Effects of curcumin and leaf extract of *Withania somnifera* on chronomics of various age induced alterations in suprachiasmatic nucleus of male Wistar rats

A thesis submitted to University of Hyderabad for the award of the degree
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In
Animal Sciences

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CERTIFICATE

This is to certify that the thesis entitled **"*Effects of curcumin and leaf extract of Withania somnifera on chronomics of various age induced alterations in suprachiasmatic nucleus of male Wistar rats*"** submitted by Mr**. Kowshik K** bearing registration number **13LAPH02** in partial fulfillment of the requirements for award of **Doctor of Philosophy** in the School of Life Sciences is a bona fide work carried out by him under my supervision and guidance.

This thesis is free from plagiarism and has not been submitted previously in part or in full to this or any other University or Institution for award of any degree or diploma.

Further, the student has the following publications before submission of the thesis for adjudication and has produced the evidence for the same in the form of acceptance letter or the reprint in the relevant area of his research.

A. Publications:

1. Kowshik Kukkemane, Anita Jagota (2019) Therapeutic effects of curcumin on age-induced alterations in daily rhythms of clock genes and *Sirt1* expression in the SCN of male Wistar rats. **Biogerontology** 20:405-419 (ISSN: 1573-6768, Springer Nature); ***(Part of thesis: Objective 1)***
2. Anita Jagota, Kowshik K (2017) Therapeutic Effects of Ashwagandha in Brain Aging and Clock dysfunction. (eds Kaul S and Wadhwa R) in "Science of Ashwagandha: Preventive and Therapeutic Potentials." Springer Nature, pp 437–456 (ISBN: 978-3-319-59192-6, Springer Nature)
3. Anita Jagota, Neelesh Babu Thummadi*, Kowshik Kukkemane* (2019) Circadian regulation in Hormesis: Health and Longevity (eds Rattan S and Marios Kariazis) in "The Science of Hormesis in Health and Longeivity." Chapter 22 pp 223-233. Academic press (* equal contribution) (ISBN: 978-0-12-814253-0, Elsevier)
4. Anita Jagota, Kowshik Kukkemane*, Thummadi NB* Biological Rhythms and Aging. In "Models, Molecules and Mechanisms in Biogerontology." Springer Nature (in Press) (* equal contribution)

and

has made presentations in the following conferences:

B. <u>Presentations in Conferences:</u>

1. ***International conference on 'Neurochemistry and Neuropharmacology: From bench to bedside' (SNCI – 2019)*** "Age associated alterations in chronomics of serotonin metabolism: Beneficial effects of *Withania somnifera*" **Oral Presentation**, March 2019, JSS college of pharmacy, Mysuru, Karnataka, India, as part of 32nd Annual meeting of Society for Neurochemistry, India (SNCI). **(International)**
2. ***International Conference on 'Translational Neurosciences and its Application in Protection of Mental Health' (IAN – 2017)*** "Effects of Withania somnifera leaf extract on the age induced alterations in daily clock gene expression rhythms in the SCN of male Wistar rats" **Poster Presentation**, October 2017, Ravenshaw University, Cuttack, Odisha, India, as a part of 35th Annual conference of Indian Academy of Neurosciences (IAN). **(International)**
3. **International conference on 'Recent trends in Neurological and Psychiatric *Research' (SNCI – 2016)*** "Age induced alterations in clock gene expression rhythms in the SCN of male Wistar rats: Curcumin intervention" **Oral Presentation**, December 2016, CSIR-CCMB, Hyderabad, Telangana, India, as part of 30th Annual meeting of Society for Neurochemistry India (SNCI). **(International)**
4. **Academia Sinica-UOH (Indo-Taiwan) joint workshop on Frontiers in Life Sciences** "Therapeutic effects of *Withania somnifera* on the age induced alterations in clock gene expression rhythms in the SCN of male Wistar rats" **Poster Presentation,** September 2016, School of Life Sciences, University of Hyderabad, Hyderabad, India. **(International)**

Further, the student has passed the following courses towards the fulfillment of course work required for the award of Ph.D.

Course Code	*Name*	*Credits*	*Pass/Fail*
AS 801	Seminar 1	1	Pass
AS 802	Research Ethics & Management	2	Pass
AS 803	Biostatistics	2	Pass
AS 804	Analytical Techniques	3	Pass
AS 805	Lab Work	4	Pass

Supervisor Head of the Department Dean of the School

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DECLARATION

I, **Kowshik K,** hereby declare that this thesis entitled **"*Effects of curcumin and leaf extract of Withania somnifera on chronomics of various age induced alterations in suprachiasmatic nucleus of male Wistar rats*"** submitted by me under the guidance and supervision of ***Prof. Anita Jagota*** is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this university or any other University or Institute for the award of any degree or diploma.

Date: **Name: Kowshik K**

Signature of the Student

Reg. No.: 13LAPH02

Dedicated to my brother Karthik K.

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Kowshik K.

Contents

CHAPTER - I

INTRODUCTION AND REVIEW OF LITERATURE

BIOLOGICAL RHYTHMS

Living organisms have evolved under constantly changing environment due to the daily and yearly revolution of earth that has driven the development of biological rhythms. Biological rhythms are cyclical changes that exist at genetic, physiological and behavioral levels across taxa which enable the organisms to predict and adapt to variety of climatic conditions. Based on the time scales, these are categorized as circannual, circalunar, circatidal, infradian, ultradian and circadian rhythms (Stevenson 2018).

CIRCADIAN RHYTHMS

Circadian (from Latin, around a day) rhythms are cyclical events in physiology and behavior of organisms that have a near 24 hours (h) periodicity. These rhythms are external manifestation of an internal time keeping system that processes time of the day and enables living beings to anticipate the external environment contributing to organismal fitness and survival (Reppert and Wever 2002; Jagota 2006; Honma 2018). Characteristically circadian rhythms are (i) Endogenous - ability to persist in the absence of any external cue (ii) Entrainable - ability to be synchronized in the presence of an external cue (iii) with a periodicity of nearly 24 h (iv) temperature compensated and (v) are susceptible to phase-shifts (Wilking et al. 2013). The external environmental cues such as day-night cycles, availability of food, geo-magnetic fields, social cues etc. are referred as '*zeitgebers'* or 'time givers' and light is the principal zeitgeber to circadian system. In the absence of these cues, circadian rhythms progressively deviate and persist with an endogenous periodicity of slightly more than or less than 24 h resulting in 'free running' rhythms. Any of the time cues such as light can phase-shift the circadian rhythms and phase response curve (PRC) helps to visualize the direction (advance/delay) and extent of phase-shifts (Beale et al. 2016; Honma 2018). Sleep-wake cycles, melatonin secretion, body temperature, bowel movements etc. are few of the examples among numerous physiological and behavioral parameters that follow circadian pattern (Duffy and Wright 2005).

Several researchers have been deciphering various aspects of circadian rhythms for last 70 years beginning with foundational studies conducted by Colin Pittendrigh and Jürgen Aschoff, and the importance of the field of chronobiology was recognized with the award of the 2017 Nobel Prize in Physiology and Medicine to Jeffrey C. Hall, Michael Rosbash and Michael W. Young 'for their discoveries of molecular mechanisms controlling the circadian rhythm' (Vitaterna et al. 2001; Hastings et al. 2018).

CIRCADIAN TIME KEEPING SYSTEM

Circadian rhythms in mammals are orchestrated by well-organized, hierarchically structured circadian time keeping system (CTS) which majorly consists of 'afferent pathways' that perceive environmental inputs, 'a master pacemaker' that integrates time of day information, and 'efferent pathways' that transmit timing information to metabolic and physiological processes, followed by subsequent feedback from peripheral oscillators (Fig. 1) (Albrecht 2012).

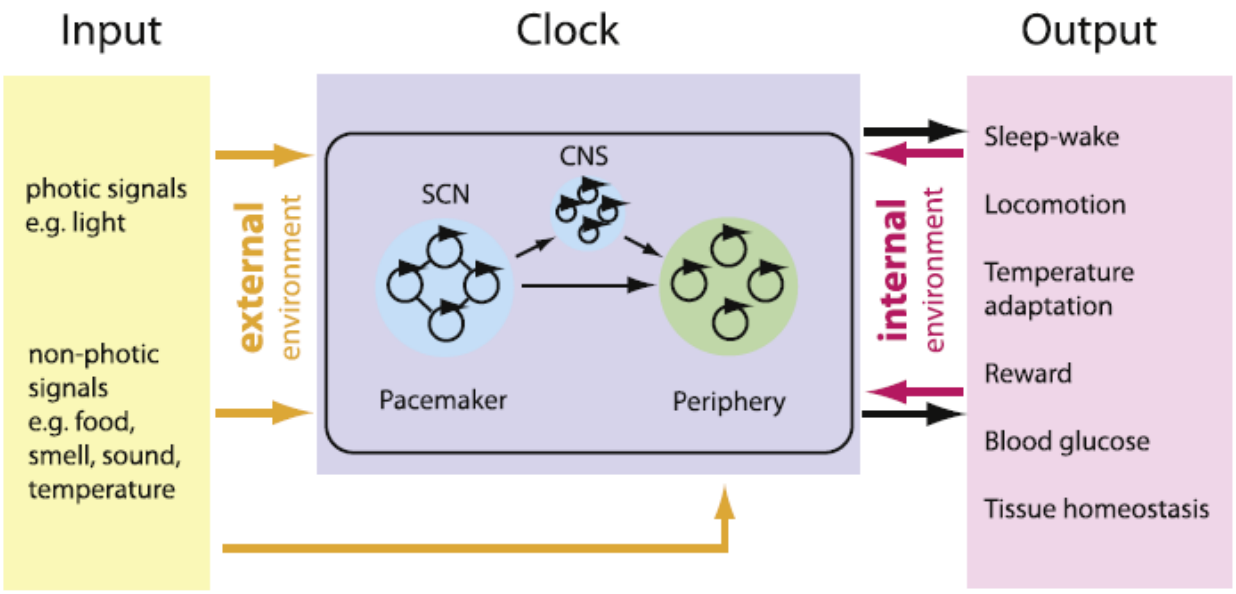


Fig. 1 Basic organization of the circadian time keeping system. Input to the clock from the external environment, integration of information by the clock and output from the clock to temporally regulate physiology (Albrecht 2012).

Suprachiasmatic nucleus: the master clock

The central circadian pacemaker also referred as the master clock 'suprachiasmatic nucleus' (SCN) is located above the optic chiasm, bilateral to 3rd ventricle in the anterior hypothalamus of the mammalian brain (Fig. 2). This bi-lobed nuclei consisting of approximately 20,000 neurons is at the top of hierarchical organization of CTS (Jagota 2006; Albrecht 2012). While the surgical and electrolytic experiments leading to ablation of physiological rhythms confirmed SCN as the master pacemaker, the prolonged persistence of rhythmicity in SCN cultures ascertained the autonomous nature of this clock (Silver and Rainbow 2013). Interestingly, several functional studies suggested SCN as an

intricate assemblage of multiple oscillators that are coupled in-phase by electrical and neurochemical signaling. Studies conducted in hamsters with exposure to constant light resulting in splitting of activity rhythms and the identification of 'morning and evening oscillators' illustrated the multi-oscillatory nature of SCN (de la Iglesia et al. 2000; Jagota et al. 2000).

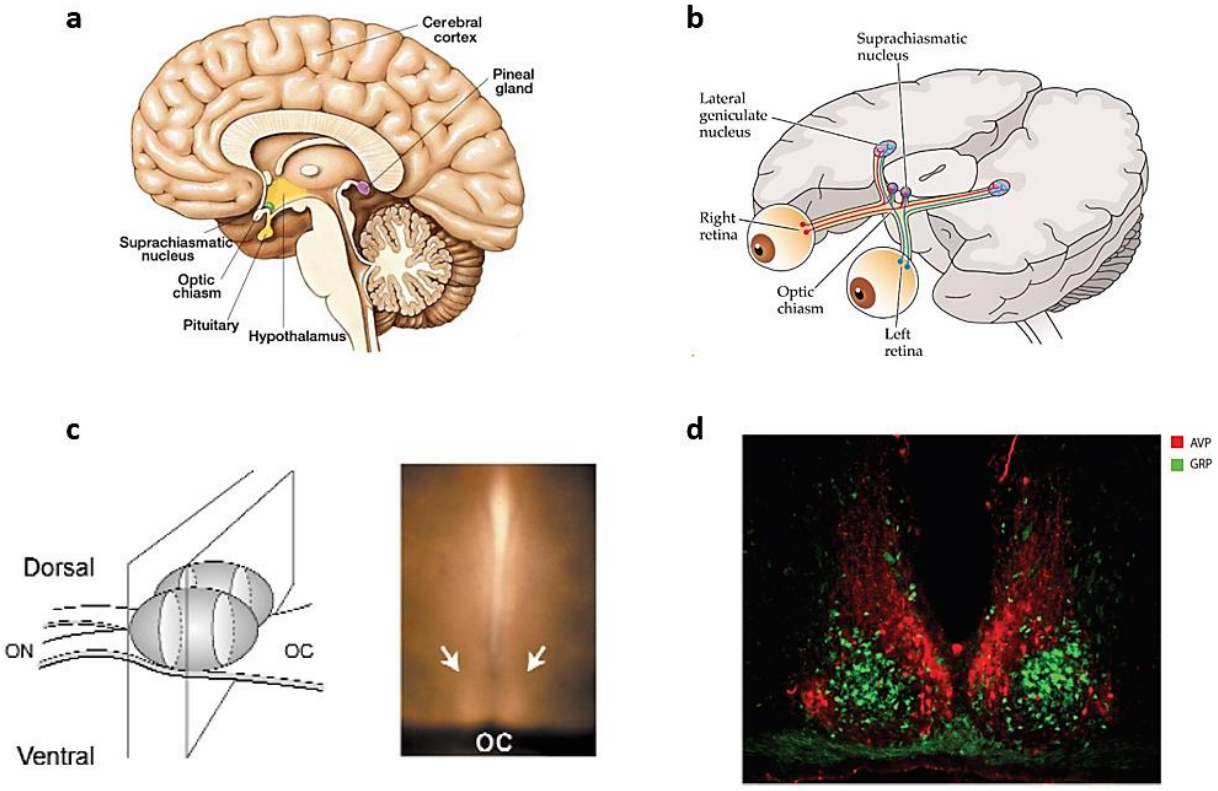


Fig. 2 (a) and **(b)** Location of suprachiasmatic nucleus (SCN) in the mammalian brain, **(c)** Coronal brain section containing the SCN as observed under dissecting microscope, **(d)** Coronal section of the SCN showing ventrolateral core region with gastrin releasing peptide (GRP) neurons (green) and the dorsomedial shell region with arginine vasopressin (AVP) neurons (red) (Jagota et al. 2000; Jagota 2006; Welsh et al. 2010).

Further, the heterogeneous nature of SCN is marked at anatomical level based on the distribution of specific neurotransmitters and innervation from retino-hypothalamic tract. The ventrolateral 'core' (VL-SCN) region which expresses vasoactive intestinal peptides (VIP) receives retinal input whereas, the dorsomedial 'shell' (DM-SCN) region enriched with arginine vasopressin (AVP) does not receive retinal input (Fig. 2d). Neuronal population in VL-SCN also contain gastrin releasing peptide (GRP), calretinin and

neurotensin (NT) positive cells. Whereas, DM-SCN contain met-enkephalin and angiotensin II expressing neurons in addition to AVP. VIP functions as a coupling agent and plays key role in SCN synchrony. The core neurons integrate the external input that will subsequently be communicated to rest of the SCN wherein shell neurons generate high amplitude oscillations of clock genes expression which determine the endogenous period of the clock. The low amplitude oscillations of clock genes in core region accommodate environmental perturbations by facilitating phase shifts. With its complex organization, the SCN serves as a relay between external environment and internal physiology (Jagota 2006; Dibner et al. 2010; Colwell 2011).

Afferent Pathways to the SCN: reception of information from the external environment

Time cues from the exterior surroundings can reach the SCN via three major afferent or input pathways: the retino-hypothalamic tract (RHT), the geniculo-hypothalamic tract (GHT) and the retino-raphe pathway (RRP) (Fig. 3a). Among these three pathways, RHT mediates photic signals whereas GHT and RRP mediate non-photic signals (Dibner et al. 2010).

Light (blue light in the specific wavelength range of 460 – 480 nm), the major zeitgeber to the CTS, is perceived by retina of the eye where the non-image forming intrinsically photoreceptive retinal ganglion cells (ipRGC) expressing melanopsin photo-pigments directly convey the photic information to the VL-SCN via RHT. Excitatory neurotransmitter glutamate, aspartate and pituitary adenylyl cyclase-activating peptide (PACAP) are involved in this transmission. Upon their release, several signaling pathways such as extracellular signal-regulated kinase (ERK) pathway gets activated leading to phosphorylation of cAMP response element binding protein (CREB) which will ultimately result in induction of clock genes.

RHT also innervates intergeniculate leaflets (IGL), projections from where extends to the SCN as GHT and indirectly communicates photic information by releasing gamma-aminobutyric acid (GABA) and neuropeptide Y (NPY).

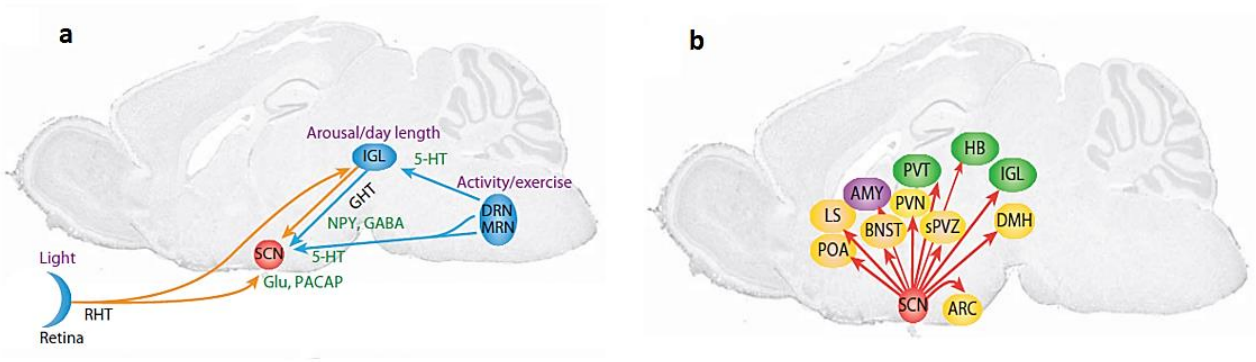


Fig. 3 (a) Major afferent pathways to the SCN. Photic inputs are represented by orange arrows; non-photic inputs are represented by blue arrows. RHT – retino-hypothalamic tract, GHT – geniculo-hypothalamic tract, IGL – intergeniculate leaflet, DRN – dorsal raphe nuclei, MRN – median raphe nuclei, Glu – glutamate, PACAP – pituitary adenylyl cyclase activating peptide, 5-HT – serotonin, NPY- neuropeptide Y, GABA – γ amino butyric acid. **(b)** Major efferent pathways from the SCN (red arrows). sPVZ – subparaventricular zone, PVN – paraventricular nucleus of hypothalamus, DMH – dorsomedial hypothalamus, POA – preoptic area, BNST – bed nucleus of the stria terminalis, LS – lateral septum, ARC – acuate nucleus, AMY – amygdala, IGL – inter-geniculate leaflet, HB – habenula, PVT – paraventricular nucleus of thalamus (Dibner et al. 2010).

In addition, IGL receives non-photic signals such as information on motor activity from dorsal raphe nuclei (DRN) indicating the integration of photic and non-photic signals entraining the SCN. Third afferent input, the RRP involves serotonergic nerve fibres emanating from median raphe nuclei (MRN) and DRN ending in VL-SCN overlapping with retinal afferents. Serotonin receptors such as 5-$HT_{1A/7}$, 5-HT_{1B} and 5-HT_{2C} are involved in modulating phase shifts in addition to entraining the clock to non-photic cues in the RRP (Jagota 2006; Dibner et al. 2010).

Efferent Pathways from the SCN: transmission of information to the body

The intrinsic time information from the SCN is communicated to other regions of the brain and peripheral clocks through various neural and humoral signals. Anterograde and retrograde tracers have elucidated the axonal projections from the SCN terminating in several brain sites (Fig. 3b). SCN efferents innervate subparaventricular zone (sPVZ), preoptic area (POA), lateral septum (LS), dorsomedial hypothalamus (DMH), arcuate nucleus (ARC) and bed nucleus of the stria terminalis (BNST) within the hypothalamus, whereas in the thalamus they project to IGL and paraventricular nucleus (PVN).

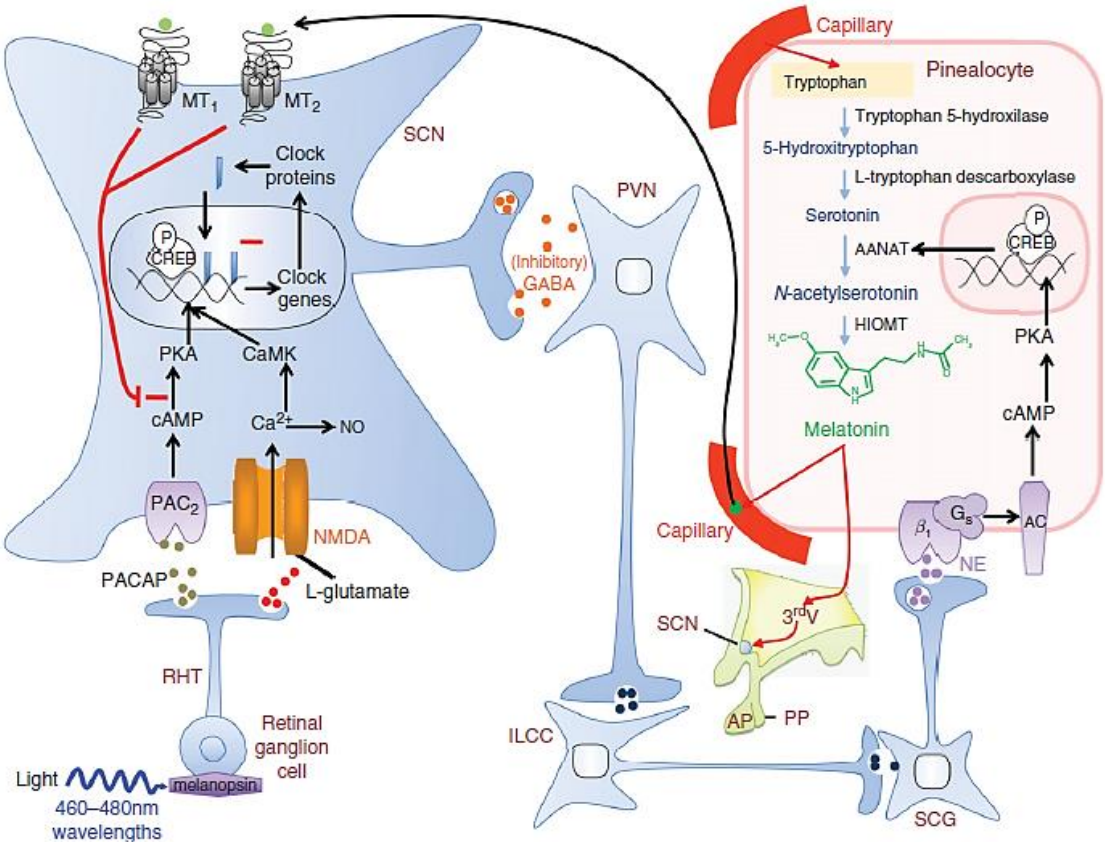


Fig. 4 Diagrammatic illustration of circadian time keeping system in mammals. Temporal regulation of pineal melatonin synthesis and release. RHT – retino-hypothalamic tract, PACAP – pituitary adenylyl cyclase activating peptide, cAMP – cyclic adenosine monophosphate, PKA – protein kinase A, NO – nitric oxide, CaMK – calmodulin kinase, PVN – paraventricular nucleus, ILCC – inter-mediolateral cell column, SCG – superior cervical ganglion, NE – norepinephrine, AC – adenylate cyclase, AANAT – arylalkylamine N-acetyl transferase, HIOMT – hydroxyindole-*O*-methyl transferase, AP – anterior pituitary, PP – posterior pituitary (Reiter et al. 2010).

In addition, there are reports suggesting SCN efferents innervating the amygdala (AMY) and habenula (HB). Though there are explicit subdivisions among the SCN projections to specific brain areas, the projection pattern may vary in species specific manner.

The communication from the SCN is majorly via neurotransmitters such as GABA and glutamate, however, there are number of other molecules such as AVP, VIP, prokineticin 2 (PK2), cardiolipin like cytokine and transforming growth factor α (TGFα) which are out put signals of the SCN. In addition, melatonin (internal zeitgeber, hormonal message of

darkness) biosynthesis and secretion from pineal gland is directly regulated by the SCN (Fig. 4) (Reiter et al. 2014).

