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Impact of Circadian Disruption on CART mRNA Expression in Nucleus Accumbens: Insights from Constant Light Exposure and Wheel-Running Activity in Rats

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Abstract

Background: *Cocaine Amphetamine Related Transcript (CART) is expressed in the nucleus accumbens (NAc), a region that serves as extra-SCN circadian oscillators. This study examined whether CART mRNA in the NAc follows a diurnal rhythm and how circadian disruption affects its expression.*

Methods: *Rats (n=28) were monitored for 30 days in a 12:12 light-dark cycle (LD). On day 30, 14 rats were sacrificed (7 in the morning, 7 in the evening). The remaining rats (n=14) were exposed to constant light (LL) from day 30 to day 60 and then sacrificed (7 in the morning, 7 in the evening). CART mRNA levels were measured via real-time PCR.*

Results: *In the LD group, CART mRNA was higher in the evening than in the morning (P-value<0.001). In the LL group, evening levels remained elevated but were reduced compared to LD (P-value<0.01). Morning mRNA levels in the LL group were lower than in LD (P-value<0.05).*

Conclusions: *Constant light exposure downregulated CART mRNA, suggesting impaired circadian regulation in the NAc. This disruption may alter neurochemical signaling, affecting behavioral and mood-related processes. The findings highlight CART's role in circadian coordination and its susceptibility to rhythm disturbances, which could influence mood and activity patterns.*

Keywords: Neuropeptide CART, Circadian rhythm, Nucleus accumbens, Wheel running.
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Introduction

The hypothalamus of ovine was originally used by Spiess et al. in 1981 to isolate and sequence the Cocaine Amphetamine Related Transcript (CART) which was initially named somatostatin-like peptide [1]. About 14 years later, however, it was renamed CART once Douglass et al. realized that this peptide was upregulated in the rat brain after administration of cocaine and amphetamine [2]. CART peptides are suggested to be neuropeptides since they are expressed exclusively in neurons in the brain [3]. There is much evidence which suggests that CART neuropeptides act through G protein-coupled receptors, particularly through the Gi/o pathway [4]. The interaction between CART and its receptor(s) leads to a variety of functions. Among the best-known functions modulation of mesolimbic dopamine related reward and reinforcement [5, 6], maintenance of energy homeostasis [6], regulation of the hunger-satiety cycle [7], modulation of the HPA axis [8], induction of arousal and alertness [9], and finally involvement in the cycle of sleep-wake regulation [7, 9] could be mentioned. Both CART peptides and its mRNA are broadly expressed in the central nervous system [10], while CART mRNA is one of the most plentiful mRNAs in the rat NAc [11]. Circadian rhythms are internal timekeeping systems, synchronized by environmental cues like light, feeding, and activity, to regulate behavioral and physiological functions. In mammals, the central clock in the suprachiasmatic nucleus (SCN) coordinates peripheral clocks across the body to maintain metabolic and temporal balance [12]. Although the SCN was traditionally considered the sole central pacemaker in the mammalian brain, emerging evidence indicates the presence of multiple extra-SCN circadian oscillators in the brain, including the NAc, which drive rhythms in physiology and behavior [13, 14]. NAc acts as a circadian oscillator, integrating sensory and circadian information to regulate motivated behaviors, with diurnal variations in neuronal activity, dopamine signaling, and molecular clock functions [15, 16]. Research conducted on rodent models and human post-mortem samples demonstrates substantial circadian rhythmicity within the NAc. Using the PER2:LUC mouse model, ex vivo bioluminescent rhythms were detected in the NAc, supporting findings of diurnal variations in molecular clock gene mRNA and protein expression in the striatum [17, 18]. RNA-sequencing analyses of human post-mortem NAc tissue reveal robust rhythms in canonical clock genes (e.g., Bmal1, Npas2, Period, Cryptochrome, and Rev-erb α) and enrichment of Circadian Rhythm Signaling pathways among the top rhythmic genes. Functional studies primarily focus on medium spiny neurons (MSNs) and their subtypes, but recent findings also highlight robust circadian rhythms in NAc astrocytes [19].

