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RESEARCH ARTICLE

Aging renders desynchronization between clock and immune genes in male Wistar rat kidney: chronobiotic role of curcumin

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Abstract Suprachiasmatic nucleus (SCN) contains the central clock that orchestrate circadian rhythms in physiology and behavior in mammals. Tightly interlocked transcriptional and translational feedback loops (TTFLs) comprising of various clock genes such as *Clock, Bmal1, Periods, Cryptochromes* etc. in the SCN, send the timing signals to peripheral clocks that governs local metabolism with similar TTFLs. Peripheral clocks in kidney regulates several circadian rhythms like blood pressure, immunity etc. However, aging leads to circadian and inflammatory disorders in kidney. Though there are increasing evidences on age associated perturbations, studies elucidating the rhythmic expression of clock and immune genes across aging in kidney are obscure. We therefore studied changes in daily rhythms of clock and immune genes in kidney. In this study we measured mRNA expression of clock genes *rBmal1, rPer1*, *rPer2*, *rCry1*, *rCry2*, *rRev-erbα*, *rRorα,* and inflammatory genes *rNfκb1*, *rTnfα*, *rIl6*, *rTlr4* and *rTlr9* in 3, 12 and 24 months male Wistar rat kidney using qRT-PCR. From our study, we did not observe significant changes in clock genes expression except *rRorα*, but immune genes showed significant phase alterations as well as increase in mean 24 h levels. Pearson correlation analysis of data showed desynchronization between immune and clock genes expression. We further studied the effect of administration of curcumin which has anti-aging, anti-inflammatory, antioxidant etc. properties, and evaluated its chronobiotic properties. We here report differential effects of curcumin administration on daily rhythms of clock and immune genes expression.

Keywords Curcumin · Kidney · Clock genes · Immune genes · Aging · Peripheral clock

Introduction

In mammals, Suprachiasmatic nucleus (SCN) contains the central clock that synchronizes physiology, behavior and metabolism to the external environmental cues (*Zeitgebers*) (Jagota 2012; Roenneberg and Merrow 2016). SCN regulates circadian rhythms by core clock genes viz. *Clock, Bmal1, Periods, Cryptochromes, Rev-erbα*, *Rorα* etc. whose expression is orchestrated at transcriptional and translational levels to establish compact feedback loops that eventually result in ~ 24 h periodicity (Jagota 2012; Takahashi 2017).

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Such circadian clock machineries are present in almost every other cell in mammals and are considered as peripheral clocks, which are capable of exhibiting tissue specific rhythms (Schibler et al. 2015) like immune responses, energy metabolism etc. but are under the regulation of central clock (Dibner et al. 2010). Misaligned circadian rhythms may lead to various physiological, metabolic and behavioral disorders (Hatori et al. 2017).

Peripheral clocks in immune cells (Arjona and Sarkar 2005) temporally gate immune responses. Various inflammatory genes such as *Nfκb*, *Tnfα*, *Il6*, toll like receptors (tlrs) etc. playing important role in inflammation and immune response are known to show rhythmic expression (Scheiermann et al. 2013). CLOCK was shown to elevate transcription of NFκB mediated immune responsive genes (Spengler et al. 2012), while PER1 and CRY1 were shown to inhibit the activation of NFκB (Sugimoto et al. 2014; Yang et al. 2015). Further, CLOCK/BMAL1 complex has been reported to bind to the promoter of *Tlr9* to initiate its transcription (Silver et al. 2012). However, circadian regulation of immunity is cell and tissue specific. For example: recruitment of leukocytes to tissues is observed at zeitgeber time (ZT)-13 and to blood at ZT-5; *Tlr9* shows peak expression at ZT-11 in macrophages, at ZT-19 in spleen, at ZT-15 to ZT-19 in B cells (Silver et al. 2012; Tognini et al. 2018). Moreover, it has been well-established that desynchronizing circadian clock can adversely modulate the immune responses (Curtis et al. 2014). But most of these unveiled interactions between clock and immunity were confined to immune cells.

It has been reported recently that in kidney *Bmal1, Cry1, Cry2* play critical role in circadian regulation of blood pressure (Doi et al. 2010; Hara et al. 2017; Douma et al. 2018). Further, CLOCK/BMAL1 complex was shown to regulate Na^+/H^+ exchanger NHE3 (Saifur Rohman et al. 2005). PER1 has been reported to regulate expression of renal epithelial sodium channel αENaC (Gumz et al. 2009). Various sodium transporters, water channels, vasopressin receptors were also shown to be under circadian regulation (Stow and Gumz 2011). Aging, as a global process was reported to deregulate circadian pattern of blood pressure that may lead to adverse conditions (Hart and Charkoudian 2014). In addition, aging was reported to be associated with chronic inflammation (Bolignano et al. 2014; Xi et al. 2014). Further, with aging increased activity of NFκB had been attributed to chronic kidney diseases (CKD) (Chen et al. 2016). As there are increasing evidences on immune perturbations with aging, we wanted to understand age-associated crosstalk between circadian and immune systems in peripheral clock kidney.

Curcumin, an active constituent of turmeric is known for its pleiotropic properties like anti-inflammatory, anti-oxidant, anti-cancerous, anti-microbial, anti-aging etc. (Hewlings and Kalman 2017). We have reported recently differential chronobiotic property of curcumin on clock genes expression in aged rat SCN (Kukkemane and Jagota 2019). We here report chronobiotic role of curcumin on age-induced alterations of expression of clock and inflammatory genes in peripheral clock kidney.

Methodology

Animals

All the studies were done with male Wistar rats. The rats were individually housed in standard polypropylene cages and maintained at 23 ± 1 °C; relative humidity 55 ± 6%; with LD, 12:12 [lights on: 06:00 AM (Zeitgeber time (ZT)-0) and lights off: 6:00 PM (ZT-12)] for 2 weeks prior to experiments. Food and water was provided ad libitum. Cage changing was done at random intervals. Dim red light was used for handling the animals in the dark (Mattam and Jagota 2014). All the experiments were performed as per Institutional Animal Ethics (approval number: IAEC/UH/151/2016/05/AJ/P12/Rats Wistar/M-144 dated 16/06/2016).

Animals were divided into three age groups: Group A—3 months (m), Group B—12 m and Group C—24 m. Each group (n = 48) was subdivided into three groups (I) Control (C) (II) Vehicle treatment (VT) and (III) Curcumin treatment (CT) with n = 16 in each sub group.

Control: Group A (I), B (I) and C (I) animals (n = 48) did not receive any treatment.

Vehicle treatment: Group A (II), B (II) and C (II) animals (n = 48) were administered with 0.5% carboxy methyl cellulose (CMC) orally at ZT-11 for 15 days.

Curcumin treatment: 100 mg/ml w/v of curcumin (Sigma) was suspended in 0.5% CMC. Required

amount of curcumin was mixed freshly with CMC and stirred for at least 30 min. For 15 days, Group A (III), B (III) and C (III) animals (n = 48) were administered with curcumin (300 mg/kg body weight) orally at ZT-11, since it showed differential restoratory properties in rat central clock SCN (Kukkemane and Jagota 2019).

Tissue preparation

Animals of group A (I, II, III), B (I, II, III) and C (I, II, III) were sacrificed at ZT-0, 6, 12 and 18 (n = 4 at each time point; n = 16 for four time points) on 16th day and kidneys were removed carefully and snap frozen in liquid N_2. Tissues were stored at – 80 °C until further by use.

RNA extraction and cDNA synthesis

Whole tissues were grinded with liquid N_2 and 50 mg (dry weight) was used for RNA isolation from each sample. RNA extraction was carried out using TRI reagent (Sigma) according to manufacturer's protocol (Vinod and Jagota 2017). RNA was dissolved in 50 µl nuclease free water. Concentration and purity of extracted RNA were quantified by using Nano drop spectrophotometer (Thermo Fischer) (Chomczynski and Sacchi 2006). cDNA was synthesized using Bioline cDNA synthesis kit following manufacturer's protocol. cDNA was diluted to 1:20 in RNase free water for the further studies (Mattam and Jagota 2014).

Quantitative reverse transcriptase PCR (qRT-PCR)

The expression of clock genes (*rBmal1, rPer1*, *rPer2*, *rCry1*, *rCry2*, *rRev-erbα* and *rRorα*) and immune genes (*rNfκb1*, *rTnfα*, *rIl6*, *rTlr4* and *rTlr9*) mRNA transcripts were quantified using qRT-PCR by the SYBR Green (Applied Biosystems, Foster, USA) detection method (Mattam and Jagota 2014). Details of the primers used are given in Table 1.

Dissociation curves for all the genes studied showed a single peak (Supplementary Fig. 1) representing specific amplified target gene. Threshold cycle (Ct) values were obtained from the exponential phase of amplification plots. The relative quantitative expression of clock genes were obtained by normalizing each target gene expression with *Cyclophilin A* (ΔCt = target gene Ct—*Cyclophilin A* Ct) in each sample and used $2^{-\Delta Ct}$ method for analysis (Mattam and Jagota 2014).

