is in-phase with the neuronal activity of the peri-SCN areas. These areas showed higher neuronal activity during the night and lower activity during the day (Fig 4 A). However, in the doxorubicin treated mouse, both locomotor activity and neuronal activity in the peri-SCN are disrupted and not in phase (Fig. 4 A). Hence, we performed the same behavior-neuronal correlation method and found a positive correlation between neuronal activity and behavior in baseline condition (Fig. 4 A). Next, we compared the correlation between baseline and treated condition, we found the correlation in doxorubicin treated mice showed less or no positive correlation compared with baseline and control condition (Fig. 4 C, E, G). When we compared the control mice, the correlation after sine-wave fit is very close to 1 under both baseline and post-treated conditions (Fig. 4 B, D, F). This may indicate that the circadian timing system is disrupted downstream of the SCN, with a reduction or loss of connectivity from the SCN to the periphery in the doxorubicin induced fatigue mice.

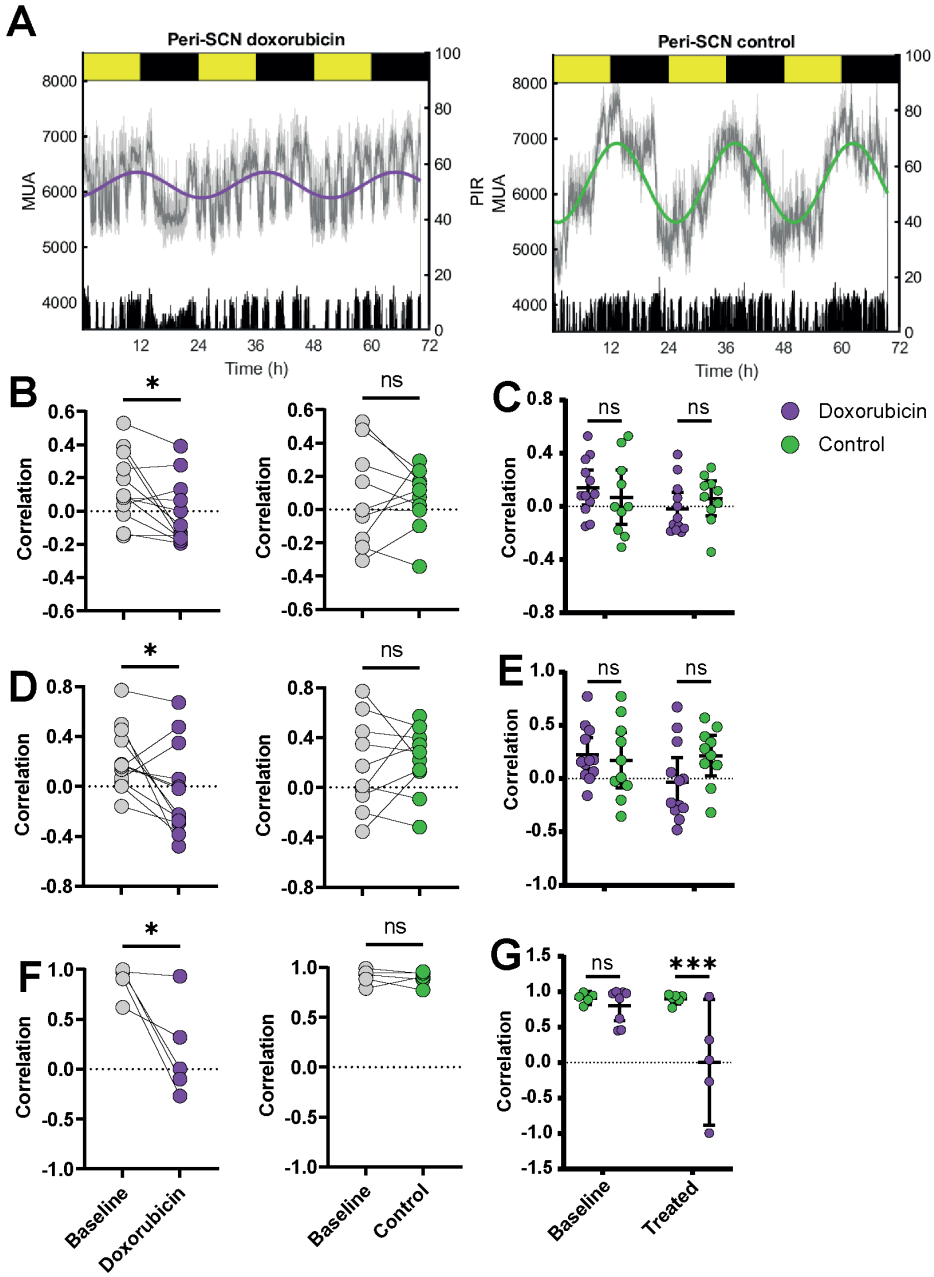

Figure 4 Doxorubicin treated mice showed misalignment of peri-SCN electrical activity and behavioral activity (**A**) Representative sine-wave fit multi-unit activity and locomotor activity of doxorubicin (left) under ? conditions. (**B - C**) peri-SCN-behavior correlation of raw data of doxorubicin (left) and control (right) groups under both baseline and post-treatment (LD) conditions. (**D-E**) peri-SCN-behavior correlation of low-pass data of doxorubicin (left) and control (right) groups under both baseline and post-treatment (LD) conditions. (**F-G**) peri-SCN-behavior correlation of sine-wave fit data of doxorubicin (left) and control (right)

groups under both baseline and post-treatment (LD) conditions. Values are shown as mean ± SEM. Yellow indicate the light phase, black indicate the dark phase.

3. 6. Misalignment of behavioral and peri-SCN neuronal activity in doxorubicin treated mice under constant dark

SCN neurons have a distinct, topographically organized coupling mechanism which allows them to remain synchronized to one another even in constant conditions. Thus, we performed multi-unit recording under constant darkness to determine the extent to which doxorubicin affects rhythms in the absence of light information. The peak time of SCN neuronal activity was around CT 6 and showed no difference between doxorubicin and control treated mice (Fig. 5 C). The peri-SCN peak time showed more variation in the doxorubicin-treated mice compared to the control mice, which showed a stable peak time around CT 18, similar to the peak time of locomotor activity (Fig. 5 B, D). The SCN of the doxorubicin treated mice maintained its near 24 hour rhythmic neuronal activity, but the inhibition of SCN neuronal activity during increased locomotor activity is less strong compared with control mice (Fig.5 E). In contrast, the peri-SCN timing shows an effect of doxorubicin mainly in the circadian timing between behavior and the peri-SCN area. (Fig 5F)

Also here we see a reduction in correlation between behavior and SCN neuronal activity in the doxorubicin treated mice, which becomes significant in the correlation of the raw data (Fig. 5 and Supplementary Fig.6). The n in the doxorubicin group under constant darkness is lower due to the loss of doxorubicin treated animals in the course of the experiment due to illness/ euthanasia. This may give a survival bias to the data, rendering more positive results in the treatment group, and clearly reduces the power of the statistical analysis.

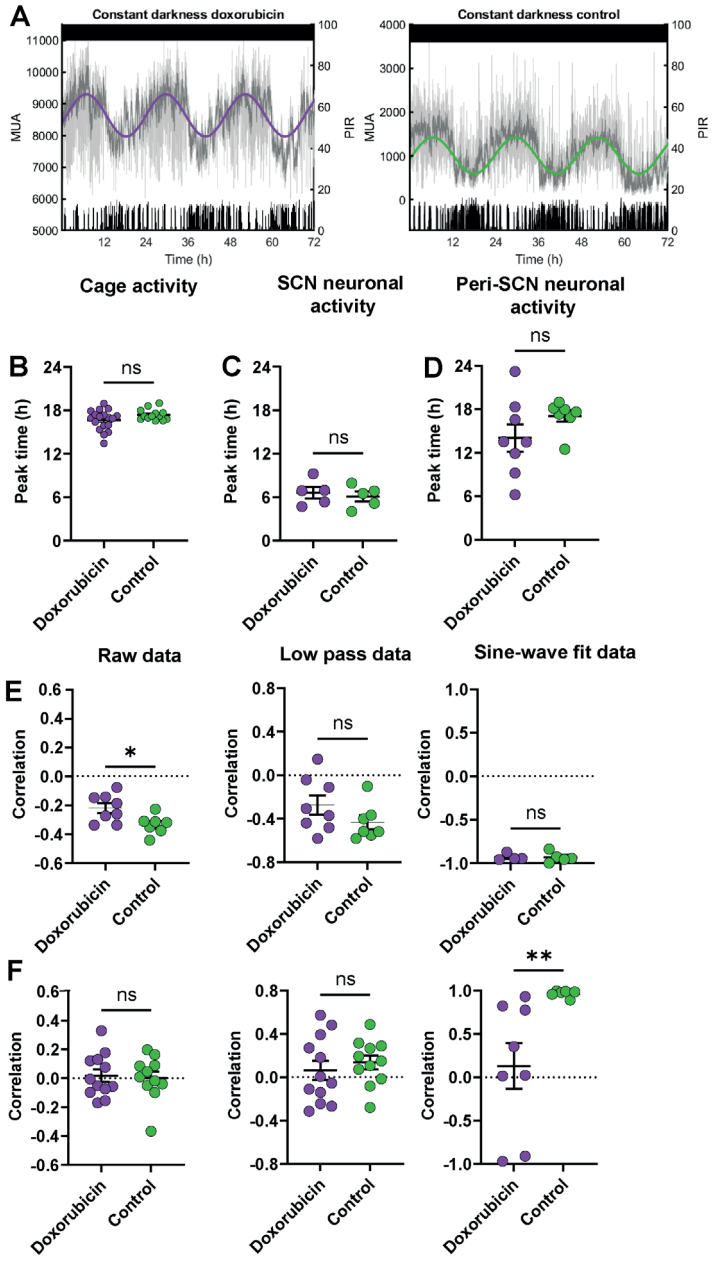

Figure 5 Doxorubicin treated mice showed less correlation of SCN and behavior under constant darkness (**A**) Representative sine-wave fit multi-unit activity and locomotor activity of doxorubicin and control treated mouse under constant darkness conditions. (**B**) Peak time of cage activity of doxorubicin and vehicle treated mice under post-treatment conditions (**C**) Peak time of SCN neuronal activity of doxorubicin and vehicle treated mice under post-treatment conditions. (**D**) Peak time of peri-SCN neuronal activity of doxorubicin and vehicle treated mice under post-treatment conditions. (**E**) SCN-behavior correlation of

raw data, low-pass data and sine-wave fit data of doxorubicin and control groups under post-treatment conditions. (**F**) peri-SCN-behavior correlation of raw data, low-pass data and sine-wave fit data of doxorubicin and control groups under post-treatment conditions. Values are shown as mean ± SEM.

