CHAPTER -3

Evaluation of NAC effect on central clock related circadian rhythm

3. Introduction

Growing evidence on the subject of circadian rhythms, health and disease comes from disciplines including molecular biology and epidemiology (Karatsoreos, 2012; Reppert and Weaver, 2002). The suprachiasmatic nucleus (SCN) plays a major role in regulating circadian rhythms. The SCN controls the rhythmic release of pineal melatonin via noradrenergic neurons in the end (Lall et al., 2012), which regulates the sleep wake cycle. Numerous studies suggest that several genes and transcription factors play vital role, as the molecular basis for well-adjusted synchrony among diverse physiological systems are coordinated through the SCN (Grundschober et al., 2001; Reppert and Weaver., 2002). Our recent reports on the SCN shows that exposure to ecologically relevant levels of chronic dim light (5 lux) altered core circadian clock in the SCN at both the molecular and gene level. Notably, of the several circadian parameters, the amplitude of core clock genes (Clock, Bmal1, Per1, Per2 and Cry1, Cry2) was altered in SCN under chronic dim light condition (P. Rajput et al., 2023).

Several studies conducted in the last decade highlighting the role of principal and peripheral clocks in regulating the mitochondrial functions. Circadian clock has been shown to control variety of mitochondrial activities in the liver, heart muscle, and skeletal muscles and in *in-vitro* studies, including mitochondrial dynamics (fission-fusion) and bioenergetics (oxidative phosphorylation and ATP production)

(Neufeld-Cohen et al., 2016), substantiate this notion. According to Peek et al., 2013, clock proteins have been shown to beregulate mitochondrial redox system through the NAD^+ dependent deacetylase SIRT1, through the deacetylation of the activator and repressor of core clock machinery, SIRT1 regulates the daily rhythmicity of core clock genes (Asher et al., 2008; Nakahata et al., 2008). It also has been reported that in isolated liver mitochondria, genetic deletion of the clock activator Bmal1 reduced oxygen consumption rate (OCR) (Peek et al., 2013; Jacobi et al., 2015). The cyclic pattern of mitochondrial respiration under LD conditions is also disrupted by the continuous dim light tested in SCN (Rajput et al., 2023). Diurnal variations are seen in mitochondrial respiration. In animal models, disrupting the molecular clock results in altered respiration and a loss of mitochondrial rhythmicity. SCN, the central pacemaker innervates several centers in the body, and can directly or in directly can synchronize the clocks in them using multiple communication cascades, including metabolic, physiological, biochemical and hormonal cues. For example, several neurological (Cavanagh and Mathias., 2008; Detanico et al., 2009; Piato et al., 2008; Vogelzangs et al., 2013) and metabolic illnesses (Sardon Puig, Laura, et al., 2018) have been reported to be caused by abnormal mitochondrial respiration as a result of a disrupted circadian rhythm.

In particular, we demonstrated that mitochondrial energy metabolism (oxidative phosphorylation, adenosine triphosphate (ATP) generation), mitochondrial DNA, and CORT all exhibit periodic rhythms under LD. This rhythmicity gets altered by chronic exposure to artificial dim light (Rajput et al., 2023). We have selected a mitochondrial modulator N-acetylcysteine (NAC) to address its effect on the arrhythmic functioning of mitochondria and evaluating its impact on the abnormal

mitochondrial function brought on by chronic dim LL exposure in mice. NAC is a dietary supplement and medication that has received FDA approval for use as a mucolytic and in the treatment of paediatric acetaminophen overdose (Waring., 2012). The reason NAC was chosen is because it is a cysteine analogue, a GSH precursor and an antioxidant with several therapeutic applications, and is frequently used to alleviate the hepatotoxic effects of acetaminophen abuse by encouraging GSH production (Corcoran and Wong., 1986) or by directly scavenging free radicals (Aruoma et al., 1989), NAC can protect against free radicals' harmful effects. Studies show that NAC is a glutamate modulator, shows no side effects that are associated with most NMDA antagonists (Costa-Campos et al., 2013; Smaga et al., 2012). To our knowledge, there are no reports describing the role of NAC in regulating the mitochondrial functions in the principal circadian clock (SCN). We propose that NAC may regulate the SCN by enhancing mitochondrial activity and brain GSH levels during the disturbed circadian rhythms (here chronic dim LL). The present study is sought to determine the chrono-biotic effects of NAC on chronic circadian rhythm disturbance in mice exposed to artificial chronic dim LL. We also examined the role of melatonin, one of the most extensively used medications for irregular circadian rhythm as a reference in clinical studies, to know how melatonin affected the altered mitochondrial function as a result of dysregulated circadian rhythm.

3.1 Materials and methods

3.1.1 Animals

Swiss albino adult male mice (25-30g, eight week-old) were procured from the central animal house facility (Institute of Medical Sciences, Banaras Hindu University). They were housed in groups of six in polypropylene cages (41 × 28.2 ×

15.3 cm). All the cages were placed in a light and climate controlled environment (25 ± 1^0C, relative humidity 55±5%, and under 12:12 h light/dark (LD) cycle) for seven days before the start of the experiment. Lighting schedules within the experimental chambers were controlled with the help of an electronic timer (Havells, India). Lights were switched on at 06:00 h Zeitgeber time (ZT 0) and were switched off at 18:00 h (ZT 12). Light intensity at the cage floor level was approximately 150 lux (Testo 540) during the day (L) and 0 lux during the night (D). The mice were fed a standard rodent diet (Pashu-Aahar, Varanasi, India). Entry to the experimental chamber for cleaning and providing animal food occurred at random hours, once in 10 days. Food and tap water were provided *ad libitum*. All the experimental protocols were conducted following the principles of laboratory animal care (The Committee for the Purpose of Control and Supervision of Experiments on Animals [CPCSEA], India) guidelines, as well as law approved by the Institutional Animal Ethical Committee (IAEC), Banaras Hindu University (IMS-BHU, No. Dean/2019/IAEC/1254).

3.1.2 Drugs

N-acetylcysteine (NAC) and melatonin (MEL) were acquired from Sigma-Aldrich (St Louis, MO). All drugs were solubilised in saline (NaCl 0.9%).

3.1.3 Study design

Study design figure 1 illustrates all the animals were divided into two sets after acclimatization. One set of animals were housed in a light control environment in a 12:12 hour light/dark (LD 12:12 h, L=150 lux, D=0 lux) chamber to entrain them for 21 days. The second were housed in dim light 24 hour (LL 24:00 h, LL=5 lux)

condition for 21 days. Mice were treated daily one hour after the light switch off with NAC 20 mg/kg, 40 mg/kg, 80 mg/kg and melatonin 1 mg/kg. NAC and melatonin were given orally from day 14 to day 21. On the day 22nd animals were sacrificed by cervical dislocation, and brain was micro-dissected to isolate SCN. Half SCN was isolated and bioenergetics was performed immediately and another half was stored at -80 °C until further use.

