

Deviant circadian rhythmicity, corticosterone variability and trait testosterone levels in aggressive mice

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Abstract

Although aggression has been linked to disturbances of circadian rhythm, insight into the neural substrate of this association is currently lacking. The suprachiasmatic nucleus (SCN) of the hypothalamus, the master circadian clock, is regulated by clock genes and known to influence the secretion of corticosterone and testosterone, important hormones implicated in aggression. Here, we investigated deviations in the regulation of the locomotor circadian rhythm and hormonal levels in a mouse model of abnormal aggression. We tested aggressive BALB/cJ and control BALB/cByJ mice in the resident-intruder paradigm and compared them on their locomotor circadian rhythm during a 12 h light/12 h dark cycle and constant darkness. State (serum) corticosterone and trait (hair) corticosterone and testosterone levels were determined, and immunohistochemistry was performed to assess the expression of important clock proteins, PER1 and PER2, in the core and shell of the SCN at the start of their active phase. Compared with BALB/cByJ mice, aggressive BALB/cJ mice displayed: (1) a shorter free-running period in constant darkness; (2) reduced state corticosterone variability between circadian peak and trough but no differences in corticosterone trait levels; (3) lower testosterone trait levels; (4) higher PER1 expression in the SCN shell with no changes in PER2 in either SCN subregion during the early dark phase. Together, these results suggest that aggressive BALB/cJ mice have disturbances in different components encompassing the circadian and hormonal cycle, emphasizing their value for future investigation of the causal relationship between SCN function, circadian clocks and aggression.

KEYWORDS

aggression, circadian rhythms, clock, corticosterone, suprachiasmatic nucleus, testosterone

Abbreviations: AR, androgen receptor; BSA, bovine serum albumin; CORT, corticosterone; DD, constant darkness conditions; LD, 12 h light/12 h dark cycle; PBS, phosphate-buffered saline; PFA, paraformaldehyde; RIP, resident-intruder paradigm; SCN, suprachiasmatic nucleus; ZT, Zeitgeber time.

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1 | INTRODUCTION

An individual's internal circadian clock is essential to predict daily changes in the surrounding environment associated with the transition from day to night and to adapt behavioural and physiological processes appropriately. Cycles of change that repeat approximately every 24 h are known as circadian rhythms and include examples such as the sleep/wake cycle, changes in body temperature and release of hormones such as glucocorticoids (Eastman et al., 1984; Nunez & Stephan, 1977; Refinetti et al., 1994). Altered sleep patterns, disturbances in the sleep/wake cycle and low and high glucocorticoid levels have been observed in individuals suffering from aggressive behaviour and conduct disorder (Hawes et al., 2009; Lindberg et al., 2003, 2006), suggesting that disruption of the circadian clock system might underly its pathology.

Circadian rhythms are generated by the suprachiasmatic nucleus (SCN) of the hypothalamus (Abe et al., 1979; Refinetti et al., 1994) and are regulated by several genes known as clock genes that influence their own transcription and translation through series of negative feedback loops (Dunlap, 1999; Reppert & Weaver, 2001). The SCN is divided into two subregions: the core and the shell (Dunlap, 1999). The SCN core receives direct photic input from the retina through the retino-hypothalamic tract and thereby displays a strong response to light stimuli, which can reset the phase of SCN cells. In contrast, the SCN shell contains neurons with strong autonomous daily rhythms that persist even in the absence of environmental cues (known as 'free running' rhythms; Yan et al., 1999). Particularly, the clock genes *Per1* and *Per2* have been proposed to play important roles in the maintenance of circadian rhythmicity, because in the absence of light stimuli, dysfunction of the typically oscillating *Per1* and *Per2* expression in the SCN has been found to destabilize the circadian clock and lead to severely disrupted rhythmicity over time (Bae et al., 2001).

Initial evidence suggests a possible link between circadian rhythm components and aggression. Hormones implicated in aggressive behaviour such as glucocorticoids and testosterone (Haller & Kruk, 2006; Jasnów et al., 2000; Stagkourakis et al., 2020) have been found to influence clock gene rhythms (Karatsoreos et al., 2011; Segall et al., 2006). Whereas testosterone has been proposed to play a role in several parameters of circadian rhythmicity known to be SCN-dependent, including the period and precision of daily activity onset (Karatsoreos et al., 2007), glucocorticoids have been found to regulate peripheral clock genes outside the SCN (Segall et al., 2006). What is more, recent rodent findings highlighted that SCN activity directly modulates the daily

rhythm of aggression by regulating function of the subparaventricular zone of the hypothalamus, which subsequently influences activity in the ventromedial hypothalamus, a region known to regulate aggression (Todd et al., 2018). Although this emphasizes a possible involvement of disrupted circadian clocks in aggression and recent reviews have proposed hypotheses on the underlying mechanisms (Hood & Amir, 2018; Mogavero et al., 2018), empirical studies investigating the contribution of circadian disturbances to abnormal aggression are lacking.

In the present study, we aimed to assess potential deviations in the regulation of circadian rhythms and hormonal levels in a mouse strain known to display extreme and abnormal forms of aggressive behaviour: the BALB/cJ mice. Compared with their non-aggressive counterparts, that is, the genetically related BALB/cByJ mice, BALB/cJ mice display elevated levels of maladaptive aggression in the resident-intruder test. That is, whereas aggressive behaviour towards an intruder per se is not abnormal, BALB/cJ mice break several behavioural natural rules. In short, aggressive intent is typically signalled by threat behaviour to allow withdrawal of the opponent (1), the level of aggressiveness is not extreme to minimize risks and energy losses (2), aggression should be attenuated upon submission of the opponent (3) and attacks are targeted to non-vulnerable body parts (4) (Haller, 2018). These behavioural features are preserved in the laboratory (Haller et al., 2001, 2005) but not in aggressive BALB/cJ mice, which keep displaying heightened aggressive behaviour, even after submission of the intruder, and they attack vulnerable body parts in smaller non-life-threatening intruders (Jager et al., 2020; van Heukelum, Drost, et al., 2019; van Heukelum, Mogavero, et al., 2019; Velez et al., 2010).