3. 7. Doxorubicin induces weight loss but does not induce significant inflammation changes in spleen and kidney

Mice treated with doxorubicin failed to maintain or increase body mass after the first injection (Supplementary Fig.6 A). Five weeks after treatment, mice treated with doxorubicin had a relatively smaller spleen and kidney compared with the control group, but did not differ in the relative heart weight (Fig.6 A and B; Supplementary Fig.6. B). Doxorubicin did not alter the kidney mRNA expression of multiple markers of inflammation. The gene expression of pro-inflammatory cytokine and chemokine (IL-10 and IFN-gamma) could not be detected in either vehicle or doxorubicin treated groups, IL-1beta and IL-6 were not detected in the spleen in both groups. Five weeks after treatment, *Cox2* and *Ifnb* expression were significantly increased in the spleen (Fig. 6 B), whereas *Tnfa* was decreased (Fig. 6 B). Doxorubicin did not change the kidney *Il1b*, *Il6*, *Cox2*, *Cxcl10*, *Ifnb*, *Tnfa* gene expression five weeks post treatment (Fig. 6 D)

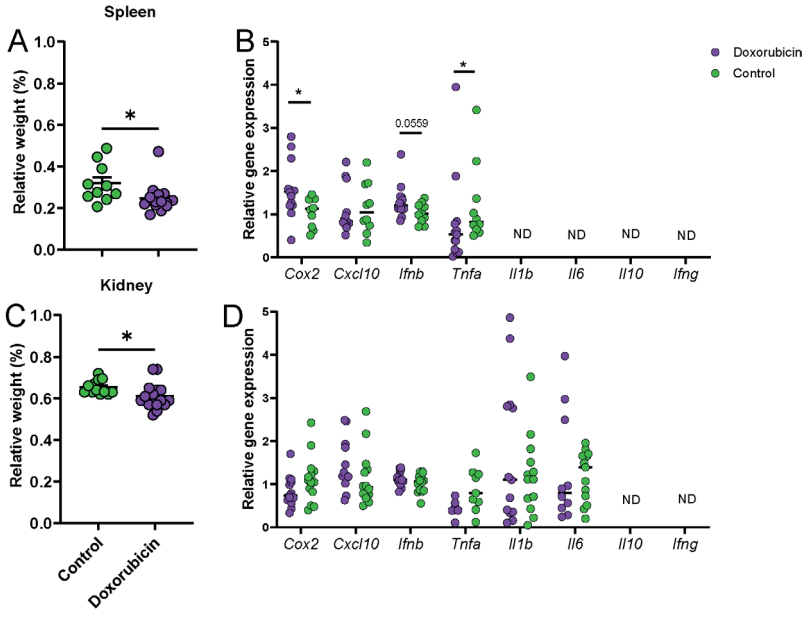

Figure 6 Organ weight change, peripheral inflammation and brain lipids after doxorubicin treated. (**A-B**) Relative weight change of spleen and kidney in control and

doxorubicin treated groups. (**C**) Peripheral inflammatory related gene expression of spleen at long-term time points. (**D**) Peripheral inflammatory related gene expression of kidney at long-term time points. Values are shown as mean ± SEM.

4. Discussion

The present study describes for the first time how doxorubicin, a commonly used chemotherapeutic drug, affects the rest-activity behavior rhythm in relation to neuronal activity rhythms of the brain. We find that SCN neuronal activity is intact after doxorubicin treatment, but that peri-SCN brain activity and locomotor activity of the mice are disturbed and desynchronized from the SCN circadian rhythm. The effects we find are unlikely to be caused by inflammatory mechanisms as inflammatory markers did not show large effect changes due to treatment.

We first examined the effects of doxorubicin on voluntary wheel running under LD conditions. The treated mice did not show differences during the light phase and at the beginning of the dark phase compared with control mice. However, the treated mice ran less under the dark phase, especially during the second half of the dark phase, as previously described.[11] In the present study, we additionally show that doxorubicin treated mice also display increased rest and a significant increase in the fragmentation of the rest/activity cycle compared with baseline condition. Fatigue has been commonly reported in cancer patients. Accordingly, in response to the chemotherapeutic agents, there were studies that showed impaired molecular circadian clocks in the SCN, liver, adrenals, and other peripheral organs of chemotherapy induced fatigue mice a few days after treatment.[8,27] Therefore, fatigue like symptoms and a disrupted circadian clock may be quite common in patients and animal models under treatment.[22,28]

In subsequent experiments, with multi-unit recording in SCN slices, we wanted to assess in treated mice the effect of doxorubicin on neuronal activity in the central circadian clock. Recent studies have shown that in paclitaxel chemotherapy induced fatigue. However, the SCN slices cultured from these mice did not exhibit changes in period of PER2::LUC rhythms. This is similar to what we found in the *ex vivo* MUA experiment. This may indicate that acute or chronic application of chemotherapy will not change the rhythm in SCN slices. However, we do find the peak timing to be different after treatment, with larger variability in peak time after doxorubicin treatment. Previously, we suggested that the output from the SCN is decreased or changed by

the chemotherapy treatment,[11] which may explain both the changes in behavioral and hormonal rhythms (e.g., CORT rhythm).[6,29,30] To function as a central pacemaker, the SCN not only has to display a strong timekeeping property itself, but it also needs to be able to synchronize other brain areas and peripheral clocks.

To investigate the effect of this increased variability in SCN neuronal activity rhythms in an intact mouse model, we perform *in vivo* recording. Our previous study showed long-lasting fatigue-like symptoms in the model.[11] Thus we also performed MUA neuronal recordings at least three weeks after the last injection. As previously, we found that the 24 h rhythmic behavior was much more disrupted after doxorubicin treatment compared with baseline condition in the same mouse, but to our surprise, the SCN maintained its 24 hour neuronal activity (Fig. 2 A and B). Many studies suggest or hypothesize that the central clock's circadian timing system is disrupted in fatigue models or patients (ref). Here we show that even though the mice show disrupted circadian behavior, its 24-h rhythmic electrical activity in the SCN was mostly intact.

We therefore analyzed in more detail the relationship between neuronal activity and behavioral activity and correlated them with each other. We have performed this analysis on three different levels, from the one-on-one 10 seconds epochs up to the fitted 24-h/circadian sine wave. Assuming that the 10-sec epochs represent more the feedback of activity to the SCN (van Oosterhout 2014), whereas the sine wave represents the influence of the SCN circadian pacemaker on the behavioral 24-h timing, we are able to draw some conclusions. The fatigue model mouse showed a less negative correlation between the 10-sec behavior and the 10-sec SCN neuronal activity after doxorubicin treatment. These results suggest that the SCN decreases the sensitivity of the network to the input of the behavior or the input of the behavior is insufficient to influence the SCN, which can protect the SCN 24 hour electrical rhythmicity to disturbances of inappropriately timed behavior under post-chemotherapy conditions. As the effect of doxorubicin was not observed in the master clock, the neuronal activity in the peri-SCN areas was analyzed as well. Intriguingly, the peak time of peri-SCN area neuronal activity was changed, indicating that the circadian timing was disrupted in these peri-SCN areas after doxorubicin treatment. This change is in line with the changes in the peak time of the rest-activity behavioral rhythm. The correlation between the 10-sec behavioral and peri-SCN neuronal activity

did not change, but fatigue mice lost the significant correlation in sinusoidal rhythmic neuronal activity after the doxorubicin treatment. This suggests that the SCN normally coordinates peripheral clocks to ensure proper optimum function, but this function is compromised in the absence of effective SCN outflow after chemotherapy.

The environmental light/dark cycle exerts a powerful synchronizing effect on the endogenous circadian clock of the SCN and supports its functioning. In order to assess the effect of doxorubicin in the absence of this support, the running wheel activity of mice in constant darkness was determined. The fatigue mice shortened their locomotor activity period under DD condition more than control mice. Our results were slightly different compared with a previous study which used another chemotherapy agent, paclitaxel, which lengthened the wheel running circadian period the first week aftertreatment.[8] In contrast, other groups have reported no effects on circadian period directly after γ-radiation in DD.[31] All these different results suggest that different therapies may have different effects, and that the effect may also depend on the time between treatment and measurement. Moreover, the strength of the clock also decreased under constant darkness condition, which is in line with our previous finding.[11] The more profound effects of doxorubicin on the reduced strength of the clock in constant darkness compared to a LD cycle demonstrates the strong supporting influence of the light dark cycle on clock functioning.

