Therapeutic effects of hydro-alcoholic leaf extract of *Withania somnifera* on age-induced changes in daily rhythms of *Sirt1, Nrf2* and *Rev-erbα* in the SCN of male Wistar rats

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Abstract The temporal expression pattern of the circadian clock genes are known to be altered/attenuated with advance in age. *Withania somnifera* (WS) essentially consists of numerous active constituents including withanolides is known to have antioxidant, anti-inflammatory and adaptogenic properties. We have earlier demonstrated therapeutic effects of hydro-alcoholic leaf extract of WS on the age-induced alterations in the levels and daily rhythms of various clock genes such as *rBmal1, rPer1, rPer2* and *rCry1*. We have now studied effects of hydro-alcoholic leaf extract of WS on the age-induced alterations in the levels and daily rhythms of expression of SIRT1 (an NAD$^+$ dependent histone deacetylase and a modulator of clock) and NRF2 (a clock controlled gene and a master transcription factor regulating various endogenous antioxidant enzymes) in addition to *rRev-erbα* in SCN of adult [3 months (m)], middle-aged (12 m) and old-aged (24 m) male Wistar rats. The daily rhythms of *rNrf2* expression showed 6 h phase delay in middle age and 12 h phase advance in old age. WS restored *rSirt1* daily rhythms and phase in old age whereas it restored the phase of *rNrf2* in the SCN of both middle and old aged animals. At protein level, SIRT1 expression showed phase advances in 12 m and 24 m whereas NRF2 daily rhythms were abolished in both the age groups. WS restored the phase and daily rhythms of SIRT1 as well as NRF2 in 12 m old rats. However, *rRev-erbα* expression was found insensitive to WS treatment in all the age groups studied. Pairwise correlation analysis demonstrated significant stoichiometric interactions among *rSirt1, rNrf2* and *rRev-erbα* in 3 m which altered with aging significantly. WS treatment resulted in differential restorations of such interactions.

Keywords Aging · Ashwagandha · Circadian clock · NRF2 · SCN · SIRT1

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Introduction

Circadian time keeping system (CTS) in mammals consists of endogenous, entrainable molecular clocks that regulate physiological processes in coordination with 24 hour (h) solar day (Jagota et al. 2000; Reppert and Wever 2002). Suprachiasmatic nucleus (SCN) functions as the master pacemaker and regulates peripheral clocks present in all tissues (Jagota 2012).

Progressive deterioration of CTS during aging and in age related diseases has been evident with numerous reports (Mattis and Sehgal 2016; Popa-Wagner et al. 2017). However, the underlying mechanisms remain elusive as there are reports showing absence of cell loss or pathological conditions in SCN with aging (Hofman and Swaab 2006). Studies reporting the diminished daily rhythms of AVP and decreased VIP expression in elderly (Hofman and Swaab 1994; Vosko et al. 2007), decreased sensitivity of SCN towards melatonin in old aged rodents (Touitou 2001; Jagota and Kalyani 2010), decreased pineal melatonin secretion in elderly and declined melatonin feedback (Wu et al. 2007; von Gall and Weaver 2008) suggests that these could be influencing age associated dysfunction of CTS. Altered cytokine levels, activation of microglia and astrocytes in SCN of aged rodents suggested a role for neuroinflammation in age associated circadian dysfunction (Beynon and Coogan 2010; Deng et al. 2010). Reports demonstrating disturbed sleep architecture and core body temperature indicates the inability of CTS in entraining to the external environment upon aging (Hofman and Swaab 2006; Arellanes-Licea et al. 2014). Alterations in the expression of clock genes and proteins in SCN and several other brain regions of old aged rodents have been reported (Wyse and Coogan 2010; Duncan et al. 2013; Bonaconsa et al. 2014). We have earlier reported significant age-induced alterations in daily rhythms of serotonin metabolism (Jagota and Kalyani 2010; Reddy and Jagota 2015) and clock gene expression in SCN (Mattam and Jagota 2014). Additionally, alterations in daily rhythms and levels of antioxidant enzymes, leptin, NO and *Socs1* in peripheral clocks in addition to altered gross locomotor activity with aging had demonstrated the deterioration of CTS at several levels upon aging (Manikonda and Jagota 2012; Reddy and Jagota 2014; Vinod and Jagota 2016, 2017; Jagota and Mattam 2017; Thummadi and Jagota 2019).

Several compounds from natural sources have potential therapeutic effects on clock related disorders (Chen et al. 2013; He et al. 2016). Nobelitin, resveratrol and curcumin are anti-oxidants reported to exert beneficial effects in aging and several metabolic ailments by targeting clock components (Jagota and Reddy 2007; Gloston et al. 2017). We have earlier reported the beneficial role of melatonin and recently of curcumin in restoring the age related

alterations in clock gene expression (Mattam and Jagota 2014; Kukkemane and Jagota, 2019; Thummadi and Jagota, 2019). *Withania somnifera* (WS), popularly known as Ashwagandha, is categorized as a 'Medhya Rasayana' herb in Ayurveda owing to its extraordinary neuro-stimulatory properties. With several active constituents such as various withanolides and alkaloids, it is known to possess anti-oxidant, adaptogenic, neuroprotective, neuro-regenerative and anti-neuroinflammatory properties (Wadhwa et al. 2016; Jagota and Kowshik 2017; Manchanda et al. 2017; Gupta and Kaur 2018; Pandey et al. 2018; Savage et al. 2018).

Circadian clocks operate via interlocking transcriptional translational feedback loops (TTFL) consisting of CLOCK:BMAL1 heterodimers transcriptionally activating the repressors PER:CRY as well as other clock controlled genes. In addition to this major loop, two auxiliary loops involving RORs, REV-ERBs, DBP and NFIL3 function together to generate overt rhythms with a periodicity of approximately 24 h (Takahashi 2017). There has been accumulating evidence on reciprocal interactions between TTFL and subcellular pathways involved in redox metabolism (Ray and Reddy 2016). *Nampt*, the gene responsible for biosynthesis of redox cofactor NAD^+ is under the regulation of clock. SIRTUIN1 (SIRT1), a NAD^+ dependent deacetylase is involved in transcriptional activation of *Bmal1* and also in feedback regulation of TTFL by deacetylating PER2 and BMAL1 thus acting as clock modulator (Pritchett and Reddy 2017). In addition, nuclear erythroid 2-related factor 2 (NRF2) the master transcription factor regulating antioxidant system is a clock effector molecule, as it is under the control of BMAL1-CLOCK (Lee et al. 2013; Pekovic-Vaughan et al. 2014).

We have reported earlier the restoratory effects of hydro-alcoholic leaf extract of WS leaves on age-induced alterations in various clock genes such as *rPer1, rPer2, rCry1, rCry2* and *rBmal1* in the SCN of middle and old aged rats (Jagota and Kowshik 2017). In middle aged rats, the phase of *rBmal, rPer1, rCry1* and daily pulse of *rPer2* showed restoration upon WS treatment. In old age, WS restored the phase of *rPer1* in addition to the restoration of *rPer2* and *rBmal1* mean 24 h levels (Jagota and Kowshik 2017). We have studied earlier age-induced changes in *rRev-erbα* and *rSirt1* (Kukkemane and Jagota 2019). We have

now further studied the effect of aging on levels and daily rhythms of clock effector NRF2 in the SCN.