While BALB/cJ and BALB/cByJ mice show clear differences in aggressive behaviour, they are genetically greatly similar; genetic differences have only been identified in 11 DNA copy number variants (Velez et al., 2010). The genetic similarity between the two strains allows us to conduct controlled comparisons to investigate circadian rhythm deviations in an animal model of abnormal aggression.

We hypothesized that aggressive BALB/cJ mice would display an aberrant behavioural circadian rhythm and levels of corticosterone (CORT) and testosterone, as well as differences in *PER1* and *PER2* expression in the SCN subregions during the start of their active phase, when the expression of both clock proteins is most prominent (Field et al., 2000; Guissoni Campos et al., 2017). To test our hypotheses, we assessed the release of CORT during circadian peak and trough, and aggression-induced levels in response to a resident-

intruder encounter. In addition, we examined long-term (i.e., trait) secretion of CORT and testosterone in the hair of the mice. Behavioural expression of the circadian rhythm was assessed by measuring spontaneous locomotor activity patterns, first under standard 12 h light/12 h dark (LD) conditions and then under free running conditions during constant darkness (DD) to assess autonomous daily rhythms in the absence of environmental cues.

2 | MATERIALS AND METHODS

2.1 | Animals

We tested male BALB/cJ ($n = 12$) and BALB/cByJ ($n = 12$) mice (8 weeks old at the start of testing), obtained from the Jackson Laboratory (Bar Harbor, ME, USA) in the resident intruder paradigm (RIP) to assess aggression and in the PhenoTyper (Noldus IT, Wageningen, The Netherlands) automated home cage environment to assess the circadian rhythm of locomotion. For the RIP, male C57BL/6J ($n = 16$, Charles River Laboratories, Erkrath, Germany, and $n = 8$, Janvier, France) mice were used as intruders. All mice were housed in an enriched environment (High Makrolon[®] cages with Enviro Dri[®] bedding material and Mouse Igloo[®]) with ad libitum access to dry food and water. They were maintained on a reversed 12–12 h light–dark (LD) cycle with lights on at 8:00 PM (Zeitgeber Time 0—ZT0). In line with the typical RIP protocol, experimental mice were housed individually at least 1 week prior to the start of the RIP, whereas intruder mice were housed in groups of four. RIP testing occurred during the dark phase between ZT13 and ZT14. All animal procedures followed the ARRIVE Guidelines 2.0 and were conducted in compliance with EU and national regulations as well as local animal use ethical committees (European Directive 2010/63/EU) and approved by the Ethics Committee on Animal Experimentation of Radboud University (RU-DEC number 2016-0094).

2.2 | Procedures

Upon arrival, all mice were allowed to adapt for 2 weeks to the new environment before behavioural testing started. Subsequently, mice were individually housed in the PhenoTyper automated home cage environment to assess the circadian rhythm of locomotion in a 12–12 h LD cycle. After 8 days in the PhenoTypers, the mice were individually housed in the standard High Makrolon[®] cages for 10 days before the start of the 5-day RIP. Following the RIP interaction on the last day, a tail bleed was performed on each animal (exactly 25 min after the start of the RIP test) to collect blood for the assessment of peak CORT levels associated with the aggression task. The blood collection lasted less than a minute, and because CORT rises in plasma are delayed, it is improbable that the stress of the blood collection impacted the results. A tail bleed was also performed a week after to take baseline measurements of CORT at different time points to assess its circadian peak and trough. Three weeks after the last RIP interaction, mice were housed again in the PhenoTypers but this time to assess the circadian rhythm of locomotion in DD. After 9 days in DD, the mice were allowed to recover in a 12–12 h LD cycle for 3 weeks before they were transcardially perfused for subsequent immunohistochemical analysis. At sacrifice, the hair of the animals was collected for the assessment of hair hormone levels. The experimental timeline can be found in Figure 1.

2.3 | Behavioural testing

2.3.1 | RIP

Aggression testing was done for five consecutive days in the home cage of the BALB/cJ and BALB/cByJ mice in a dark room with red overhead lighting, following the same procedures as previously described (van Heukelum, Mogavero, et al., 2019). Each day, confrontation with a 2 weeks younger unknown intruder of lower weight was allowed for 5 min, following 5 min separation by a glass

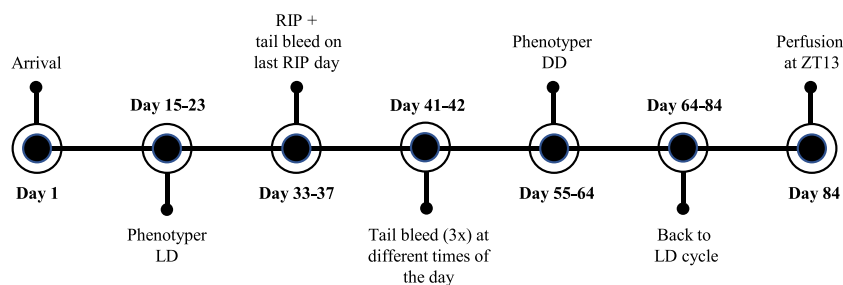


FIGURE 1 Schedule of the experimental design. Timeline of the study from day of arrival (Day 1) until sacrifice (Day 78) of BALB/cJ and BALB/cByJ mice. Abbreviations: LD = 12–12 h light–dark cycle; RIP = resident intruder paradigm; DD = constant darkness; ZT = Zeitgeber time

screen. Behaviour was videotaped using an infrared camera for later analysis of attack behaviour.