Having established that the NAc is a site enriched in CART, many studies have been conducted to understand the origin, function, biological properties, and signaling of the intra-NAc of CART peptide. In general, these studies fall into

three categories. A number of studies aim to identify the projection of CART -containing neurons in the NAc. For example, Hubert et al. (2010) demonstrated that the substantia nigra pars reticulata and ventral pallidum are nuclei that receive CART-containing accumbens efferent [20]. Another part of the studies investigates the changes in the levels of CART and its mRNA in the NAc after treatments/interventions and it also examines the possible mechanisms involved [21-23]. Another group of studies evaluates the effects of intra-NAc administration of the peptide CART and/or its antibody following treatments/interventions [24-27]. Despite the large amount of research being conducted for better understanding of the function of CART in the NAc and other tissues, our knowledge in this area is still in its infancy. However, in spite of our limited knowledge, it is known that CART plays such an important role in biological processes and homeostasis that the body keeps its quantity stable throughout life. Although the average production of the peptide CART remains stable whole life,[28] it has been found that this production follows the diurnal circadian rhythm. Reports by Vicentic et al. (2004) suggest that CART levels in peripheral blood exhibit a diurnal rhythm, without clarification of the sources from which circulating CART are derived [29]. Studies have shown that not only does the expression of CART follow the circadian rhythm, but it is influenced by changes of it. For instance, as circadian rhythm disruptors, sleep deprivation of 10 days in rats noticeably suppressed levels of mRNA for the CART gene both in the arcuate nucleus as well as dorsal hypothalamus [30].

The design of the current study was based on the findings of previous studies that showed the property of rhythmicity,[29] the effect and also the influenceability of the peptide CART on the circadian rhythm [30]. The aim of the present study was to determine whether the peptide CART follows a diurnal pattern, specifically in the NAc as CART enriched site, involved in the coordination of the circadian rhythm. Additionally, the study aimed to evaluate how circadian rhythm disruption influences CART mRNA expression in the NAc, providing insights into potential implications for circadian-related disorders and their clinical management.

Materials and Methods

To carry out this experiment, 28 adult male Wistar rats, from 250 to 300 grams, were provided by Animal Centre of Faculty of Medicine in Shahroud University of Medical Sciences. After being transferred to the laboratory, all rats were left undisturbed for one week to acclimate to laboratory conditions. The animals were placed under standard laboratory conditions with room temperature of 21°C and humidity of 50-45% and were provided with desirable amount of food and water. There was also a 12-hour light/dark cycle for the normal light/dark group (LD) (lights were turned on at 7:00 am and turned off at 7:00 pm) and 24-hour continuous light for the disturbed circadian rhythm group (LL). Rats were maintained according to the guidelines of the National Institutes of Health in the United States. Ethics code is IR.SHMU.REC.1398.058.

To determine the activity/rest pattern, animals were housed in an individual running wheel cage [31]. The baseline activity/rest of the animals was monitored continuously for 30 days on a 12:12 light/dark cycle. Data was collected by a home-made digitized system then visualized and plotted using ImageJ plug-in "ActogramJ". Results revealed that none of the rats needed to be excluded from the study since no one showed abnormal pattern of activity/rest. Therefore, all subjects (n=28) were included in study. Then, half of the total included rats (n=14) were decapitated between Zeitgeber time (ZT) 0–2 (where ZT 0=lights-on and ZT 12=lights-off) for morning groups and ZT 12-14 for evening groups. Animals decapitated [32] in the morning were grouped as (LD, morning) and others decapitated in the evening as (LD, evening). Afterward, to model circadian rhythm disruption, the remaining animals (n=14) were exposed to constant light (LL) for 30 consecutive days/nights [33] (from day 31 to 60). On 60th experimental day, half of LL animals (n=7) were decapitated in the morning (LL, Morning) and the other half (n=7) in the evening (LL, Evening). In fact, half of the animals in the LL and LD groups were decapitated in the morning and the other half in the evening to determine the mRNA expression of CART at two time points during the day. Thus, each group (LL and LD) was divided into two groups (morning and evening) based on the time of brain sampling. Four groups (n=7 per group) as follows:

1. (LD, Morning): Rats were left undisturbed for 30 days and remained on a 12/12-h LD cycle. On the 30th day, the animals were decapitated at 7 a.m.