Here we report the effect of hydro-alcoholic extract of WS leaves (with 2% withanolides) on age-induced alterations in daily rhythms and levels of one of the core clock gene *rRev-erbα* in addition to clock modulator SIRT1 and clock effector NRF2 in rat SCN.

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 with body weights in the range of 150–200 g, 320–380 g and 400–450 g respectively were used for the study. Rats of respective age groups were procured from National Center for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad, India. Each age group (n = 48) was further separated into three groups of 16 animals each as, (A) Control (B) WS leaf extract treated (WST) and (C) Vehicle treated (VT). Rats were housed individually in polypropylene cages and maintained at 23 ± 1 °C, and 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. All the animals were provided food and water ad libitum. Cages were changed at random intervals. Dim red light was used for handling animals in the dark. All the experiments were performed as per Institutional Animal Ethics Committee (IAEC) guidelines (approval number IAEC/UH/151/2016/05/AJ/P12/Rats Wistar/M-144).

WS leaf extract administration

The hydro-alcoholic leaf extract of *Withania somnifera* which was found to have 80% free radical scavenging activity at 1 mg/ml concentration was used in the present study (Jagota and Kowshik 2017).

Carboxy methyl cellulose (CMC) (0.5%) as vehicle was prepared freshly, to this 100 mg/ml w/v of Hydro-alcoholic leaf extract of WS (Herbochem, India) was added and suspended by constant stirring for at least 30 min. 100 mg/kg body weight of WS leaf extract was administered orally at ZT-11 for 15 days to Group IB, IIB and IIIB. Group IC, IIC and IIIC were similarly administered with 0.5% CMC (1 ml/kg body weight).

SCN tissue preparation

On 16th day animals of all three age groups were decapitated at ZT-0, 6, 12 and 18 (n = 4 at each time point) and brains were dissected out carefully. 500 μ brain slices were made using rat brain slicer (Zivic Instruments; Pittsburg USA) and the SCN was carefully punched out (Mattam and Jagota 2015).

RNA extraction and cDNA synthesis

RNA extraction and cDNA synthesis was done as per method of Kamphuis et al. 2005. Total RNA was extracted from SCN using TRI reagent following the manufacturer's protocol (Sigma). Isolated RNA was dissolved in 20 μl RNase free water. Concentration and purity of extracted RNA was quantified by measuring the optical density (OD) at 280 nm and by 260/280 ratio respectively with Nano drop spectrophotometer (Thermo Fischer). cDNA synthesis was performed using Bio-Rad iScript cDNA synthesis kit and was finally diluted 1:20 in RNase free water. Aliquots of 4 μl was used for real time quantification (Mattam and Jagota 2014).

Quantitative reverse transcriptase PCR (qRT-PCR)

Expression of *rSirt1*, *rNrf2*, *rRev-erbα* transcripts were measured by qRT-PCR by the SYBR Green (Applied Biosystems, Foster, USA) detection method (Mattam and Jagota 2014). Gene specific primers for *rSirt1, rNrf2* and *rRev-erbα* were designed using IDT PrimerQuest tool (www.idtdna.com/PrimerQuest) (Kukkemane and Jagota 2019). Primer sequences used in the present study were:

rSirt1	Forward—5′CTTGGAGCAGGTTGCAGGAAT3′ Reverse—5′GGACACCGAGGAACTACCTGAT3′
rNrf2	Forward—5′CTACTCCCAGGTTGCCCACATT3′ Reverse—5′GCTCTCAACGTGGCTGGGAATA3′
rRev-erbα	Forward—5′GGTGACCTGCTCAATGCCATGTT3′ Reverse—5′CGAGCGGTCTGCAGAGACAAGTA3′

β-actin   Forward—
          5′AGCCATGTACGTAGCCATCC3′
          Reverse—
          5′CTCTCAGCTGTGGTGGTGAA3′

Dissociation curves for all the genes showed a single peak representing specifically amplified target (Supplementary Fig. 1). Threshold cycle (Ct) values were obtained from the exponential phase of amplification plots. The relative quantitative expression of clock genes were obtained by normalizing their expression in relation to expression of β-*actin* (ΔCt = target gene Ct − β-*actin* Ct) in each sample that is equal to $2^{-\Delta Ct}$ (Livak and Schmittgen 2001).

Protein extraction

Individual SCN were homogenized with four volumes of RIPA buffer (1% deoxycholate, 0.1% SDS, 1% TritonX-100, 150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4) containing protease inhibitor cocktail (Roche). Homogenate was placed in 4 °C with intermittent agitation for 2 h. Centrifuged at 12,000 rpm for 20 min at 4 °C. Supernatant was collected and protein estimation was carried out by Bradford's method (Bradford 1976).

Western blot analysis

Protein samples (30 μg) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis using miniVE vertical electrophoresis system (Amersham Biosciences) with 5% stacking gel (pH 6.8) and 10% resolving gel (pH 8.8) (Laemmli, 1970). Prestained protein markers (Puregene) were loaded to assess the molecular weight of protein bands. Proteins were blotted to PVDF membrane by wet transfer method (towbin buffer: 25 mM Tris base, 192 mM Glycine, 20% methanol and 0.02% SDS) over night at 4 °C. Membranes were incubated for 1 h in blocking solution (7.5% nonfat milk powder in Tris buffered saline (pH 7.4) containing 0.05% Triton X (TBST)). Probing was done by incubating the membranes in mouse monoclonal antibody against SIRT1 and NRF2 (1:5000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4 °C. Secondary antibody incubations were done at room temperature for one hour using Goat anti-mouse IgG conjugated with horse radish peroxidase (HRP) (1:10,000) (Santa Cruz

Biotechnology, Inc. Santa Cruz, CA). Signals were developed using femtoLUCENT-HRP kit (G Biosciences) and captured using Chemidoc imaging system (Biorad) and processed using Image lab software (Biorad). Probing of β-TUBULIN (1:5000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was done as loading control. Densitometry analysis were performed by using Image J software.

Data analysis

Statistical analysis

Jandel Scientific Sigma stat software and GraphPad prism were used for the data analysis and generation of figures respectively. Multiple comparisons of all parameters obtained at various time points within the age group were analyzed by one way ANOVA followed by Post hoc Dunkan's test. Student's t test was performed to compare control and treated groups. Correlation plots were drawn using corrplot package in R-program (Haarman et al. 2014). Effect of WS treatment on pair wise correlation was analyzed between mean light (ZT-0, 6, 12) and dark (ZT-12, 18, 24/0) phase levels of *rRev-erbα, rSirt1* and *rNrf2* in various age groups 3, 12 and 24 m in rat SCN (Kukkemane and Jagota 2019).

The temporal pattern of mRNA expression data plotted in Fig. 1 was analyzed using "CosinorWin'' software (Table 1). This software normalizes the data and generates cosine fitted curves and gives specific values for characteristics of rhythms such as mesor (circadian rhythm-adjusted mean based on the parameters of a cosine function), amplitude (half the total rhythmic variability in a cycle within 24 h period), acrophase (the time at which peak of a rhythm occurs) and 'r' (correlation coefficient of the rhythm) (Nachiyar et al. 2011; Manikonda and Jagota 2012).