Attack behaviour was scored manually in terms of attack latency, attack duration and tail rattles using the program The Observer XT 11 (Noldus). An attack was defined as a bite directed at the back, tail, belly, neck or face of an intruder (van Heukelum, Mogavero, et al., 2019). All recordings were scored by the same researcher who was blinded to the strain of the animal (BALB/cJ and BALB/cByJ mice have the same appearance). Aggression scores on all 5 days are shown in Figure S1. A previous study has indicated that trait aggression is best captured by the first 3 days of the RIP, because also non-aggressive animals learn over time to anticipate intruders entering their home cage, leading to learnt aggression towards an intruder (van Heukelum, Drost, et al., 2019). Because we wanted to examine trait/unprovoked aggression rather than learnt/strategic aggression, average scores for the first 3 days were used to quantify mean aggression scores per animal.

2.3.2 | Phenotyper

To measure the circadian rhythm of locomotion, mice were individually housed in the PhenoTyper (Noldus IT, Wageningen, The Netherlands) automated home cage environment during two different conditions: in a 12–12 h LD cycle and in DD. Due to a limited amount of PhenoTyper home cages, the animals were divided in three cohorts of $n_{cJ} = 4$ and $n_{cByJ} = 4$. Already in the first cohort, significant differences were observed between BALB/cJ and BALB/cByJ mice in the circadian rhythm of locomotion following 9 days in DD. Therefore, for the other two cohorts, after the 9 days in DD, BALB/cJ ($n = 8$) and BALB/cByJ ($n = 7$) mice were kept for five extra days in the PhenoTypers to measure their re-entrainment to the 12–12 h LD cycle. Long-term continuous locomotor activity in mice was assessed with built-in digital infrared light sources in the top unit of each cage, as previously described (de Visser et al., 2006), and recorded by EthoVision 3.0 (Noldus IT, Wageningen, The Netherlands). The circadian rhythm of locomotion was assessed by measuring distance moved in 15 min intervals and were plotted in actograms. When the animals were exposed to DD, the animals showed the expected advance in the circadian rhythms, as observed before (Dubocovich et al., 2005; Valentinuzzi et al., 1997). The free-running period of each animal was calculated by determining the onset of activity for every day in DD and by fitting a regression line (LeGates & Altimus, 2011). The amplitude of the rhythms in LD and DD conditions was obtained with the cosinor method. Here, a cosine

wave with known period (i.e., the free-running period) was fitted to the data using the least squares method (Refinetti et al., 2007).

2.4 | Hormone measurements

2.4.1 | Plasma CORT measurements

The tail snip method (Kim et al., 2018) was used to collect 10 μ l of blood from the tail of the mice for CORT measurements at different timepoints. Here, we compared baseline CORT levels at circadian peak (ZT11) with circadian trough (ZT23), and we measured CORT after the last RIP interaction (at ZT14) and compared it with CORT levels at baseline at the same time (at ZT14) on another day. All blood samples were centrifuged at 3500 rpm for 25 min at 4°C, and the plasma was stored at –20°C until further analysis. Plasma CORT concentrations were measured *in duplo* using a radioimmunoassay kit (Corticosterone RIA Kit, MP Biomedicals, LLC) with a sensitivity of 7.70 ng/ml, following the manufacturer's instructions. For the analysis, the average of the two measurements was taken. In case the variation coefficient between the two measurements was greater than 20% and with an F value greater than 5.0, the measurements were considered unreliable and excluded from further analysis. This resulted in the exclusion of four BALB/cJs and one BALB/cByJ for the comparison of CORT following the last RIP interaction and the corresponding baseline measurements. No measurements were excluded for the comparison of baseline CORT levels at ZT11 and ZT23.

2.4.2 | Hair CORT and testosterone measurements

Hair samples were collected by shaving all the hair from the back of all animals just before they were sacrificed. Samples were enclosed in aluminium foil and sent to the laboratory of Prof. Dr. C. Kirschbaum at the TU Dresden (Dresden LAB Service GmbH) for the analysis of CORT and testosterone concentrations. The hormone levels were assessed with the liquid chromatography with tandem mass spectrometry (LC–MS/MS) method as previously described (Gao et al., 2013, 2019).

2.5 | Perfusion and tissue preparation

All BALB/cJ and BALB/cByJ mice were sacrificed between 9:00 AM and 9:30 AM (ZT13 and ZT13.5). Mice

were deeply anaesthetized with isoflurane (3–5%) and 0.15 ml of i.p. sodium pentobarbital (90 mg/kg) and perfused with 0.1 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde solution (PFA). Brains were removed, fixed overnight in 4% PFA and then kept in 0.1 M PBS at 4°C. One day before sectioning, brains were placed in 0.1 M PBS plus 30% sucrose to ensure cryoprotection. Coronal sections (30 µm) were obtained on a freezing microtome (Microm HM 4500 Sliding Microtome, Thermo Scientific) and stored at 4°C in containers filled with 0.1 M PBS + 0.01% sodium azide.

2.6 | Immunohistochemistry

For immunofluorescence procedures, free-floating sections were rinsed in 0.1 M PBS for 10 min, followed by a 30-min pre-incubation in PBS-BT. PBS-BT solution consisted of 0.1 M PBS supplemented with 10% bovine serum albumin (BSA) and 0.3% Triton X-100. Rabbit anti-PER1 (1:1000, Sigma-Aldrich, ab2201) and rabbit anti-PER2 (1:1000, Abcam, ab180655) were incubated overnight in PBS-BT at 4°C in separate series of sections. After the overnight incubation, sections were washed three times for 10 min in PBS, followed by 2 h incubation with secondary antibodies at room temperature. The secondary antibody was goat anti-rabbit Alexa Fluor 555 (1:500, Thermo Scientific). Sections were then washed again three times for 10 min, incubated shortly in DAPI (1:10,000 in PBS) and mounted on gelatin-coated slides. When the sections were dry, they were coverslipped using Vectashield (Thermo Scientific).

