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Full Length Article

Chronic circadian phase advance in male mice induces depressive-like responses and suppresses neuroimmune activation

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ABSTRACT

Altered working and sleeping schedules during the COVID-19 pandemic likely impact our circadian systems. At the molecular level, clock genes form feedback inhibition loops that control 24-hr oscillations throughout the body. Importantly, core clock genes also regulate microglia, the brain resident immune cell, suggesting circadian regulation of neuroimmune function. To assess whether circadian disruption induces neuroimmune and associated behavioral changes, we mimicked chronic jetlag with a chronic phase advance (CPA) model. 32 adult male C57BL/6J mice underwent 6-hr light phase advance shifts every 3 light/dark cycles (CPA) 14 times or were maintained in standard light/dark cycles (control). CPA mice showed higher behavioral despair but not anhedonia in forced swim and sucrose preferences tests, respectively. Changes in behavior were accompanied by altered hippocampal circadian genes in CPA mice. Further, CPA suppressed expression of brain-derived neurotrophic factor (BDNF) and pro-inflammatory cytokine interleukin-1 beta in the hippocampus. Plasma corticosterone concentrations were elevated by CPA, suggesting that CPA may suppress neuroimmune pathways via glucocorticoids. These results demonstrate that chronic circadian disruption alters mood and neuroimmune function, which may have implications for shift working populations such as frontline health workers.

1. Introduction

Urbanization in recent decades has irreversibly changed our sleep and work schedules. Flexible working schedules expose workers to artificial light at various times of day and perturbs sleep, disrupting the circadian system (Deacon and Arendt, 1996; Espitia-Bautista et al., 2017). Frequent artificial light exposure and sleep restrictions were increasingly experienced under the recent COVID-19 pandemic, especially by shift workers doing essential work and virtual commuters interacting with people in other time zones. Although globalization offers many benefits to social and economic development, it also may cause physiological costs to individuals experiencing circadian disruption.

The circadian system uses endogenous and exogenous cues to maintain daily sleep-wake, metabolic, and hormone rhythms (Edgar et al., 1993). The suprachiasmatic nucleus (SCN) in the anterior hypothalamus acts as the primary oscillator to establish temporal cooperation in almost all cells in the body. The SCN synchronizes outputs to the 24-hr cycle on earth by creating anticipatory responses to exogenous signals, such as light, heat, and additional stressors (Tamiya et al., 2016; Herzog et al., 2017; Ramkisoensing and Meijer, 2015). In turn, external cues, most notably light, allow the SCN to re-synchronize circadian rhythms to the environment (Ramkisoensing and Meijer, 2015). At the molecular level, core circadian clock genes *Bmal1*, *Clock*, *Per*, and *Cry* form a feedback inhibition loop which is necessary for daily circadian oscillation and this feedback loop controls cycle length (Bae et al., 2001). Additionally, REV-ERBα/β stabilizes rhythms through regulation of BMAL1 (Yin and Lazar, 2005) and may be able to adjust rhythms to exogenous factors (Jolley et al., 2014). Despite the SCN's role as the primary oscillator, circadian clocks exist in nearly every organ and tissue including peripheral organs such as the liver (Tahara and Shibata, 2016), and in brain regions such as the hippocampus (Debski et al., 2020). Specifically, the hippocampus is a brain region involved in emotional regulation (Hajszan et al., 2010; Altar, 1999), it is sensitive to stress, glucocorticoids (McEwen, 2008) and chronic light treatment (Bedrosian et al., 2011; Chen et al., 2021), and diseases such as epilepsy reset hippocampal circadian oscillations (Debski et al., 2020). Abrupt changes of external signals that are experienced during global travel or rotating shift work, including changes in timing of light exposure and food intake, may lead to desynchronization of internal circadian rhythms and cause mood disturbances (Reinberg and Ashkenazi, 2008). The schism between

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physiological and social rhythms, coined "social jetlag," is also associated with depressive symptoms (Islam et al., 2020).

In addition to influencing sleep and circadian rhythms (Jahrami et al., 2021; Conroy et al., 2021; Cellini et al., 2020), the COVID-19 pandemic and the subsequent lockdown in many countries has resulted in more-negative mood such as anxiety and depression (Conroy et al., 2021; Ingram et al., 2020; Morin et al., 2020). Circadian disruption influences the development and maintenance of common mood and behavioral disorders. Depressive disorders often include a circadian component including disturbances in daily rhythms of the sleep-wake cycle (Li et al., 2013), neurotransmitters (Li et al., 2013), and hormone concentrations (Cleare et al., 1995). For example, patients with bipolar disorder exhibit blunted or abnormal circadian oscillations of body temperature, plasma cortisol, blood pressure, and melatonin rhythms that can be stabilized by antidepressants or disease recovery (McClung, 2007), whereas disruption to the circadian system increases susceptibility to mood disorders (Daut and Fonken, 2019; Ben-Hamo et al., 2016).

Exaggerated innate immune responses in the brain are associated with psychiatric disorders including depression, anxiety, and post-traumatic stress disorders. The circadian system regulates neuro-immune processes and circadian disruption may induce neuroimmune changes. For example, the clock gene REV-ERBα regulates the immune system through the expression and activation of the NLRP3 inflamma-some (Pourcet et al., 2018), and ventral midbrain REV-ERBα inhibition produces mania-like behaviors (Chung et al., 2014). Neuroinflammatory cytokines such as TNFα (Taishi et al., 1997), phagocytic regulatrors such as CD68 (Fonken et al., 2016), and brain-derived neurotrophic factor (BDNF) (Schaaf et al., 2000) all exhibit circadian variations. Further, the brain's resident immune cell, microglia, is under the control of the circadian system: microglia show diurnal rhythms in immune responses (Fonken et al., 2015). In aged rats, diminished microglia rhythms result in blunted and elevated diurnal cytokine expression upon immune challenges (Fonken et al., 2016). However, it remains unclear whether and how neuroimmune mechanisms modulate circadian disruption-induced behavioral changes in healthy young animals.

Here, we hypothesized that modeling "chronic jetlag" in mice would alter mood and related neuroimmune pathways. We implemented a model of 6-hr phase advance of the light phase every 3 days; this pro-duces a 22-hr-like (T22-like) cycle (Casiraghi et al., 2012) that in mice is at the lower limit of the entrainment range (Ouk et al., 2019; Walbeek and Gorman, 2017). Overall, our results show that mice undergoing chronic advance shifts have a more depressive-like phenotype. At the molecular level, chronic advance shifts suppress baseline expression of pro-inflammatory cytokines, potentially via activation of the hypothal-amus-pituitary-adrenal axis (HPA axis).