Table 1 Forward and reverse primers of various clock and immune genes

Gene	Forward primer	Reverese primer
Reference gene		
rCyclophilin A	CGCGTCTGCTTCGAGCTGTTT	GTCACCACCCTGGCACATGAAT
Clock genes		
rBmal1	GGCTTCTTTGGTACCAACATG	AATCCATCTGCTGCCCTGAGAAT
rPer1	GGGCCAAGAAAGATACGTCGTCAG	ACACCACGCTCTCTGCCTTATTG
rPer2	AGCCACAGCCTGAACTAGAGACA	TCCTTGGTGAGGCCTAGCTTCT
rCry1	GGCGGAAACTGCTCTCAAGGA	CCAACACTCTGTGCGTCCTCTT
rCry2	CCATCGTCAACCACGCAGAGA	GGGACAGATGCCAACAGACAGAG
rRev-erbα	GGTGACCTGCTCAATGCCATGTT	CGAGCGGTCTGCAGAGACAAGTA
rRorα	TAGGATGTGCCGTGCCTTT	CAGGAGCGATCTGCTGACAT
Immune genes		
rNfκb1	TACGATGGGACGACACCTCTACAC	GGTCTGCTCCTGCTGCTTTGA
rTnfα	GTCGTAGCAAACCACCAAGC	CCTTGAAGAGAACCTGGGAGTAG
rIl6	TCTCCGCAAGTAAGTGAAGGC	GCGTGGAGGAAAGGGAAAGA
rTlr4	GGCCTCCCTGGTGTTGGATTT	TGGCTACCACAAGCACACTGAC
rTlr9	CTGGGACGTCTGGTACTGTTTC	CCGCACTCGAAGCTCGTTAT

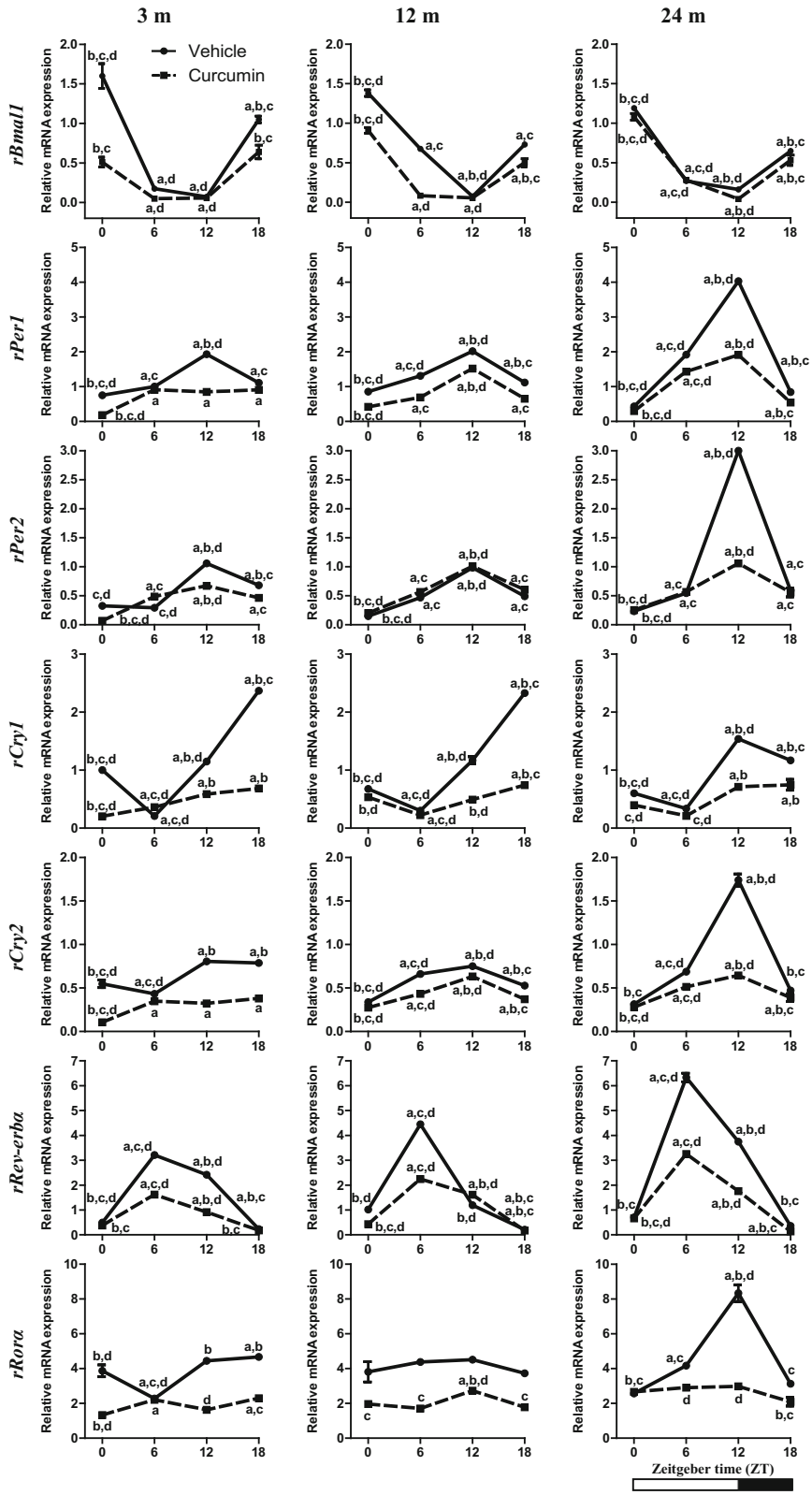
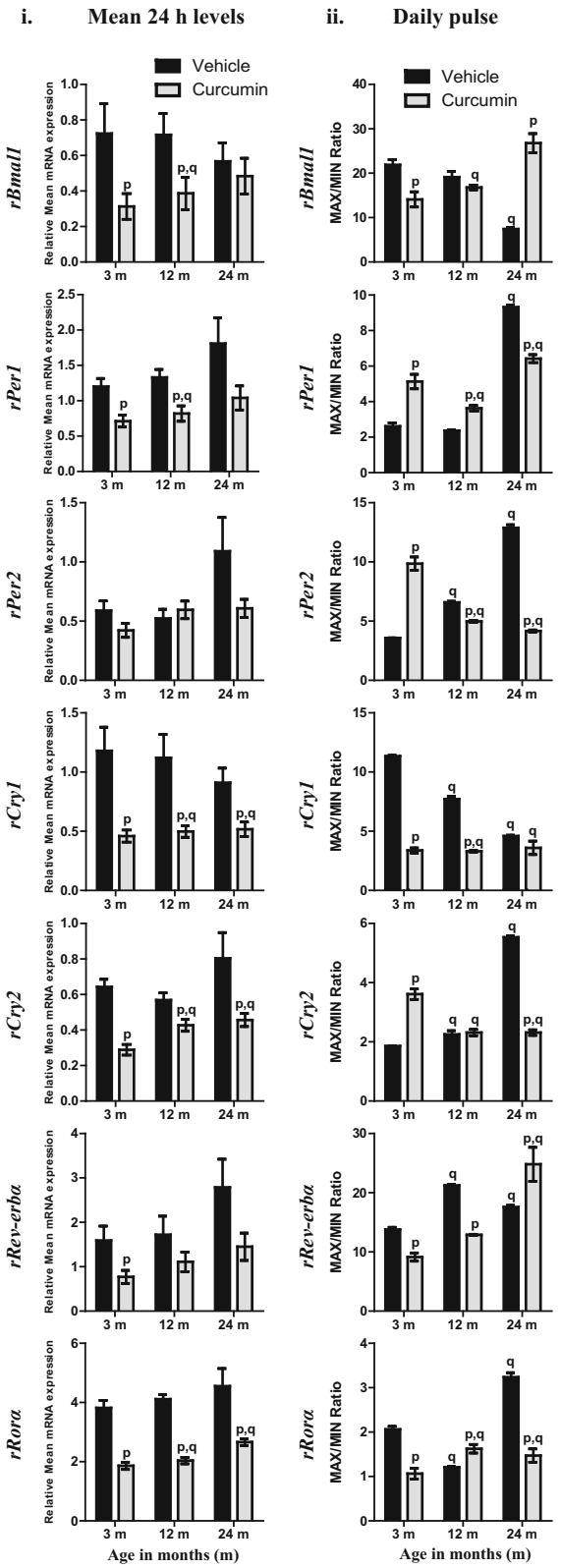


Fig. 1 Effect of curcumin administration on daily rhythms of *rBmal1, rPer1*, *rPer2*, *rCry1*, *rCry2*, *rRev-erbα* and *rRorα* mRNA expression in 3, 12 and 24 m old rat kidney. Each value is mean ± SEM (n = 4), $p < 0.05$ and expressed as relative mRNA expression. $p_a < 0.05$; $p_b < 0.05$, $p_c < 0.05$ and $p_d < 0.05$ (where 'a', 'b', 'c' and 'd' refers to comparison with ZT-0, ZT-6, ZT-12 and ZT-18 respectively within the group)



◀**Fig. 2** Effect of curcumin administration on (i) Mean 24 h levels and (ii) Daily pulse of *rBmal1, rPer1*, *rPer2*, *rCry1*, *rCry2*, *rRev-erbα* and *rRorα* expression in 3, 12 and 24 m old rat kidney. Each value is mean ± SEM (n = 4), $p < 0.05$ and expressed as mean relative gene expression. $p_p < 0.05$ (where 'p' refers to comparison with age-matched vehicle treated group). $p_q < 0.05$ (where 'q' refers to comparison with 3 m vehicle treated group)

Data analysis

Statistical analysis: GraphPad Prism software was used for the data analysis. Multiple comparisons of four time points within each age group were analyzed by one way ANOVA followed by Post hoc Tukey's test. Student's t test was performed to compare the mean 24 h levels and daily pulse between respective vehicle treated groups and curcumin treated groups.