Stainings were imaged using a Zeiss fluorescent microscope (Axio Imager.A2, Carl Zeiss Microscopy GmbH). Images were acquired using a 20× magnification using the Zen imaging software (Zeiss) applying identical settings for all samples within each experiment (brightness, exposure time, etc.). All image analyses were performed using Image J (Fiji). The localization of the SCN was determined by the specific strong staining of PER1 or PER2 together with DAPI (see Figure 5c,d). To assess signal intensity, the average optical density for the PER1/2 fluorophore was obtained for a fixed square area of 5600 µm² in the core and shell for each image. Background staining was measured in a non-immunoreactive region adjacent to each SCN and subtracted from the immunoreactive intensity within the SCN core and shell (Cheng et al., 2019). Background staining did not significantly differ between groups ($p > 0.6$ for both PER1 and PER2). At least two images of the SCN (Bregma –0.46 until –0.58 mm) were measured per animal, and data were averaged to obtain single intensity measures per animal. Two BALB/cJ mice plus one BALB/cByJ were

excluded from the PER1 analysis, and three BALB/cJ plus two BALB/cByJ mice were excluded from the PER2 analysis because not enough good quality/undamaged SCN slices were available. The researcher performing these analyses was blinded to the strain of the animal being analysed.

2.7 | Statistical analysis

Two BALB/cByJ mice were excluded from all the analyses, one because he unexpectedly died in his home cage before the end of the study and one because he was an extreme outlier in aggressive behaviour (deviating more than 3 interquartile ranges from the group's median). The outlier BALB/cByJ mouse was more aggressive than the most aggressive BALB/cJ mouse and as such not representative of the overall BALB/cByJ group. On the last resident intruder day (Day 5), this outlier BALB/cByJ mouse was also the only mouse that caused bleeding in the intruder mouse. Because this unpredictable result could impact the results of the other measurements, we considered it fitting to exclude this animal from all further analyses. This gave a final sample of $n_{cJ} = 12$ and $n_{cByJ} = 10$ for the analyses.

All variables were checked for normality with the Shapiro–Wilk's W test in combination with inspection of histograms and Q – Q plots. Due to non-normal distribution of majority of the data, non-parametric Mann–Whitney U tests were performed to check strain differences using SPSS23-software (SPSS inc., Chicago, USA). To test the relationship between aggressive behaviour, locomotor circadian rhythm parameters, hormone levels and clock gene expression, we performed non-parametric partial correlations controlling for. The false discovery rate method (Benjamini & Hochberg, 1995) was used to correct p values for multiple comparisons (indicated by 'p_{corr}') when behavioural differences between strains were tested using more than one behavioural readout and when more than two variables were correlated to test a specific hypothesis. The significance level tested was set at $p \leq 0.05$, and all tests were two-tailed. Figures depict medians and interquartile ranges.

3 | RESULTS

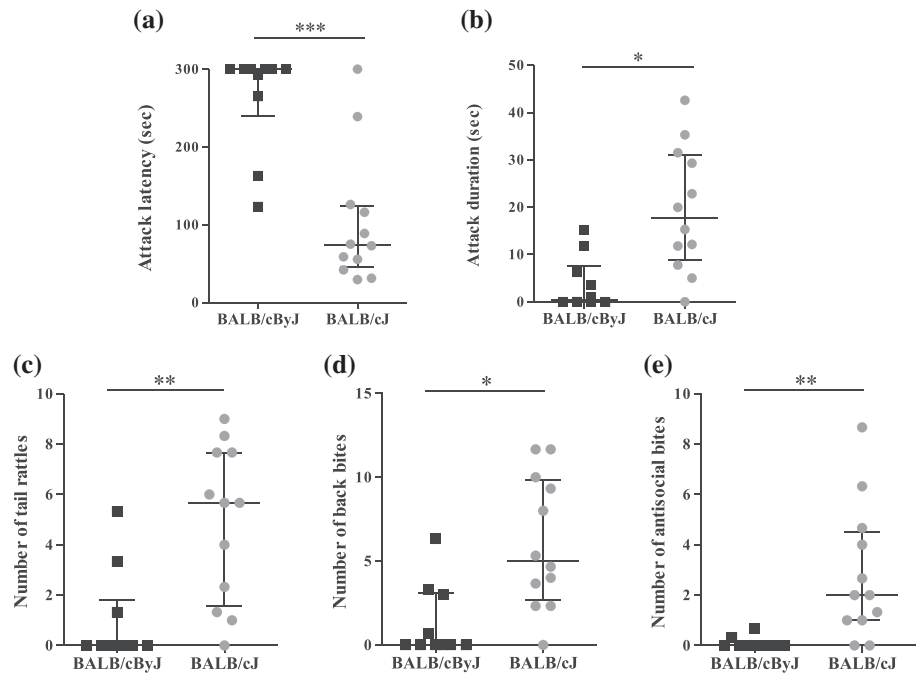
3.1 | BALB/cJ mice are more aggressive than BALB/cByJ mice

We used the 5-day resident-intruder paradigm to confirm that BALB/cJ mice engaged in more aggressive behaviour than the control BALB/cByJ mice. In line with previously

FIGURE 2 Aggressive behaviour in the resident intruder paradigm (RIP). BALB/cJ attacked faster (a), for longer (b), showed more tail rattles (threat behaviour; c) and bit more often in socially accepted places (i.e., the back; d) and anti-social places (vulnerable body parts such as tail, face, neck or belly; e). For all variables, median and interquartile range (IQR) values of the first three RIP days are displayed.