2. (LD, Evening): Rats were left undisturbed for 30 days and remained on a 12/12-h LD cycle. On the 30th day, the animals were decapitated at 7 p.m.

3. (LL, Morning): After 30 days on a 12/12-h LD cycle, the rats were kept in constant light (LL) for 30 days. On day 60, the animals were decapitated at 7 a.m.

4. (LL, Evening): After 30 days of 12/12-h LD cycle, rats were kept in constant light (LL) for 30 days. On the 60th day, the subjects were decapitated at 7 p.m.

The setup of the experiment is shown in Figure 1.

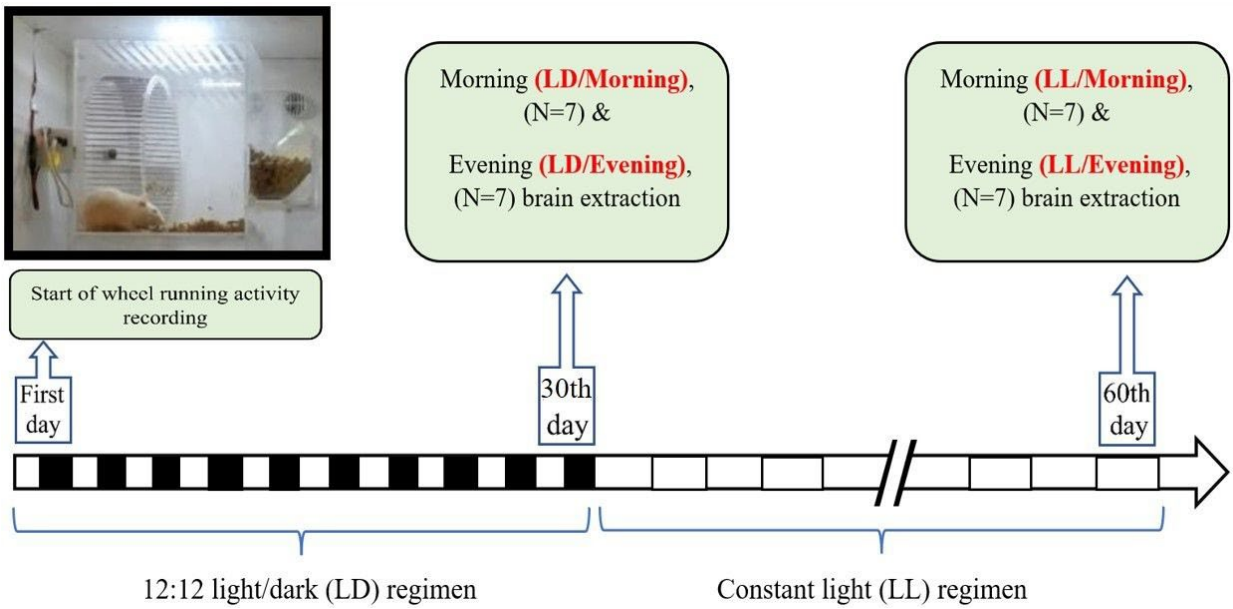

Figure 1. The setup of the experiment (created with biorender.com). Animals were housed individually with voluntary wheel-running devices to monitor baseline activity/rest patterns for 30 days under a 12:12 light/dark (LD) cycle. Data collection and visualization were performed using a digitized system. On the 30th day, 14 rats were randomly assigned to two groups based on decapitation timing: LD Morning (ZT 0–2): Animals were decapitated between 7:00–9:00 a.m.LD Evening (ZT 12–14): Animals were decapitated between 7:00–9:00 p.m.