Results

Age-induced alterations in daily rhythms and levels of NRF2 in SCN

Expression of *rNrf2* transcripts showed daily rhythmicity with maximum expression at ZT-12 and minimum at ZT-0 in 3 m group. Aging altered the daily rhythm of *rNrf2* with 6 h phase delay in 12 m
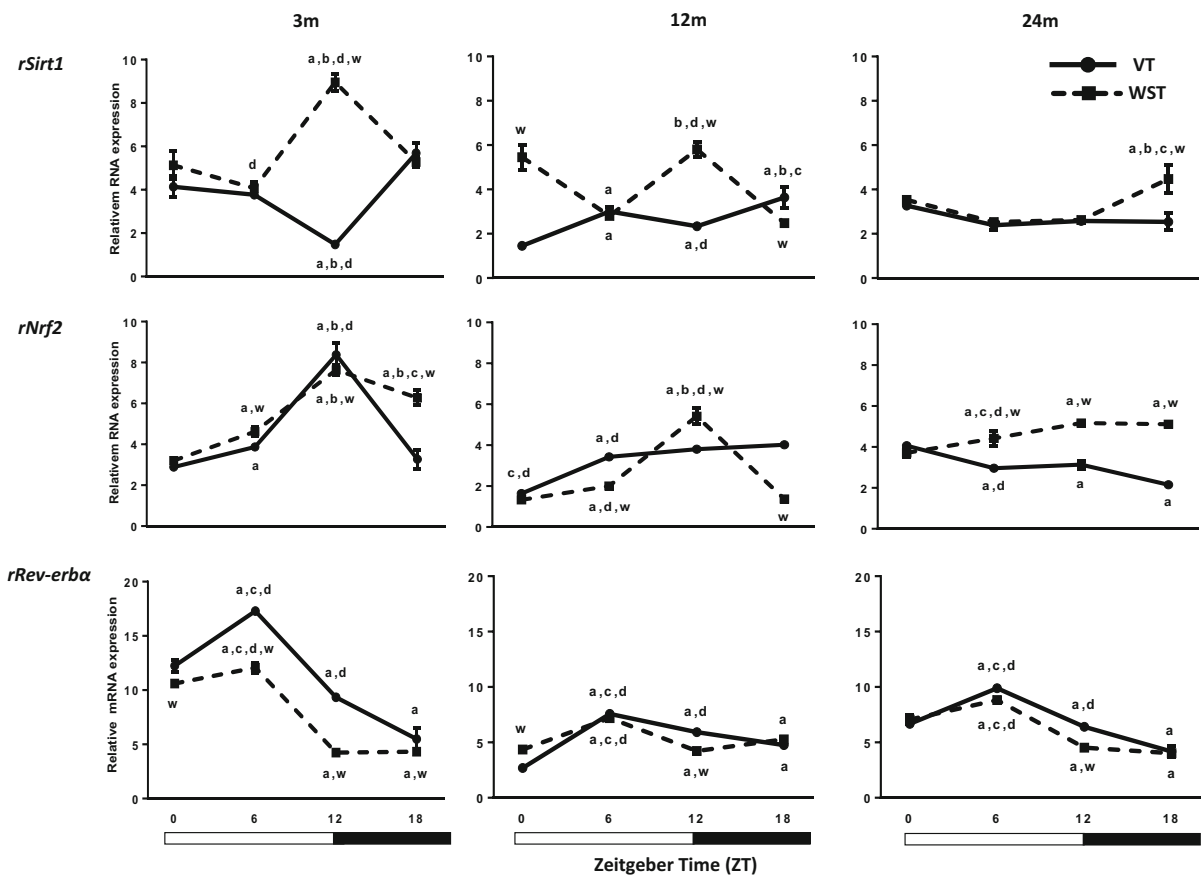
Fig. 1 Effect of administration of hydro-alcoholic leaf extract of *Withania somnifera* on daily rhythms of *rSirt1*, *rNrf2* and *rRev-erbα* mRNA expression in the SCN of 3, 12 and 24 months (m) old rats. Each value is mean ± SEM (n = 4), $p < 0.05$ and expressed as relative gene expression. $p_a < 0.05$; $p_b < 0.05$, $p_c < 0.05$ 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). $p_w < 0.05$ (where 'w' refers to comparison of gene expression levels at same time point in the age matched vehicle group)

and 12 h phase advance in 24 m groups along with diminished amplitudes in both the age groups compared to 3 m controls (Fig. 1). Further, cosinor analysis showed a decline in *rNrf2* mesor from 4.6 in 3 m to 3.0 in 24 m (Table 1). *rNrf2* mean 24 h levels appeared to be declining upon aging though the decrease was not statistically significant ($p \geq 0.05$) (Fig. 2i). But daily pulse decreased by 1.2 folds from 3 to 12 m and by a significant 1.5 folds from 3 to 24 m in VT animals ($p < 0.05$) (Fig. 2ii). Similar to mRNA, NRF2 protein expression in SCN of adult animals showed a rhythmic pattern with maximum levels at ZT-12. Aging severely altered the daily rhythm of NRF2 with abolishment of rhythms in both 12 m and 24 m groups (Fig. 3i, ii). However, NRF2 mean 24 h levels as well daily pulse did not vary significantly upon aging (Fig. 3ivA, ivB).

Effect of *Withania somnifera* leaf extract on age-induced alterations in SIRT1 and NRF2 expression levels and daily rhythms

rSirt1 mRNA expression in SCN of 3 m animals followed a rhythmic pattern with maximum expression at ZT-18 and minimum expression at ZT-12. In 12 m vehicle treated animals, amplitude of the rhythm was reduced though peak expression was comparable to 3 m vehicle control. However in 24 m old control animals, *rSirt1* transcript expression was arrhythmic (Fig. 1). At protein level as well SIRT1 expression in 3 m control animals followed a rhythmic pattern with maximum expression at ZT-12. In middle aged animals, SIRT1 daily rhythm showed a 6 h phase advance in comparison to 3 m controls with peak expression at ZT-6 and minimum at ZT-12. Similarly

Table 1 Effect of *Withania somnifera* treatment on characteristics of daily rhythms of *rSirt1*, *rNrf2* and *rRev-erbα* transcripts in SCN of 3, 12 and 24 months old rats

Sl. no	Genes	Characteristics	Age					
			3 m		12 m		24 m	
			VT	WST	VT	WST	VT	WST
1	*rSirt1*	Mesor	3.7	5.8	2.6	4.1	2.6	3.2
		Amplitude	1.6	2.0	0.5	0.2	0.3	1.0
		Acrophase	15:36	13:10	14:22	9:10	23:11	19:41
		r	− 0.24	0.62	0.15	0	0.66	− 0.61
2	*rNrf2*	Mesor	4.6	5.4	3.2	2.5	3.0	4.6
		Amplitude	2.7	2.3	1.1	2.0	0.6	0.7
		Acrophase	0:24	22:39	13:10	23:25	2:45	13:52
		r	0.86	0.75	0.72	0.82	0.08	0.52
3	*rRev-erbα*	Mesor	11.0	7.8	5.2	5.2	6.7	6.1
		Amplitude	6.0	5.0	2.1	0.9	2.8	2.7
		Acrophase	5:40	3:22	9:15	5:44	5:49	4:80
		r	− 0.88	− 0.19	0.11	− 0.56	− 0.98	− 0.55

