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

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

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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.

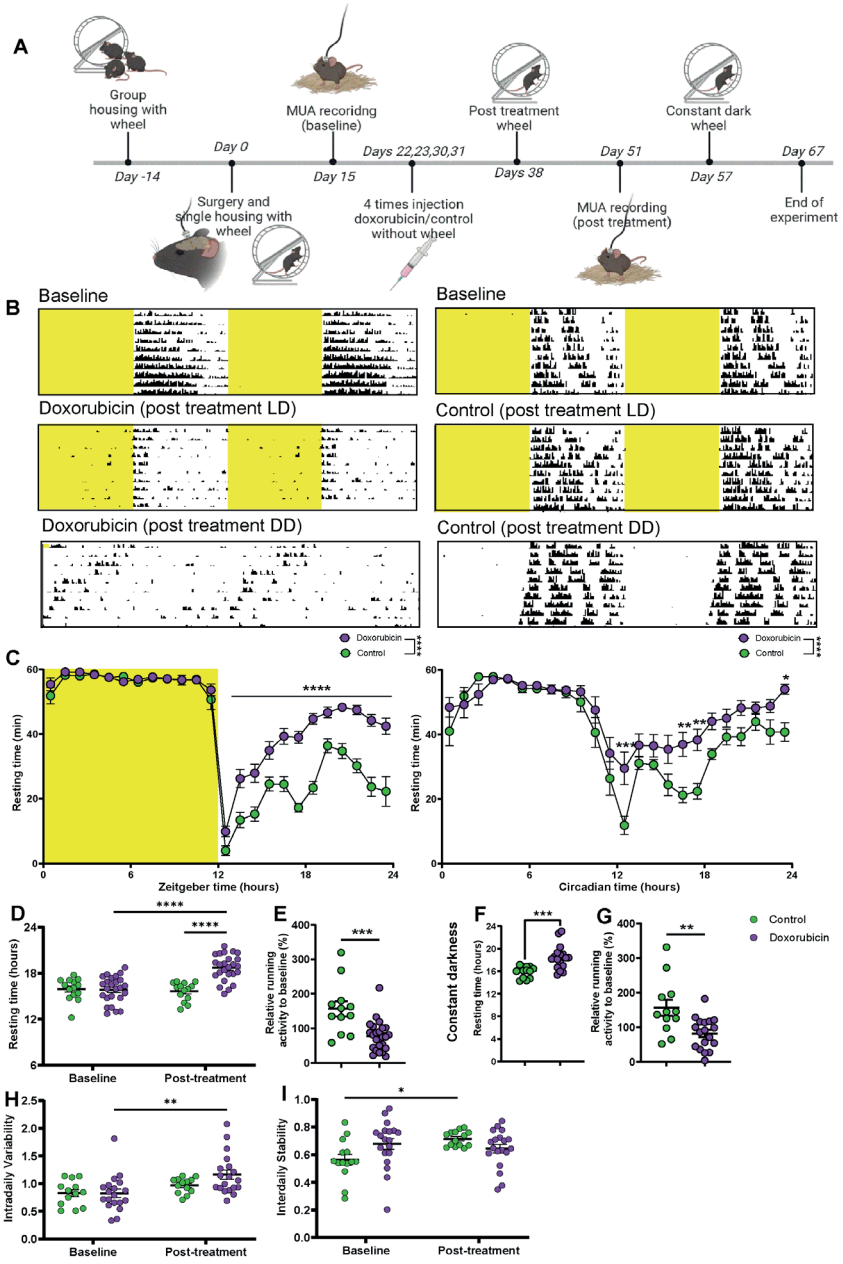

Figure 1 Doxorubicin treated mice showed fatigue like symptoms and disrupted clock. (A) Experimental design of the running wheel and multi-unit neuronal activity recording under light-dark and constant darkness conditions. (B) Representative double-plotted actograms of vehicle (right) and doxorubicin (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).

