Biogerontology (2014) 15:257–268
DOI 10.1007/s10522-014-9495-2

RESEARCH ARTICLE

Differential role of melatonin in restoration of age-induced alterations in daily rhythms of expression of various clock genes in suprachiasmatic nucleus of male Wistar rats

Ushodaya Mattam · Anita Jagota

Received: 28 January 2014 / Accepted: 28 February 2014 / Published online: 12 March 2014
© Springer Science+Business Media Dordrecht 2014

Abstract Aging is associated with changes in several basic parameters of circadian rhythms in mammals leading to circadian dysfunction. The hypothalamic Suprachiasmatic nucleus (SCN) regulates neuronal, endocrine and behavioral rhythms through the expression of various clock genes and release of melatonin from pineal gland. In the present study, we investigated the effect of aging on daily rhythms of various clock genes such as *rPer1, rPer2*, *rCry1, rCry2* and *rBmal1* in the SCN of male Wistar rats. The m-RNA expression levels of these genes were studied by using quantitative Polymerase Chain Reaction (qPCR) in 3 age groups [3 (adult), 12 and 24 month (m)] at variable time points (Zeitgeber time (ZT)—0, 6, 12 and 18). The m-RNA expression for all genes studied was rhythmic in SCN of adult rats with maximum for *rPer1* at ZT-6, *rPer2, rCry1* and *rCry2* at ZT-12 and *rBmal1* at ZT-18. However in 12 and 24 m, the phases of expression of these genes were significantly altered with abolition of daily rhythms of *rCry1, rCry2* and *rBmal1* in 24 m. Melatonin, messenger of darkness, an endogenous synchronizer of rhythm, an antioxidant and an antiaging drug, declines with aging. We therefore studied the effects of melatonin administered subcutaneously at 1 h before the onset of darkness (ZT-11) for 11 days on age induced desynchronization in expression of these genes. We report here differential restoration of daily rhythm, phase, levels and stoichiometric interaction of m-RNA expression of these genes in various age groups in rat SCN with melatonin treatment.

Keywords Suprachiasmatic nucleus · Aging · Clock genes · Melatonin · Circadian rhythms

Introduction

Biological clock system consists of three components: environmental inputs such as light entrainment, a timing system of approximately 24 h (pacemaker) (Welsh et al. 2010), and clock outputs such as melatonin release whose primary function is to transduce light and dark information to whole body physiology (Arendt 2005). Melatonin is a regulator of sleep-wake cycle, an effective antioxidant, endogenous synchronizer and mitochondrial function protector. The hypothalamic suprachiasmatic nucleus (SCN), the light entrained circadian clock localized in the brain, regulates neuronal, endocrine and behavioral rhythms (Welsh et al. 2010) through a network of interconnected transcriptional and translational feedback loops. These feedback loops autoregulate the expression of both positive and

Electronic supplementary material The online version of this article (doi:10.1007/s10522-014-9495-2) contains supplementary material, which is available to authorized users.

U. Mattam · A. Jagota (✉)
Neurobiology and Molecular Chronobiology Laboratory, Department of Animal Sciences, School of Life Sciences, University of Hyderabad, Hyderabad 500046, Andhra Pradesh, India
e-mail: ajsl@uohyd.ernet.in; anita_jagota@yahoo.com

negative clock components and their protein products through coordinated expression of various clock genes such as *clock, bmal1, per1, per2*, *per3*, *cry1, cry2*, *rorα, rev-erbα* etc. CLOCK and BMAL1 are the positive transcriptional activators, which bind to enhancer sequences of clock controlled genes (*CCGs*) and negative transcriptional factors (PER and CRY). PER and CRY proteins generate circadian rhythm by disrupting the activity of CLOCK/BMAL1 complex (Ko and Takahashi 2006; Mohawk et al. 2012).

Age-related changes in the SCN may lead to circadian dysfunction such as decline in circadian neural activity (Nakamura et al. 2011), decrease in the amplitude of the circadian body temperature rhythms (Weinert 2010), altered serotonin rhythms in SCN (Jagota and Kalyani 2010), neuropeptide content and GABAergic network of the SCN (Hofman and Swaab 2006; Palomba et al. 2008) as well as altered SCN sensitivity (von Gall and Weaver 2008; Jagota and Kalyani 2010; Manikonda and Jagota 2012). Melatonin production, amplitude and its pulsatile release from pineal gland decreases upon aging (Karasek 2004). Disturbed circadian melatonin rhythm have profound effects on the health and well-being of the elderly subjects (Poeggeler 2005; Wu and Swaab 2005). Disruption of *Per2* and *Bmal1* in mice has been related by some workers with alterations in behavioral rhythms, development of malignant tumors, metabolic syndrome (Kunieda et al. 2006) and premature aging (Kondratov et al. 2009). Neonatal SCN tissue implantation have been reported to improve circadian rhythmicity and longevity in aging hamsters (Hurd and Ralph 1998)

Modulation of mammalian circadian system by melatonin has been reported by many workers (Reiter et al. 2010) with an entrainment effect on activity rhythms, phase shifts and synchronization of rhythmicity (Jagota 2012). In addition melatonin effects has been reported to restore pinealectomy induced changes (Kolker et al. 2002) and suppression of behavioral phenotype of the CLOCK mutant (Shimomura et al. 2010). Melatonin was also reported to have two distinct effects on the SCN: phase shifting and acute inhibition of SCN electrical activity (Liu et al. 1997; Jin et al. 2003). In addition we have reported earlier the differential effects of melatonin in restoration of daily rhythms of serotonin (Jagota and Kalyani 2010), antioxidant enzymes and lipid peroxidation (Manikonda and Jagota 2012). We report here aging results in differential alterations in daily rhythms of expression of various clock genes (*rPer1, rPer2*, *rCry1, rCry2* and *rBmal1*) and therapeutic effects of melatonin in restoration of such age induced alterations.

Materials and methods

Animals: Male Wistar rats of three age groups: Group I: 3 months (m), Group II: 12 m and Group III: 24 m, i.e. with life spans 12.5, 50 and 95–100 % respectively were studied. Average life span for rats is approximately 24 m though maximum can be even 30 m. Each group (n = 48) was further divided into three groups of 16 animals each, i.e., (A) control (B) melatonin treated (MT) and (C) vehicle control. All rats were kept individually in polypropylene cages contained within well ventilated light proof environmental cabinets isolated in animal facility. They were maintained at room temperature 23 ± 1 °C and relative humidity 55 ± 6 % in LD 12:12 [lights on: 06:30 A.M. Zeitgeber time (ZT-0) and lights off: 6:30 P.M. (ZT-12)] for 2 weeks prior to experiment. Food and water were provided ad libitum. During handling of animals in dark dim red light was used. Cage changing was done at random intervals. All experiments were performed as per Institutional Animal Ethics.

SCN tissue preparation

Animals were sacrificed by decapitation and the brains were dissected out carefully at various time points. 500 μ brain slices were made using rat brain slicer (Zivic Instruments; Pittsburg, USA) and the SCN was carefully punched out with the help of a sharp scalpel (Jagota and Reddy 2007).

