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Circadian disruption and psychostimulants dysregulates plasma acute-phase proteins and circulating cell-free mitochondrial DNA

Tamizhselvi Ramasamy [a,b], Mayur Doke [a], Jay P. McLaughlin [c], Thangavel Samikkannu [a,*]

[a] Department of Pharmaceutical Sciences, Irma Lerma Rangel School of Pharmacy, Texas A&M University, College Station, TX, 77843, USA
[b] School of Biosciences and Technology, Vellore Institute of Technology, Vellore, 632014, Tamilnadu, India
[c] Department of Pharmacodynamics, College of Pharmacy, University of Florida, Gainesville, FL, 32611, USA

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ABSTRACT

Background: Previous studies have indicated a close link between the inflammatory response, exacerbated by circadian disruption and psychostimulants such as cocaine and methamphetamine (METH). Indicators of this inflammation include cortisol and acute-phase proteins (APPs) like C-reactive protein (CRP), complement C3 (C3), and serum amyloid A (SAA). The connection between these inflammation markers and circulating mitochondrial DNA (mtDNA) has been gaining attention. However, the specific influence of cocaine and METH on APP, cortisol, and mtDNA levels in mice with disturbed circadian rhythm has yet to be explored, which is the main aim of this research.

Methods: In our study, we employed 10–12-week-old male C57BL/6J mice, which underwent an imposed 6-h phase advance every six days for a total of eight cycles. This process led to the formation of mice with disrupted circadian rhythm and sleep disorders (CRSD). We administered 11 dosages of cocaine and METH 15 mg/kg and 20 mg/kg, respectively to these CRSD mice over the course of 22 days. Quantitative assessments of CRP, C3, SAA, cortisol, and cell-free circulating mtDNA were conducted using enzyme-linked immunosorbent assay (ELISA), Western Blot, and quantitative real-time polymerase chain reaction (qRT-PCR) techniques.

Results: The experiment revealed that disruption in circadian rhythm alone or cocaine or METH on their own increased CRP, C3, SAA, and cortisol levels in comparison with the control group. CRSD mice, exposed to cocaine and METH, showed a significant rise in CRP, C3, and SAA, while those without exposure remained stable. We also found a reduction in circulating cell-free mtDNA in all CRSD mice, regardless of cocaine and METH exposure.

Conclusions: The findings of our study affirm that the levels of CRP, C3, SAA, and cortisol, which reflect inflammation, are enhanced by circadian disruption, cocaine, and METH, and these levels show a strong correlation with the content of circulating cell-free mtDNA. Furthermore, it also shows the potential link between the disruption of the circadian clock and the inflammatory response triggered by cocaine and METH.

1. Introduction

Circadian rhythm sleep disorder (CRSD) has been commonly considered a consequence of neuroinflammatory processes and a cause of neurodegenerative disorders (Musiek, 2015). Substance abuse disrupts numerous mental and physical processes, including the regulation of the circadian clock/sleep associated with the suprachiasmatic nucleus (SCN). At molecular level, the relationship is bidirectional and complex. Sleep disruption leads to an increased risk of drug abuse; conversely, drug abuse may also cause CRSD and is associated with neurodegenerative diseases (Du et al., 2019; Hasler et al., 2012). A neurobiological mechanism connecting oscillations of the circadian system with sleep disturbance links psychostimulant cocaine use to the innate immune system(Schierenbeck et al., 2008; Wang et al., 2019), and generates acute phase proteins (APPs), which are rapidly increased during inflammatory processes.

APPs such as C-reactive protein (CRP), complement C3 (C3) and serum amyloid A (SAA) are well-studied systemic biomarkers from liver in response to inflammatory signal (Jain et al., 2011). APP is released immediately following an increase in proinflammatory cytokines IL-6

* Corresponding author. Department of Pharmaceutical Sciences Irma Lerma Rangel School of Pharmacy, Texas A&M University, 159 Reynolds Medical Buildings, MS1114 College Station, Texas, 77843, USA.
E-mail address: thangavel@tamu.edu (T. Samikkannu).

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and TNF-α. Elevated levels of circulating CRP correlate with changes in brain metabolites and brain vulnerability (Masi et al., 2013). CRP is an important regulator of neuroinflammatory processes involved in the development of dementia in Parkinson's disease (Eagan et al., 2012; Song et al., 2013). C3 secreted by astrocytes interacts with the cognate C3 receptor on microglia and thus plays an important role in immune activation and neuroinflammation (Lian et al., 2016; Wei et al., 2021). Additionally, a significant reduction in neurodegeneration and cognitive neural disorder improvements are observed in mice with C3 inhibition (Boulos and Bray, 2018). The SAA protein accumulates in the brain by crossing the blood-brain barrier and triggers the neuronal inflammatory response along with amyloid aggregation attributed to Alzheimer's disease (Jang et al., 2019). Furthermore, an increase in cortisol enhances IL-6-mediated APP release in plasma and thus modulates inflammatory responses (Ouanes and Popp, 2019). Physiologically, cortisol has very distinct and intriguing rhythms. The circadian pattern of the cortisol level oscillates with the biological clock: levels are high in the morning and relatively low in the evening (Nader et al., 2009).