Does the disruption of circadian rhythms in constant darkness observed in fatigue mice result from an alteration of the SCN endogenous clock or from downstream effects? Although in most of the previous studies, a potential dysfunction of the SCN was not directly assessed in either cancer related fatigue models or therapy related fatigue models, previous studies in PER2:LUC recording suggest that the PER2 rhythm is not affected in cultured slices.[8] Consequently, in the current study, the electrical neuronal activity from both SCN and peri-SCN area were recorded under DD conditions. The SCN maintained its near 24 hours rhythm under DD under post doxorubicin treatment. However, the correlation between the behavior and raw SCN electrical activity was less negative compared to control, which indicates that the output of the SCN became less effective. The peri-SCN areas were not able to generate robust circadian rhythms under DD condition, which indicates that the circadian timing system is weak in doxorubicin induced fatigue mouse model. Taken together the results suggest that circadian alterations of

behavioral rest/activity in the doxorubicin induced fatigue mouse model are unlikely to involve dysfunction in the endogenous SCN clock. The fatigue like symptoms are likely generated either downstream from the SCN or by insufficient communication between the central and peripheral clock.

We first observed doxorubicin-induced loss of body mass starting after the last injection and persisting for up to five weeks post-treatment. This decrease is in line with our previous study and other studies using doxorubicin. [11,15] As we expected, four injections of doxorubicin are not enough to induce cardiotoxicity in the heart, which means the fatigue is not induced by cardiovascular dysfunction as heart size and weight did not differ between the groups.[32] Chemotherapy can induce the release of several cytokines or growth factors that can also modify the circadian clock (ref).[12] Increased peripheral inflammation is associated with fatigue in both patients and animal models (ref).[8,33] Moreover, as doxorubicin has been demonstrated to induce increased signaling of the pro-inflammatory cytokines TNF-α and IL-6, it is possible that chemotherapy-induced inflammation may contribute to circadian misalignment in cancer patients.[7,34,35] We therefore investigated the effects of doxorubicin on inflammatory markers in the spleen and kidney. Peripheral inflammation in the kidney was not observed in our chemotherapy induced fatigue mouse model. However, doxorubicin decreased spleen mass,[36] and it mediated the physiological downregulation of TNF-α and upregulation of IFN-beta in spleen, indicative of an adverse impact on immune cell health in the spleen. COXs are immune-sensitive metabolic enzymes required for the biosynthesis of autacoids using essential fatty acids that are enriched in the spleen.[36] Doxorubicin induced dysregulation of COX-2 and caused defective immunometabolism. Overall the effects are small but they may contribute to the fatigue like symptom in our mouse model.

The aim of this study was to investigate the effects of doxorubicin on the circadian timing system. We show that the toxicity of doxorubicin results in circadian misalignment of the central circadian clock and the peripheral brain areas and rest-activity behavior. Timing and alignment of circadian rhythms are important to the health and well-being of all organisms, including humans. [37]. The current study suggests that misalignment of neuronal and behavioral rhythms underly cancer related fatigue. And underscore the findings that treatments of cancer related fatigue aimed to align the circadian phase, such as bright light and melatonin, have shown promising results in treatment of cancer related fatigue. Therefore these kind of treatments can be viewed as a

promising non-invasive treatment to alleviate fatigue symptoms induced by different therapies.

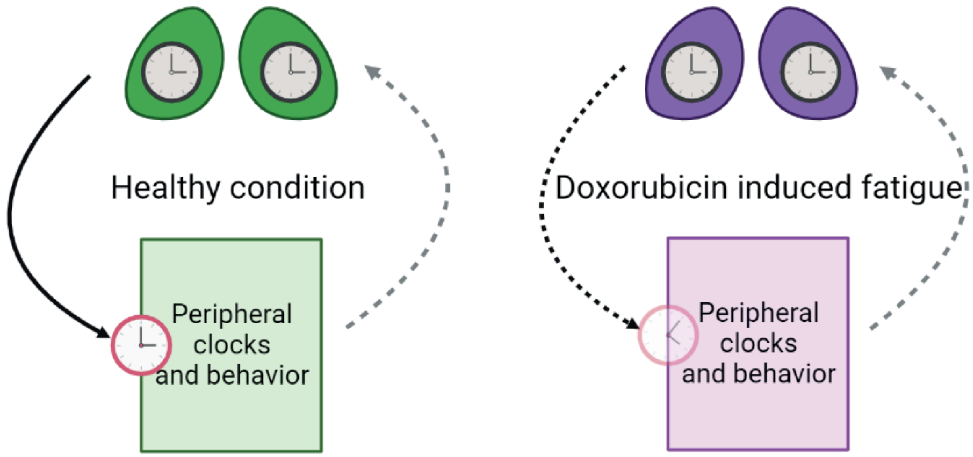

Figure 7. Circadian misalignment in chemotherapy induced fatigue model

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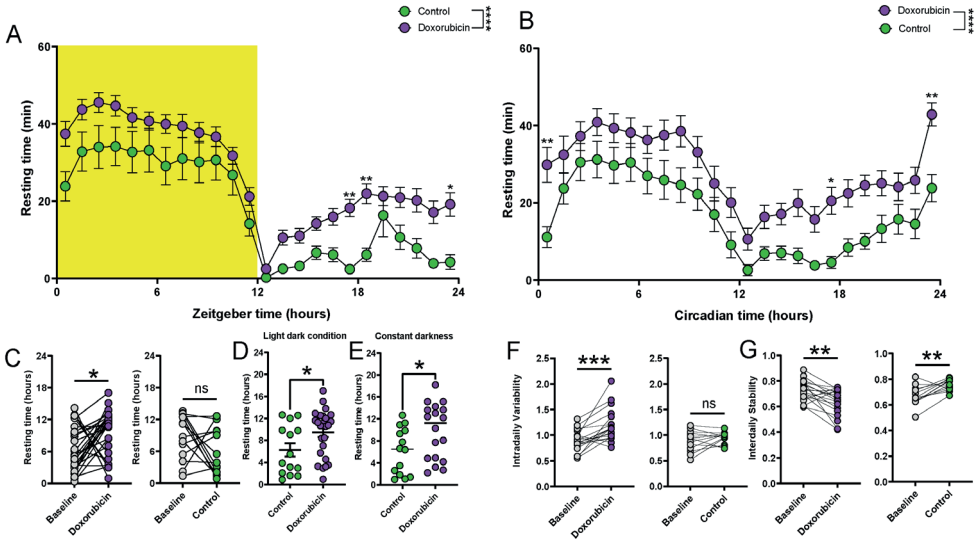
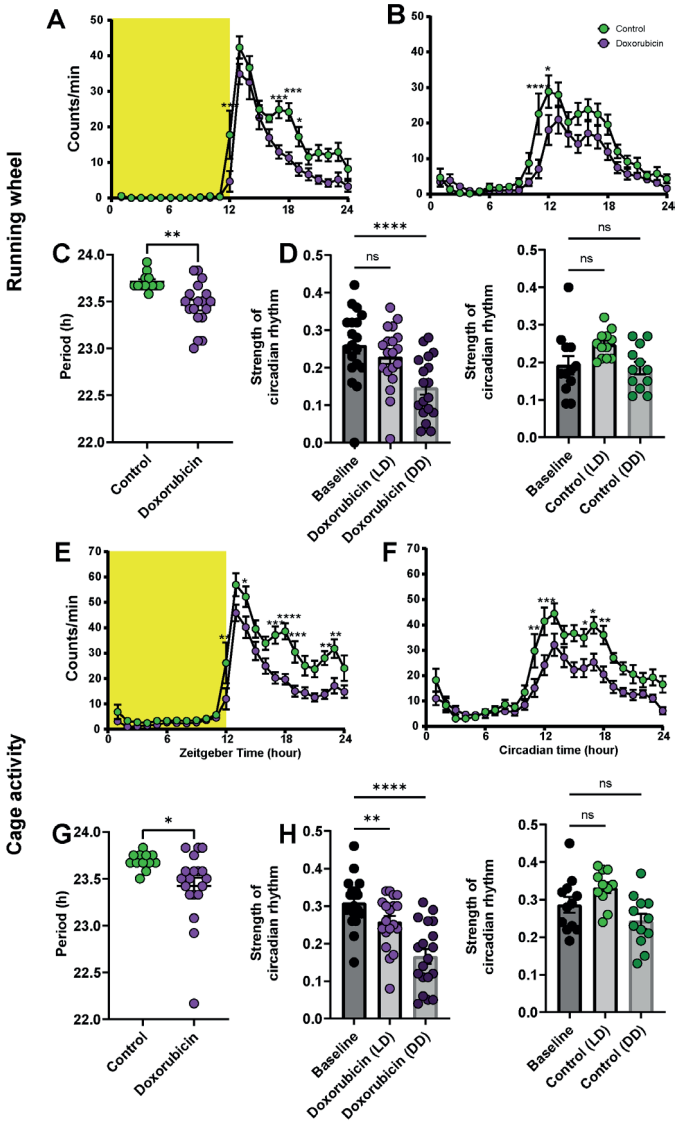
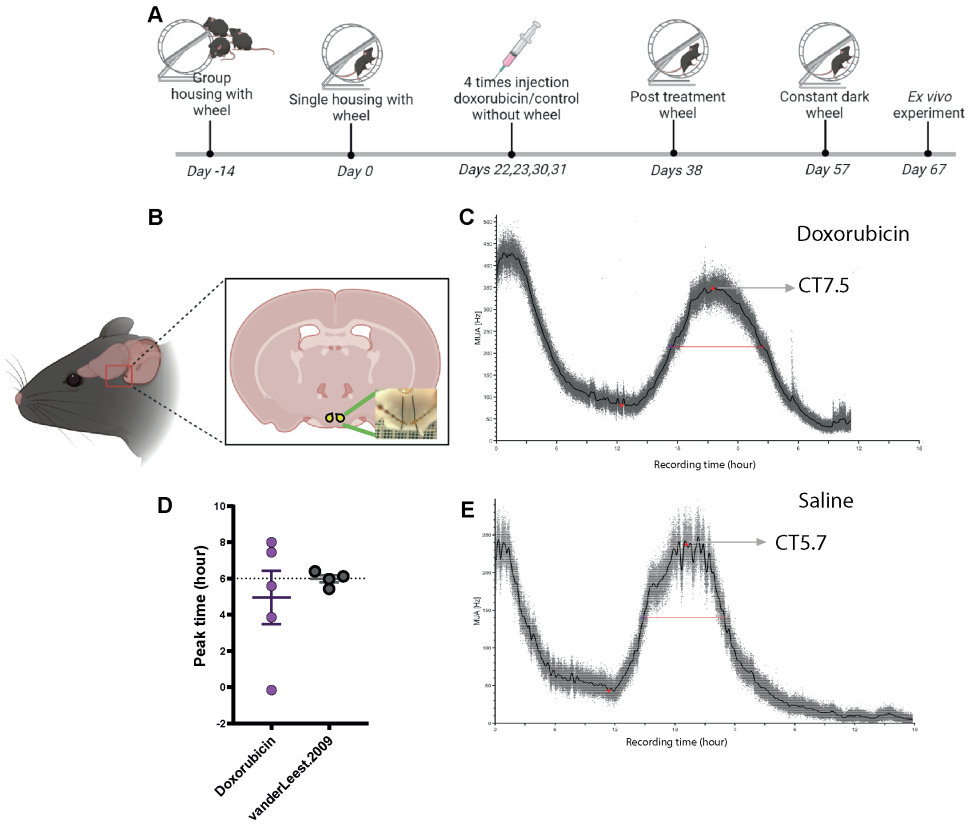
Supporting information



