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Adrenalectomy prevents the effects of social defeat stress on PER2 rhythms in some peripheral tissues in male mice

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ABSTRACT

While stress does not affect the phase or period of the central pacemaker in the suprachiasmatic nucleus, it can shift clocks in peripheral tissues. Our previous studies showed significant delays of the PER2 rhythms in lung and kidney following social defeat stress. The mechanism underlying these effects is not fully understood, but might involve glucocorticoids (GC) released during the stressor. In the present study, we performed social defeat stress in adrenalectomized (ADX) mice to see if the induction of endogenous GC is necessary for the stress-induced phase shifts of peripheral clocks. We used mice that carry a luciferase reporter gene fused to the circadian clock gene Period2 (PER2::LUC) to examine daily rhythms of PER2 expression in various peripheral tissues. Mice were exposed to 5 consecutive daily social defeat stress in the late dark phase (ZT21–22). Running wheel rotations were recorded during 7 baseline and 5 social defeat days, which showed that social defeat stress suppressed locomotor activity without affecting the phase of the rhythm. This suppression of activity was not prevented by ADX. One hour after the last stressor, tissue samples from the liver, kidney and lung were collected and cultured for ex vivo bioluminescence recordings. In the liver, PER2 rhythms were not affected by social defeat stress or ADX. In the kidney, social defeat stress caused a $>$ 4 h phase delay of the PER2 rhythm, which was prevented by ADX, supporting the hypothesis of a crucial role of GC in this stress effect. In the lung, social defeat stress caused an 8 h phase delay, but, surprisingly, a similar phase delay was seen in ADX animals independent of defeat. The latter indicates complex effects of stress and stress hormones on the lung clock. In conclusion, the findings show that repeated social defeat stress in the dark phase can shift PER2 rhythms in some tissues (lung, kidney) and not others (liver). Moreover, the social defeat stress effect in some tissues appears to be mediated by glucocorticoids (kidney) whereas the mechanism in other tissues is more complex (lung).

1. Introduction

Circadian rhythms in physiology and behavior are a common phenomenon in most of the species on this planet (Froy, 2010; Panda et al., 2002; Reppert and Weaver, 2002). In mammals, the endogenous circadian system is composed of a central clock located in the suprachiasmatic nucleus of the hypothalamus (SCN) and peripheral clocks, which reside in extra-SCN brain areas and peripheral tissues (Dibner et al., 2010). Daily light/dark cycles can reset the SCN, which in turn through neuronal, physiological and endocrine signals synchronize the peripheral clocks. In this way, the whole endogenous clock system as well as rhythmic biological processes are coordinated and running in pace with the external geophysical time (Buijs et al., 2013; Dibner et al., 2010; Schibler et al., 2015).

A disturbance of the circadian system can have adverse effects on performance, well-being, and health (Bass and Lazar, 2016; Roenneberg and Merrow, 2016; Takahashi et al., 2008). For example, a higher incidence of cancer and metabolic disorders is often seen in humans exposed to chronic shift work or frequent jet lags (Bishehsari et al., 2016). Existing evidence suggests that stress may be another important cause for disturbance of the endogenous circadian system (Meerlo et al., 2002; Ota et al., 2021). While studies in laboratory rodents show that stress may not have major effects on the period and phase of the master clock in the SCN and its output rhythms such as the body temperature rhythm and activity rhythm, it is capable of shifting peripheral clocks in a time-of-day and tissue-specific manner (Meerlo et al., 2002; Ota et al.,

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2021). Particularly, uncontrollable stressors such as defeat or restraint can phase-shift the rhythms in clock gene expression in, among others, liver, lung, kidney, pituitary, and adrenals (Bartlang et al., 2014; Kong et al., 2022; Ota et al., 2020; Razzoli et al., 2014; Tahara et al., 2015). Also, in vitro studies showed phase shifts of clock gene expressions in peripheral tissues but not in the SCN slices upon exposure to the stress hormone corticosterone or the synthetic analogue dexamethasone (Balsalobre et al., 2000; Ota et al., 2020). These findings suggest that the effects of real stressors on peripheral clocks are perhaps mediated by release of glucocorticoids from the adrenal cortex.

The biological effects of glucocorticoids in most tissues depend on their interaction with glucocorticoid receptors (GR) in the cytoplasm, which then triggers various cellular responses and modulates the transcription of many genes, including Per1, Per2, and other clock genes, by binding to glucocorticoid responsive elements (GRE) or a similar functional GR binding sequence (GBS) in the promoter region of these genes (Cheon et al., 2013; Dickmeis et al., 2013; Scheschowitsch et al., 2017; So et al., 2009). This mechanism of action might explain why, in contrast to peripheral clocks, the master clock in the SCN does not respond to stress because the adult SCN lacks receptors for glucocorticoids (Balsalobre et al., 2000; Rosenfeld et al., 1988).

Thus, stressors might affect the peripheral clock system by activating the hypothalamic-pituitary-adrenal (HPA) axis and stimulating the release of glucocorticoids from the adrenal cortex, which in turn bind to GRs and GREs of clock genes (Ota et al., 2020; Ota et al., 2021). However, no studies so far directly tested the role of endogenous GC in stress-induced phase shifts of peripheral clocks in vivo. Therefore, in the present study we examined the effect of stress in mice that were adrenalectomized to remove the main source of glucocorticoids. We used transgenic PER2::LUC knock-in mice, which produce a PER2::LUC fusion protein that allows for prolonged and continuous tracking of PER2 expression using measurement of luciferase-driven bioluminescence (Kong et al., 2022; Ota et al., 2020; Yamazaki and Takahashi, 2005; Yoo et al., 2004). Sham-operated and adrenalectomized adult male mice were subjected to social defeat stress during the late dark phase for 5 consecutive days. On the last day of defeat, mice were sacrificed and peripheral tissues (liver, lung, kidney cortex) were collected for ex vivo assessment of phase, period, and amplitude of PER2 expression.