Each Value is derived from the data collected at ZT 0, 6, 12 and 18 (n = 4 at each time point) by using "Cosinorwin'' software which normalizes the data generating Cosine fitted curves and gives specific values for the characteristics of rhythms such as mesor (circadian rhythm-adjusted mean based on the parameters of a cosine function), amplitude (half the total rhythmic variability in a cycle within 24 h period), acrophase (the time at which peak of a rhythm occurs) and 'r' (correlation coefficient of the rhythm)

in 24 m old control animals, SIRT1 rhythms were phase advanced by 12 h when compared to 3 m group with maximum and minimum expression at ZT-0 and ZT-6 respectively (Fig. 3i, ii). Mean 24 h levels of SIRT1 did not vary significantly between 3 and 12 m, however in 24 m there was a significant 1.4 folds increase compared to 3 m ($p < 0.05$) (Fig. 3iiiA). Similarly, there was no significant change in daily pulse of SIRT1 from 3 to 12 m. But it decreased by a significant 1.3 folds from 3 to 24 m in vehicle group ($p < 0.05$) (Fig. 3iiiB).

With WS administration to adult animals there was a 6 h phase advance in *rSirt1* in comparison to 3 m vehicle group, with significantly higher expression at ZT-12 ($p < 0.05$). WS administration to 12 m animals resulted in 6 h phase advance in comparison to both 12 m and 3 m VT. Interestingly, WS resulted in restoration of *rSirt1* expression rhythm in 24 m animals with peak expression at ZT-18, indicative of a phase restoration (Fig. 1). The change in mean 24 h levels upon WS treatment was not significant in any of the age groups compared to age matched control animals (Fig. 2i). Similarly, daily pulse of *rSirt1* in all

the three age groups were comparable to respective controls with no significant difference (Fig. 2ii).

SIRT1 protein expression in 3 m group treated with WS followed a rhythmic pattern though there was a 6 h phase advance compared to age matched controls with maximum expression at ZT-6 and minimum at ZT-12. WS administration in 12 m animals resulted in 6 h phase delay in peak expression in comparison to 12 m VT thus restoring the phase. However, in 24 m WST animals SIRT1 expression peaked at ZT-18, which is an 18 h phase delay in comparison to age matched controls and 6 h phase delay when compared to 3 m controls (Fig. 3i, ii). Mean 24 h SIRT1 levels upon WS treatment to 12 m animals increased by 1.6 folds when compared to 3 m vehicle controls and by 1.3 folds when compared to age matched controls (p < 0.05). Similarly, there was a 1.5 fold increase in 24 m WS animals compared to adult controls (p < 0.05) (Fig. 3iiiA).

With WS treatment, *rNrf2* transcripts showed rhythmic expression in 3 m SCN similar to the age matched vehicle group with peak at ZT-12 and nadir at ZT-0. Interestingly, it advanced the peak expression by 6 h in 12 m animals thus restoring the phase in

i. Mean 24 h levels
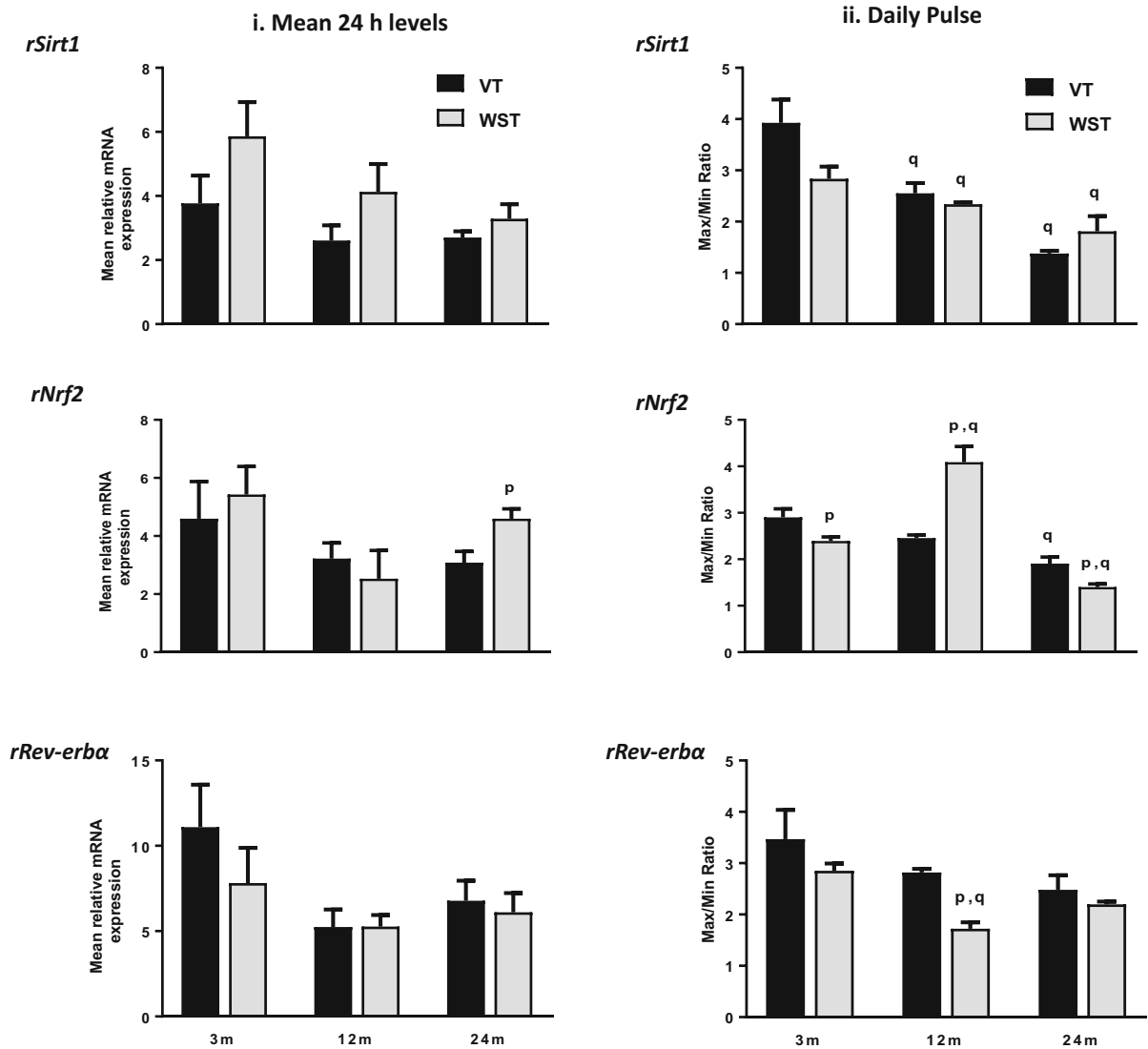
ii. Daily Pulse

Fig. 2 Effect of administration of hydro-alcoholic leaf extract of *Withania somnifera* on **i**. mean 24 h levels and **ii**. Daily Pulse (maximum/minimum ratio) of *rSirt1*, *rNrf2* and *rRev-erbα* genes in the SCN of 3, 12 and 24 months (m) old rats. Each value is mean $\pm$ SEM, $p < 0.05$ and expressed as mean relative gene expression. $p_p < 0.05$ (where 'p' refers to comparison with age matched vehicle group). $p_q < 0.05$ (where 'q' refers to comparison with 3 m vehicle group)