2. Materials and methods

2.1. Animals

Thirty-two male C57BL/6J (abbreviated C57) mice aged 8–10 weeks were purchased from Jackson Labs (Stock 00664). All mice were housed

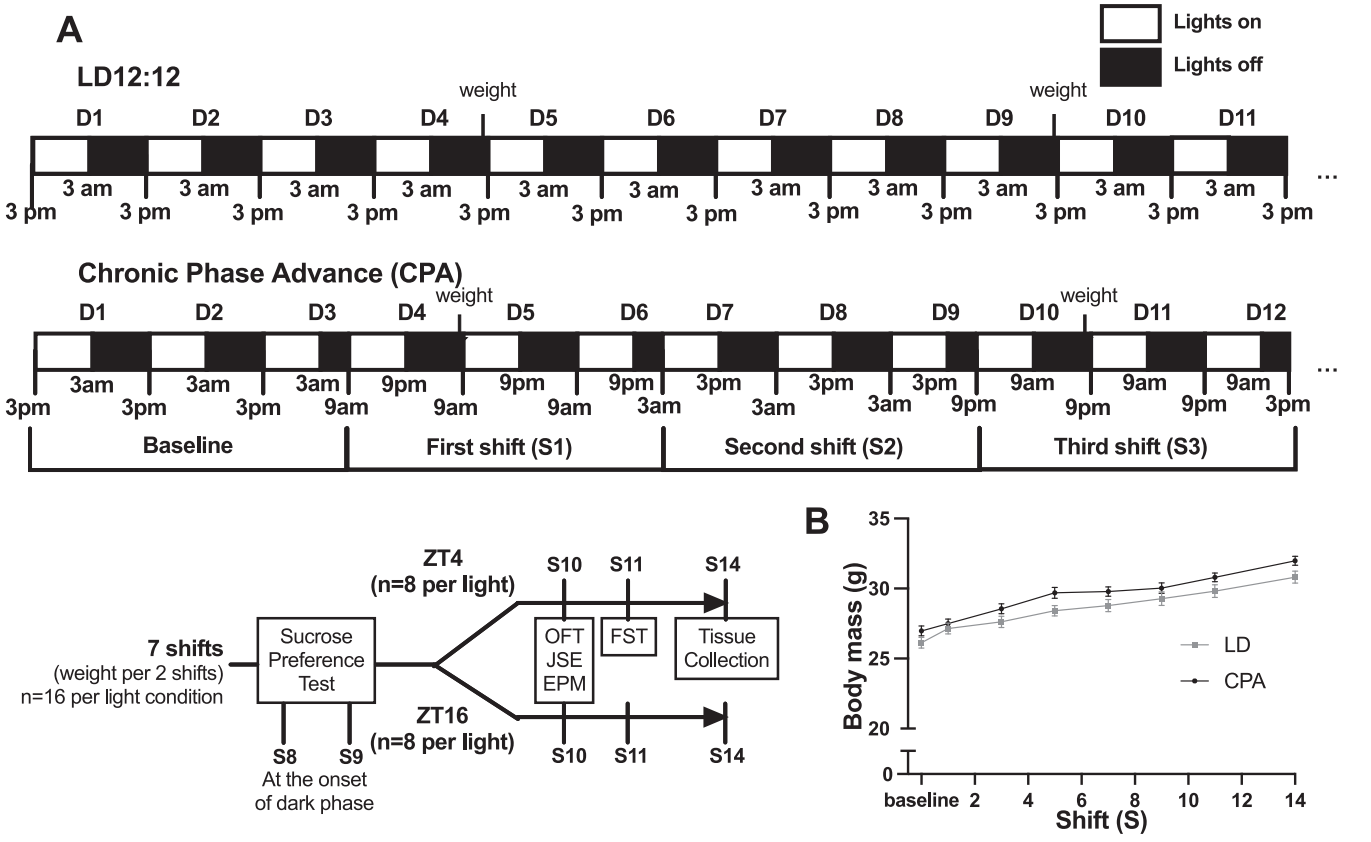

Fig. 1. CPA did not influence total weight gain. (**A**) Schematic timeline. Male mice were randomly assigned into control light (LD, 12:12 light/dark) or chronic phase advance (CPA) groups. Lights for CPA group were turned on 6-h in advance every three light/dark cycles (shortening of the third dark phase for 6 h). After 7 shifted light cycles (S7), affective behaviors were assessed at ZT4 or ZT16 respectively, except for sucrose preference which was assessed during the dark phase. Mice were then allowed to recover from behavioral test batteries. All mice were euthanized on the third day of S14. Controls were tested and euthanized at the relative ZTs of their own light schedule on the same day as CPA animals. (**B**) CPA transiently influenced weight gain. CPA mice weighed slightly more at some time points, although not statistically significant after adjusting for multiple comparisons. By S14, CPA mice gained as much weight as control mice. (OFT, open field. JSE, juvenile social exploration. EPM, elevated plus maze. FST, forced swim test. Body mass: repeated measures ANOVA, n = 16 per group.)

in ventilated light-controlled Sun Calc chambers (Lab Products, Inc.) with 12-hr light (150 lux white light)/12-hr dark (0 lux) cycle unless otherwise specified. White light was generated by built-in light strips controlled by Sun Calc chambers under a user-defined program. Upon arrival all mice were pair-housed under an average room temperature of 21 ± 1 °C with corncob bedding, 5 cm square nestlets, and unlimited access to standard lab diet and water. Mice acclimated to the facility housing environment for a week before initiation of the experimental light manipulations. All procedures were approved by the University of Texas at Austin Institutional Animal Care and Use Committee (IACUC) and were performed according to the local and country guidelines governing animal care.

2.2. Chronic phase advance (CPA)

Zeitgeber Time (ZT) is the time associated with external cues such as light/dark cycles. ZT0 in this study represents the beginning of the light phase and ZT12 represents the beginning of the dark phase. To model chronic jetlag, sixteen mice were subject to 6-h light phase advancements every 3 light/dark cycles (Fig. 1A). A "shift" (abbreviated as "S") was defined as the beginning of an advanced light phase to the end of the third following dark phase. The control groups of sixteen mice for this study were housed under 12:12 light/dark cycles simultaneously throughout the experiments under otherwise identical conditions in distinct light-controlled Sun Calc chambers. Body mass was tracked every two shifts, at ZT0 on the 2nd day of a shift, except for S11 when mice were weighed before behavioral tests. Control mice were weighed every 5 days at ZT0.

2.3. Behavioral testing

Mice are nocturnal animals that are active during the dark phase. To characterize the potential circadian variations in behaviors, affective behaviors were assessed during S8–S11, at either mid-light phase (ZT4, n = 8) or mid-dark phase (ZT16, n = 8) on the 2nd or 3rd day after the shift, and then compared to the control groups tested on the same day at ZT4 (n = 8) or ZT16 (n = 8) of their own schedules, respectively (see Fig. 1 for the schematic timeline). All behavioral tests were conducted under dim red light (0.4 lux–2 lux at animal eye level) to avoid the influence of light exposure. Mice acclimated to the test room for at least 40 min in their home cages before each test.

2.3.1. Sucrose anhedonia test

The sucrose anhedonia test involved one training day (2nd night of S8) and two testing days (3rd night of S8 and 2nd night of S9). Preference for 2% sucrose was assessed in the home cage. Two 50 mL Falcon tubes containing either sucrose solution or tap water were introduced into the home cage for 6 h starting at lights off. The bottles were weighed before and after the tests to calculate sucrose preference: [sucrose consumption/total liquid consumption × 100%]. A cage of two mice was represented as one data point for liquid intake (averaged) and sucrose preference.