These circulating APP and cortisol levels influence circulating cell-free (cf)-mitochondrial DNA (mtDNA) in inflammation (Trumpff et al., 2019). Recently, cf-mtDNA has been identified as a potential indicator for inflammation and various other physiological reactions. We were intrigued by its potential and sought to investigate its role in our study. Our aim was to quantify the variations in cf-mtDNA levels, triggered by disruptions in circadian rhythm and exposure to psychostimulants, to gain deeper insight into their influence on inflammatory responses within the mouse body. Circulating cf-mtDNA consists of short mtDNA fragments circulating in bodily fluids rather than being contained within the cell. The release of cf-mtDNA is associated with oxidative stress, as there is a decline in the damage-associated molecular patterns observed in neurodegenerative diseases, while the opposite is observed in cancer (An et al., 2019). Circulating cf-mtDNA consists of short mtDNA fragments circulating in bodily fluids rather than being contained within the cell. The release of cf-mtDNA is associated with oxidative stress, as there is a decline in the damage-associated molecular patterns observed in neurodegenerative diseases, while the opposite is observed in cancer (An et al., 2019). In addition, our previous studies have also shown that cocaine and METH significantly increased the level of APPs in cocaine and METH users' plasma(Samikkannu et al., 2013a).However, the relationship between CRSD-associated cocaine or methamphetamine (METH) exposure-associated inflammatory dysfunction is largely unknown. The present study aims at examining the levels of APPs, cortisol, and mtDNA in mice exposed to cocaine and METH and their interactive role in promoting CRSD progression. To briefly clarify, our study intends to investigate the inflammatory response linked to substance addiction, specifically cocaine and methamphetamine, and Circadian Rhythm Sleep Disorder (CRSD). We believe it is especially important to continue investigating the interactions between these two factors given prior research that suggests a connection between cocaine or METH usage and CRSD. Additionally, we want to comprehend how substance addiction and CRSD interact to affect the inflammatory response, which is why we concentrated our research on the CRSD + Cocaine and CRSD + methamphetamine groups.

2. Materials and methods

2.1. Animal model

The animal model used for this study was based on 10- to 12-week-old C57BL/6J male mice sourced from the Jackson Laboratory, Bar Harbor, Maine. These mice were initially housed in polycarbonate cages within a vivarium. The environment was controlled to maintain an optimal temperature of 23 °C and followed a 12-h light and 12-h dark (LD) photocycle. Throughout the experiment, the mice had unrestricted access to food and water. Circadian Time 0 (CT0) designates the start of the subjective "day," when lights would be turned on, and Circadian Time 12 (CT12), which designates the start of the subjective "night," when lights would be turned off, in a typical, undisturbed circadian rhythm. The cycle of day and night in the environment is modeled by this pattern. For the purpose of inducing Circadian Rhythm Sleep Disorder (CRSD) in a group of mice for our study, we disturbed this cycle. We did this by moving their cycle of light and dark forward by 6 h. This indicates that when the subjective day (CT0) for the control group began the subjective day (CT0) for the CRSD group had already begun by 6 h. Similar to how it was 6 h into the subjective night for the CRSD group when the subjective night began (CT12) for the control mice. In real terms, the CRSD group's "day" would begin at 12:00 a.m. (midnight) if the control group's did so at 6:00 a.m. (lights on). Similar to how it would be midnight (12:00 a.m.) for the CRSD group when the control group started their "night" at 6:00 p.m. (lights off). This change caused a disruption in the CRSD group's circadian rhythms by moving their complete light-dark cycle forward by 6 h compared to the control group. This adjustment to the light-dark cycle continued over a period of eight weeks.

In contrast, the control group of mice was kept under a consistent 12-h light and 12-h dark cycle throughout the duration of the study, without any alterations. The timeline for the circadian mouse model study, including illustrations of the day-night cycles for the normal and CRSD mice, is presented in Fig. 1A, B, and 1C.

2.2. Treatment schedule and method

Animals were randomly grouped into 6 groups (n = 6 animals per group) as follows: group 1: control (normal 12 h light/12 h dark); group 2: circadian disruption (CRSD; animals were exposed to a 6 h phase advance every six days for eight cycles); group 3: cocaine alone- 15 mg/kg (11 doses, every other day for 22 days); group 4: METH alone-20 mg/kg; group 5: CRSD with cocaine −15 mg/kg (CRSD animals treated with 11 doses of cocaine every other day for 22 days); and group 6: CRSD with METH-20 mg/kg (CRSD animals treated with 11 doses of METH every other day for 22 days) (Fig. 1D). Intraperitoneal injections were used to treat the animals in the study. The control animals were given saline alone (no drugs). The final optimum concentration and time were established and used in subsequent experiments. At the end of the treatment, the animals were decapitated, and the plasma was collected for further analysis. All experiments were performed in compliance with protocols approved by the local Basel Committee for Animal Care and Texas A&M University Animal Use Committee.

2.3. Measurement of CRP, SAA, and C3 protein levels in plasma samples by ELISA

Blood collected (cardiac puncture) was centrifuged and stored at −80 °C. CRP, SAA and C3 protein levels in plasma samples were determined using the Immunoassay Kit #E-90 CRP, #E-90 SAA and #E-90 C3 (Immunology Consultant Laboratory, Portland, OR, USA). This colorimetry-based immunoassay kit uses a monoclonal antibody (mAb) raised against full-length human CRP, SAA and C3 to measure and quantify the total protein levels.

2.4. Measurement of CRP, SAA, and C3 protein levels in plasma samples by western blot

Plasma CRP, SAA2 and C3 protein levels was done by Western blotting in randomly selected samples from the METH, cocaine, CRSD, CRSD with cocaine, and CRSD with METH groups. Equal amounts of proteins were resolved on 4–15% polyacrylamide gel and transferred to a nitrocellulose membrane and incubated with respective antibodies. Immunoreactive bands were visualized using a chemiluminescence Western blotting system (Amersham Piscataway, NJ, USA).