Pearson correlation analysis was performed using R-program (Kukkemane and Jagota 2019). Pair wise correlations were analyzed in light (ZT-0, 6, 12) and dark (ZT-12, 18, 24/0) phase separately among *rBmal1, rPer1*, *rPer2*, *rCry1*, *rCry2*, *rRev-erbα*, *rRorα, rNfκb1*, *rTnfα*, *rIl6*, *rTlr4* and *rTlr9* genes in 3, 12 and 24 m vehicle treated (VT) and curcumin treated (CT) kidney samples.

Gene to gene network analysis: We used weighted correlation network analysis (WGCNA) data mining package in R program to understand the gene to gene network alterations with the aging and curcumin treatment and the network images were developed using 'Cytoscape' software.

Results

Aging differentially alters clock genes expression in kidney

In the present study, we have measured the mRNA expression of clock genes *rBmal1, rPer1, rPer2, rCry1, rCry2, rRev-erbα, rRorα* at four different time points ZT-0, 6, 12, 18 in three age groups 3, 12, 24 m controls (C) and VT animals. There was no change in expression of all the genes between controls and vehicle treated animals. In all the age groups studied, *rBmal1* showed significant daily rhythms with peak at ZT-0 and trough at ZT-12. Mean 24 h levels did not show significant change among age groups studied.

Daily pulse i.e. Max/Min ratio did not show significant change in 12 m, but showed a significant decrease ($p < 0.05$) in 24 m in comparison to 3 m rat kidney. *rPer1* did not alter its expression pattern in all the age groups with maximum expression at ZT-12 and minimum expression at ZT-0. Mean 24 h levels were not significantly changed among all age groups. Daily pulse did not change in 12 m but showed significant increase ($p < 0.05$) in 24 m in comparison to 3 m rat kidney. *rPer2* also expressed maximum at ZT-12 in all age groups but minimum at ZT-6 in 3 m and ZT-0 in both 12 and 24 m rat kidney. Mean 24 h levels did not show significant difference among age groups studied. Daily pulse was significantly increased ($p < 0.05$) in 12 and 24 m in comparison to 3 m animals (Figs. 1 and 2).

In 3 and 12 m animals, *rCry1* showed maximum expression at ZT-18 and minimum at ZT-6. But in 24 m, maximum expression was at ZT-12 with phase advance of 6 h, minimum expression was at ZT-6. Mean 24 h levels did not show significant change among age groups studied. Daily pulse was decreased significantly ($p < 0.05$) in 12 and 24 m in comparison to 3 m animals. *rCry2* showed maximum expression at ZT-12 in all age groups but minimum at ZT-6 in 3 m and at ZT-0 in 12 and 24 m animals. Mean 24 h levels were not significantly different among the age groups. Daily pulse showed significant increase ($p < 0.05$) in 12 and 24 m with respect to 3 m animals. *rRev-erbα* expressed maximum at ZT-6 and minimum at ZT-18 in all the age groups. Mean 24 h levels also did not show significant difference among age groups studied. Daily pulse increased significantly ($p < 0.05$) in 12 and 24 m in comparison to 3 m animals. *rRorα* - showed maximum expression at ZT-18, minimum at ZT-6 in 3 m. Interestingly, in 12 m rhythmicity was abolished. In 24 m, maximum expression was observed at ZT-12 and minimum at ZT-0. Mean 24 h levels did not show significant change among age groups studied. Daily pulse showed significant decrease ($p < 0.05$) in 12 m but significantly increased ($p < 0.05$) in 24 m with respect to 3 m animals (Figs. 1 and 2).

Inflammatory genes exhibit mid-age perturbations in kidney

The mRNA expression of various pro-inflammatory genes like *rNfκb1, rTnfα, rIl6* as well as pattern recognition receptor genes *rTlr4* and *rTlr9* were studied in 3, 12 and 24 ms (m) control (C) and vehicle treated (VT) animals. No change was observed between controls and vehicle treated animals. *rNfκb1* showed maximum expression at ZT-12 and minimum expression at ZT-6 in 3 m animals. In 12 m, maximum expression was at ZT-6 and minimum at ZT-18 with 6 h phase advance in comparison to 3 m animals. In 24 m, maximum expression was at ZT-12 and minimum expression at ZT-18. Mean 24 h levels showed significant increase ($p < 0.05$) with aging. Daily pulse was significantly decreased ($p < 0.05$) in 12 m but increased in 24 m in comparison to 3 m animals. *rTnfα* showed maximum expression at ZT-12 and minimum expression ZT-6 in 3 m animals. In 12 and 24 m, maximum expression was at ZT-6 i.e. 6 h phase advance with respect to 3 m, but minimum expression was at ZT-12 and ZT-0 respectively. Mean 24 h levels significantly increased ($p < 0.05$) in 12 and 24 m with respect to 3 m animals. Daily pulse also showed significant increase ($p < 0.05$) in 12 and 24 m in comparison to 3 m. *rIl6* expressed maximum at ZT-12 and minimum at ZT-0 in 3 m animals. In 12 m, maximum expression was observed at ZT-6 with phase advance of 6 h and minimum at ZT-18. In 24 m, maximum and minimum expressions were at ZT-12 and ZT-0 respectively. Mean 24 h levels did not show significant change among age groups studied. Daily pulse showed significant decrease ($p < 0.05$) in 24 m in comparison to 3 m animals (Figs. 3 and 4).

rTlr4 showed rhythmic expression with maximum at ZT-12 and minimum at ZT-6 in 3 m animals. In 12 m, maximum expression showed phase advance of 6 h and minimum expression was observed at ZT-12. In 24 m, maximum expression was at ZT-12 and minimum expression at ZT-18. Mean 24 h levels were increased significantly ($p < 0.05$) in 12 and 24 m in comparison to 3 m animals. Daily pulse also showed significant increase ($p < 0.05$) in 12 and 24 m animals. *rTlr9* showed maximum expression at ZT-12 and minimum at ZT-6 in 3 m animals. In 12 m, maximum expression was at ZT-6 with phase advance of 6 h and minimum at ZT-18. In 24 m, maximum expression was at ZT-12 and minimum expression at ZT-0. Mean 24 h levels showed significant increase ($p < 0.05$) in comparison to 3 m animals. Daily pulse did not show significant change among age groups studied (Figs. 3 and 4).

Correlation analysis of daily rhythms of clock genes in kidney: age-induced alterations

Pairwise correlations were analyzed among clock genes in light phase (LP) and dark phase (DP) of 3, 12 and 24 m animals (Fig. 5). In light phase of 3 m, a significant negative correlation between *rBmal1* and *rRev-erbα* ($p < 0.001$) was observed. Also a negative correlation was observed between *rBmal1* and *rPer1* ($p < 0.01$). Within the *rPer1,2* genes and within the *rCry1,2* genes there was a significant positive correlation ($p < 0.001$). However, *rPer1,2* showed significant positive correlation with *rCry2* ($p < 0.001$). *rRev-erbα* showed negative correlation ($p < 0.05$) with *rRorα*. We also observed that *rRorα* showed positive correlation ($p < 0.001$) with *rCry1,2*. In dark phase of 3 m, negative correlation persisted between *rBmal1* and *rRev-erbα* and the negative correlation between *rBmal1* and *rPer1,2* genes became more significant ($p < 0.001$). Positive correlation ($p < 0.001$) persisted between *rPer1,2* genes but positive correlation between *rCry1,2* was abolished. Positive correlation between *rPer1,2* and *rCry2* genes persisted. Negative correlation between *rRorα* and *rRev-erbα* was abolished. But positive correlation between *rRorα* and *rCry1,2* genes persisted. Moreover, there was a positive correlation between *rRev-erbα* and *rPer1,2* genes ($p < 0.001$).