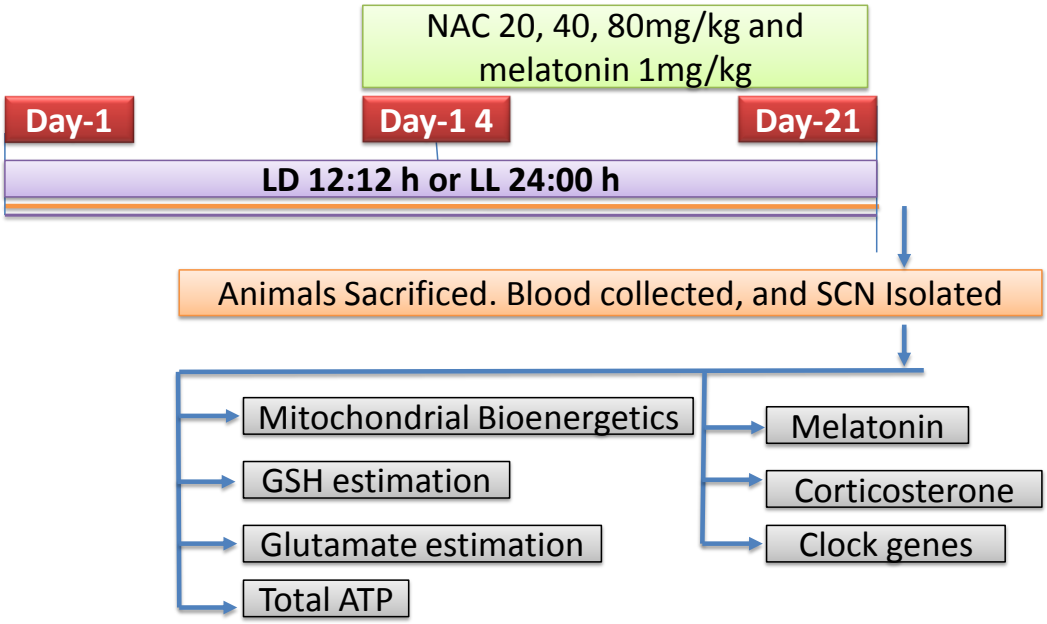


Figure 3.1 Study design

3.1.4 Locomotor activity measurement

Healthy adult mice (25-30 g) were singly housed in a polycarbonate cage (11.5 cm width × 21.5 cm length × 30 cm height) equipped with a running wheel in a light and climate-controlled chamber, described above. Wheel running locomotor activity was recorded with the help of a Chronobiology Kit (Stanford Software Systems, Santa Cruz, CA) in 6 min bin size. Both group animals were housed in different experimental chambers with their respective lighting condition. Daily

locomotor activity was recorded till the end of the experiment (three weeks) under LD, dim LL and all the treatments conditions. Food and water were available *ad libitum* in each cage. Actograms and rhythms were constructed, and the last ten days' data were analyzed using Clock Lab software (version 6.1.10) (Actimetrics, USA). The endogenous period of animals was quantified using a chi-square periodogram (Clocklab functions), and was further verified by following the protocols of (Kumar and Singaravel, 2014).

3.1.5 Isolation of mitochondria from brain suprachiasmatic nuclei (SCN)

Isolation of mitochondria from the brain (SCN) was done by differential centrifugal method (Berman and Hastings, 1999) with some slight modifications (Rajput & Krishnamurthy, 2022). Briefly, the brain was dissected and SCN quickly isolated keeping on ice. Three SCN were pooled to make sufficient tissue for mitochondria isolation and homogenized with 8 to 10 strokes in isolation buffer (215 mM mannitol, 75 mM sucrose, 0.1 %w/v bovine serum albumin, 20 mM HEPES buffer and 1 mM of EGTA in 100 ml of distilled water and pH adjusted 7.2 with KOH) using a glass Teflon tissue homogenizer (Thomas Scientific; USA). The homogenate was centrifuged at 1,300 X g for 5 min at 4°C. The supernatant was transferred to another tube and centrifuged at 14,000 X g for 10 min at 4°C to pellet mitochondria. The supernatant was discarded, and mitochondria were washed by resuspending the pellets in an isolation buffer without EGTA and centrifuging at 14,000 X g for 10 min. Mitochondria were resuspended in 1 ml of respiration buffer, and protein concentration was determined using the Lowry assay (Lowry *et al.*, 1951) on a microplate reader (Biotek; Gen5, USA).

3.1.5 Measurement of mitochondrial respiration

Mitochondrial respiration was assessed as previously described by (Samaiya and Krishnamurthy 2015; Rajput & Krishnamurthy, 2022) with a miniature Clark-type electrode in a sealed, thermostatically controlled chamber at 37^0C. Briefly, the mitochondria were added to the chamber respiratory states were evaluated with suitable substrates and inhibitors. Purified mitochondrial protein was suspended in respiration buffer in a final volume of 250 µL. State II respiration was initiated by addition of pyruvate/malate; (P/M), shows a basal rate of respiration. State III respiration was initiated by the addition of adenosine diphosphate; (ADP), the high level of oxygen utilization indicates that ADP is getting converted into ATP. State IV was measured by addition of oligomycin. State V was measured by addition of FCCP. This causing uncoupling of the ETC to ATP synthesis and represents the maximum rate of respiration. Rotenone was then added to shut down complex I mediated respiration. State V was determined by the addition of succinate. This is the maximum rate of respiration via complex II since FCCP is present in the system. The respiratory control rate (RCR) was calculated by dividing state III respiration (presence of ADP) to state IV respiration (absence of ADP).

3.1.6 Measurement of GSH

GSH was fluorometrically measured using ELISA assay Kit (ab65322, Abcam). Briefly all the materials equilibrated to room temperature and prepared reagents prior to use. All the standards, controls and samples were measured in triplicate. Reaction wells are setup, for standard 100 µL. Sample 50 µL was added to sample well and volume was cover with the lysis buffer. After that 2 µl of GST

reagent was added followed by 2 µl of MCB into each sample and standard well and mix the plate well and incubated at 37°C for 1 hour. Fluorescence was measured in a fluorescence plate reader at Ex./Em. = 360±20 nm/460±20 nm.

3.1.7 Measurement of glutamate

Glutamate was measured colorimetrically using ELISA assay Kit (KA1909). Briefly all reagents and samples were allowed to reach room temperature and mix thoroughly by gentle inversion before use. 100 µL of the diluents were then added to all the wells and covered with the adhesive foil and shake for 10 min at room temperature (20 - 25 °C) on a shaker at approx 600 rpm. Subsequently, derivatization was performed using 25 µL of the extracted standards, controls and samples into the appropriate wells of the reaction plate. 10 µL of NaOH was added into all wells followed by 50 µL of the equalizing reagent and 10 µL of the D-Reagent into all wells. Plate was then covered with adhesive foil and shakes again for 2 hours same as before. After incubation, 75 µL of the Q-Buffer was pipetted into all wells and shaken for 10 min at RT (20-25 °C) on a shaker approx 600 rpm 50 µL of the Glutamate Antiserum was then added into all wells and mix shortly. Covered the plate with adhesive foil and incubated for 15 - 20 hours (overnight) at 2-8°C. Foil was then removed and the contents of the wells were aspirated. The plate was washed 3 times by adding 300 µL of wash buffer, discarding the content and blotting dry each time by tapping the inverted plate on absorbent material. 100 µL of the Enzyme Conjugate was added into all wells and incubate for 30 min at RT (20-25°C) on a shaker (approx 600 rpm). 100 µL of stop solution were added to each well and the microtiter plate was shaken to ensure a homogeneous distribution of the solution. Absorbance was read within 10 minutes at 450 nm, using a microplate reader.