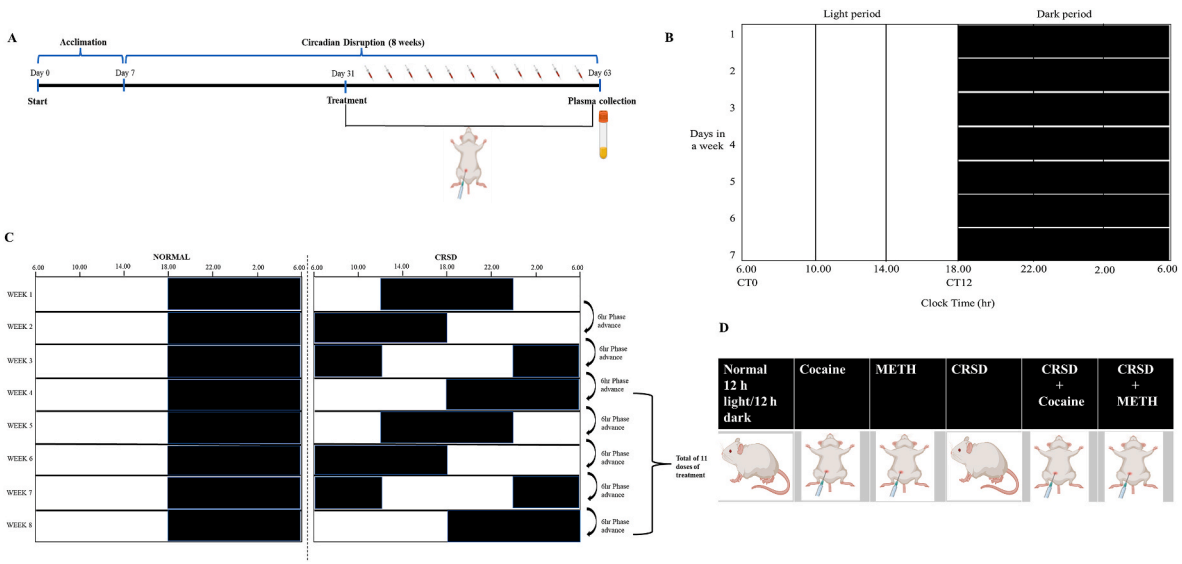

Fig. 1. Experimental design and treatment schedule for CRSD mouse model study. (A). Experimental design used to evaluate the effects of circadian disturbances and cocaine or METH exposure on inflammation markers. (B) Normal day-night cycle for normal(control) mice group (C) Graphical representations of the stable and rotating light schedules used to induce circadian disturbances in CRSD mice group. (D) The classification of normal, CRSD and treatment groups. Created with BioRender.com.

2.5. Analysis of mtDNA

cf-DNA was isolated from the plasma using QI Amp Blood Mini Kit (Qiagen, Hilden, Germany). For total DNA, the Qubit Fluorometer and Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) were used. mtDNA was analyzed using a real-time polymerase chain reaction (RT-PCR) Realplex 4 Master Cycler (Eppendorf, Hamburg, Germany). PCR was performed using the QuantiFast SYBR Green PCR Kit (Qiagen, Hilden, Germany), designed for the absolute quantification of low-copy DNA targets. The following program was used: 1 × 5 min at 95 °C; and 40 × 10 s at 95 °C for denaturation, 30 s at 60 °C for annealing and 30 s at 75 °C for extension. For mitochondrial DNA, the following primers for the *cytochrome c* gene were used: F: 5′-CCCAGCTACTACCATCATTCAAGT-3'; R: 5′-GATGGTTTGGGA-GATTGGTTGATGT-3′(Homolová et al., 2020).

2.6. Metabolomics analysis of plasma samples

Plasma samples were centrifuged at 14,000×*g* for 10 min at 4–8 °C, and the supernatant was made up to a final 80% (vol/vol) methanol solution. The samples were centrifuged, and the supernatant was filtered through Target2™ PVDF syringe filters and lyophilized. The mass spectrometry was performed as described previously (Doke et al., 2022).

2.7. Integrative bioinformatic analysis of inflammation markers

The protein-protein interaction was analyzed by using STRING database which uses known and predicted protein-protein interactions (Szklarczyk et al., 2021). Metabolomics data was analyzed with MetaboAnalyst 5.0 web interface of MetaboAnalystR package (Pang et al., 2022).

2.8. Statistical analysis

Statistical calculations were carried out using GraphPad Prism 9 (GraphPad Software Inc., La Jolla, CA, USA). The effect of METH, cocaine or their combination on circadian disruption compared was determined via comparison with control animals and using two-way analysis of variance and 2way-ANOVA as well as the nonparametric Mann–Whitney *U* test. The results of comparisons between the control group and the CRSD with cocaine or CRSD with METH groups were considered significant at $P < 0.05$. Pearson's correlations were used to assess the correlations between the APPs – (CRP, C3, and SAA and cortisol) and expression of plasma circulating cf-mtDNA. Significance level-ns $P > 0.05$, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ and ****$P \leq 0.0001$.