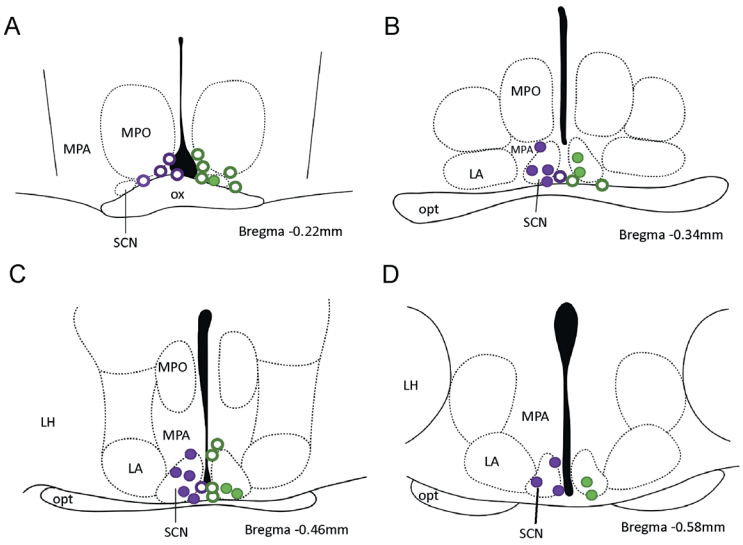
Supply Figure 1 Doxorubicin treated mice showed fatigue like symptoms and disrupted clock in cage activity. Average resting time/h of cage activity over 10 days under post-treatment LD condition (A), and post-treatment DD condition (B). (C) Average resting time/day over 10 days under baseline and post-treatment LD condition. (D) Relative running wheel activity over 10 days under LD of doxorubicin and control groups. (E) Average resting time/day over 10 days under post-treatment DD condition. (F) Intradaily variability under baseline and post-treatment LD condition. (G) Interdaily stability under baseline and post-treatment LD condition. Values are shown as mean ± SEM. Yellow indicate the light phase.



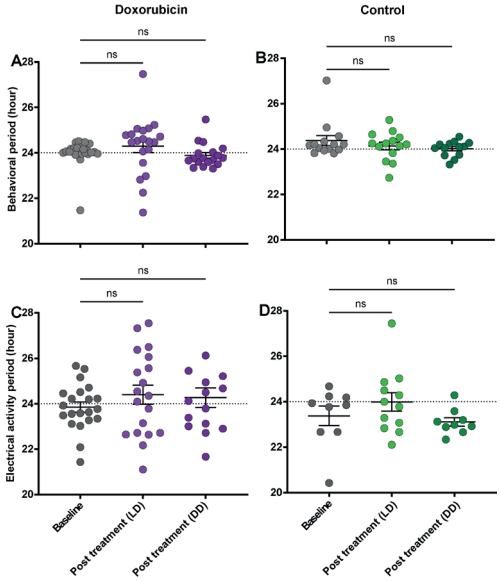
Supply Figure 2 Doxorubicin treated mice showed decreased running wheel and cage activity, decreased tau and decreased strength of the circadian clock after the doxorubicin treated. Average running wheel counts/h over 10 days under post-treatment LD condition (A), and post-treatment DD condition (B). (C) Circadian period of running wheel in doxorubicin and control groups. (D) Strength of the circadian rhythm (Qp) under baseline and post treatment conditions in doxorubicin and control groups. Average cage activity counts/h over 10 days under post-treatment LD condition (E), and post-treatment DD condition (F). (G) Circadian period of cage activity in doxorubicin and control groups. (H) Strength of the circadian rhythm (Qp) under baseline and post treatment conditions in doxorubicin and control groups. Values are shown as mean ± SEM. Yellow indicate the light phase.



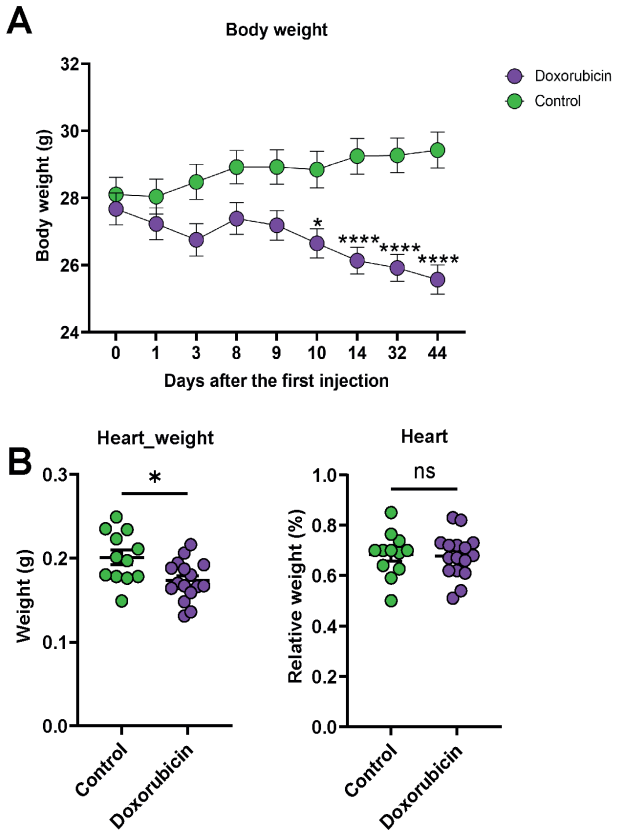
Suppl Figure 3. Doxorubicin treated brain slices showed unstable electrical peak time. A. Experimental design. B. Schematic figure of the location of SCN in the brain slice. C. Representative MUA recording from doxorubicin treated mouse. D. Peak time of the MUA activity. E. Representative MUA recording from control treated mouse.



Suppl Figure 4. Histology of the MUA electrodes. A. Brain lesion of the electrodes in bregma – 0.22 mm. B. Brain lesion of the electrodes in bregma – 0.34 mm. C. Brain lesion of the electrodes in bregma – 0.46 mm. D. Brain lesion of the electrodes in bregma – 0.58 mm. Open circle: peri-SCN, circle: SCN. Purple: doxorubicin mice, green: control mice.



Suppl Figure 5. Tau of PIR and MUA under LD and DD conditions. Tau of the locomotor activity under baseline, post-treatment conditions in doxorubicin (A) and control (B) groups. Tau of the MUA activity under baseline, post-treatment conditions in doxorubicin (C) and control (D) groups.



Suppl Figure.6 Body weight change and heart weight. A. Relative body weight changes after doxorubicin and control. B. Heart weight and relative changes in control and doxorubicin groups. Values are shown as mean ± SEM.

6
General Discussion

General discussion

This thesis consists of two parts. The overall theme is the study of pharmacological treatments and their effects on sleep and the circadian clock. The first part, consisting of **chapter 2** and **3**, focuses on the long-term effects of caffeine, ketamine and sleep deprivation (SD) on vigilance states, electroencephalogram (EEG) power density spectra and locomotor activity under constant dark conditions. For this first part, we asked two questions: 1. How long do the effects of the treatments last?; 2. What are the similarities and differences in the effects of SD and ketamine on sleep and the EEG? This latter question is of interest because both treatments are known for their rapid antidepressant effect. The second part, **chapters 4** and **5**, explores the mechanism of cancer related fatigue (CRF) induced by chemotherapy. Here are three questions we tried to answer: 1. Is CRF a clock problem or a sleep problem? 2. How does neuronal activity in the suprachiasmatic nucleus (SCN) respond to chemotherapy? 3. As a follow up question, we investigated the alignment between activity of the central clock versus the brain, and the bodily function and behavior.

1 Drugs that influenced sleep

1.1 Acute caffeine had a long lasting effect on REM sleep and neuronal activity of the hypothalamus

Caffeine is a commonly used central nervous system (CNS) stimulant which is known to increase waking and alertness. Most studies report only short-term effects of caffeine administration. However, the effect of caffeine may last longer, particularly under conditions of low light levels or constant darkness. In **chapter 2**, we investigated the long-term effect of acute caffeine administration on sleep, EEG power spectrum and neuronal activity of peduncular part of the lateral hypothalamus (PLH).