2. Materials and methods

2.1. Animals and housing

Adult two to five-month-old male PER2::LUC knock-in mice with a C57BL/6 background from our breeding colony were used as experimental animals. The animals were individually housed in cages with a running wheel. Male CD-1 mice were used as aggressors for the social defeat stress (4–8 months of age, Charles River, Sulzfeld, Germany). These CD-1 mice were individually housed in a different room in which the social defeats took place. All mice were housed under a 12 h:12 h LD cycle with lights-on at 13:00 (regular light ~30 lx) and lights-off at 01:00 (dim red light <9 lx). Ambient temperature was kept at 21 ± 1 °C and relative humidity at 50 ± 2 %. Food and drinking water were provided ad libitum. The experiments were conducted under the Dutch rules and regulations and approved by the Central Authority for Scientific Procedures on Animals (CCD, license number: AVD 105002016589).

2.2. Experimental design

To study effects of stress on peripheral clocks, we applied the well-established social defeat model (e.g., Meerlo et al., 1996; Meerlo et al., 1997; Ota et al., 2020; Kong et al., 2022). This models is based on territorial aggression displayed by male rodents defending their home cage against intruders, which normally only occurs when the intruder is a male, not a female. For this reason, the current study was done in male subjects only. Male PER2::LUC male mice were randomly assigned to one of the following four groups: **Group 1, ADX + Social Defeat**: mice were adrenalectomized and subjected to 5 daily repeated social defeat stress; **Group 2, ADX + Control**: mice were adrenalectomized and only subjected to brief daily handling when the animals of group 1 were exposed to stress; **Group 3, Sham + Social Defeat**: mice were subjected to sham ADX surgeries and 5 daily repeated social defeat stress; **Group 4, Sham + Control:** mice were subjected to sham ADX surgeries and only brief daily handling. The two groups of ADX mice were provided with saline (0.9 %) + CORT (3 mg/L) + aldosterone (10 μg/L) as drinking water, while the sham groups received tap water as drinking water. The low levels of CORT and aldosterone in the drinking water of the ADX mice were necessary for maintenance of health and survival after stress(Hausler et al., 1992; Lehmann et al., 2013); yet, these mice did not have the high stress-induced peaks in CORT.

To assess whether the low levels of CORT in itself might affect circadian organization, we decided to add an additional group: **Group 5, ADX + noCORT**. This group of mice was adrenalectomized but not exposed to stress (only brief daily handling). These mice received saline (0.9 %) only as drinking water. In all groups, running wheel rotations were recorded before and during the social defeat phase of the experiment to confirm stable daily rhythms and to assess the effects of repeated social defeat. One hour after the last defeat, mice were sacrificed and trunk blood was collected for corticosterone (CORT) analysis, and samples of other tissues were collected for culturing and measurement of PER2::LUC rhythms.

2.3. Adrenalectomy

All surgeries took place under isoflurane anesthesia (2.5 %) during the inactive phase of the animals. In the ADX groups, dorsal incisions of the skin and muscle layer were made on both sides, the blood vessels of both adrenals were cauterized (Bovie DEL1 Change-A-Tip Deluxe High Temp Cautery Kit) to prevent bleeding, and subsequently the whole adrenals were removed. Mice in the sham-operated groups underwent the same procedure except for cauterization and removal of adrenals. After surgery, the mice were allowed to recover for 14 days before starting the social defeat experiment.

2.4. Social defeat stress

Social defeats took place in the late dark phase (ZT21–22), which is when repeated social defeat stress shifted peripheral clocks in our previous studies (Ota et al., 2020; Kong et al., 2022). The social defeat sessions took place in the home cage of the aggressors (CD-1 mice), which were housed in a different room than the experimental mice. These cages measured 35 × 30 × 30 cm (L*W*H), with a separation compartment of 10 × 30 × 30 cm (L*W*H). Transport of the experimental mice and social defeat procedures took place under dim red-light conditions (<9 lx), similar to the red light illuminating the home room of the experimental animals during the dark phase. The social defeat protocol was the same as used in our previous studies (Kong et al., 2022; Ota et al., 2020; Ota et al., 2018). Briefly, each social defeat session had a total duration of 20 min, divided into 3 phases. Phase 1 (5 min) was the initiation phase and separated by a perforated acrylic wall, only allowing olfactory and visual contact. Phase 2 (10 min) was the actual phase of physical interaction and defeat. If during this phase, the intruder received more than10 attacks in <10 min, the animals were separated and the remaining time was added to Phase 3. In Phase 3 (5 min), the mice were again separated by the perforated divider. At the end of the procedure, experimental animals were returned to their home cage. Each day, the experimental mice in the social defeat stress groups were exposed to a new aggressor to avoid habituation and reduced aggression. Every day when the mice in the social defeat groups were exposed to the stress protocol, the associated control mice were gently picked up and handled for body weight measurement, and their bedding

was disturbed.

2.5. Running wheel activity

Running wheel rotations were recorded and stored in 2 min bins by an automated computer system (Circadian Activity Monitor System (CAMS), designed by Cooper, INSERM U486; (Ota et al., 2018). Data were extracted with Actoview (version 4.0) and further exported to Excel for calculation of daily and hourly activity counts (Mulder et al., 2013; Ota et al., 2018). Wheel rotations were analyzed for two time-blocks consisting of 7 baseline days and 5 social defeat days, respectively. Total activity per day across baseline and social defeat days and hourly running wheel activities were normalized and expressed as percentage (%) of the average daily baseline activity. Twenty min of running wheel rotations of the social defeat hour were deleted from the non-defeated control mice since the defeat animals were in the social defeat room and not recorded during the social defeat protocol.

2.6. Plasma corticosterone

One hour after the last defeat, mice were euthanized under dim red light by cervical dislocation followed by decapitation. At this time point, stress-induced levels of corticosterone have reached peak levels and effects of adrenalectomy should be clearly measurable (Lehmann et al., 2013).

Trunk blood was collected in EDTA-coated tubes (Sarstedt, Numbrecht, Germany), placed on ice, and centrifuged at 4 °C (RPM = 14,000, 10 min) to obtain plasma which was then stored at −20 °C. Plasma CORT was determined by radioimmunoassay using an ImmuChem Double Antibody 125I RIA kit (MP Biomedicals, LLC, USA).