The remaining 14 rats were exposed to constant light (LL) conditions for 30 consecutive days (Day 31–60) to induce circadian rhythm disruption. On the 60th day the LL animals were divided into two groups based on decapitation timing: LL Morning (ZT 0–2): Decapitated between 7:00–9:00 a.m.LL Evening (ZT 12–14): Decapitated between 7:00–9:00 p.m.

In order to anesthetize animals, Sodium-pentobarbital, 3.5%, 3 ml/kg, was injected intraperitoneally. Using a standard small guillotine, five minutes right after the injection, subject rats were decapitated. Following that, the rat total brain tissue was first extracted and then immersed in extremely cold PBS (phosphate-buffered-saline). Afterward, the tissue was put on a sterile dish that was placed on ice. In order to separate hemispheres from each other, a sagittal cut was first performed and after that NAc were extracted from them. Shortly, a coronal cut of 1 mm was performed in front of the forceps minor of the corpus callosum so as to isolate PFC (cut 1) in accordance with Paxinos and Watson rat brain atlas (2007). Another cut (cut 2) was also done just in front of column of fornix. The area which is between cut 1 and 2 contains NAc. By using a proper device, the NAc was first punched and then isolated. This isolated tissue was placed immediately in individual micro-tubes and immersed quickly in some liquid nitrogen. In final step, after some time, microtubes were transferred to a freezer with -80 ˚C [34].

RNeasy Lipid Tissue Mini Kit (Qiagen) carried out total RNA from samples extraction in accordance with manufacturer's protocol. By a Pico drop Microliter Spectrometer the quantification of extracted RNA was performed and its integrity was also confirmed using gel electrophoresis (1.2% agarose; Gibco/ BRL). 1 µg of extracted RNA was actually reverse-transcribed into first-strand cDNA so as to synthesize complementary DNA (cDNA) by using Qiagen (QuantiTect Reverse Transcription Kit) in a final volume (20 µl) and then was kept at -20 for further processes [35].

Target gene expression was normalized by beta-actin which acted as the internal standard gene. Both Oligonucleotide primers of real-time PCR amplification of CART as well as beta-actin genes were provided from Qiagen company primer bank.

In all real-time PCR reactions 2 µl cDNA was utilized in a final volume of 25µl. Power SYBR-Green PCR Master Mix (Life Technologies) was used to complete reactions on StepOnePlus Real-Time PCR System (Applied Biosystems). CART primer pairs' optimized annealing temperature was 61.5. As it was reported in previous sensitization studies, by using beta-actin acting as the reference gene founded data was normalized. The samples were then examined in duplicate and the mean data were also calculated for the following steps. Samples' target gene expression was quantified using standard curve methodology. CART gene's quantity measurement in each sample was first done by defining the cycle where the sample fluorescence met a predetermined threshold significantly above the background. Following that the cycle number was referred to a standard curve which existed in every run of the reaction. The collected data were normalized against beta-actin (the housekeeping gene) for all of observed groups. PCR products specificity was verified by reaching a single peak in melt curve. In order to check the length validation, PCR products were also visualized on 2.5 per cent agarose gel [36].

Statistical analysis data were analyzed using statistic software (Graph Pad version 10). The mean values were calculated in each experimental group. The data were also expressed as: SEM±means (standard error of the mean). A one-way analysis of variance (ANOVA) was used to compare the differences among groups, which was then followed by a post hoc test of Tukey. A value with P-value<0.05 was taken as statistically significant. In order to evaluate noticeable distinctions in relative expression levels between LD and LL group's gene expression experiments data were analyzed using REST (Relative Expression Software Tool- XL version 2). Pair-wise fixed reallocation randomization test is used by the software to determine the importance of results. Gene normalization and quantification alike are also performed at the same time using REST software.

Results

Rest-activity pattern was recorded for 60 consecutive days using wheel running placed on each animal cage. Data was sampled with 3 minutes' intervals. A double plot actogram was then plotted using ImageJ plug-in "ActogramJ" program. The rats whose running-wheel activity rhythms are shown in Figure 2 were exposed to a light–dark cycle for 30 days (LD) and to constant light (250 lux) for 30 days (LL). In the first 30 days' animals exhibited synchronized rest-activity rhythms with light-dark cycle and, in the next 30 days under constant light, free-running rhythms were first shown and then the rhythms deteriorated.