3.1.8 Measurement of total ATP

Total ATP content was measured using fluorometric ATP Assay Kit (ab83355, Abcam). Briefly, approximately 10 mg of tissue was washed in cold PBS, and then homogenized in 100 µL ice cold 2N perchloric acid (PCA) with a Dounce homogenizer (Thomas Scientific) using 10-15 passes. Samples were incubated on ice for 30-45 min, and then centrifuged at 13,000 g for 2 min at 4°C. Supernatant was transferred to a fresh tube, and volume was brought to 500 µL by adding ATP assay buffer. PCA was precipitated by adding 100 µl of ice cold 2M KOH and sample was vortexed. A 5 µL aliquot was used for testing using pH paper, and pH was adjusted between 6.5 to 8 by addition of 0.1 M KOH or PCA. Samples were centrifuge at 13,000 g for 15 min at 4 °C and supernatant was collected and measured fluorometrically at Ex/Em = 535/587 nm (Spark 10M, and Plate: Thermo Fisher Scientific-Nunclon 96 Flat Black with transparent bottom, NUN96fb). To calculate the dilution factor introduced by the deproteinization step (DDF), following formula was applied: DDF=Sample volume (PCA + Assay Buffer) + volume KOH /initial sample volume in PCA. Concentration of ATP (nmol/µL) in the test samples was calculated using the formula ATP concentration = (BV× D) ×DDF

Where:

B = Amount of ATP in the sample well calculated from standard curve (nmol).
V = Sample volume added in the sample wells (µL).
D = Sample dilution factor if sample is diluted to fit within the standard curve range (prior to reaction well set up)

3.1.9 Measurement of plasma melatonin level

Melatonin was measured using a mouse melatonin ELISA assay kit (KLM0120). Briefly, a standard curve was prepared by adding 50 µl prepared standards. Diluted samples were added to respective wells and 50 µl biotinylated melatonin antibody working solution was added to all the wells. The plate was then covered with a sealer and incubated for 45 minutes at 37°C. After incubation, the plate was aspirated and washed 4 times with diluted wash buffer (1X) and blot residual buffer by firmly tapping plate upside down on absorbent paper. Any liquid from the bottom outside of the microtiter wells wiped off as any residue can interfere in the reading step. Then 100µl Streptavidin: HRP Conjugate working solution was added to all wells and mixed well and again plate was covered with a sealer and incubated for 30 minutes at 37 °C. The plate was aspirated and washed again same as above. 90 µl of TMB substrate was added to all the wells and Incubate the plate at 37 °C for 10 minutes and then 50 µl of Stop solution were added to all wells. Absorbance was read at 450 nm with a microplate within 10-15 minutes after addition of Stop solution.

3.1.10 Estimation of corticosterone

Blood samples of each group (*n=4*) were centrifuged at 4°C for 10 min. Plasma was removed at different for corticosterone analysis. Hormone levels were measured in triplicate. The plasma CORT was quantified in a high-performance liquid chromatography (HPLC) with ultraviolet (Diode, UV) detector system (Agilent Technology, USA), according to (Woodward & Emery, 1987) with minor modification.

3.1.11 Quantification of clock genes

Total RNA was isolated from the SCN (n = 4) tissue using the TRIzol method (Invitrogen, CA) following the manufacturer's instructions. RNA quality was checked using a Nanodrop 2000 Spectrophotometer (Thermo Scientific) using a 260/280 ratio. RNA (1μg) was converted into cDNA using the Verso cDNA synthesis kit (Thermo Scientific). Quantitative real-time PCR (RTq-PCR) was performed on diluted cDNA samples with SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich), using the CFX Connect real-time PCR System (Bio-Rad, USA). Cycle conditions were used; 50℃ for 2 min, 95℃ for 10 min, and 40 cycles of 95℃ for 15s and 60℃ for 1 min. Relative gene expression of individual samples run in triplicate was calculated by comparison to a relative standard curve and standardized by comparison to the GAPDH signal. The primers were designed using Integrated DNA Technology (IDT, India). (Table 1) shows the amplification length and temperature. The primers were validated for linearity and specificity of amplification before the experiment. All reactions were performed in triplicate. The results are expressed relative to a GAPDH, used as an internal control.

Table 3.1. List of primers

Primer name	Primer sequences	Annealing temp. (Tm)^{0}C	Product size(bp)
Clock	F: ACAACGCACACATAGGCCTTC R: TGGTGGTGCCCTGTGATCTA	57.9 58.2	21 20
Bmal1	F: CGTGCTAAGGATGGCTGTTC R: CTTCCCTCGGTCACATCCTA	56.1 55.8	20 20
Per1	F:TGCACTTCGGGAGCTCAAACTTC R: GTCCATGGCACAAGGCTCACC	59.7 61.0	23 21
Per2	F: AACAAATCCACCGGC R: CTCCGGTGAGACTCC	49.6 51.1	15 15
Cry1	F: AACGTCCCGAGCTGTAGCGGT R: GACGCTTCCCACTGCTGAGGC	63.2 63.0	21 21
Cry2	F: TGCCTCTCCTGCCGCCTCTT R: TGCGGTCCCAGGGGATCTGG	63.8 64.2	20 20
GAPDH	F: ATCCACTGGTGCTGCCAAG R: CCGTTCAGCTCT GGGATGAC	58.1 57.9	19 20

3.1.12 Statistical analyses

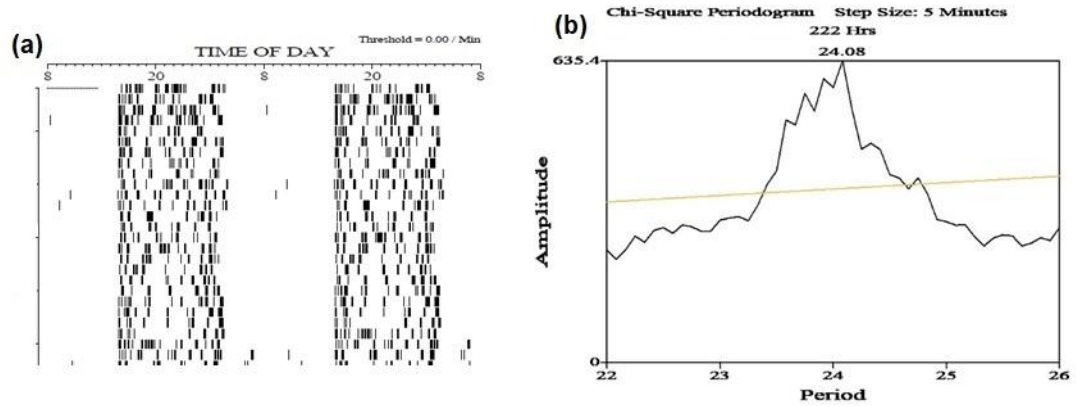
To determine significant changes in mitochondrial bioenergetics, CORT, GSH, Glutamate, Total ATP and clock genes, one-way analysis of variance (ANOVA) were performed. Data were considered as statistically significant if ($p \leq 0.05$), a post-hoc Tukey test for multiple comparisons was performed to evaluate the statistical difference between the different groups. Body weight and feed intake was assessed using two-way ANOVA followed by bonferoni post-test. Data analyses and figures were drawn by using Graph Pad Prism 5.0 (La Jolla, CA) and Microsoft Excel 2007 software. Data are reported as mean ± SD throughout the text. All experiments were performed as per existing institutional guidelines.