3. Results

3.1. Variation in plasma APP levels analyzed by ELISA

ELISA results of APP levels in plasma samples from the CRSD, cocaine and METH groups were compared with the control group. The CRSD with cocaine and CRSD with METH groups were compared with the CRSD group. Plasma CRP level was elevated in the CRSD ($F(5, 25) = 12.68$, pANOVA <0.0001), cocaine ($F(5, 25) = 12.68$, pANOVA = 0.0017), METH ($F(5, 25) = 12.68$, pANOVA = 0.0230), CRSD + cocaine ($F(5, 25) = 12.68$, pANOVA <0.0001) and CRSD + METH ($F(5, 25) = 12.68$, pANOVA <0.0001) (Fig. 2A). However, CRP levels among CRSD mice and CRSD with cocaine were insignificant. Interestingly, CRP levels in CRSD mice and CRSD with METH were significant ($F(5, 25) = 12.68$, pANOVA = 0.0395). Moreover, we also observed similar trends of elevated levels of C3 and SAA in cocaine, METH and CRSD-exposed mice. The C3 ($F(5, 25) = 16.74$, pANOVA = 0.024) and SAA ($F(5, 25) = 26.98$, pANOVA = 0.0004) levels in the plasma of CRSD mice were elevated than control as in Fig. 2B and C respectively. In addition, cocaine ($F(5, 25) = 16.74$, pANOVA <0.0001) and METH ($F(5, 25) = 16.74$, pANOVA = 0.0053) exposed mice showed higher levels of C3 (Fig. 2B). Similarly, SAA levels were increased in cocaine ($F(5, 25) = 26.98$, pANOVA = 0.0061) and METH ($F(5, 25) = 26.98$, pANOVA = 0.0005) exposed mice (Fig. 2C). Interestingly, when we compared CRSD mice group with CRSD + Cocaine, we identified that C3 ($F(5, 25) = 16.74$, pANOVA = 0.0156) and SAA ($F(5, 25) = 26.98$, pANOVA = 0.0001) levels increased (Fig. 2B and C). Similarly, we noted similar observations in CRSD group with CRSD + METH, C3 ($F(5, 25) = 16.74$, pANOVA = 0.012) and SAA ($F(5, 25) = 26.98$, pANOVA = 0.0239) levels were higher compared to control (Fig. 2B and C). The plasma CRP levels in the CRSD with cocaine and CRSD with METH groups were decreased when compared with those in the CRSD group, whereas the C3 and SAA levels were increased compared with those in CRSD