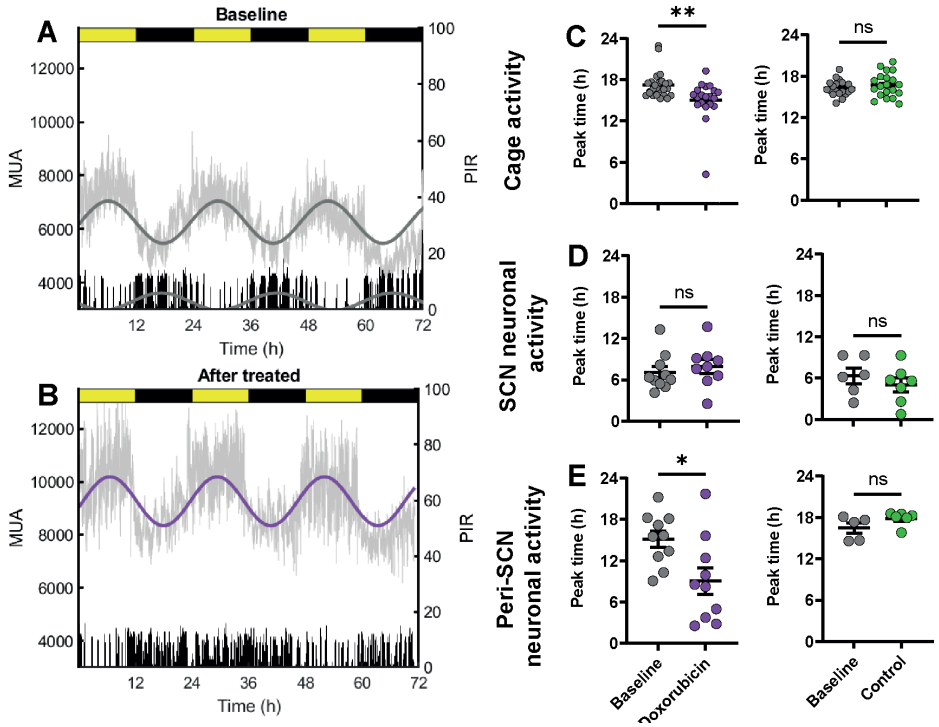

Figure 2 Timing of locomotor activity and peri-SCN are affected after doxorubicin treated in vivo. (**A**) Representative multi-unit recording and locomotor activity of doxorubicin treated mouse under baseline condition. Light grey: raw data. (**B**) Representative multi-unit recording and locomotor 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.