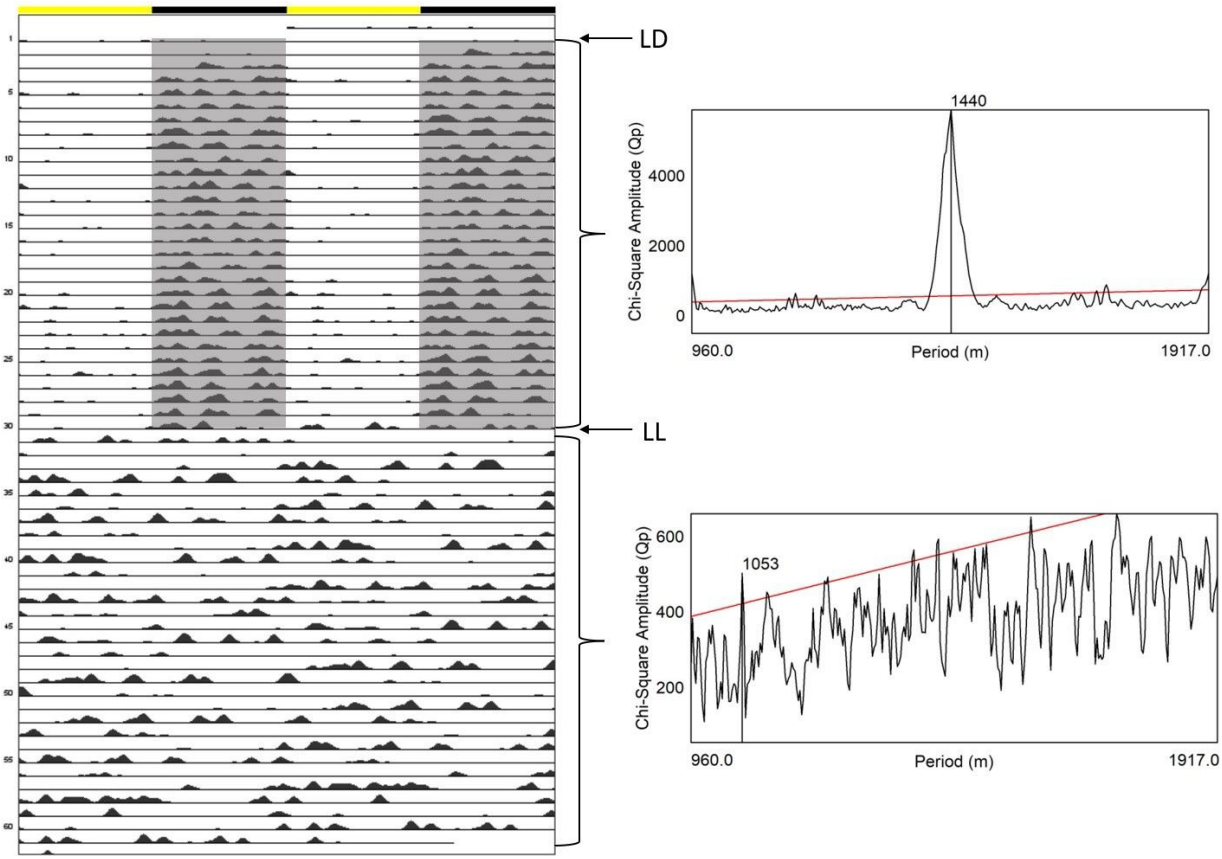

Figure 2. Representative double-plotted actograms of circadian rhythms of wheel running in rat maintained under LD (30 days) and LL (30 days) and corresponding chi square periodogram analyses. Each line of the double-plotted actograms represents 48 h. Dark bars indicate the dark hours and yellow bars indicate the light hours of the light/dark cycle. Dark shadow rectangle indicates aligned phase. chi square periodogram analysis of the LL animals confirmed the absence of significant rhythmic components with a circadian period using a 95% confidence threshold. The circadian rhythm LD rat has a prominent rhythm with a period of 1440 minutes (24 hour).