comparison to 3 m vehicle control. Cosinor analysis also confirmed this restoration with respect to resetting of acrophase value to 23:25 (Table 1). Similarly in 24 m animals, WST delayed the peak *rNrf2* expression by 12 h thus restoring the phase (Fig. 1). In addition to restoring *rNrf2* mesor in 24 m (Table 1), WS resulted in a significant 1.5 fold increase in mean 24 h levels of *rNrf2* in 24 m compared to the age matched control group ($p < 0.05$) indicative of a restoration when compared to 3 m (Fig. 2i). WS

administration decreased the daily pulse by 1.3 folds in both 3 and 24 m animals, but increased it by 1.7 folds in 12 m animals in comparison to respective VT groups ($p < 0.05$) (Fig. 2ii).

NRF2 protein expression rhythm upon WST in 3 m was similar to the age matched vehicle controls with peak at ZT-12 and minimum at ZT-18. Interestingly, WS restored the rhythm of NRF2 expression in 12 m animals with maximum expression at ZT-12, thus restoring the phase in comparison to 3 m. Similarly in
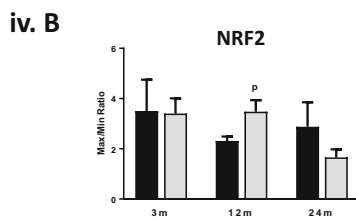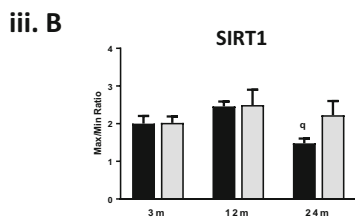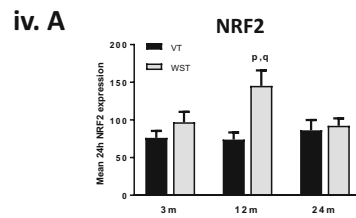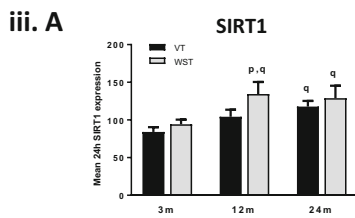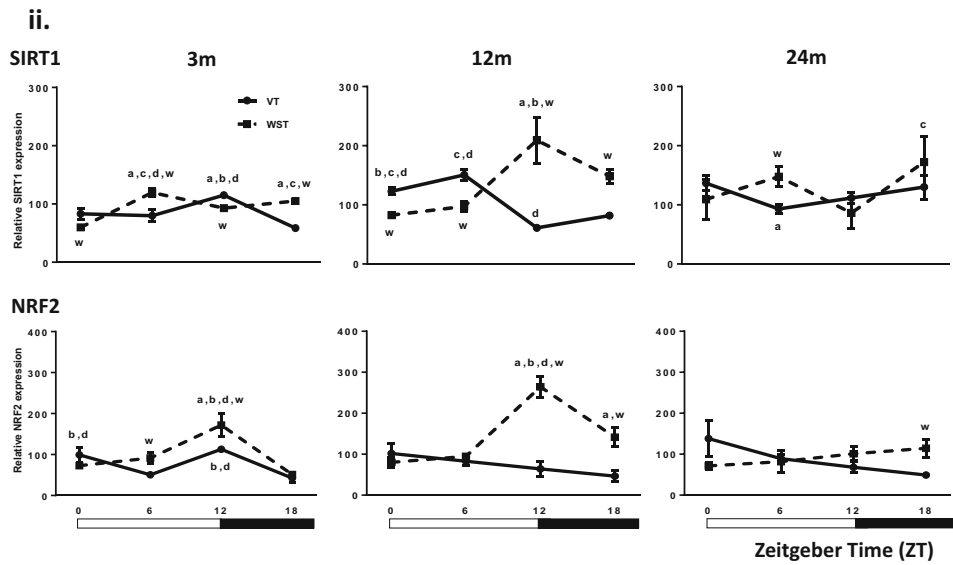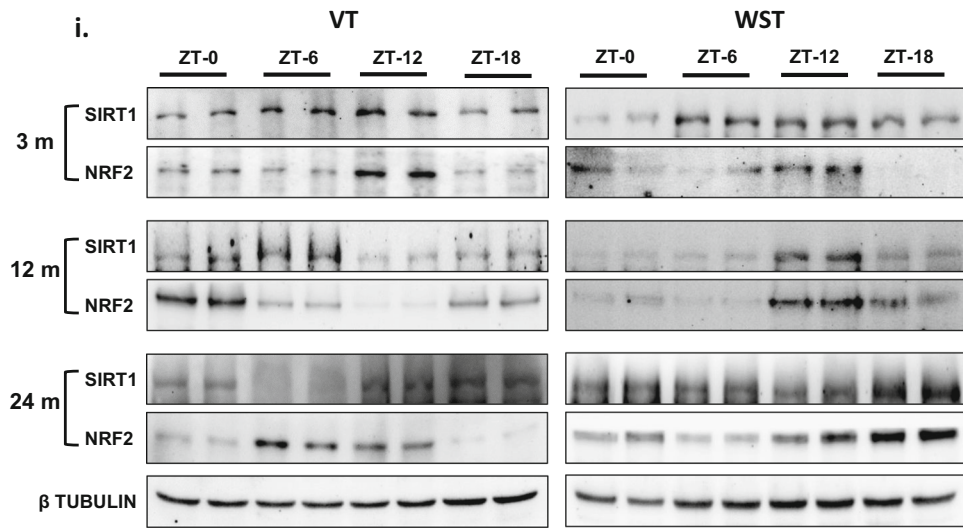
◄**Fig. 3 i** Immunoblots showing daily rhythms in SIRT1 (120 kDa) and NRF2 (61 kDa) expression in SCN of 3, 12 and 24 months (m) aged rats at ZT-0, 6, 12 and 18. β-TUBULIN (55 kDa) was probed as loading control. VT- vehicle treated; WST- *Withania somnifera* treated. **ii** Daily rhythms of SIRT1 and NRF2 expression in the SCN of 3, 12 and 24 months (m) old rats. Each value is mean ± SEM (n = 4), $p < 0.05$ and expressed as normalized mean density. $p_a < 0.05$; $p_b < 0.05$, $p_c < 0.05$ 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). $p_w < 0.05$ (where 'w' refers to comparison of protein expression levels at same time point in the age matched vehicle group). **iiiA** Effect of administration of hydro-alcoholic leaf extract of *Withania somnifera* on mean 24 h levels of SIRT1 and **iiiB** Daily Pulse (maximum/minimum ratio) of SIRT1 in the SCN of 3, 12 and 24 months (m) old rats. **ivA** Effect of administration of hydro-alcoholic leaf extract of *Withania somnifera* on mean 24 h levels of NRF2 and **ivB** Daily Pulse of NRF2 in the SCN of 3, 12 and 24 months (m) old rats. Each value is mean ± SEM, $p < 0.05$ and expressed as normalized mean density. $p_p < 0.05$ (where 'p' refers to comparison with age matched vehicle group). $p_q < 0.05$ (where 'q' refers to comparison with 3 m vehicle group)

24 m animals, WST resulted in restoration of NRF2 daily rhythm though there was a 6 h phase delay in comparison to 3 m (Fig. 3i, ii). A significant 2.0 fold and 1.9 fold increase in mean 24 h levels of NRF2 was observed in 12 m WST compared to 12 and 3 m vehicle groups respectively ($p < 0.05$) (Fig. 3ivA). WS administration increased the daily pulse by 1.5 folds in 12 m animals in comparison to age matched control group ($p < 0.05$). However, the change observed in other age groups were not statistically significant (Fig. 3ivB).