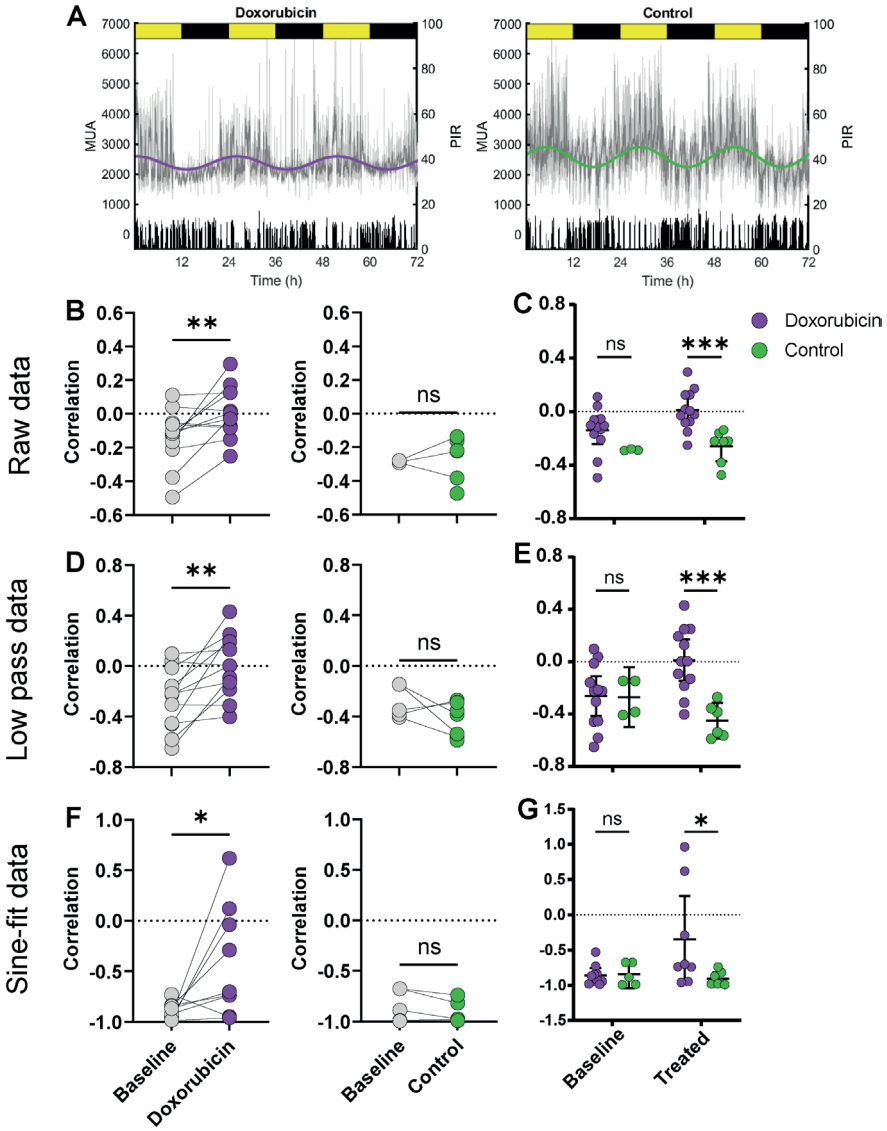

Figure 3 Doxorubicin treated mice showed misalignment of SCN electrical activity and behavioral activity (**A**) Representative sine-wave fit multi-unit activity and locomotor activity of doxorubicin (left) and vehicle (right) treated mouse under post treatment conditions. Light grey: raw data; dark 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.