2.7. Tissue culturing and measurement of PER2 rhythms

The procedures for tissue preparation and in vitro measurement of PER2 expression were similar to a previously described procedure, with minor adaptations(Ota et al., 2020; Yamazaki and Takahashi, 2005). In brief, immediately after decapitation and blood collection, the head and body of the mouse were placed on ice for transportation to the culture room where dissection and collection of peripheral tissues were done in the light. Samples were taken from liver, kidney and lung. Tissues were first placed in a plate (60 × 15 mm, Greiner bio-one) with chilled cutting medium and further dissected into smaller pieces (1–9 mm^3) by disposable scalpels under a long working distance microscope. These pieces of tissues were then placed in separate dishes (35 × 10 mm, Greiner bio-one) with the pre-warmed recording medium. The cutting and recording medium used in the present study was the same as published standards (Ota et al., 2020; Yamazaki and Takahashi, 2005). Finally, the dishes were sealed by grease (Molykote® 111 Compound) and cover glasses (40 mm in diameter, thickness No.1, VWR) and placed in a LumiCycle photon top counter machine for 5–7 days for ex vivo culturing (~37 °C) and PER2 rhythms recording (Yoo et al., 2004).

Raw bioluminescence data were processed and analyzed with LumiCycle software (Version 3.002; Actimetrics Inc., Evanston, IL). The first 12 h of data in culture were excluded because the tissue bioluminescence during this period may exhibit fluctuations due to dissection and culture medium exposure (Bartlang et al., 2014; Kong et al., 2022; Ota et al., 2020; Stokkan et al., 2001). Bioluminescence rhythm data were detrended by subtracting a centered 24-h running mean (RM) from each data point. As a result, the first and last 12-h data had to be deleted since no 24-h RM data can be calculated over these time windows. The final range of detrended data selected for analysis was 24 to 96 h after the start of bioluminescence recording. These detrended data were further processed by fitting a dampened LM sin fit curve in the LumiCycle software. Only the samples with a "goodness of fit" value >70 % were selected for further analysis. Onset1, defined as the first positive crossing of the detrended data through 0 in the selected time range of the recording (24–96 h, Fig. 2 and 3) was selected as the phase marker and expressed in hours after the last lights on perceived by the mouse (ZT0).

For calculation of the bioluminescence rhythm amplitude, we slightly extended the data range beyond the 24-96 h to ensure that it included three complete circadian bioluminescence cycles. The amplitude of cycle 1 is calculated as the bioluminescence counts of (first peak – first trough) /2 of the baseline-subtracted data, and so forth for the amplitude of cycle 2 and 3.

2.8. Statistics

The normalized total daily running wheel counts were subjected to three-way repeated-measures ANOVA with between-subjects factors SOCIAL DEFEAT (social defeat vs control) and ADX (ADX vs Sham), and within-subjects factor TIME (baseline days 1–7 days and social defeat days 1–5). To test for differences in daily profiles of activity, the normalized hourly running wheel counts were subjected to three-way repeated-measures ANOVA with SOCIAL DEFEAT (social defeat vs control) and ADX as between-subjects factors, and TIME (hours of the day) as a within-subjects factor.

The amplitude of PER2::LUC rhythms was analyzed using three-way repeated-measures ANOVA with between-subjects factors SOCIAL DEFEAT (social defeat vs control) and ADX (ADX vs Sham), and within-subjects factor CYCLES. Period and phase of PER2::LUC rhythms were analyzed using three-way ANOVA with factors TISSUE (liver vs kidney vs lung), SOCIAL DEFEAT (social defeat vs control) and ADX (ADX vs Sham) followed by Tukey's test. The significance threshold was set at $\alpha = 0.05$. Eta squared (η^2) were calculated for one-way ANOVA and partial eta squared (η_p^2) were calculated for three-way ANOVA and repeated measures ANOVA, using SPSS Statistics for Windows, version 26.0.0.1 (R02) (SPSS Inc., Chicago, Ill., USA), and a $\eta^2 = 0.01$ is considered small, $\eta^2 = 0.06$ is considered medium, and $\eta^2 = 0.14$ is considered large.

3. Results

3.1. Locomotor activity

Fig. 1a shows representative activity recordings of two sham adrenalectomized mice with or without social defeat stress, and two adrenalectomized mice with or without social defeat stress under regular 12:12 LD cycles. The running wheel rotations per day expressed as percentage of the mean daily totals during the baseline is shown in Fig. 1b. There was no difference in daily activity between groups during baseline. For the 5-day social defeat phase of the experiment, three-way repeated-measures ANOVA indicated an effect of DAY ($F(2.1, 54.73) = 7.5$, $p = 0.001$, $\eta_p^2 = 0.67$), SOCIAL DEFEAT ($F(1,26) = 5.74$, $p = 0.02$, $\eta_p^2 = 0.55$) and a DAY x SOCIAL DEFEAT interaction ($F(4,104) = 3.28$, $p = 0.01$), but no effect of ADX ($F(1,26) = 0.02$, $p = 0.89$, $\eta_p^2 = 0.01$).

The average daily activity profiles of mice during the 7 baseline and 5 social defeat days are depicted in Fig. 1c and d. The activity showed a time-of-day variance, but the profiles were not different among the four groups during baseline days. During the social defeat days, in addition to the time of day variance, three-way repeated-measures ANOVA also indicated an effect of SOCIAL DEFEAT ($F(1,21) = 8.52$, $p = 0.01$, $\eta_p^2 = 0.40$) and an HOUR × SOCIAL DEFEAT interaction ($F(23,483) = 3.69$, $p < 0.001$, $\eta_p^2 = 0.53$), but no effect of ADX ($F(1,21) = 0.002$, $p = 0.96$, $\eta_p^2 < 0.01$).

3.2. PER2::LUC rhythms

Fig. 2a-c shows representative bioluminescence traces of liver, kidney, and lung in each group. Although the amplitude of the oscillation is different, all tissues showed clear and robust PER2 rhythms during the recording period.