Effect of *Withania somnifera* (WS) leaf extract on age-induced alterations in the expression levels and daily rhythms of *rRev-erbα*

Effect of WS leaf extract administration was studied on *rRev-erbα* mRNA expression levels and daily rhythms in SCN of 3, 12 and 24 m old rats.

In the SCN of 3 m animals treated with WS the maximum expression of *rRev-erbα* was at ZT-6 and minimum expression was at ZT-12. In comparison to 3 m SCN *rRev-erbα* expression showed no change in phase upon WS treatment (Fig. 1). Mean 24 h levels as well as daily pulse of *rRev-erbα* did not show any significant change upon WS treatment compared to 3 m vehicle group (Fig. 2i, ii).

In 12 m WST group *rRev-erbα* showed maximum expression at ZT-6 and minimum at ZT-12 with no significant change when compared to age matched as well as 3 m controls (Fig. 1). However, cosinor analysis suggested a delay in *rRev-erbα* acrophase in 12 m (9:15) as compared to 3 m (5:40) controls (Table 1) and WS administration to 12 m resulted in its restoration (5:44). Mean 24 h levels of *rRev-erbα* did not show significant difference in 12 m WST group in comparison to 12 m and 3 m vehicle group (Fig. 2i). However, *rRev-erbα* showed significant 2 folds and 1.6 folds decrease in daily pulse in comparison to 3 and 12 m vehicle controls respectively ($p < 0.05$) (Fig. 2ii).

In 24 m WST SCN, *rRev-erbα* showed maximum expression at ZT-6 and minimum at ZT-18 which is similar to 3 m and 24 m vehicle control (Fig. 1). Similarly, there was no significant change in mean 24 h levels and daily pulse of *rRev-erbα* compared to both 3 m and 24 m vehicle control groups (Fig. 2i, ii).

Pearson correlation analysis

In light phase (LP) of 3 m rats treated with vehicle, *rRev-erbα* showed significant positive correlation with *rSirt1* ($p < 0.05$). Whereas in the dark phase (DP), there was a negative correlation between *rRev-erbα* and *rSirt1*. Further, a significant negative correlation was observed between *rRev-erbα* and *rNrf2* in the LP which was lost in the DP. Interestingly, a significant negative correlation between *rSirt1* and *rNrf2* was observed in LP as well as DP in 3 m SCN ($p < 0.05$) (Fig. 4). However, with WS administration, the positive correlation between *rRev-erbα*–*rSirt1* became negative whereas the negative correlation between *rRev-erbα*–*rNrf2* persisted in 3 m LP ($p < 0.05$). The negative correlation between *rRev-erbα* and *rSirt1* sustained where as a significant negative correlation between *rRev-erbα* and *rNrf2* appeared in dark phase of 3 m rats treated with WS ($p < 0.05$). Interestingly, the significant negative correlation between *rSirt1* and *rNrf2* observed in both LP and DP of 3 m SCN turned into a weak positive correlation upon WST in this age group (Fig. 4).

The negative correlations existed between *rRev-erbα* and *rNrf2* as well as *rSirt1* and *rNrf2* in 3 m turned as positive correlations in 12 m in both the phases. In addition the significant positive correlation between *rRev-erbα* and *rSirt1* observed in adult age

VT   **WST**
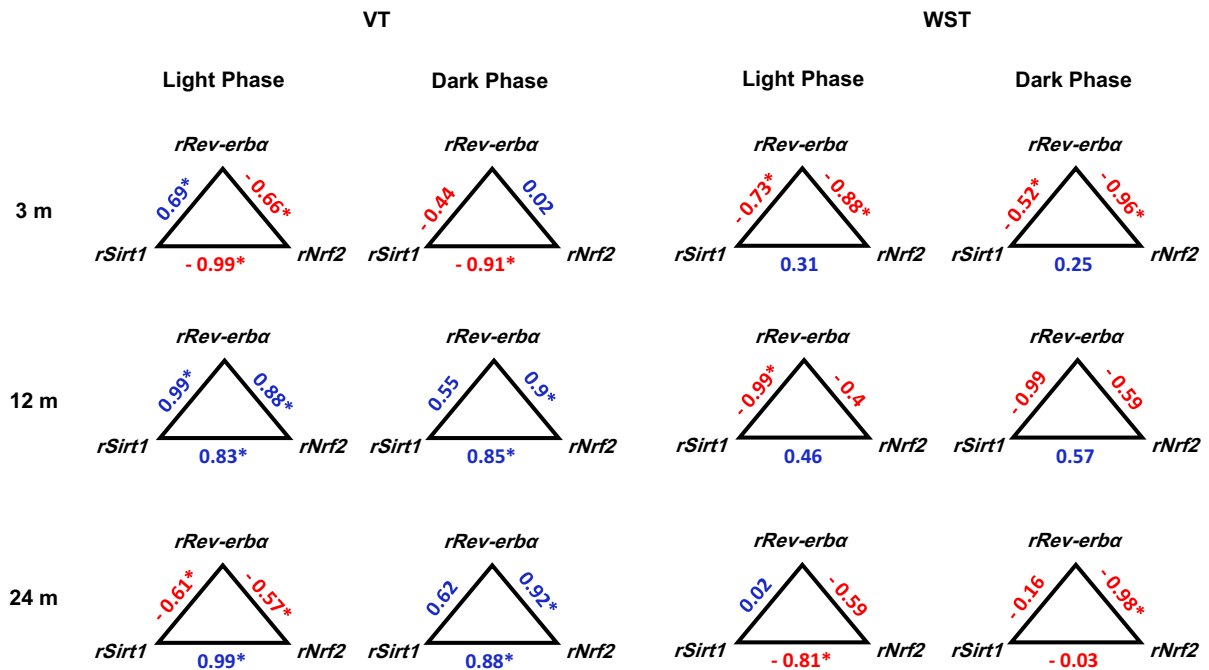
Fig. 4 Effect of administration of hydro-alcoholic leaf extract of *Withania somnifera* on pair wise correlation between mean light (ZT-0, 6, 12) and mean dark (ZT-12, 18, 24/0) phase values of *rRev-erbα*, *rSirt1* and *rNrf2* in SCN of 3, 12 and 24 months (m) aged rats. '* (Asterisk)' Indicates statistically significant correlation value ($p < 0.05$)

persisted in 12 m LP. In 12 m DP, a significant positive correlation was observed between *rRev-erbα* and *rNrf2* ($p < 0.05$) (Fig. 4). WS treatment to 12 m animals restored the negative correlations between *rRev-erbα–rNrf2* in LP and *rRev-erbα–rSirt1* in DP. Further, the significant positive correlation between *rSirt1–rNrf2* observed in both the phases of middle aged rats turned into weak positive correlations upon WST (Fig. 4).