* $p_{\text{corr}} < 0.05$; ** $p_{\text{corr}} < 0.01$;

*** $p_{\text{corr}} < 0.001$



published studies (Jager et al., 2020; van Heukelum, Drost, et al., 2019; van Heukelum, Mogavero, et al., 2019; Velez et al., 2010), BALB/cJ mice showed increased levels of aggression compared to BALB/cByJ mice on all aggression measures, and this was most pronounced on the first 3 days (Figure S1), when trait aggression is captured best (van Heukelum, Drost, et al., 2019). Therefore, data of these days were averaged to quantify mean aggression scores per animal. Non-parametric Mann–Whitney U tests revealed that BALB/cJ mice attacked the intruder faster ($U = 10.0$, $n_{\text{cJ}} = 12$, $n_{\text{cByJ}} = 10$, $p_{\text{corr}} < 0.001$, $\eta^2 = 0.494$, Figure 2a) and for longer duration ($U = 14.5$, $n_{\text{cJ}} = 12$, $n_{\text{cByJ}} = 10$, $p_{\text{corr}} = 0.012$, $\eta^2 = 0.409$; Figure 2b). BALB/cJ mice also displayed increased threat display shown as tail rattles ($U = 15.0$, $n_{\text{cJ}} = 12$, $n_{\text{cByJ}} = 10$, $p_{\text{corr}} = 0.006$, $\eta^2 = 0.400$; Figure 2c) and increased number of bites both in non-vulnerable (back bites, $U = 17.0$, $n_{\text{cJ}} = 12$, $n_{\text{cByJ}} = 10$, $p_{\text{corr}} = 0.018$, $\eta^2 = 0.365$; Figure 2d) and vulnerable body parts (antisocial bites, $U = 12.0$, $n_{\text{cJ}} = 12$, $n_{\text{cByJ}} = 10$, $p_{\text{corr}} = 0.006$, $\eta^2 = 0.455$; Figure 2e).

3.2 | BALB/cJ mice have a shorter free-running period with no differences in amplitude, overall activity levels and re-entrainment to the LD cycle

To measure the circadian rhythm of locomotion in aggressive BALB/cJ mice compared with the control BALB/cByJ mice, animals were individually housed in the Noldus PhenoTyper automated home cage environment, and their level of activity was tracked under LD

and DD conditions. During the LD cycle, both groups displayed similar circadian patterns of locomotor activity with activity onset at the start of the dark phase at ZT12 and the lowest amount of activity when the lights were on (Figure 3a). There were also no differences in the amplitude and in the overall activity levels (measured by the distance travelled per hour) during LD and DD (Table 1). However, in DD conditions, BALB/cJ mice had a significantly shorter free-running period ($U = 1.0$, $n_{\text{cJ}} = 12$, $n_{\text{cByJ}} = 10$, $p < 0.001$, $\eta^2 = 0.688$; Figure 3b). Following DD, all animals immediately re-entrained to the LD cycle within a single day (see Figure S2 for an hourly overview of activity in the first 24 h after the lights were switched on).

3.3 | BALB/cJ mice have lower CORT variability between circadian peak and trough, and lower testosterone levels

We next sought to assess differences in hormone levels between the two strains by measuring plasma CORT levels at different time-points during standard LD conditions and long-term (i.e., trait) CORT and testosterone levels in the hair. We found that at circadian peak (at 7:00 h [ZT11]; the time preceding the steepest increase in locomotor activity in both strains; see Figure 3b), BALB/cJ mice had lower levels of CORT compared with BALB/cByJs ($U = 25.0$, $n_{\text{cJ}} = 12$, $n_{\text{cByJ}} = 10$, $p_{\text{corr}} = 0.021$, $\eta^2 = 0.242$), whereas at circadian trough (at ZT23), they had higher CORT levels ($U = 21.5$, $n_{\text{cJ}} = 12$, $n_{\text{cByJ}} = 10$, $p_{\text{corr}} = 0.021$, $\eta^2 = 0.293$; Figure 4a,

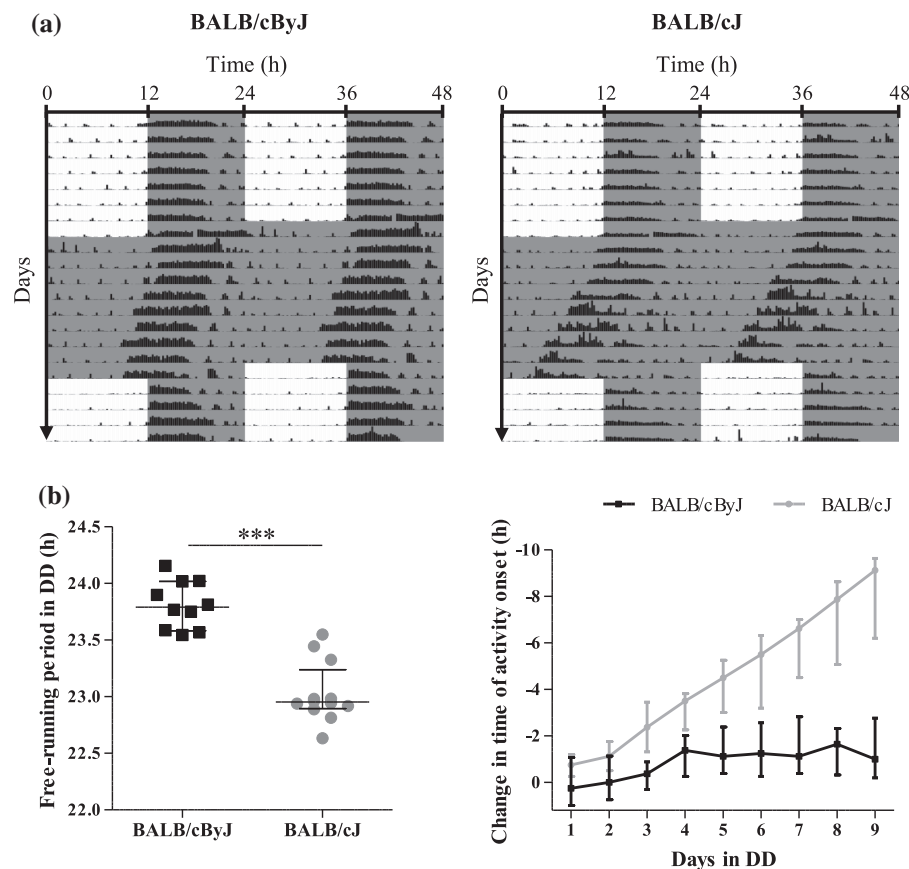


FIGURE 3 Circadian rhythm of locomotion. (a) Sample double plotted actograms from a representative control BALB/cByJ and an aggressive BALB/cJ mouse. The shaded area reflects the dark phase of the 12 h light/12 h dark (LD) and constant darkness (DD) cycles. During DD conditions, BALB/cJ showed a shorter free-running period with a greater daily change in time of activity onset (b). Displayed are median and interquartile range (IQR) values. *** $p < 0.001$