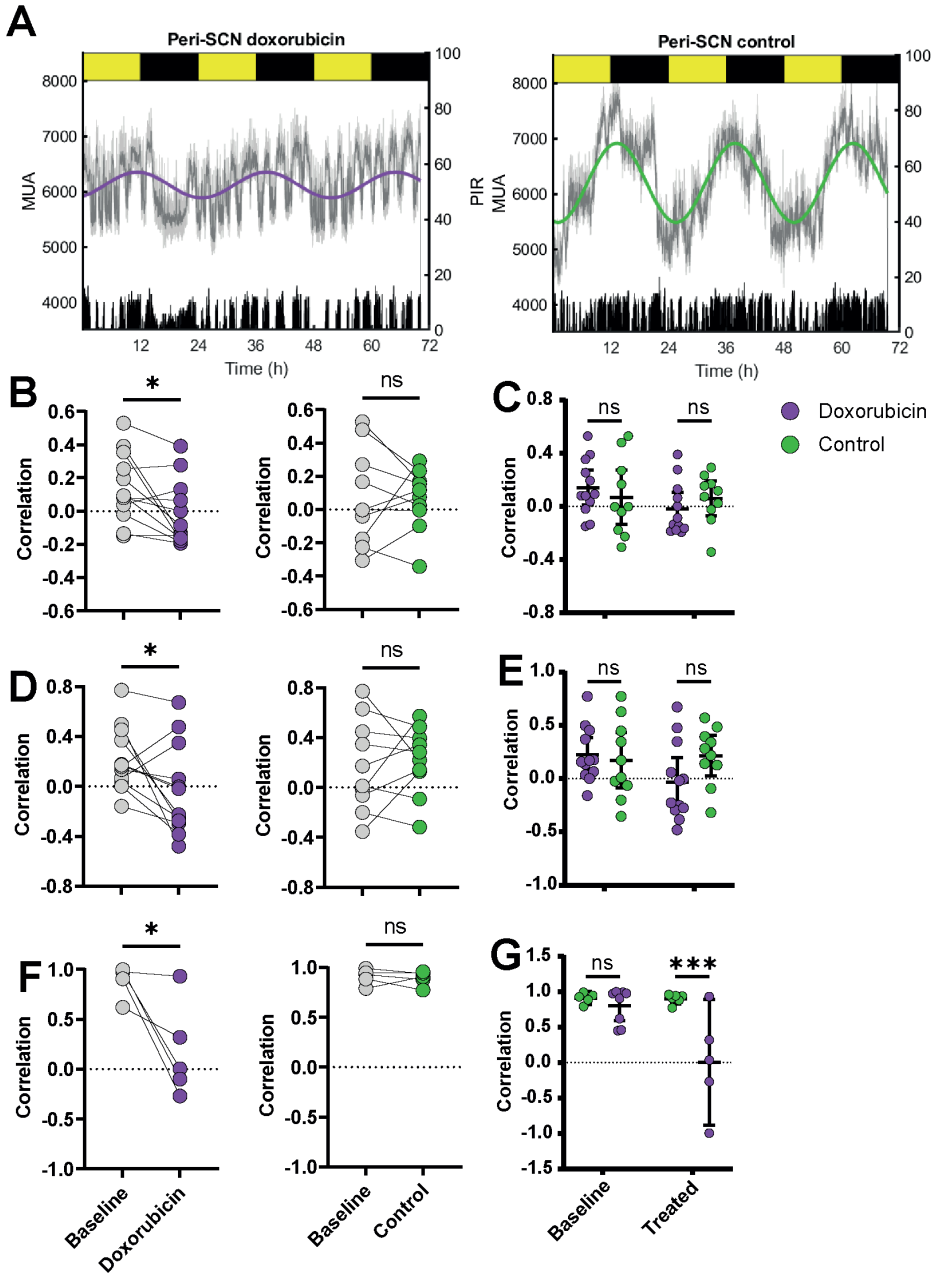

Figure 4 Doxorubicin treated mice showed misalignment of peri-SCN electrical activity and behavioral activity (**A**) Representative sine-wave fit multi-unit activity and locomotor activity of doxorubicin (left) under ? conditions. (**B - C**) peri-SCN-behavior correlation of raw data of doxorubicin (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.