Melatonin administration

30 μg/kg body weight of melatonin was administered in 10 % ethanol in physiological saline subcutaneously at 1 h before the onset of darkness (ZT-11) for 11 days to Group IB, IIB and IIIB (Pazo et al. 2002; Jagota and Kalyani 2010). Group IC, IIC and IIIC animals were similarly injected with 10 % ethanol in physiological saline. On 12th day rats of variable age groups were sacrificed at ZT-0, 6, 12, and 18 ($n = 4$ at each time point) and SCN was dissected out.

RNA extraction and cDNA synthesis

Total RNA was extracted from SCN (1-2 mg) tissue by TRI reagent, following the manufacturer's protocol (Sigma). Isolated RNA was then dissolved in 20 µl diethylpyrocarbonate (DEPC)-treated water. The amount of extracted RNA was quantified by measuring the optical density (OD) at 260 and 280 nm with Nano drop spectrophotometer (Thermo Fischer) (Chomczynski and Sacchi 2006). Extracted RNA (2 µg) was then used for cDNA synthesis using Bio-Rad iScript cDNA synthesis kit following manufacturer's instructions. The cDNA was then diluted to 1:20 in RNase free water and aliquots of 8 µl were used for the further analysis (Kamphuis et al. 2005).

Quantitative reverse transcriptase PCR (qRT-PCR)

The m-RNA expression of *rPer1, rPer2, rCry1, rCry2* and *rBmal1* were measured by relative qRT-PCR by the SYBR Green (Applied Biosystems, Foster, USA) detection method. 40 ng of cDNA from each sample was used for qRT-PCR analysis. All the clock genes studied and *β-actin* were amplified separately using the same group of 1st strand cDNA template from each sample. Successful reverse transcription was confirmed for all samples by performing PCR amplification of the internal control β-actin. Real-time specific primers for the clock genes studied as well as for internal control β-actin were selected as per Kamphuis et al. 2005. Primer sequences for various clock genes used in the present study were β-actin: forward-AGCCATGTACGTAGCCATCC, reverse-CTCTCAGCTGTGGTGGTGAA, *rPer1*: forward-TCTGGTTCGGGATCCACGAA, reverse-GAAGAGTCGATGCTGCCAAAG, *rPer2*: forward-CACCCTGAAAAGAAAGTGCGA, reverse-CAACGCCAAGGAGCTCAAGT, *rCry1*: forward-AAGTCATCGTGCGCATTTCA, reverse-TCATCATGGTCGTCGGACAGA, *rCry2*: forward-GGATAAGCACTTGGAACGGAA, reverse-ACAAGTCCCACAGGCGGT, *rBmal1*: forward-CCGATGACGAACTGAAACACCT, reverse-TGCAGTGTCCGAGGAAGATAGC. PCR amplification was carried out using 2× Power SYBR Green PCR Master Mix (Applied Biosystems) in the ABI-PRISM 7500 real time PCR machine (Applied Biosystems). The PCR reaction setup includes 10 µl of 2× Power SYBR Green PCR Master Mix (Applied Biosystems), 1 µl of 10 pmol forward primer, 1 µl of 10 pmol reverse primer and 8 µl (40 ng) of cDNA in a total reaction volume of 20 µl. Manufacturer's universal thermal cycling conditions were followed for gene amplification. Dissociation curves showed a single amplified product and the absence of primer-dimer formation. Cycle threshold (Ct) values were obtained from the exponential phase of PCR amplification. The expression of clock genes were normalized with the expression of β-actin (ΔCt = target gene Ct − β-actin Ct) that is the relative quantity of target m-RNA expression in each sample is equals to $2^{-\Delta Ct}$ (Livak and Schmittgen 2001).

Data analysis

Statistical analysis: Data was analyzed using Jandel Scientific Sigma stat software by one way ANOVA followed by Post hoc Dunkan's test for multiple comparisons of all parameters determined at variable time points within an age group. The melatonin treated groups were compared with respective control groups by Student's t test.

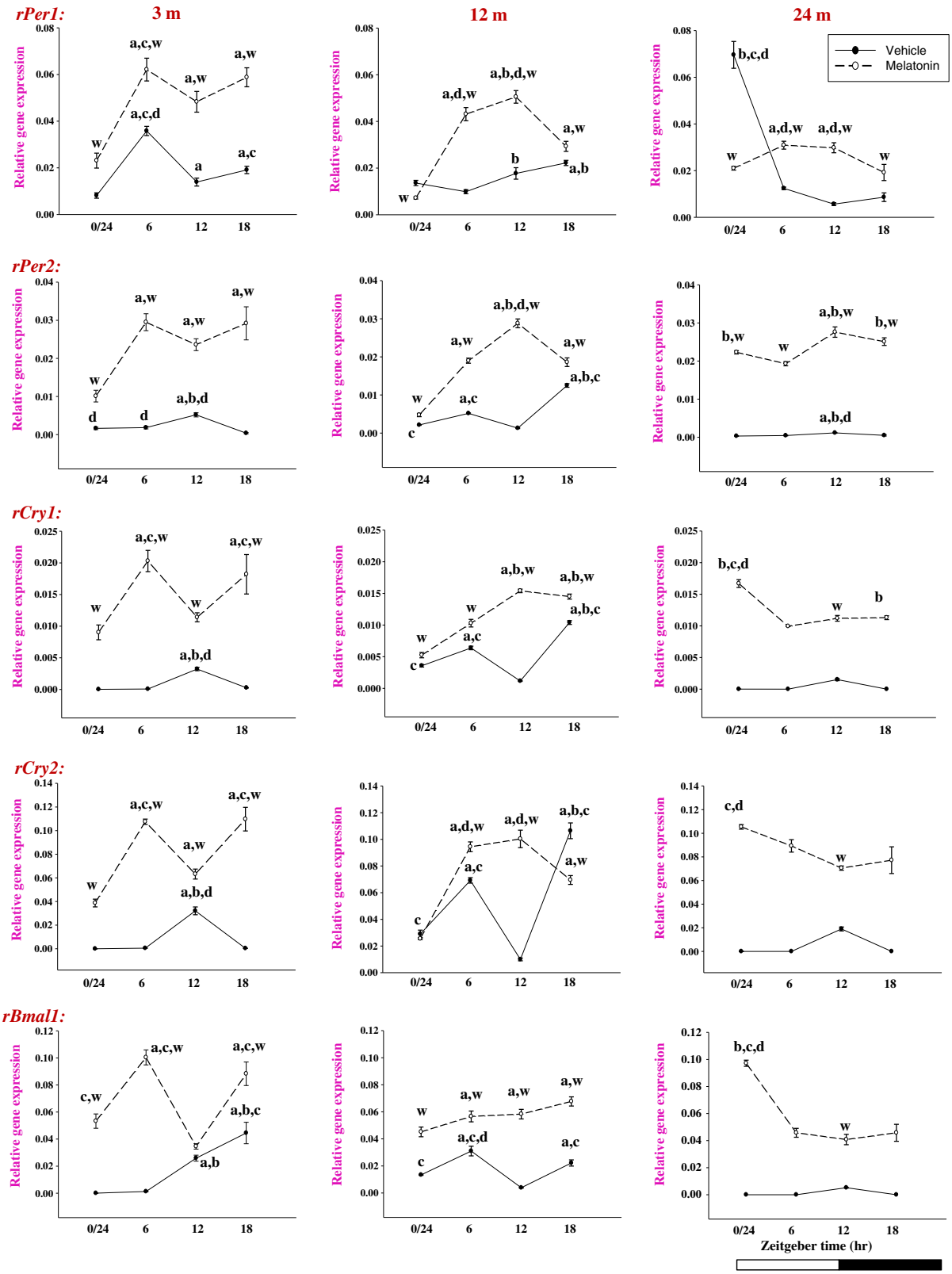
In addition correlation analysis was also done between various parameters (Muradian et al. 2002; Manikonda and Jagota 2012). Effect of melatonin administration on pair wise correlation between mean light (ZT-0, 6,12) and dark (ZT-12, 18, 24/0) phase levels of *rPer1, rPer2, rCry1, rCry2* and *rBmal1* in various age groups 3, 12 and 24 m in rat SCN was also done.