2.3.2. Open field test

An open field test was completed at either ZT4 or ZT16 on the 2nd day of S10. The mice were placed facing the wall in a random corner of a beige plexiglass box divided into quadrants (4 × 50 cm × 50 cm × 40 cm); each served as test arena for one mouse. Movements were recorded for 5 min using a camera mounted on the ceiling. The total distance traveled and the time spent in the innermost 30% central area of the arena were automatically quantified using Ethovision XT14 (Noldus Corp., Leesburg, VA, USA). Decreased time spent in the center may indicate an increase in anxiety-related behavior (Bailey et al., 2009).

2.3.3. Juvenile social exploration

The juvenile social exploration test was performed immediately after open field. A 3-4 week-old juvenile male mouse was introduced as a social stimulus into the open field arena containing the test adult mouse. Behaviors were recorded with the ceiling camera for 5 min as soon as the juvenile stimulus mouse was placed into the box. Videos were scored by an observer blinded to the experimental groups using Ethovision software. Only interactions initiated by the adult mouse were scored and the time adult mice spent investigating the juvenile (chasing, sniffing, fighting, and grooming) were quantified. Lower investigation time may indicate a more anxiety-like phenotype (Christianson et al., 2008). Test arenas were cleaned with 70% ethanol after each trial.

2.3.4. Elevated plus maze

The elevated plus maze test was conducted at either ZT4 or ZT16 on the 3rd day of S10. The plus-maze apparatus was raised 39 cm off of the floor and included a center, two arms open to the environment (30.5 cm × 5 cm), and two arms enclosed by walls (30.5 cm × 5 cm × 18.5 cm wall height). The mice were placed in the center of the plus-maze, and movements were recorded for 5 min with the ceiling camera. The time the mice (center body point) spent in the open arms and number of entries into the open arms were automatically measured by Ethovision software. Lower open arm entries or time spent in the open arms may indicate a more anxiety-like phenotype (Bailey et al., 2009).

2.3.5. Forced swim test

Forced swim was performed at either ZT4 or ZT16 on the 2nd day of S11. A fan was used as a white noise generator to mask any interfering sound during the tests. The mice were placed in a glass beaker (15 cm inner diameter × 25 cm height) filled with 25 °C ± 0.5 °C of water 9 cm to the top. Behaviors were recorded for 7 min with the ceiling camera once the mouse was placed on the water surface. The mice were taken out of the water and placed back in their home cages with a piece of paper towel. The videos were manually scored by an observer blinded to the experimental groups with Ethovision software. Latency to the first floating event was recorded and then the time and frequency the mice spent floating were scored for the last 5 min. Increased floating time and frequency may indicate a more depressive-like phenotype and learned helplessness (Slattery and Cryan, 2012).

2.4. Tissue collection

Mice were euthanized at ZT4 or ZT16 respectively on the 3rd day of S14. Mice were transferred into the procedure room in their home cage one cage at a time, anesthetized with 5% isoflurane, and immediately decapitated. Trunk blood was collected within 90 s of first touching the cage, and serum was isolated from trunk blood by centrifuge and stored at −80 °C. The brains were freshly dissected on ice. Brain regions of interest were flash frozen on dry ice and stored at −80 °C. Peripheral organs including white adipose tissue surrounding the gonads and adrenal glands were removed and weighed.

2.4.1. qPCR

RNA was extracted from half of the hippocampus using a homogenizer and TRIzol/chloroform extraction method. 3 μg of RNA was reverse transcribed to cDNA with SuperScriptII Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. Relative mRNA expressions of pro-inflammatory markers interleukin-1β (*Il1β*), *Nfkbia* (indicative of *NFκB*), *Tnf*, microglia phagocytic marker *Cd68*, anti-inflammatory gene arginase 1 (Arg*1*), brain-derived neurotrophic factor (*Bdnf*), and circadian clock genes *Per1*, *Per2*, *Nr1d1*, and *Nr1d2* were determined relative to housekeeping gene *Gapdh* as previously described (Chen et al., 2021). All genes were determined in duplicates, analyzed using the $2^{\Delta\Delta Ct}$ method, and normalized such that the level of the control ZT4 group was set to a value of 1. There were no group differences in *Gapdh* mRNA expression.

2.4.2. ELISA

Serum corticosterone concentrations were determined in duplicate

with Corticosterone ELISA kit (Arbor Assay, sensitivity = 20.9 pg/mL, limit of detection = 17.5 pg/mL, mean intra-assay CV = 3.73%) per manufacturer guidelines. 1 outlier was identified and excluded.

2.5. Statistics

Statistical analyses were conducted with GraphPad Prism 9.0. Equality of variance and normal distribution were tested using Brown-Forsythe test and Shapiro-Wilk test, respectively. Data that failed to meet the assumption of ANOVAs were square-root transformed. Unpaired *t*-test, two-way ANOVA and repeated-measures ANOVA were performed when appropriate with light and ZT as the independent variables. Multiple Mann-Whitney tests were performed if data were non-Gaussian distributed after transformation. Outliers were identified using Grubb's test for outliers. Significance level is set at $p < 0.05$. Post-hoc Tukey's analyses were conducted based on prior hypotheses. Post-hoc analyses for body mass, weight gain, and sucrose preference were conducted with Bonferroni's test for multiple comparisons by comparing control cell mean with CPA cell mean at the same time point. All data were presented as mean ± S.E.M.

3. Results

3.1. CPA altered clock gene profiles in the hippocampus

Body mass was monitored every other cycle. There was no group difference in body mass at the beginning of the experiment. CPA mice weighed more at some time points (time × light interaction, $F_{(7,210)} = 2.69$, $p < 0.05$, Fig. 1B), although this was not statistically significant at any single timepoint after adjusting for multiple comparisons. Therefore, we compared the relative weight gained in each shift. There was a similar time × light interaction in weight gain ($F_{(6,210)} = 4.353$, $p < 0.05$, data not shown). CPA initially lowered weight gain at S1 (mean control vs CPA, 1.03 g vs 0.52 g, $p < 0.05$), followed by a transient rebound in weight gain at S3 (mean control vs CPA, 0.47 g vs 1.06 g, $p < 0.05$). By the end of the study at S14, weight gain among CPA and control mice was equivalent. Epididymal fat pads were weighed as an index of white adipose tissue composition and at the conclusion of the study no group differences were noted (mean control vs CPA, 0.74 g vs 0.70 g, $p > 0.05$, data not shown). These results suggest that shifts in the LD cycle may transiently influence weight gain in mice.

The hippocampus is a brain region involved in emotional regulation (Anacker and Hen, 2017; Sandi and Bisaz, 2007; Campbell and Macqueen, 2004) and exhibits circadian rhythms (Chun et al., 2015). In order to determine how the hippocampus responds to CPA, we collected the hippocampus from control and CPA mice at two time points at the end of S14. Core clock genes *Per1* and *Per2* are necessary for the rhythmicity of clock genes and clock-controlled genes (CCGs) (Zheng et al., 2001). As expected based on the two timepoint selected, there was no time-of-day difference in *Per1* mRNA in the control mice. After 14 shifts, CPA elevated Per1 expression at both ZT4 and ZT16 (main effect of light, F (Deacon and Arendt, 1996; Ben-Hamo et al., 2016) = 8.78, $p < 0.05$, Fig. 2A). In contrast, a time-of-day difference in *Per2* mRNA expression was observed in the control mice but was abolished after 14 shifts in the CPA mice (light × ZT interaction, F (Deacon and Arendt, 1996; Ben-Hamo et al., 2016) = 6.16, $p < 0.05$; Post-hoc, control ZT4 vs ZT16, $p < 0.05$, Fig. 2B). Although with two time points it is not possible to interpret the phase angle, the changes in *Per1* and *Per2* levels indicate altered core clock gene expressions in the hippocampus, which may lead to changes in gene oscillations in downstream target clock genes and CCGs.