3.2 Result

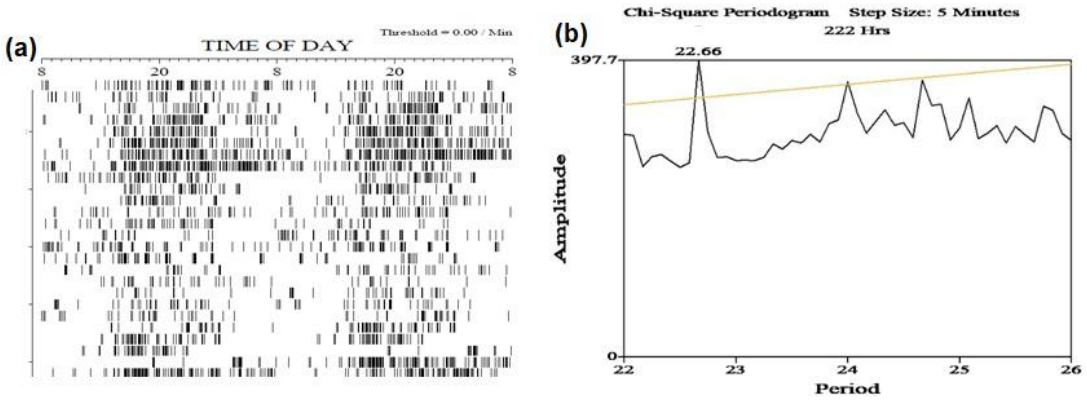
3.2.1 Locomotors activity dose dependently improved by NAC, hampered in continuous dim light condition

Actogram and periodograms from an animal held in LD 12:12 h and LL 24 h for 21 days (Fig 3.2 a and b) shows bimodality with the period length (τ=24.08). The bimodal rhythm of locomotor activity in mice was follows the circadian rhythm pattern. When mice were subjected to LL 24:00 h, the rhythm becomes unimodal free running, and period length spontaneously shortens (τ = 22.66 h; Fig 3.3 a and b). NAC improved the locomotors activity dose dependently. 20mg/kg (τ = 23.16 h; Fig 3.4 a and b), 40 mg/kg (τ = 23.50 h; Fig 3.5 a and b), 80 mg/kg (τ = 23.75 h; Fig 3.6 a and b), and melatonin (τ = 23.91 h; Fig 2.7 a and b).

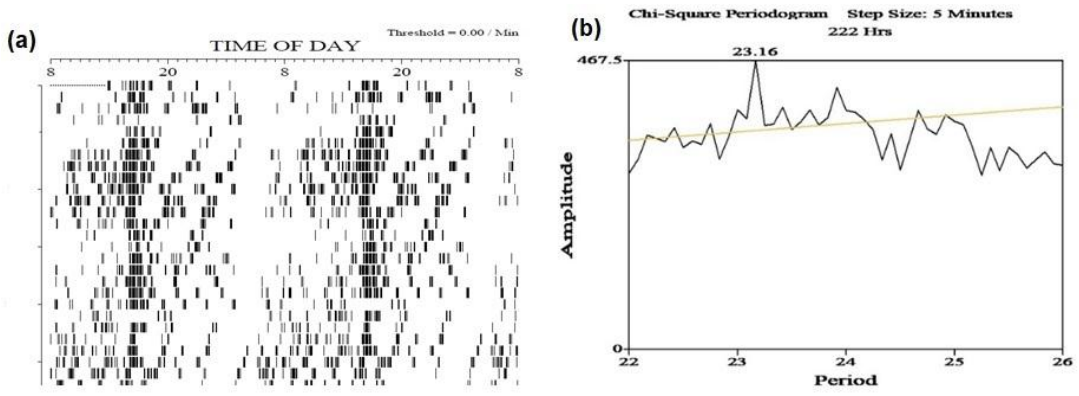
3.2 (*LD)*



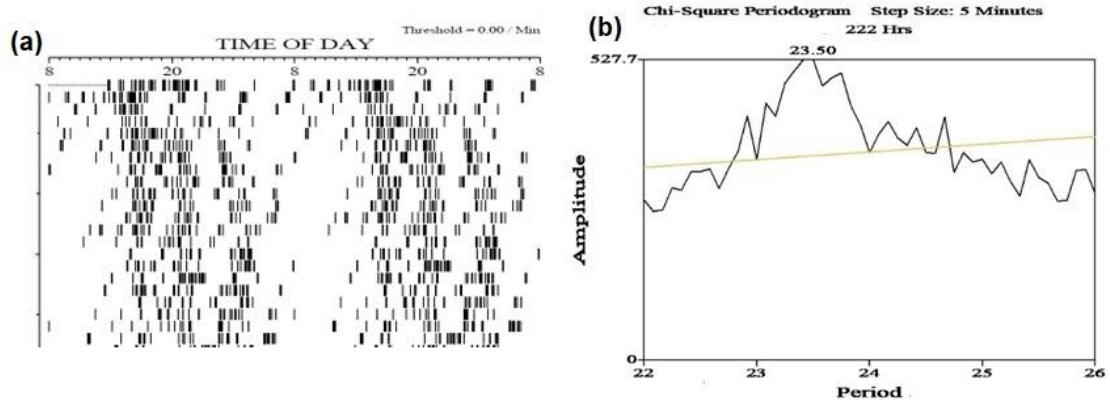
3.3 (LL)



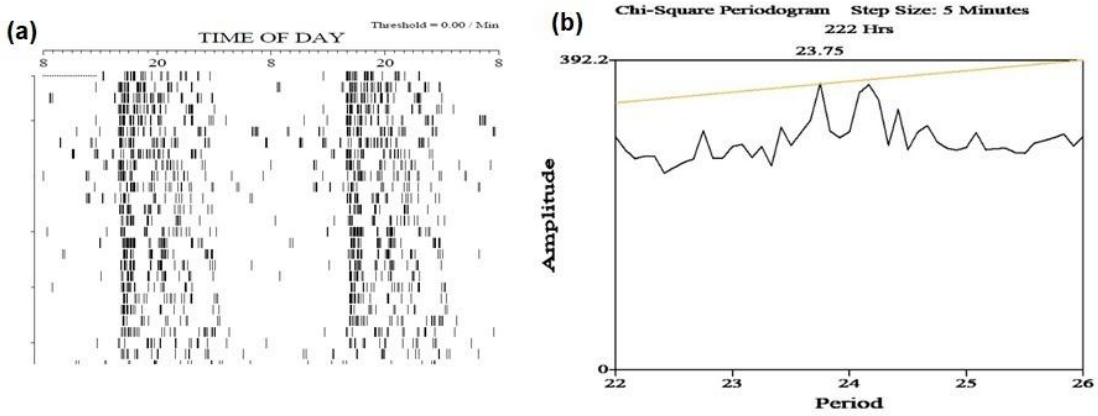
3.4 (LL+ NAC20 mg/kg)