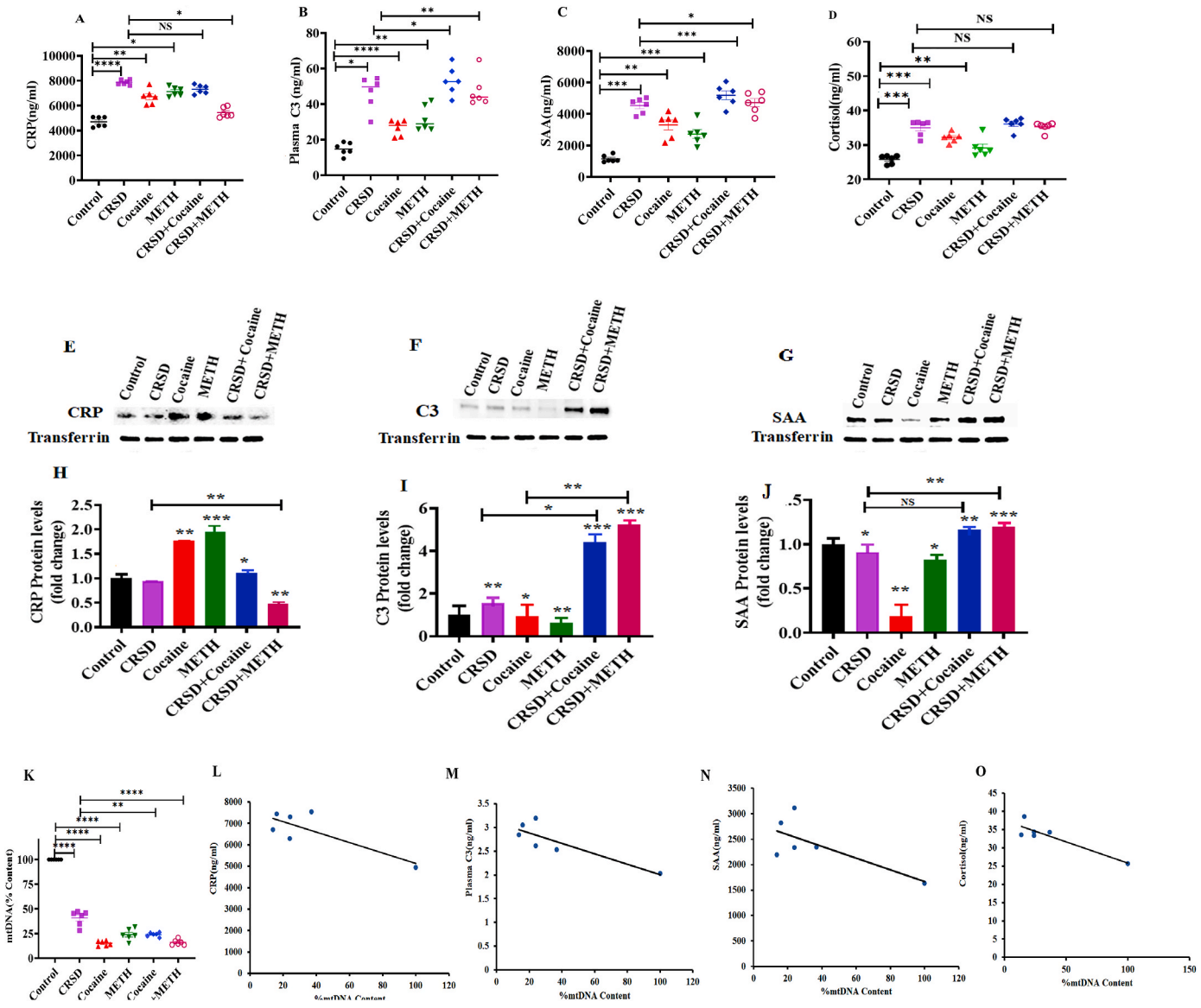

Fig. 2. (A–D) Estimated plasma levels of acute-phase proteins in the CRSD with cocaine and CRSD with METH groups. The plasma levels of C-reactive protein (CRP) (A), C3 (B), SAA (C) and Cortisol (D) were estimated by ELISA. The protein levels are indicated in ng/ml. (E–G) Plasma levels of acute-phase proteins in the CRSD with cocaine and CRSD with METH groups estimated by Western blot. The plasma levels of C-reactive protein (CRP) (E), C3 (F), and SAA (G) were elucidated by Western blot. Transferrin protein was used as the loading control. (H–J) Corresponding densitometry of WB. (K–O) Plasma levels of cell-free (cf)-mtDNA in the CRSD with cocaine and CRSD with METH groups. The plasma levels of cf-mtDNA were estimated using the QI Amp Blood Mini Kit (K). cf-mtDNA is represented as the % content against the control group. The relationship between acute-phase proteins and cf-mtDNA. mtDNA content was compared by linear regression to plasma C-reactive protein (CRP) expression (L), plasma C3 levels (M), plasma SAA levels (N) and plasma cortisol levels (O). The data are presented as the mean ± standard error of the mean. NS-Not significant, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

animals. In plasma, a significant effect of circadian disruption and cocaine and METH exposure on cortisol levels was observed. The CRSD (F (5, 25) = 14.25, pANOVA = 0.0007), cocaine (F (5, 25) = 14.25, pANOVA = 0.0009) and METH (F (5, 25) = 14.25, pANOVA = 0.0045) groups showed an increase in cortisol levels, whereas the difference between the CRSD with cocaine and CRSD with METH groups was much less obvious, but there was no statistically significant difference from the levels in the CRSD group (Fig. 2D).

3.2. Variation in plasma APP levels

The plasma levels of CRP, C3 and SAA protein were analyzed by Western blot to validate ELISA results. The protein expression of CRP, C3 and SAA in the CRSD, cocaine, METH, CRSD with cocaine and CRSD with METH groups was compared with the control group (Fig. 2E-G). Statistically significant differences in the CRSD, METH and cocaine groups (Densitometry; Fig. 2H–I). The densitometric evaluations of the statistically significant differences were observed among the CRSD, METH, and cocaine groups in comparison with the control group. Concordance between the CRP, C3 and SAA ELISA and western blotting analysis was observed. Determination of CRSD's effect with and without cocaine and METH exposure on cortisol levels revealed an elevated level of plasma C3 (F (5, 10) = 9.605, pANOVA = 0.0023) in the CRSD group compared to the control group (Fig. 2F and I). Intriguingly, METH (F (5, 10) = 11.248, pANOVA = 0.0004) and cocaine (F (5, 10) = 11.248, pANOVA = 0.0034) groups showed elevated protein levels of CRP while C3 and SAA proteins level were decreased compared to the control group. (Fig. 2E–G; 2H-2J). Mainly, the METH downregulates the C3 protein level (F (5, 10) = 9.605, pANOVA = 0.0053) (Fig. 2F and I). Additionally, cocaine downregulated the SAA protein level (F (5, 10) =

18.64, pANOVA = 0.0042) (Fig. 2G and J). The plasma CRP level in the CRSD with cocaine (non-significant) and CRSD with METH groups (F (5, 10) = 11.248, pANOVA = 0.0038) was decreased compared with that in the CRSD group (Fig. 2E and H), whereas the C3 (F(5, 10) = 9.605, pANOVA = 0.0023) (Fig. 2F and I) and SAA (F(5, 10) = 18.64, pANOVA = 0.0068) (Fig. 2G and I) levels were increased in CRSD with METH group compared with those in the CRSD group.

3.3. Analyzing circulating cf-mtDNA for correlation of APP and cytochrome c

To analyze the factors associated with plasma circulating cf-mtDNA, RT-PCR targeting the mitochondrial gene *cytochrome c* was performed (Supplementary file S1). CRSD (F (5, 25) = 10.04, pANOVA <0.0001), cocaine (F (5, 25) = 10.04, pANOVA <0.0001) and METH (F (5, 25) = 10.04, pANOVA <0.0001) exposure altered the mtDNA levels in plasma. We also observed that the CRSD with cocaine and CRSD with METH groups showed decreased cf-mtDNA levels in plasma compared to the control group (Fig. 2K). Interestingly, the CRSD with cocaine (F (5, 25) = 10.04, pANOVA = 0.0071) and CRSD with METH (F (5, 25) = 10.04, pANOVA <0.0001) groups showed significant differences compared to the CRSD group (Fig. 2K). The levels of the APPs - CRP, C3, and SAA and cortisol were inversely correlated with the mtDNA levels and specifically, C3 (Pearson's correlation r = −0.8553, p = 0.0299) (Fig. 2M) and cortisol (Pearson's correlation r = −0.8957, p = 0.0157) (Fig. 2O) level were significantly correlated with reduction cf-mtDNA. These observations strengthen the evidence that sleep onset is a major factor contributing to the synchronization of APP and cortisol rhythms.