Results

Age induced alterations in daily rhythms and levels of expression of various clock genes

Expression levels of various clock genes such as *rPer1, rPer2, rCry1, rCry2* and *rBmal1* were measured at different time points such as ZT-0, 6, 12 and 18 in 3, 12 and 24 m rat SCN.

The daily rhythm in *rPer1* gene expression in 3, 12 and 24 m rat SCN was observed. Maximum and minimum expression levels of *rPer1* were observed at ZT-6 and ZT-0 in the 3 m, at ZT-18 and ZT-6 in the 12 m and ZT-0 and ZT-12 in the 24 m old animals respectively. The maximum expression of *rPer1* was


rPer1:
3 m
12 m
24 m
Vehicle
Melatonin
Relative gene expression
rPer2:
rCry1:
rCry2:
rBmal1:
Zeitgeber time (hr)
0/24
6
12
18

◀ **Fig. 1** Effect of melatonin administration on levels and rhythmicity of *rPer1, rPer2, rCry1, rCry2* and *rBmal1* genes in the aging rat SCN in 3, 12 and 24 months. Each value is mean ± SEM ($n = 4$), $p \leq 0.05$ and expressed as relative gene expression. $p_a \leq 0.05$; $p_b \leq 0.05$, $p_c \leq 0.05$ and $p_d \leq 0.05$ (where a, b, c and d refers to comparison with ZT-0, ZT-6, ZT-12 and ZT-18 respectively within the group) $p_w \leq 0.05$ (where w refers to comparison of gene levels at same time point in vehicle group in same age group)

phase delayed in 12 m but phase advanced in 24 m rat SCN by 12 and 6 h respectively in comparison to 3 m SCN. The *rPer1* maximum expression levels were also delayed by approximately 6 h in the 24 m in comparison to the 12 m animals (Figs. 1, 2). The *rPer1* mean 24 h levels decreased in 12 m and increased in 24 m but the change from 3 to 24 m was not significant. However, daily pulse in 24 m increased significantly by about 2.65 and 5.4 fold compared to 3 and 12 m respectively (Fig. 3; $p \leq 0.05$).

The *rPer2* expression in SCN was rhythmic with maximum and minimum levels at ZT-12 and 18; ZT-18 and 12; ZT-12 and 0 in 3, 12 and 24 m respectively (Figs. 1, 2). The *rPer2* mean 24 h levels in 24 m were decreased by 0.27 and 0.11 fold compared to 3 and 12 m respectively, whereas daily pulse in 12 m has been increased by about 2.78 and 1.86 fold compared to 3 and 24 m respectively (Fig. 3; $p \leq 0.05$).

The *rCry1* and *rCry2* expression showed daily rhythm in both the 3 and 12 m old rat SCN. However in 24 m old animals, the expression of both *rCry1* and *rCry2* could be detected only at ZT-12. The maximum and minimum levels of *rCry1* and *rCry2* were observed at ZT-12 and ZT-0 respectively in 3 m whereas in 12 m the expression peak was at ZT-18 with 6 h phase delay (Figs. 1, 2). Mean 24 h levels of *rCry1* were increased in 12 m by about 5.95 fold compared to 3 m and then decreased in 24 m by about 0.28 fold compared to 12 m. Daily pulse of *rCry1* in 12 m significantly decreased by about 0.02 fold compared to 3 m. Mean 24 h levels of *rCry2* increased in 12 m by about 6.54 fold compared to 3 m and then decreased in 24 m by about 0.35 fold compared to 12 m. Daily pulse of *rCry2* in 12 m has been decreased by about 0.07 fold compared to 3 m (Fig. 3; $p \leq 0.05$).

Daily rhythm of *rBmal1* was observed in 3 m with maximum levels at ZT-18 and minimum at ZT-0, in 12 m daily rhythm persisted and maximum levels were observed at ZT-6 and minimum at ZT-12 indicating approximately 12 h advance compared to 3 m. *rBmal1* levels could be detected only at ZT-12 in 24 m but not at any other time points studied (Figs. 1, 2). There was no significant difference in mean 24 h levels of *rBmal1* of 3 and 12 m SCN; however levels decreased significantly by 0.3 fold by 24 m. Daily pulse of *rBmal1* in 12 m decreased by about 0.005 fold compared to 3 m (Fig. 3; $p \leq 0.05$).

Pair wise correlation analysis (Fig. 4; $p \leq 0.05$) revealed the statistically significant positive correlation between *rPer2* and *rCry1; rPer2 and rCry2; rCry1* and *rCry2* in both light and dark phase in 3 and 12 m. This correlation was abolished in both light and dark phase of 24 m. Positive correlation between *rPer2 and rBmal1; rCry1 and rBmal1; rCry2* and *rBmal1* was observed in light phase of both 3 and 24 m. However in dark phase in 3 m there was significant reduction in the positive correlation of *rBmal1* with *rCry1* and *rCry2* and became negative with *rPer2*. However in 12 m dark phase such correlation was not significantly different from light phase though these were abolished in both light and dark phase of 24 m. Negative correlation between *rPer1 and rCry1; rPer1 and rCry2* in light phase present in 12 m was found to be abolished in 24 m.

Effect of melatonin administration on age induced alterations of various clock gene expression levels and daily rhythms

Effect of melatonin administration was studied on *rPer1, rPer2, rCry1, rCry2* and *rBmal1* gene expression levels and daily rhythms in SCN in 3, 12 and 24 m old rats. There were no significant differences in control and vehicle treated animals such as IA and IC, IIA and IIC, IIIA and IIIC.