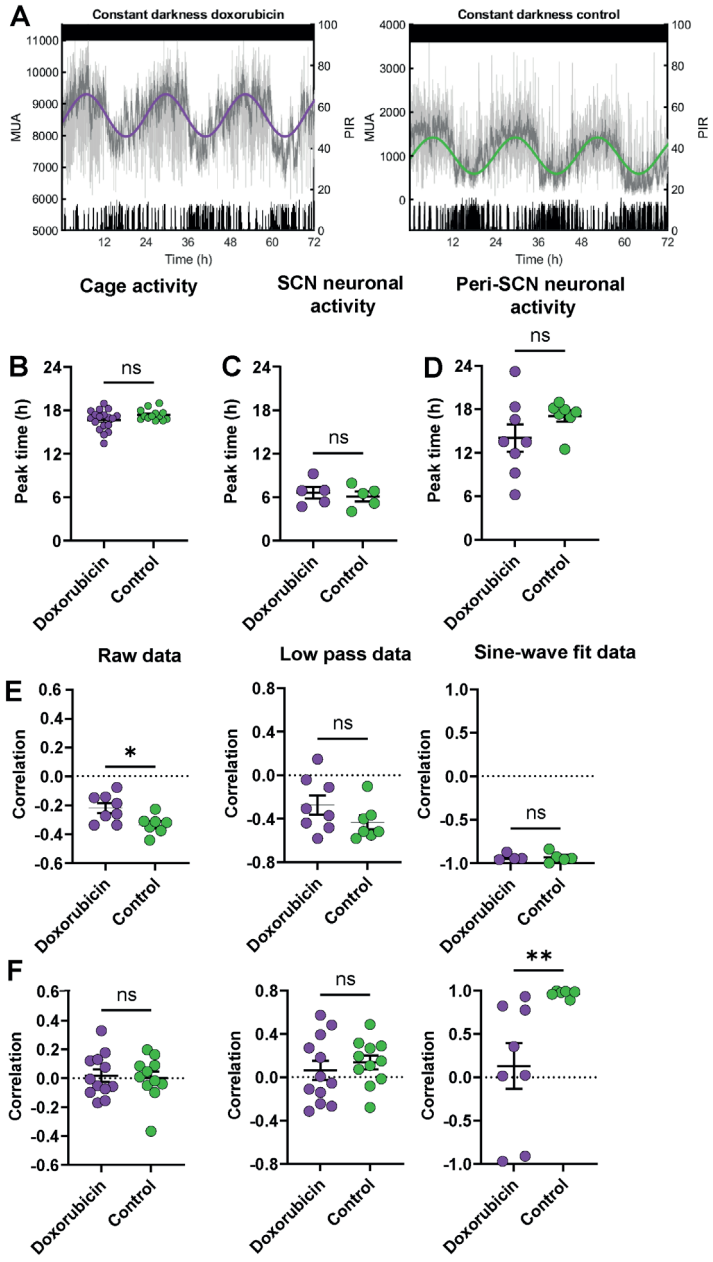

Figure 5 Doxorubicin treated mice showed less correlation of SCN and behavior under constant darkness (**A**) Representative sine-wave fit multi-unit activity and locomotor activity of doxorubicin and control treated mouse under constant darkness conditions. (**B**) Peak time of cage activity of doxorubicin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

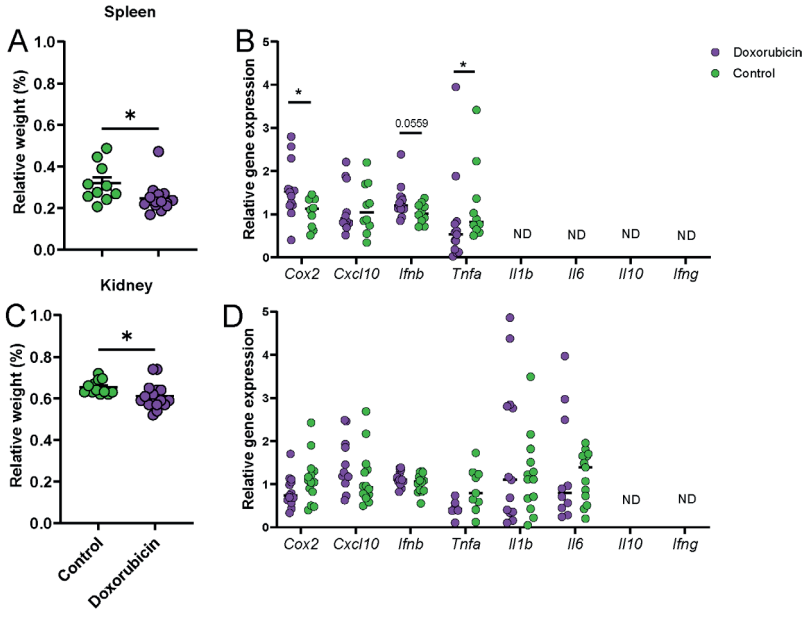

Figure 6 Organ weight change, peripheral inflammation and brain lipids after doxorubicin treated. (**A-B**) Relative weight change of spleen and kidney in control and

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.