After treatment with caffeine, we found that waking was increased and sleep was delayed, on the first day after treatment, which is the normal and well-known response to caffeine. However, we also observed that the amount of activity in the peduncular part of the lateral hypothalamus (PLH) and the amount of REM sleep was still reduced on the second day. Research on the acute effect of caffeine (100mg, a dose that is at the lower limit of the effective dose range) at bedtime in human has shown prolonged sleep latency

and decreased sleep pressure [1]. The dosage in our study, which is equal to 3 – 4 mg/kg in human (around 200 - 300 mg), is considered to be a medium to a high dose of caffeine. Studies in humans showed that higher doses (400 mg) further prolong sleep latency, decrease total sleep time, sleep efficiency and reduce the amount of SWS and REM sleep [2]. More recent research in adolescents showed that a single dose of 80 mg caffeine (content in 250 ml of energy drink) is also enough to induce alertness at a subjective level and slightly reduced SWS [3]. Consistent with the notion of the arousal effect of acute caffeine in humans, animal studies give more insights into the changes in adenosine receptors, neurotransmitters and brain activity [4-6]. It was shown that caffeine mediates its effect by an antagonistic action on adenosine receptors [7].

Adenosine is thought to increase extracellularly after prolonged waking, and has been proposed as an indicator of the need to sleep. During the spontaneous sleep-wake cycles, extracellular adenosine levels to increase during waking and decrease during sleep in several brain regions [8]. Moreover, microdialysis studies in rats revealed that adenosine level increased by more than 200% in the basal forebrain after a 6 hour sleep deprivation (SD) [9]. In a recent study, Peng et al. first designed a genetically encoded G protein–coupled receptor (GPCR)–activation-based (GRAB) sensor for adenosine ($GRAB_{Ado}$). This sensor was expressed in the basal forebrain and the fluorescence was measured by fiber photometry [10]. With the high temporal resolution of this adenosine sensor, a higher concentration of extracellular adenosine during REM sleep was found, even higher than compared with waking [10]. This may indicate that the REM sleep state is more sensitive to adenosine antagonists, like for example, caffeine, than previously thought, which may have led to the prolonged influence of caffeine or caffeine metabolites on REM sleep in our study.

Interestingly, when caffeine was taken daily, the changes in waking which we observed in acute administration in both animals and humans were not visible [11,12]. Studies in human showed that SWA in NREM sleep during night was not different after nine successive days of chronic caffeine consumption (3 x 150 mg, daily) compared with a placebo. Moreover, chronic caffeine did not change the sleep structure in these healthy subjects [12]. Thus, caffeine intake might differently affect the homeostasis of sleep when taken in an acute and chronic manner.

In acute caffeine experiments, it is well documented that caffeine not only has an effect on sleep, but also on the circadian clock [7]. The SCN is sensitive to caffeine in both humans and rodents, suggesting there is a pathway where adenosine and its receptors are involved in influencing the SCN network [13-16].

In our results, caffeine caused an increase in neuronal activity in the PLH. It has been demonstrated that systemically administered caffeine induced c-Fos expression in several brain areas, including the cortex and hypothalamus, that control energy homeostasis [17]. Caffeine activated the orexin neurons in the lateral hypothalamus (LH) in rats [18]. There is also evidence indicating that injection of orexin A into the rostral LH of rats induces running independently of feeding behavior [19]. This may indicate that caffeine can increase activity in the LH and may be active by the orexin neurons, which may be in line with our finding of increased REM sleep and increased neuronal activity in the PLH after acute administered caffeine.

1.2 Similarities between ketamine and sleep deprivation on sleep, differences in waking

Disrupted sleep is more likely to increase the risk of developing mental illness, like anxiety and depression [20]. In the *diagnostic and statistical manual of mental disorders-IV-TR*, both insomnia and hypersomnia are common symptoms of depression, indicating that sleep disruption is common in depression [21,22]. The implication is that disrupted sleep can be a symptom or consequence of mental problems and is suggestive for a bi-directional link between sleep and mental health. Sleep deprivation (SD) and low dosages of ketamine are two rapid-acting antidepressant treatment strategies. In **chapter 3** of this thesis, the effect of both ketamine and sleep deprivation on sleep and EEG power density were compared.

Although both treatments are rapidly acting anti-depressant treatments that also influence sleep and the sleep EEG [23,24], there are not many studies comparing the two treatments on these effects [25]. In chapter 3, we compared these two treatments in the same animal model, and determined the similarities and differences in their effect on sleep and waking and the EEG power spectrum. The sleep-wake changes after SD and ketamine were in line with what was known from previous studies. In our study, however, we show that the EEG power spectrum of waking immediately after ketamine

is different compared to during SD, which suggests that SD and ketamine induce two different qualities of waking. Further, we analyzed the subsequent NREM and REM sleep power spectrum and noticed that both ketamine and SD increase slow wave activity in the NREM sleep EEG, which suggests that both treatments had a similar effect on sleep homeostatic processes in the recovery period after the initial waking period. Although ketamine induced hypoactivity, the waking EEG was not different compared with saline during waking after sleep onset. This may indicate that the active waking induced by ketamine only lasts for 2-3 hours; after which the waking power spectrum is less influenced by ketamine. When we compared the waking and NREM sleep power spectrum, we noticed that the peak frequency is faster after SD than the baseline condition and ketamine treatment, this may suggest that the cortex is activated differently during the recovery period after SD compared to ketamine. In contrast to the strong acute sleep disturbance, ketamine administration and SD were not associated with long-term changes in sleep-wake architecture, however, both SD and ketamine were associated with long-term changes in EEG power density, especially in the NREM and REM sleep EEG. The common actions of SD and ketamine on sleep consist of an acute increase in NREM sleep SWA and a reduction in REM sleep. With the similar features of these two rapid antidepressants, it may help to target these similarities to develop new treatments against depression.

2. Is cancer related fatigue a clock problem? Or a sleep problem?

Cancer related fatigue is a debilitating side effect of cancer and cancer treatments. However, the mechanism underlying cancer-related fatigue is unclear. In **Chapter 4** we investigated how sleep and clock controlled behavior changes under chemotherapy induced fatigue. Furthermore, **chapter 5** is a follow-up study that further investigates the effect of chemotherapy on the master clock and peripheral circadian activity in the brain and body.

Fatigue is a common and distressing symptom reported by cancer patients before, during, and after cancer treatments, and it has negative effects on the quality of life [26-29]. Chemotherapy is nonspecific, thus it not only eliminates tumor cells, but also damages normal cells and tissue and is known to cause side effects through that. Cytotoxics, including but not limited to doxorubicin, etoposide, 5-fluorouracil and paclitaxel cause fatigue symptoms both in human and mouse models [30-35]. Different types of chemotherapy

also had different side effects, even if they came from the same drug family. Furthermore, patients who received dose-dense or standard-dose taxane treatments presented no significant difference in fatigue scores, indicating that fatigue severity changed significantly over time with different chemotherapy treatment types [36,37]. Sleep problems are commonly mentioned in patients with cancer, and CRF may influence sleep efficiency, maintaining sleep and waking [38,39]. In chapter 4, we applied three different anthracycline cancer drugs to investigate the effects on circadian controlled wheel running activity and the sleep-wake cycle in a fatigue model. Wheel running activity is not only a voluntary behavior but also a circadian controlled behavior. Therefore, it can be applied as a measure for both fatigue and daily circadian rhythms in mice. Voluntary running, compared with forced activity like treadmill running, is a naturally occurring and spontaneous behavior in rodents, more related to the level of fatigue of the animal [40]. The fatigue-like behavior induced by doxorubicin showed similar symptoms as found in cancer patients in their daily activity (Figure 1) whereas the other treatments (aclarubicin and etoposide) did not induce these long-term symptoms. To our surprise, the sleep-wake cycle was not disrupted and the EEG power spectrum was not differing substantially from the baseline condition in the chemo-induced fatigue mice. This may indicate that objective sleep is less disrupted in a chemotherapy induced fatigue model, and suggests that there may be an association between impaired circadian rhythms and fatigue severity. We found that as the animal showed fatigue symptoms, their circadian rhythms became less strong. Compared with sleep problems reported by the patients and studied in animal models, this is one of the few studies investigating the relationship between CRF and circadian rhythm, but, the fatigue symptoms may be more complex in cancer patients and survivors.

It is generally accepted that fatigue is related to the loss of muscle mass, but research has found that fatigue like symptom, such as decreased wheel-running distance is not correlated with muscle contractile properties or motor coordination, indicating that fatigue is more associated with behavioral activity motivation rather than muscle dysfunction [32,37,41].

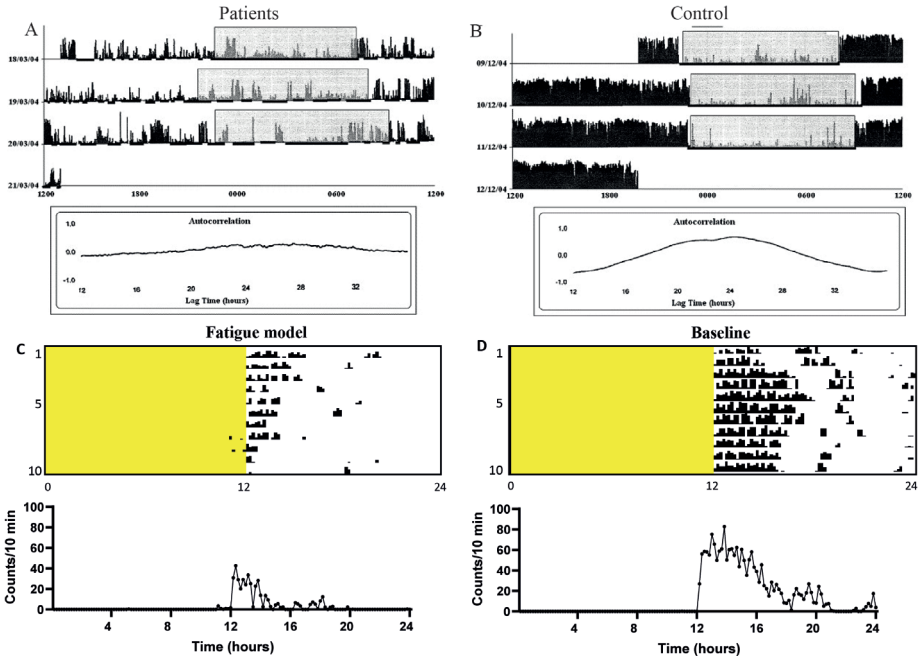


Figure 1. The actogram of patients and animal models. A. B are the representative actograms of cancer patients and healthy control. Grey block indicated the sleep period. Adapted from Fernandes et al., 2006 [43]. C. D are the representative actograms after chemotherapy and the baseline recording of the same animal. The lower panels are the average of 10 days running activity, data was sampled in a 10 min bin size. Yellow indicated the light phase.