Maximum and minimum expression levels of *rPer1* in 3 m MT were at ZT-6 and ZT-0 respectively, same as that of 3 m vehicle group. In 12 m MT daily rhythm of *rPer1* persisted and maximum levels were observed at ZT-12 instead of ZT-18 which has been observed in case of 12 m vehicle group, thus phase of the rhythm has been advanced by approximately 6 h compared to 12 m vehicle group, leading to partial restoration of the phase of rhythm which is comparable to 3 m vehicle group. In 24 m MT *rPer1* daily rhythm persisted and maximum levels were observed at ZT-6 instead of ZT-0 which has been observed in case of

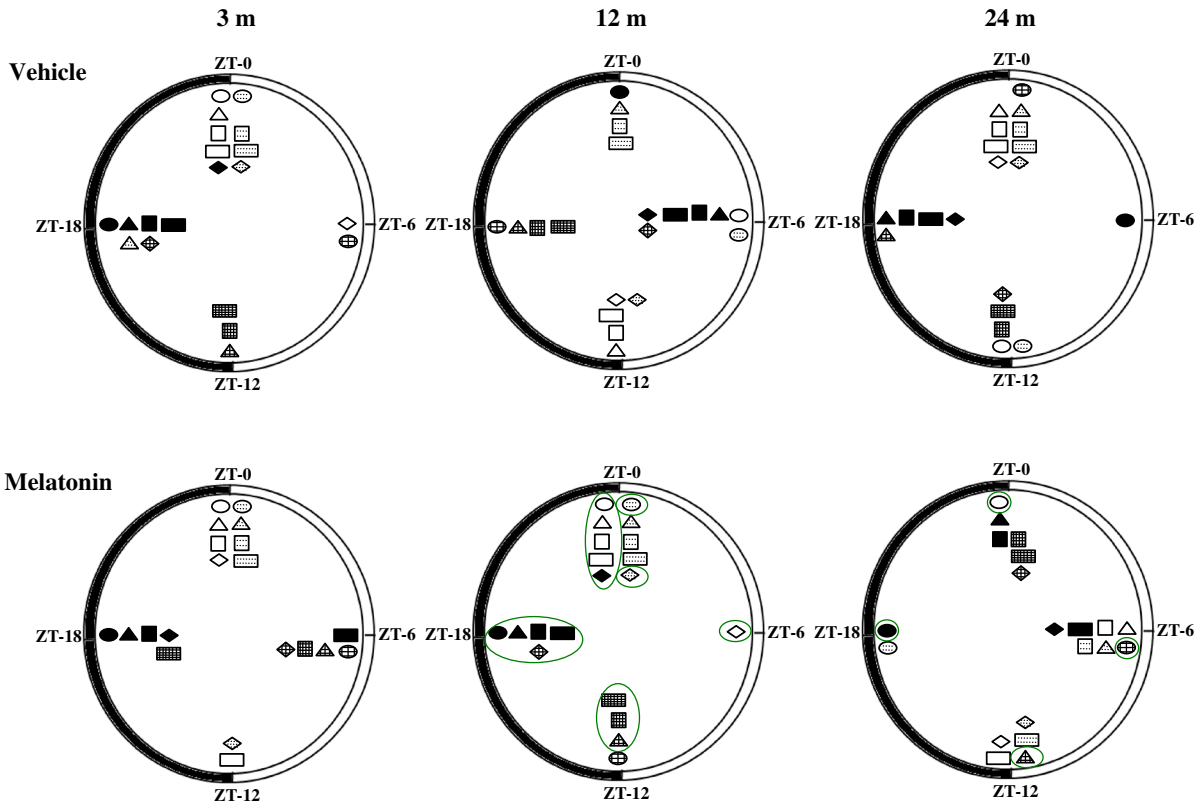


Fig. 2 Effect of melatonin administration on various clock gene expression onset and offset of *rPer1, rPer2, rCry1, rCry2 and rBmal1* gene expression rhythm are indicated by ⬭, △, □, ▭, ◇ and ●, ▲, ■, ▬, ◆ respectively. The maximum and minimum gene expression for these genes have been indicated by ⊕, ⧋, ▦, ▩, ✥ and ⬭, △, □, ▭, ◇ respectively. *Green* markings indicate phase restoration for onset and offset as well as time point of maximum and minimum gene expression with melatonin administration in 12 and 24 m in comparison to 3 m vehicle. (Color figure online)

24 m vehicle group, thus phase of the rhythm has been delayed by approximately 6 h compared to 24 m vehicle group, leading to restoration of the phase of rhythm that is comparable to 3 m vehicle group (Figs. 1, 2). Upon melatonin administration the *rPer1* mean 24 h levels increased by 2.5 and 2.05 fold in 3 and 12 m compared to 3 and 12 m vehicle group respectively, whereas daily pulse could be restored in 12 m MT compared to 12 m vehicle group (Fig. 3).

In 3 m MT, the maximum and minimum expression levels were at ZT-6 and ZT-0 respectively in case of *rPer2, rCry1* and *rCry2,* thus advancing the phase of the daily rhythm by approximately 6 h compared to 3 m vehicle group. Maximum expression levels in 12 m MT for *rPer2, rCry1* and *rCry2* were observed at ZT-12 instead of ZT-18 in 12 m vehicle group indicating approximately 6 h phase advance with MT. Thus there was phase restoration comparable to 3 m vehicle group. Daily rhythm and pattern of *rPer2* persisted in 24 m MT and the levels were increased at ZT-12 compared to the same time point in 24 and 3 m vehicle group. Melatonin could restore the daily rhythm of *rCry1* and *rCry2* in 24 m and at ZT-0, maximum expression levels were observed which indicates approximately 12 h phase advance and 6 h phase delay compared to 3 and 12 m vehicle group respectively (Figs. 1, 2). Mean 24 h levels of *rPer2* and *rCry1* increased in all the three age groups by melatonin compared to age matched vehicle groups. The increase was about 10.5, 3.42 and 39.33 fold in case of *rPer2* and 16.51, 2.15 and 8.1 fold in case of *rCry1* in 3, 12 and 24 m MT compared to 3, 12 and 24 m vehicle group respectively. However, daily pulse of *rPer2, rCry1* and *rCry2* has been decreased in 12