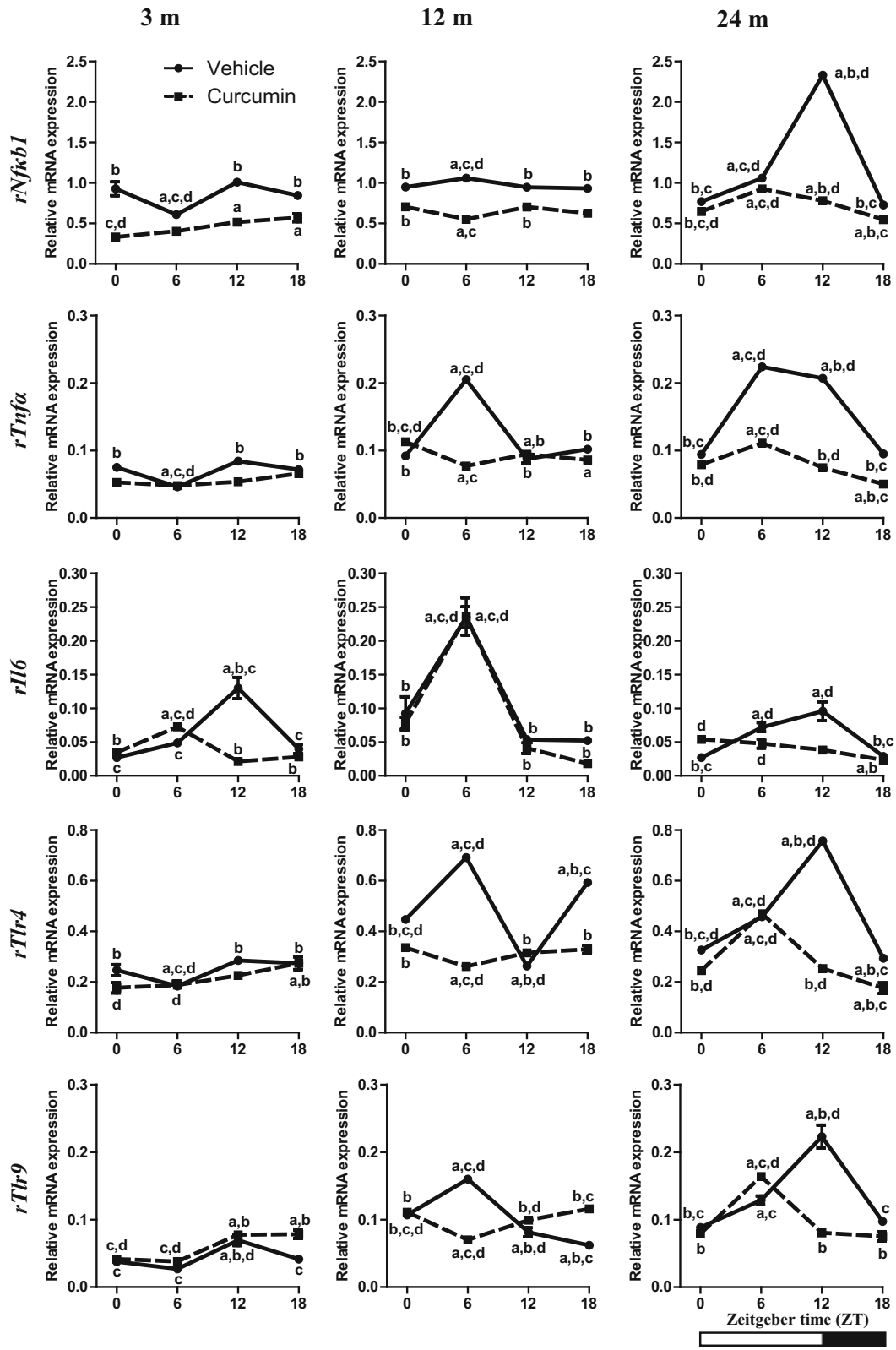
In light phase of 12 m, correlation between *rBmal1* and *rRev-erbα* was abolished. However, negative correlation between *rBmal1* and *rPer1* genes persisted ($p < 0.001$). Also, positive correlation between *rPer1,2* genes persisted ($p < 0.001$), whereas correlation between *rCry1,2* genes was abolished. Interestingly, a significant positive correlation appeared between *rPer1,2* and *rCry1* and positive correlation with *rCry2* ($p < 0.001$) persisted. Negative correlation between *rRorα* and *rRev-erbα* was abolished but positive correlation between *rRorα* and *rCry2* persisted ($p < 0.001$). In dark phase of 12 m, there was no correlation between *rBmal1* and *rRev-erbα,* but negative correlation between *rBmal1* and *rPer1* genes persisted ($p < 0.001$). Positive correlation between *rPer1,2* genes persisted ($p < 0.001$) but between *rCry1,2* genes correlation was abolished. Positive correlation persisted between *rPer1,2* and *rCry2* ($p < 0.001$). A positive correlation appeared between *rRorα* and *rRev-erbα* ($p < 0.01$). *rRorα* also showed positive correlation with *rCry2* ($p < 0.001$).

Fig. 3 Effect of curcumin administration on daily rhythms of ▶ *rNfκb1*, *rTnfα*, *rIl6*, *rTlr4* and *rTlr9* mRNA expression in 3, 12 and 24 m old rat kidney. Each value is mean ± SEM (n = 4), $p < 0.05$ and expressed as relative mRNA expression. $p_a < 0.05$; $p_b < 0.05$, $p_c < 0.05$ and $p_d < 0.05$ (where 'a', 'b', 'c' and 'd' refers to comparison with ZT-0, ZT-6, ZT-12 and ZT-18 respectively within the group)

In light phase of 24 m, negative correlation between *rBmal1* and *rRev-erbα* persisted ($p < 0.001$). Significant negative correlation persisted between *rBmal1* and *rPer1* genes ($p < 0.001$). Significant positive correlation persisted within and in between *rPer1,2* and *rCry1,2* genes ($p < 0.001$). *rRorα* did not show correlation with *rRev-erbα,* but showed positive correlation with *rCry1,2* ($p < 0.001$). In dark phase of 24 m, correlations between clock genes were significantly affected where *rBmal1* showed significant negative correlation ($p < 0.001$) with all the other clock genes. A significant positive correlation ($p < 0.001$) appeared among all the clock genes.

Correlation analysis of daily rhythms of immune genes in kidney: age-induced alterations

Pairwise correlation among immune genes in light phase (LP) and dark phase (DP) were analyzed in 3, 12 and 24 m animals (Fig. 5). In light phase of 3 m, *rNfκb1* showed significant positive correlation ($p < 0.001$; $p < 0.01$) with all other immune genes except *rIl6*. In dark phase of 3 m, *rNfκb1* showed positive correlation ($p < 0.001$) with all the immune genes except *rTlr4*. In light phase of 12 m, *rNfκb1* showed significant positive correlation ($p < 0.001$) with all other immune genes. In dark phase of 12 m, *rNfκb1* showed negative correlation with *rTnfα* and *rTlr4* ($p < 0.001$; $p < 0.01$) but significant positive correlation with *rTlr9* ($p < 0.001$) persisted and correlation with *rIl6* was abolished. In light phase of 24 m, positive correlation of *rNfκb1* with all the immune genes ($p < 0.001$; $p < 0.05$) persisted. In dark phase of 24 m, *rNfκb1* showed a significant positive correlation ($p < 0.001$) with all the immune genes.


3 m
12 m
24 m
Vehicle
Curcumin
rNfκb1
rTnfa
rIl6
rTlr4
rTlr9
Relative mRNA expression
Zeitgeber time (ZT)

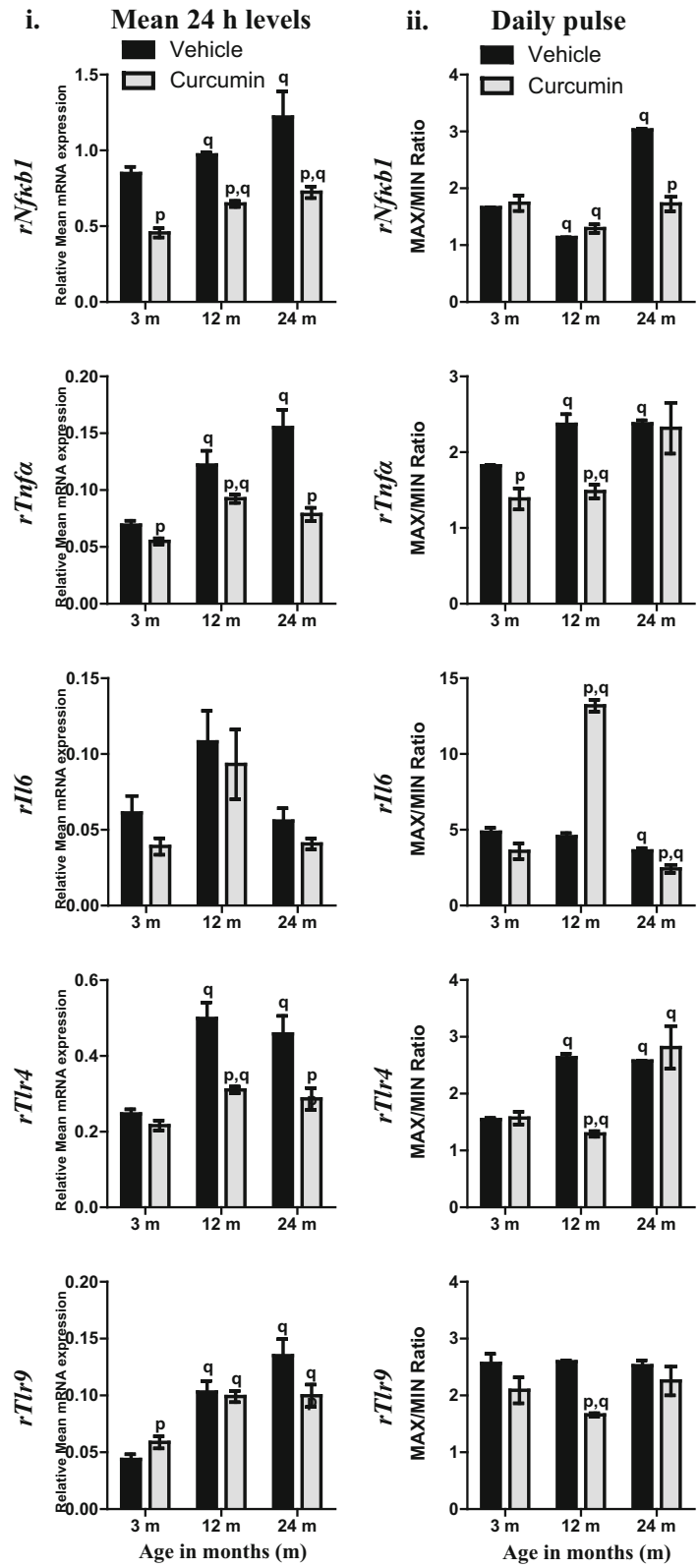


Fig. 4 Effect of curcumin administration on (i) Mean 24 h levels and (ii) Daily pulse of *rNfκb1*, *rTnfα*, *rIl6*, *rTlr4* and *rTlr9* expression in 3, 12 and 24 m old rat kidney. Each value is mean ± SEM (n = 4), $p < 0.05$ and expressed as mean relative gene expression. $p_p < 0.05$ (where 'p' refers to comparison with age-matched vehicle treated group). $p_q < 0.05$ (where 'q' refers to comparison with 3 m vehicle treated group)