TABLE 1 Details of circadian parameters for BALB/cByJ and aggressive BALB/cJ mice

	BALB/cByJ ($n = 10$)	BALB/cJ ($n = 12$)
Rhythm amplitude LD (m/h)	73.12 (32.46)	58.91 (27.50), n.s.
Distance travelled LD (m/h)	72.12 (18.73)	69.40 (14.28), n.s.
Rhythm amplitude DD (m/h)	97.69 (78.21)	101.22 (51.71), n.s.
Distance travelled DD (m/h)	110.34 (58.23)	95.24 (44.05), n.s.

Note: Displayed are medians (and IQR values). All comparisons were not significant (n.s.).

Abbreviations: DD, constant darkness; LD, 12 h light/12 h dark cycle.

left panel). The difference in CORT level between circadian peak and circadian trough was therefore smaller for the BALB/cJs ($U = 23.0$, $n_{cJ} = 12$, $n_{cByJ} = 10$, $p_{corr} = 0.021$, $\eta^2 = 0.271$).

We also sought to investigate the CORT response to an aggressive encounter. Therefore, we measured CORT after the last RIP interaction (at ZT14) and compared it with CORT levels at baseline at the same time (at ZT14) on another day (Figure 4a, right panel). Similar to the result of baseline CORT levels at ZT11, BALB/cJ mice

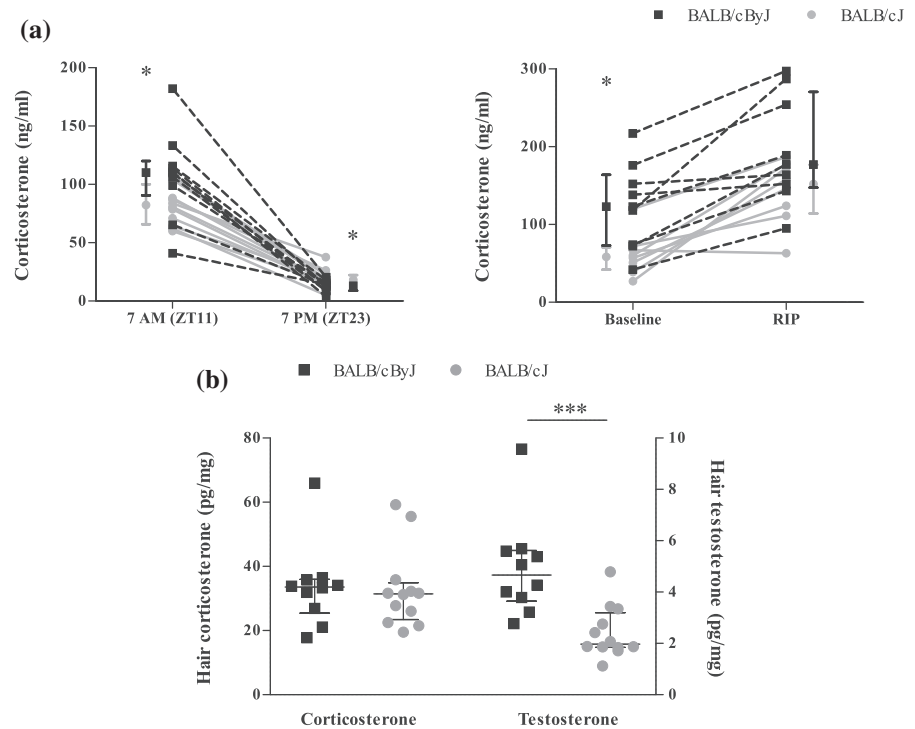
had lower baseline CORT levels at ZT14 ($U = 9.0$, $n_{cJ} = 8$, $n_{cByJ} = 9$, $p_{corr} = 0.024$, $\eta^2 = 0.397$). Neither absolute CORT levels after the RIP test ($U = 21.0$, $n_{cJ} = 8$, $n_{cByJ} = 9$, $p_{corr} = 0.251$), nor the RIP-induced increase in CORT levels from baseline (measured by subtracting baseline CORT levels from the RIP CORT levels; $U = 31.0$, $n_{cJ} = 8$, $n_{cByJ} = 9$, $p_{corr} = 0.673$) differed between strains. However, because of learnt aggression in the BALB/cByJ mice, also most readouts of aggressive behaviour did not differ anymore between strains on the last RIP interaction (Figure S1), which might explain the absence of a difference. No correlation was found between the RIP-induced increase in CORT levels and abnormal aggressive behaviour on that day ($p > 0.5$).

When analysing long-term hormone levels in the hair, CORT levels did not differ between the two strains ($U = 49.0$, $p = 0.497$), but BALB/cJ mice had significantly lower hair testosterone levels ($U = 9.0$, $n_{cJ} = 12$, $n_{cByJ} = 10$, $p < 0.001$, $\eta^2 = 0.514$; Figure 4b).