Chemotherapy induced fatigue also depends on the type of cytotoxic drugs patients receive. In our study, we tested three different cytotoxins, aclarubicin, doxorubicin and etoposide. Only the mice treated with doxorubicin showed clear fatigue like symptoms both during treatment and after treatment. One previous study found that female C57Bl/6J mice displayed significantly reduced wheel running activity under repeated etoposide administration, and etoposide may induce CRF by increasing interleukin-6 concentration in serum [31]. In our study, we did notice that the mice treated with etoposide showed a slower recovery of wheel running activity after the etoposide compared to aclarubicin, but we did not find the chronic fatigue phenotype like after doxorubicin. Aclarubicin treatment did not induce fatigue like symptoms in our animal model. It was also reported that patients who received combinations of cyclophosphamide, fluorouracil, doxorubicin experienced more severe fatigue than those who received only paclitaxel in breast cancer patients [42]. Despite that these cytotoxics belong to the same drug family as those used in our study, they still induced different levels of fatigue both in our animal

model as well as in cancer patients.

In summary, in chapter 4 we used an animal model which can mimic the pathological features (decreased voluntary activity) of chemotherapy induced CRF. This allowed us to test the side effects of different cytotoxics from the same drug family. CRF appeared strongly dependent on the type of chemotherapy, and hopefully this knowledge will lead to an additional criterium for selecting cancer drugs for treatment. This would strongly contribute to the quality of life for (ex-) patients.

2.2 Cancer related fatigue and internal misalignment

The results we obtained in chapter 4 indicated that CRF is related to a disrupted circadian clock. In chapter 5, to understand how doxorubicin disrupts the clock and clock controlled running behaviors, we set up a follow-up study, to explore the neuronal activity of both SCN and peri-SCN brain areas before and after the doxorubicin treatment.

There is recent evidence showing that cancer and cancer treatment may influence the central circadian clock [44,45]. One study used the PER2::LUC rhythms in SCN slices and adrenal glands after paclitaxel and showed that paclitaxel shortened SCN slice circadian rhythms, increased the amplitude of adrenal gland oscillations in PER2::luciferase cultures, and increased the concentration of pro-inflammatory cytokines and chemokines released from the SCN [46]. However, in a brain slice, without all the inputs to and outputs from of the SCN, it is challenging, if not impossible, to predict the full consequences for the *in vivo* condition.

Thus, we applied multi-unit activity (MUA) recordings in the SCN and in the peri-SCN areas (hypothalamus areas, surrounding SCN), which allowed us to measure the neuronal activity in both brain areas. To our surprise, the SCN maintained its normal neuronal 24-hour rhythm in the mice that showed fatigue like symptoms. We further checked the MUA recording of the peri-SCN, and we observed a disrupted circadian rhythm in the electrical discharge of the neurons. Therefore, the data showed that the clock is still ticking normally but that the output of the SCN is disturbed after the doxorubicin treatment. Together, this new evidence, highlights that the rhythm of the SCN remained strong in mice with CRF but shows a reduced synchronization with the peripheral brain areas. We proposed that the cause of fatigue-like symptoms

originate from a reduced output of the circadian clock with consequently a reduced waking promoting effect (Figure.2)

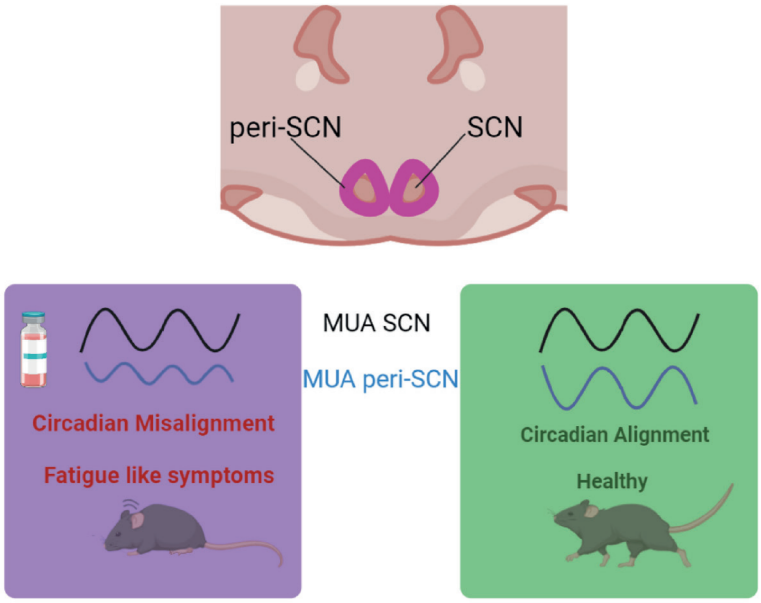


Figure 2. Internal misalignment and cancer related fatigue

2.3 What is the origin of the reduced signaling capability by the SCN?

The running wheel data showed that the 24 h rhythmic behavior was much more disrupted after doxorubicin treatment compared with the baseline condition in the same mouse, but to our surprise, the SCN maintained its 24 hour neuronal activity. One recent study hypothesized that the central clock is disrupted in CRF mice [46]. Here our study showed that even though the mice show disrupted circadian behavior, its 24-h rhythmic electrical activity in the SCN was intact. Partial lesions of the SCN in squirrel monkeys, even though only 20% of the SCN remained, still showed persisting rhythmicity in body temperature (Tb) [47]. Similar findings were obtained in male Long-Evans rats after partial SCN lesion [48]. The resilience of circadian timekeeping of the SCN, demonstrated by these findings may be a unique neurophysiological property of the SCN. Besides the evidence shown by the lesion studies, also in several animal disease models, the SCN clock still functions even though the behavior is arhythmic. In the mouse models of fragile X syndromes, the *Fmr1* knock-out and *Fmr1/Fxr2* double knock-out mice still showed rhythmic SCN

activity in *in vitro* electrophysiology recording which indicated that the output is affected [49]. Similarly, a mouse model of Huntington's disease replicates the disrupted behavioral rhythm of the human disease, but the *Per2* rhythm within SCN does not seem to be affected in the BACHD (expressing the entire human huntingtin gene with 97 mixed CAA–CAG repeats) transgenic mouse model [50]. Interestingly, in SCN brain slices of animals treated with paclitaxel, a commonly used chemotherapy in clinical, the *Per2* rhythm was intact even though the mice showed disrupted behavioral rhythm and fatigue symptoms after treatment [46]. Likewise, in aging mice, the behavioral activity and rhythmicity is reduced, despite normal electrical rhythmicity in the SCN [51]. These studies suggest that circadian timing control in the SCN might be maintained under particular disease states, including CRF.

Behavioral activity rhythms are driven by the SCN, but vice versa, activity itself feeds back to the SCN electrical activity [52]. Behavioral activity maintains a phase-locked relation to the activity of SCN neurons in anti-phase in nocturnal animals. The fatigued mice showed a reduction in this phase locking meaning that the SCN rhythm is only loosely coupled to behavior. Intriguingly, the peak time of peri-SCN area neuronal activity was changed, indicating that the circadian timing was disrupted in these peri-SCN areas after doxorubicin treatment. This change was in phase with the changes in the peak time of the rest-activity behavioral rhythm. The peri-SCN areas were not able to generate robust circadian rhythms under DD condition, which indicates that the circadian timing system is weak in doxorubicin induced fatigue mouse model.

3. Concluding remarks and future Perspectives

In the first part of this thesis, we observed a long-lasting effect of caffeine on REM sleep and theta activity in REM sleep, not previously observed. We obtained these results under constant dark conditions, which may be important, considering the interactive effects of light and caffeine on sleep and the circadian clock. In future research, it may be important to consider lighting levels when investigating the effects of caffeine in humans and rodents. Moreover, since this long-term effect of caffeine was observed, the time between different treatments in the case of caffeine should maybe also be longer in future experiments.

SD is a widely documented rapid antidepressant treatment, but SD alone has

only a transient effects on mood. However, the effect can be sustained by treating the patient with selective serotonin reuptake inhibitors and circadian related interventions that include a phase advance of the sleep-wake cycle and bright light therapy [53]. Ketamine raised attention these last two decades because of its rapid antidepressant effect. Hence, considering the similar fast effects, comparing SD and ketamine may actually give us more insight into the mechanism of the antidepressant effect of both. In chapter 3 we showed that both the acute effect and long-lasting effect share similarities in the sleep EEG power spectrums of both, and that differences between the two are mainly found in waking. Moreover, from the similarities we observed in our study, we may be able to explain the rapid antidepressant effect on different levels, such as sleep homeostasis, and glutamatergic mechanism [53]. Although the neurophysiology mechanisms involved in rapid antidepressants, and how chronobiological and sleep interventions improve the rapid antidepressant effect, are still unknown, inducing these shared effects on sleep and chronobiological variables may be beneficial for the patients.

With regard to chemotherapy and the circadian clock, our study only showed differential effect of cytotoxic effects on chronic fatigue. Doxorubicin, a frequently used drug in the clinic had major effects whereas aclarubicin had only minor effects on fatigue like symptoms. Although we did not find any changes in sleep-wake rhythm a few weeks after the treatment, there are some studies that showed disrupted sleep after chemotherapy [34]. This may, however, be related to disturbance in the communication of the circadian clock with the rest of the brain, as we showed in Chapter 5. Future studies should examine both the acute and chronic effects of these treatments in mice, to see whether the same mechanisms driving the acute response to the chemotherapy are also responsible for the long-term fatigue in the CRF mouse model and to see whether these effects can be prohibited.