In 24 m SCN, there were negative correlations between *rRev-erbα–rSirt1* and *rRev-erbα–rNrf2* in LP and positive correlations between these set of genes in DP. Further, there appeared a significant positive correlation between *rNrf2–rSirt1* in both LP and DP of 24 m vehicle group which was actually significant negative interaction in respective phases of 3 m control ($p < 0.05$) (Fig. 4). Interestingly, with WS administration, the negative correlation between *rRev-erbα–rNrf2* and *rSirt1–rNrf2* was restored in LP of 24 m SCN. However in dark phase, WS resulted in significant negative correlations between *rRev-erbα–rNrf2* and a weak negative correlation between *rRev-erbα–rSirt1* (Fig. 4).

Discussion

Studies from our group and other researchers have shown that the expression of clock genes in the rat SCN show daily rhythms. *rBmal1,* the positive loop element, peak at mid-subjective night (ZT-18). In contrast, the negative loop elements *rPer1* and *rRev-erbα* show maximum expression at mid-subjective day (ZT-6) and *rPer2, rCry1, rCry2* peak levels were towards the end of light phase (ZT-12) (Preitner et al. 2002; Mattam and Jagota 2014; Jagota and Kowshik 2017; Kukkemane and Jagota 2019).

Interestingly, WS did not alter the phase of *rRev-erbα* in any of the age groups studied however in another recently reported study with curcumin treatment 6 h phase advance in both adult and middle aged animals was reported (Kukkemane and Jagota 2019).

We reported earlier the sensitivity of CTS towards hydro-alcoholic leaf extract of WS in differential restoration of the expression of core clock genes in middle and old aged rat SCN (Jagota and Kowshik 2017). In middle-aged animals, the WS treatment had resulted in restorations in the phase of daily rhythms of *rBmal1*, *rPer1* and *rCry1* whereas old-aged rats

showed restoration in phase of daiy rhythms of *rPer1* and *rCry1* (Jagota and Kowshik 2017). These restorations were similar to the results observed with administration of melatonin and curcumin (Mattam and Jagota 2014; Kukkemane and Jagota 2019). Clock genes, especially *Period* genes undergo transcriptional activation by binding of CREB to CREs present in their promoter region (Travnickova-Bendova et al. 2002). Interestingly, the active components of WS known as withanolides, with their steroidal structure (Das et al. 2002), could function like estradiol to activate signaling cascades including CREB phosphorylation (Konar et al. 2011). Thus, the phase restorations observed with WS administration could be due to withanolides induced activation of cAMP and MAPK pathway contributing to CREB phosphorylation (Jagota and Kowshik 2017).

The pairwise correlation analysis of various clock transcripts in light and dark phases suggested age-induced alterations in stoichiometric interactions among clock genes (Mattam and Jagota 2014) and a differential restoratory role for WS leaf extract (Jagota and Kowshik 2017). Most importantly, WST had restored the significant negative correlation between *rPer1* and *rBmal1* in both LP and DP of 24 m animals (Jagota and Kowshik 2017) which could be of great significance considering the importance of anti-phasic expression of *Period* and *Bmal1* (Abe et al. 1998; Yan et al. 1999). Interestingly, both melatonin and curcumin had also resulted in restoration of negative correlation between *rPer1* and *rBmal1* in SCN of old aged rats (Mattam and Jagota 2014; Kukkemane and Jagota 2019).

As our results showed a differential effect of aging and WS leaf extract on components of molecular clock, we hypothesized a possible role for circadian modulator SIRT1. The modulation of circadian clock by SIRT1/PGC-1α via RORα ultimately regulating *Bmal1* expression is well known and has been reported to deteriorate with aging (Chang and Guarente 2013). There are numerous reports demonstrating natural antioxidants like resveratrol and curcumin activating/upregulating SIRT1, there by bringing beneficial effects (Park et al. 2014; Jia et al. 2016; Grabowska et al. 2017). Hydro-alcoholic extract of WS being a strong antioxidant, may also exert similar effect on SIRT1 and influence biological clock.

Our results showed daily rhythmicity in transcript levels of *rSirt1* with peak expression at mid subjective
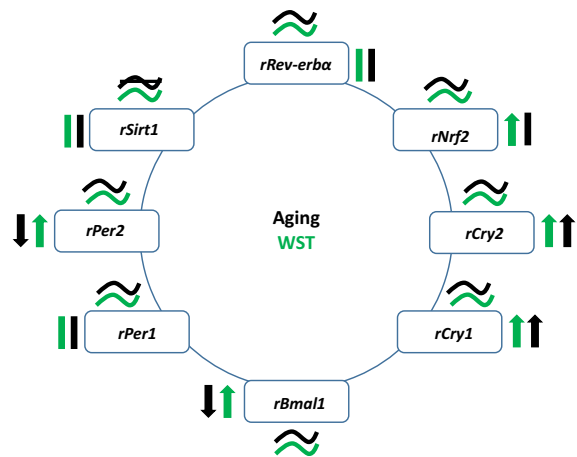
Fig. 5 Schematic summarization of the effect of *W. somnifera* treatment on age-induced alterations in daily rhythms and mean 24 h levels of various clock genes, *Sirt1* and *Nrf2* in the SCN of male Wistar rat. Increase (↑), decrease (↓), no change (|), rhythmic (∿) and non-rhythmic (━). The data for *rBmal1, rPer1, rPer2, rCry1* and *rCry2* was obtained from our earlier report (Jagota and Kowshik 2017)