The biosynthesis and release of melatonin is governed by a neural circuit originating in the SCN. In this multi-synaptic neural circuit, GABAergic axons of SCN neurons projects to hypothalamic PVN. Efferents from PVN descend via the brain stem to spinal cord and synapse with preganglionic sympathetic neurons of the inter-mediolateral cell column (ILCC). Axons of these nerve cells project onto a set of cells in superior cervical ganglion (SCG) eventually terminating in pinealocytes. Release of norepinephrine from superior cervical post-ganglionic neurons stimulates melatonin's synthesis and release. Melatonin secreted into circulation and also to cerebrospinal fluid (CSF) of the 3rd ventricle influences the master clock via MT1 and MT2 melatonin membrane receptors (Jagota 2006; Reiter et al. 2010).

Molecular architecture of the circadian clock

The mammalian circadian clock machinery is cell autonomous and the molecular mechanism governing this machinery relies on three tightly inter-regulating transcriptional/translational feedback loops (TTFL) (Takahashi 2017). The transcriptional activators such as CLOCK (and its paralogue NPAS2) and BMAL1 (also referred as ARNTL) along with several other dedicated transcription factors exist at the core of these feedback loops. At the beginning of a subjective day, BMAL1-CLOCK heterodimer binds to the E-box elements of *Period (Per1, 2, 3), Cryptochrome (Cry1, 2)* along with several clock controlled genes (CCGs) initiating their transcription. Upon reaching critical levels towards the end of subjective day, PER-CRY proteins interact, hetero-dimerize and translocate to the nucleus during the subjective night to block BMAL1- CLOCK activity. This leads to the repression of their own transcription and also of other CCGs.

Phosphorylation of PER-CRY by serine/threonine kinases such as casein kinase 1ε (CK1ε) and casein kinase 1δ (CK1δ) play key role by governing the stability and localization of these clock elements. Interestingly, the site of phosphorylation facilitates their nuclear transport. CK1ε and CK1δ play decisive role in determining the intrinsic period of the circadian clock by regulating the rate of PER-CRY turnover and nuclear entry. Phosphatases PP1 and PP5 counter-regulate the activity of these casein kinases. The E3 ubiquitin ligase complexes regulate turnover of PER and CRY proteins by targeted ubiquitylation resulting in proteasome mediated degradation. As the repression on

CLOCK-BMAL1 gets relieved upon degradation of repressor complex, the cycle commences again with a periodicity of 24 h.

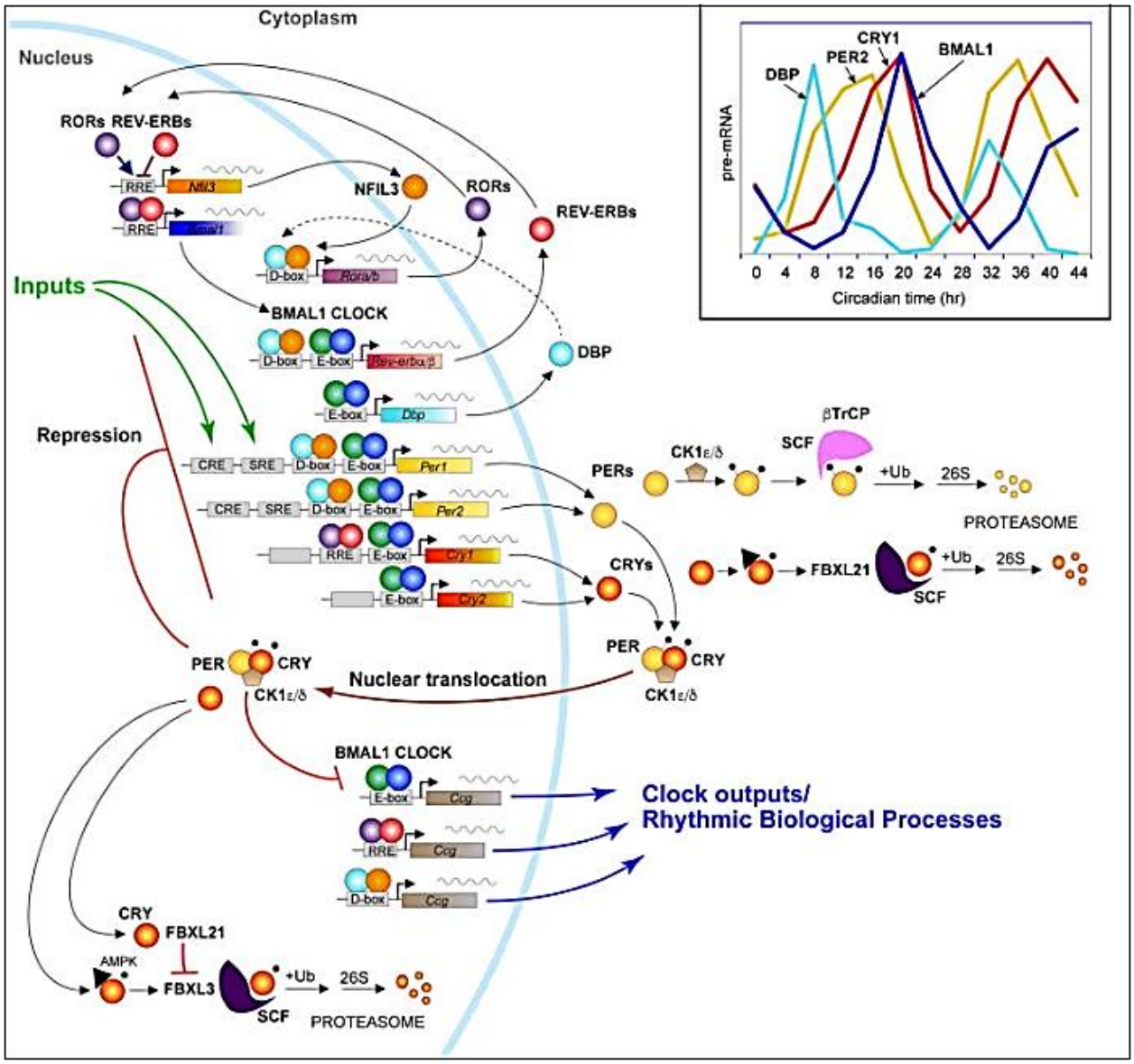


Fig. 5 Molecular architecture of the circadian clock machinery in mammals. Interconnected autoregulatory transcriptional translational feedback loops (TTFL) function together to regulate 24 h endogenous periodicity of the clock. mRNA profiles of clock genes in mouse liver (Top right). AMPK – AMP activated protein kinase, Ccg – clock controlled genes, CRE – cAMP response element, CK1 – casein kinase 1, DBP – D-box binding protein, RRE – ROR responsive element, SRE – serum response element, SCF – SKP1-cullin-F-box protein, FBX – F-box protein, Ub – ubiquitin (Takahashi 2017).

Along with the core CLOCK-BMAL1/PER-CRY loop, auxiliary loops involving *Retinoic acid receptor - related orphan receptor alpha (Rorα), Rorβ* and *Rev-erbα* also known as *Nr1d1* (Nuclear receptor subfamily 1, group D member 1), *Rev-erbβ* (*Nr1d2*) further function to stabilize the clock mechanism. RORs and REV-ERBs are directly under the transcriptional regulation of CLOCK-BMAL1. ROR responsive elements (RREs) present in the *Bmal1* promotor region are the targets for these factors wherein, REV-ERBs suppress and RORs activate *Bmal1* transcription. This leads to the rhythmic expression of BMAL1 in antiphase with rhythmic PER expression. In addition to these, RORs and REV-ERBs rhythmically regulate the repressor *Nfil3* (Nuclear factor interleukin 3) contributing to another auxiliary loop. NFIL3 in turn represses D-box binding protein (DBP) to modulate rhythmic ROR expression. These three interlocked TTFLs together generate robust transcriptional rhythms underpinning the 24 h circadian machinery (Fig. 5) (Partch et al. 2014; Takahashi 2017; Honma 2018).

Regulation of peripheral clocks

The mammalian CTS with central pacemaker SCN at the top of hierarchical array of oscillators is composed of peripheral clocks which exist in most of the tissues as well as organs. The canonical clock genes and the molecular mechanism involving TTFLs exist ubiquitously and not restricted to hypothalamic SCN (Balsalobre 2002; Mohawk et al. 2012; Schibler et al. 2015). However, the entrainment of these peripheral oscillators by the SCN results in coordinated rhythmicity at the organismal level (Fig. 6). Output signals from the SCN synchronize pineal, pituitary, adrenal gland and other peripheral clocks. Rhythmic release of pineal melatonin, aldosterone and corticosterone (from adrenal glands) in to circulation play crucial role in entraining all the other oscillators in the periphery.

SCN mediates rhythmic secretion of pituitary hormones via neuroendocrine signals where they in directly or indirectly target peripheral clocks with the secretion of gonadotropins or by modulating the release of adrenal steroids (Menaker et al. 2013). In addition to these, SCN directs temporal regulation of body temperature and rest/activity cycles which further aid in the entrainment of peripheral oscillators. Further, food entrainable oscillator (FEO) and methamphetamine sensitive circadian oscillator (MASCO) are known to contribute to the circadian machinery though information on their anatomical location and molecular mechanisms are limited. The intricate nature of CTS is highlighted by the presence of

hierarchical multi-oscillator network comprising the central clock SCN, extra SCN oscillators and peripheral clocks distributed in entire body (Menaker et al. 2013; Honma 2018).

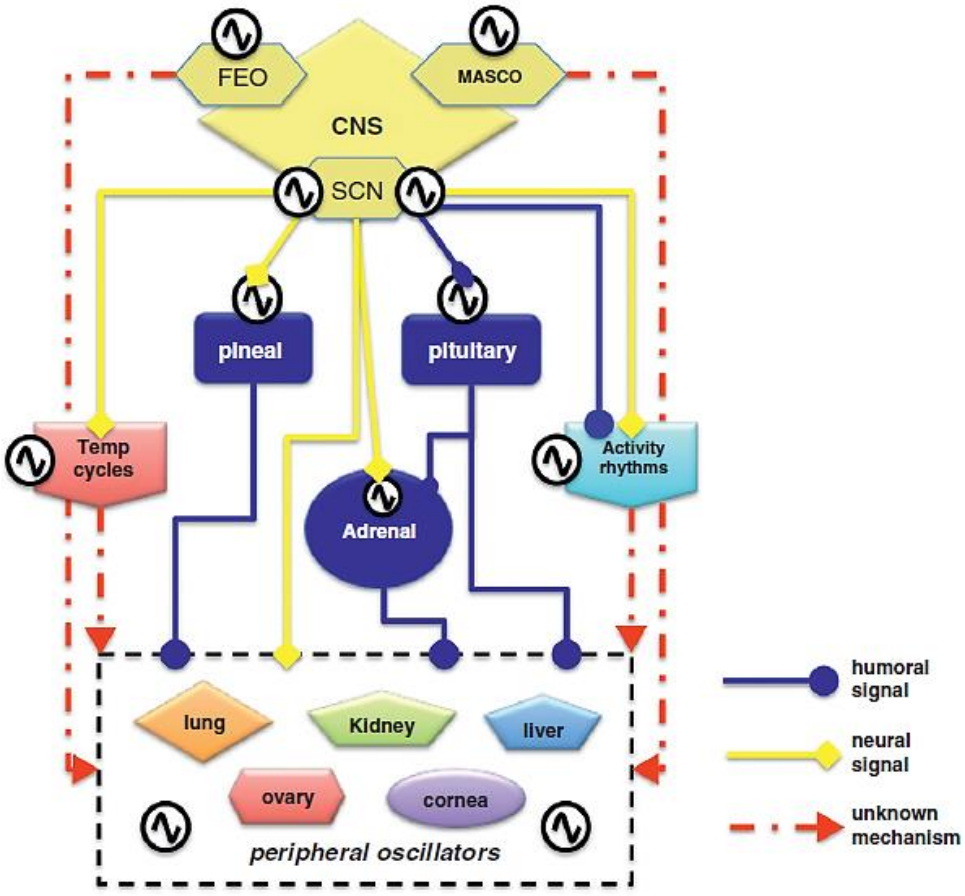


Fig. 6 Diagrammatic representation of networks inter-connecting central and peripheral clocks in mammals. CNS – central nervous system, SCN – suprachiasmatic nucleus, FEO – food entrainable oscillator, MASCO – methamphetamine sensitive circadian oscillator (Menaker et al. 2013).

Interactions between circadian clocks and cellular metabolism

Though circadian clocks are sustained by TTFLs consisting dedicated clock genes and proteins, several epigenetic mechanisms contribute to the robust functioning of the clock. In mammals, sirtuins (SIRTs), homologs of yeast silent information regulator 2 (Sir2) modulate circadian clock at various levels. With seven members in the family (SIRT1 – 7), these nicotinamide adenine dinucleotide (NAD^+)-dependent histone deacetylases

(HDACs) play crucial roles in regulation of clock function in addition to controlling various physiological functions including energy metabolism, stress and nutrient sensing, genome integrity maintenance and aging (Masri and Sassone-corsi 2014). The enzymatic activity, cellular targets and function of these SIRTs vary in addition to their sub-cellular localization. SIRT1 shuttles amid the cytoplasm and the nucleus, SIRT2 is localized within the protoplasm whereas, SIRT3 – 5 are mitochondrial, SIRT6 is located in nucleus and SIRT7 in nucleolus.

Among the seven members, role of SIRT1 in circadian regulation is extensively studied and several reports suggested their importance in central and peripheral clock functioning (Jagota et al. 2019). Most importantly, SIRT1's regulatory role in influencing the *Bmal1* expression as well as amplitude of the circadian machinery was elucidated by knockout and over-expression studies in mice SCN (Chang and Guarente 2013). The brain specific deletion of SIRT1 had resulted in increased BMAL1 acetylation, lengthened circadian period and dampened expression of *Bmal1*, *Per2* which were reversed in mice over expressing SIRT1 suggesting its role in clock function (Chang and Guarente 2013). Nuclear receptor RORα interacts with peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) to positively regulate *Bmal1* expression (Liu et al. 2007). Both SIRT1 and PGC-1α cooperatively bind at the RREs and SIRT1 mediates this transcriptional activation of *Bmal1* by deacetylating PGC-1α (Fig. 7a). On the other hand, SIRT1 activity follows a circadian pattern which implies the temporally regulated nature of this HDACs function. *Nampt*, the gene coding for the rate limiting enzyme NAMPT (in salvage pathway) which biosynthesizes NAD^+ is a CCG which is under the transcriptional control of canonical positive regulators of the clock. CLOCK, BMAL1 and SIRT1 bind in close proximity to the E-box elements located within the *Nampt* promotor in a circadian fashion. Thus the production of NAD^+ is circadian and in turn the activity of SIRT1 is also circadian. The circadian fashion of NAMPT-SIRT1-PGC-1α loop is driven by CLOCK-BMAL1 and this loop modulates the amplitude of BMAL1 and subsequently other clock elements (Fig. 7a and Fig. 7c) (Nakahata et al. 2008; Ramsey et al. 2009; Chang and Guarente 2013). Earlier studies conducted in peripheral clock liver and mouse embryonic fibroblasts revealed novel mechanisms of clock interactions involving SIRT1. SIRT1 was shown to promote PER2 degradation by directly deacetylating the latter, influencing circadian periodicity (Asher et al. 2008). It was also found to be deacetylating BMAL1, which is important for temporal transcriptional control. Moreover, recruitment of SIRT1

by CLOCK-BMAL1 complex to the promotors of CCGs, where subsequent deacetylation of specific lysine residues (Lys^{9} and Lys^{14}) of H3 histones results in regulation of transcriptional activation (Fig. 7b) (Nakahata et al. 2008).

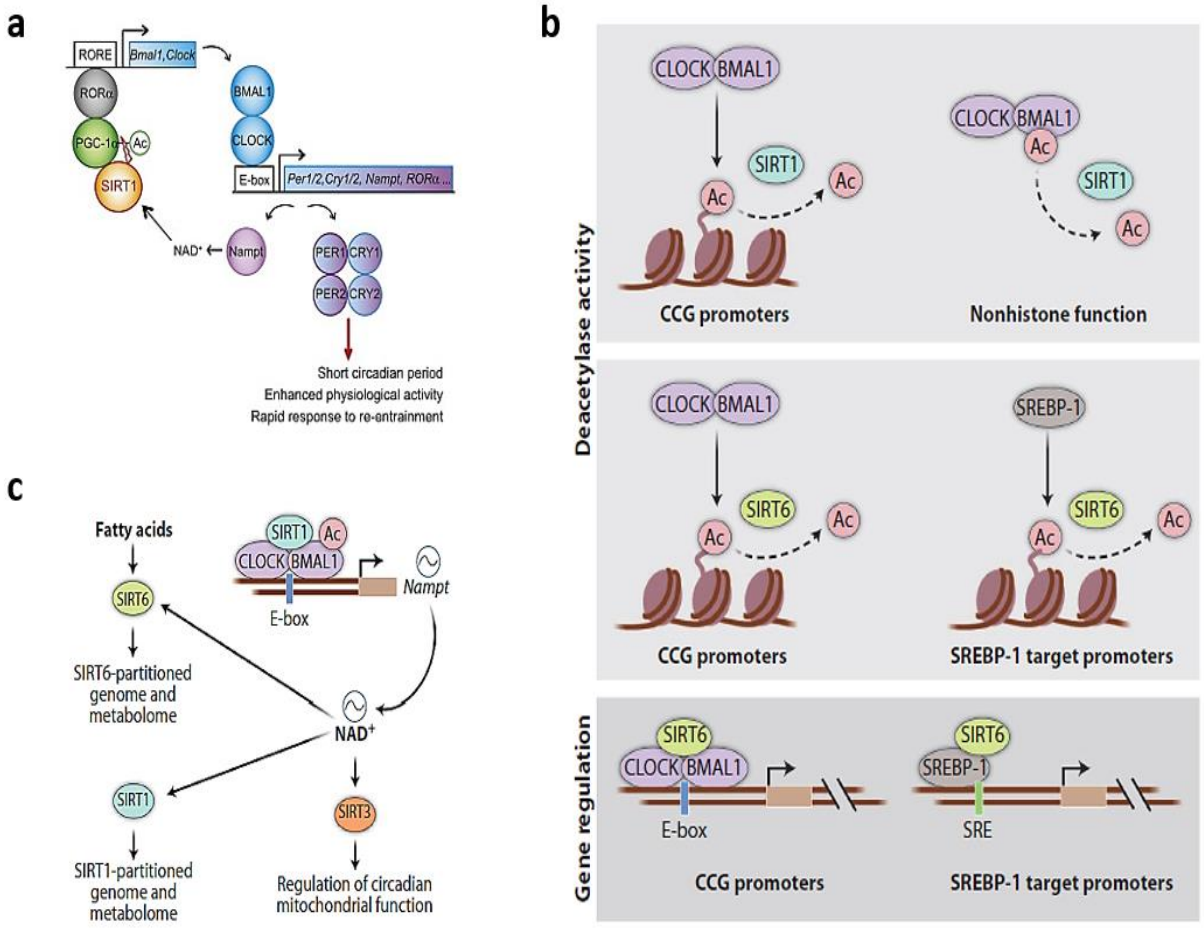


Fig. 7 (a) Diagrammatic representation of SIRT1-mediated circadian gene activation model in the SCN. **(b)** SIRT1 and SIRT6 mediated nuclear regulation of circadian gene expression. **(c)** Circadian regulation of NAD+ synthesis and SIRTUIN activation (Chang and Guarente 2013; Masri and Sassone-corsi 2014).

SIRT6 which is also localized in nucleus participates in the regulation of circadian gene expression (Fig. 7b) (Masri and Sassone-corsi 2014). Though SIRTs are primarily dependent on NAD^{+} for their activity (Fig. 7c), emerging data suggests the modulation by other metabolites such as free fatty acids (FFA). SIRT1 indirectly contributes to fatty acid metabolism by regulating the circadian activity of acetyl-CoA-synthetase 1 which produces acetyl-CoA, thus setting in complex intertwining regulatory mechanisms. Collectively,

these observations suggest that the feedback loops constituted majorly by SIRTs mediate the reciprocal interactions between circadian clocks and metabolism.

SEROTONIN

Serotonin [5-Hydroxytryptamine (5-HT)], a biogenic amine which was discovered 70 years ago, is known to function as a neurotransmitter communicating the signals and information among neurons (Berger et al. 2009). The essential amino acid L-tryptophan that is consumed in the diet is the precursor of serotonin. The biosynthesis of serotonin takes place in various sites including the pineal gland, raphe nucleus located in the brain stem, retina, para-follicular cells of thyroid, neuro-epithelial bodies of the lung, enterochromaffin cells of the gut as well as in platelets (Sanchez et al. 2008). The biosynthesis of serotonin in mammals is catalyzed by the enzyme tryptophan hydroxylase (TPH) which exists as isomers TPH1 and TPH2. In pinealocytes of the pineal gland and in intestinal enterochromaffin cells TPH1 is involved in serotonin synthesis. Whereas, in raphe nuclei and myenteric plexus TPH2 carries out the 5-HT production. While less than 10 % of the total serotonin is originated from brain and pineal gland, approximately 90 % of it is synthesized in enterochromaffin cells (Ruddick et al. 2006).

Serotonin is important in the modulation of most of the brain functions, physiology and behavior (Fig. 8), dysregulation of serotonergic system has been linked with several psychiatric and neurological disorders (Berger et al. 2009).

One of the major component among the many physiological and behavioral aspects that are influenced by serotonin is CTS. Serotonin is implicated in functioning of circadian clock especially in the retino-raphe pathway (RRP) and plays key role in photic as well as non-photic entrainment of the central clock (Yao et al. 2010; Ciarleglio et al. 2011). The central pacemaker SCN receives densest serotonergic terminal innervations in the brain and the RRP involving serotonin signaling has been described in earlier section (Afferent pathways to the SCN).

Serotonin modulates a range of sensory, motor and neurological functions via its receptors. The serotonin receptors are categorized in to seven classes and such as 5-HT_1 to 5-HT_7 that belong to the super family of GPCRs (guanine nucleotide triphosphate (GTP)-binding protein)-coupled receptors).

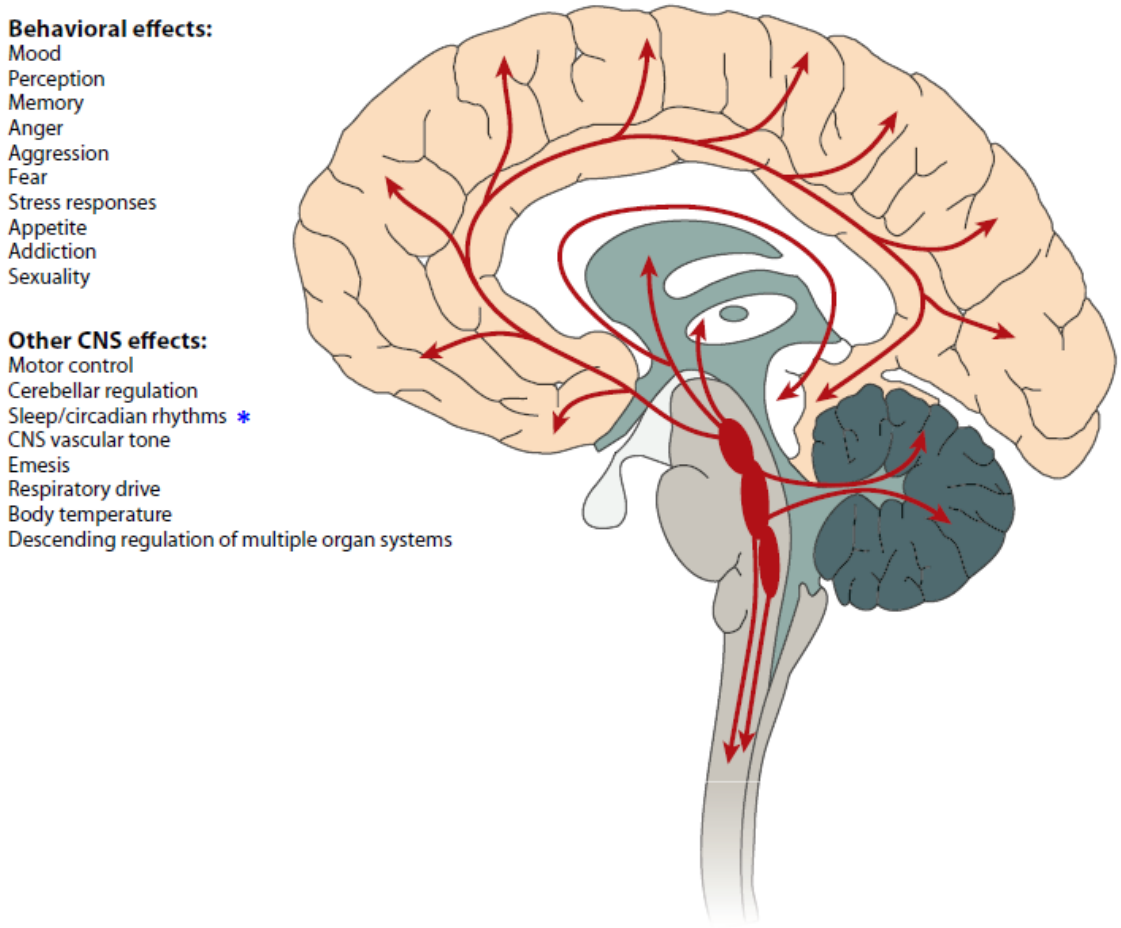


Fig. 8 Central serotonergic pathways regulating various physiological and behavioral effects. In the central nervous system (CNS), serotonin is almost exclusively produced in neurons originating in the raphe nuclei located in the midline of the brainstem. These serotonin-producing neurons form the largest and most complex efferent system in the human brain. The most caudal raphe innervate the spinal cord, while the more rostral raphe, the dorsal raphe nucleus and the medial raphe nucleus, innervate much of the rest of the CNS by diffuse projections. Indeed, virtually every cell in the brain is close to a serotonergic fiber, and nearly all behaviors as well as many other brain functions are regulated by serotonin (Berger et al. 2009).