Supporting this idea, CPA also altered other hippocampal circadian clock genes. BMAL1 and CLOCK form the positive limb of the feedback inhibition loop. At our collecting times in the control hippocampus there were no differences in *Bmal1* mRNA expression. However, CPA induced a *Bmal1* time-of-day difference, with a lower level at ZT4 (light × ZT interaction, $F_{(1,\ 28)} = 4.88$, $p < 0.05$. Post-hoc, CPA ZT4 vs ZT16, $p < 0.05$, Fig. 2C). REV-ERBα is a transcriptional repressor that negatively regulates the core clock components BMAL1, CLOCK, and CRY1 (Preitner et al., 2002). At our collecting times, control hippocampus did not exhibit a time-of-day difference in *Rev-erbα* mRNA expression, but after CPA the hippocampal *Rev-erbα* level was lower at ZT4 (light × ZT

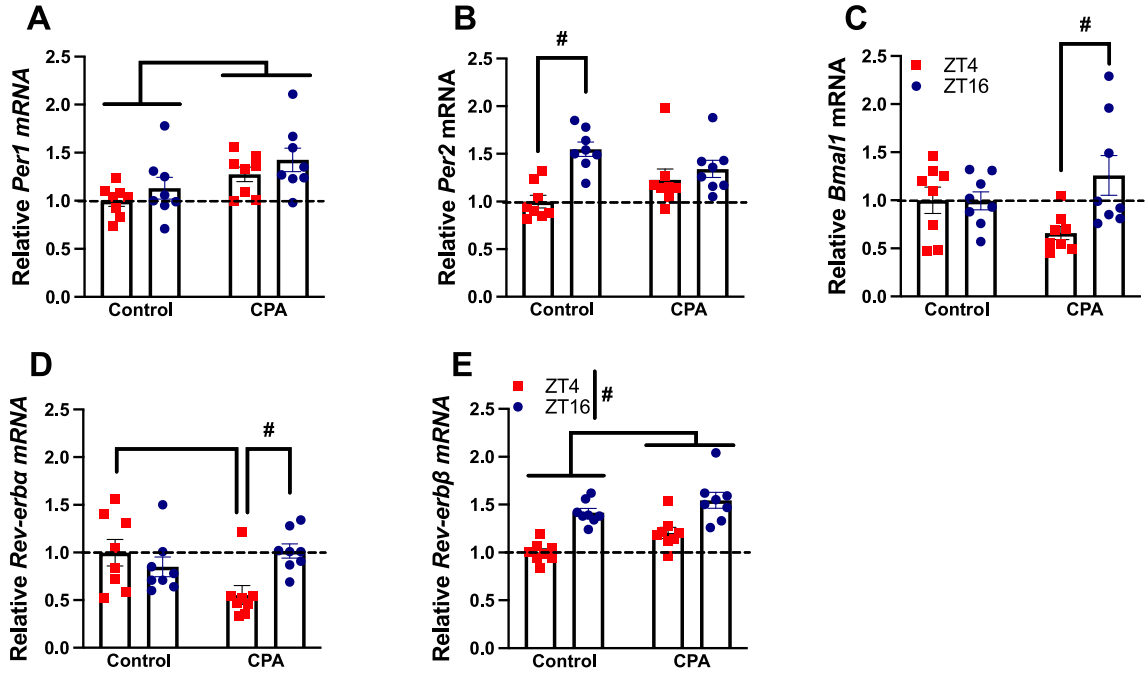

Fig. 2. CPA altered hippocampal central clock gene profiles at S14. CPA led to (**A**) elevated mRNA expression of *Per1*, (**B**) blunted diurnal variation of *Per2*, (**C**) time-of-day difference in *Bmal1* mRNA expression, (**D**) suppressed *Rev-erbα* expression at ZT4, and (**E**) elevated *Rev-erbβ* expression. (n = 8 per group, two-way ANOVA, *$p < 0.05$ control vs CPA, #$p < 0.05$ ZT4 vs ZT16, mean ± S.E.M.)

interaction, $F_{(1,28)} = 8.24$, $p < 0.05$. Post-hoc, ZT4 control vs CPA, $p < 0.05$, Fig. 2D). REV-ERBβ is generally considered functionally redundant to REV-ERBα (Ikeda et al., 2019). Interestingly, CPA elevated *Rev-erbβ* mRNA levels at both ZT4 and ZT16 (main effect of light, $F_{(1,28)} = 8.12$, $p < 0.05$, Fig. 2E). Taken together, these data suggest that after repeated phase advance, the hippocampus was unable to re-adjust to the new light cycle within 3 days, resulting in altered expression of hippocampal circadian genes at ZT4 and ZT16 of the new light cycle.

3.2. CPA increased depressive-like behaviors

We examined if the disrupted circadian genes as a result of the chronic circadian disruption could influence mood-related behaviors. Anxiety- and depressive-like behaviors were assessed between S8–S11 with a battery of tests. Behavioral tests were conducted either on the 2nd or the 3rd day after a shifted light phase. On the 2nd day of S10, locomotion and exploratory behaviors were tested with an open field immediately followed by a juvenile social exploration test. In the open field test, CPA mice had a higher central tendency during their inactive phase (light × ZT interaction, $F_{(1,28)} = 6.03$, $p < 0.05$; *post-hoc*, ZT4 control vs CPA, $p < 0.05$, CPA ZT4 vs ZT16, $p < 0.05$, Fig. 3A) and a decreased number of fecal boli (main effect of light, $F_{(1,25)} = 5.17$, $p < 0.05$, Fig. 3B). CPA did not alter locomotion ($p > 0.05$, Fig. 3C) and social exploratory behaviors at either ZT4 or ZT16 ($p > 0.05$, Fig. 3D). Anxiety-like behaviors were also tested on the 3rd day post-shift in an elevated plus maze. CPA did not influence open arm exploration (Fig. 3E). These results suggest a mixed anxiety phenotype with a reduction in anxiety-like behavior in the open field test, but not other behavioral tasks.

Anhedonia and behavioral despair are two major symptoms of depression. To avoid time-after-shift influences, here we used a three-day sucrose preference paradigm to assess anhedonia: mice were given a two-bottle sucrose/water choice for two consecutive dark phases in S8, one during the 2nd night, another during the following night. Mice were tested again on the 2nd night of S9. In the first sucrose exposure, control mice showed a high preference for sucrose solution (87%) over plain water. CPA mice also preferred sucrose over water, although at a lower level than the controls (light × ZT interaction, $F_{(2,28)} = 3.77$, $p < 0.05$; *post-hoc*, test1 control vs CPA, $p < 0.05$, Fig. 4A). CPA mice also had lower total liquid intake (light × ZT interaction, $F_{(2,28)} = 5.50$, $p < 0.05$; *post-hoc*, test1 control vs CPA, $p < 0.05$, Fig. 4B). All mice elevated sucrose prefrence after the single training exposure, with over 90% of preference to sucrose in both groups.