Fig. 2d-f shows the mean amplitude of the PER2-coupled bioluminescence rhythms in the liver, kidney, and lung. Although a clear

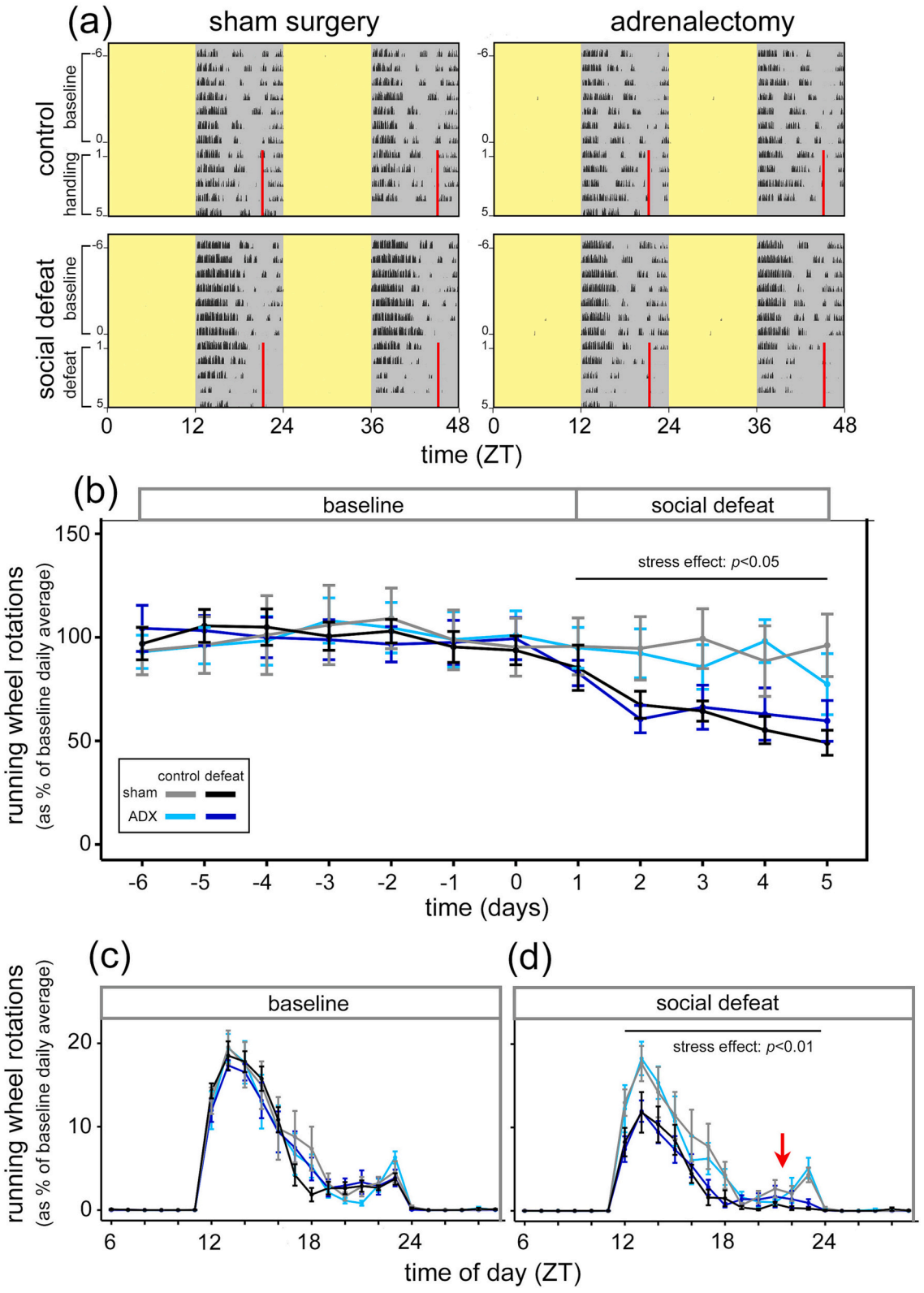

Fig. 1. Effects of repeated social defeat stress and adrenalectomy on running wheel activity.
(a) Representative actograms of mice from sham control, sham stress, ADX control and ADX stress groups. Red lines indicate when brief daily handling or social defeat stress occurred. Activity suppression can be seen after the daily defeat in the stressed mice but not after brief handling in the control mice. (b) Total running wheel activity per day (as percentage of daily baseline) during 7 baseline days (−6 to 0) and 5 social defeat days (1 to 5). Social defeat suppressed activity in both ADX and sham mice. (c) and (d) Wheel running activity per hour (as percentage of daily baseline) during baseline and stress days, respectively. Social defeat suppressed the hourly activity in both ADX and sham mice mainly in the first half of the dark phase. Data represented as percentage (%) of mean daily baseline activity. Symbols represent mean ± SEM. The red arrow indicates when the daily stress or handling occurred. Sample size: ADX control: n = 7, ADX defeat: n = 7, sham control: n = 9, sham defeat: n = 7.