But 5-HT_3 is an exception as it is different other receptors being a ligand-gated ion channel distributed in CNS and in the PNS (peripheral nervous system) (Donaldson et al. 2013). On the basis of their action mechanism, GPCRs are classified as Gs (Stimulatory- Ca^{2+} channel activation, adenylyl cyclase (AC) activation), Gi (Inhibitory- K^+ channel activation, AC inhibition), Go (Ca^{2+} channel inhibition), Gq (phospholipase C (PLC) activation).

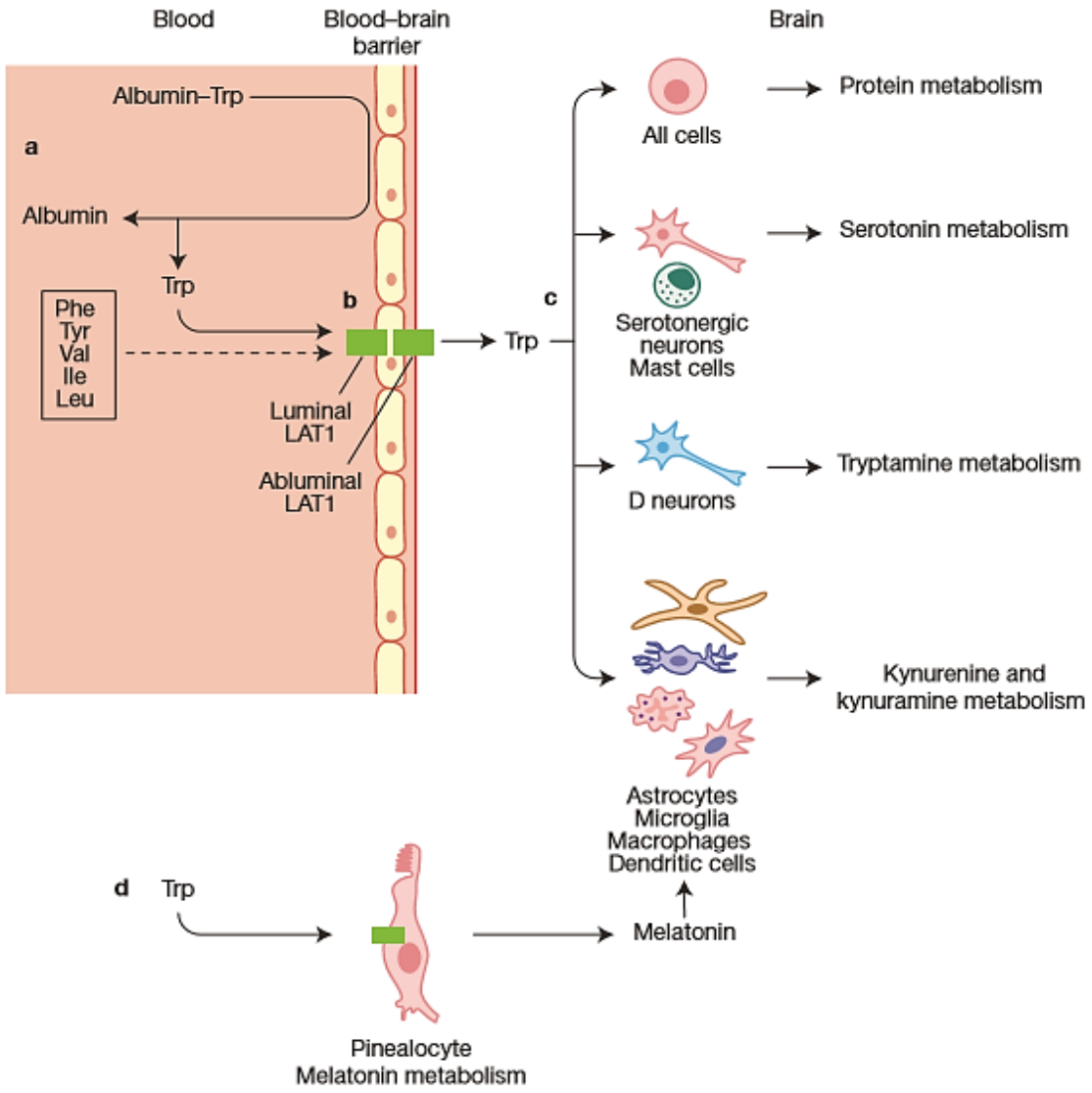


Fig. 9 Overview of tryptophan metabolism in the central nervous system (CNS) (a) Availability of unbound tryptophan for uptake into the brain. (b) Free tryptophan is transported across the BBB via the L-type amino acid transporter (LAT1), at the luminal and abluminal surfaces of endothelial cells. (c) From CSF, tryptophan is up taken by all cells for protein synthesis, and specialized cells that synthesize neuroactive metabolites of tryptophan, including serotonin (in serotonergic neurons and mast cells), tryptamine (in 'D' neurons that express aromatic amino acid decarboxylase), and kynurenines (in astrocytes, microglia, macrophages and dendritic cells). (d) Melatonin synthesized in the pineal gland has ready access to the brain, where it is involved in neural signaling through G-protein-coupled receptors, but also has antioxidant effects and can serve as a precursor for synthesis of other molecules with antioxidant effects, including kynuramines (Ruddick et al. 2006).

Subtypes of 5-HT_1 receptors belong to Gi/o type which inhibit AC and designated as 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E} and 5-HT_{1F}. Whereas, 5-HT_2 receptors subtypes are Gq type which activate PLC and denoted as 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}. The ligand gated 5-HT_3 receptor is a sodium (Na^+)-potassium (K^+) ion channel. 5-HT_4 and rest of the receptors belong to Gs type that activate AC (Donaldson et al. 2013).

Serotonin and its receptors are important in regulating all major organ systems including CNS, pulmonary, cardiovascular, gastrointestinal and genitourinary systems. 5-HT virtually modulates appetite (appetite suppressant effect), sleep, mood (antidepressant action), body temperature and circadian rhythms via its receptors (Berger et al. 2009). In circadian system, serotonin is known to mediate phase shifting effects. It can bring about phase-advances in the pacemaker when applied in the photo phase and phase-delays during the scotophase. 5-HT_7 receptors in the SCN have been identified to be involved in these phase shifting effects (Ursin 2002).

In addition to these, serotonin is also known to have a suppressive effect on oxidative stress in the brain signifying its importance in aging and associated neurodegenerative conditions as demonstrated in a recent study. A novel regulatory function of serotonin in mitochondrial biogenesis and in combating oxidative stress via SIRT1/PGC-1α axis with the involvement of 5-HT_{2A} receptors was reported in rodent cortical neurons (Fanibunda et al. 2019).

Biosynthesis and metabolism of serotonin

The essential amino acid L-tryptophan is central to many crucial biochemical processes that takes place in every living cell (Le Floc'h et al. 2011). Tryptophan metabolism produces several molecules which are biologically active including various neuromodulators of CNS. The ingested tryptophan gets absorbed and a major fraction of it binds with albumin in the plasma forming a complex which fails to traverse the blood–brain barrier (BBB). Unbound free tryptophan in circulation gets transported across the BBB into the cerebrospinal fluid (CSF), where it is readily accessible to the CNS cells for utilization in numerous metabolic pathways (Fig. 9).

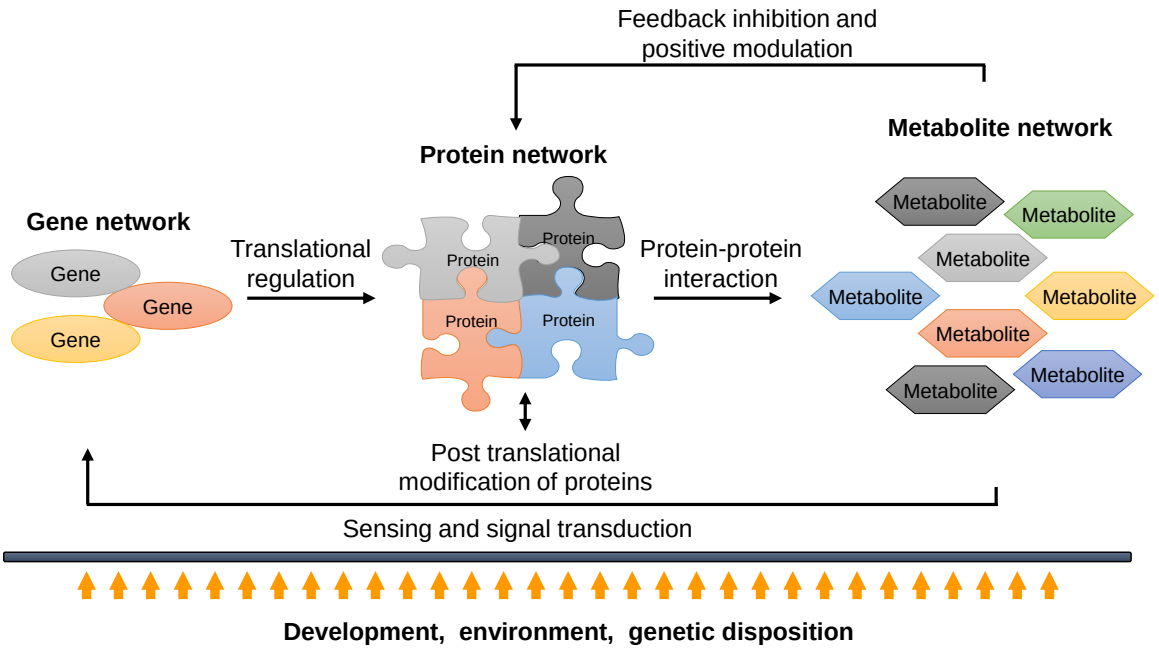


Fig. 10 Diagrammatic representation of the gene-protein-metabolite network (Weckwerth 2003).

Out of the many tryptophan dependent cellular processes, protein synthesis consumes major part of tryptophan. Kynurenine and 3-hydroxyanthranilic acid pathway is the major catabolic route of tryptophan whereas, the biosynthesis of serotonin is a minor but vital metabolic route (3% of ingested tryptophan is used for serotonin biosynthesis in the entire body and 1% is used for the same in brain) (Kema et al. 2000; Richard et al. 2009). The synthesis/metabolism of serotonin and such neuroactive molecules constitute a metabolite network which is of great significance as the metabolome is closely tied to an organism's genotype, its physiology and its environment, representing the overall physiological status of the organism (Fig. 10) (Weckwerth 2003).

L-tryptophan enters the indole metabolism pathway to be converted to serotonin which further forms major metabolites such as melatonin, 5-HIAA, 5-MIAA and 5-MTOH. In another limb of this pathway tryptophan is also converted to NAT (Fig. 11) (Reiter et al. 2010; Lewczuk et al. 2014).

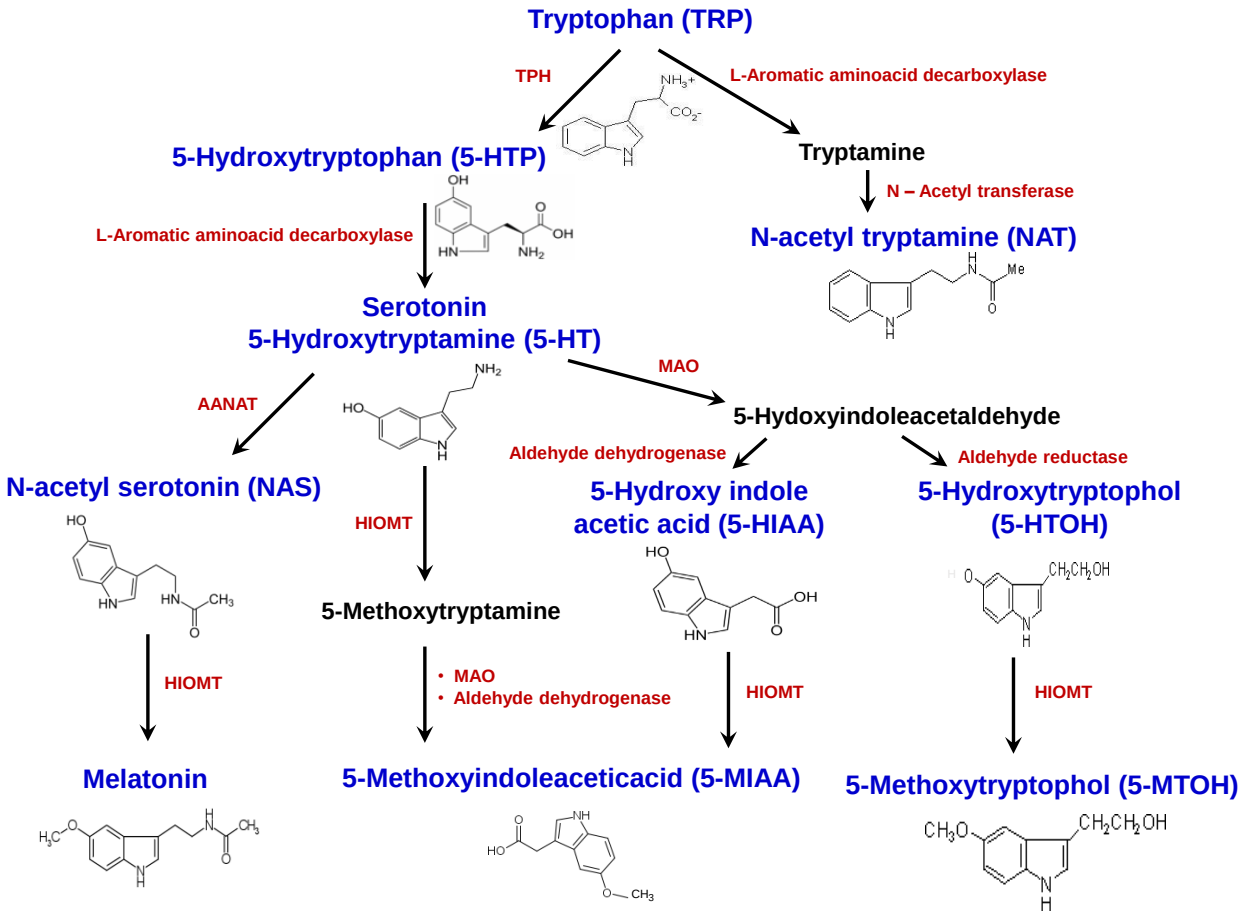


Fig. 11 Serotonin biosynthesis and metabolism. TPH – Tryptophan hydroxylase, MAO – Monoamine oxidase, AANAT – Aryl alkylamine N-acetyl transferase, HIOMT – Hydroxy indole-O-methyl transferase (Lewczuk et al. 2014).

Tryptophan, serotonin and their related metabolites are neuromodulators which are involved in modulation of several important processes at endocrine, physiological and behavioral levels. Alterations in levels of these components lead to the manifestation of various disorders. Tryptophan deficiency results in pathology of various psychological and physiological processes including mood disorders, motion sickness, cognition, sleep, visual discrimination, aggression, anxiety, memory consolidation, appetite, visual perception, hemodynamics etc. (Richard et al. 2009; Le Floc'h et al. 2011). In various pathology of liver including chronic alcoholism related pathologies, significant increase in 5-HTP levels has been noted as a result of metabolic shift. 5-HTP has been known to function as an antidepressant and benefits patients with anxiety and panic disorders (Shaw et al. 2009). Serotonin syndrome, a condition originating from hyperserotonemia is associated with cardiac fibrosis and is known to be lethal (Berger et al. 2009). While higher

5-HT levels is linked to autism, low levels lead to mood and memory related ailments. Clinical studies have associated decreased titers of 5-HT and 5-HIAA with aggression, depression, mania and suicide (Kema et al. 2000; Berger et al. 2009). Moreover, excessive titers of 5-HIAA in urine has been established as a marker for carcinoid tumors (Mansencal et al. 2010). NAS, the intermediate between serotonin and melatonin, with its potent antioxidant effect is known to maintain the optimal fluidity of biological membranes by regulating lipid peroxidation (García et al. 2001). The terminal components of serotonin metabolism such as 5-HTOH, 5-MTOH and 5-MIAA are considered as important biomarkers in several pathological conditions (Das et al. 2008). In addition, their importance as endogenous antioxidants has been reported earlier. 5-MTOH has been shown to be involved in human developmental processes, regulation of phagocytosis, and based on species, sex and photoperiod as pro/anti gonadotropin (Zawilska et al. 1998). Moreover, researchers have probed the contribution of 5-MTOH in the regulation of seasonal variations in reproductive behavior of diurnal rodents (Saxena et al. 1991).

Serotonin undergoes enzymatic modifications so as to be excreted out of the body. Enzymes uridine diphosphate glucuronyltransferase and serotonin sulfotransferase convert serotonin into serotonin-O-glucuronide and serotonin-O-sulfate respectively in liver and subsequently these are excreted in urine. Serotonin sulfotransferase is also present in brain, lungs and kidneys. While 5-HIAA is excreted in free form, 5-HTOH is predominantly excreted in a conjugate form in urine (Kema et al. 2000).

MELATONIN

Melatonin or N-acetyl-5-methoxytryptamine is a biogenic amine enzymatically produced from serotonin (Fig. 11). This multitasking neuro-hormone also referred as the ‘messenger of darkness’ is one of the major products of the pineal gland. In addition to pineal gland, extra pineal sites including retina, lacrimal gland, skin, Harderian gland, ovary, lymphocytes, bone marrow and most importantly gastrointestinal tract are known to synthesize melatonin (Karasek 2007; Pandi-Perumal et al. 2008). Based on the site of its synthesis and the organ/tissue of target, melatonin can function as a biological modulator, neurotransmitter, hormone or cytokine (Slominski et al. 2012). Melatonin is considered as direct output of central pacemaker SCN as its biosynthesis and secretion from the pineal gland follows circadian pattern with a surge in the night (Reiter et al. 2014). The multisynaptic pathway originating in SCN terminates in pinealocytes to temporally

regulate the melatonin release (Fig. 4). Upon release, melatonin in CSF and in circulation functions as an internal *zeitgeber* or endogenous synchronizer of rhythms (Pevet and Challet 2011). As the title 'messenger of darkness' suggests, melatonin fetches the information of night-time to the organism and thereby prepares it to assume the right niche.

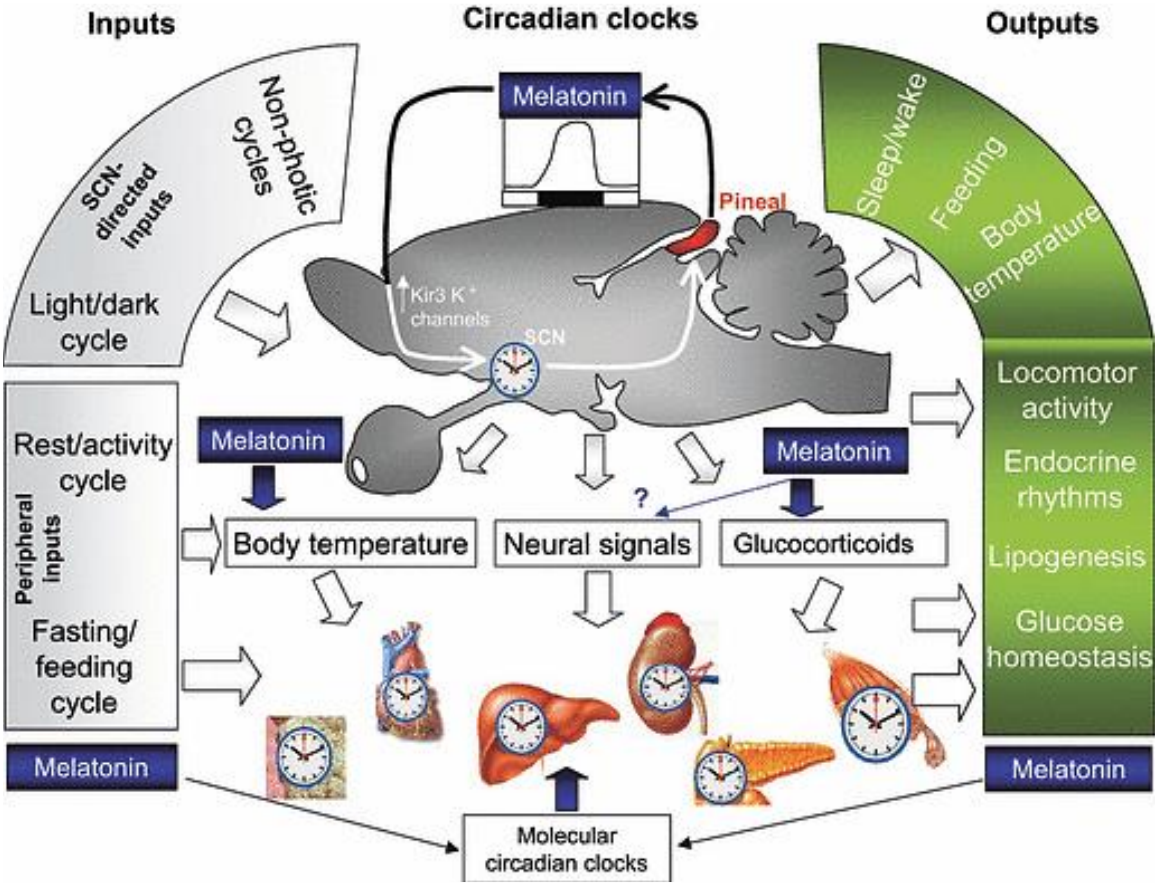


Fig. 12 Overview of the roles of melatonin in the circadian multi-oscillator system. Kir3 K^+ channels - type 3 inward rectifier K^+ channels, SCN - suprachiasmatic nucleus (Hardeland et al. 2012).

A diurnal animal would prefer to rest while the nocturnal one choose to forage upon melatonin surge (Pandi-perumal et al. 2008). The daily and seasonal time information is transduced throughout the organism by circadian variation in melatonin release (Johnston

et al. 2006). Melatonin mediates a large array of physiological functions in a time dependent manner (Fig. 12).

Apart from its function as an internal time giver, melatonin is extensively involved in the regulation of numerous physiological processes including retinal physiology, ovarian physiology, neuroprotection, neuro-immuno-modulation, sexual development, seasonal reproduction, regulation of blood pressure, osteoblast differentiation and various psychological conditions. Most importantly, its inevitable role in free radical scavenging has been studied extensively. Further, melatonin facilitates sleep propensity by modulating the core body temperature during the night via its vasodilatory property. As it promotes sleepiness, melatonin functions as a soporific agent (Rasmussen et al. 2003; Witt-Enderby et al. 2003).

Melatonin being a pleotropic molecule has been demonstrated to exert wide array of therapeutic effects when administered exogenously. The beneficial effect of melatonin has been studied in several pathologies and disorders including circadian/sleep disorders, seasonal affective disorders (SAD), depression, obesity, disorders of the immune system and sexual dysfunction. Melatonin and its metabolites such as cyclic 3-hydroxymelatonin, AFMK and AMK exhibit vigorous free radical scavenging activity thereby elicit efficient cyto-protective response. Its antioxidant role is also implicated in neutralizing the mitochondrial oxidative stress as well (Reiter et al. 2010). Moreover melatonin has been shown to prevent oxidative damage and cell death in trauma, ischemia in addition to function as an anti-apoptotic agent in several neurodegenerative disorders (Reiter et al. 2007; Esposito et al. 2008). Interestingly an earlier study had demonstrated the role of melatonin in ameliorating the menopause associated depression (Bellipanni et al. 2005).

MELATONIN RECEPTORS

The photoperiodic information from the SCN to other peripheral clocks and feedback to the master clock is mediated through specific receptors with high-affinity to melatonin referred as ‘Melatonin receptors’. These receptors belong to G-protein coupled receptor (GPCR) subfamily (Jockers et al. 2016). Melatonin receptor 1a (MT1) and melatonin receptor 1b (MT2) are the two members of the melatonin receptor subfamily which are known to be present in mammals (Reppert et al. 1995; Cecon et al. 2017). A third receptor for melatonin ‘MT3’ which is cytoplasmic quinone reductase II has been reported in hamster and rabbit (Slominski et al. 2012). In humans melatonin receptors have been

localized in several brain regions and most of the peripheral tissues (Fig. 13). Cerebellum, cerebral cortex, thalamus, SCN and hippocampus express MT1 and MT2 receptors in addition to retina. They have been also localized in human non-neural tissues such as kidney, adipocytes, coronary arteries and blood vessels (Sallinen et al. 2005). In rodents these receptors have been identified in different regions of the CNS including SCN and in most of the tissues/organs in the periphery such as retina, heart, lungs, liver, spleen, pancreas and kidney (Sallinen et al. 2005; Ishii and Tanaka 2009; Slominski et al. 2012). The expression of these receptors is reported to alter with species, photoperiodic conditions, endocrine status, neural developmental stages and time of day (Venegas et al. 2013).