Behavioral despair was assessed with a forced swim test on the 2nd day of S11. Latency to immobility was quicker when mice were tested during their active phase (main effect of ZT, $F_{(1,28)} = 4.53$, $p < 0.05$, Fig. 4C). CPA mice overall had higher immobility (main effect of light, $F_{(1,28)} = 10.0$, $p < 0.05$, Fig. 4D). No time-of-day difference in floating duration was observed. Taken together, these data suggest that CPA induced a depressive-like phenotype regardless of testing time.

3.3. CPA suppressed neuroimmune pathways

Mood disturbances can arise due to changes in neuroimmune pathways (Dantzer et al., 2008; Drexhage et al., 2011; Jin et al., 2019). To test if CPA-induced mood disturbances are accompanied by changes in the neuroimmune system, we collected the hippocampus after 14 shifts and measured neuroinflammatory markers and neurotrophic factors. BDNF is one crucial neurotrophic factor in the CNS. After 14 shifts, CPA suppressed BDNF mRNA expression specifically at ZT4 (light × ZT interaction, $F_{(2,28)} = 15.63$, $p < 0.05$; *post-hoc*, ZT4 control vs CPA, $p < 0.05$, Fig. 5A). Further, a BDNF time-of-day difference was observed in the controls, but not after CPA exposure (*post-hoc*, control ZT4 vs ZT16, $p < 0.05$, Fig. 5A). CPA also suppressed IL-1β (main effect of light, $F_{(1,26)} = 9.01$, $p < 0.05$, Fig. 5B), and abolished time-of-day difference in NFκBia (Mann-Whitney, control ZT4 vs ZT16, $U = 4$, $n_1 = 16$, $n_2 = 15$, $p < 0.05$; CPA ZT4 vs ZT16, $U = 18$, $n_1 = n_2 = 16$, $p > 0.05$, Fig. 5C). CPA did not significantly alter TNF, although similar to IL-1β, it trended towards being suppressed, particularly at ZT4 (Fig. 5D). Time-of-day differences in expression were also observed in the phagocytic gene CD68 (main effect of ZT, $F_{(1,28)} = 7.79$, $p < 0.05$, Fig. 5E) and Arg1 (main effect of ZT, $F_{(1,28)} = 4.302$, $p > 0.05$, Fig. 5F) and CPA did not significantly alter expression. These results suggested that CPA disrupted and suppressed some neuroimmune activity.

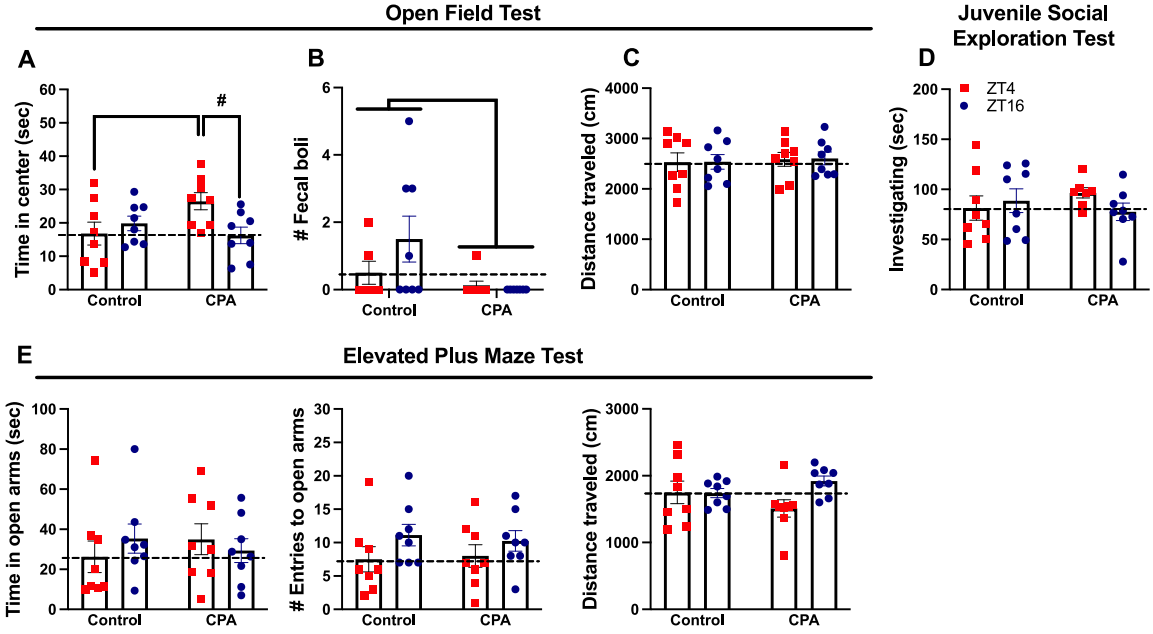

Fig. 3. CPA reduced anxiety-like behavior at ZT4 in an open field test. Anxiety-like behaviors were assessed at S10 with a battery of tests. (**A**) CPA increased central tendency and (**B**) decreased number of fecal boli in the open field test but (C) did not influence locomotor activity. CPA did not influence (D) time spent in social investigation in the juvenile social exploration test. (E) Open arm exploration or locomotion on the plus maze was not influenced by CPA in the elevated plus maze test. ($n = 8$ per group, two-way ANOVA, *$p < 0.05$ control vs CPA, #$p < 0.05$ ZT4 vs ZT16, mean ± S.E.M.)