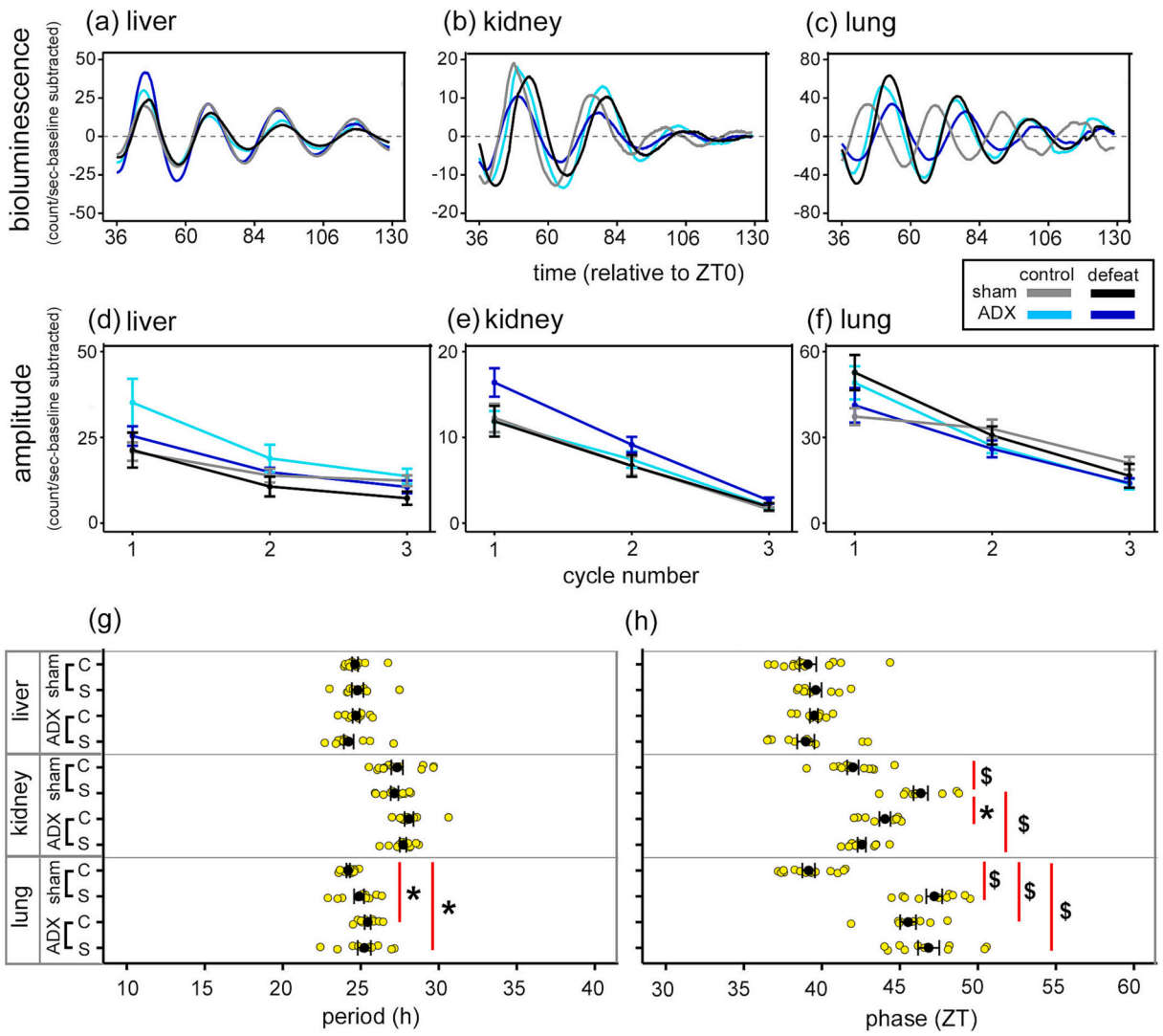

Fig. 2. Effects of repeated social defeat stress and adrenalectomy on PER2 expression in peripheral tissues.
Representative ex vivo PER2::LUC bioluminescence traces from liver (a), kidney cortex (b), and lung (c) samples taken from control mice and social defeat mice with either adrenalectomy or sham operation. Values are plotted as 24-h running mean baseline subtracted photon counts per second. Recordings are from 12 h–108 h after the start of the culture, but time is recalculated to represent ZT before sacrifice (relative to ZT0 of the dissection day). Average amplitude of PER2::LUC bioluminescence rhythms in liver (d), kidney (e), and lung (f) samples from control mice and social defeat mice with either adrenalectomy or sham operation. Amplitude values are plotted as mean photon counts per second of (peak – trough)/2 of each cycle. Average period (g) and phase (h)values of PER2::LUC rhythms from liver, kidney cortex, and lung from control mice and social defeat mice with either adrenalectomy or sham operation. The phase was calculated as the time of the first onset of the trace, defined as its first incremental baseline crossing of the sampling time (24 h–96 h of recording). C: control, S: social defeat. Yellow dots represent individual mice and black dots represent group means (± SEM). Red lines indicate the comparison pairs. * $p < 0.05$, $ $p < 0.001$. Sample size: liver, ADX control: n = 10, ADX social defeat: n = 13, sham control: n = 14, sham social defeat: n = 10; kidney, ADX control: n = 11, ADX social defeat: n = 12, sham control: n = 14, sham social defeat: n = 11; lung, ADX control: n = 10, ADX social defeat: n = 11, sham control: n = 14, sham social defeat: n = 11.

dampening of the rhythm was found in all tissues, neither social defeat nor adrenalectomy affected the amplitude of the rhythm.

Fig. 2g and h show the period and phase of the PER2-coupled bioluminescence rhythms in different tissues. For the period of the PER2 rhythm, three-way ANOVA revealed a significant overall effect of ADX ($F(1,129) = 5.86$, $p = 0.02$, $\eta_p^2 = 0.03$), as well as a TISSUE x ADX interaction ($F(2,129) = 3.98$, $p = 0.02$, $\eta_p^2 = 0.05$). However, post-hoc Tukey test did not indicate significant differences between the groups for any of the three tissues.

For the phase of the PER2 rhythm, three-way ANOVA indicated a significant effect of SOCIAL DEFEAT ($F(1,129) = 57.46$, $p < 0.001$, $\eta_p^2 = 0.31$), and this effect is dependent on the TISSUE (TISSUE x SOCIAL DEFEAT interaction, $F(2,129) = 26.4$, $p < 0.001$), on ADX (ADX x SOCIAL DEFEAT interaction, $F(1,129) = 71.28$, $p < 0.001$, $\eta_p^2 = 0.27$) and the combination of those two (TISSUE x ADX x SOCIAL DEFEAT interaction, $F(2,129) = 10.78$, $p < 0.001$, $\eta_p^2 = 0.16$). Post-hoc Tukey tests showed that the phase of the PER2 rhythm in the liver samples was not different among the groups. In the samples of kidney cortex, the phase of the PER2 rhythm was significantly delayed in the samples of defeat sham-operated mice as compared to control sham-operated mice (Onset1 = 46.33 ± 0.46 h and 41.99 ± 0.37 h, respectively; $p < 0.001$). This defeat-induced phase delay was reversed in the ADX group, as indicated by the comparison of the ADX stress group (42.55 ± 0.26 h) with both the ADX control (44.05 ± 0.35 h, $p = 0.51$), and the sham control ($p > 0.99$), as well as the comparison of ADX defeat group with sham defeat group ($p < 0.001$). In lung, the phase of the PER2 bioluminescence rhythm showed a highly significant delay of ~8 h in sham-operated defeated mice compared to sham-operated control mice (47.20 ± 0.51 h and 39.15 ± 0.40 h, respectively; $p < 0.001$). The lung samples of both adrenalectomized groups of mice had phases that were not

significantly different from that in the sham defeated mice (Onset 1 of ADX control = 45.52 ± 0.51 h, $p = 0.39$; Onset 1 of ADX stress = 46.84 ± 0.68 h, $p > 0.99$), but their phases were both significantly delayed relative to the sham control mice (both $p < 0.001$). Thus, adrenalectomy in itself was associated with highly significant phase delay relative to the sham control mice, similar in magnitude to the delay seen in defeat sham mice (no difference in phase between ADX control and ADX stress, $p = 0.75$).