Correlation analysis between immune genes and clock genes in kidney

Pairwise correlation between clock and immune genes in light phase (LP) and dark phase (DP) were analyzed in 3, 12 and 24 m animals (Fig. 5). In light phase of 3 m, *rNfκb1* and *rTnfα* showed significant positive correlation with *rRorα* and *rCry1,2* ($p < 0.001$; $p < 0.01$) and negative correlation with *rRev-erbα* ($p < 0.05$). *rIl6* showed significant positive correlation ($p < 0.001$) with *rPer1,2* genes. *rTlr4* and *rTlr9* showed significant positive correlation with *rRorα* and *rCry1,2* genes ($p < 0.001$; $p < 0.01$; $p < 0.05$). *rTlr9* also showed positive correlation with *rPer1,2* genes. In dark phase of 3 m, *rNfκb1, rTnfα*, *rIl6, rTlr4* and *rTlr9* showed significant positive correlation with *rRev-erbα* and *rPer1,2* genes ($p < 0.001$; $p < 0.01$; $p < 0.05$).

In light phase of 12 m, *rNfκb1* and *rTnfα* changed to negative correlation ($p < 0.001$) with *rCry1,* but significant positive correlation ($p < 0.001$) with *rRev-erbα* was appeared. *rIl6, rTlr4, rTlr9* changed to negative correlation with *rCry1* and *rPer1,2* genes ($p < 0.001$; $p < 0.01$; $p < 0.05$). In dark phase of 12 m, *rTnfα* showed positive correlation with *rCry1* ($p < 0.01$). *rIl6* and *rTlr4* showed negative correlation with *rPer1,2* genes ($p < 0.01$; $p < 0.05$). *rTlr4* changed to negative correlation ($p < 0.001$) with

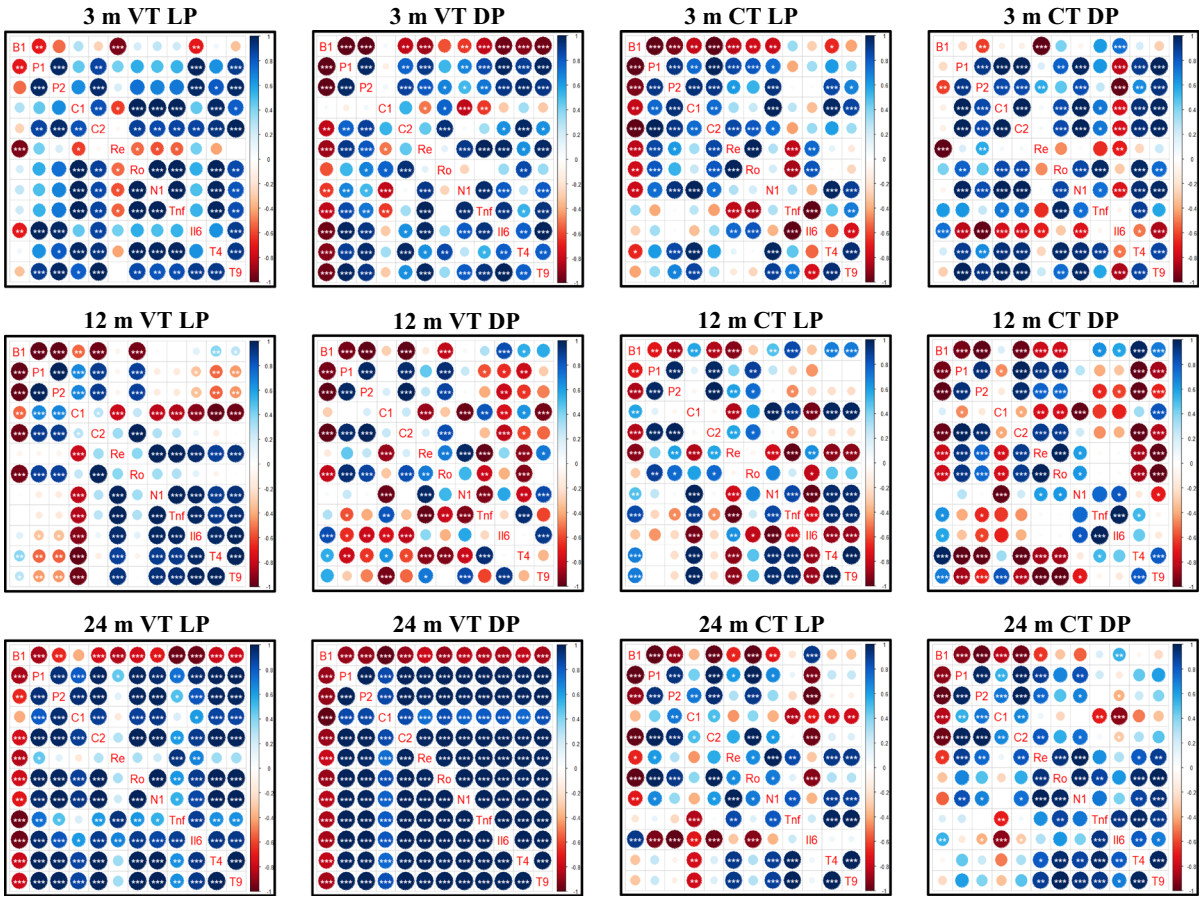


Fig. 5 Effect of curcumin administration on Pair wise correlations among *rBmal1, rPer1*, *rPer2*, *rCry1*, *rCry2*, *rRev-erbα*, *rRorα, rNfκb1*, *rTnfα*, *rIl6*, *rTlr4* and *rTlr9* in light (ZT-0, 6, 12) and dark (ZT-12, 18, 24/0) phase of 3, 12 and 24 m old rat kidney (*LP* light phase, *DP* dark phase, *VT* vehicle treated, *CT* curcumin treated). Intensity of color and size of circle represents correlation coefficient values between the genes. Where, positive correlations are indicated by shades of blue, negative correlations by shades of red and white indicates no correlation. '*', '**', '***' indicates statistically significant correlations ($p < 0.05$), ($p < 0.01$). ($p < 0.001$) respectively. (B1—*rBmal1*; P1—*rPer1*; P2—*rPer2*; C1—*rCry1*; C2—*rCry2*; Re—*rRev-erbα*; Ro—*rRorα*; N1—*rNfκb1*; Tnf—*rTnfα*; Il6—*rIl6*; T4—*rTlr4*; T9—*rTlr9*)