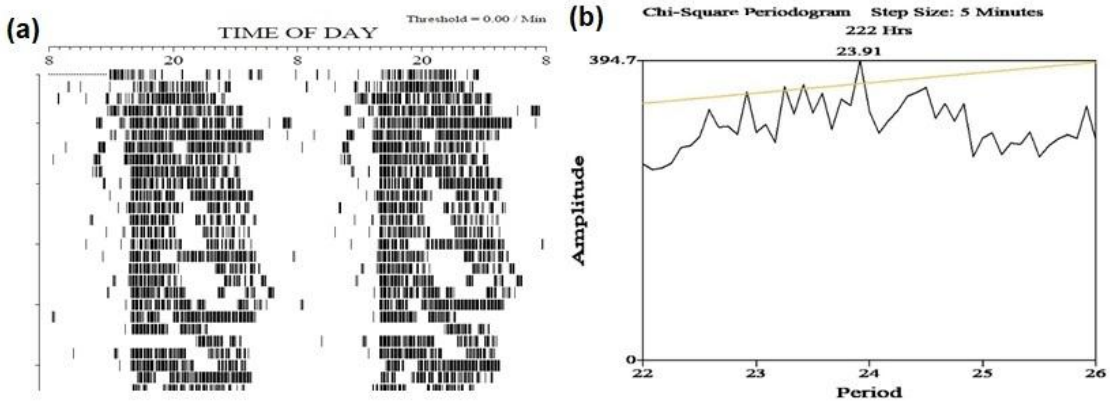
3.5 (LL+ NAC40mg/kg)



3.6 (LL+ NAC80mg/kg)



3.7 (LL+MEL1mg/kg)



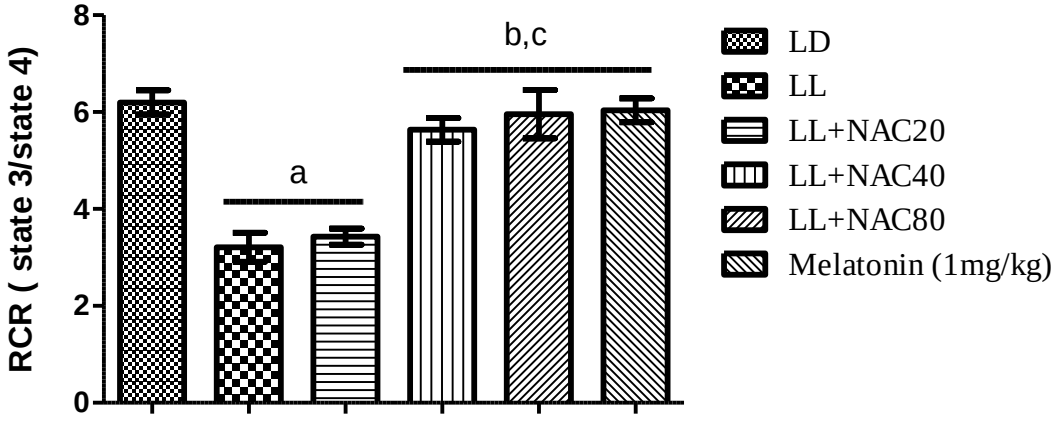
Wheel running locomotor activity rhythm is in mice is affected by lighting schedule. Double plotted actograms depicting the wheel running locomotor activity of mice held under (2.1; 12:12 h LD) (a) actogram (b) periodogram. (2.2; 24:00 h LL, (a) actogram and (b) periodograms (3) LL+ NAC20 mg/kg (a) actogram (b) periodogram (4) LL+ NAC40mg/kg (a) actogram (b) periodogram (5) LL+

NAC40mg/kg (a) actogram (b) periodogram and (6) LL+ Melatonin 1mg/kg (a) actogram (b) periodogram profiles respectively. All the parameters, wheel running rhythm, and periodograms are disrupted by chronic dim LL. Activity pattern under normal LD conditions was rhythmic and robust whereas, it became arrhythmic under dim LL.

3.2.1 NAC improved mitochondrial RCR and states in the SCN

Impairment in cellular respiration in LL results into reduced ATP production making cells prone to oxidative stress (Zamzami et al., 1997). The effect of various doses of NAC on LL exposed mice, RCR (state 3/state 4 respirations) and changes in oxygen consumption in different states of mitochondrial respiration in SCN is shown in Fig.3.8 a and b, respectively. Mitochondrial bioenergetics was hampered in mice exposed to LL. One-way ANOVA revealed that NAC 40 mg and 80 mg/kg was significantly improved RCR ($F_{5, 23} = 82.64$, $p < 0.05$, Fig 3.8 a), and different states of mitochondrial respiration, namely, state 2 ($F_{5, 23} = 16.456$, $p < 0.05$), state 3 ($F_{5, 23} = 35.67$, $p < 0.05$), state 4 ($F_{5, 23} = 18.96$, $p < 0.05$), state 5 complex I ($F_{5, 23} = 28.63$, $p < 0.05$), and state 5 complex II respiration ($F_{5, 23} = 5.561$, $p < 0.05$, Fig 3.8 b), but 20 mg/ kg was not effective to restore the mitochondrial function.

(a)



(b)

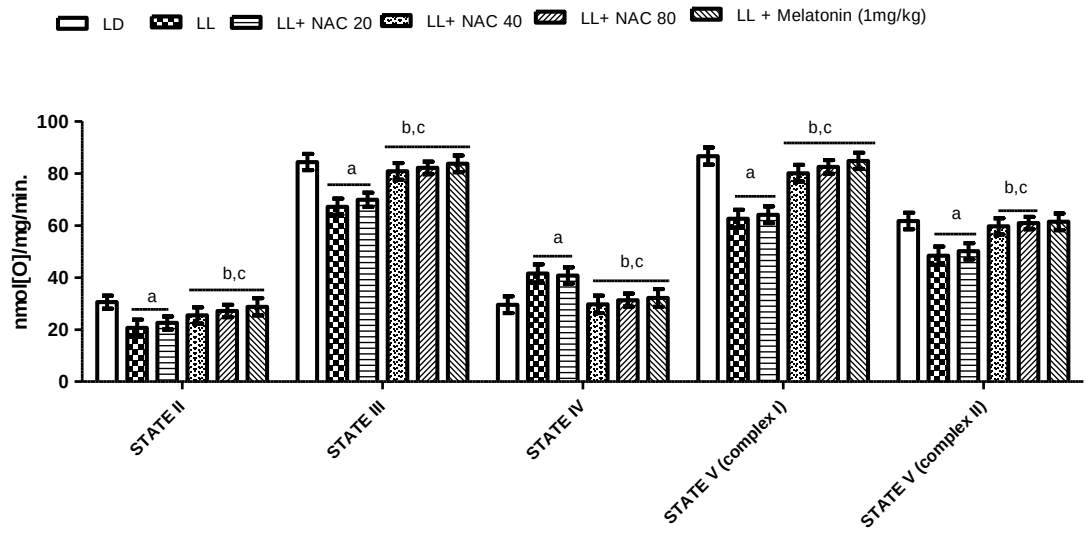
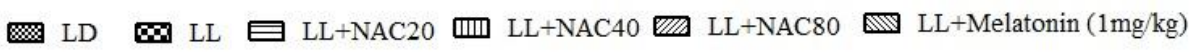


Figure 3.8 (a) Respiratory control ratio (RCR) and (b) states in SCN mitochondria. Bars represent groups mean ± SD (n=4). $^{a}p \leq 0.05$ compared to LD, $^{b}p \leq 0.05$ compared to LL, $^{c}p<0.05$ compared to LL+NAC20. One-way ANOVA followed by Tucky posttest.