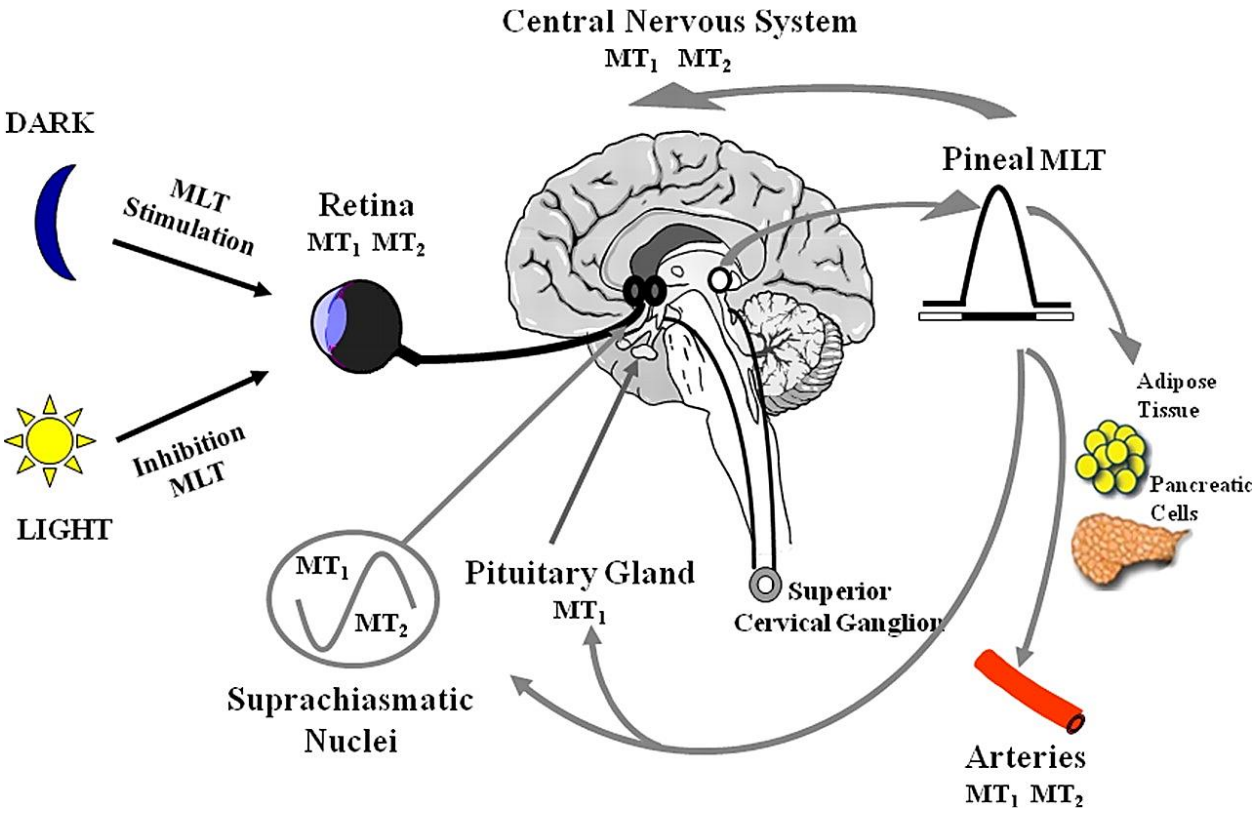


Fig. 13 Regulation of melatonin production and receptor function (Dubocovich et al. 2010).

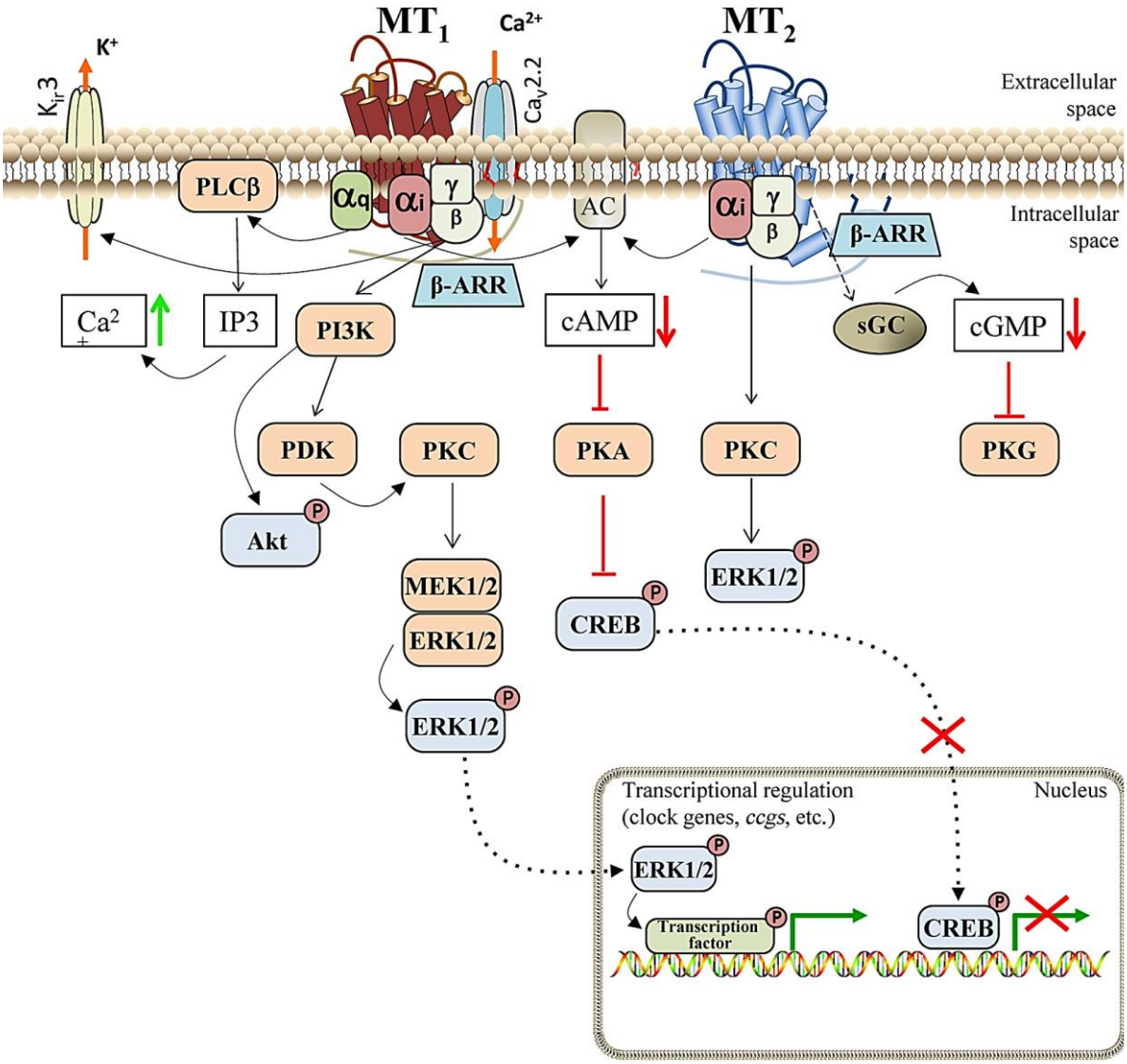


Fig. 14 Melatonin receptor signaling pathways. Melatonin activation of MT1 receptors triggers $G\alpha_i$ activation, decreasing the levels of the secondary messenger cAMP, and $G\beta\gamma$-dependent activation of PI3K/Akt, PKC and ERK pathways. MT1 coupling to Gq leads to PLC activation and increase in intracellular Ca^{2+}. Melatonin-induced modulation of neuronal action potential is mediated by MT1-dependent activation of the potassium and calcium ion channels ($K_{ir}3$ and $Ca_v2.2$). The physical interaction of MT1 receptors with $Ca_v2.2$ channels tonically inhibits $Ca_v2.2$-mediated calcium entry through $G\beta\gamma$ subunits. Melatonin activation of MT2 receptors triggers $G\alpha_i$-dependent cAMP and ERK signaling pathways and inhibits cGMP levels. Melatonin induced β-arrestin recruitment to both MT1 and MT2 receptors, but β-arrestin-dependent down-streaming signaling is not yet reported. β-ARR - β-arrestin, $Ca_v2.2$ - voltage-gated calcium channel, *ccgs* - clock-controlled genes, CREB - cAMP-responsive element binding, $K_{ir}3$ - G protein-coupled inwardly rectifying potassium channel, sGC - soluble GC (Cecon et al. 2017).

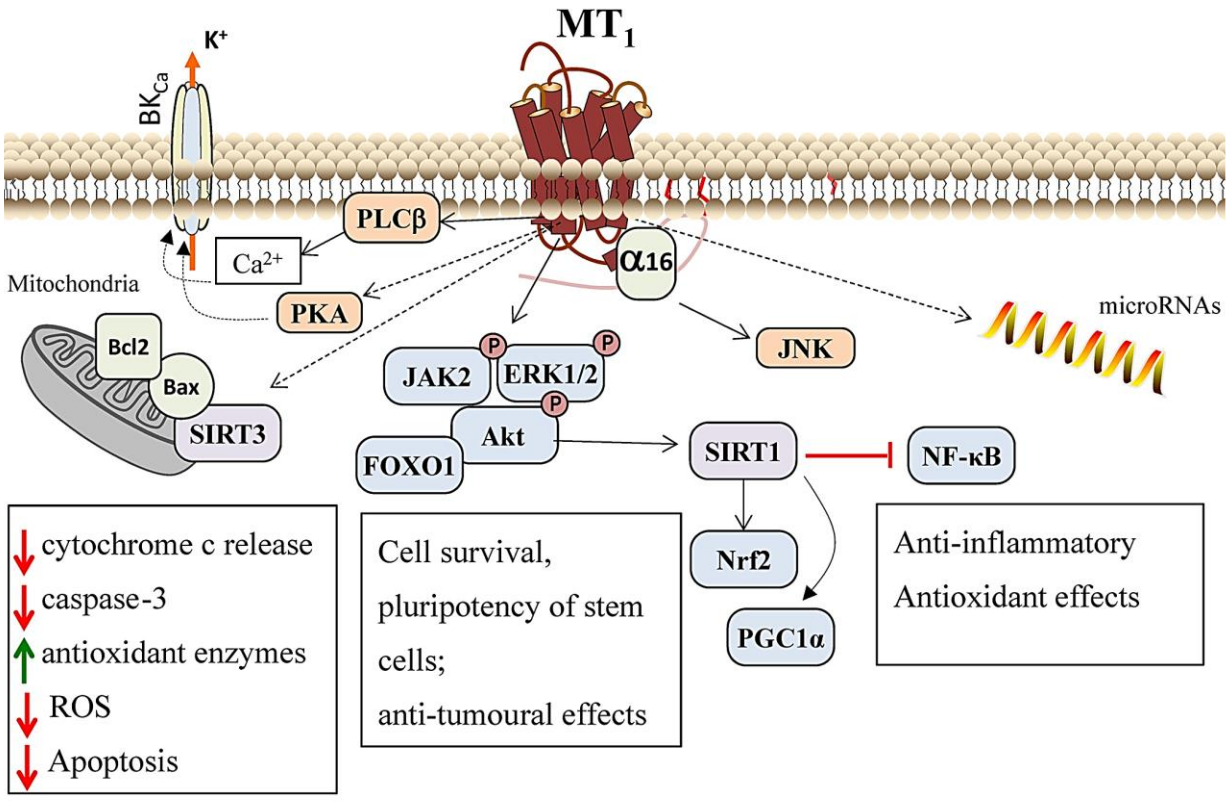


Fig. 15 Extended, context-specific melatonin receptor signaling pathways. Depending on the cell type or the presence of cell stressors, melatonin can activate additional melatonin receptor-dependent signaling cascades. These pathways have been reported mainly for MT1 receptors, but the participation of MT2 receptors cannot be excluded. Melatonin modulation of mitochondrial function is reported under oxidative stress condition and in neurodegenerative diseases. Proposed signaling pathways involve the regulation of the activity and/or translocation of Bcl2/Bax and SIRT proteins. Activation of JAK2, ERK and the Akt/FOXO1 complex are suggested to mediate melatonin-induced cell survival and to modulate pluripotency/differentiation of stem cells, while melatonin-induced inhibition of these pathways is reported in cancer cells. MT1-dependent activation of SIRT1 might underlie melatonin's anti-inflammatory and anti-oxidative effects through regulation of transcription factors like Nrf2, PGC1α and NF-κB. MT1-coupling to G_{16} protein occurs in hematopoietic cells and triggers the JNK pathway (Cecon et al. 2017).

These receptors are primarily coupled to Gαi/o proteins and thus majorly function via modulating the intracellular levels of the secondary messenger cAMP upon activation by melatonin (Fig. 14). Melatonin receptors play regulatory role in several physiological and behavioral aspects including sleep, seasonal reproduction, glucose homeostasis, retinal physiology, immune function, vasodilation and vasoconstriction (Dubocovich et al. 2010; Jockers et al. 2016; Laurent et al. 2017; Silva et al. 2019).

Melatonin mediates chronobiotic effects through melatonin receptors located on the SCN and stimulation of these receptors result in synchronizing the amplitude and phase of circadian rhythms throughout the body (Dubocovich 2007; Pevet and Challet 2011). MT1 and MT2 receptors follow rhythmic expression pattern (Poirel et al. 2002; Venegas et al. 2013) and their function in modulating circadian clocks has been inevitable (Pandi-perumal et al. 2008). The endogenous pineal melatonin feeds back to the master clock SCN through MT1 and MT2 receptors and regulate neuronal firing and circadian rhythms (Dubocovich and Markowska 2005).

Within SCN these receptors are known to function through multiple pathways. Activation of MT1 in mouse SCN inhibits PACAP mediated CREB phosphorylation whereas studies in rat SCN revealed MT2 mediated stimulation of PKC activity (Dubocovich and Markowska 2005). Further, the MT1 receptor mediated inhibition of neuronal firing in SCN has been well studied and this inhibition resulting in altered clock response during night is known to have specific physiological significance in promoting sleep (Zhdanova 2005) Furthermore, melatonin's phase shifting effects on circadian rhythms of neuronal firing in SCN is observed to be temporally gated to dusk (day-night transition) and dawn (night-day transition) and involves the activation of MT2 receptors (Hunt et al. 2001). Studies have also demonstrated the involvement of MT1 receptors in phase shifting locomotor activity and re-entrainment to specific photoperiodic conditions (Dubocovich and Markowska 2005). Moreover, MT1 receptors have direct role in transmitting photoperiod information and in turn influence reproductive behavior (Yasuo et al. 2009). In addition, studies have shown suppressive role for pineal melatonin on *Per1* transcript expression in pituitary gland through activation of MT1 receptor during night phase (von Gall et al. 2002). Genetic deletion studies in mice showed the importance of these two receptors for circadian phenotype, regulation of clock genes, neuronal activity, (Dubocovich et al. 2005; Dubocovich 2007; Jockers et al. 2008), mental health, learning and memory (Wang et al. 2005; Weil et al. 2006; Comai et al. 2015).

Melatonin is also known to activate a number of cascades of MT receptor dependent signaling pathways depending on the location of the receptors and physiological status (Fig. 15). In several neurodegenerative diseases and in conditions of increased oxidative stress, melatonin is known to modulate mitochondrial function via Bcl2/Bax and SIRT regulation. MT receptor mediated regulation of SIRT1/NRF2, SIRT1/PGC1α and

SIRT1/NF-κB axis might underpin melatonin's anti-oxidative and anti-inflammatory effects (Cecon et al. 2017).

AGING

Aging is an inevitable unidirectional process which is associated with decrease in 'buffering capacity' or 'homeodynamic space' that eventually leads to the progressive decline of metabolism, physiology and behavior, ultimately leading to death (Rattan 2008; Jagota et al. 2019). The systematic study of various aspects of aging specifically termed as 'gerontology' has gained global interest in past decades due to the extended life span of humans as a result of enormous progress made in medicine and health care (Rattan 2018). The phenomenon of aging has been explained through several different theories such as "the programmed theory" (which includes programmed longevity, endocrine theory and immunological theory), "damage or error theory" and "the free radical theory."

While programmed longevity theory postulates programmed switching of particular genes leading to senescence resulting in overt manifestations, endocrine theory assumes evolutionarily conserved hormonal signaling such as insulin/IGF-1 signaling pathway that regulate the process of aging (Jagota and Thummadi 2017). Whereas, immunological theory hypothesizes the pre-programmed deterioration of immune system. The damage or error theory postulates that the accumulated damages or errors at several levels over the period of time would cause aging and is linked to metabolic disorders, instability of genome, erosion of telomeres, alterations in epigenetics and in inter/intra-cellular communications, proteostasis loses, cellular senescence, deregulated nutrient sensing, stem cell exhaustion, mitochondrial dysfunction and DNA damage. Of all the macromolecules that are being damaged as the age progress, DNA is very important because the damage is largely irreversible unlike other macromolecules and also slowing down of DNA repair mechanisms promotes aging (López-Otín et al. 2013).

However, the free radical theory of aging is one of the widely accepted theory which proposes progressive accumulation of oxidative damage to biomolecules due to an imbalance between prooxidants such as reactive nitrogen species (RNS), reactive oxygen species (ROS) and antioxidants (Fig. 16) (Reiter et al. 2018). The redox status/balance could be an imperative element in influencing the extent of general health maintenance as well as in impacting the frequency of various age-linked neurodegenerative conditions

including Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD) and also sleep disorders (Sohal and Orr 2012).

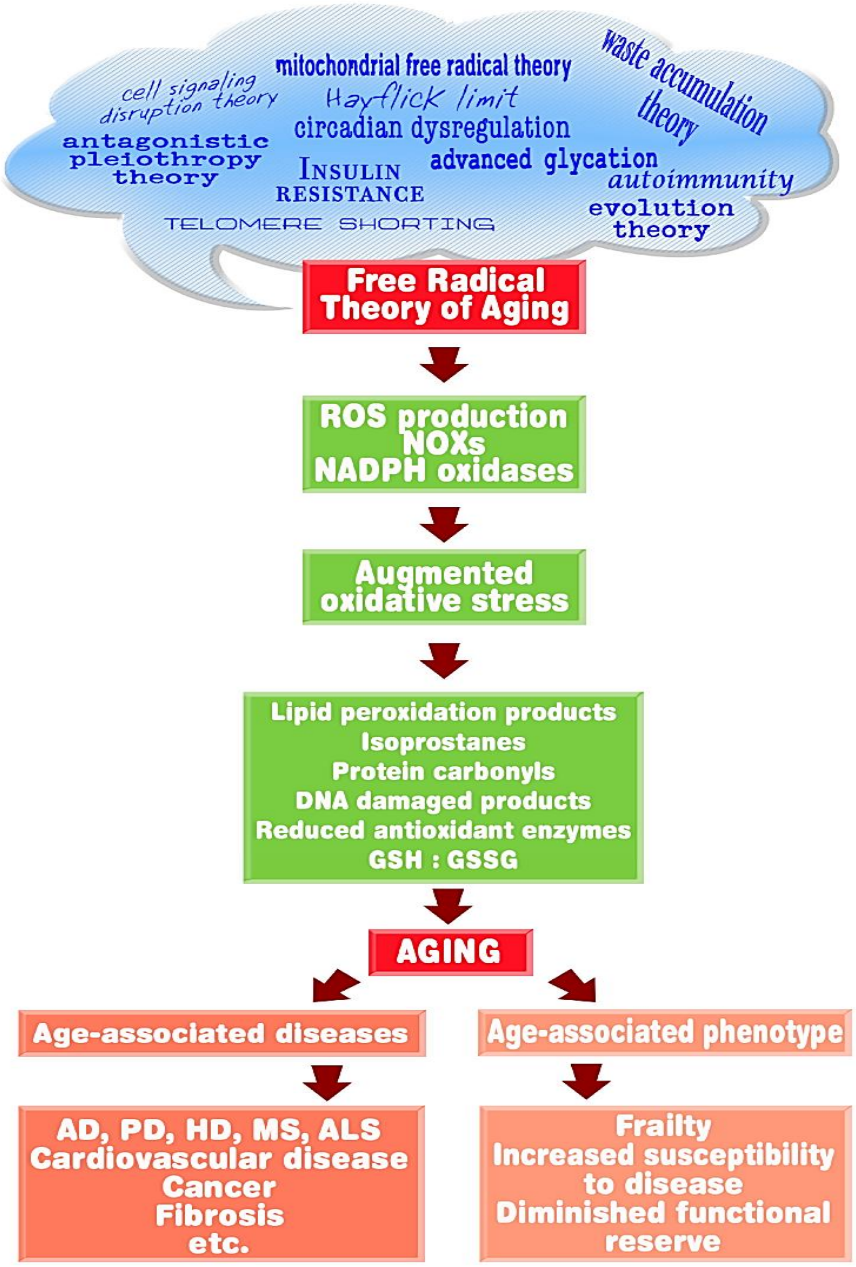


Fig. 16 Schematic representation that links free radicals and the associated oxidative damage with the progression of the aging phenotype and the onset and development of age-related diseases. The cloud at the top lists many of the iterations of the free radical theory of aging that have been introduced over the last 60 years. ROS = Reactive oxygen species; AD = Alzheimer disease; PD = Parkinson disease; HD = Huntington disease; MS = Multiple sclerosis; ALS = amyotrophic lateral sclerosis (Reiter et al. 2018).

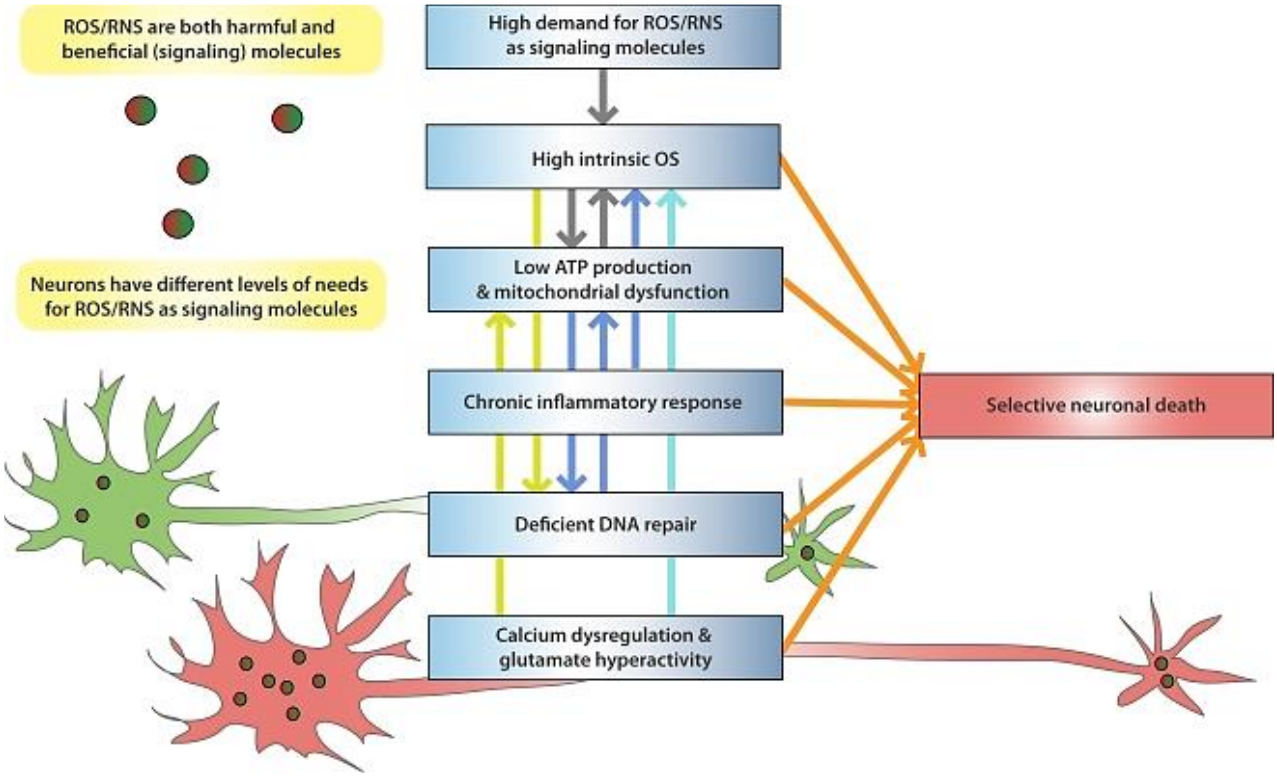


Fig. 17 Molecular and cellular factors that contribute to the selective vulnerability of neurons to oxidative stress. Vulnerable neurons are characterized by low ATP production and mitochondrial dysfunction, because of the high oxidative stress (OS) and factors such as calcium dysregulation. Low ATP production can affect DNA repair, which, when combined with high DNA oxidation, can cause change of genomic activity and decreased metabolic activity in mitochondria. Chronic inflammatory response exist in vulnerable neurons, which can further elevate OS within them. Calcium dysregulation and glutamate hyperactivity are closely connected to OS generation and underlie many adverse conditions that are characterized by SNV. There is emerging evidence that directly connects these factors, such as low calcium-buffering capacity and glutamate-mediated selective neurodegeneration, to the selective vulnerability of neurons (Wang and Michaelis 2010).