night and nadir at day-night transition in the SCN of adult rats. This is similar to a previous report where the maximum *Sirt1* mRNA levels were at dark phase in SCN of adult mice (Chang and Guarente 2013). Interestingly, *rSirt1* and *rRev-erbα* showed anti-phasic expression pattern which is corroborated by correlation study where, a negative correlation is observed between the two in DP. This also corroborates the anti-phasic expression of RORs and REV-ERBs, of which the former is regulated by SIRT1 (Pritchett and Reddy 2017). SIRT1 protein expression was also rhythmic and in agreement with the earlier report from mice SCN (Chang and Guarente 2013). Aging did not affect mean 24 h levels of *rSirt1* transcripts as it appeared same in all the age groups, which is consistent with a study where age-related alterations in *Sirt1* mRNA levels were region specific in mouse brain (Lafontaine-Lacasse et al. 2010). Interestingly, SIRT1 mean 24 h protein levels were significantly increased in old aged SCN which is corroborated by an earlier report where SIRT1 levels were significantly higher in several brain regions of 24 m old rats (Braidy et al. 2015). However, *rSirt1* daily rhythms were found to be abolished in 24 m SCN and similar to curcumin WS resulted in its restoration (Kukkemane and Jagota 2019). The reduced mean 24 h levels and attenuated rhythm of *rBmal1* in 24 m old rat could be due to the
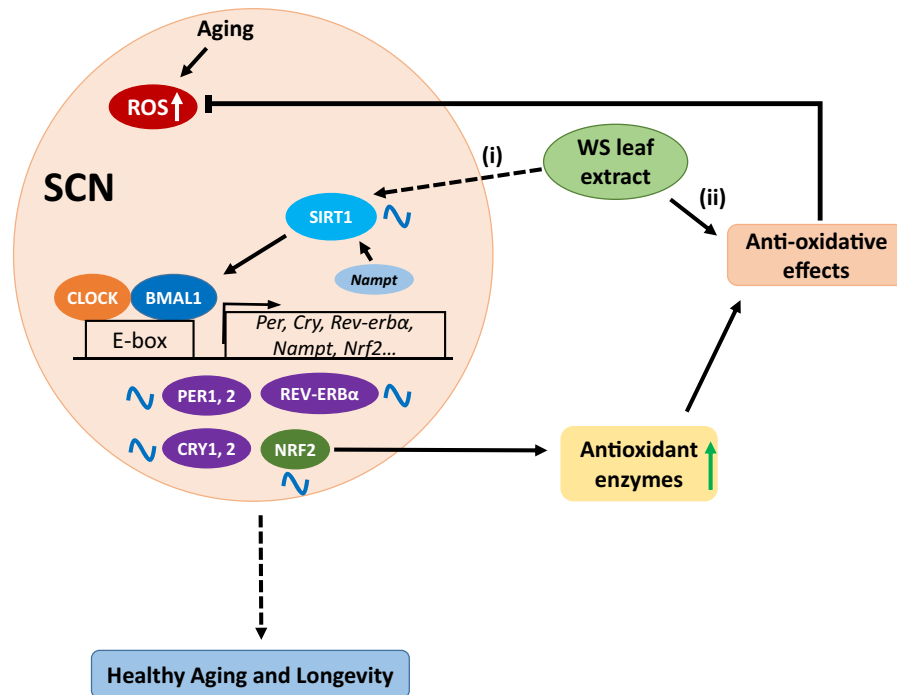
Fig. 6 The proposed mechanism of beneficial effect of hydro-alcoholic leaf extract of *Withania somnifera* (WS) against age-induced alterations in SCN. With advance in age ROS levels are known to increase and rhythms/levels of clock genes are altered. (i) The WS leaf extract may modulate expression of core clock genes via SIRT1, a modulator of circadian clock leading to restorations in rhythms/levels of clock gene expression. Such modulations may restore the rhythmic expression of *Nrf2* a clock controlled gene and the master transcription factor regulating the antioxidant enzymes. Additionally, (ii) the endogenous antioxidant enzymes along with potential antioxidant WS leaf extract may neutralize the reactive oxygen species (ROS) (Gupta and Kaur 2018). Overall WS leaf extract may involve these mechanisms to ameliorate age linked circadian dysfunction and may lead to healthy aging and longevity

abolished *rSirt1* daily rhythm in the same age group (Jagota and Kowshik 2017). Immunoblots suggested attenuated SIRT1 rhythms in old aged rats with altered phase. Restorations observed in mean 24 h levels of *rBmal1* in 24 m WST rats is consistent with the restoration of *rSirt1* transcript rhythms as well as partial restoration in phase of SIRT1 protein expression in the same group (Jagota and Kowshik 2017). These restoratory effects contributing to healthy aging also corroborates the previous reports where SIRT1 expression enhanced the tolerance of neuronal cells against oxidative stress (Patel et al. 2014).

Several anti-oxidant enzymes have been reported to follow rhythmic patterns in their activity and levels in peripheral clocks such as liver as well as brain regions such as hippocampus (Fontana 2009; Manikonda and Jagota 2012). The master transcription factor regulating antioxidant system, NRF2 is directly regulated by BMAL1-CLOCK (Lee et al. 2013; Pekovic-Vaughan

et al. 2014). Recent reports show that aging alters both expression and activity of several antioxidant enzymes in regions of brain as well as other organs (Manikonda and Jagota 2012; Zhang et al. 2015; Lacoste et al. 2017). However, the information on age-associated changes in NRF2 expression in SCN is very limited, though tissue and species specific alterations are recorded from different animal models (Zhang et al. 2015). Our results showed a temporal expression pattern in both *rNrf2* transcripts as well as its protein product (NRF2) in the SCN of adult rats, where the peak expression was at ZT-12, which is the end of photic phase. Interestingly, the pattern of expression is similar to earlier reports of *Nrf2* mRNA in peripheral clock liver (Xu et al. 2012). Similar to *rSirt1*, mean 24 h levels of *rNrf2* did not vary upon aging. However, there was a 6 h phase delay in 12 m and 12 h phase delay in 24 m vehicle groups in comparison to 3 m control group. Similarly, the amplitude of the rhythm

decreased significantly upon aging (Table 1). Further, at protein level NRF2 expression was found to be arrhythmic both in 12 and 24 m rats. Interestingly, WS treatment resulted in restoration of phase of *rNrf2* in 12 m and restored the mean 24 h levels in 24 m WST group. Also, WS restored the rhythmic NRF2 expression in SCN of both middle and old aged animals along with restoring the phase in 12 m. This correlates with the restored phase of *rBmal1* in 12 m WST and restored mean 24 h *rBmal1* levels in 24 m WST (Jagota and Kowshik 2017), thus corroborating earlier reports linking NRF2 and BMAL1 (Lee et al. 2013; Pekovic-Vaughan et al. 2014; Wende et al. 2016). Our results indicate WS leaf extract might have potentially regulated *rBmal1* and other clock transcripts via SIRT1 in SCN of aged rats (Jagota and Kowshik 2017). BMAL1 in turn might have influenced NRF2 which would further lead to robust antioxidant defense as reported for tea polyphenols by some researchers (Qi et al. 2017).

Our earlier report (Jagota and Kowshik 2017) and present study together indicate that hydro-alcoholic leaf extract of WS exhibits robust chronomodulatory effect considering the restorations in daily rhythms, mean 24 h levels and daily pulse of various clock genes, SIRT1 and NRF2 (Fig. 5). Based on these results we propose that the hydro-alcoholic leaf extract of WS may have a therapeutic advantage on age-induced alterations in circadian clock via two major pathways involving SIRT1 and anti-oxidative effects (Fig. 6).

Age-related circadian ailment is a global health challenge and there is a necessity to test prospective drug candidates from natural sources. The high antioxidant activity (Jagota and Kowshik 2017) and presence of active components such as Withanolides A–E, Withanolide glycosides, Sitoindoside VII, VIII, IX (Glycowithanolides), Withaferin A and several steroidal alkaloids (Panchawat 2011) could be responsible for differential restoration of levels and rhythms of various clock genes, clock modulator (SIRT1) and clock effector, a master transcription factor (NRF2). The present study demonstrates sensitivity of WS hydro-alcoholic leaf extract in restoration of the stoichiometric interactions between these molecules. Further studies with components of the hydro-alcoholic leaf extract of WS and variable doses will help in identifying specific components responsible for

therapeutic, antioxidant and neuroprotective properties of WS in age associated circadian dysfunction.

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