We further examined whether the phase delay of the PER2-coupled bioluminescence rhythms in the lung samples of non-defeat ADX mice could be an effect of the ADX per se or, alternatively, might be a consequence of the low-level CORT that ADX mice received in their drinking water. For this matter, we introduced a new group of mice that were adrenalectomized but did not receive any CORT in their drinking water (saline only, ADX noCORT; Fig. 3). The amplitudes of the PER2 bioluminescence rhythm in the ADX no-CORT group were not different from that in the ADX control group and the sham control group (Fig. 3b). The period of the PER2-bioluminescence rhythm in the ADX no-CORT mice (24.95 ± 0.21 h) was not different from the period in the ADX control group with low-level CORT (25.44 ± 0.19 h, $p = 0.15$) but it was significantly longer than the period in the sham control group (24.19 ± 0.11 h, $p = 0.01$; Fig. 3c). Moreover, although mice in the ADX no-CORT group displayed highly variable Onset1 phases (50.49 ± 1.7 h), these were delayed ~10 h compared to sham-operated control ($p < 0.001$) and ~ 2 h with ADX control mice with low-level CORT ($p = 0.01$, Fig. 3a, d), suggesting that the major phase delay seen in the ADX control mice was not related to the low-level of CORT these animals received in their drinking water.

3.3. Plasma CORT measurements

All CORT levels were measured in trunk blood collected in the late dark phase from ZT21–23, which in the social defeat groups were one hour after the last daily defeat stress (Fig. 4). One-way ANOVA indicated a significant difference among groups ($F(4, 48) = 22.02$, $p < 0.001$, $\eta^2 = 0.65$). The social defeat stress was associated with significant elevated CORT levels in the sham-operated animals (302.38 ± 59.74 ng/mL, $p < 0.001$) but not in ADX animals (89 ± 16.3 ng/mL, $p = 0.38$), as compared with sham control (33.05 ± 5.37 ng/mL). Plasma CORT levels in the ADX no-CORT (saline drinking), the ADX control (3 mg/L CORT), and the ADX defeat groups were not different from each other (20.17 ± 4.73, 43.85 ± 10.89, and 89 ± 16.3 ng/mL respectively). The slightly, but not significantly, higher plasma CORT levels in the ADX stress group might be the result of social defeat animals drinking more after the stress exposures than non-defeat animals (data not shown).

4. Discussion

In the present study, we demonstrated that the effects of social defeat stress, ADX, or the interaction of both on peripheral clocks in male mice were highly tissue-specific. While PER2 rhythms in the liver were resistant to social defeat and ADX, the rhythms in kidney and lung were affected by both social defeat and ADX, although the effect of ADX was different for these two organs. The PER2 rhythm in the kidney cortex was phase delayed by over 4 h following 5 daily social defeat stresses, and this delay was largely reversed by removal of the adrenal glands. In the lung, PER2 rhythms displayed strong phase delays of ~8 h after both social defeat and ADX, which might imply that both high peak levels of CORT and a lack of CORT affect the phase of the peripheral clock in this tissue. Together the findings support the hypothesis that at least in some tissues, the induction of GC is required in social defeat-induced phase shifts of peripheral clocks (kidney) whereas the role of GC signaling in other tissues appears to be more complex (lung) (Fig. 4b).

During the social defeat stress days, we observed a significant suppression of locomotor activity in defeat mice (Fig. 1), in line with our previous studies (Meerlo et al., 1996; Meerlo et al., 1997; Ota et al., 2020). This social defeat stress-induced suppression of activity might be the result of a motivational deficit and is sometimes viewed as depressive-like behavior (Kong et al., 2022; Ota et al., 2018). In the current study, adrenalectomy did neither affect the basal level of activity nor did it prevent the social defeat-induced suppression of activity. The latter result is in agreement with several other studies reporting no effects of ADX on the amount of movement or profiles of running wheel activity in mice (Mulder et al., 2014; Tsuchiya et al., 2018; van den Buuse et al., 2004). In contrast, other studies reported a decrease in running wheel activity after ADX, and an increase in activity after administration of the synthetic glucocorticoid (dexamethasone) at the beginning of the light or the dark phase in rats (Malek et al., 2007;