3.4. Analyzing the association between metabolites and inflammation markers in plasma

We analyzed if inflammation alters metabolites of cocaine and METH in CRSD mice and performed an untargeted detection method based on plasma LC–MS/MS metabolite profiling (Fig. 3A). A total of 43 metabolites were significantly altered in CRSD, and cocaine group compared to control (Supplementary file S2). Among 45 metabolites, the most significant common 17 metabolites were altered among CRSD, and

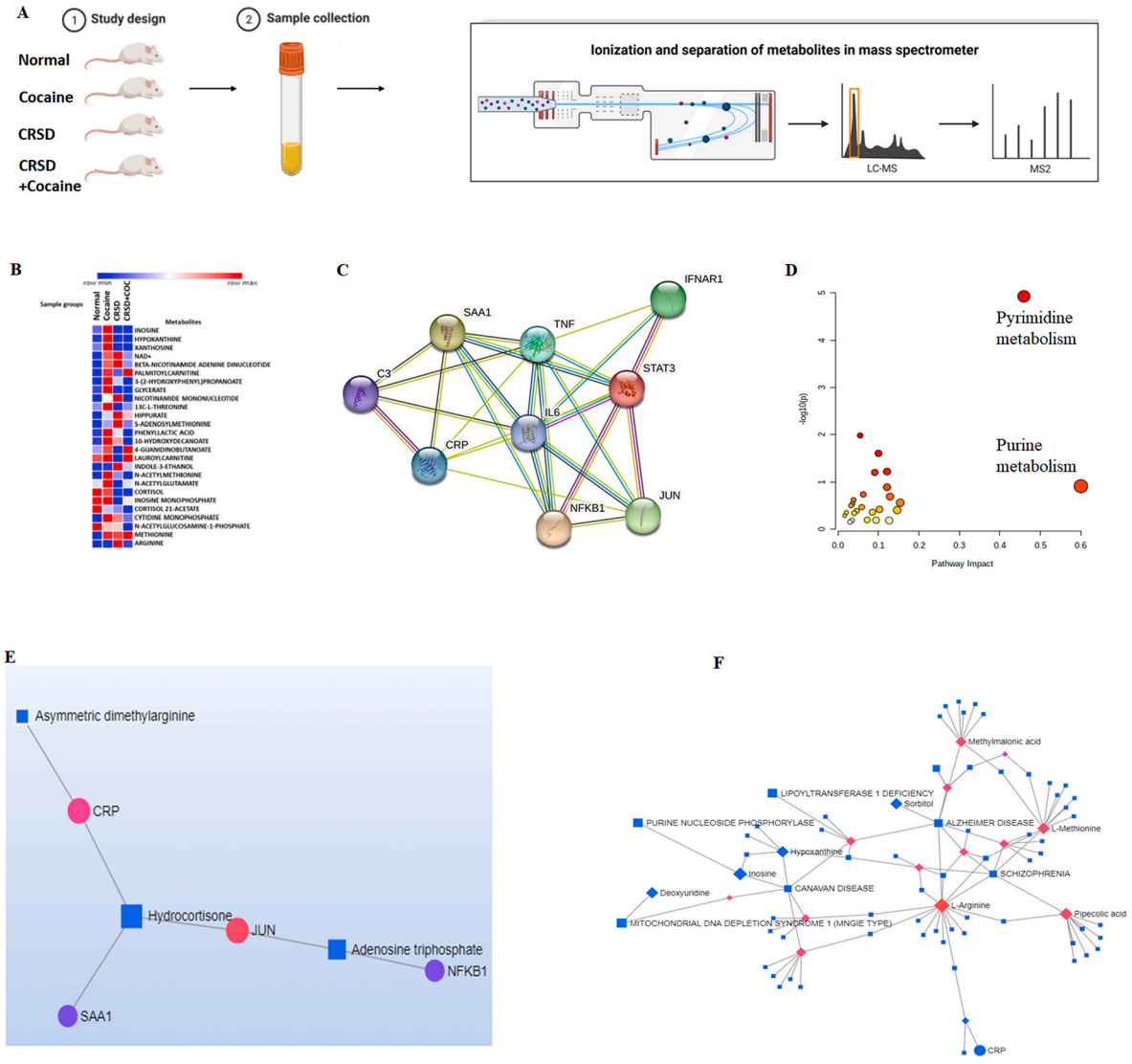

Fig. 3. Inflammation marker analysis. (A)Metabolites detection in the plasma samples of cocaine and CRSD group mice by using mass spectrometry analysis. (B) Heatmap showing the top upregulated metabolites (red) and downregulated metabolites (blue) across the control, circadian disruption (CRSD), cocaine, and CRSD with cocaine treatment groups. (C) Protein-protein interaction between inflammation markers by STRING. (D) Functional analysis of the common significantly altered metabolites. The pathway impact is shown based on−log10 (pvalue). (E) The MetaboAnalyst analysis between inflammation markers-genes and metabolites. (F) The MetaboAnalyst analysis between inflammation markers-genes- metabolites-diseases. Created with BioRender.com.(For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

cocaine group compared to control, which corroborates with our earlier results (Doke et al., 2022). Metabolomic analysis shows altered levels of uridine monophosphate ($p < 0.008$), adenosine 5′-diphosphate ($p < 0.044$), and inosine ($p < 0.019$) in CRSD + cocaine group compared to cocaine group. Moreover, Cortisol-21-acetate, NAD+, Methionine and Hippurate were among the topmost significantly altered metabolites identified in cocaine, CRSD and CRSD + cocaine compared to control (Fig. 3B).