3.2.2 NAC dose dependently improved the GSH and glutamate level in the SCN

We measured the amount of GSH and glutamate in the SCN under LD, LL and NAC treatment regimen to determine the anti-oxidants level. One-way ANOVA showed significant dose-dependent increases in GSH ($F_{5, 23}= 173.5$, $p \leq 0.05$, Fig 3.9 a) and significant dose dependent reduction in glutamate ($F_{5, 23} = 223$, $p \leq 0.05$, Fig 3.9 b) with all the three doses of NAC.



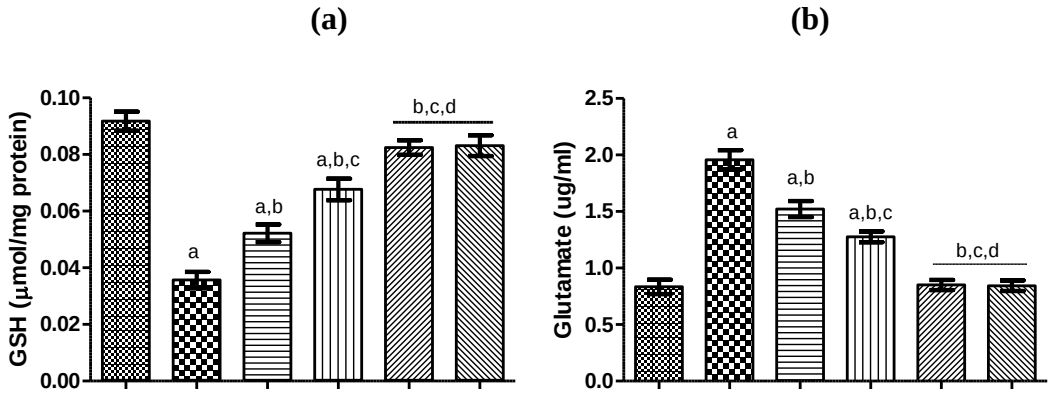


Figure 3.9 (a) GSH and (b) Glutamate level in the SCN. Bars represent groups mean±SD (n=4). [a]p<0.05 compared to LD, [b]p<0.05 compared to LL, [c]p<0.05 compared to LL+NAC20 and [d]p<0.05 compared to LL+NAC40. One-way ANOVA followed by Tucky post hoc test.

3.2.3 NAC dose dependently improved total ATP content

Total ATP was fluorimetrically measured in SCN. Mice exposed to continuous light were found to have compromised with their ATP level. One-way ANOVA showed significant dose-dependent improvement in ATP level ($F_{5, 23} = 42.57$, $p \leq 0.05$, Fig 3.10) with all the three doses of NAC.

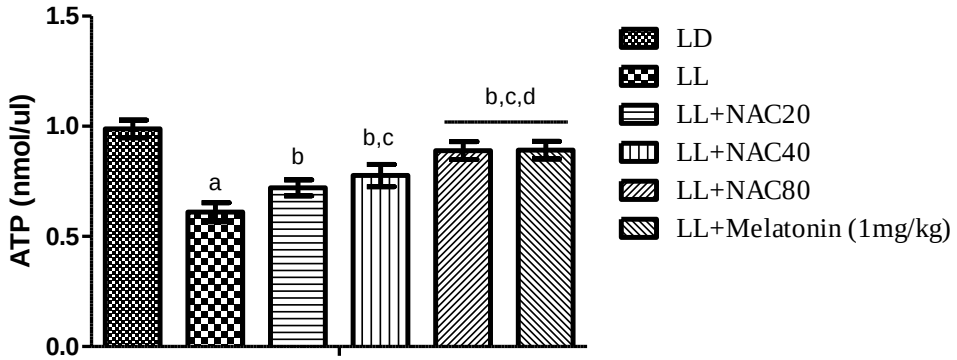


Figure 3.10. ATP level in the SCN. Bars represent groups mean ± SD (n = 4). [a]p<0.05 compared to LD, [b]p<0.05 compared to LL, [c]p<0.05 compared to LL+NAC20 and [d]p<0.05 compared to LL+NAC40. One-way ANOVA followed by Tucky posttest.

3.2.4 NAC improved melatonin production

Changes in melatonin level in LL 24:00 h exposed mice are shown in Fig 3.11. One-way ANOVA revealed that there was significant decrease ($p \leq 0.001$) in melatonin was found in LL mice and that was attenuated by the NAC 20, 40 and NAC 80mg/kg ($F_{5, 23} = 64.99$, $p \leq 0.05$). The NAC 20 was less effective than two others doses of NAC.

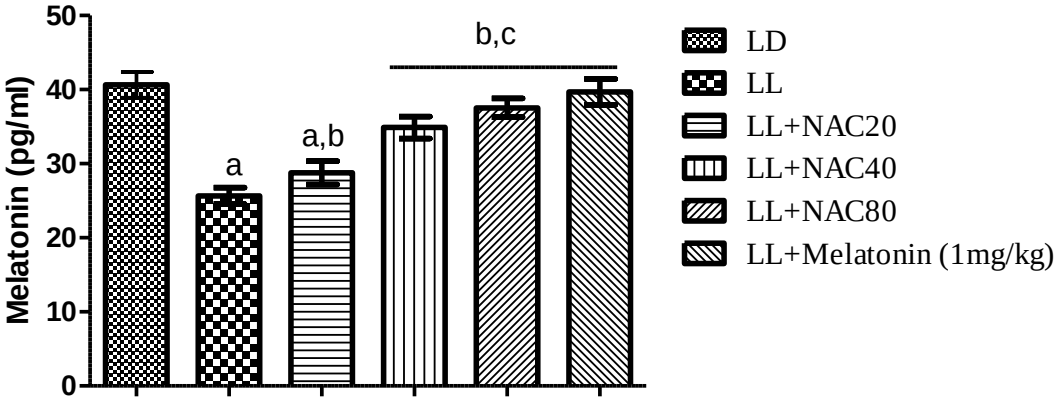


Figure 3.11 Blood plasma melatonin levels. Bars represent groups mean±SD (n = 4). [a]$p<0.05$ compared to LD, [b]$p<0.05$ compared to LL, [c]$p<0.05$ compared to LL+ NAC 20. One-way ANOVA followed by Tukey post hoc test.