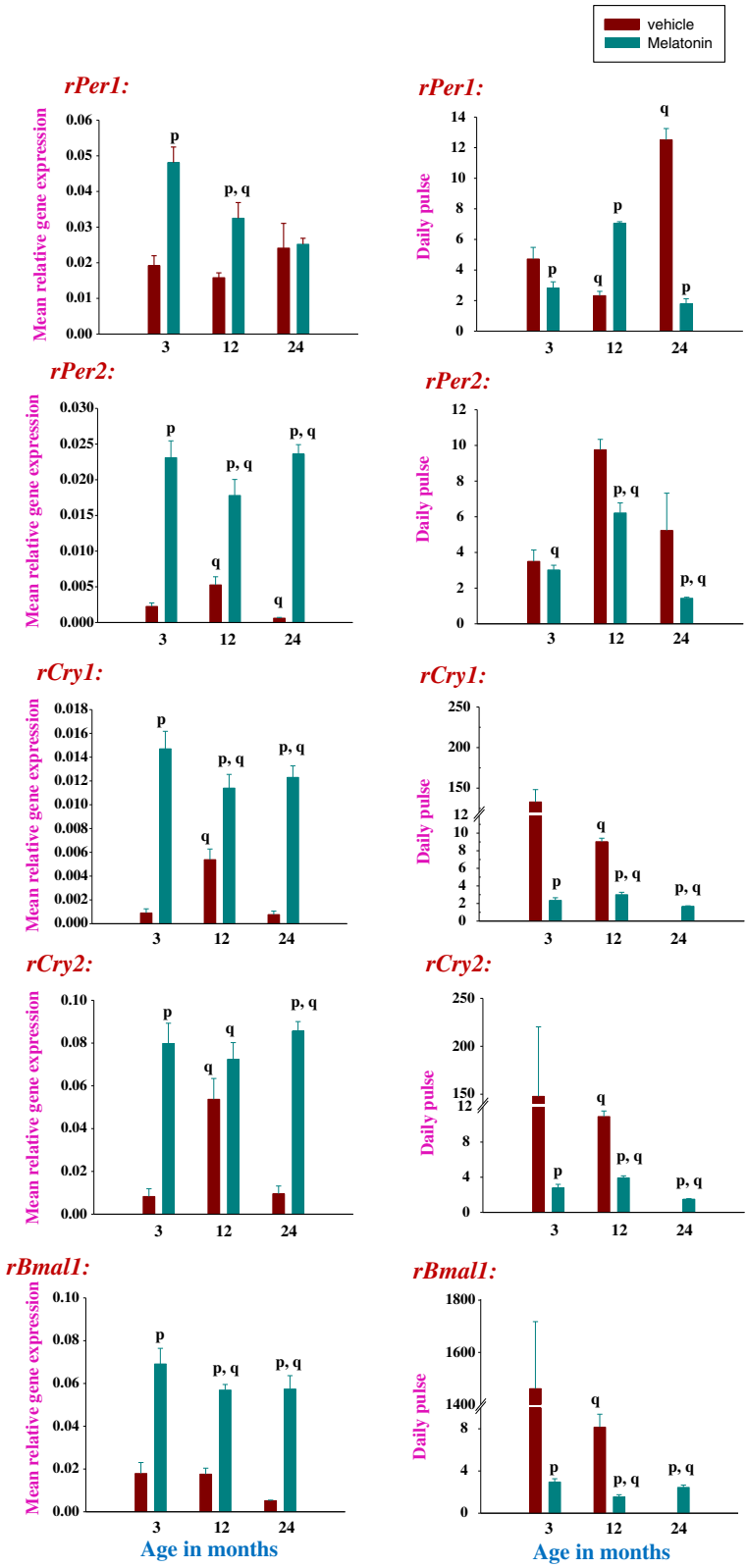


Fig. 3 Effect of melatonin administration on mean 24 h levels and Daily Pulse of *rPer1, rPer2, rCry1, rCry2 and rBmal1* genes in the aging rat SCN in 3, 12 and 24 months rat SCN. Each value is mean ± SEM, $p \leq 0.05$ and expressed as mean relative gene expression. $P_p \leq 0.05$ (where *p* refers to comparison with age matched vehicle group). $P_q \leq 0.05$ (where q refers to comparison with 3 m vehicle group)

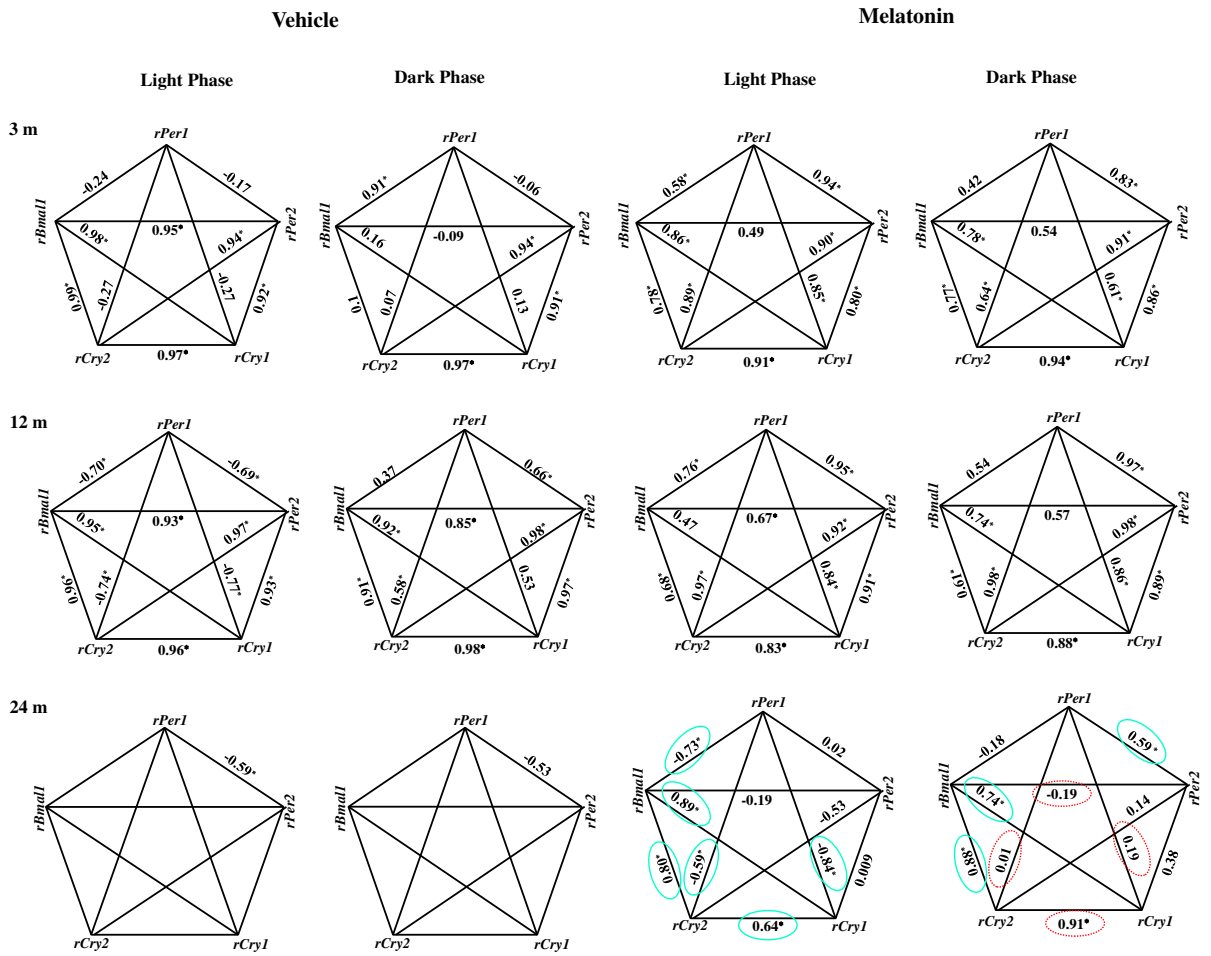


Fig. 4 Effect of melatonin administration on pair wise correlation between mean light (ZT-0, 6,12) and mean dark (ZT-12,18, 24/0) phase values of *rPer1, rPer2, rCry1, rCry2* and *rBmal1* in various age groups 3, 12 and 24 months (m) in rat SCN. Each value is correlation coefficient values between the parameters. * Indicates statistically significant value between parameters ($p < 0.05$). Negative value indicates negative correlation between parameters. *Red colour* dotted markings indicate restoration of correlation coefficient values between the parameters with melatonin administration in 24 m in comparison to 3 m vehicle whereas *Cyan colour* indicate restoration closer to 12 m vehicle. (Color figure online)