Statistical analysis of the mRNA level of the gene CART in the NAc region of the brain showed that the level in the LD group was significantly higher in the evening than in the morning (P-value>0.001) (Figure 3. A), similarly, mRNA level of the gene CART was dramatically higher in the animals of the LL group in the evening than in the morning (P-value>0.01) (Figure 3. B). Further comparisons were made between the animals in the LD group and LL group in the morning and evening. Statistical analysis showed that mRNA level of the CART gene measured in the morning in the LL group was significantly lower than in LD group (P-value>0.05) (Figure 3. C). In addition, the result of the evening examination

of the mRNA level of the CART gene was significantly lower in the NAc of the LL animals than in the LD group (P-value>0.01) (Figure 3. D).

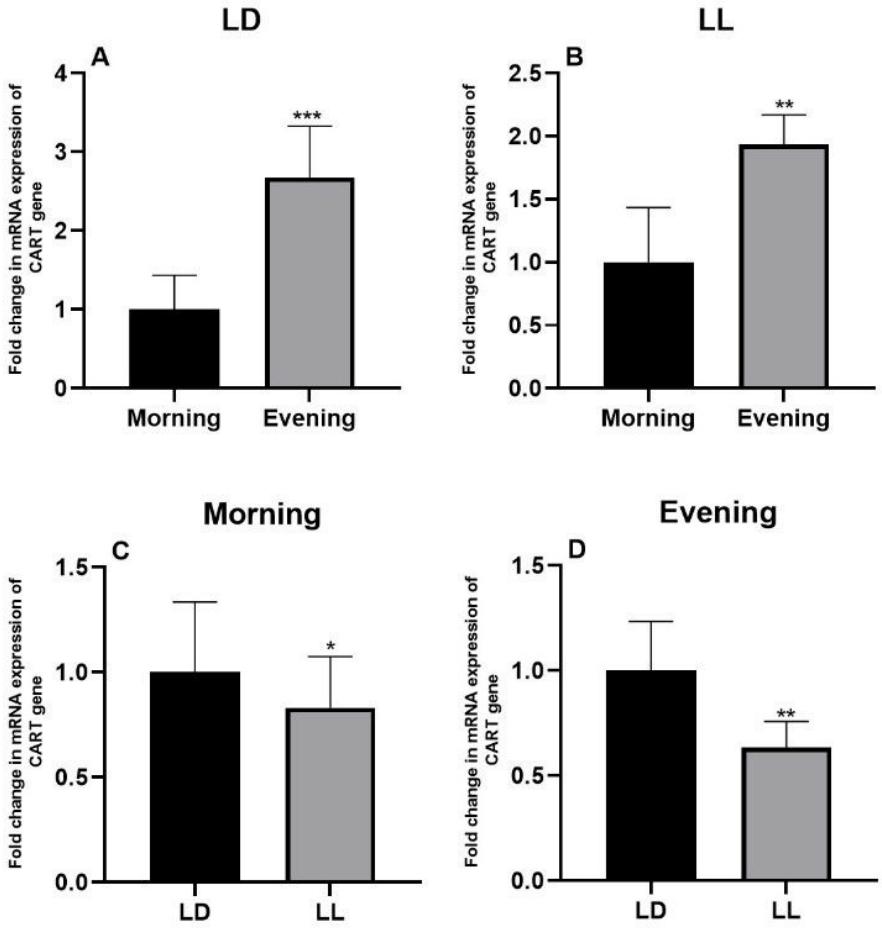

Figure 3. Morning and evening mRNA expression of CART gene (A) in the NA of LD groups after 10 days' exposure to normal 12-hour light/dark cycle. morning and evening mRNA expression of CART gene (B) in the NA of LL groups after 30 days' exposure to normal 12-hour light/dark cycle following by 50 days' exposure to constant lighting protocol. Comparison morning mRNA expression of CART gene in the NA of LD groups and LL animals (C). Comparison evening mRNA expression of CART gene in the NA of LD groups and LL animals (D). Bars represent fold differences of mean normalized expression values±SEM (n=4). ***P-value<0.001, **P-value<0.01, *P-value<0.05

Discussion

The results demonstrated that from day 30 onwards, animals subjected to constant light conditions exhibited disrupted locomotor activity patterns, as observed in double-plotted actograms. These findings highlight the significant role of physical activity in regulating the "circadian syndrome" and disorders associated with circadian rhythms.