Large number of preclinical and clinical studies corroborate free radical mediated damage to the biological macromolecules with advance in age, paralleled with declining endogenous enzymatic/non-enzymatic antioxidant defense (Jagota 2012; Zhang et al. 2015).

It has been well recognized that Nuclear factor erythroid-derived 2-like 2 (NRF2) governs the transcriptional activation of numerous enzymes with anti-oxidative/detoxification response thus functioning as a master transcription factor. Emerging evidences show that the decline in NRF2/EpRE signaling underlies the age associated decrease in antioxidant

enzyme responses (Zhang et al. 2015). In a recent study researchers demonstrated the importance of NRF2 mediated antioxidant defense as its suppression resulted in premature aging (Kubben et al. 2016). However it is interesting to note that these antioxidant pathways and thereby antioxidant responses are fundamentally under the regulation of circadian clocks. NRF2, the central player in induction of antioxidant enzymes is an E-box containing CCG which relies on the transcriptional regulation mediated by CLOCK-BMAL1 (Tahara et al. 2017). These interlinked networks between clock elements and cellular stress defense mechanisms further signifies the complexity of aging and related physiological conditions.

Although ROS and RNS are deleterious at higher levels, they are crucial as signaling molecules at physiological concentrations for several neuronal mechanisms such as long term potentiation (LTP), synaptic plasticity as well as formation of memories. Selective neuronal vulnerability (SNV) or selective neuronal death is one of the important contributing mechanisms underlying brain aging and age related neurodegenerative disorders as a consequence of duality in ROS/RNS function (Fig. 17). While several brain regions (eg. Hippocampal CA1) require higher levels of ROS/RNS, they are also at increased predisposition to be damaged by oxidative stress, thus being selectively vulnerable. Though brain aging and age linked pathologies such as AD and PD, involves numerous factors that result in neuronal damage including protein aggregation, mitochondrial dysfunction, glutamate-induced excitotoxicity, calcium dysfunction and genomic instability, accumulation of oxidative stress plays the most crucial role (Wang and Michaelis 2010).

AGE ASSOCIATED CIRCADIAN DYSFUNCTION

Age has a significant effect on the circadian timing system which influences the temporal organization of circadian physiology and behavior. In humans, fragmented sleep and progressive advance in sleep phase has been recorded in elderly (Fig. 18) (Jagota 2005; Roenneberg et al. 2007; Espiritu 2008). Similarly, the amplitude of feeding rhythms, secretion of hormones and body temperature is reported to drop with age (Pandi-Perumal et al. 2005; Weinert 2010). In aged animals, decline in locomotor activity rhythms and disrupted sleep-wake cycles suggests age-associated circadian alterations (Farajnia et al. 2012; Banks et al. 2015). Reports from mice have demonstrated that the aged animals are more vulnerable to negative effects of photoperiodic phase shifts as the adaptability of

circadian system is compromised with aging (Azzi et al. 2014). In animal models, age-associated variations in circadian rhythms have been reported for but not limited to activity-wakefulness, body temperature, drinking behavior, locomotor activity patterns, and serotonin rhythms (Witting et al. 1994; Weinert 2000; Jagota and Kalyani 2010; Reddy and Jagota 2014). In addition, core clock in aged mice showed diminished response to the external stimuli suggesting CTS deterioration (Biello 2009; Lupi et al. 2012).

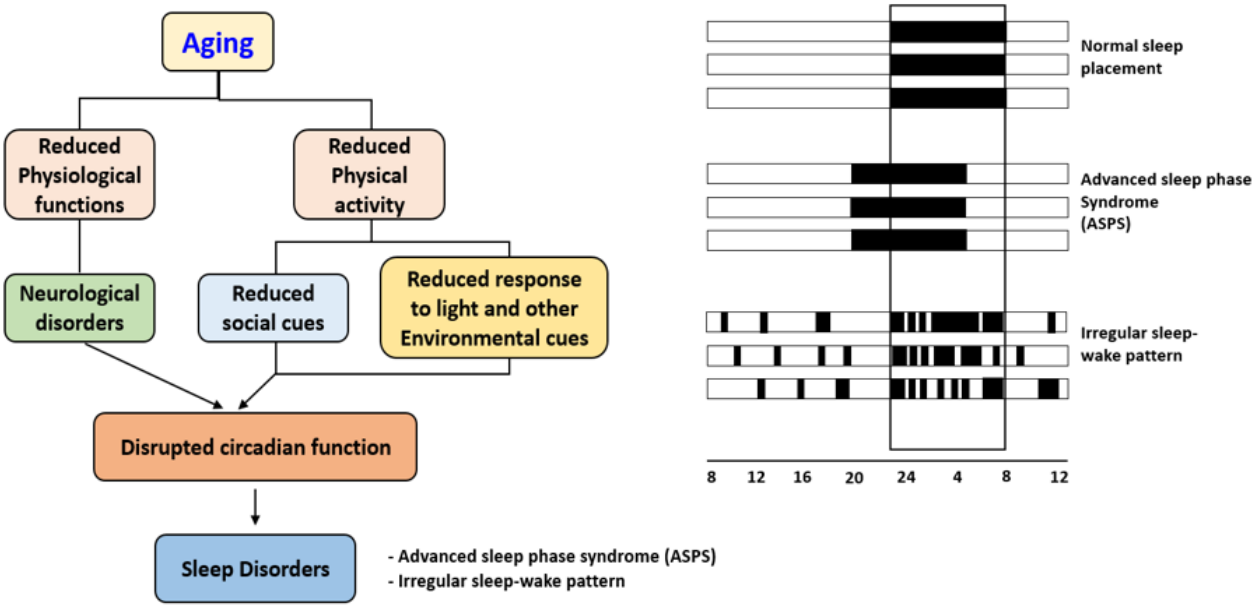


Fig. 18 Schematic representation of age associated circadian dysfunction leading to sleep disorders (Jagota 2005).

Age associated alterations in central and peripheral clocks

As the SCN communicates directly and indirectly to various peripheral clocks, circadian clock and aging may be interconnected by pathology at the level of the SCN and SCN output signals (Mattis and Sehgal 2016). Though there appears no reduction in cell number in aged SCN (Hofman and Swaab 2006), age related loss of amplitude in SCN electrical activity (Nakamura et al. 2016) suggests alterations in cellular properties, neuronal circuitry and clock genes (Banks et al. 2016). At single cell level, the neurons of aged SCN shows diminished amplitude of potassium currents and resting membrane potential as a

result of possible alterations in large conductance calcium-activated potassium channels (BK channels) (Farajnia et al. 2012, 2015).

Age associated alterations in cellular communication in SCN has been evident with reports showing age dependent loss of neuronal connectivity, marked by decline in synaptic spines and shortened dendrites (Palomba et al. 2008). Moreover, alterations in expression of neuropeptides arginine-vasopressin (AVP) and vasoactive-intestinal polypeptide (VIP) reported upon aging would hamper the SCN output as they are essential for intracellular coupling within the SCN (Cayetanot et al. 2005; Maywood et al. 2006). Disrupted GABAergic signaling in aged SCN indicates clock deterioration (Palomba et al. 2008). Weakened melatoninergic feedback to the SCN is suggested by reports showing diminished MT1 receptor expression in aged human SCN (Wu et al. 2007; von Gall and Weaver 2008). Collectively, these changes in electrical activity, loss of neuronal connectivity and altered signaling molecules compromise the internal synchronization and thereby SCN output leading to impaired co-ordination of peripheral clocks with advance in age. Age linked circadian disruption is significantly contributed by desynchrony between SCN and oscillators in peripheral tissues. Phase-shifting studies in elderly involving exposure to different LD regimens showed decline in the ability to re-entrain in several parameters such as rhythms of activity, rest/sleep and body temperature (Hood and Amir 2017). Similarly, phase advance study using PER2::LUC mouse demonstrated that oscillators in esophagus, thymus gland and lungs in older mice took longer time period to get entrained to specific LD regimen, in contrast to younger counterparts (Sellix et al. 2012).

Aging is also known to be resulting in declined total melatonin secretion (Fig. 19a). Studies in humans, primates, and hamsters indicate that the usual nighttime surge in elderly is dropped and phase advanced compared to younger adults (Hood and Amir 2017). In addition, diminished pineal melatonin synthesis and SCN expression of melatonin receptors have been reported in individuals with Alzheimer's or Parkinson's disease (Videnovic et al. 2014; Videnovic and Zee 2015). Severe alterations in daily rhythms and levels of serotonin metabolism in SCN of aged rats and in rotenone induced PD (RIPD) rat model has been reported from our lab (Jagota and Kalyani 2010; Mattam and Jagota 2015; Reddy and Jagota 2015). Similarly, cortisol, a hormone under clock control which also synchronizes peripheral clocks was observed to show age related reduction in amplitude

and advance in phase (Hood and Amir 2017). In a recent study analyzing hepatic transcriptome, it was observed that 2,626 genes (44.8%) were exclusively oscillatory in young mice where as in old mice only 1,626 genes (28.4%) were rhythmic (Sato et al. 2017). Further, age dependent decline in cyclic global protein acetylation was observed in peripheral clock, liver (Sato et al. 2017). Recently from our laboratory we have reported the age associated day-night variations of proteins in SCN, substantia nigra (SN) and pineal gland of rats (Jagota and Mattam 2017). In SCN the number of proteins showing day-night variations was fond to be decreased from 32 (in young adults) to 9 (in old age). Similarly, SN also showed a decrease from 59 to 9. However, in pineal the number of protein spots increased from 51 to 62 (Jagota and Mattam 2017). Our earlier studies investigating daily rhythms of lipid peroxidation and anti-oxidant enzyme activities in rats showed age dependent variations in liver (Manikonda and Jagota 2012). Further, reports from our group has demonstrated differential variations in daily rhythms and levels of NO and *Socs1* levels in several peripheral clocks of aged rats suggesting desynchrony (Vinod and Jagota 2016, 2017).

Age associated alterations in clock genes and proteins

The canonical genes and proteins constituting the TTFL of the core clock machinery show significant variations upon aging. We have reported severe alterations in rhythms and levels of various clock genes in the SCN of mid and old aged rats (Mattam and Jagota 2014). Similarly, studies in aged mice SCN showed alterations in *Rev-erb a*, *Dec1* and *Dbp* expression (Bonaconsa et al. 2014). Earlier studies in mice showed altered levels of CLOCK and BMAL1 expression in several brain regions including SCN, hippocampus, and amygdala by middle age (Wyse and Coogan 2010). Additionally, altered expression of *Per2* and *Bmal1* transcripts in various brain regions of aged hamsters has been reported (Duncan et al. 2013). Studies on Per1:luc rats showed slight age-dependent variations in *Per1* expression in the SCN, whereas robust changes in peripheral oscillators (Yamazaki et al. 2002). Though, reports on rhythmic BMAL1 and PER2, 3 expression in cortex of elderly humans suggests persistence of clock function in old age (Lim et al. 2013), altered PER1, 2, 3 rhythms in leucocytes indicated desynchrony (Hida et al. 2009). A detailed account on age-linked variations in expression of core clock gene in the SCN is discussed elsewhere (Banks et al. 2016).

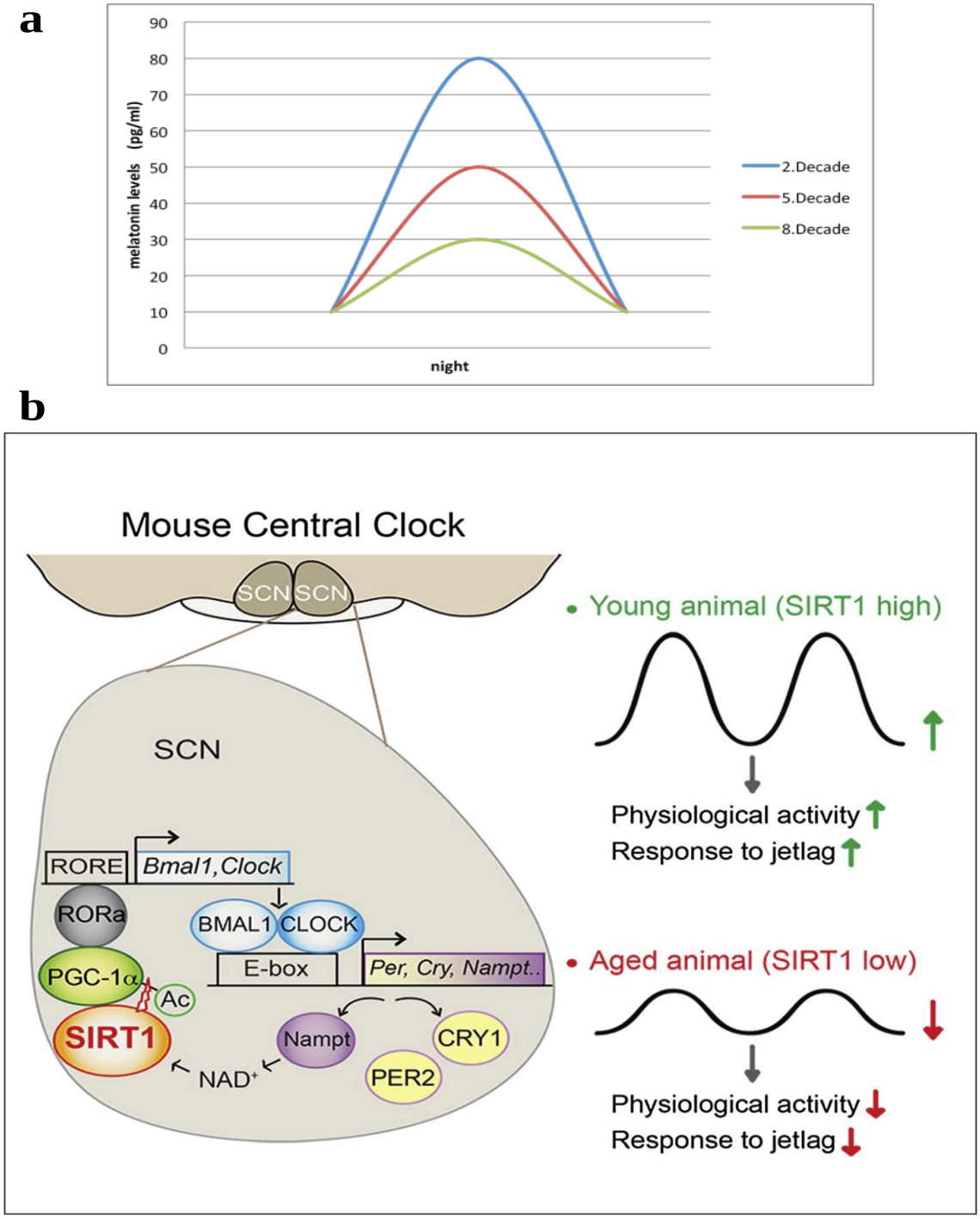


Fig. 19 (a) Peak melatonin levels at night tend to decrease with advanced age in humans. **(b)** Age associated SIRT1 mediated decline in circadian clock function (Chang and Guarente 2013; Emet et al. 2016).

Mechanisms underlying modulation of clock genes expression in aging might be involving the cross-talk between CTS and system regulating metabolism (Popa-Wagner et al. 2017).

The NAD^+ dependent protein deacetylase SIRT1 is known to play a substantial role in life span, age related disorders and most importantly in the age-associated circadian clock deterioration (Fig. 19b) (Chang and Guarente 2013; Fanny et al. 2015). The role of SIRT1 in modulation of circadian clock is well known and studies in the SCN of aged mice have demonstrated decline in SIRT1 levels concomitantly with levels of BMAL1 and PER2 (Asher et al. 2008; Nakahata et al. 2008, 2009; Ramsey et al. 2009). Further, *Sirt1* knockout mice resulted in senescent-like phenotype and *Sirt1* overexpression resulted in anti-aging phenotype with respect to alterations in clock (Chang and Guarente 2013). In addition, role of SIRTs in age associated epigenetic changes in the clock has also gained substantial importance (Orozco-Solis and Sassone-Corsi 2014).

Age associated alterations in melatonin receptors

Several studies in animal models and postmortem brain samples have indicated alterations in expression of MT1 and MT2 receptors in physiological aging and age related neurodegenerative disorders (Jockers et al. 2016). Significant reduction in MT1 receptor expression has been recorded in the postmortem SCN samples of aged humans (Wu et al. 2007). MT1 and MT2 expression were found to be altered in pineal, hippocampus and cortex of AD patients and in the SCN of patients with depression (Savaskan et al. 2005; Wu et al. 2013; Sulkava et al. 2018). Earlier studies in mice SCN had shown decreased MT1 mRNA expression during the light phase and reduced responsiveness to melatonin upon aging (Benloucif et al. 1997; von Gall and Weaver 2008). Similarly, an age-related decrease in melatonin binding in rat and mice brain regions has been described earlier (Laudon et al. 1988; Benloucif et al. 1997). Moreover, several peripheral oscillators including heart, spleen, liver and kidney showed age linked decrease in expression of MT1 and MT2 mRNA and MT1 protein in rats (Sanchez-Hidalgo et al. 2009).

Overall the progressive accumulation of numerous alterations at physiological, molecular and endocrine systems together culminate in various pathologies of aging including the deterioration of CTS.

THERAPEUTIC INTERVENTIONS AGAINST AGE ASSOCIATED CIRCADIAN DYSFUNCTION

Researchers across the globe have been addressing aging and age associated pathologies through various approaches. One of the widely studied molecules which shows significant benefit in age linked circadian dysfunction is melatonin. Melatonin, being a multitasking neuro-hormone and chronobiotic agent also possesses extraordinary antioxidant property, targets multiple signaling pathways to improve clock function (reviewed in Jagota 2012). Our group has extensively studied the beneficial effects of exogenous melatonin in age linked alterations in various parameters of the circadian clock in Wistar rats. Our reports suggested that melatonin differentially restores the daily rhythms in various antioxidant enzyme activities and lipid peroxidation in peripheral clock liver of middle and old aged rats (Manikonda and Jagota 2012). Melatonin had shown phase and rhythm restorations of canonical clock genes in addition to restorations in daily rhythms of serotonin metabolism and protein profiles in SCN (Mattam and Jagota 2014; Reddy and Jagota 2015; Jagota and Mattam 2017). Moreover, age related changes in daily NO and *Socs1* rhythms in several peripheral oscillators were also found to be restored differentially (Vinod and Jagota 2016, 2017). In addition to melatonin various clock enhancing small molecules (CEM) have gained vast attention in recent years to address the challenges of disorders of the circadian system (Chen et al. 2013, 2018). However, numerous drug candidates or herbal formulations such as Nobelitin, resveratrol, ginseng, wolfberry extract, curcumin, Ashwagandha etc. from natural sources which possess several pharmacologically beneficial properties have gained enormous interest in managing age related pathologies (Gocmez et al. 2016; Wadhwa et al. 2016).

CURCUMIN

Curcumin is an active component and yellow pigment present in turmeric which is derived from rhizomes of *Curcuma longa* (Zingiberaceae family) and known for centuries for its medicinal properties in Asia (Fig. 20). Diferuloylmethane is the chemical name of this bioactive polyphenol (Shen et al. 2013). Curcumin, a well-established antioxidant with potential free radical scavenging activity, can efficiently neutralize hydroxyl radicals, superoxide anions and also elevate the levels of endogenous antioxidants including glutathione. It can impact and alleviate the enzymatic antioxidant defense in the brain by

activating enzymes such as glutathione S-transferase (GST), glutathione peroxidase (GPx), catalase and superoxide dismutase (SOD). Curcumin also rescues the cells from several other detrimental effects that may originate from lipid peroxidation, protein carbonylation, protein oxidation and DNA damage (Fig. 21) (Cheng et al. 2013; Maiti and Dunbar 2018). Studies have proven that along with anti-oxidative stress, anti-inflammatory and anti-cancerous properties, curcumin exhibits strong neuro-protective action against a wide spectrum of neurodegenerative diseases in animal models (Calabrese et al. 2008; Hatcher et al. 2008). Curcumin is proven to be pharmacologically safe at high doses and is reported to be able to cross blood brain barrier (Tsai et al. 2011; Lao et al. 2015).

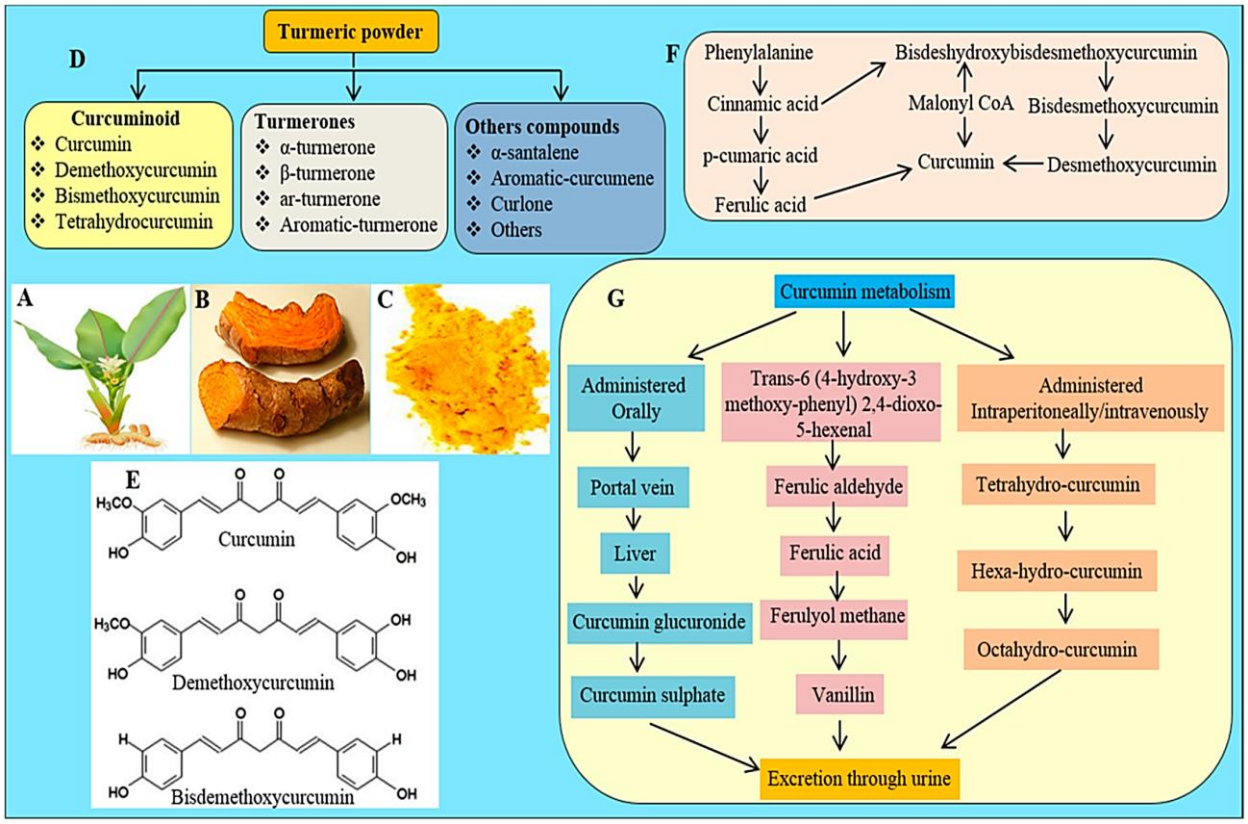


Fig. 20 Chemical structure of curcumin and its derivatives. **(A–C)** Curcuma longa, its rhizomes and turmeric extract **(D**) Different chemical components of turmeric extract **(E)** Chemical structure of principal ingredients of curcuminoid **(F)** pathway of curcumin biosynthesis **(G)** curcumin metabolism in our body (Maiti and Dunbar 2018).

The hydrophobic and lipophilic nature of curcumin is advantageous as it enhances its absorption/bioavailability in brain which is naturally rich in lipid content (Maiti and Dunbar 2018). The beneficial effects of curcumin in lifespan extension has been

extensively studied in various model organisms including *Caenorhabditis elegance, Drosophila melanogaster* and *Mus musculus*. While curcumin has suppressive effect on lipid peroxidation and ROS, it can also inhibit pro-inflammatory conditions via targeting various signaling pathways such as NF-kB /mTOR pathway. Through such mechanisms curcumin has been shown to substantially contribute to reverse aging and associated pathological conditions (Shen et al. 2013). Tetrahydro-curcumin (THC) which is an active metabolite of curcumin is reported to possess significantly higher antioxidant/ anti-inflammatory properties and has also shown beneficial effects on life span in *Drosophila* and mice under aging conditions (Shen et al. 2013).