Earlier studies have documented that cocaine exposure, sleep disturbances and psychiatric disorder are significantly linked to elevated levels of inflammatory mediators such as C3, CRP and cortisol; inflammatory signaling pathways- NF-Kb-TNF-α; JUN transcription factors; JAK-STAT pathways. To investigate the relationship between inflammation markers, we performed protein-protein interaction between C3, CRP, SAA1, NF-Kb, TNF-α, IL-6, JAK, JUN and STAT3 (Fig. 3C) (Supplementary file S3). Furthermore, we investigated the interaction between significantly altered metabolites and inflammatory genes which were shown to be affected by cocaine exposure and sleep disturbances by using the metabolomic database MetaboAnalyst5.0. Fig. 3D shows the pathway impact is shown based on −log10 (p-value). Pyrimidine and purine metabolic pathways were affected significantly. Furthermore, gene-metabolite interaction analysis demonstrated a very close connection between genes CRP, JUN, SAA1 and NFKB1 with metabolites-hydrocortisone, adenosine triphosphate and asymmetric dimethylarginine (Fig. 3E). Fig. 3F shows the interaction between gene-metabolite-disease. Gene-metabolite interactions mainly pointed out towards Alzheimer's disease, Parkinson's disease, and mitochondrial DNA depletion syndrome-1 (MNGIE) (Supplementary file S4 & S5).

4. Discussion

Several clinical studies with various cohorts have reported that circadian rhythm disturbance can accelerate drug addiction (Hasler et al., 2012). Clinical studies have also demonstrated that illicit drug use is associated with sleep disorders and impairs both immune and neuronal functions (Logan et al., 2014). To understand the similar effects of circadian disturbance and drug abuse on inflammation, we generated a CRSD mouse model using phase shifts and investigated the influence of psychostimulant (cocaine/METH) exposure on APP, cortisol and mtDNA levels. To understand the similar effects of circadian disturbance and drug abuse on inflammation, we generated a CRSD mouse model using phase shifts and investigated the influence of psychostimulant (cocaine/METH) exposure on APP, cortisol and mtDNA levels with ELISA assays. We measured the APPs CRP, C3 and SAA in plasma from the CRSD, cocaine alone, METH alone, CRSD with cocaine, and CRSD with METH groups using ELISA and Western blot. The expression of all three APPs, CRP, C3 and SAA, was significantly upregulated compared with that in the control group. We also observed that APP levels showed a slight increase in the CRSD with cocaine and CRSD with METH groups, and this increase was significant not only when compared with the levels in the cocaine or METH alone groups but also when compared with the CRSD group. In contrast to the untreated CRSD group, our study's CRSD-meth group showed a significant drop in CRP levels, and the treated control animals showed a rise in CRP levels compared to the untreated controls which is determined by Western blot experiments. This finding points to a complex interplay between methamphetamine use, circadian rhythm sleep disruption, and the inflammatory response. Methamphetamine and other psychostimulant substances may cause varied inflammatory reactions depending on the physiological and neurological circumstances, according to previous research (Doyle et al., 2015). The decrease in CRP levels seen in the CRSD-meth group may therefore be explained by the neurobiological alterations brought on by circadian disruption, which may influence the body's response to methamphetamine (Yu et al., 2015). The recognized inflammatory response to methamphetamine in a typical circadian setting is reflected by an increase in CRP levels in control rats treated with METH (Re et al., 2022). The precise processes underlying these varying reactions to methamphetamine under both normal and disrupted circadian settings require further study.

We noticed disparities between the Western blot and ELISA findings specifically for the CRSD group, which we recognize might be attributed to the distinct roles these proteins play both physiologically and pathologically. The Western blot results revealed a decline in C3 protein levels when control mice were exposed to METH, and a considerable decrease in SAA protein levels when control mice were exposed to cocaine. Intriguingly, these findings starkly contrasted with the ELISA results, which indicated a significant increase in these protein levels in the treated mice as opposed to the control. According to Ricklin et al. (2010), the C3 protein, a vital part of the complement system, is essential for boosting immunological response and inflammation. As a result, a drop in C3 protein levels after exposure to METH would indicate a muted immunological response (Ricklin et al., 2010). Similar to how SAA, a multifunctional protein involved in the dampening inflammatory response and immunological response (Ye and Sun, 2015), exhibited a significant decrease with cocaine exposure. These seemingly incongruous findings may be explained by the varied physiological and pathological roles that these proteins play.

The current finding that elevated APP levels after psychostimulant exposure and circadian misalignment is consistent with our earlier report (Samikkannu et al., 2013b). Most studies have shown that CRP, C3 and SAA are involved in regulating inflammatory mechanisms and are linked to cognitive impairment and neurodegenerative diseases (Luan and Yao, 2018). Furthermore, these APPs are major players in chronic inflammation caused by circadian disturbance, trigger immune dysfunction, and subsequently promote disease progression. Involvement of CRP, SAA and C3 in sleep disorders shows that a brief increase in their levels is sufficient to induce neurodegeneration, although the CRP, C3 and SAA levels in cocaine and METH users were not compared with those in people with circadian misalignment or sleep disorders (Shivshankar et al., 2020). In contrast, both decreased clock gene expression and psychostimulant preference lead to excessive release of cytokines (McClung et al., 2005). Indeed, the synthesis of APPs is mediated by cytokines, which are the main factors of the immune system and are rhythmically expressed in the SCN, which may regulate clock gene activation (Cermakian et al., 2014). APPs and cortisol could be possible systemic cues influencing circadian misalignment, and psychostimulants' drug abuse may underlie the link between the immune response and circadian misalignment.

In addition, several studies have explored cortisol-drug abuse associations as well as the effect of circadian rhythm on cortisol levels, which are the risk factors for developing psychostimulant use problems, to psychomotor dysfunction (Marceau and Abel, 2018). Circadian misalignment is associated with the development of cocaine and METH addiction and leads to dysregulation of immune responses of the central nervous system (CNS). Cortisol levels are elevated in cocaine and METH abusers (Pirnia et al., 2016). Confirming that, we found increased plasma cortisol levels in the CRSD with and without cocaine and METH groups when compared with those in the control group. High cortisol levels in cocaine dependence affect cognitive and neuroendocrine function, and studies with METH have shown persistent immune activation and neuroinflammation along with elevated cortisol levels(Fox et al., 2009). Our findings suggest that elevated levels of cortisol could be a chronological biomarker of circadian rhythm disturbance triggered by psychostimulants.