In 2019, Pathak et al. demonstrated that mutations in the GCN2 gene in eukaryotes result in alterations in the light/dark cycle, impair nocturnal locomotor behavior, and reduce the rhythmic expression of per1 and per2 genes in the SCN [37]. On the other hand, the disruption in the actogram double-plotted cycle for wheel running in rats with circadian rhythm disorder can be attributed to the effect of wheel-running exercise on the body clock. This exercise shortens the free-running period, likely due to increased locomotor activity. The mechanism may involve serotonin's role in modulating circadian cycles [38, 39]. Consistent with previous research, our findings demonstrate that prolonged wheel-running exercise leads to a shortening of the free-running period, particularly under conditions of circadian rhythm disruption. This effect, likely mediated by increased locomotor activity and potentially influenced by serotonin signaling, underscores the complex interplay between physical activity and the regulation of the circadian system. These results suggest that wheel running not only serves as a behavioral adaptation to environmental shifts but also acts as a modulator of intrinsic circadian

mechanisms, with strain-specific and temporal factors contributing to the observed outcomes.

CART expression is known to be modulated by chronic stress and antidepressant interventions, as demonstrated by its downregulation following chronic mild stress [40] and upregulation after electroconvulsive stimulation in the nucleus accumbens [41]. Moreover, administration of exogenous CART peptides has been shown to reduce immobility time in the forced swim test, supporting its antidepressant-like effects [42]. Recent evidence also suggests that *CART* gene may serve as a molecular marker for antidepressant response, particularly in ketamine-treated mice, where increased CART expression in the anterior cingulate cortex correlates with behavioral improvement [43]. These findings underscore the potential involvement of CART in mood regulation.

The current study has revealed that mRNA expression of CART peptides in NAc follows circadian rhythms. In addition, our measurement of CART peptide mRNA levels shows that its levels are about 2 times higher in the evening than in the morning. It is important to point out that mice and rats are nocturnal rodents, i.e., they are active during the dark period and rest during the day [44]. Therefore, the higher level of CART peptide mRNA in the evening reflects the stronger gene expression during the active/dark phase of the rat compared with the inactive/light phase. The main finding of the present study is that the disruption of circadian rhythms by continuous light leads to a down-regulation of CART both in the morning and in the evening. Interestingly, our study has shown that CART is still subject to circadian control despite the reduced expression under continuous light. This diurnal rhythm, which was observed under constant light conditions, suggests the endogenous nature of this modulation even in the absence of the light/dark cycle as an external cue. However, it is not clear whether continued disruption of the circadian rhythm can disrupt the resilience of endogenous regulation in the long term. Further research is needed to clarify this question. Significant rhythmizing of various gene expressions and/or protein productions in NAc neurons has been addressed several times in the literature [45]. Studies in rodents have shown that concentrations of dopamine and its metabolites in the NAc exhibit robust diurnal rhythms [46-49]. In addition, diurnal variations in Cryptochrome protein levels in the NAc have been reported [50]. Diurnal variation in the NAc is also shown in core clock genes which include Npas2, Clock as well as Period [51]. In addition to Npas2 and Bmal1, Rev-erbα and RORα as their transcriptional regulators indicate remarkable diurnal variation in the NAc, too. This circadian regulation reaches the regulation of Sirt1, Nampt, and also, the level of the mitochondrial coenzyme NAD +, all of which show diurnal variations in NAc [52]. Surprisingly, this is also evident in gene expression analysis at the time of death in human postmortem tissue, where all canonical clock genes including Bmal1, Npas2, Period, Cryptochrome, and Rev-erbα are strongly rhythmic in NAc from healthy donors [53, 54]. A study which has been published recently has found that robust rhythms are also shown in NAc astrocytes in particular [55]. After using next generation total RNA sequencing in NAc astrocytes across diurnal time, it was found that not only do NAc astrocytes express robust rhythms in canonical clock genes unanimously, but also have significant diurnal rhythms in approximately 43% of the total NAc astrocyte transcriptome [55]. Together with previous findings of neuronal rhythmicity, this lately characterized astrocyte-specific rhythmicity underscores the complicated nature of circadian regulation in NAc cells. Focus of previous studies mentioned above has been on the rhythmicity of clock genes in NAc, and a limited number of studies address the rhythmicity of expression of other molecules. However, with increasing evidence for the existence of rhythmicity in expression of genes, there is still the possibility of discovering other circadian oscillators in the NAc as extra-SCN circadian oscillators region. We found that expression of the CART gene (as a non-clock gene) in rat NAc is subject to diurnal variation. Our results support the further studies which have shown that CART follows a circadian pattern in blood and brain of rats alike [29, 56]. It should be acknowledged that CART gene promoter contains an E-box [57] being implicated in circadian rhythmicity;[58] hence, the regulation of expression, based on a daily cycle, is not entirely surprising. However, the pattern of CART expression rhythmicity in cells of various brain regions may be different. Therefore, the determination of these different rhythms may be valuable for the discovery of the biological and pharmacological functional roles in which the peptide CART is involved.