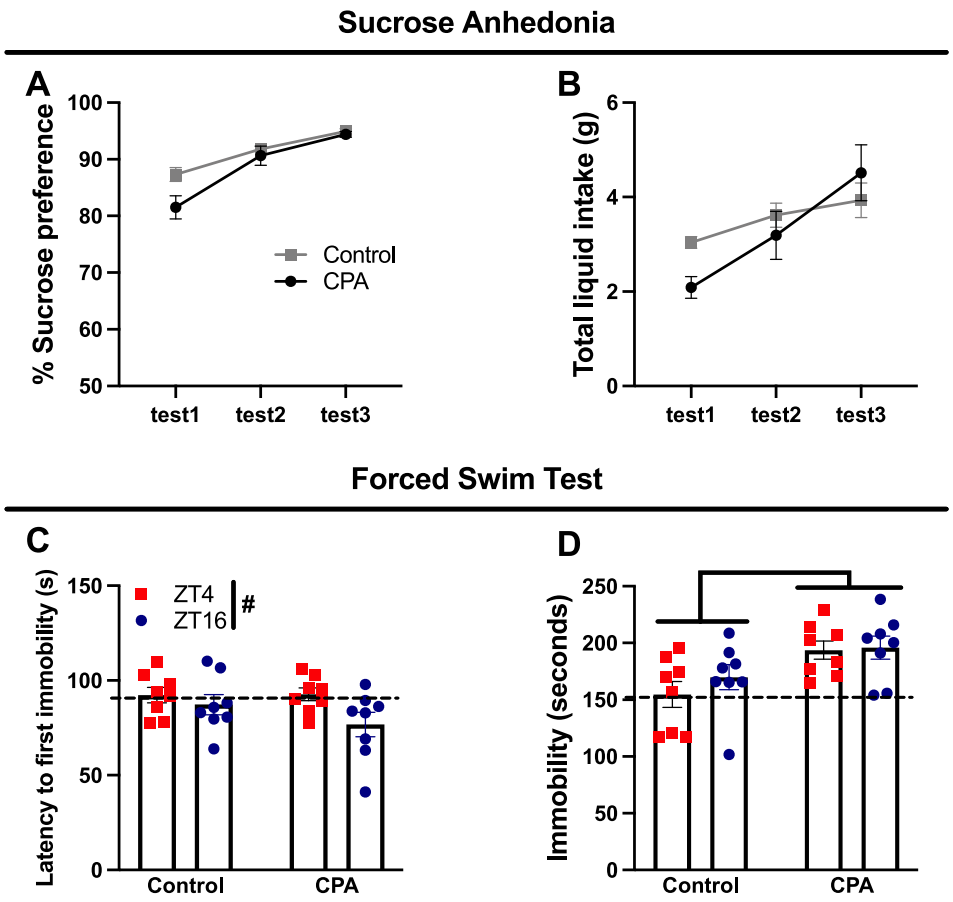

Fig. 4. CPA increased depressive-like behaviors. (**A-B**) Sucrose anhedonia tests were conducted between S8–S9. CPA mice had lower (**A**) preference to sucrose and (**B**) total liquid intake on the training session, but quickly adjusted and learned to prefer sucrose in the following test sessions. (**C-D**) A forced swim test was conducted at S11. (**C**) Both control and CPA mice tested in their dark phase had lower latency to immobility. (**D**) CPA mice were more immobile overall. (Sucrose anhedonia, n = 4 per group, average was taken to present each cage as one data point, repeated measure ANOVA. Forced swim, n = 8 per group, two-way ANOVA. *p < 0.05 control vs CPA, #p < 0.05 ZT4 vs ZT16, mean ± S.E.M.)

Activation of the HPA axis can suppress pro-inflammatory cytokines by increasing plasma corticosterone concentrations (Nadeau and Rivest, 2002). To determine if CPA leads to activation of the HPA axis, we assessed corticosterone concentrations in serum and analyzed the mass of the adrenal gland, an essential stress-responsive organ (Ulrich-Lai et al., 2006). There was no difference in adrenal gland mass ($p > 0.05$, Fig. 6A). The control mice did not show time-of-day difference in serum corticosterone concentration (mean ZT4 vs ZT16, 57.22 ng/mL vs 64.75 ng/mL, $p > 0.05$). CPA induced an increase in serum corticosterone concentrations (main effect of light, $F_{(1,27)} = 8.37$, $p < 0.05$, Fig. 6B). Taken together, these results suggest that CPA induced depressive-like phenotype which is accompanied by elevated corticosterone concentrations and suppressed neuroimmune activity.

4. Discussion

In this study, male mice that underwent chronic phase advance shifts had an increased depressive-like phenotype and mixed results in anxiety-related behaviors. At the molecular level, chronic phase advance shifts suppressed baseline expression of pro-inflammatory markers, potentially via elevated corticosterone concentrations. Our work, as well as others, collectively demonstrate that CPA shifts alter mood and neuroimmune function (Ben-Hamo et al., 2016; Daut et al., 2019; Castanon-Cervantes et al., 2010).

Dissociation of internal rhythms, or internal desynchronization, leads to negative health consequences such as cardiovascular and metabolic disease (Morris et al., 2012), cancer (Van Dycke et al., 2015), and mood disorders (Ben-Hamo et al., 2016). This may be regulated by internal desynchrony between clocks: the top circadian oscillator in the SCN can quickly adjust to 6-hr advance shifts of the light cycles, whereas the other central and peripheral tissues may adjust at a different speed (Yamazaki et al., 2000). We showed that after the initial changes in weight gain, mice quickly adapted to the new cycles in the following shifts. Recent studies suggest that mice could quickly adjust their behavioral rhythms to repeated 6-hr shifts (Wolff et al., 2013), and their core body temperature rhythm is mostly re-entrained after 3 days (Castanon-Cervantes et al., 2010). However, internal desynchronization may still occur at the organ and molecular levels. PER2:LUC bioluminescence rhythms suggest desynchronization in central and peripheral tissues after CPA (Wolff et al., 2013). Here we demonstrated that after repeated 6-hr phase advance, the hippocampus is unable to re-adjust to the 6-hr-advanced phase within three days. Of note, because only two collection time points were included in this study, we cannot examine clock gene rhythmicity. However, the gene expression profiles from our control mice were in line with other groups' works in the hippocampal regions (see Carver et al. for Per1/2, Rev-erbα, Shimizu et al. for Bmal1 and Rev-erbα, and Debski et al. for Per1/2, Bmal1, Rev-erbα/β) (Debski et al., 2020; Carver et al., 2014; Shimizu et al., 2016). Specifically, in the hippocampus Per2 peaks during mid-dark phase ($\Phi = 18.42 \pm 1.02$ (Carver et al., 2014)), Rev-erbα peaks during mid-day ($\Phi = 8.09 \pm 0.55$ (Carver et al., 2014)), and Bmal1 around dawn (Debski et al., 2020; Shimizu et al., 2016). The loss of a time-of-day difference in Per2 mRNA expression and the gain of a difference in Bmal1 and Rev-erbα observed in this study (Fig. 2B–D) could potentially be explained by misaligned rhythms as a result of the CPA. Chronic internal desynchronization may lead to uncoupled circadian rhythms between the central pacemaker and extra-SCN rhythms, including the glucocorticoid (GC) rhythm, and thus alter physiological and psychological functions that are sensitive to GCs.

The HPA axis has a bidirectional relationship with the circadian system. The HPA axis is under the control of the circadian system: plasma