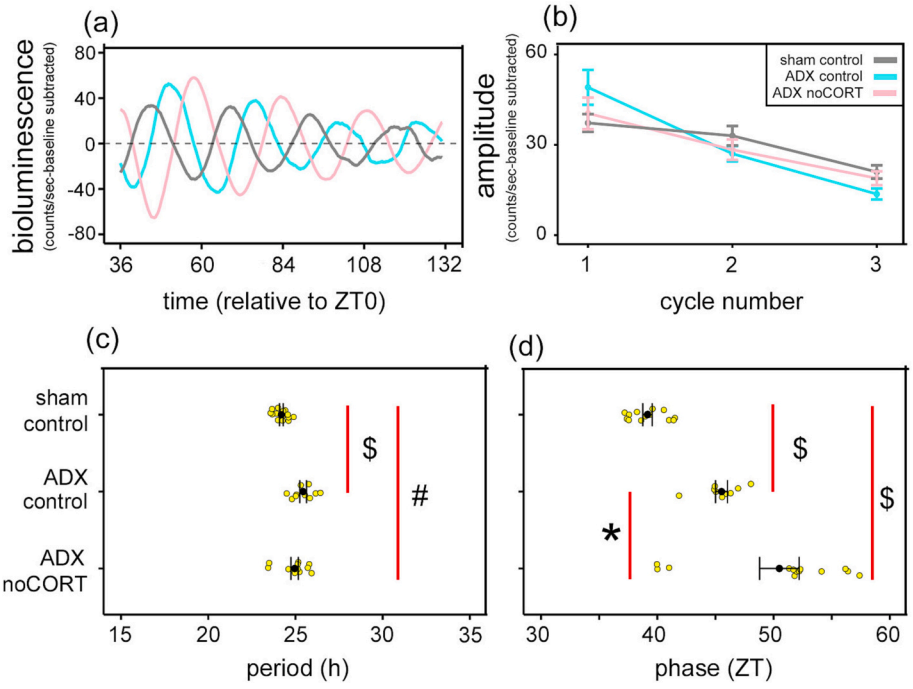

Fig. 3. Effects of adrenalectomy and exogenous CORT on PER2 expression in the lung.
Representative ex vivo PER2::LUC bioluminescence traces from lung (a) samples were taken from sham control mice, ADX control (3 mg/L CORT drinking), and ADX noCORT (saline only) mice. Values are plotted as 24-h running mean baseline subtracted photon counts per second. Recordings are from 12 h–108 h after the start of the culture, but time is recalculated to represent ZT before sacrifice (relative to ZT0 of the dissection day). Average amplitude of PER2::LUC bioluminescence rhythms in samples of the lung (b) from sham control mice, ADX control, and ADX noCORT mice. Amplitude values are plotted as mean photon counts per second of (peak – trough)/2 of each cycle. Period (c) and phase (d) values of PER2::LUC rhythms from the lung from sham control mice, ADX control, and ADX noCORT mice. The phase was calculated as the time of the first onset of the trace, defined as its first incremental baseline crossing of the sampling time (24 h–96 h of recording, see traces in panel (a)). Yellow dots represent individual mice and black dots black dots represent group means (± SEM). Red lines indicate the comparison pairs. * $p < 0.05$, # $p < 0.01$, $ $p < 0.001$. Sample size: ADX control: n = 10, sham control: n = 14, ADX noCORT: n = 13.

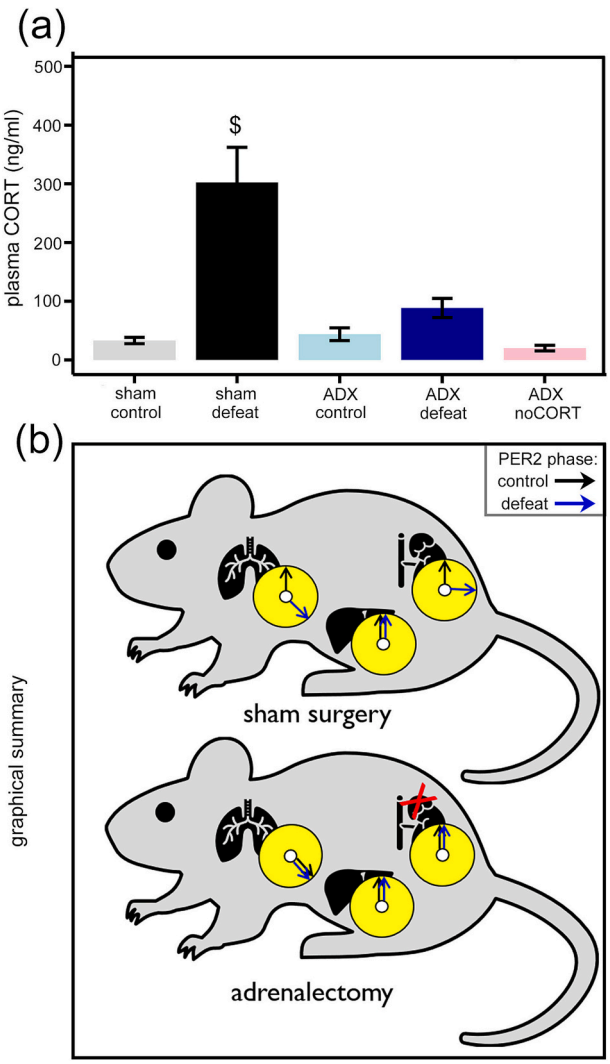

Fig. 4. Plasma CORT measurement in different groups (a) and graphic summary (b).

(a) Plasma CORT levels collected 1 h after the last defeat or control handling session (mean ± SEM; $ $p < 0.001$) indicates that CORT levels in sham mice exposed to stress were significantly higher than the levels in the other groups. No significant differences in CORT levels were found among the other four groups. (b) Graphic summary of main findings. Upper mouse represents sham and lower represents an adrenalectomized mouse (red cross depicts surgically removed adrenals). Clock symbols in the different tissues: black arrow indicates PER2 phase in control mice, blue arrow indicates that in social defeat mice. In the liver, neither stress nor adrenalectomy affected the phase of the PER2 rhythm. In the kidney, stress shifted the PER2 rhythm in sham operated mice, which was prevented in adrenalectomized mice. In the lung, both stress and adrenalectomy mice had a comparable delayed PER2 phase. Sample size: ADX control: n = 10, ADX defeat: n = 13, sham control: n = 10, sham defeat: n = 8, ADX noCORT: n = 12.

Moberg and Clark, 1976; Sage et al., 2004). One possible explanation for this discrepancy may be the different animal models that were used (mice vs rats). More comprehensive studies on the effects of ADX on running wheel activity of rodents are needed in the future, but at least in the present study, the shifted PER2 rhythm in tissues of the ADX mouse was not a result of locomotor activity changes.

In the present study, the 5-day social defeat stress in the late dark phase significantly shifted PER2 rhythms in the kidney and lung, but not in the liver (Fig. 2), which is in line with our previous study using the 10-day chronic social defeat protocols(Kong et al., 2022).

The resistance of the liver to effects of social defeat is not because the liver clock is resistant to glucocorticoid signaling, since various studies have shown phase shifts of liver clock gene expression in response to GC treatment both in vivo and in vitro (Ota et al., 2020; Tahara et al., 2015). Perhaps in the current study and Kong et al. (2022), we did not find effects of stress because of stable feeding and locomotor rhythms under entrained 12:12 LD conditions that may have dominated the liver PER2 rhythms over stress-related GC effects. This might explain the discrepancy with our earlier study showing social defeat-induced shifts in liver PER2 in mice under DD conditions (Ota et al., 2020). Indeed, signals associated with feeding/fasting cycles are the main synchronizers for liver clock, but not for lung and kidney (Manella et al., 2021).