The circadian regulation of CART expression in the nucleus accumbens appears to parallel the rhythmic profile of CART peptide levels and daily corticosterone secretion [59, 60]. Evidence suggests that CART expression may be modulated by core clock genes such as *Per1*, which influences time-dependent behavioral responses to psychostimulants [61]. Notably, disruptions in *Per1* expression have been linked to altered reward sensitivity, implying a potential regulatory link between circadian genes and CART-mediated processes. Moreover, interactions between *Per1* and NMDA receptor signaling pathways may further contribute to light-induced disturbances in CART rhythmicity [62]. These findings suggest that constant light exposure may interfere with CART expression through dysregulation of clock gene networks and associated neurotransmitter systems.

Previous studies have shown that the diurnal rhythm of CART expression is sensitive to metabolic status, feeding cues, and circadian regulation. In regular-fed animals, CART levels in the nucleus accumbens (NAc) display a robust diurnal rhythm that is abolished under fasting conditions [59]. This disruption may be mediated in part by leptin signaling, as fasting reduces circulating leptin levels and the CART promoter contains a STAT-binding site responsive to leptin [63, 64]. Leptin crosses the blood–brain barrier [65] and regulates anorexigenic peptides, including CART, in hypothalamic circuits involved in energy balance [66]. CART's physiological role in appetite regulation has been demonstrated by multiple studies: immunoneutralization of CART increases food intake [67, 68], and global CART knockout leads to hyperphagia and weight gain [69, 70]. Moreover, CART neurons in the hindbrain suppress feeding behavior through projections to the hypothalamus [71], and CART signaling in the nucleus tractus solitarius (NTS) is critical for satiety [72]. Disrupted circadian rhythms have been linked to overeating, weight gain, and obesity [73-75], and reduced CART expression in SCN has been observed in clock gene mutant rodents with associated metabolic dysregulation [76]. In our study, constant light exposure disrupted CART rhythmicity, particularly in the NAc

brain region implicated in both circadian and feeding circuits. Collectively, these findings suggest that reduced CART expression following circadian disruption may promote hyperphagia and contribute to metabolic disturbances. Future investigations should assess the causal role of CART in circadian rhythm–induced obesity, particularly in mesolimbic reward pathways.

Ethical Considerations

This article was approved by the ethical committee of Shahroud University of Medical Sciences, Iran (Ethical Code: IR.SHMU.REC.1398.058).

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Conflict of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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