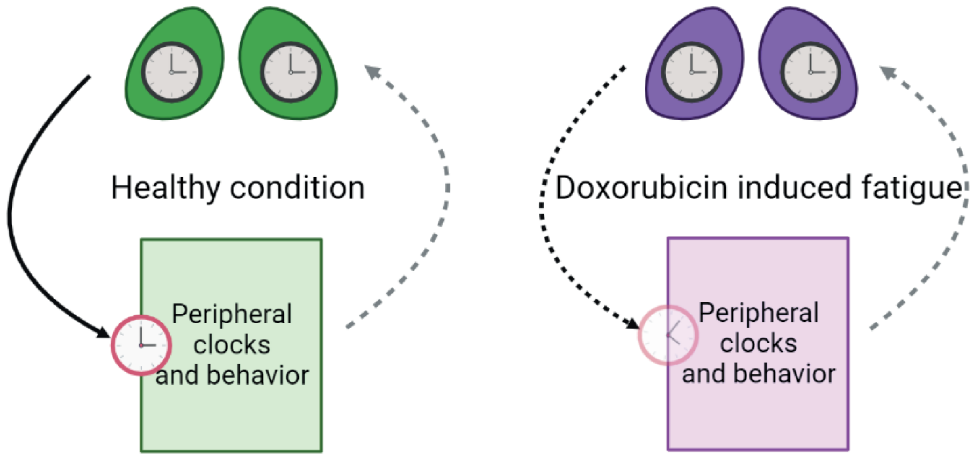

Figure 7. Circadian misalignment in chemotherapy induced fatigue model

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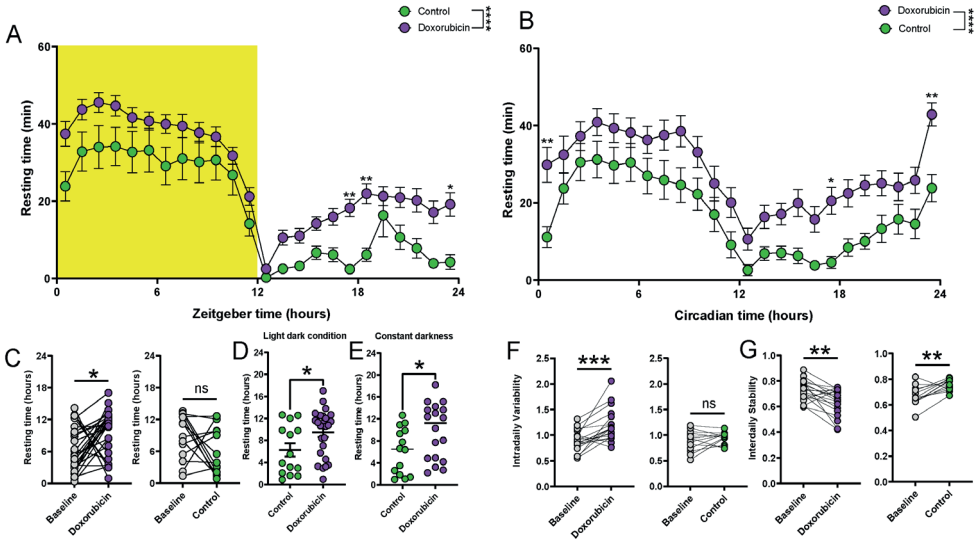
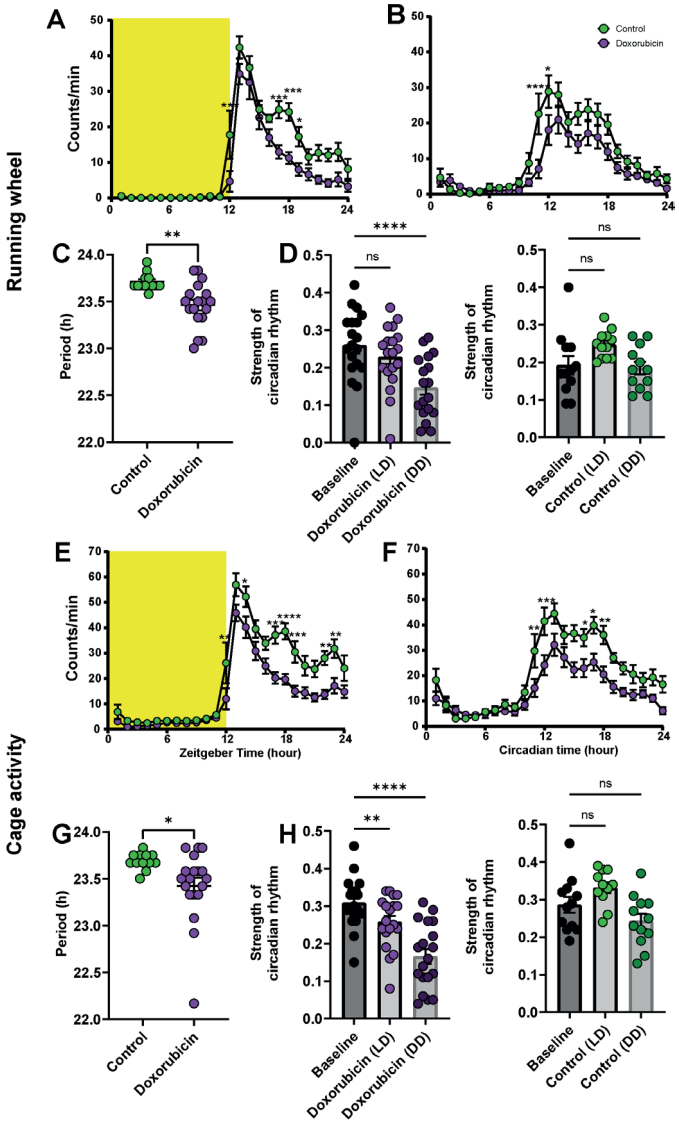
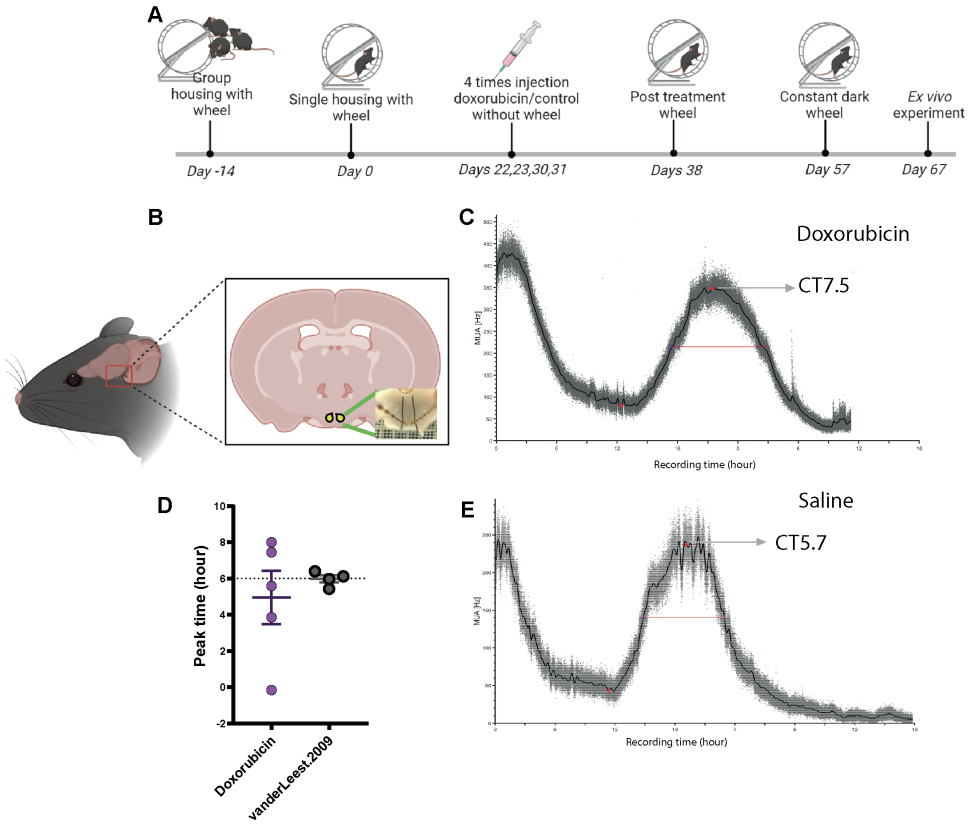
Supporting information