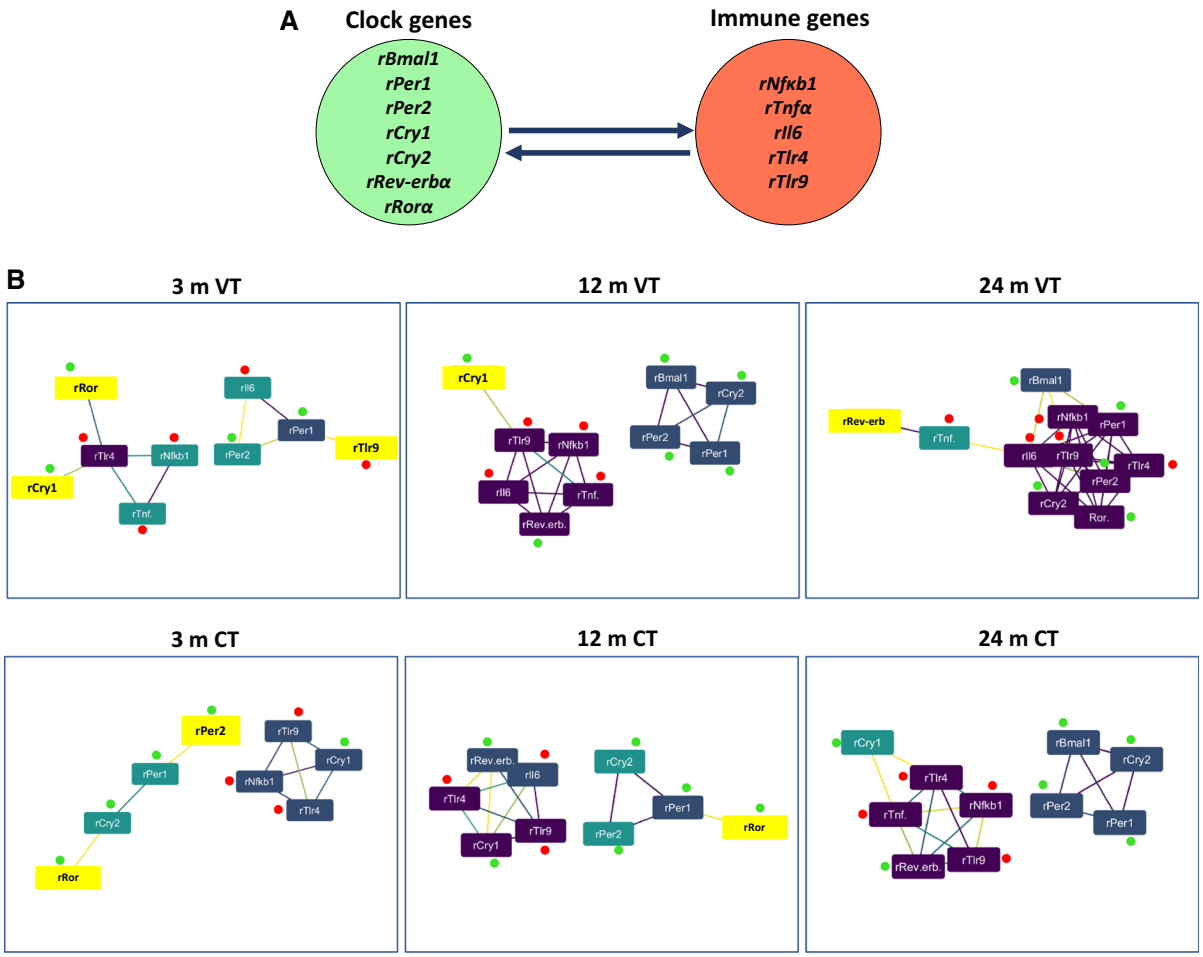


Fig. 6 WGCNA analysis between clock and immune gene clusters: **a** clock and immune gene clusters (**b**) effect aging on gene to gene network in 3, 12 and 24 m old rat kidney (upper Panel) and effect of curcumin administration (lower Panel). Color of node indicates no. of interactions (highest—purple; intermediate–sion and least—yellow). Color of edge indicates the strength of interaction (strongest—purple; sion—intermediate and weakest—yellow). Green and red dots indicate clock and immune genes respectively

rRev-erbα, but positive correlation between *rRev-erbα* and *rTlr9* persisted ($p < 0.05$).

In light phase of 24 m, positive correlation between *rNfκb1* and *rCry1,2* genes persisted ($p < 0.001$). Positive correlation of *rIl6, rTlr4* and *rTlr9* with *rPer1,2* and *rCry1,2* genes persisted ($p < 0.001$; $p < 0.05$). In dark phase of 24 m, all immune genes showed significantly altered correlations with all the clock genes where significant positive correlation appeared with all clock genes but showed negative correlation with *rBmal1* ($p < 0.001$).

Gene to gene network interactions altered with aging in kidney

WGCNA analysis demonstrated in 3 m, *rCry2* and *rRorα* showed interactions with *rTlr4*; *rPer1* showed interaction with *rIl6* and *rTlr9*. In 12 m, *rRev-erbα* showed interactions with all immune genes except *rTlr4*. In 24 m, interaction between *rRev-erbα* and *rTnfα* persisted; *rPer1,2* showed interaction with all immune genes except *rTnfα*, whereas, *rBmal1* showed interaction with *rNfκb1* and *rIl6* (Fig. 6).

Effect of curcumin administration on daily rhythms of clock genes expression in kidney

With curcumin treatment, *rBmal1* showed maximum expression at ZT-18 i.e. 6 h phase advance in 3 m animals with respect to 3 m vehicle group and minimum expression was at ZT-6. In 12 and 24 m, maximum expression persisted at ZT-0 and minimum at ZT-12 with curcumin treatment in comparison to age-matched vehicle groups. Curcumin administration reduced mean 24 h levels significantly ($p < 0.05$) in 3 and 12 m in comparison to age-matched vehicle groups, but did not show any change in 24 m. Daily pulse showed significant decrease in 3 m, did not vary in 12 m, but restored in 24 m animals with significant increase ($p < 0.05$) in comparison to age-matched vehicle group. *rPer1* showed maximum expression at ZT-6 in 3 m CT group with phase advance of 6 h in comparison to 3 m vehicle group, minimum expression was at ZT-0. In both 12 and 24 m, curcumin treatment showed similar expression as in age-matched vehicle groups. Mean 24 h levels were significantly decreased ($p < 0.05$) in 3 and 12 m with respect to age-matched vehicle groups, but in 24 m CT the decrease was not significant. Daily pulse showed significant increase ($p < 0.05$) in 3 and 12 m with curcumin treatment in comparison to age-matched vehicle groups. In 24 m CT animals, significant decrease ($p < 0.05$) was observed in comparison to 24 m vehicle group. In 3 m, curcumin treatment did not alter *rPer2* maximum expression but minimum expression was shifted from ZT-6 to ZT-0. In 12 and 24 m, curcumin administration did not change the expression pattern of *rPer2* with respect to age-matched vehicle groups. There was no significant change in mean 24 h levels of *rPer2* in 3, 12 and 24 m in comparison to age-matched vehicle groups. Daily pulse was significantly increased in 3 m but decreased in 12 and 24 m CT ($p < 0.05$) with respect to age-matched vehicle groups (Figs. 1 and 2).

In 3 m, maximum expression of *rCry1* persisted at ZT-18 but minimum expression was observed at ZT-0 with curcumin treatment. In 12 m, curcumin did not alter daily rhythm in comparison to vehicle group. In 24 m, curcumin restored maximum expression at ZT-18 in comparison to 3 m vehicle group. With curcumin administration mean 24 h levels were significantly decreased ($p < 0.05$) in all age groups with respect to age-matched vehicle groups. Daily pulse was also reduced significantly ($p < 0.05$) in all age groups. *rCry2* showed maximum expression at ZT-6 and minimum expression at ZT-0 in 3 m CT group. However, in 12 and 24 m, expression patterns were similar to the age-matched vehicle groups. Mean 24 h levels were significantly decreased ($p < 0.05$) in all age groups with curcumin administration. Daily pulse was increased in 3 m, unaltered in 12 m and decreased in 24 m ($p < 0.05$) upon curcumin treatment. In case of *rRev-erbα*, curcumin treatment did not change daily rhythm pattern in all the age groups with respect to age-matched vehicle groups. Mean 24 h levels showed significant decrease in 3 m but did not show significant change in 12 and 24 m group in comparison to age-matched vehicle groups. Curcumin treatment significantly decreased ($p < 0.05$) daily pulse in 3 and 12 m with respect to age-matched vehicle groups. However, curcumin restored daily pulse in 12 m in comparison to 3 m vehicle group. But in 24 m, daily pulse increased ($p < 0.05$) in comparison to 24 m vehicle group. *rRorα* showed maximum expression at ZT-6 and minimum expression at ZT-0 in 3 m CT group. In 12 m, curcumin restored rhythmicity with maximum expression at ZT-12 and minimum expression at ZT-6. In 24 m, maximum expression persisted at ZT-12 but minimum expression was at ZT-18 with curcumin administration. Mean 24 h levels were significantly decreased ($p < 0.05$) with curcumin in all age groups with respect to age-matched vehicle groups. Curcumin decreased daily pulse in 3 and 24 m ($p < 0.05$), however increased ($p < 0.05$) in 12 with respect to age-matched vehicle groups (Figs. 1 and 2).

Effect of curcumin administration on daily rhythms of inflammatory genes expression in kidney

Curcumin administration resulted in alleviation of expression of inflammatory genes in kidney. Curcumin administration phase delayed *rNfκb1* about 6 h with maximum expression at ZT-18 and minimum expression at ZT-0 in 3 m. In 12 m, *rNfκb1* showed restoration in comparison with 3 m vehicle group with maximum expression at ZT-12 and minimum expression at ZT-6. In 24 m, *rNfκb1* was phase advanced by 6 h with maximum expression at ZT-6 and minimum expression at ZT-18 with respect to 24 m vehicle group. Being an anti-oxidant curcumin significantly

reduced mean 24 h levels in all age groups ($p < 0.05$). Daily pulse did not show significant change in 3 and 12 m with respect to their age-matched vehicle groups. However, in 24 m, curcumin decreased ($p < 0.05$) and restored daily pulse with respect to 3 m vehicle group. *rTnfα* showed phase delay of 6 h with maximum expression at ZT-18, minimum expression at ZT-6 in 3 m CT. In 12 m, maximum expression was observed at ZT-0 which is 6 h phase advance with respect to 12 m vehicle group and minimum expression was at ZT-6. In 24 m, expression pattern was not changed with respect to 24 m vehicle group. Curcumin significantly reduced ($p < 0.05$) the mean 24 h levels in all the age groups. But in 24 m, curcumin restored mean 24 h levels in comparison with 3 m vehicle group. In 3 and 12 m, daily pulse was reduced significantly ($p < 0.05$) in comparison to age-matched vehicle group. In 24 m, daily pulse remained unaltered with respect to 24 m vehicle group. *rIl6* showed 6 h phase advance with maximum expression at ZT-6 and minimum expression at ZT-12 in 3 m CT group. In 12 m, curcumin treatment did not change expression pattern with respect to 12 m vehicle group. But in 24 m, maximum expression was observed at ZT-0 which is 12 h phase advance in comparison to 24 m vehicle group and minimum expression at ZT-18. Curcumin administration did not alter mean 24 h levels in all age groups. Daily pulse remained unaltered in 3 m, significantly increased in 12 m ($p < 0.05$), and significantly decreased in 24 m ($p < 0.05$) with cucumin administration (Figs. 3 and 4).