3.4 | BALB/cJ mice display higher PER1 expression in the SCN shell during the early dark phase

PER1 and PER2 expression levels were determined in the SCN core and shell during the early dark phase. We

FIGURE 4 Corticosterone and testosterone levels. Serum corticosterone levels at the circadian peak and trough for all individual animals (a). Left panel: BALB/cJ mice had lower corticosterone levels at circadian peak (ZT11) but higher levels at circadian trough (ZT23). Right panel: BALB/cJ mice had lower corticosterone levels at baseline (ZT14) but no significant difference at ZT14 following the RIP. There was also no difference in the increase in corticosterone from baseline to resident intruder paradigm (RIP). (b) BALB/cJ and BALB/cByJ mice had no differences in hair corticosterone levels, but BALB/cJ mice showed lower hair testosterone levels than BALB/cByJ mice. Displayed are median and interquartile range (IQR) values. * $p < 0.05$; *** $p < 0.001$



chose this timepoint because it corresponds to the start of the active phase of the mice, and PER1 and PER2 expression levels are both known to be high from ZT10–15 (Field et al., 2000; Guisconi Campos et al., 2017), facilitating the detection of potentially deviant expression. No differences in PER2 expression between the two strains were observed in either the SCN core or shell ($U = 35.0$, $n_{cJ} = 9$, $n_{cByJ} = 8$, $p = 0.923$ for both; Figure 5b). Also for PER1, expression in the SCN core did not differ ($U = 37.0$, $n_{cJ} = 10$, $n_{cByJ} = 9$, $p = 0.549$), but BALB/cJ mice displayed significantly higher PER1 expression in the SCN shell compared with BALB/cByJs ($U = 13.0$, $n_{cJ} = 10$, $n_{cByJ} = 9$, $p = 0.008$, $\eta^2 = 0.359$; Figure 5a).

Subsequent partial Spearman's correlation analyses between abnormal aggression (antisocial bites from D1–3), free-running period, CORT variability (amplitude difference between circadian peak and trough), hair testosterone levels and PER1 expression in the SCN shell revealed no significant relationships ($p > 0.2$ for all comparisons controlling for strain).

4 | DISCUSSION

In the present study, we sought to investigate whether abnormal aggression as observed in the BALB/cJ mouse model is associated with alterations in the regulation of the circadian rhythms and hormonal levels of CORT and testosterone, known modulators of—and known to be modulated by—clock genes. BALB/cJ mice, displaying

excessive aggressive behaviour, were found to show aberrant circadian rhythmicity in the absence of external Zeitgebers. Moreover, even in the presence of these Zeitgebers, the mice displayed increased PER1 clock protein levels in the SCN shell during the early dark phase, as well as lower CORT variability between circadian peak and trough and lower testosterone trait levels.

We validated that BALB/cJ mice, compared with BALB/cByJ controls, exhibit elevated aggressive behaviour and increases in abnormal forms of attacks directed towards vulnerable body parts of the intruder (Jager et al., 2020; van Heukelum, Drost, et al., 2019; van Heukelum, Mogavero, et al., 2019). Aggressive behaviour has previously been linked with physiological changes including altered hormonal function of glucocorticoids and androgens (Haller & Kruk, 2006; Jasnow et al., 2000; Stagkourakis et al., 2020). Here, we observed that BALB/cJ mice had lower circadian peak and higher circadian trough plasma CORT levels compared with BALB/cByJ mice, whereas their long-term hair CORT levels were similar, suggesting that BALB/cJ mice may have a blunted or distorted circadian rhythm of CORT without changes in overall CORT secretion. These results are consistent with results from the clinic, where low baseline morning cortisol as well as low cortisol variability between morning and afternoon has been observed in antisocial and aggressive behaviour groups (Das et al., 2018; McBurnett et al., 2000; Virkkunen, 1985). As for testosterone, long-term hair levels were significantly reduced in aggressive BALB/cJ mice. In rodents, a causal

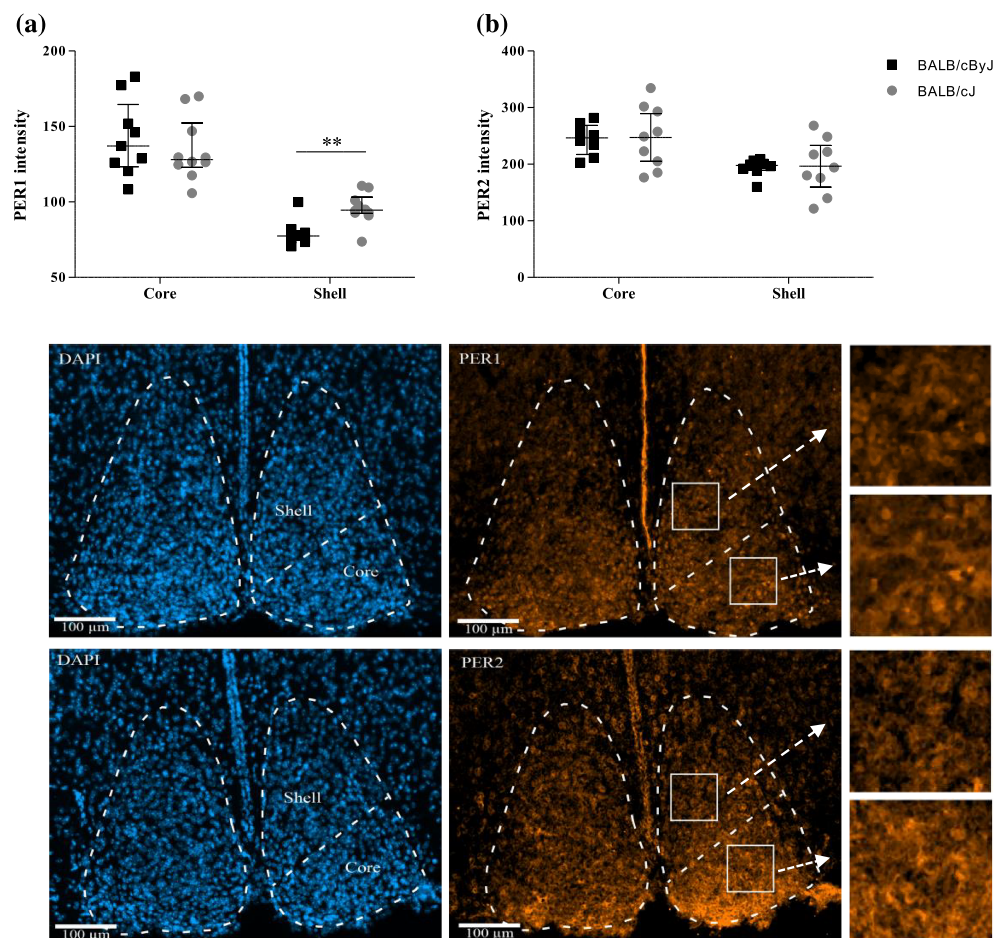


FIGURE 5 PER1 and PER2 expression in the suprachiasmatic nucleus (SCN) core and shell. BALB/cJ mice displayed increased PER1 expression in the SCN shell but not core (a). There were no differences in PER2 expression between the two strains for both the SCN core and shell (b).