Curcumin treatment influences neurogenesis, cognitive function, as well as learning and memory in rodent models of aging suggesting its multiple beneficial effects (Dong et al. 2012; Shen et al. 2013; Vidal et al. 2017). Reports demonstrating prevention of mitochondrial dysfunction in senescence accelerated mouse model and regulation of sirtuins, further support anti-aging effects of curcumin (Eckert et al. 2013; Grabowska et al. 2017). Our group had earlier showed restorative effects of curcumin on alcohol induced alterations in levels and rhythms of serotonin and one of its immediate metabolite 5-HIAA in SCN and pineal of rats (Jagota and Reddy 2007). Moreover, recent report from our group demonstrated the chronobiotic role of curcumin against aging rendered alterations in clock and immune related genes in a peripheral clock of male Wistar rat suggesting the therapeutic role of curcumin in CTS dysfunction (Thummadi and Jagota 2019).

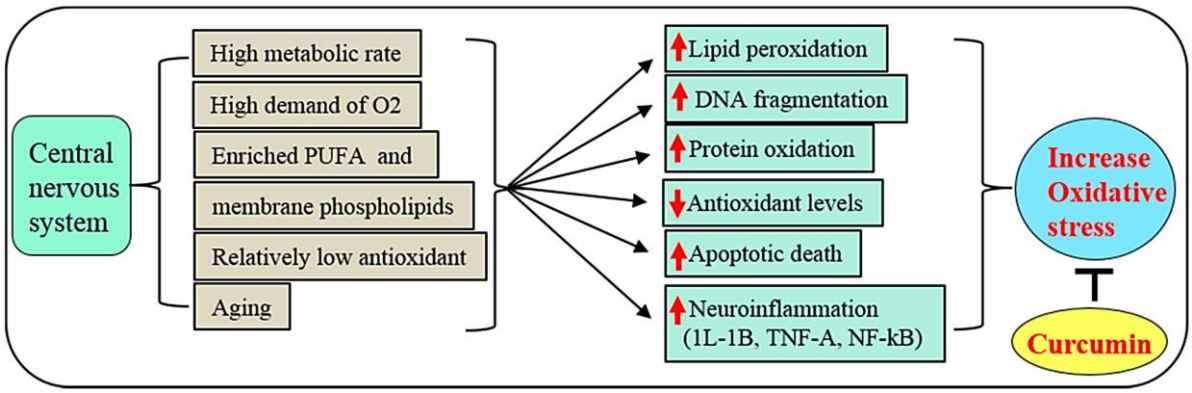


Fig. 21 Amelioration of oxidative stress by curcumin in brain. The CNS is vulnerable to oxidative stress due to high metabolic rate, which causes higher O_2 demand. This leads to an increase in oxidative stress in the brain. Curcumin, as a potent free radical scavenger, ameliorates these effects (Maiti and Dunbar 2018).

WITHANIA SOMNIFERA

Withania somnifera (WS), known as Ashwagandha or Indian winter cherry or Indian Ginseng has been used in Indian traditional medicines since documented history, classified as a 'rasayana herb' that promotes health, enhance longevity and create a sense of well-being. It is widely distributed in the dry regions of India, Middle East, North Africa and the Mediterranean regions. The root extract of *W. somnifera* is claimed to possess revitalizing, life prolonging, aphrodisiac and sedative properties. In Ayurveda, it is extensively used for the treatment of disorders such as, rheumatoid arthritis, dehydration and chronic fatigue. The fruits and leaves are used in treating ulcers and tumors. Clinical trials corroborates the benefits of *W. somnifera* in case of anxiety, inflammation and rheumatism (Berghe et al. 2012). The biologically active constituents in WS are alkaloids (ashwagandhin, cuscohygrine, anahygrine, topine etc.), steroidal compounds, including ergostane type steroidallactones, withaferin A, withanolides A–Y, withasomniferin A, withasomnidienone, withasomnierose A–C, withanone etc.

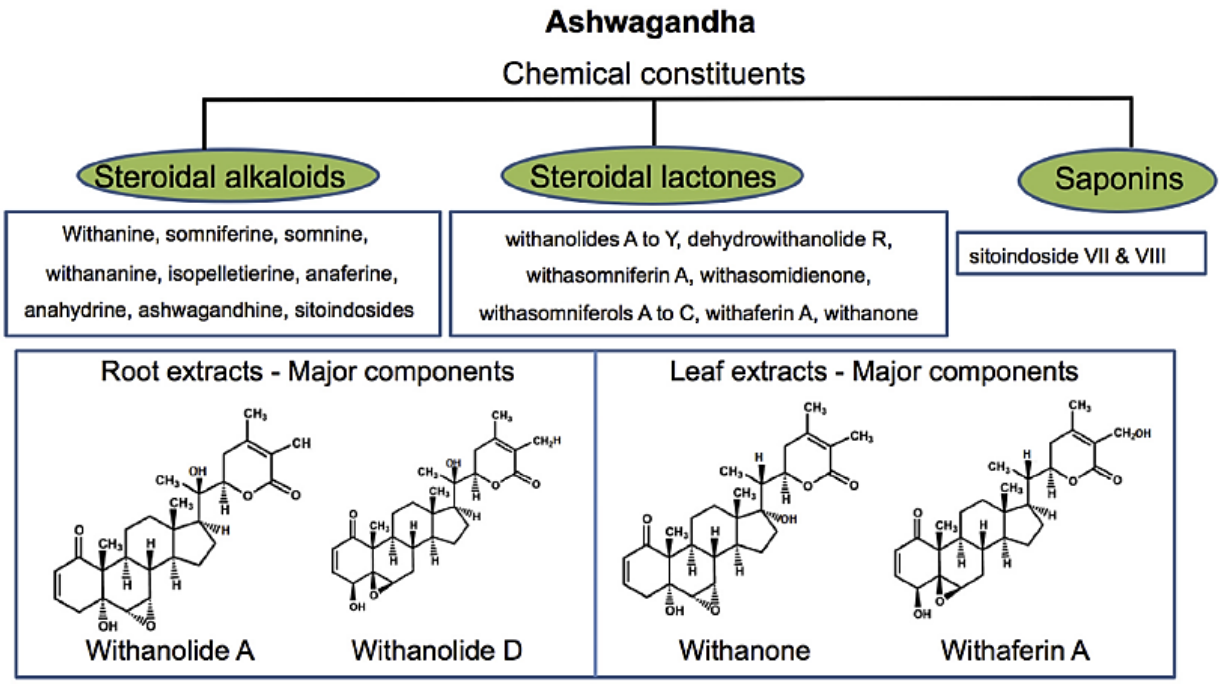


Fig. 22 Schematic diagram showing the classification of chemical constituents of Ashwagandha plant and active components of roots and leaves (Wadhwa et al. 2016).

A detailed account on nootropic potential as well as therapeutic effects of Ashwagandha leaf extracts on various brain pathologies such as Alzheimer's disease (AD), Parkinson's disease (PD), Neuroblastoma and Glioma has been reviewed elsewhere (Wadhwa et al. 2016). Its action on Hypothalamic-Pituitary-Adrenal (HPA) axis modulating the imbalance between neuroendocrine and immune system so as to regulate the body physiology under stressful conditions makes it a key adaptogen (Verma and Kumar 2011). Earlier studies have reported the antioxidant and anti-inflammatory properties of WS (Panchawat 2011; Grover et al. 2012) in addition to regulation of lipid peroxidation, alteration in glutathione content, glutathione-S-transferase activity, catalase activity and amelioration of nitrite levels (Kumar and Kalonia 2007; Khan et al. 2015). Clinical trials and animal research support the use of WS for treatment of anxiety, stress, depression, insomnia, cancer, cognitive and age-associated neurodegenerative disorders (Mishra et al. 2000; Shah et al. 2006; Konar et al. 2011; Sehgal et al. 2012). Treatment with *W. somnifera* root extract improved the body weight, behavioral alterations such as reduced locomotion and anxiety in sleep deprived mice (Kumar and Kalonia 2007).

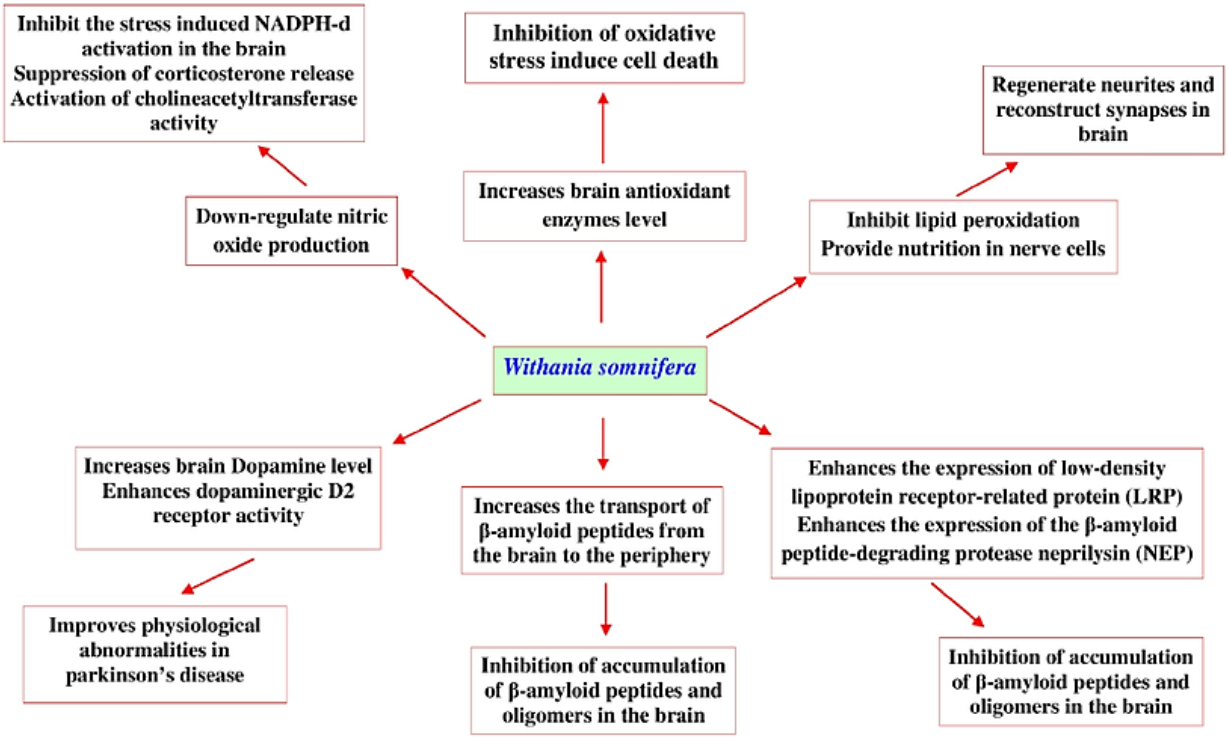


Fig. 23 Neuroprotective mechanisms of *Withania somnifera* (Kumar et al. 2015)

Further, the possible involvement of GABAergic mechanism in improving electrophysiological parameters and sleep promoting effects of *W. somnifera* in sleep disturbed rats has been reported (Kumar and Kalonia 2008). In addition, studies in sleep deprived rats have explored the neuroprotective effects of aqueous leaf extract of WS (Manchanda et al. 2016).

The inhibitory effects of Withaferin A on cholinesterase is of interest in treating cognitive deficits and in AD and related dementias (Schliebs et al. 1997). Withanoside IV and its metabolite somnione was proven to improve memory and prevent neurodegeneration induced by amyloid beta (25-35) in mice. In addition, in cultured rat cortical neurons damaged by amyloid beta (25-35), somnione showed synaptic reconstruction, axonal and dendritic regeneration potential (Kuboyama et al. 2006). Similarly, studies have investigated the potential of WS in improving the catecholamines and antioxidant enzyme activities, motor function in rodent models of PD (Ahmad et al. 2005; Rajasankar et al. 2009). In MB-PQ induced PD mice, root extract of WS enhanced Bcl2 levels and declined Bax levels thus affecting the apoptosis of dopaminergic neurons (Prakash et al. 2014). Clinical studies have demonstrated the beneficial precognitive effects of WS on bipolar disorder (Chengappa et al. 2013). Withanolide A induces significant regeneration of both axons and dendrites in memory deficit mice with neuronal atrophy and synaptic loss (Kulkarni and Dhir 2008). The GABAergic activity of WS on $GABA_A$ and GABAρ, the main inhibitory receptors of mammalian CNS, has been reported adding on to the mechanism of its adaptogenic properties (Candelario et al. 2015). In addition to this, WS leaf extract downregulated senescence in human fibroblasts and the root extract could extend the life span in *C. elegance* (Widodo et al. 2009; Kumar et al. 2015). Though the anti-inflammatory, anti-oxidant and neuroprotective roles of WS have been extensively studied, the information on its potential in treating the age induced circadian disruption is very limited. An overview of beneficial role of Ashwagandha in age related clock dysfunction is vividly discussed in (Jagota and Kowshik 2017).

With this literature background the objectives of the present work were designed with the aim of understanding the effects of curcumin and leaf extract of *Withania somnifera* on chronomics of various age induced alterations in SCN of male Wistar rats.

OBJECTIVES

Objectives

To study the effect of **curcumin** and ***Withania somnifera*** leaf extract on chronomics of age induced alterations in SCN of male Wistar rats:

1. Various clock genes (*rPer1*, *rPer2*, *rCry1*, *rCry2*, *rBmal1* and *rRev-erbα*), clock modulator SIRT1 and effector NRF2 expression.
2. Understanding role of SIRT1 using its inhibitor Nicotinamide (NAM) *ex vivo.*
3. Components of Serotonin metabolism: TRP, 5-HTP, 5-HT, 5-HIAA, NAS, MEL, 5-MIAA, 5-MTOH, 5-HTOH and NAT
4. Melatonin receptor expression: MT1 and MT2
5. Gross locomotor activity

CHAPTER - II

METHODOLOGY

Experimental Animals

Male Wistar rats of three age groups: 3 months (m), 12 m and 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 various age groups were procured from NCLAS, National Institute of Nutrition, Hyderabad, India. 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. During the light phase animals were exposed to light intensity of 300 lux. The 12:12 LD cycles were mechanically regulated by a 24 h timer (Legrand, Germany). 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 the directions of Institutional Animal Ethics Committee (IAEC) (Mattam and Jagota 2014).

Animals of each age groups were majorly divided into four groups, (1) Control (2) curcumin treated (CT), (3) *Withania somnifera* treated (WST) and (4) vehicle treated (VT) which were further divided as follows:

I. Gene expression studies (Objective 1)

Control – 3 m (n = 16; n = 4 per time point)
12 m (n = 16; n = 4 per time point)
24 m (n = 16; n = 4 per time point)

VT- 3 m (n = 16; n = 4 per time point)
12 m (n = 16; n = 4 per time point)
24 m (n = 16; n = 4 per time point)

CT- 3 m (n = 16; n = 4 per time point)
12 m (n = 16; n = 4 per time point)
24 m (n = 16; n = 4 per time point)

WST- 3 m (n = 16; n = 4 per time point)
12 m (n = 16; n = 4 per time point)
24 m (n = 16; n = 4 per time point)

II. *ex vivo* SCN explant culture (Objective 2)

Control – 3 m (n = 16; n = 4 per time point)

Nicotinamide (NAM) - 3 m (n = 16; n = 4 per time point)

CT- 3 m (n = 16; n = 4 per time point)

CT + NAM - 3 m (n = 16; n = 4 per time point)

WST- 3 m (n = 16; n = 4 per time point)

WST + NAM- 3 m (n = 16; n = 4 per time point)

III. Serotonin metabolism studies (Objective 3)

Control – 3 m (n = 24; n = 6 per time point)
12 m (n = 24; n = 6 per time point)
24 m (n = 24; n = 6 per time point)

VT- 3 m (n = 24; n = 6 per time point)
12 m (n = 24; n = 6 per time point)
24 m (n = 24; n = 6 per time point)

CT- 3 m (n = 24; n = 6 per time point)
12 m (n = 24; n = 6 per time point)
24 m (n = 24; n = 6 per time point)

WST- 3 m (n = 24; n = 6 per time point)
12 m (n = 24; n = 6 per time point)
24 (n = 24; n = 6 per time point)

IV. Melatonin receptors analysis (Objective 4)

Control – 3 m (n = 16; n = 4 per time point)
12 m (n = 16; n = 4 per time point)
24 m (n = 16; n = 4 per time point)

VT- 3 m (n = 16; n = 4 per time point)
12 m (n = 16; n = 4 per time point)
24 m (n = 16; n = 4 per time point)

CT- 3 m (n = 16; n = 4 per time point)
12 m (n = 16; n = 4 per time point)
24 m (n = 16; n = 4 per time point)

WST- 3 m (n = 16; n = 4 per time point)
12 m (n = 16; n = 4 per time point)
24 m (n = 16; n = 4 per time point)

Melatonin treatment (MEL) - 3 m (n = 16; n = 4 per time point)
12 m (n = 16; n = 4 per time point)
24 m (n = 16; n = 4 per time point)

V. Gross locomotor activity studies (Objective 5)

CT- 3 m (n = 4)
12 m (n = 4)
24 m (n = 4)

WST- 3 m (n = 4)
12 m (n = 4)
24 m (n = 4)

Curcumin administration

Carboxy methyl cellulose (CMC) (0.5%) as vehicle was prepared freshly, to this 100 mg/ml w/v of Curcumin (Sigma) was added and suspended by constant stirring for at least 30 min. Curcumin was administered orally 300 mg/kg body weight at ZT-11, 1 h before the onset of darkness for 15 days. Vehicle treatment (VT): the age matched vehicle groups were similarly administered with 0.5 % CMC (3 mL/kg body weight) (Zhao et al. 2008; Reeta et al. 2009).

Withania somnifera administration

Hydro-alcoholic leaf extract of WS (Herbochem, India) was suspended in 0.5 % CMC (100 mg/ml w/v). All the age groups received a dose of 100 mg/kg body weight of WS leaf extract orally at ZT-11 for 15 days (Rajasankar et al. 2009).

Melatonin administration

30 µg/Kg body weight of melatonin (Sigma) in 10 % ethanol made with physiological saline was administered subcutaneously, at ZT-11 for 11 days. The vehicle controls were administered with 10 % ethanol in physiological saline subcutaneously at ZT-11 (Pazo et al. 2002; Mattam and Jagota 2014).

SCN tissue preparation

After the end of individual treatments, animals of all three ages were decapitated at four time points, ZT-0, 6, 12 and 18. 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 for further experiments (Mattam and Jagota 2015).

SCN explant culture

3 m old Wistar rats were acclimatized in standard laboratory conditions for two weeks as mentioned earlier. After decapitation (at ZT-12), brain was dissected out and immediately sectioned using vibratome (Compresstome® VF-200, Precisionary Instruments) under sterile conditions. 700 µ coronal brain section containing SCN was collected. SCN explants were placed in culture dishes containing DMEM media + 25 units/mL penicillin + 25 µg/mL streptomycin. Cultures were maintained at 37 °C, with 5% CO_2 up to 54 h (Rijo-ferreira et al. 2018).

Nicotinamide (NAM) at 10 mM concentration was used to selectively inhibit SIRT1. Experimental groups were: (i) control, (ii) 10 mM Nicotinamide (NAM) treated, (iii) Curcumin treated (CT) (20 µM), (iv) CT + NAM, (v) WS treated (WST) (0.2% in media), (vi) WST+NAM. All treatments were given after 24 h of culturing and SCN were collected 12 h after treatment at every 6 h interval (n=4 per time point) (Lee et al. 2007; Peled et al. 2012; Gupta and Kaur 2016).

RNA extraction and cDNA synthesis

RNA extraction and cDNA synthesis was done as reported earlier (Mattam and Jagota 2014). Total RNA was extracted from SCN using TRI reagent following the manufacturer's protocol (Sigma). In brief, individual SCN tissues were homogenized with 400 µl of TRI reagent followed by 5 minutes (min) incubation at room temperature (RT). To the homogenate, 100 µl of chloroform was added and mixed well. After 5 minutes of incubation at RT, the samples were subjected to centrifugation for 15 min at 12,000 x g at 4°C. The aqueous phase at the top was carefully collected without contaminating with lower organic phases and transferred to a fresh Eppendorf tube containing 125 µl of isopropyl alcohol. At this step the samples were incubated at - 20°C overnight for better RNA yield. The precipitated RNA was pelleted down by centrifugation at 12,000 x g for 10 min at 4°C. RNA pellets were washed twice with 75% ethanol by mixing and centrifuging at 12,000 x g for 5 min each at 4˚C. Total RNA isolated was dissolved in 20 µl RNase free water. Concentration and purity of the same were quantified by measuring the optical density at 280 nm and by 260/280 ratio respectively with Nano drop spectrophotometer (Thermo Fischer) (Chomczynski and Sacchi 2006).

cDNA synthesis was performed with 1 µg RNA input using iScript cDNA synthesis kit (Bio-Rad) by incubating the reverse transcription mixture at 25 ℃ for 5 min; at 42℃ for 30 min and finally at 85℃ for 5 min. cDNA were finally diluted 1:20 in RNase free water. Aliquots of 4 µl was used for real time quantification (Kamphuis et al. 2005).

Quantitative real time PCR (qRT-PCR)

Expression of canonical clock genes such as *rPer1*, *rPer2*, *rCry1*, *rCry2, rBmal1*, *rRev-erbα,* clock modulator *rSirt1* and effector *rNrf2,* melatonin receptor transcripts MT1 and MT2 were measured by qRT-PCR by SYBR Green detection method (Step One Plus, Applied Biosystems, Foster, USA) (Mattam and Jagota 2014). Primer sequences used in present study for various clock genes were as reported earlier from our group (Mattam and Jagota 2014). In addition, gene specific primers for *rRev-erbα, rSirt1* and *rNrf2* were designed using IDT PrimerQuest tool (www.idtdna.com/PrimerQuest). Primer sequences for melatonin receptors were based on previous reports (Sanchez-Hidalgo et al. 2009). Table 1 shows all the gene specific primer sequences used in the present study.

Table 1: Various gene specific primer sequences used for qRT-PCR experiments

Genes	Primer Sequences
β-*actin*	Forward – 5'AGCCATGTACGTAGCCATCC3' Reverse – 5'CTCTCAGCTGTGGTGGTGAA3'
Per1	Forward – 5'TCTGGTTCGGGATCCACGAA3' Reverse – 5'GAAGAGTCGATGCTGCCAAAG3'
Per2	Forward – 5'CACCCTGAAAAGAAAGTGCGA3' Reverse – 5'CAACGCCAAGGAGCTCAAGT3'
Cry1	Forward – 5'AAGTCATCGTGCGCATTTCA3' Reverse – 5'TCATCATGGTCGTCGGACAGA3'
Cry2	Forward – 5'GGATAAGCACTTGGAACGGAA3' Reverse – 5'ACAAGTCCCACAGGCGGT3'
Bmal1	Forward – 5'CCGATGACGAACTGAAACACCT3' Reverse – 5'TGCAGTGTCCGAGGAAGATAGC3'
Rev-erbα	Forward – 5'GGTGACCTGCTCAATGCCATGTT3' Reverse – 5'CGAGCGGTCTGCAGAGACAAGTA3'
Sirt1	Forward – 5'CTTGGAGCAGGTTGCAGGAAT3' Reverse – 5'GGACACCGAGGAACTACCTGAT3'
Nrf2	Forward – 5'CTACTCCCAGGTTGCCCACATT3' Reverse – 5'GCTCTCAACGTGGCTGGGAATA3'
MT1	Forward – 5'CAGTACGACCCCCGGATCTA3' Reverse – 5'GGCAATCGTGTACGCCG3'
MT2	Forward – 5'ATGTTCGCAGTGTTTGTGGTTT3' Reverse – 5'CTGCAAGGCCAATACAGTTGA3'

Dissociation curves for all the genes showed a single peak representing specifically amplified target (Fig. 24). Threshold cycle (Ct) values were obtained from the exponential phase of amplification plots. The relative quantitative expressions of target 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).