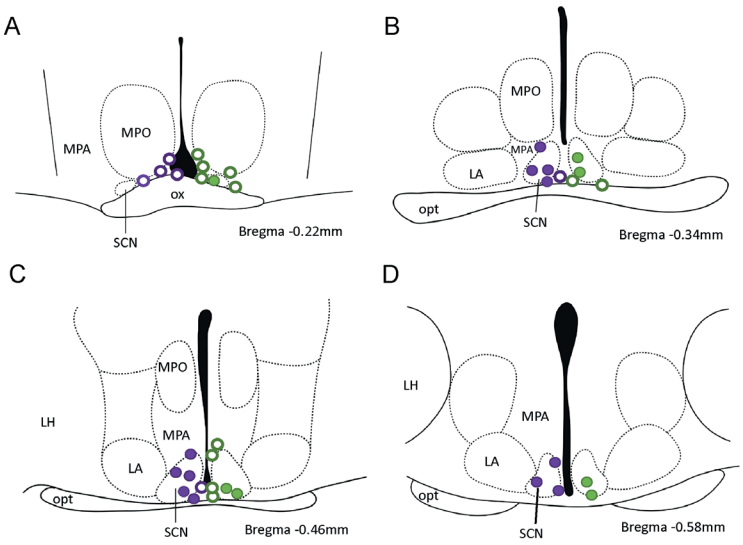
Supply Figure 1 Doxorubicin treated mice showed fatigue like symptoms and disrupted clock in cage activity. Average resting time/h of cage activity over 10 days under post-treatment LD condition (A), and post-treatment DD condition (B). (C) Average resting time/day over 10 days under baseline and post-treatment 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.