In 3 m curcumin treated group, *rTlr4* showed maximum expression at ZT-18 with phase delay of 6 h in comparison to 3 m vehicle group, and minimum expression was observed at ZT-0. In 12 m, maximum expression was observed at ZT-0 with the phase advance of 6 h and minimum was observed at ZT-6. In 24 m, maximum expression was observed at ZT-6 with phase advance of 6 h and minimum expression was observed at ZT-18. Curcumin administration did not change mean 24 h levels in 3 m, but significantly reduced in 12 and 24 m ($p < 0.05$) with respect to age-matched vehicle groups. Interestingly, in 24 m, curcumin restored the mean levels in comparison to 3 m vehicle group. Daily pulse did not alter in 3 m and 24 m, but significantly reduced in 12 m ($p < 0.05$) with respect to age-matched vehicle groups. With curcumin treatment *rTlr9* showed rhythmic expression with maximum at ZT-18 i.e. 6 h phase delay with respect to 3 m vehicle group and minimum at ZT-6. In 12 m, maximum expression was observed at ZT-18 with phase delay of 12 h and minimum expression at ZT-6. In 24 m, maximum expression was observed at ZT-6 with phase advance of 6 h and minimum expression was observed at ZT-18. With curcumin treatment, mean 24 h levels were significantly increased in 3 m ($p < 0.05$), remained unaltered in 12 m and significantly decreased in 24 m ($p < 0.05$) with respect to their age-matched vehicle groups. Daily pulse was unaltered in 3 and 24 m, significantly reduced in 12 m ($p < 0.05$) with curcumin treatment (Figs. 3 and 4).

Correlation analysis of clock genes with curcumin administration in kidney

In light phase of 3 m CT group, negative correlation of *rBmal1* with *rPer1* genes and *rRev-erbα* ($p < 0.001$) persisted. Within and between *rPer1,2* and *rCry1,2* genes positive correlation persisted ($p < 0.001$; $p < 0.01$). *rRorα* changed to positive correlation with *rRev-erbα* ($p < 0.001$) but positive correlation with *rCry2* ($p < 0.001$) persisted. In dark phase of 3 m CT group, negative correlation of *rBmal1* with *rRev-erbα* ($p < 0.001$) persisted. Positive correlation persisted between *rPer1,2* and *rCry1,2* genes ($p < 0.001$). *rRorα* showed insignificant negative correlation with *rRev-erbα* but significant positive correlation ($p < 0.001$) with *Cry1,2* genes persisted (Fig. 5).

In light phase of 12 m CT group, negative correlation of *rBmal1* with *rRev-erbα* was restored and negative correlation with *rPer1* gene ($p < 0.001$; $p < 0.01$) persisted. Positive correlation between *rPer1,2* genes persisted ($p < 0.001$). Curcumin administration abolished correlation between *rCry1,2* genes. *rRorα* showed significant positive correlation with *rCry2* ($p < 0.05$) but not with *rRev-erbα* and *rCry1*. In dark phase of 12 m CT group, negative correlation of *rBmal1* with *rRev-erbα* was restored and negative correlation with *rPer1* gene ($p < 0.001$) persisted. Positive correlation between *rPer1,2* genes ($p < 0.001$) persisted. Interestingly, *rCry1* showed negative correlation with *rCry2* ($p < 0.05$). *rRorα* showed positive correlation with *rCry2*, *rRev-erbα* ($p < 0.001$) and negative correlation with *rCry1* ($p < 0.01$) (Fig. 5).

In light phase of 24 m CT group, negative correlation of *rBmal1* with *rPer1* genes and *rRev-erbα* ($p < 0.001$) persisted. Positive correlation within the *rPer1,2* and *rCry1,2* genes persisted ($p < 0.001$; $p < 0.05$). In dark phase of 24 m CT group, negative correlation of *rBmal1* with *rPer1* and *rRev-erbα* ($p < 0.001$) persisted. Positive correlation within and between *rPer1,2* and *rCry1,2* genes persisted ($p < 0.001$; $p < 0.01$) (Fig. 5).

Correlation analysis of immune genes in kidney upon curcumin administration

In light phase of 3 m CT group, curcumin administration abolished correlation between *rNfκb1* and *rTnfα,* but showed significant negative correlation between *rTnfα* and *Il6* ($p < 0.001$). Positive correlation of *rNfκb1* with *Tlr9* and *Tlr4* ($p < 0.001$) persisted. In dark phase of 3 m CT group, positive correlation of *rNfκb1* with *rTnfα*, *rTlr9* and *rTlr4* ($p < 0.001$; $p < 0.05$) persisted. But *rNfκb1* changed to negative correlation with *rIl6* ($p < 0.001$).

In light phase of 12 m CT group, positive correlation of *rNfκb1* with *rTnfα*, *rTlr4* and *rTlr9* ($p < 0.001$) persisted. Significant negative correlation was observed between *rTnfα* and *rIl6* ($p < 0.001$). Significant negative correlation was observed between *rIl6* and *rTlr4,9* ($p < 0.001$). In dark phase of 12 m CT group, curcumin treatment resulted in abolition of correlation of *rNfκb1* with *rTnfα* and *rTlr4*. However, positive correlation was restored between *rNfκb1* and *rIl6* ($p < 0.05$); *rTnfα* and *rIl6* ($p < 0.001$). Interestingly, negative correlation appeared between *rNfκb1* and *rTlr9* ($p < 0.05$). Positive correlation between *rTnfα* and *rIl6* ($p < 0.001$) was restored, and positive correlation between *rTlr4* and *rTlr9* ($p < 0.001$) was restored.

In light phase of 24 m CT group, positive correlation of *rNfκb1* with *rTnfα* and *rTlr4,9* ($p < 0.001$; $p < 0.01$) persisted. Curcumin administration resulted in abolition of correlation between *rNfκb1* and *rIl6*, and resulted in restoration in comparison to LP of 3 m vehicle group. Correlation between *rIl6* and *rTlr4,9* abolished. In dark phase of 24 m CT group significant correlation of *rNfκb1* with *rTnfα* abolished, but positive correlation with *rTlr4,9* ($p < 0.001$; $p < 0.01$) persisted. Positive correlation of *rIl6* with *rTlr4,9* ($p < 0.01$; $p < 0.05$) persisted.

Effect of curcumin on pairwise correlation between inflammatory genes and clock genes in kidney

In light phase of 3 m CT group, positive correlation of *rCry1,2* genes with *rNfκb1* ($p < 0.001$; $p < 0.05$) persisted but abolished with *rTnfα*. Positive correlation of *rTlr4* and *rTlr9* with *Cry1* ($p < 0.001$) persisted. Curcumin resulted in abolition of correlation between *rIl6* and *rPer1,2* genes. In dark phase of 3 m CT group, significant positive correlation of *rPer1,2* genes with *rNfκb1* ($p < 0.001$) persisted. Positive correlation of *rPer1,2* with *Tlr4* and *Tlr9* persisted ($p < 0.001$; $p < 0.01$) (Fig. 5).

In light phase of 12 m CT group, *rNfκb1, rTnfα* and *rTlr4,9* changed to positive correlation with *rCry1* and *rRev-erbα*. Positive correlation of *rTlr4* and *rTlr9* with *rPer1,2* genes abolished. In dark phase of 12 m CT group, *rTlr9* showed significant negative correlation with *rPer1,2* genes ($p < 0.001$). Correlation of *rCry1* with *rTnfα, rIl6* and *rTlr4* abolished. Correlation between *rRev-erbα* and *rTnfα* abolished (Fig. 5).

In light phase of 24 m CT group, correlation between *rTlr4,9* and *rPer1,2* genes abolished. *rIl6* showed negative correlation with *rPer1,2* genes ($p < 0.001$). Correlation between *rNfκb1* and *rCry1* abolished. *rIl6* and *rTlr4,9* changed to negative correlation with *rCry1*. In dark phase of 24 m CT group, there was abolition of correlation between *rTlr4.9* target gene C and *rPer1,2* genes. *rTnfα, rIl6* changed to negative correlation with *rCry1*. Correlation between *rCry1* and *rNfκb1* abolished with curcumin treatment (Fig. 5).

Curcumin differentially altered gene to gene network interactions in kidney

WGCNA analysis demonstrated in 3 m CT, *rCry1* showed interactions with *rNfκb1*, *rTlr4,9*. In 12 m CT, interactions of *rRev-erbα* with *rIl6* and *rTlr9* persisted and *rBmal1* lost the interactions with other clock genes. In 24 m CT, the interactions between clock and immune genes showed similarity with the interactions observed in 12 m VT, where *rRev-erbα* showed interactions with all immune genes except *rIl6*; *rBmal1*, *rPer1,2, rCry2* showed interactions with each other (Fig. 6).