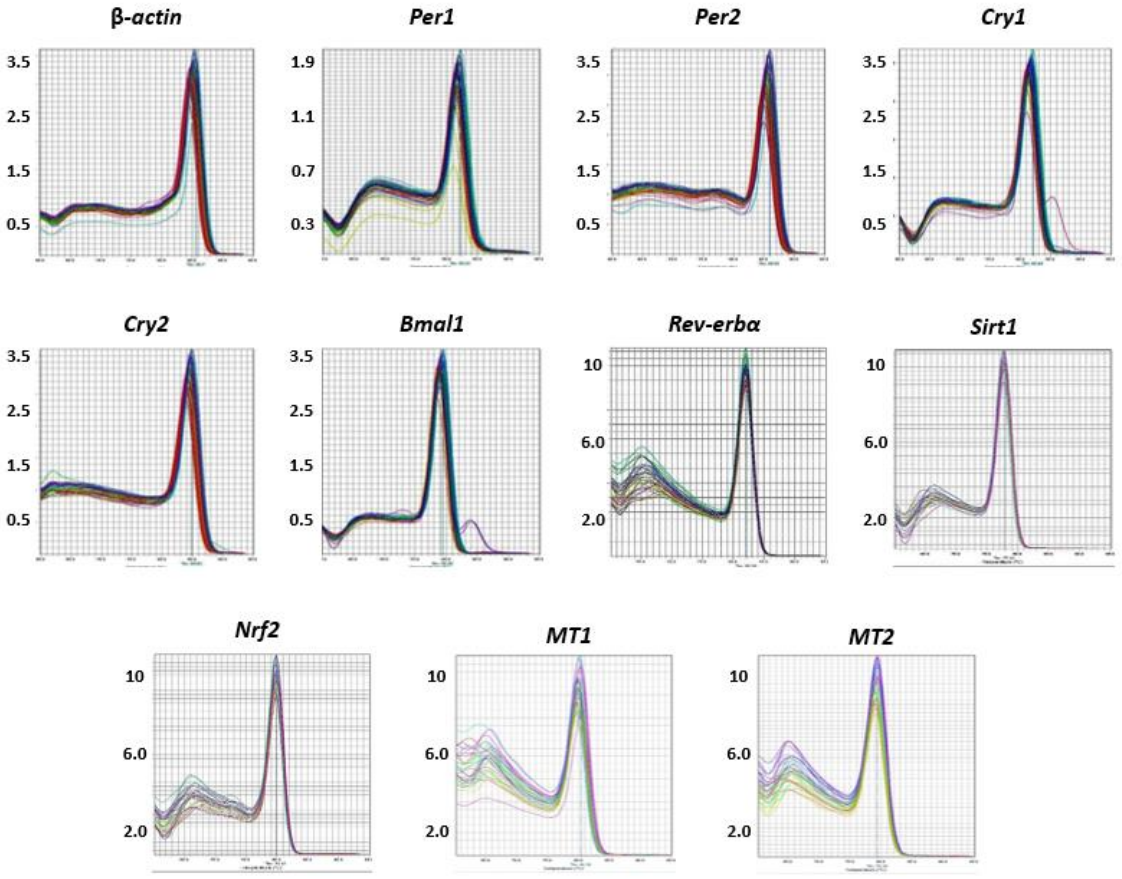


Fig. 24 Representative Dissociation curves showing specific amplification of target genes

SIRT1 ACTIVITY ASSAY

To measure the SIRT1 activity in SCN, SCN homogenate (containing 10 µg of protein aliquots), 100 µM of SIRT1 substrate (Enzo Life Sciences, USA), and SIRT1 assay buffer (50 mM Tris-HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mg/mL BSA) were incubated at 37 °C for 30 min in triplicates and the reaction was stopped using Fluor de Lys developer II solution (Enzo Life Sciences, USA). The plate was read by a fluorimeter (infinite M200 pro, TECAN) with 360 nm as excitation wavelength and 460 nm as emission wavelength (Qin et al. 2018).

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, 50mM Tris, pH 7.4) and protease inhibitor cocktail (Roche). Homogenate was placed in 4°C with intermittent

agitation for 2 h. Samples were centrifuged for 20 min at 12,000 rpm at 4 °C. Collected supernatant was quantified for protein by Bradford's method (Bradford 1976).

WESTERN BLOT ANALYSIS

Protein samples (30 µg) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using miniVE vertical electrophoresis system (Amersham Biosciences) with 5% stacking gel (pH 6.8) and 10% resolving gel (pH 8.8) (Laemmli 1970). Pre-stained 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: 25mM Tris base, 192mM 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)). Membranes were probed by incubating the membrane in rabbit polyclonal antisera raised against MT1 (1:1000, Boster biological technology Co. Ltd.) as well as MT2 (1:1000 dilution, Santa Cruz Biotechnology, inc., Santa Cruz, CA) overnight at 4˚C. After washing five times (5 min each) in TBST, incubated with secondary antibody, goat anti-rabbit IgG, conjugated with Horse radish peroxidase (HRP) (1:2000 dilution, Santa Cruz Biotechnology, Inc.). Similarly, probing with mouse monoclonal antibody against SIRT1 and NRF2 (1:5000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was carried out 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) (1:10000) (Santa Cruz Biotechnology, Inc. Santa Cruz, CA). Signals were developed using femtoLUCENT-HRP kit (G Biosciences) and captured using Chemidoc imaging system (Biorad touch plus) and analyzed 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 (Sanchez-Hidalgo et al. 2009).

Estimation of 5-HT metabolism components by Reversed-phase high-performance liquid chromatography (RP-HPLC) with electrochemical (EC) detection method:

Measurement of TRP, 5-HTP, 5-HT, 5-HIAA, NAS, MEL, 5-MIAA, 5-MTOH, 5-HTOH and NAT levels in SCN were carried out using the RP-HPLC-EC detection method as described in Reddy and Jagota (2015). In brief, SCN samples were homogenized with 100 µl of 0.1 N perchloric acid containing sodium bisulfate (1 mM). After homogenization the

samples were sonicated for approximately 10 seconds and centrifuged at 12,800 g for 10 minutes. The supernatant was filtered through 0.22 µ syringe filters; the clear supernatant was applied to the chromatography system (Waters 2695, Alliance system with Empower 2.0 software). The concentration of each compound was estimated by comparing the peak area with respective standard curve generated by using commercially available standards (Sigma) (Mefford and Barchas 1980; Mattam and Jagota 2015; Reddy and Jagota 2015).

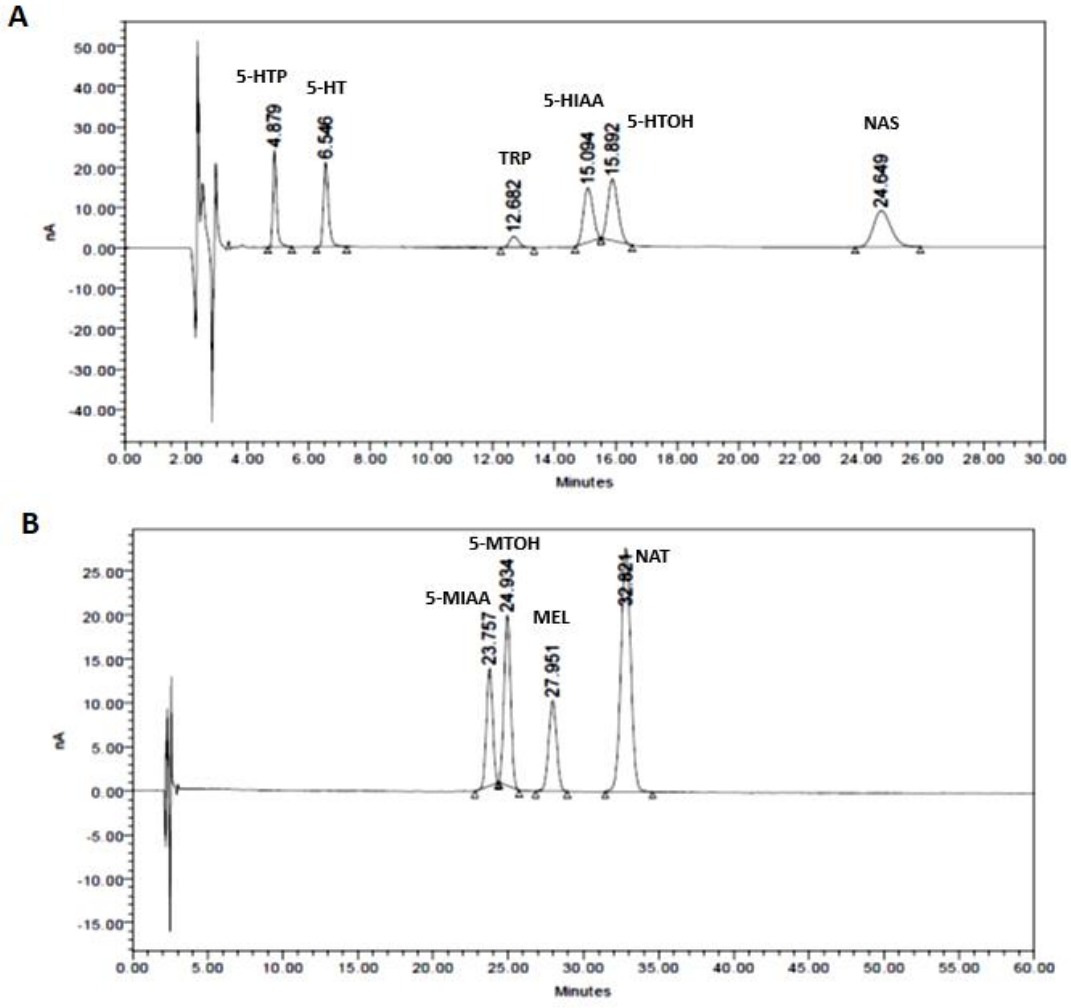


Fig. 25 Standard representative chromatograms showing various components of serotonin metabolism. **(A)** 10% methanol; 0.1N Sodium acetate; 0.1N citric acid, 50 mg/L EDTA was used for detection of compounds- 5-HTP, 5-HT, 5-HIAA, TRP, 5-HTOH and NAS. **(B)** 25% methanol; 0.1N Sodium acetate; 0.1N citric acid, 50mg/L EDTA was used for detection of compounds- 5-MIAA, 5-MTOH, MEL and NAT. nA refers to nanoampere (Unit of electric current).

Gross locomotor activity recording

Animals all the three age groups (n = 4 in each age group) were housed individually in cages connected with infra-red (I.R) motion detector sensors (IR 28 kit, INT, India). The gross locomotor activity recordings were monitored and data was collected using Chronobiology Kit for four weeks (Stanford Software Systems, USA) (Mammen and Jagota 2011; Reddy and Jagota 2014). After completion of two weeks of activity recording under control conditions, rats of respective treatment groups were administered with curcumin and WS for 15 days.

Data analysis

Jandel Scientific Sigma stat 11.0 software and GraphPad prism 7.0 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 between control and treated groups. Correlation analysis was carried out and correlation plots were generated using 'corrplot' package in R-program (Haarman et al. 2014). Effect of curcumin and WS treatments on pair wise correlations between the genes were analyzed in mean light (ZT-0, 6, 12) and dark (ZT-12, 18, 24/0) phases. Similarly correlations between components of serotonin metabolism were analyzed in three different age groups (Mattam and Jagota 2014; Reddy and Jagota 2015).

CHAPTER - III

RESULTS

OBJECTIVE 1

a. Effect of curcumin on age induced alterations in chronomics of clock gene expression

Effect of curcumin administration was studied on *rPer1, rPer2, rCry1, rCry2, rBmal1* and *rRev-erbα* mRNA expression levels and daily rhythms in SCN of 3, 12 and 24 m old rats. There were no significant difference between control and vehicle treated groups. All the clock genes studied showed rhythmic expression pattern in the SCN of CT animals and age matched VT groups (Table 2).

Upon CT, *rPer1* mRNA showed a rhythmic expression profile in 3, 12 and 24 m old animals. The maximum and minimum expression levels of *rPer1* in the age groups studied were at ZT-6 and ZT-0 respectively. In 12 m and 24 m CT animals, a phase advance of 12 h and a phase delay of 6 h were observed in comparison to their respective age matched VT groups. Comparing with 3 m VT animals this is indicative of a phase restorative effect (Fig. 26). Mean 24 h levels of *rPer1* did not show any significant difference upon aging in VT groups. However, there was a 2.6 fold and 1.9 fold increase in mean 24 h levels of *rPer1* in 3m and 24 m CT respectively in comparison to 3 m VT ($p < 0.05$) (Fig. 27). Daily pulse of *rPer1* showed a significant decrease of 2.1 and 1.9 folds in 3 and 12 m CT group respectively in comparison to 3 m vehicle group ($p < 0.05$). In 24 m animals, curcumin decreased the daily pulse of *rPer1* by 3.8 folds ($p < 0.05$), thereby bringing a restoration (Fig. 28).

rPer2 expression rhythm in 3 m CT was in phase with 3 m VT with maximum and minimum expression at ZT-12 and ZT-0 respectively. In 12 m CT group *rPer2* mRNA expression was maximum at ZT-12 showing a phase advance of 6 h in comparison to 12 m VT thus restoring the phase with respect to 3 m VT. However, in 24 m CT animals, *rPer2* showed maximum expression at ZT-6 and minimum at ZT-12 with a phase advance of 6 h compared to 24 m as well as 3 m VT (Fig. 26). Mean 24 h levels of *rPer2* showed no significant change in 3 m CT in comparison to 3 m vehicle group.

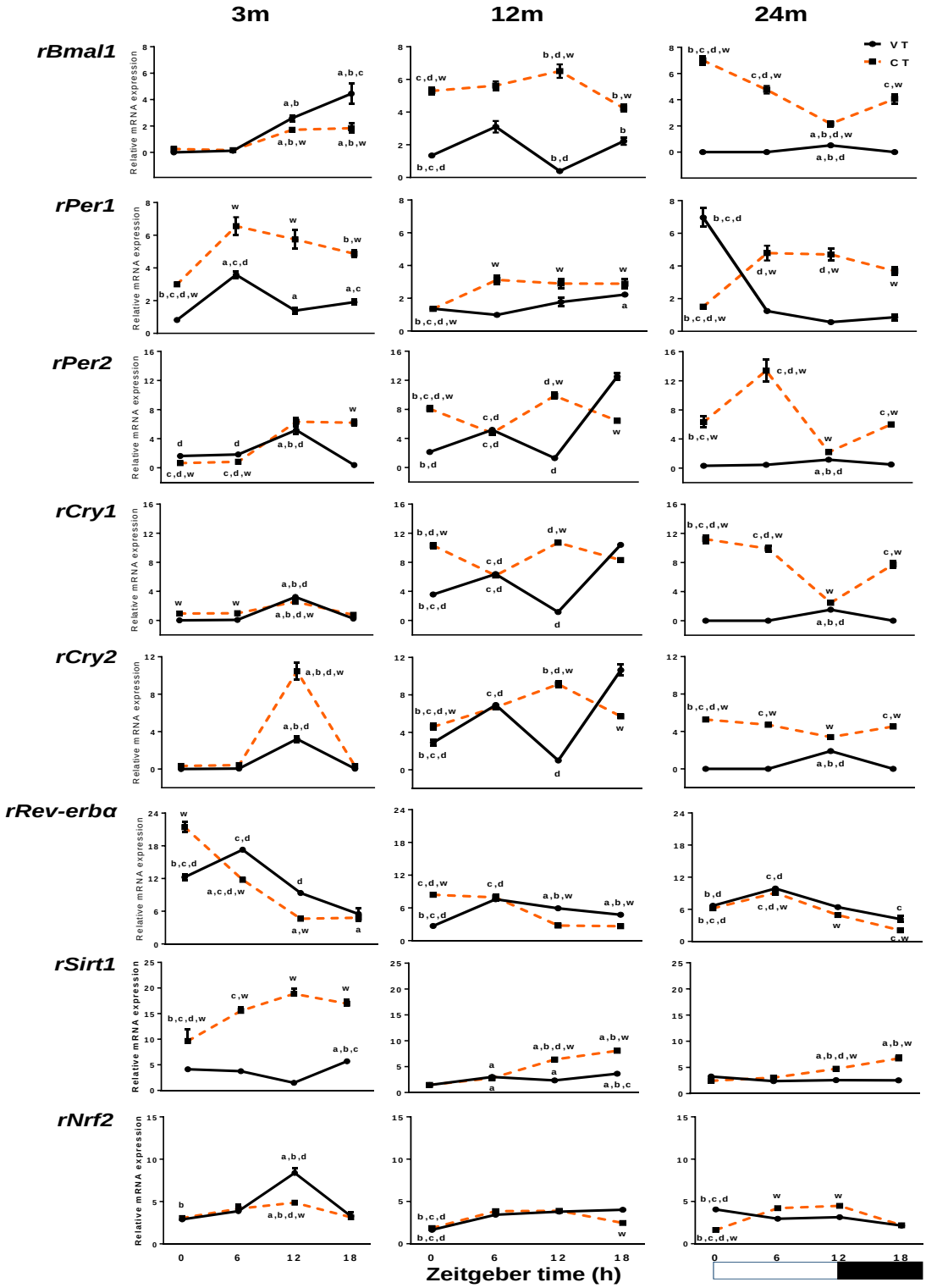


Fig. 26 Effect of curcumin on daily rhythms of clock genes, *rSirt1* and *rNrf2* mRNA expression in 3, 12 and 24 months (m) old rat SCN. Each value is mean ± SEM (n = 4), $p < 0.05$ and expressed as relative gene expression. $p_a < 0.05$; $p_b < 0.05$, $p_c < 0.05$ and $p_d < 0.05$ (where 'a', 'b', 'c' 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 treated group).

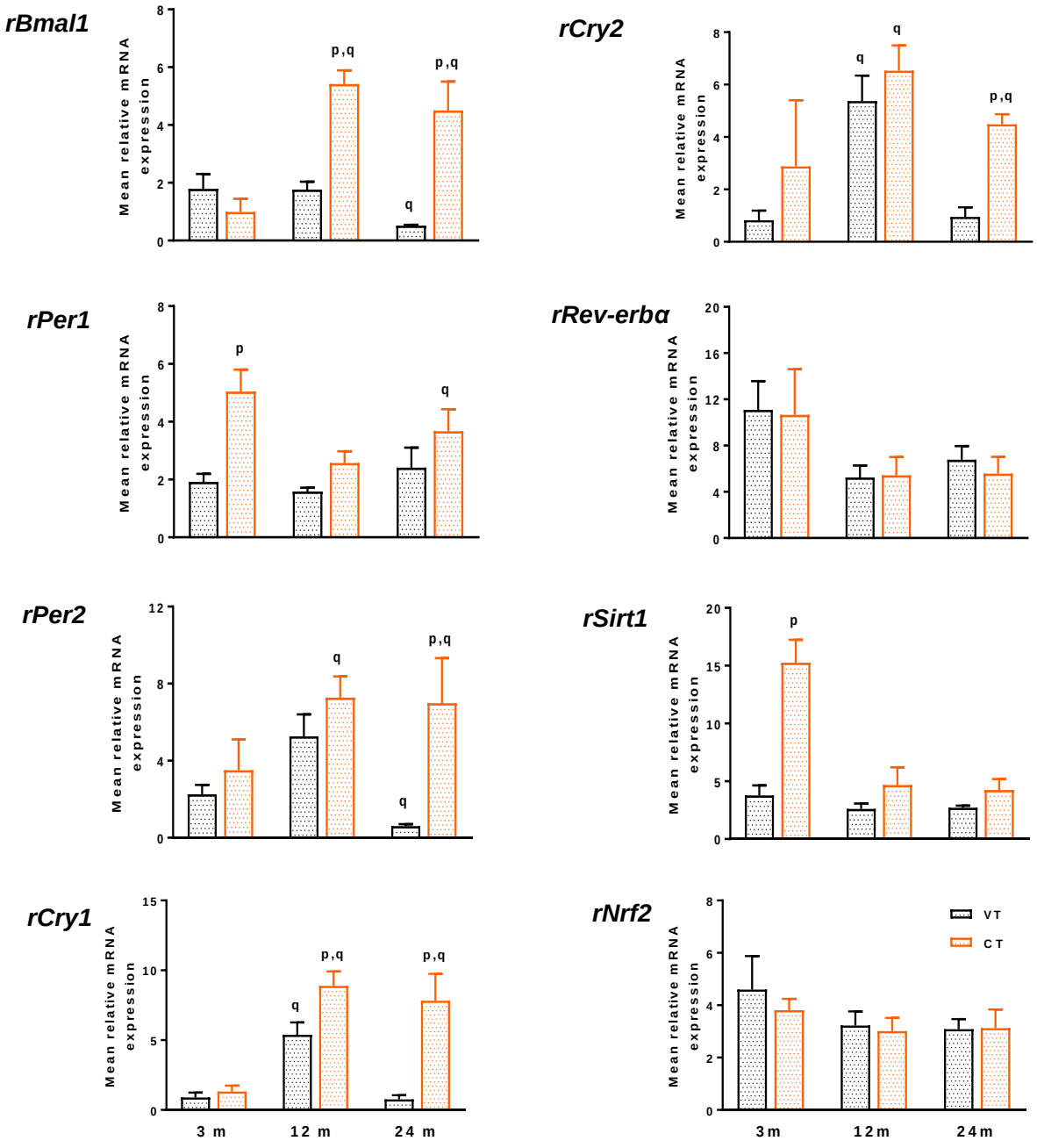


Fig. 27 Effect of curcumin administration on mean 24 h levels of *rPer1*, *rPer2*, *rCry1*, *rCry2*, *rBmal1*, *rRev-erbα*, *rSirt1* and *rNrf2* expression in 3, 12 and 24 months (m) old rat SCN. Each value is mean ± SEM, $p < 0.05$ and expressed as mean relative gene expression. $p_p < 0.05$ (where 'p' refers to comparison with age matched vehicle group). $p_q < 0.05$ (where 'q' refers to comparison with 3 m vehicle treated group).

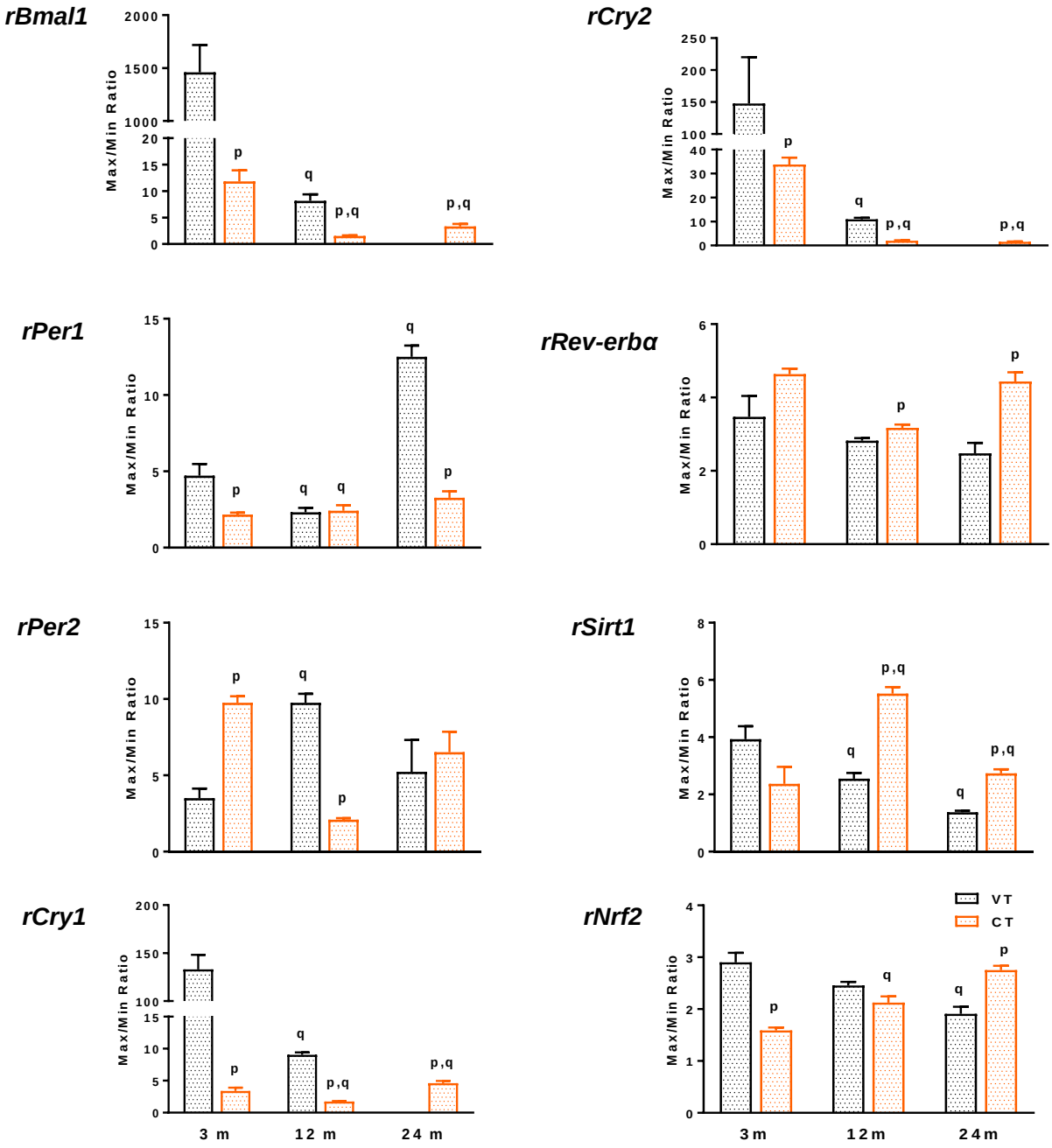


Fig. 28 Effect of curcumin administration on daily pulse of *rPer1*, *rPer2*, *rCry1*, *rCry2*, *rBmal1*, *rRev-erbα, rSirt1* and *rNrf2* expression in 3, 12 and 24 months (m) old rat SCN. Each value is maximum/minimum ratio of relative gene expression. $p_p < 0.05$ (where 'p' refers to comparison with age matched vehicle group). $p_q < 0.05$ (where 'q' refers to comparison with 3 m vehicle treated group).