and 24 m MT compared to 12 and 24 m vehicle group respectively by about 0.63 and 0.27 fold in case of *rPer2*, 0.01 and 0.33 fold in case of *rCry1* and 0.01 and 0.36 fold in case of *rCry2* (Fig. 3).

Maximum and minimum expression levels for *rBmal1* in 3 m MT group were observed at ZT-0 and ZT-12 indicating approximately 12 h phase advance compared to 3 m vehicle group. Maximum levels were observed at ZT-18 in 12 m MT that is 12 h phase advance compared to 12 m vehicle group, and leading to daily rhythm phase restoration that is comparable to 3 m vehicle group. Restoration of *rBmal1* daily rhythm has been achieved in 24 m upon melatonin administration with maximum levels at ZT-0, showing approximately 6 h phase delay compared to 3 m vehicle group (Figs. 1, 2). Upon melatonin administration the mean 24 h levels significantly increased by 3.86, 3.23 and 11.25 fold in 3, 12 and 24 m MT compared to 3, 12 and 24 m vehicle group respectively. However, daily pulse is significantly decreased in 3 and 12 m MT by about 0.002 and 0.18 fold compared to 3 and 12 m vehicle group respectively (Fig. 3; $p \leq 0.05$).

Correlation analysis (Fig. 4; $p \leq 0.05$) revealed that there was no change in the pair wise correlation between *rPer2 and rCry1*; *rCry1 and rCry2; rPer2 and rCry2* in both light and dark phase in 3 and 12 m.

In 24 m, positive correlation between *rCry1 and rCry2* has been restored as compared to 3 and 12 m vehicle group. Significant positive correlation between *rPer1 and rCry1; rPer1 and rCry2* were observed in both light and dark phase of 3 and 12 m, which has become negative correlation in the light phase of 24 m.

Discussion

The maximum levels of m-RNA expression of *rPer1* were observed in light phase, *rPer2, rCry1* and *rCry2* at onset of darkness and *rBmal1* in subjective midnight in 3 m rat SCN. Thus *rBmal1* was phase opposed to *rPer1*. The expression of *rPer1, rPer2, rCry1, rCry2* and *rBmal1* in 3 m rat SCN was found to be in agreement with reports of earlier workers (Abe et al. 1998; Yan et al. 1999; Park and Kang 2004).

The daily rhythm of *rPer1* in 24 m rat SCN showed 6 h phase advance compared to 3 m. The maximum levels of *rPer2* expression were observed at ZT-12 in both 3 and 24 m rat SCN, though with significant decrease in 24 m. The alterations in *rPer1* and *rPer2* in aged rats could be compared to aged mice (Weinert et al. 2001). Unaltered mean 24 h levels of *rPer1* and reduced mean 24 h levels and altered expression profile of *rBmal1* in 12 and 24 m rat SCN were similar to unaltered *Per1* levels and decreased and altered expression profile of *Bmal1* observed in the SCN of aging hamsters (Kolker et al. 2003; Wyse and Coogan 2010; Duncan et al. 2013). Approximately 6 h phase delay was observed in the daily rhythm of *rPer2* with a phase advancement of 6 h for *rCry1* and *rCry2* with arrhythmicity in *rBmal1* expression in 24 m rat SCN in the present study could be related to age-related pathologies observed in old age. Interestingly *Bmal1* knockout animals have been reported to be arrhythmic (Bunger et al. 2000) with reduced lifespan and a number of age related pathologies (Kondrotov et al. 2006).

The phase delay of 12 h for *rPer1* and 6 h for *rPer2*, *rCry1* and *rCry2* with 12 h phase advance for *rBmal1* was found in 12 m rat SCN. In 24 m old rat SCN *rPer1* and *rPer2* daily rhythm persisted but *rCry1*, *rCry2* and *rBmal1* daily rhythm was abolished. Though *rPer1* and *rPer2* daily rhythm persisted (Fig. 1), the mean levels as well as daily pulse had been significantly altered in 24 m (Fig. 3). In addition to this there was robust change in the daily onset and offset of rhythm as well as time points of maximum and minimum levels of expression of various genes in 12 and 24 m rat SCN (Fig. 2). Such desynchronization and alteration could be responsible for age related pathologies as described by some researchers (Kondrotov et al. 2006).

There was increase in mean 24 h levels of *rPer1*, *rPer2, rCry1, rCry2* and *rBmal1* m-RNA expression upon melatonin administration in 3, 12 and 24 m rat SCN. Interestingly some workers have reported significant increase in *Bmal1* and AVP mRNA expression by 14.6 and 14.8 % respectively during the whole circadian period in adult rat SCN after melatonin injection (Poirel et al. 2003). Reduced amplitude in the activity-rest rhythm and altered sleep quality in aged ring doves was reported to improve after melatonin administration (Garau et al. 2006). A unique single melatonin injection in the late subjective day ZT-12 (CT-11.5) had been reported to inhibit the SCN metabolic activity (Cassone et al. 1987). In addition phase shifting the clock in vivo (Warren et al. 1993) as well as phase advancing the light induced expression of *c-fos* in the adult rat SCN were also reported upon melatonin administration (Sumova and Illnerova 1996).

Increase in the amplitude of the daily rhythms of clock genes upon melatonin administration could be related to increased amplitude of the circadian pacemaker system by melatonin through feedback regulation (Armstrong and Redman 1991). Melatonin could also effect clock gene expression such as *Rev-erbα, Bmal1* and *nuclear orphan receptor genes* in the adult rat SCN as reported by some workers (Poirel et al. 2003; Agez et al. 2009). Phase shifting of the circadian rhythm by melatonin in the SCN has been reported to be mediated through Protein Kinase C (PKC) activation (Dubocovich and Markowska 2005). Resetting of circadian rhythms could be through PKC mediated phosphorylation of CLOCK (Shim et al. 2007) and PKCα had been shown to interact with BMAL1 in a circadian manner. CLOCK and BMAL1 could be ultimately affected by melatonin induced activation of PK-C (Robles et al. 2010) and thus in turn regulating the expression of *Per*, *Cry, Clock, Bmal1* and other *CCGs*.