Discussion

In the present study, all the clock genes studied showed significant daily rhythms in kidney of 3 m old animals (Fig. 1). Elevated levels of *rBmal1* at dark phase are corroborated with previous studies in different peripheral clocks across different species (Christiansen et al. 2016; Yang et al. 2016). This emphasizes the importance of well organised synchrony between the clocks for a better survival of an organism (Hatori et al. 2017). Interestingly, in 3 m rat SCN, *rBmal1* maximum expression was seen at ZT-18 (Mattam and Jagota 2014) which is 6 h earlier to kidney, this further demonstrate the relation between master and slave clocks (Balsalobre 2002). *rPer1* and *rPer2* showed offset at ZT-12 (Fig. 1) and is in agreement with previous studies in different tissues and species (Pizarro et al. 2013; Yang et al. 2016). However, in SCN, only *rPer2* showed peak expression at ZT-12 (Mattam and Jagota 2014). Expression pattern of *rCry1, rCry2* and *rRev-erbα* (Fig. 1) also corroborates to the previous studies (Takeda et al. 2012; Yang et al. 2016; Astafev et al. 2017). In the present study, *rPer1* did not show any change in expression with aging. Interestingly, mRNA levels of αENaC (alpha subunit epithelial Na^+ channel), essential for regulation of salt and water reabsorption was reported to be under PER1 regulation (Gumz et al. 2009), did not vary with aging (Haloui et al. 2013). *rBmal1, rPer2, rCry2* and *rRev-erbα* also did not show significant variations across the age groups studied (Fig. 1). However, in SCN *rBmal1* and *rPer1* showed variations as age progress (Mattam and Jagota 2014). This implies that SCN is more sensitive towards age-related attritions than peripheral clock kidney. However, in kidney only *rRorα* exhibited mid-age perturbations, where the rhythmicity was completely abolished. Interestingly, all the clock genes did not show significant variation in mean 24 h levels with aging (Fig. 2). On the other hand, SCN displayed an increase in the mean 24 h levels of *rPer2*, *rCry1* and *rCry2* in 12 m (Mattam and Jagota 2014) further signifying the sensitivity of central clock in mid-age.

rBmal1 showed significant negative correlation with *rPer1* and *rRev-erbα* in both light phase and dark phase of 3 m (Fig. 5). These tightly regulated interactions are essential for the sustained metabolism in organisms (Solt et al. 2011). However, *rBmal1* did not show any correlation with rRor α in LP and DP of 3 m (Fig. 5), which is in agreement with the minimal role of rRor α on circadian clock in kidney (Takeda et al. 2012). Positive correlation between *rCry1* and *rCry2* in LP did not vary with aging in kidney in present study (Fig. 5) but abolished in SCN (Mattam and Jagota 2014). Negative correlation between *rBmal1* and *rPer1* observed in kidney was not observed in SCN 3 m (Mattam and Jagota 2014). This emphasizes the fact that central and peripheral clocks exhibit several variations though similar transcriptional feedback loops are involved in both (Schibler et al. 2015).

Recently, we reported restoratory effect of curcumin administered at ZT-11 on *rPer1*, *rPer2, rCry1* and *rCry2* in 12 m and *rPer1* in 24 m rat SCN (Kukkemane and Jagota 2019). Here we explored the chronobiotic properties of curcumin on peripheral clock kidney for the first time. Curcumin administration did not show significant changes in expression pattern of *rBmal1* and *rRev-erbα*. However, *rPer1*, *rCry1* and *rCry2* were altered with curcumin treatment only in 3 m but remained unaltered in 12 and 24 m animals with respect to their age-matched vehicle groups. Further detailed study is required to understand the underlying mechanism. Interestingly, only *rRor-α* showed sensitivity towards curcumin in all age groups studied (Fig. 1).

Time dependent immune responses are well documented in several immune cells; and there are remarkable evidences to show that these are cell and tissue specific circadian regulations (Curtis et al. 2014). NFκB1, an important regulatory transcription factor in inflammation, plays a central role in inducing transcription of *Tnfα*, *Il6* and several other cytokines and also involved in apoptosis, cellular growth and differentiation (Hoesel and Schmid 2013). Several studies showed that these inflammatory genes in immune cells show diurnal expression in rodents (Keller et al. 2009; Cermakian et al. 2013; Curtis et al. 2014). Interestingly, in our study, we observed that *rNfκb1, rTnf-α* and *rIl6* showing significant daily rhythms in 3 m kidney (Fig. 3) and corroborates to the previous reports in immune cells (Curtis et al. 2014). In addition, LPS induced phase shift of circadian rhythms in SCN were observed to be through TNFR1 receptors (Paladino et al. 2014). In this context, it would be of greater importance to understand role of *Tnfα* on circadian rhythms in kidney to address renal chrono-inflammatory aberrations. *Tlr9* contains

canonical E-boxes at its promoter site where CLOCK/BMAL1 complex can induce the expression, but its circadian rhythms are cell and tissue specific (Silver et al. 2012). In our study, we observed both *rTlr9* and *rTlr4* showing peak expression at ZT-12 (Fig. 3) which is similar to expression seen in inflammatory cells (Silver et al. 2012, 2018). Interestingly, all the immune genes studied showed phase advance of 6 h in 12 m but remained unaltered in 24 m in comparison to 3 m kidney (Fig. 3). Further studies are essential in understanding the mid-age perturbations in chrono-immune system.

Curcumin administration had profound effect on daily rhythms of all the immune genes. Interestingly, curcumin had similar chronomodulatory effects on *rNfκb1* and *rTnfα* in all age groups (Fig. 3). This could be because of curcumin's regulation on *rTnfα* through NFκB1. Several researchers demonstrated the role of elevated pro-inflammatory molecules like NFκB1 and TNFα in various renal disorders (Tilstra et al. 2011; Wang et al. 2017). Here we report that *rNfκb1* and *rTnfα* expressions were significantly elevated with aging (Fig. 4) which corroborates to previous studies (Tilstra et al. 2011; Xi et al. 2014). Curcumin being an anti-inflammatory molecule reduced the expression of *rNfκb1* and *rTnfα* in all age groups and restored *rTnfα* in 24 m kidney (Fig. 4). Further, we also report the gradual increase in transcription of *rTlr4* and *rTlr9* with aging which supports previous studies (Xi et al. 2014). Curcumin reduced the mean 24 h levels of *rTlr4*, which is in agreement with previous studies (Zhu et al. 2014) and restored in 24 m animals (Fig. 4). It has been reported that the anti-inflammatory action of curcumin could be through the activation of Nrf2 which was shown to attenuate inflammatory responses (Wardyn et al. 2015). Interestingly, in 3 m, curcumin significantly increased *Tlr9* levels but remained unaltered in 12 and 24 m with respect to age-matched vehicle groups (Fig. 4).

Pairwise correlation analysis revealed the change of positive correlation of *rNfκb1* with *rTnfα* and *rTlr4* in DP of 12 m kidney (Fig. 5); this suggests the deregulated interactions between inflammatory genes with aging. *rNfκb1* showed significant positive correlation with other immune genes in 24 m, this further suggests altered inflammatory status with aging. Curcumin showed significant alterations in correlations among the immune genes as it reduced the expression of several immune genes (Fig. 5).

We also correlated clock genes and immune genes in order to understand the possible interactions with each other. *rPer1* showed positive correlation with *rIl6* in LP and DP of 3 m animals (Fig. 5), whereas PER1 negatively regulates IL-6 expression in spinal astrocytes (Sugimoto et al. 2014). *Tlr9* shows *Per2* dependent circadian expression in macrophages (Silver et al. 2012), we also observed positive correlation between *rPer2* and *rTlr9* in LP and DP of 3 m animals (Fig. 5). REV-ERBα shows inhibitory action on TLR4 expression in human macrophages (Fontaine et al. 2008), but in our study we observed insignificant negative correlation between *rRev-erbα* and *rTlr4* in LP of 3 m animals (Fig. 5). In LP and DP of 12 m, these correlations were altered (Fig. 5), suggesting desynchrony between immunity and circadian clock. CRY proteins were proposed to inhibit *Il6* expression by blocking NFκB activity in fibroblasts and macrophages (Narasimamurthy et al. 2012). Interestingly, in our study we observed a positive correlation between *rCry2* and *rIl6* (Fig. 5). In another study, overexpression of CRY1 reduced the TLR4 expression in atherosclerosis mouse model (Yang et al. 2015). But, we observed a positive correlation between *rCry1* and *rTlr4* in LP of 3 m animals (Fig. 5). Though our study involves mRNA expression, the study at protein level may yield a better understanding on such interactions. In DP of 24 m, all the immune genes showed significant positive correlation with all clock genes except *rBmal1*, this provides significant basis for desynchronised clock and immune systems with aging (Fig. 5). WGCNA analysis between clock and immune gene clusters demonstrated that in young age clock and immune genes exhibit interactions in two different groups with *rTlr4* and *rPer1* being the hub genes and intensity of the interactions are medium to weak (Fig. 6). With aging the *rRev-erbα* showed maximum interactions with immune and with increased strength of interactions. In 24 m, interactions between clock and immune genes were increased with increased intensity of strength. This suggests that increased inflammatory status with aging might be because of subtle change in clock system. However, with curcumin treatment in 24 m, interactions between clock and immune genes showed similarity with 12 m VT, suggesting the potential of curcumin as chronobiotic to regulate both clock and immune system (Fig. 6).

The present study demonstrates that aging renders desynchronization between the expression of clock and immune genes in kidney. Curcumin administration resulted in differential restoration of immune gene expressions and their correlation with clock genes in aged kidney. Our results have given novel insights of curcumin as a chronobiotic on immune genes to further establish it as a potent drug against age associated chrono-immune attritions.

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