3.2.5 NAC decreases the CORT level in LL exposed mice

Changes in CORT level in LL 24:00 h exposed mice are shown in Fig 3.12. One-way ANOVA revealed that there were significant increases ($p \leq 0.001$) in CORT level in LL mice. Post-test revealed that NAC 40 and NAC 80 attenuated the CORT ($F_{5, 23} = 52.56$, $p \leq 0.05$) at same extent and NAC 20 was less effective ($p \leq 0.05$) against alleviated CORT.

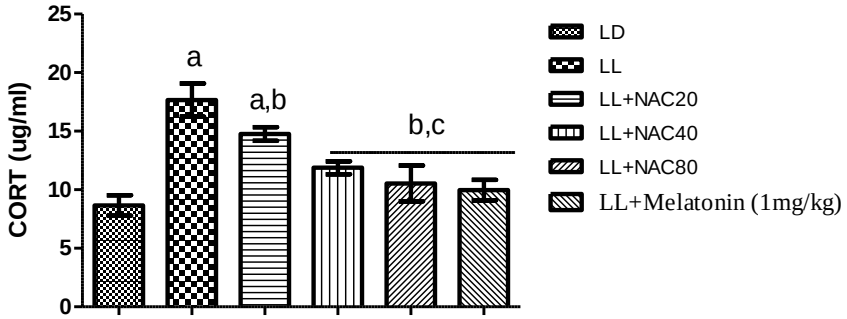
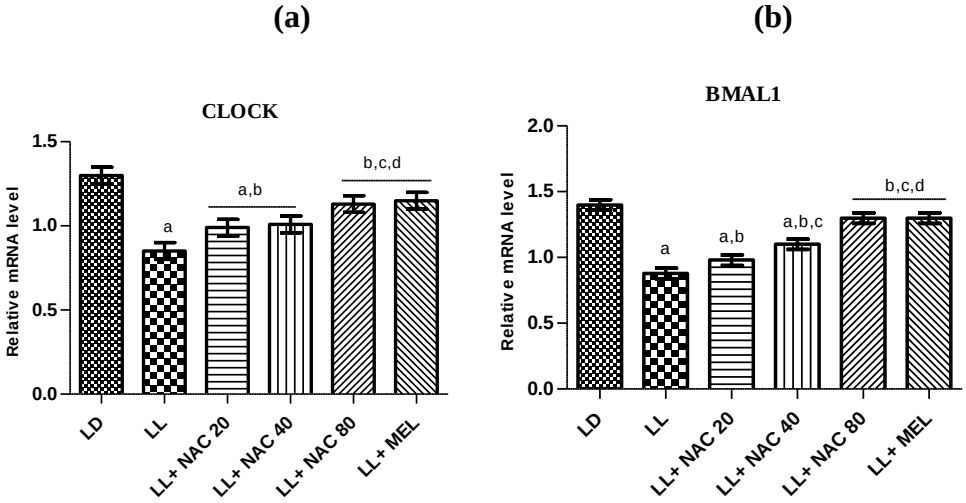


Figure 3.12 Plasma CORT level. Bars represent groups mean ± SD (n=4). [a]$p < 0.05$ compared to LD, [b]$p<0.05$ compared to LL, [c]$p<0.05$ compared to LL+ NAC 20. One-way ANOVA followed by tukey post hoc test.

3.2.6 NAC dose dependently upregulated the clock genes

Expression of core clock genes was quantified at the maximum responsive zone, ZT/CT15 (during the active period of mice), where maximum RCR and CORT levels were observed from the chapter 2. Result showed continuous light exposure significantly ($p \leq 0.05$) down regulated the levels of all the clock genes (by 1.3 to 1.4-fold). We determined the effects of NAC at different doses on clock gene expression. One-way ANOVA revealed significant dose dependent effects of NAC on the expression levels of core clock genes *Clock,* ($F\ 5, 23= 38.81$, $p \leq 0.05$, Fig 3.13a), *Bmal1* ($F\ 5, 23= 106.2$, $p \leq 0.05$, Fig 3.13b), *Cry1* ($F\ 5, 23= 337.7$, $p \leq 0.05$, Fig 3.13c), *Cry2* ($F\ 5, 23= 126.1$, $p \leq 0.05$, Fig 3.13d), *Per1* ($F\ 5, 23= 141$, $p \leq 0.05$, Fig 3.13e), *and Per2* ($F\ 5, 23= 155$, $p \leq 0.05$, Fig 3.13f) in the SCN.