Phase advancement in the daily rhythm pattern of *rPer2, rCry1* and *rBmal1* upon melatonin administration

in 3 m old SCN was observed. Earlier it has been reported that single melatonin injection at CT-11 may have effect 24 h later but not immediately on clock gene expression thus relating to post-translational than transcriptional mechanisms (Yasuo et al. 2002; Poirel et al. 2003). Interestingly, the phase advance of *rPer1, rPer2, rCry1* and *rCry2* m-RNA expression in 12 m melatonin treated SCN could be compared to the phase advancing effect of melatonin on *c-fos* rhythm (Sumova and Illnerova 1996).

The correlation analysis revealed positive correlation between the negative clock components i.e. between *rPer2* and *rCry1*; *rPer2* and *rCry2*; *rCry1* and *rCry2* in both light and dark phase of 3 m. This positive correlation persisted in 12 m, though was abolished in 24 m in both light and dark phase. It has been reported that the negative clock components (PER and CRY) forms a repressor complex which regulates the expression of their own genes as well as CCGs (Mohawk et al. 2012). The disruption of positive correlation between these negative regulators in 24 m could be correlated to the abolished daily rhythm of *rCry1, rCry2* and *rBmal1*. Such differential alterations in the daily rhythm pattern and levels of these clock genes could be correlated to the age related phase desynchronization at the network level and electrophysiological arrhythmia at the single cell level (Farajnia et al. 2012) and changes in sleep and circadian timing of SCN in mice (Biello 2009). Melatonin administration resulted in partial restoration of correlation between *rPer2* and *rCry1*; *rPer2* and *rCry2* and significantly robust restoration of positive correlation between *rCry1* and *rCry2*. The restoration of such correlation could be responsible for daily rhythm restoration of *rCry1* and *rCry2* in 24 m MT group. Negative correlation between *rPer1* and *rCry2*; *rPer1* and *rCry1* observed in light phase of 24 m MT group was similar to light phase of 12 m. Thus melatonin restored clock gene expression in 24 m comparable to 12 m indicating differential restoration of the stoichiometric interactions among various clock genes.

Thus exogenous melatonin administration for 11 days at ZT-11 resulted in differential restoration of the phase of *rPer2, rCry1, rCry2* and *rBmal1* daily rhythm in 12 m old SCN but not in 24 m. We have reported earlier the restoration of daily rhythms of Serotonin in middle age group rat SCN with MT (Jagota and Kalyani 2010). The daily rhythms of lipid peroxidation and antioxidants could also be restored with MT in rat liver 12 m group (Manikonda and Jagota 2012). This could be due to reduced sensitivity of SCN to melatonin with aging rat (von Gall and Weaver 2008), due to decrease in melatonin receptors (Sanchez-Hidalgo et al. 2009). Melatonin had been reported to act via non genomic action i.e. receptor independent action. This property could be responsible for restoration of levels and rhythms of various clock gene expressions. In addition daily rhythm of *rCry1, rCry2* and *rBmal1* were restored in 24 m old SCN thus indicating differential effects of melatonin in restoring levels and rhythms of various CCGs.

Acknowledgments The work was supported by DBT grant (BT/PR3974/MED/30/813/2012) to AJ. UM is thankful to DBT for fellowship.

References

Abe H, Honma S, Namihira M, Tanahashi Y, Ikeda M, Honma K (1998) Circadian rhythm and light responsiveness of BMAL1 expression, a partner of mammalian clock gene Clock, in the suprachiasmatic nucleus of rats. Neurosci Lett 258:93–96

Agez L, Laurent V, Guerrero YH, Pevet P, Masson-Pevet M, Gauer F (2009) Endogenous melatonin provides an effective circadian message to both the suprachiasmatic nuclei pars tuberalis of the rat. J Pineal Res 46:95–105

Arendt J (2005) Melatonin: characteristics, concerns, and prospects. J Biol Rhythms 20:291–303

Armstrong SM, Redman JR (1991) Melatonin: a chronobiotic with anti-aging properties? Med Hypotheses 34:300–309

Biello MS (2009) Circadian clock resetting in the mouse changes with age. AGE 31:293–303

Bunger MK, Wilsbacher LD, Moran SM, Clendenin C, Radcliffe LA, Hogenesch JB, Simon MC, Takahashi JS, Bradfield CA (2000) Mop3 is an essential component of the master circadian pacemaker in mammals. Cell 103: 1009–1017

Cassone VM, Roberts HM, Moore YR (1987) Melatonin inhibits metabolic activity in the rat suprachiasmatic nuclei. Neurosci Lett 81:29–34

Chomczynski P, Sacchi N (2006) Single-step method of RNA isolation by acid guanidinium thiocyanatephenol-chloroform extraction: twenty-something years on. Nat Protoc 1:581–585

Dubocovich ML, Markowska M (2005) Functional MT1 and MT2 melatonin receptors in mammals. Endocrine 27: 101–110

Duncan MJ, Prochot JR, Cook DH, Tyler SJ, Franklin KM (2013) Influence of aging on Bmal1 and Per2 expression in extra—SCN oscillators in hamster brain. Brain Res 1491:44–53

Farajnia S, Michel S, Deboer T, Vanderleest HT, Houben T, Rohling JH, Ramkisoensing A, Yasenkov R, Meijer JH (2012) Evidence for neuronal desynchrony in the aged suprachiasmatic nucleus clock. J Neurosci 32:5891–5899

Garau C, Aparicio S, Rial VR, Nicolau CM, Esteban S (2006) Age related changes in the activity-rest circadian rhythms and c-fos expression of ring doves with aging. Effects of tryptophan intake. Exp Gerontol 41:430–438

Hofman MA, Swaab DF (2006) Living by the clock: the circadian pacemaker in older people. Ageing Res Rev 5:33–51

Hurd MW, Ralph MR (1998) The significance of circadian organization for longevity in the golden hamster. J Biol Rhythms 13:430–436

Jagota A (2012) Age-induced alterations in biological clock: therapeutic effects of melatonin. In: Thakur MK, Rattan SIS (eds) Brain aging and therapeutic interventions. Springer, Netherlands, London, pp 111–129

Jagota A, Kalyani D (2010) Effect of melatonin on age induced changes in daily serotonin rhythms in suprachiasmatic nucleus of male wistar rat. Biogerontology 11:299–308

Jagota A, Reddy MY (2007) The effect of Curcumin on ethanol induced changes in Suprachiasmatic nucleus (SCN) and Pineal. Cell Mol Neurobiol 27:997–1006

Jin X, von Gall C, Pieschl RL, Gribkoff VK, Stehle JH, Reppert SM, Weaver DR (2003) Targeted disruption of the mouse Mel(1b) melatonin receptor. Mol Cell Biol 23:1054–1060