The social defeat stress-induced delays of the PER2 rhythm in the kidney samples were prevented by ADX, which supports our hypothesis that these social defeat effects are mediated by adrenal glucocorticoid release. This interpretation is in agreement with studies showing that administration of glucocorticoids by itself can phase shift peripheral clocks in vitro and in vivo (Balsalobre et al., 2000; Cheon et al., 2013; So et al., 2009). Moreover, it has been established that glucocorticoids binding to their receptors can act on specific response elements in the promotor region of core clock genes, such as Per1 and Per2, and thereby directly affect the expression of these genes (Dickmeis, 2009; Dickmeis et al., 2013; Segall and Amir, 2010; So et al., 2009). Together these findings suggest that real life stressors may affect the peripheral kidney clock through glucocorticoid stress hormones released from the adrenals directly acting on clock gene expression.

Confirming our previous study, repeated social defeat stress in the late dark phase resulted in a large ~8 h phase delay of the lung clock (Kong et al., 2022). Whether this shift is mediated by social defeat-induced release of glucocorticoids as well remains uncertain. In the current study, both the ADX social defeat and ADX non-defeat mice showed a 6-8 h phase delay of the lung clock relative to control animals with intact adrenals and no difference in phase relative to stressed animals with intact adrenals (Fig. 2). The finding that in the adrenalectomized animals there was no difference between defeat and non-defeat mice in the phase of the lung PER2 rhythm might indicate that ADX prevented the stress effect. However, then presumably this effect was masked by a phase delaying effect of ADX itself. The large phase delay seen in the ADX non-defeat animals relative to the sham-operated non-defeat animals appears to be mediated by the ADX itself and not the additive low-level CORT in their drinking water. In fact, the phase delay in ADX animals receiving no CORT in their drinking water was even larger than the one in mice that received low-level CORT in their drinking water (Fig. 3). Thus, if anything, in the lung, the circadian and low physiological level of CORT reduced the effect of adrenalectomy. Interestingly, in the adrenalectomized mice that received no CORT in their drinking water, the phase of the PER2 rhythm in the lung was not only delayed, it was also highly variable (Fig. 3), which might suggest that the circadian and low physiological levels of CORT are important to maintain phase stability in this tissue. Together, it seems that the PER2 rhythm in the lung is very sensitive to manipulations of GC rhythms in the body and it would be interesting to uncover the underlying mechanisms of this.

The phenomenon that peripheral clocks respond differently to GC manipulation has been noticed before. For example, Pezuk et al. (2012) found that adrenalectomy delayed the peak phase of the steady-state rhythm of PER1::LUC in the kidney by 7.5 h and in the liver by 9 h even though there was no change in the LD cycle. Hydrocortisone treatment in ADX animals restored the phase of PER1::LUC in the kidney, but in the liver it caused the loss of phase synchrony (Pezuk et al., 2012). Also, Chun et al. (2018) showed that in rats adrenalectomy attenuated acute (30 min) restraint stress-induced Per1 mRNA in the paraventricular nucleus of hypothalamus and ventral orbital cortex, but not in the medial prefrontal cortex.

Not only different tissues, but also the different clock genes in those tissues respond differently to GC signaling. Sotak et al. (2016) found that adrenalectomy had a significant inhibitory effect on the level of Per1

mRNA in visceral adipose tissue (VAT), liver, and jejunum, but not in kidney and splenocytes. Similarly, adrenalectomy downregulated mRNA levels of Per2 in splenocytes and VAT, Per3 in the jejunum, RevErbα in VAT and Dbp in VAT, kidney, and splenocytes, whereas the mRNA amounts of Per1 and Per2 in kidney and Per3 in VAT and splenocytes were up-regulated (Sotak et al., 2016). Interestingly, another article reported in rats that ADX phase delayed the Per2 mRNA rhythms but advanced the Per1 mRNA rhythms in prefrontal cortex. While in-phase CORT injections restored Per1, Per2 and Bmal1 mRNA rhythms in ADX rats, anti-phasic CORT injections reversed the Per2 rhythms but silenced the daily rhythms of Per1 and Bmal1 mRNA transcriptions (Woodruff et al., 2016). Our current study uses only PER2 expression as the indicator of peripheral rhythms, but it could be interesting to see how the rhythms of other clock genes are affected by social defeat stress or adrenalectomy in the future.

A possible explanation for the tissue-specific responses to stress and GC manipulation might involve differences in glucocorticoid receptor densities or differences in downstream of the receptors. Glucocorticoids such as corticosterone bind to GR, which in turn anchor in the GRE or GBS in the promoter region of Per1 and Per2 and other clock genes to activate their transcriptions (Balsalobre et al., 2000; Cheon et al., 2013; Dickmeis et al., 2013; Oster et al., 2017; So et al., 2009). The expression of GR together with local cellular CORT concentrations and exposure of the GRE in different tissues could all influence the effects of GC on clock gene expression (Oakley and Cidlowski, 2013; Scheschowitsch et al., 2017).

Another possible explanation for variation between tissue clocks in their response to stress might be the modulation of some tissues by direct sympathetic nervous system inputs. Supporting this, it was found that forced exercise (a moderate stressor) at ZT4 induced phase advances of PER2 in the submandibular gland, which could be prevented by ADX itself, while the phase advance in kidney and liver could only be prevented by the combination of ADX and adrenergic receptor blockers (Sasaki et al., 2016).

Lastly, there is a concern that ex vivo PER2-coupled bioluminescence rhythms can be modulated by the dissection time or culturing procedures and may not always exactly represented the in vivo phase in living animals (Leise et al., 2020). Importantly, in the current study we did not compare PER2 rhythms in tissue samples dissected at different times of day. We only directly compared the bioluminescence rhythms in samples collected at the same time of day, i.e., samples from defeated mice and control mice (with or without adrenals) dissected at the same time in the late dark phase (ZT22–23). We are therefore confident that our conclusions on the effects of stress and adrenalectomy are not confounded by comparing samples collected at different times of day. Whether different tissues or organs are differently affected by culturing procedures (e.g., temperature or media changes) remains to be established.

5. Conclusion

Together, our experiment suggests that uncontrollable stress can have a major impact on peripheral clocks. However, both the magnitude of the effect and the underlying physiological mechanism is strongly tissue dependent. In some tissues, this may involve glucocorticoid released from the adrenals during stress. Consequently, stress results in a major reorganization of the circadian system by inducing strong alterations of the phase relationships between different tissues and organs. An important unanswered question is whether this circadian reorganization is a way of coping and adapting to repeated stressors or a sign of maladaptation and a potential road to disease.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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