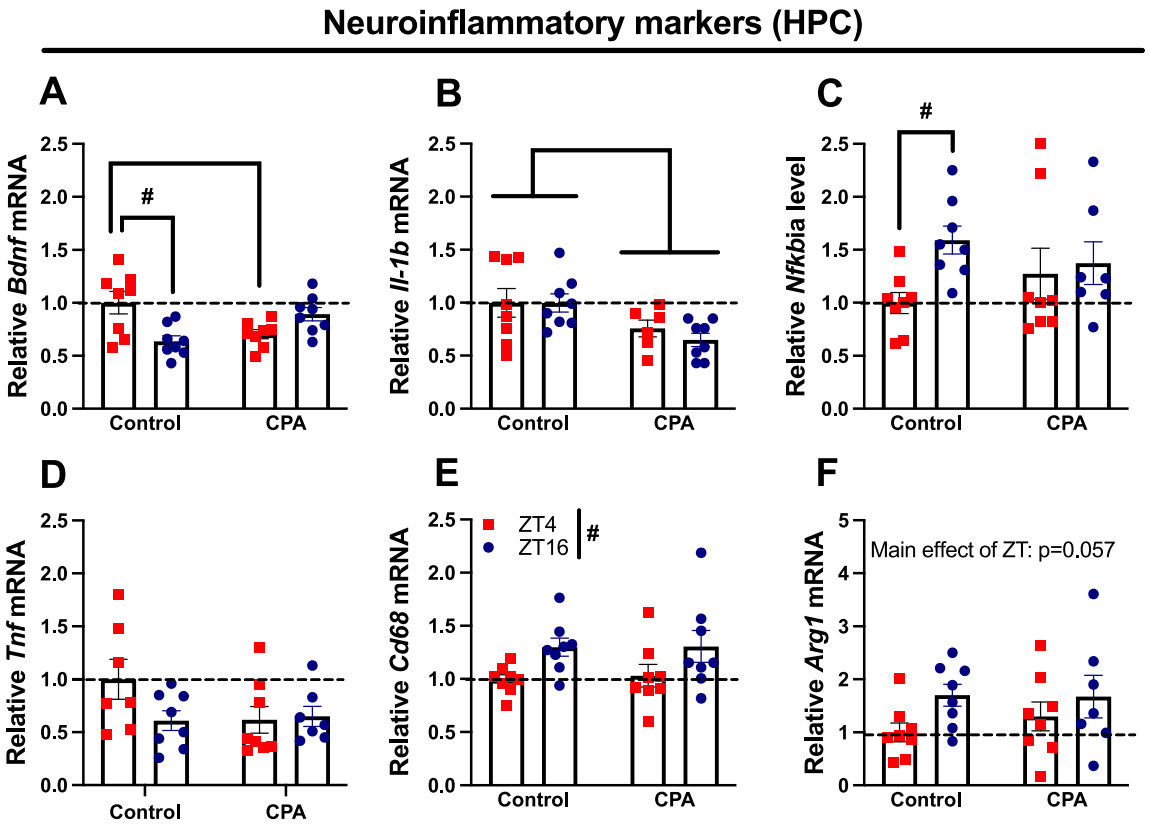

Fig. 5. CPA suppressed the neuroimmune system by activation of the HPA axis. (**A-F**) Hippocampal inflammatory markers were quantified at two times of the third day of S14. (**A**) BDNF levels were higher during the light phase in the controls. This daily fluctuation was abolished in CPA mice. (**B**) CPA decreased hippocampal IL-1β levels at both ZT4 and ZT16. (**C**) NFκBia levels were higher during the dark phase in the controls. This daily fluctuation was abolished after CPA. (**D**) TNF had a trend towards being suppressed by CPA specifically at ZT4. (**E**) CD68 levels were higher during the dark phase in the controls and were unaltered after CPA. (**F**) CPA induced higher ARG1 levels during the dark phase. (n = 6–8 per group, two-way ANOVA or Mann-Whitney U tests (NFκBia), *p < 0.05 control vs CPA, #p < 0.05 ZT4 vs ZT16, mean ± S.E.M.).

corticosterone, the major GC in mice, has a diurnal rhythm and peaks at the beginning of the active phase (Oster et al., 2006). In turn, GCs also regulate circadian re-entrainment (Karatsoreos and McEwen, 2011). Activation of GC receptors shifts the circadian clock based on the timing of administration (Tsuchiya et al., 2018). Removal of the adrenal glands by adrenalectomy (Pezuk et al., 2012) and transplantation with clock-deficit adrenal glands both accelerate re-entrainment after advance phase shift (Kiessling et al., 2010), whereas rhythmic GC administration in adrenalectomized mice restores the slower re-entrainment profile (Sage et al., 2004). Thus, it is possible that the GC rhythm is more resistant to changes of the light phase and it acts as a gate that slows other tissues from shifting to the new light cycles, further contributing to the internal desynchronization. As expected at our collecting times (ZT4 and ZT16, see Fig. 6B), control mice did not exhibit a time-of-day difference in serum corticosterone concentration. CPA mice exhibited elevated corticosterone concentrations at both time points. The elevated CORT is not likely explained by a phase delay in CORT rhythm (the lag between internal CORT rhythm and Zeitgeber), which would lead to higher CORT at ZT16 and lower at ZT4 (Fig. 6C). It similarly appears to not be explained by an accelerated CORT rhythm either. Mice entrained to the applied T22-like light cycles would show accelerated internal rhythms that oscillate less than every 24hrs, but greater than 22hrs (Jud et al., 2005). However, no matter what internal circadian time (CT) it was, in any given 12-hr-apart time points, at one of the two timepoints the mouse would have decreased CORT compared to the control, which is not supported by data here. Thus, instead of phase delay or accelerated CORT rhythm, the changes in CORT could be better explained by a global elevation of serum CORT concentration after CPA, which could be a result of the persistently activated HPA axis as a consequence of chronic lag. This increase in serum corticosterone has previously been observed with more drastic shifts such as repeated 12-hr shifts (Sakellaris et al., 1975). Interestingly, chronic phase delay also abolishes the rhythmicity in corticosterone concentration, but induces a lower corticosterone concentration (Zeman et al., 2016). We did not observe hypertrophy in adrenal glands, possibly because a change in tissue volume takes longer to occur (Karin et al., 2020).

In addition to elevated corticosterone concentrations, CPA altered neuroinflammatory profile in the hippocampus. BDNF is suppressed in certain neuropsychiatric disorders, neurodegenerative disorders, and by long-term stress and inflammation (Lima Giacobbo et al., 2019). CPA suppressed BDNF mRNA expression. Prior work has associated suppressed BDNF with microglia activation and corresponding pro-inflammatory cytokine production (Audet and Anisman, 2013). Thus, we assessed microglia phagocytic gene CD68, pro-inflammatory markers IL-1β, NFκB, and TNF, and anti-inflammatory gene Arg1 mRNA expression. Contrary to our hypothesis, instead of inducing pro-inflammatory responses, CPA actually suppressed pro-inflammatory cytokine gene expression in the hippocampus. These results agree with previous findings that neuroinflammatory responses can be suppressed by both exogenous (Frank et al., 2010) and endogenous (Nadeau and Rivest, 2002) GCs. Noticeably, GCs can also prime the neuroimmune system to produce more cytokines upon a subsequent immune challenge with lipopolysaccharide (LPS) (Frank et al., 2010). We previously showed the entraining effect of GCs on microglia rhythms (Fonken et al., 2015), suggesting that the circadian rhythmicity of neuroimmune processes can be altered by GCs. Similarly, Castanon-Cervantes et al. showed