** $p < 0.01$. (c, d) Examples of PER1 (c) and PER2 (d) images with the corresponding DAPI staining. The area enclosed within the dashed lines shows the SCN, which is subdivided into the core and shell. The squares in the PER1 and PER2 images indicate the fixed area in the core and shell that was selected for intensity measurements in all images of all animals

positive relationship has been found between testosterone and aggression, with castration reducing aggressive behaviour (Payne, 1973; Saylor, 1970). In addition, during aggressive encounters, a greater testosterone response has been observed to predict increased aggression (Scotti et al., 2009; Stagkourakis et al., 2020). Importantly, in the present study, we only measured overall secretion of testosterone in the hair, but not acute responses to the aggressive encounters, which may be particularly important in this association. Unfortunately, the small amount of blood obtained by tail bleed did not suffice for additional analyses, refraining us from testing whether increased aggressive behaviour in BALB/cJ mice was accompanied by a greater increase in testosterone response. Concerning trait levels of testosterone, both high and low testosterone have been associated with aggression (Jasnow et al., 2000; Stagkourakis et al., 2020). It has been suggested that when testosterone levels are low, there might be an increase in androgen receptor (AR) sensitivity as a possible compensatory mechanism (Jasnow et al., 2000). It might therefore be interesting for future studies to assess AR expression inside and outside of the SCN.

While in the standard LD conditions there were no differences in the circadian rhythm of locomotor activity, in DD, BALB/cJ mice had a shorter free-running period compared with BALB/cByJ mice with no differences in amplitude of locomotor behaviour nor overall activity levels. Upon re-exposure to light, both BALB/cJ and BALB/cByJ mice immediately re-entrained to the LD conditions, indicating a similar ability of both strains to adapt to environmental light cues. Intriguingly, in the SCN, the master circadian clock, we observed increased PER1 levels of BALB/cJ mice during the early dark phase of LD conditions, specifically in the shell. Because during DD, autonomous rhythmic oscillation of *Per1* and *Per2* expression occurs only in the shell (Yan et al., 1999), it is tempting to speculate that these deviations in PER1 expression relate to the aberrant free-running period of the mice as observed under DD. Conversely, neurons within the SCN core follow weak autonomous daily rhythms of clock gene expression but strongly respond to light stimuli (Yan et al., 1999) as it receives direct photic input from the retina through the retino-hypothalamic tract, which is able to reset the phase of SCN cells (Yan et al., 2007). Notably, PER proteins influence their own

expression through a series of auto-regulatory transcriptional/post-translational feedback loops, where their degradation reinitiates the cycle ensuring that it approximately lasts 24 h. Accelerated degradation of PER proteins is expected to shorten the period of a cycle (Hastings et al., 2008). As such, the shorter free-running period in aggressive BALB/cJ could be related to accelerated PER protein degradation, which should be assessed in future studies.

However, these findings should be interpreted with caution. First of all, we only assessed PER1 and PER2 expression performing immunohistochemistry. We used this method because it most reliably enabled us to dissociate the core and shell regions of the SCN, which are considered functionally and physiologically distinct (Dunlap, 1999; Yan et al., 1999). Moreover, Western blots would require pooling of tissue of several mice because of the small volume of the mouse SCN (e.g., as done in Choi et al., 2008 and Field et al., 2000). In the future, confirmatory Western blots should however be performed next to immunohistochemistry to confirm the differences in clock protein levels in BALB/cJ mice. In addition, we only measured PER expression at one time point during the early dark phase, and we are therefore unable to draw any conclusions about the rhythmic properties of PER1 and PER2. By measuring clock gene expression at different timepoints of the day, future studies could assess whether our observed changes in SCN shell PER1 expression are concurrent with an altered circadian rhythm of PER1, either in circadian amplitude and/or phase. As for PER2, we did not observe any changes in SCN expression. Nevertheless, we cannot exclude the possibility that BALB/cJ mice have disrupted PER2 at different times of the day. Furthermore, in mice, it has been proposed that the anterior and posterior SCN contain separate oscillating cell groups with different oscillatory phases and responding differently depending on photoperiod length (Inagaki et al., 2007). In the present study, we assessed PER expression only in the central SCN (excluding the most anterior and posterior sections) and only in LD conditions, when these groups are oscillating in sync. Future studies targeting these separate cell groups in the SCN in the anterior versus posterior SCN at altered photoperiods will clarify whether *Per1* (and *Per2*) expression in these cells is similarly or differently affected in BALB/cJ mice. Another limitation of this study is that we did not assess causality of the observed associations. Future experiments inducing a disturbance in clock gene expression (for example, *Per1* and/or *Per2*) in the SCN by viral manipulation (as previously done by Landgraf et al., 2016) in BALB mice and observing the effect on aggressive behaviour, as well as circulating hormone levels could potentially illustrate such causality.

In conclusion, we observed altered circadian rhythmicity and hormone levels in the BALB/cJ mouse model of abnormal aggression, making it a valuable animal model to further disentangle the dynamic role of the SCN, circadian rhythms and clock genes in the modulation of aggressive behaviour, an association that is to date still under-investigated.

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CONFLICT OF INTEREST

The authors have no competing interests to declare.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The datasets generated for this study are available upon request to the corresponding author.

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