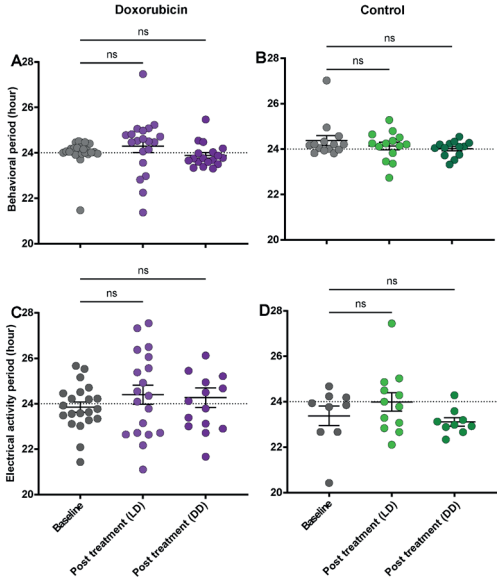
Supply Figure 2 Doxorubicin treated mice showed decreased running wheel and cage activity, decreased tau and decreased strength of the circadian clock after the doxorubicin treated. Average running wheel counts/h over 10 days under post-treatment LD condition (A), and post-treatment DD condition (B). (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.



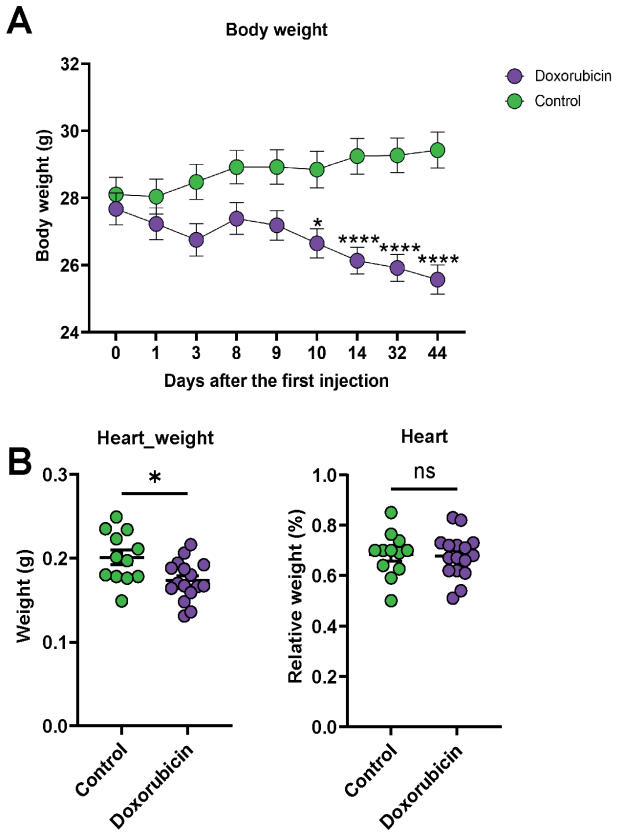
Suppl Figure 3. Doxorubicin treated brain slices showed unstable electrical peak time. A. Experimental design. B. Schematic figure of the location of SCN in the brain slice. C. Representative MUA recording from doxorubicin treated mouse. D. Peak time of the MUA activity. E. Representative MUA recording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