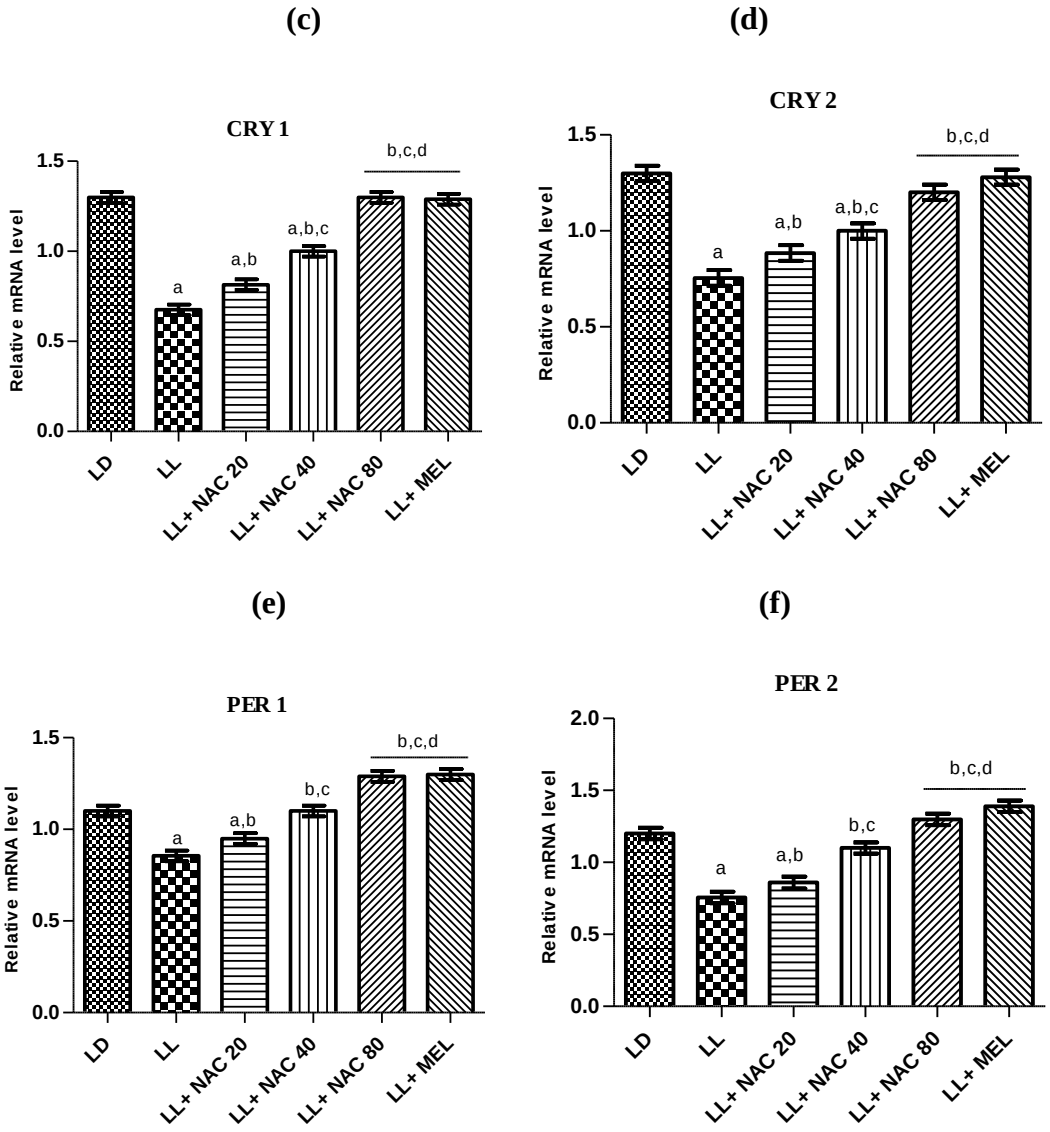


Figure 3.13 Relative mRNA expression of core clock genes in the SCN. (a) *Clock*, (b) *Bmal 1*, (c) *Cry 1*, (d) *Cry 2*, (e) *Per 1*, (f) *Per 2*. Bars represent groups mean±SD (n=4). $^{a}p<0.05$ compared to LD, $^{b}p<0.05$ compared to LL, $^{c}p<0.05$ compared to LL+NAC20 and $^{d}p<0.05$ compared to LL+NAC40. One-way ANOVA followed by Tukey post hoc test.

3.3 Discussion

This study demonstrates that mitochondrial function in the SCN decreased after chronic dim light exposure in mice. NAC at different dose (20, 40 and 80 mg/kg) and melatonin both has improved the disrupted rhythm by improving SCN mitochondrial function. Though NAC 20 mg/kg was not able to restore the altered

mitochondrial functional in LL exposed mice. While NAC 40mg/kg, and 80mg/kg as well as melatonin improved mitochondrial RCR and different states of mitochondrial respiration at the control level, substantiating that NAC similar to melatonin regulates the mitochondrial functions in the SCN. Further, in our previous finding, we have reported that mitochondrial function follows the rhythmic phenomena under LD conditions, and shows arrhythmicity under chronic dim LL.

To our knowledge there are no reports until now showing that mitochondrial respiration is rhythmic in principle clock (SCN). Only one report in whole brain homogenate shows rhythmic mitochondrial function (Simon et al., 2003). Ours is the first study to demonstrate that NAC improves mitochondrial bioenergetics in the disrupted circadian clock (SCN) due to chronic dim LL. Both NAC and melatonin improved mitochondrial function by the same mechanism i.e., through their antioxidation properties by serving as free radical scavengers. Chronic LL exposure increases free radical load in the brain results in reduced oxygen level which can cause nerve damage and neuronal cell death. NAC increases the GSH levels (main neurotransmitter responsible for proper brain functioning) (Wright., et al., 2015) and other natural antioxidants, causing increase in the oxygen levels to the tissues. Our result is supported by from the afore-mentioned study showing that NAC improved mitochondrial bioenergetics and total ATP in the SCN.

In addition to disturbed mitochondrial functions, chronic dim LL resulted in disturbed CORT rhythm. This disturbed CORT has been reported to be one of the reasons for stress, and altered core clock genes (Rajput et al., 2023). In the present study, NAC and melatonin improved principal clock function by improving CORT and endogenous melatonin. Melatonin is a neurotransmitter like compound and is the

main marker for circadian rhythm (Claustrat., et al., 1995), NAC improves endogenous melatonin level that plays essential role in regulating key circadian rhythms as well as mitochondrial function. Such protective effect of NAC may be related to its restoration of mitochondrial function, report suggests that NAC has ability to directly scavenge mitochondrial free radicals (Dekhuijzen., 2004). Another mechanism of the NAC may be that it may acts as a precursor of the principal thiol present in the cell, the tripeptide glutathione. This theory is supported by several studies describing that GSH is essential in maintaining protein thiol groups, membrane integrity and ATP synthesis (Meister, A., 1995; Ballatori, N., 2009; Miquel et al., 1995).

In our present study, chronic dim LL resulted disturbed mitochondrial function, GSH, total ATP CORT levels, suppressed melatonin and different clock gene levels in the SCN. This decrease in the GSH levels may have resulted in altered mitochondrial functions. Since, GSH is a physiological reservoir of glutamate, which is a principal excitatory neurotransmitter of the brain and participates in a various physiological and pathological processes, including learning and memory. Any disturbances in GSH levels may influence the above-mentioned functions. Thus, our present results together with afore-mentioned studies suggest that NAC may reduce both excitotoxicity and oxidative stress through its actions on glutamate reuptake (Koga et al., 2011). NAC also has the capacity to modulate glutamate-induced toxicity. The elevated synaptic glutamate observed in excitotoxic states leads to dysregulated glutamate receptor activity. Moreover, our previous study data suggests that mitochondrial dysfunction is associated with chronic dim light exposure disrupted rhythm. It is more likely that other factors such as, decreased total ATP

content and increased glutamate in the SCN may be responsible for decreased mitochondrial respiration and cytosolic oxidative stress, and are reflected as decreased GSH levels in the brain. Administration of NAC and melatonin restored GSH levels dose dependently in the SCN, and resulted in beneficial effects on mitochondrial function. Such beneficial effect of NAC on mitochondrial function seems to be associated with its ability to increased glutathione level along with maintaining glutamate level in the SCN, as shown by our data as well as several other reports (Rajput et al., 2023; Atkuri et al., 2007). Our idea is supported by several studies describing that NAC increases the pool of glutathione available in the cell for NADPH-dependent redox reactions. In turn, increased glutathione should produce additional opportunities for production of NADPH via the hexose monophosphate shunt (Winkler, and Solomon, 1986). We also found that treatment with NAC resulted in a significantly dose dependent improvement of melatonin and CORT (an indicator of disturbed rhythm) suggesting that NAC is not only improving mitochondrial function but also capable of protecting the cause of disturbed rhythm. This is in agreement with a recent study describing the protective effect of NAC on sleep (Bushana et al., 2023). On the whole, our finding suggest that NAC treatment not only significantly restored mitochondrial GSH levels but also protect brain mitochondria against oxidative damage, but also caused elevation in the levels of total ATP content. Also, NAC can directly scavenge free radicals for example hydroxyl radicals and hypochlorous radicals (Aruoma et al., 1989), ultimately causing beneficial effects on mitochondrial functions.

3.4 Summary

In conclusion, our results indicate that chronic dim light exposure decreased the GSH level and mitochondrial function in the SCN. NAC dose dependently improved mitochondrial function by improving GSH, total ATP, and maintaining the glutamate level and down regulation of clock gene caused by LL exposure. The protective effect of NAC may be related to its restoration of GSH levels in the brain and potential direct scavenging effects on free radicals. These results are consistent with maintaining glutamate level and improving total ATP, CORT, and Melatonin. NAC, a relatively inexpensive, nontoxic, natural antioxidant, nutrient supplement and commonly available agent and is useful to improve mitochondrial function altered by LL exposure.