We also found that CRP, C3, SAA, and cortisol levels were negatively correlated with the circulating mtDNA levels in the CRSD with and without cocaine and METH exposure groups (Fig. 2L–O), providing additional evidence for accelerated disease progression and immune susceptibility. A lower mtDNA copy number has been associated with inflammation and increased circulating CRP levels (Knez et al., 2017). In the present study, a decrease in plasma mtDNA content was observed in the CRSD, cocaine and METH groups (Fig. 2K). We also observed a

negative correlation between APP and cf-mtDNA, which are two well-known mediators involved in the inflammatory response, (Fig. 2L–O). Dysregulated mitochondria affect the inflammatory mechanism and lead to excessive reactive oxygen species production (ROS) along with increased mtDNA damage and lower circulating mtDNA content (Knez et al., 2017). In contrast, sleep duration is associated with elevated oxidative stress along with decreased mtDNA copy number (Kim et al., 2014). In addition, increased oxidative stress impact mitophagy in drug abuse patients, and reduction in circulating cf-mtDNA (Feng et al., 2013). The levels of mtDNA are regulated by peroxisome proliferator-activated receptor γ coactivator (PGC)-1α, a gene required for mitochondrial biogenesis (Scarpulla, 2011). Altered circadian rhythms lead to deficiency of PGC-1α and reduces the mtDNA content in mice (Chen and Yang, 2014). Also, mtDNA could play an important role in the initiation of cocaine addiction (Sadakierska-Chudy et al., 2017). The above results suggest that mitophagy and PGC-1α-mediated decreases in mtDNA levels in circadian-disturbed mice may lead to drug addiction. Moreover, cocaine addiction influences the innate immune system through the nuclear factor kappa-light-chain-enhancer of the activated B cell (NF-kB) signaling pathway and induces the release of proinflammatory cytokines such as Interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) (Bettcher et al., 2018; Maza-Quiroga et al., 2017; Morcuende et al., 2021). Cocaine addiction (Mai et al., 2018) and sleep disturbances (Liu et al., 2021) affect the JAK-STAT signaling pathway. Additionally, sleep disorder is involved with increased levels of inflammatory markers (Chiang, 2014; Sun et al., 2022).

The MetaboAnalyst 5.0 database network analysis involving significantly altered genes and metabolites from cocaine + CRSD groups revealed that purine and pyrimidine metabolism was affected the most (Supplementary file S6). Sleep disorder and depressive disorder are linked to defective energy metabolism in the brain(Doke et al., 2022; Du et al., 2021; Patkar et al., 2009). Interestingly, researchers have successfully tested the degree to which the presence of inflammatory markers in cerebrospinal fluid and plasma can mirror the increase in CNS markers of neurodegenerative disease. This can help to predict the early markers of neurodegenerative diseases (Bettcher et al., 2018). The same principle can be applied to psychostimulant addiction and sleep disorders too. Our current study can be a good example to detect the inflammation markers of specific genes and metabolites in the plasma for early diagnosis of sleep disorder and cocaine impairment. Importantly, the gene-metabolite-disease interaction network also predicted that elevated level of inflammatory markers and metabolites may lead to dementia diseases such as - Alzheimer's disease and Parkinson's disease. Another prediction of these interactions was mitochondrial DNA depletion syndrome-1 which includes Mitochondrial DNA abnormalities such as mtDNA depletion, deletion, and point mutations (Taanman et al., 2009).

Therefore, our study suggests that monitoring circulating mtDNA and inflammatory markers in the plasma of CRSD with or without METH and cocaine may be a good indicator and useful way to detect psychiatric disease progression and psychostimulant addiction-related sleep disorder.

5. Conclusion

Our study provides evidence implicating circadian misalignment and the effects of exposure to the psychostimulant's cocaine or METH on alterations in plasma APP and cortisol levels. A lower mtDNA content in plasma was inversely associated with CRSD-, cocaine- and METH-induced inflammation and associated with increased circulating APP and cortisol levels, which strengthens the association between illicit drug use and circadian misalignment. A better understanding is necessary to elucidate the pro-inflammatory mechanism at the molecular level and confirm its potential clinical usefulness.

Authorship contribution statement

Ramasamy Tamizhselvi: Performed the experiment, acquired the data, analyzed the data, and drafted the manuscript. Mayur Doek: Methodology, formal analysis, investigation. Jay P. McLaughlin: Involved in the mouse model data interpretation and revision of the manuscript. Thangavel Samikkannu: Conceptualization, resources, writing – review & editing, supervision, project administration, funding acquisition.

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Ethics approval and consent to participate

The research was ratified by the Animal Ethics Committee of Texas A&M University. All animal procedures were performed in line with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH, Bethesda, Maryland, USA). Great efforts were made to minimize the suffering of animals.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare that they do not have any potential competing interests.

Data availability

Data will be made available on request.

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Abbreviations

APP	acute-phase proteins
CRP	C-reactive protein
C3	complement 3
SAA	serum amyloid A
ELISA	enzyme-linked immunosorbent assay
RT-PCR	real-time polymerase chain reaction
mAb	monoclonal antibody
METH	methamphetamine
CRSD	circadian rhythm sleep disorder
mtDNA	mitochondrial DNA
CNS	central nervous system
ROS	reactive oxygen species production

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2023.100659.

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