A strong circadian clock that is able to signal its rhythm to the rest of the body may be essential in preventing cancer related fatigue. This is supported by the notion that patients with a robust 24-h rhythm have a higher survival outcome than those who suffer from these disruptions [54,55]. Based on the results we obtained from our animal model, we hope to raise awareness of circadian disturbances among the patients who get doxorubicin treatment. These patients may need interventions to boost their circadian rhythms during and after chemotherapy. Interventions aimed at maintaining circadian alignment with the light/dark cycle, may include exercise to boost the circadian

amplitude. Also, increased exposure to natural light or light treatment could be promoted, engaging in out-of-bed activities, scheduled food intake, and minimizing napping in the daytime, minimal light, noise and disruptions maintaining regular bedtime routines [56,57]. The response to chemotherapy may also vary with the time of day, which has led to timing of treatment, or chronochemotherapy, as a means to improve the therapeutic efficacy of cancer treatment while limiting toxicity and side effects [58] [59]. CRF is a major consequence of cytotoxic doxorubicin treatment. This serious debilitating effect can probably be prevented by better selection and/or development of the drug.

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7

Summary

The circadian timing system is highly integrated with the sleep-wake regulation system. This thesis focuses on how different pharmacological treatments influence the sleep regulation system and the circadian timing rhythm in two murine models. In the first animal model, which is presented in Chapter 2 and 3, we implanted EEG/EMG electrodes in freely moving Brown Norway rats. We chose this rat strain because it is pigmented and therefore a more representative model than the more mainstream rat strains which are usually albino rats. This study aimed to investigate the effect of caffeine, sleep deprivation and ketamine on sleep and circadian-controlled activity under constant darkness. In the second animal model, which is presented in Chapter 4 and 5, we implanted EEG/EMG or Multi-unit electrodes in chemotherapy-induced fatigue mice. This study aimed to investigate the effect of anthracycline cancer drugs on sleep, wheel running behavior, and brain neuronal activity.

In **Chapter 1**, we review the circadian timing system and physiology of sleep. The first section focuses on neuronal anatomy, physiology and the genetic bases of the circadian timing system. This is followed by a description of the neurophysiology and homeostatic regulation of sleep and the pharmacological regulation of sleep-wake function. Finally, the possible mechanism of cancer related fatigue and the relation among fatigue, sleep-wake and the circadian rhythm are discussed.

In **Chapter 2**, we studied the effects of acute caffeine administration on sleep-wake regulation and the neuronal activity in the peduncular part of the lateral hypothalamus. Most studies investigate the effect of caffeine for 24 hours but not long enough. We therefore investigated after effects of acute caffeine for two days. We found that acute administration of caffeine led to a decrease in REM sleep lasting for approximately two days. In addition, in vivo electrophysiology recordings of the peduncular part of the lateral hypothalamus showed increased neuronal activity that also lasted for two days, particularly in NREM sleep. This is evidence that caffeine influenced sleep-wake regulation substantially longer than thought until now.

In **Chapter 3**, we compared the similarity and differences between sleep deprivation and acute ketamine administration on sleep, EEG power spectrum and locomotor activity. Sleep deprivation and ketamine administration are the

few therapies that can have a rapid antidepressant effect in depressed patients. Most of the studies ignored the overlapping effects of these two therapies on sleep and circadian rhythm. The common actions of sleep deprivation and ketamine subsequently on sleep consist of an acute increase in NREM sleep SWA and a reduction in REM sleep. Although both sleep deprivation and ketamine initially keep the animals awake. Our detailed analysis show that the mechanisms of waking inducting effect is probably different and therefore not related to antidepressant effect. Therefore, we hypothesis that reducing REM sleep and increasing SWA can be part of the mechanism of the antidepressant effect.

The second animal model in this thesis we used is cancer-related fatigue mouse model. We use the C57BL/6J mouse which is commonly used in the circadian, sleep, and pharmacology field. Cancer related fatigue is a devastating side effect of cancer, cancer treatments, or both. In **Chapter 4**, we investigated three different anthracycline cancer drugs on wheel running behavior, sleep architecture and EEG power density spectrum before and after chemotherapy. Mice treated with doxorubicin, which combines both DNA- and chromatin damage activity, results in increased fatigue symptoms in tumor-free mice. In contrast, treatment with aclarubicin or etoposide which use only one of the two mechanisms failed to induce long-term fatigue, suggesting that the development of this devastating side effect is probably the result of combined DNA- and chromatin damage activity. In addition, we observed that fatigue was not the result of changes in sleep duration. We conclude that fatigue symptoms are more associated with a disrupted circadian rhythm, hinting at a deficit in SCN clock.

To further explore to what extent chemotherapy influences the circadian clock, in **Chapter 5** we examined the neuronal activity of the SCN master clock, and the peri-SCN areas located in the hypothalamus in the doxorubicin induced fatigue mouse model. Similar to what we observed in the mouse model in chapter 4, the doxorubicin treated mice showed decreased wheel running activity, decreased strength of the circadian clock, lower day-to-day stability and increased fragmentation of the rest/activity cycle. Next we investigated whether the behavior phenotype was caused by a disrupted rhythm produced by the SCN, or alternatively, by a reduced capacity of the SCN to impose the circadian rhythm, on other brain areas. We found that the doxorubicin treated mice still showed robust rhythmic SCN neuronal activity. However, the relative timing of peri-SCN areas activity and behavior was

affected by the doxorubicin treatment. This indicates that the disruption in circadian behavior is caused by the internal misalignment between the master circadian clock and the periphery.

The studies in this thesis contribute to understanding the effect of caffeine, ketamine, sleep deprivation and anthracycline in sleep-wake regulation and the circadian timing system. All the results from this thesis and future perspectives were discussed in **Chapter 6.** We are able to see how disruption of sleep and the circadian clock adversely affect health and may contribute to many diseases in modern society. In this thesis, these studies provide a better understanding of these drugs influence the circadian timing system and sleep - wake regulation and maybe new treatment approaches for antidepressant therapy and cancer related fatigue.

Samenvatting

Het circadiaanse (circa = ongeveer, dia = dag) tijdssysteem heeft een grote invloed op het slaap-waak regulatiesysteem. Dit proefschrift onderzoekt hoe verschillende farmacologische behandelingen het slaapregulatiesysteem en het circadiane tijdsritme beïnvloeden in twee muismodellen. In het eerste diermodel, dat besproken wordt in hoofdstuk 2 en 3, implanteerden we Elektro-encefalografie (EEG) en elektromyografie (EMG) elektroden in vrij bewegende 'Brown Norway' ratten. We kozen voor deze stam omdat deze dieren gepigmenteerd zijn en daardoor een representatiever model vormen dan de meer gangbare albinoratten. Het doel van deze studies was om het effect te onderzoeken van cafeïne, slaaptekort en ketamine op slaap en de circadiane activiteit in constante duisternis. In het tweede diermodel, dat wordt gepresenteerd in hoofdstuk 4 en 5, implanteerden wij EEG en EMG elektroden of multi-unit elektroden in een muismodel voor chemotherapie-geïnduceerde vermoeidheid. In deze studies beoogden wij het effect van anthracyclines, een klasse chemotherapeutica, op slaap, loopwielgedrag, en neuronale activiteit in het brein te onderzoeken.

In het **hoofdstuk 1** van dit proefschrift bespreken we het circadiaanse tijdssysteem en de fysiologie van de slaap. Het eerste deel richt zich op de anatomie in het brein, de fysiologie en de genetische fundamenten van het circadiaanse tijdsysteem. Daarna volgt een beschrijving van de neurofysiologie en de homeostatische regulatie van slaap, gevolgd door de farmacologische regulatie van het slaap-waaksysteem. Tenslotte wordt het mogelijke mechanisme onderliggend aan kanker-gerelateerde vermoeidheid beschreven. Hierbij komt de relatie tussen vermoeidheid, slaap-waak ritmiek en het circadiaanse ritme aan bod.

In **hoofdstuk 2** onderzochten wij de effecten van acute toediening van cafeïne op de slaap-waakregulatie en de neuronale activiteit in het pedunculaire deel van de laterale hypothalamus. In de meeste studies die het effect van cafeïne op de slaap na acute toediening onderzochten, was de analyse beperkt tot de eerste 24 uur. Resultaten van onze experimenten lieten zien dat acute toediening van cafeïne leidde tot een verstoring van het slaap-waak ritme die een paar uur duurde en een afname van de REM-slaap die veel langer, ongeveer twee dagen, aanhield. Bovendien vertoonden in vivo elektrofysiologische metingen van het pedunculaire deel van de laterale hypothalamus een verhoogde neuronale activiteit die bijna twee dagen aanhield, vooral in de nonREM(NREM)-slaap. Dit toont aan dat cafeïne de slaap-waakregulatie sterker beïnvloedt dan tot nu toe werd gedacht.

In **hoofdstuk 3** vergeleken we de overeenkomsten en verschillen tussen slaapdeprivatie en acute ketamine toediening op slaap, EEG frequenties en de activiteit in de kooi. Slaapdeprivatie en toediening van ketamine behoren

tot de weinige therapieën die een acuut antidepressief effect kunnen hebben op patiënten met depressie. De meeste studies negeerden de overlappende effecten van deze twee therapieën op de slaap en het circadiaanse ritme. De gemeenschappelijke effecten van slaapdeprivatie en ketamine op de slaap bestaan uit een acute toename van de NREM-slaap, lage frequentie activiteit in het EEG en een vermindering van de REM-slaap. Hoewel zowel slaapdeprivatie als ketamine de dieren aanvankelijk wakker hield, vertoonde de EEG power density (de spectrale eigenschappen van het EEG) tijdens de wakkere periode grote verschillen tussen beide therapieën en daarom niet gerelateerd aan het anti-depressie effect. Uit de resultaten concluderen wij dat vermindering van de hoeveelheid REM slaap en verhoging van de activiteit in de lagere frequenties van het EEG deel zijn van het mechanisme dat bijdraagt aan het antidepressieve effect..