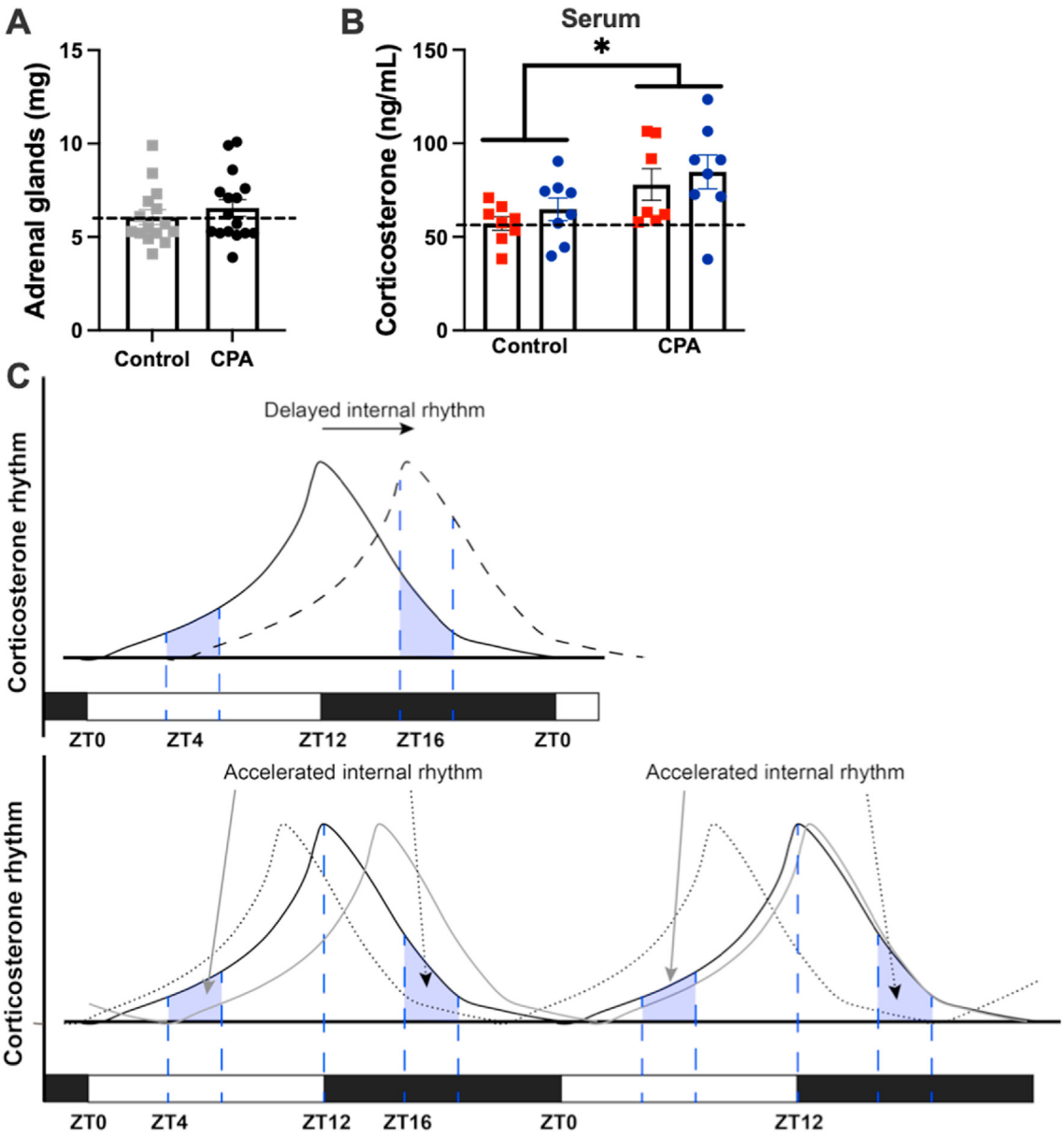

Fig. 6. CPA elevated serum corticosterone. (**A**) Adrenal gland mass was not different between control and CPA mice. (**B**) Corticosterone was determined in the serum from samples collected at ZT4 and ZT16. CPA increased serum CORT level throughout the day. (**C**) Schematic illustration of blood corticosterone daily oscillation, adapted from (Oster et al., 2006). CPA-induced elevated serum CORT may not be explained by a delayed (top) or an accelerated (bottom) internal CORT rhythm. Blue dotted lines and shadows under the curve indicate collecting time. Dotted curve in the top panel is an example of a delayed rhythm. Dotted and grey curves in the bottom panel are examples of accelerated rhythm at different circadian time (CT). (n = 7–8 per group, two-way ANOVA, *p < 0.05 control vs CPA, mean ± S.E.M.). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

that peritoneal macrophages isolated from mice that underwent 6-hr CPA release more cytokines in response to *in vitro* LPS (Castanon-Cervantes et al., 2010). Thus, it is possible that despite suppressed baseline neuroinflammatory pathways, CPA may prime the neuroimmune system and induce exacerbated pro-inflammatory responses upon a subsequent challenge. Indeed, weekly 6-hr phase advance exacerbates neuroinflammatory responses after an LPS challenge, despite no change in baseline neuroimmune profile (Ramsey et al., 2020). Future studies are needed to evaluate the priming effects of CPA on microglia activity upon an immune challenge.

Elevated serum GCs in humans is strongly associated with psychotic and melancholic depressive subtypes (Carroll et al., 2007). Our model of CPA provoked a depressive-like phenotype and produced mixed results in anxiety-related behavioral tasks. Anxiety-like behaviors were modestly reduced in an open field test but were not altered in other behavioral assays. These results are in line with other work showing that CPA produces affective and cognitive deficits in rodents. Chronic weekly 6-hr phase advance induces depressive-like behaviors, and impairs object recognition (Horsey et al., 2019) and escape learning (Daut et al., 2019). More profound changes in the light cycles, such as repeatedly reversing the LD cycle, increases serum corticosterone concentrations, and alter sensitivity to a subsequent stressor (Sakellaris et al., 1975). Of note, reductions in anxiety-like behavior are common in models of circadian disruption such as constant light (Fonken et al., 2009; Ma et al., 2007) and dim light at night (Bedrosian et al., 2011; Hogan et al., 2015) exposure, and circadian dysfunction induced by *Afh* mutant (Keers et al., 2012), *Clock* knockdown (Mukherjee et al., 2010), and *Rev-Erbα* knockout (Chung et al., 2014). One caveat of this study is that molecular profiles were characterized 3 shifts (8–9 days) after the behavior tests. However, changes in neuroimmune pathways may take place as soon as

after 4 weekly 6hr advance (Castanon-Cervantes et al., 2010; Ramsey et al., 2020), during which we performed most behavior tests. Future studies should examine the priming effects of CPA on mood and cognitive-related behaviors when followed by a second challenge such as LPS, as worsened neuroinflammation is an important contributing factor in neuropsychiatric and neurodegenerative disorders (Allison and Ditor, 2014).

One limitation of this study is that we only included male mice because it was not possible to fully power for another factor while conducting all behavioral testing within a narrow ZT window. However, future work should explore potential sex differences, as gonadal hormones regulate the SCN by modulating major SCN input pathways (Bailey and Silver, 2014). Indeed, the hippocampus of female hamsters appears to be sensitive to CPA: female hamsters show long-term cognitive deficits 4 weeks after the cessation of phase shifts (Gibson et al., 2010). We previously found that female mice during development are more susceptible to another chronic light manipulation, dim light at night, and exhibit affective behaviors and worsened neuroinflammatory responses to LPS (Chen et al., 2021). Future studies should compare the potential sex differences in neuroinflammatory responses and mood-related behaviors after CPA.

5. Conclusion

In conclusion, our study showed that in male C57BL6/J mice CPA induced higher behavioral despair in forced swim and lower anxiety in open field. Changes in behavior were accompanied by disrupted hippocampal circadian gene profile. Further, CPA suppressed expression of *Bdnf* and pro-inflammatory cytokine interleukin-1 beta in the hippocampus. Plasma corticosterone concentrations were elevated by CPA, suggesting that CPA may suppress neuroimmune pathways via glucocorticoids. Taken together, these results demonstrate that chronic circadian disruption alters mood and neuroimmune function. Future studies should further elucidate the mechanisms underlying the neuroinflammation and mood-related behavioral changes after CPA. These studies may have implications for circadian-related disorders including jetlag, shift work, and affective disorders.

Declaration of competing interest

The authors have no conflicts of interest to report.

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