Kamphuis W, Cailotto C, Dijk F, Bergen A, Buijs RM (2005) Circadian expression of clock genes and clock–controlled genes in the rat retina. Biochem Biophys Res Commun 330:18–26

Karasek M (2004) Melatonin, human aging, and age-related diseases. Exp Gerontol 39:1723–1729

Ko HC, Takahashi SJ (2006) Molecular components of circadian clock. Hum Mol Genet 15:271–277

Kolker DE, Olson SL, Dutton-Boilek J, Bennett KM, Wallen EP (2002) Feeding melatonin enhances the phase shifting response to triazolam in both young and old golden hamsters. Am J Physiol 282:1382–1388

Kolker DE, Fukuyama H, Huang DS, Takahashi JS, Horton TH, Turek FW (2003) Aging alters circadian and light-induced expression of clock genes in golden hamsters. J Biol Rhythms 18:159–169

Kondratov VR, Vykhovanets O, Kondratova AA, Antoch PM (2009) Antioxidant N-acetyl-L-cysteine ameliorates symptoms of premature aging associated with the deficiency of the circadian protein BMAL1. Aging 1:979–987

Kondrotov RV, Kondrotova AA, Gorbacheva VY, Vychovanets OV, Antoch MP (2006) Early aging and age related pathologies in mice deficient in BMAL1, the core component of the circadian clock. Genes Dev 20:1868–1873

Kunieda T, Minamino T, Katsuno T, Tateno K, Nishi J, Miyauchi H, Orimo M, Okada S, Komuro I (2006) Cellular senescence impairs circadian expression of clock genes in vitro and in vivo. Circ Res 3:532–539

Liu C, Weaver DR, Jin X, Shearman LP, Pieschl RL, Gribkoff VK, Reppert SM (1997) Molecular dissection of two distinct actions of melatonin on the suprachiasmatic circadian clock. Neuron 19:91–102

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta\ Ct}$ Method. Methods 25:402–408

Manikonda PK, Jagota A (2012) Melatonin administration differentially affects age-induced alterations in daily rhythms of lipid peroxidation and antioxidant enzymes in male rat liver. Biogerentology 13:511–524

Mohawk JA, Green CB, Takahashi JS (2012) Central and peripheral circadian clocks in mammals. Annu Rev Neurosci 35:445–462

Muradian KK, Utko NA, Mozzhukhina TG, Litoshenko AY, Pishel IN, Bezrukov VV, Fraifield VE (2002) Pair-wise linear and 3D nonlinear relationships between the liver antioxidant enzyme activities and the rate of body oxygen consumption in mice. Free Radic Biol Med 33:1736–1739

Nakamura JT, Nakamura W, Yamazaki S, Kudo T, Cutler T, Colwell CS, Block GD (2011) Age-related decline in circadian output. J Neurosci 31:10201–10205

Palomba M, Nygard M, Florenzano F, Bertini G, Kristenssion K, Bentivoglio M (2008) Decline of the presynaptic network, including GABAergic terminals, in the aging suprachiasmatic nucleus of the mouse. J Biol Rhythms 23:220–231

Park K, Kang MH (2004) Cloning and circadian expression of Rat Cry1. Mol Cells 18:256–260

Pazo D, Cardinali DP, Cano P, Reyes Toso CA, Esquifino AI (2002) Age-related changes in 24-hour rhythms of norepinephrine content and serotonin turnover in rat pineal gland: effect of melatonin treatment. Neurosignals 11:81–87

Poeggeler B (2005) Melatonin, aging, and age-related diseases: perspectives for prevention, intervention, and therapy. Endocrine 27:201–212

Poirel VJ, Boggio V, Dardente H, Pevet P, Masson-Pevet M, Gauer F (2003) Contrary to other non-photic cues, acute melatonin injection does not induce immediate change of clock gene mRNA expression in the rat suprachiasmatic nuclei. Neuroscience 120:745–755

Reiter RJ, Tan DX, Fuentes-Broto L (2010) Melatonin: a multitasking molecule. Prog Brain Res 181:127–151

Robles MS, Boyault C, Knutti D, Padmanabhan K, Weitz CJ (2010) Identification of RACK1 and protein kinase C alpha as integral components of the mammalian circadian clock. Science 327:463–466

Sanchez-Hidalgo M, de la Lastra CA, Carrascosa-Salmoral MP, Naranjo MC, Gomez-Corvera A, Caballero B, Guerrero JM (2009) Age-related changes in melatonin synthesis in rat extrapineal tissues. Exp Gerontol 44:328–334

Shim HS, Kim H, Lee J, Son GH, Cho S, Oh TH, Kang SH, Seen DS, Lee KH, Kim K (2007) Rapid activation of CLOCK by Ca^{2+}-dependent protein kinase C mediates resetting of the mammalian circadian clock. EMBO Rep 8:366–371

Shimomura K, Lowrey LP, Vitaterna HM, Buhr DE, Kumar V, Hanna P, Omura C, Izumo M, Low SS, Barrett KR, LaRue IS, Green BC, Takahashi SJ (2010) Genetic suppression of the circadian clock mutation by the melatonin biosynthesis pathway. Proc Natl Acad Sci 107:8399–8403

Sumova A, Illnerova H (1996) Melatonin instantaneously resets intrinsic circadian rhythmicity in the rat suprachiasmatic nucleus. Neurosci Lett 218:181–184

von Gall C, Weaver DR (2008) Loss of responsiveness to melatonin in the aging mouse suprchiasmatic nucleus. Neurobiol Aging 29:464–470

Warren SW, Hodges BD, Cassone MV (1993) Pinealectomized rats entrain and phase-shift to melatonin injections in a dose-dependent manner. J Biol Rhythms 8:233–245

Weinert D (2010) Circadian temperature variation and aging. Ageing Res Rev 9:51–60

Weinert H, Weinert D, Schurov I, Maywood ES, Hastings MH (2001) Impaired expression of the mPer2 circadian clock gene in the suprachiasmatic nuclei of aging mice. Chronobiol Int 18:559–565

Welsh KD, Takahashi SJ, Kay AS (2010) Suprachiasmatic nucleus: cell autonomy and network properties. Annu Rev Physiol 72:551–577

Wu Y, Swaab FD (2005) The human pineal gland and melatonin in aging and Alzheimer's disease. J Pineal Res 38:145–152

Wyse AC, Coogan NA (2010) Impact of aging on diurnal expression patterns of CLOCK and BMAL1 in the mouse brain. Brain Res 1337:21–31

Yan ST, Shigeyoshi YT, Okamura H (1999) Per1 and Per2 gene expression in the rat suprachiasmatic nucleus: circadian profile and the compartment-specific response to light. Neuroscience 94:141–150

Yasuo S, Yoshimura T, Bartell AP, Iigo M, Makino E, Okabayashi N, Ebihara S (2002) Effect of melatonin administration on *qPer2, qPer3,* and *qClock* gene expression in the suprachiasmatic nucleus of Japanese quail. Eur J Neurosci 16:1541–1546