Het tweede diermodel dat we in dit proefschrift hebben gebruikt is het muismodel voor kanker-gerelateerde vermoeidheid. Wij gebruikten de C57BL/6J muis, welke vaak wordt gebruikt in de circadiaanse, slaap en farmacologie onderzoeksvelden. Kanker-gerelateerde vermoeidheid is een ernstige bijwerking van kanker, kankerbehandelingen of beide. In **hoofdstuk 4** onderzochten we het effect van drie verschillende anthracyclines op het loopwielgedrag, de slaaparchitectuur en het EEG power density spectrum voor en na chemotherapie in tumorvrije muizen. Muizen behandeld met doxorubicine, dat als onderdeel van de werking zowel DNA schade als chromatine schade veroorzaakt, vertoonden veel vermoeidheidsverschijnselen. Behandeling met aclarubicine of etoposide, die respectievelijk chromatine schade of DNA schade als werkend mechanisme hebben, leidde daarentegen niet tot langdurige vermoeidheid in de muizen. Dit suggereert dat de ontwikkeling van deze ernstige bijwerking waarschijnlijk het gevolg is van specifieke mechanismen van kankerbehandeling. Deze vermoeidheidsverschijnselen hielden geen verband met veranderingen in de slaap, maar met een verstoorde circadiaanse klok.

Om beter te begrijpen in hoeverre chemotherapie de circadiaanse klok beïnvloedt, onderzochten wij in **hoofdstuk 5** de neuronale activiteit van de centrale circadiaanse klok, de suprachiasmatische nucleus (SCN), en de gebieden hieromheen, de peri-SCN-gebieden in de hypothalamus. De met doxorubicine behandelde muizen vertoonden verminderde loopwielactiviteit, verminderde sterkte van de circadiaanse klok, en lagere dag-tot-dag stabiliteit, vergelijkbaar met wat wij waarnamen in hoofdstuk 4. Hoewel deze muizen vermoeidheidsverschijnselen en een verstoring van het circadiaans gedrag vertoonden, lieten zij wel robuuste ritmische neuronale activiteit in de SCN zien. De relatieve timing in de neurale activiteit van peri-SCN gebieden en het gedrag van de dieren werd echter wel verstoord door de doxorubicine behandeling. Dit kan erop wijzen dat de verstoring van het circadiaanse

gedrag wordt veroorzaakt door een verstoring in de afstemming tussen de centrale circadiaanse klok en de periferie.

De studies in dit proefschrift dragen bij aan het beter begrijpen van het effect van cafeïne, ketamine, slaapdeprivatie en anthracyclines op slaap-waakregulatie en het circadiaanse tijdssysteem. Alle resultaten van dit proefschrift en toekomstperspectieven worden bediscussieerd in **hoofdstuk 6**. We bespreken hoe verstoring van zowel de slaap als de circadiaanse klok een negatieve invloed heeft op de gezondheid en hoe dit kan bijdragen aan verschillende ziekten die we zien in de moderne samenleving. Mogelijk kan ons onderzoek daarmee dienen als een aanknopingspunt voor nieuwe behandelingen van depressie en kanker-gerelateerde vermoeidheid.

总结

地球自转产生规律性昼夜交替及温度变化，为适应环境的周期性变化，生物体多数生理过程表现出为期约二十四小时的周期性波动，称为昼夜节律。在最佳的生理条件下，昼夜节律稳定，生命活动同步运作，各项生理功能达到最佳。内源性的昼夜节律是控制睡眠的主要过程之一，而昼夜节律紊乱可引起多种生理功能异常，最常见的可导致睡眠-觉醒周期异常，还有睡眠障碍、认知功能异常、高血压、糖尿病及肿瘤等一系列疾病。 本论文研究了不同的药物如何影响大鼠和小鼠模型的睡眠觉醒和昼夜节律变化规律。首先，我们使用了褐色挪威大鼠模型，这种大鼠品系具有正常分泌黑色素的功能，相较于实验室其他白化啮齿类动物，其睡眠觉醒和昼夜节律更具可信度。我们将脑电和肌电电极植入自由活动的褐色挪威鼠颅骨，以探究咖啡因、睡眠剥夺和氯胺酮在完全黑暗的条件下对睡眠和昼夜节律的影响。其次，我们在C57BL/6J模型中植入了脑电和肌电或多单元电极，并在不同光照条件下测量由于化疗引起的疲劳作用，即研究蒽环类化疗药物对小鼠的睡眠、跑轮运动和大脑神经元活动的影响。

在本论文的第一章中，我们回顾了近半个世纪以来昼夜节律系统和睡眠生理学的研究进展。 主要侧重于神经解剖学、生理学和昼夜节律的遗传基础、睡眠的神经生理学与稳态调节的关系，以及药物对睡眠-觉醒功能的调节，并讨论了癌因性疲劳的可能机制以及疲劳、睡眠-觉醒和昼夜节律之间的联系。在第二章中，我们研究了急性咖啡因给药对睡眠-觉醒调节和外侧下丘脑后叶神经部中神经元活动的影响。与大多数关于咖啡因对睡眠影响的研究仅在急性给药后的二十四小时内进行脑电分析不同的是，在我们的实验中，急性给咖啡因会导致褐色挪威鼠几小时内睡眠-觉醒紊乱和持续两天的快速眼动睡眠减少。此外，体内电生理记录显示下丘脑外侧后叶的神经元活动增加且持续近两天，尤其是在非快动眼睡眠中活动度更高。 这表明咖啡因对睡眠-觉醒调节的影响比我们想象的更为深远。在第三章中，我们比较了睡眠剥夺和急性氯胺酮给药对睡眠、脑电图功率谱和活动度的影响。睡眠剥夺和氯胺酮给药是少数可以对抑郁症患者产生快速抗抑郁作用的疗法。现有的大多数研究都忽略了这两种疗法对睡眠和昼夜节律的交叉影响。研究结果表明，睡眠剥夺和氯胺酮对睡眠的共同作用为非快动眼睡眠中慢波活动的急剧增加和快动眼睡眠的减少。虽然动物在睡眠剥夺和氯胺酮作用的初期都能保证清醒，但清醒时的脑电图功率谱显示两者之间存在很大差异。

我们在本论文中使用的第二个动物模型是癌症相关疲劳的C57BL/6J小鼠模型。癌症相关疲劳又称癌因性疲乏，是一种由于癌症或者癌症相关治疗引起的身体、情绪、认知等方面的疲劳。在第四章中，我们研究了三种不同的蒽环类化疗药物在小鼠化疗前后对跑轮运动、睡眠结构和脑电图功率密度谱的影响。结果显示，多柔比星处理的小鼠疲劳症状增加。相反，仅阿柔比星或依托泊苷治疗却未能引起长期疲劳，

这表明这种破坏性副作用的产生可能是特定药物治疗导致的结果。脑电结果也显示，这些症状与睡眠变化无关，而是与生物钟紊乱有关。在第五章中，为了进一步了解化疗对生物钟的影响，我们记录了阿霉素诱导的癌症疲劳小鼠模型中位于下丘脑的视交叉上核以及周围区域的神经元活动度。与我们在第四章内癌症相关疲劳小鼠模型中观察到的症状类似，阿霉素处理的小鼠表现出跑轮活动减少、生物钟强度降低、日常活动稳定性降低以及休息/活动周期的碎片化增加的现象。阿霉素处理的小鼠表现出疲劳症状和昼夜节律明显失调，但它们的视交叉上核神经元活动仍然具有强劲的二十四小时节律性。但多柔比星治疗却能对视交叉上核周围区域神经活动度的节律性调节产生影响。 这表明癌症相关疲劳导致的昼夜节律紊乱可能是由主生物钟和外围生物钟的相位错乱导致的内部节律失调而引起的。

第六章我们着重讨论了睡眠和生物钟的紊乱对健康产生不利影响，且可能导致许多现代社会的疾病原因，并对抗抑郁中睡眠的作用和重新调整生物钟相位来缓解癌症相关疲劳的新治疗方法进行展望。本研究有助于深入了解咖啡因、氯胺酮、睡眠剥夺和葸环类药物对睡眠-觉醒调节和昼夜节律系统的影响，并对其可能用于抗抑郁治疗和癌症相关疲劳的新疗法提供了理论基础。

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List of publications

1. **Yumeng Wang**, Tom Deboer. Long-Term Effect of a Single Dose of Caffeine on Sleep, the Sleep EEG and Neuronal Activity in the Peduncular Part of the Lateral Hypothalamus under Constant Dark Conditions. Clocks & Sleep 2022, 4(2), 260-276.

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Curriculum Vitae

Yumeng Wang was born in Heilongjiang, China, on the 21st of March, 1993. In 2011, she started her studies in the School of Life Sciences at the Northeast Agricultural University. During the last year of her bachelor's study, she visited different labs and was inspired by the firing of neurons then she decided to study neuroscience. In 2015, she obtained her bachelor's degree in Science. The same year she began her master's study, she studied Neurophysiology in the Department of Physiology, Norman Bethune Medical College at Jilin University. In 2017, she did an internship at the State Key Laboratory of Medical Neurobiology, Fudan University, which was the first time she learned how to record and analyze electroencephalogram. In the same year, she obtained a China National Scholarship, the highest-ranking scholarship for outstanding students in China. In 2018, she got her master's degree. In October 2018, she started her Ph.D. study in the lab of Neurophysiology under the Department of Cell and Chemical biology at Leiden University Medical Center in the Netherlands under the supervision of Tom de Boer and Joke Meijer. Her Ph.D. projects mainly focus on the effects of pharmacology treatments on sleep and circadian rhythms.