The increase in mean *rPer2* levels in 12 m CT were not significant compared to 12 m VT but it was a significant 3.1 fold increase ($p < 0.05$) in comparison to 3 m VT group. In 24 m CT, *rPer2* levels increased by 11.6 folds in comparison to age matched VT group and

3.1 folds when compared to 3 m VT group ($p < 0.05$) (Fig. 27). Daily pulse of *rPer2* in 3 m CT increased significantly by 2.8 folds compared to 3 m VT animals ($p < 0.05$). Interestingly, curcumin treatment decreased the daily pulse of *rPer2* by 4.6 folds in 12 m animals compared to age matched VT group thereby restoring the pulse in comparison to 3 m VT group ($p < 0.05$). The change in *rPer2* daily pulse in 24 m CT was not significant in comparison to 24 m as well as 3 m VT group (Fig. 28).

In 3 m CT SCN, *rCry1* transcripts showed rhythmic expression with maximum levels at ZT-12 and minimum at ZT-0, which is in phase with 3 m VT animals. In 12 m CT group the maximum expression of *rCry1* mRNA was at ZT-12 showing a 6 h phase advance in comparison to 12 m VT group, resulting in phase restoration in comparison to 3 m VT group. In 24 m CT, *rCry1* showed maximum expression at ZT-0 and minimum at ZT-12, thus showing a phase advance of 12 h in comparison to 3 m as well as 24 m VT group (Fig. 26). Mean 24 h levels of *rCry1* in 3 m rat SCN did not vary upon CT in comparison to 3 m VT group, but in 12 m CT, mean 24 h levels showed an increase of 1.6 and 10 folds when compared to 12 m and 3 m VT group respectively ($p < 0.05$). In 24 m rats, there was 11.1 and 8.7 folds increase upon CT compared to 24 m and 3 m vehicle group respectively ($p < 0.05$) (Fig. 27). Daily pulse of *rCry1* in 3 m CT group was decreased by 39.1 folds when compared to 3 m VT group ($p < 0.05$). In 12 m CT, the daily pulse decreased by 5.2 folds and 78.2 folds in comparison to 12 m and 3 m vehicle controls respectively ($p < 0.05$). In case of 24 m CT there was a partial restoration compared to 24 m VT, though it was decreased by 28.9 folds compared to 3 m VT ($p < 0.05$) (Fig. 28).

In SCN of 3 m CT rats, *rCry2* transcript expression was similar to 3 m VT with maximum and minimum expressions at ZT-12 and ZT-18 respectively. In 12 m, curcumin treatment restored the phase of *rCry2* with the maximum expression at ZT-12 which is a 6 h phase advance in comparison to 12 m VT. In 24 m CT group, *rCry2* maximum expression was at ZT-0 and minimum was at ZT-12 with a phase advance of 12 h in comparison to 3 m as well as 24 m VT group (Fig. 26). *rCry2* mean 24 h levels in 3 m and 12 m CT SCN did not differ significantly compared to respective age matched VT group. However in 12 m CT, mean levels increased significantly by 7.9 folds compared to 3 m vehicle group ($p < 0.05$). In 24 m CT, mean levels increased by 4.8 folds compared to 24 m VT group and 5.4 folds compared to 3 m VT group ($p < 0.05$) (Fig. 27). Daily pulse of *rCry2* in 3 m CT was decreased by 4.3 folds compared to 3 m VT. There was 5.4 and 73.8 folds decrease in 12

m CT compared to age matched and 3 m VT group respectively ($p < 0.05$). In 24 m CT, a partial restoration in *rCry2* daily pulse was observed in comparison to 24 m VT but it was a 93.8 fold decrease when compared to 3 m VT ($p < 0.05$) (Fig. 28).

The maximum and minimum expressions of *rBmal1* in 3 m CT SCN were at ZT-18 and ZT-6 respectively, which was similar to 3 m VT. There was a 6 h phase advance in *rBmal1* in 12 m CT compared to 3 m VT, whereas a phase delay of 6 h was evident in comparison to age matched VT. In 24 m CT, *rBmal1* showed maximum expression at ZT-0 and minimum at ZT-6, thus showing a phase delay of 6 h with respect to 3 m VT and phase advance of 12 h compared to 24 m VT group (Fig. 26). Mean 24 h levels of *rBmal1* in 3 m CT rat SCN showed no significant difference in comparison to 3m VT. However, *rBmal1* mean levels in 12 m CT showed 3.1 fold increase compared to age matched VT group and 3.0 fold increase compared to 3 m VT group ($p < 0.05$). Curcumin treatment in 24 m SCN increased *rBmal1* mean 24 h levels by 9 and 2.5 folds in comparison to age matched VT as well as 3 m VT respectively ($p < 0.05$) (Fig. 27). Daily pulse of *rBmal1* showed a decrease by 123.8 folds in 3 m CT compared to 3 m VT group ($p < 0.05$) (Fig 28). In 12 m CT it was a 5.4 fold and 948.7 fold decrease compared to 12 m and 3 m VT group respectively ($p < 0.05$). However in 24 m CT, daily pulse of *rBmal1* showed a partial restoration compared to age matched vehicle groups and was decreased by 437.4 folds compared to 3 m VT group ($p < 0.05$) (Fig. 28).

The *rRev-erbα* mRNA expression in SCN was rhythmic with peak and nadir at ZT-6 and ZT-18 across age groups of VT animals. Upon curcumin treatment there was a 6 h phase advance with maximum *rRev-erbα* expression at ZT-0 in both 3 and 12 m CT group. Where as in 24 m, the rhythm was in phase with age matched as well as 3 m VT (Fig. 26). Though aging did not result in phase shifts, age related alterations in amplitude of *rRev-erbα* daily rhythms were observed. Further, in vehicle group mean 24 h levels of *rRev-erbα* was decreased by 2.1 folds in 12 m compared to 3 m and by 1.6 folds in 24 m compared to 3 m though these were statistically not significant ($p > 0.05$). These levels did not vary significantly upon curcumin administration compared to their respective VT group as well as 3 m VT ($p > 0.05$) (Fig. 27). Daily pulse of *rRev-erbα* in 12 m VT showed a decrease by 1.2 folds compared to 3 m, in 24 m VT the daily pulse showed a decrease of 1.4 fold in comparison to 3 m which is statistically not significant ($p > 0.05$). However in 12 and 24

m CT animals, daily pulse of *rRev-erbα* increased by 1.1 and 1.8 folds respectively in comparison to age matched VT ($p < 0.05$) (Fig. 28).

Effect of curcumin on age induced alterations in chronomics of SIRT1 and NRF2 expression

rSirt1 transcripts were found to be rhythmically expressed in 3 and 12 m rats but not in 24 m. In 3 m and 12 m animals, the highest expression was at ZT-18 and lowest expression was at ZT-12 in 3 m and ZT-0 in 12 m respectively. In 24 m animals, the rhythmicity was abolished (Fig. 26). Mean 24 h levels of *rSirt1* transcripts did not show any significant variation across age groups ($p > 0.05$) (Fig. 27). Interestingly, daily pulse was found to be significantly declining from 3 m to 12 m by 1.5 folds and from 3 m to 24 m by 2.8 folds ($p < 0.05$) (Fig. 28). 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 nadir at ZT-12. Similarly 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. 29A and 29B). Mean 24 h levels of SIRT1 did not vary significantly between 3 m and 12 m, however in 24 m there was a significant 1.4 folds increase compared to 3 m ($p < 0.05$) (Fig. 29C). Similarly, there was no significant difference in daily pulse of SIRT1 from 3 m to 12 m. But it decreased by a significant 1.3 folds from 3 m to 24 m in vehicle group ($p < 0.05$) (Fig. 29D).

Upon CT, a rhythmic expression pattern of *rSirt1* was observed in all the age groups studied. In 3 m CT, the maximum expression was at ZT-12 and lowest expression was at ZT-0 which is a 6 h phase advance with respect to maximum expression time in 3 m VT group. In 12 m CT animals, the maximum and minimum was at ZT-18 and ZT-0 which is similar to age matched as well as 3 m VT groups. In 24 m animals, CT resulted in rhythmic *rSirt1* expression and the peak was found to be at ZT-18 which is indicative of a phase restoration (Fig. 26). Increase in mean 24 h levels of *rSirt1* upon CT with respect to age matched vehicle groups did not show a statistical significance except in case of 3 m CT (Fig. 27). The decrease in daily pulse upon CT in 3 m group was not significant compared to 3 m VT ($p > 0.05$). In 12 and 24 m CT animals, daily pulse of *rSirt1* increased significantly by 2.1 and 2 folds with respect to age matched VT group ($p < 0.05$) (Fig. 28).

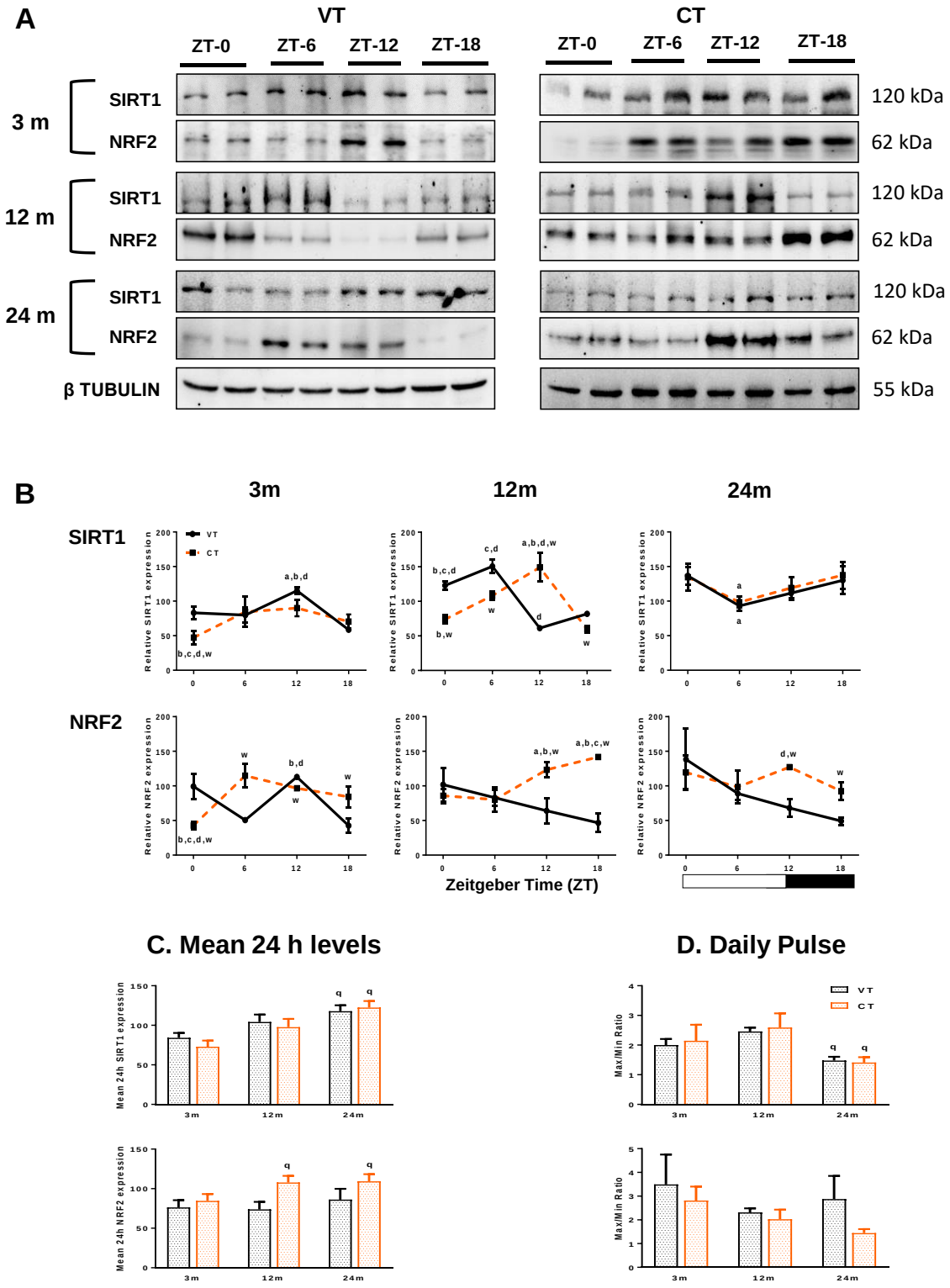


Fig. 29 (A) Immunoblots showing daily rhythms in SIRT1 and NRF2 expression in SCN of 3, 12 and 24 months (m) aged rats at ZT-0, 6, 12 and 18. β-TUBULIN was probed as loading control. VT- vehicle treated; CT- curcumin treated. **(B)** Daily rhythms of SIRT1 and NRF2 expression in the aging rat SCN in 3, 12 and 24 m and effect of curcumin. 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). **(C)** Effect of curcumin on mean 24 h levels and **(D)** Daily Pulse of SIRT1 and NRF2 in 3, 12 and 24 m rat SCN. 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).

With maximum expression at ZT-12, SIRT1 protein expression in 3 m animals upon CT followed a rhythmic pattern similar to 3 m control. In 12 m CT, with peak expression at ZT-12 curcumin delayed the SIRT1 daily rhythm by 6 h in comparison to 12 m controls, restoring the phase with respect to 3 m vehicle control. However in 24 m old CT animals, SIRT1 rhythms were in coherence with age matched vehicle control group with maximum and minimum expression at ZT-0 and ZT-6 respectively (Fig. 29A and 29B). Mean 24 h levels and daily pulse of SIRT1 did not vary significantly in any of the three age groups upon CT in comparison to respective age matched controls (Fig. 29C and Fig. 29D).

rNrf2 transcript expression was rhythmic in 3 m control group with peak at ZT-12 and nadir at ZT-0. Aging altered the daily rhythm of *rNrf2* with 6 h phase delay in 12 m and 12 h phase advance in 24 m groups in addition to diminished amplitudes in both the age groups compared to 3 m controls (Fig. 26). *rNrf2* mean 24 h levels appeared to be declining upon aging though the decrease was not statistically significant ($p > 0.05$) (Fig. 27). But daily pulse declined by 1.20 folds from 3 m to 12 m and by a significant 1.5 folds from 3 m to 24 m in VT animals ($p < 0.05$) (Fig. 28). Similar to mRNA, NRF2 protein expression in SCN of young animals showed a rhythmic pattern with maximum levels at ZT-12 (Fig. 29A and 29 B). Aging severely altered the daily rhythm of NRF2 with abolishment of rhythms in both 12 m and 24 m groups (Fig. 29B). However, NRF2 mean 24 h levels as well as daily pulse did not vary significantly upon aging (Fig. 29C and 29D).

With curcumin administration, daily rhythmicity in *rNrf2* transcripts in 3 m was similar to age matched controls with maximum expression at ZT-12 and minimum expression at ZT-0 (Fig. 26). Both in middle and old age, CT restored the phase of *rNrf2* rhythm by resetting the peak expression to ZT-12 (Fig. 26). Variation in *rNrf2* mean 24 h levels upon CT were not statistically significant compared to respective age matched controls (Fig. 27). Interestingly, CT restored the daily pulse in 24 m (Fig. 28). Rhythmic NRF2 protein expression in SCN of young animals treated with curcumin showed 6 h advance in phase

when compared to young controls (Fig. 29A and 29 B). However, CT restored the rhythm in 12 m with peak expression at ZT-18. And in old age, curcumin restored both rhythm and phase of NRF2 (Fig. 29B). NRF2 mean 24 h levels as well daily pulse did not vary significantly upon CT in comparison to respective age matched vehicle treated groups (Fig. 29C and 29D). Individual values of genes and protein expression, mean 24 h levels as well as daily pulse are tabulated in Table 2 and 3.

Correlation analysis

i. Clock genes

In light phase of 3 m CT group the positive correlation between *rBmal1* and *rPer2*; *rBmal1* and *rCry1*; *rBmal1* and *rCry2*; *rPer2* and *rCry1*; *rPer2* and *rCry2*; *rCry1* and *rCry2* was found to be persistent compared to 3 m VT group. Similarly, the negative correlation between *rRev-erbα* and all other clock genes studied persisted in 3 m CT light phase. In the dark phase, positive correlation between *rBmal1* and *rPer1*; *rCry1* and *rCry2* and negative correlation between *rRev-erbα* and *rPer1*; *rRev-erbα* and *rBmal1* was unaltered upon CT. Further, there appeared a positive correlation between *rPer1* and *rPer2*; *rPer1* and *rCry1*; *rPer1* and *rCry2*; *rBmal1* and *rPer2*; *rBmal1* and *rCry1* and a negative correlation between *rRev-erbα* and *rPer2*; *rRev-erbα* and *rCry1* in dark phase of 3 m CT which were absent in dark phase of 3 m VT group ($p < 0.05$) (Fig. 30).

The positive correlation between *rBmal1* and *rCry2*; *rPer2* and *rCry1* in the light phase of 12 m CT group was similar to the age matched as well as 3 m VT group. Curcumin treatment in 12 m animals abolished the correlation between *rPer1* and *rBmal1*; *rPer1* and *rPer2*; *rPer1* and *rCry1* in the light phase when compared to 12 m VT. There appeared a negative correlation between *rRev-erbα* and *rPer2*; *rRev-erbα* and *rCry1*; *rRev-erbα* and *rCry2*; *rRev-erbα* and *rBmal1* which were positive in 12 m VT, thus restoring the pairwise correlation compared to 3 m VT group. In 12 m CT dark phase, the correlations between *rPer2* and *rCry1*; *rPer2* and *rCry2* were similar to 12 m and 3 m VT. CT abolished the positive correlations between *rPer1* and *rPer2*; *rPer1* and *rCry1* which were present in 12 m VT group and established a negative correlation between *rRev-erbα* and *rPer1* restoring the correlations with respect to 3 m VT ($p < 0.05$) (Fig. 30).

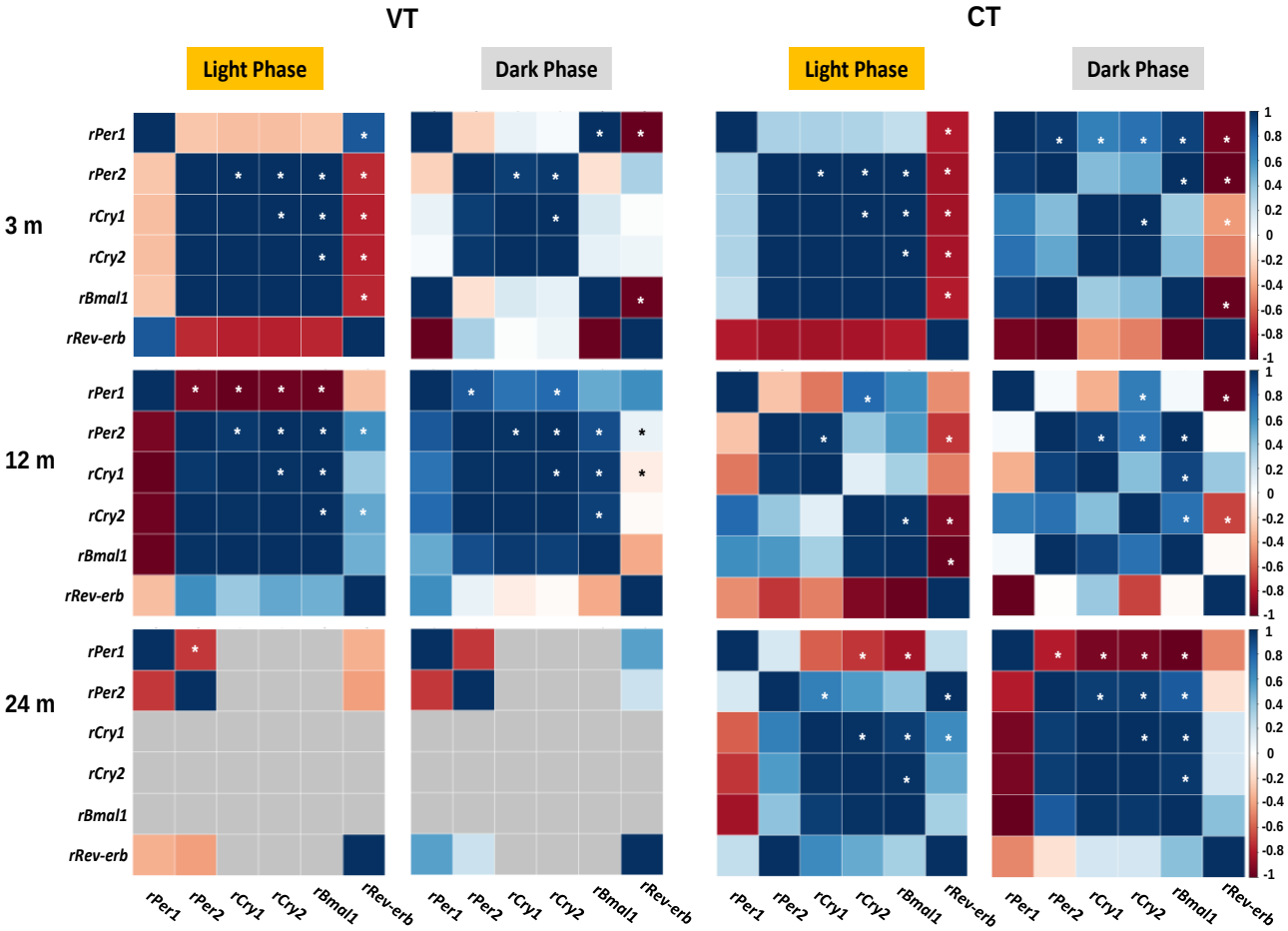


Fig. 30 Effect of curcumin treatment on pair wise correlation between mean light (ZT-0, 6, 12) and mean dark (ZT-12, 18, 24/0) phase values of *rPer1*, *rPer2*, *rCry1*, *rCry2*, *rBmal1* and *rRev-erbα* in SCN of 3, 12 and 24 months (m) aged rats. Every colored square represents correlation coefficient values between the parameters. Where, positive correlations are indicated by shades of blue, negative correlations by shades of red and white indicates no correlation. Grey squares indicate that the values were not considered for correlation analysis. '*' Indicates statistically significant correlations ($p < 0.05$).

In 24 m animals, CT restored the positive correlation between *rBmal1* and *rCry1*; *rBmal1* and *rCry2*; *rCry1* and *rPer2*; *rCry1* and *rCry2* in the light phase, which were lost in age matched vehicle group. Further, negative correlation between *rPer1* and *rCry2*; *rPer1* and *rBmal1* and positive correlations between *rRev-erbα* and *rPer2*; *rRev-erbα* and *rCry1*; *rRev-erbα* and *rCry2* were similar to 12 m VT in light phase. Similarly, in the dark phase positive correlations between *rPer2* and *rCry1*; *rCry2* and *rCry1*; *rPer2* and *rCry2* were restored in comparison to 3 m VT. The positive correlations between *rPer1* and *rBmal1* in

the dark phase of 3 m VT shifted to negative correlation upon treatment with curcumin in 24 m group ($p < 0.05$) (Fig. 30).

ii. The clock modulator rSirt1 and Clock genes

Pairwise correlation analysis in the light phase of 3 m VT groups revealed a significant and strong negative correlation between *rSirt1* and four other clock genes *rPer2, rCry1, rCry2* and *rBmal1*. And a significant positive correlation between *rSirt1* and *rRev-erbα*. However, in the dark phase negative correlations between *rSirt1* and *rCry1*; *rSirt1* and *rCry2* persisted whereas the correlations between *rSirt1* and rest of the genes were not significant (Fig. 31). In the light phase of middle aged 12 m VT animals, there was a complete abolition of significant correlations which were found in 3 m VT except the positive correlation between *rSirt1* and *rRev-erbα*. Further in the dark phase of 12 m VT groups, negative correlations between *rSirt1* and *rPer2, rCry1, rCry2* of 3 m VT group became significantly positive (Fig. 31). In 24 m old age animals, both in light as well as dark phases there existed a significant positive correlation between *rSirt1* and *rPer1*. However, the correlations between *rSirt1* and *rPer2*; *rSirt1* and *rRev-erbα* were not statistically significant in both the phases ($p < 0.05$) (Fig. 31).

In 3 m CT animals, a significant positive correlations between *rSirt1* and clock genes *rPer1*, *rPer2, rCry1*, *rCry2* and *rBmal1* were observed. And a significant negative correlation between *rSirt1* and *rRev-erbα* in both light as well as dark phases was evident (Fig. 31). In the light phase of 12 m CT group, a significant positive correlation was observed between *rSirt1* and *rCry2* as well as *rBmal1* which was not significant in 12 m VT group and which was a significant negative correlation in 3 m VT group. Further, the positive correlations found in 3 and 12 m vehicle group between *rSirt1* and *rRev-erbα* became negative in 12 m CT. However in the dark phase, there appeared a negative correlation between *rSirt1* and *rPer2*; *rSirt1* and *rCry1* similar to 3 m VT though statistically not significant.

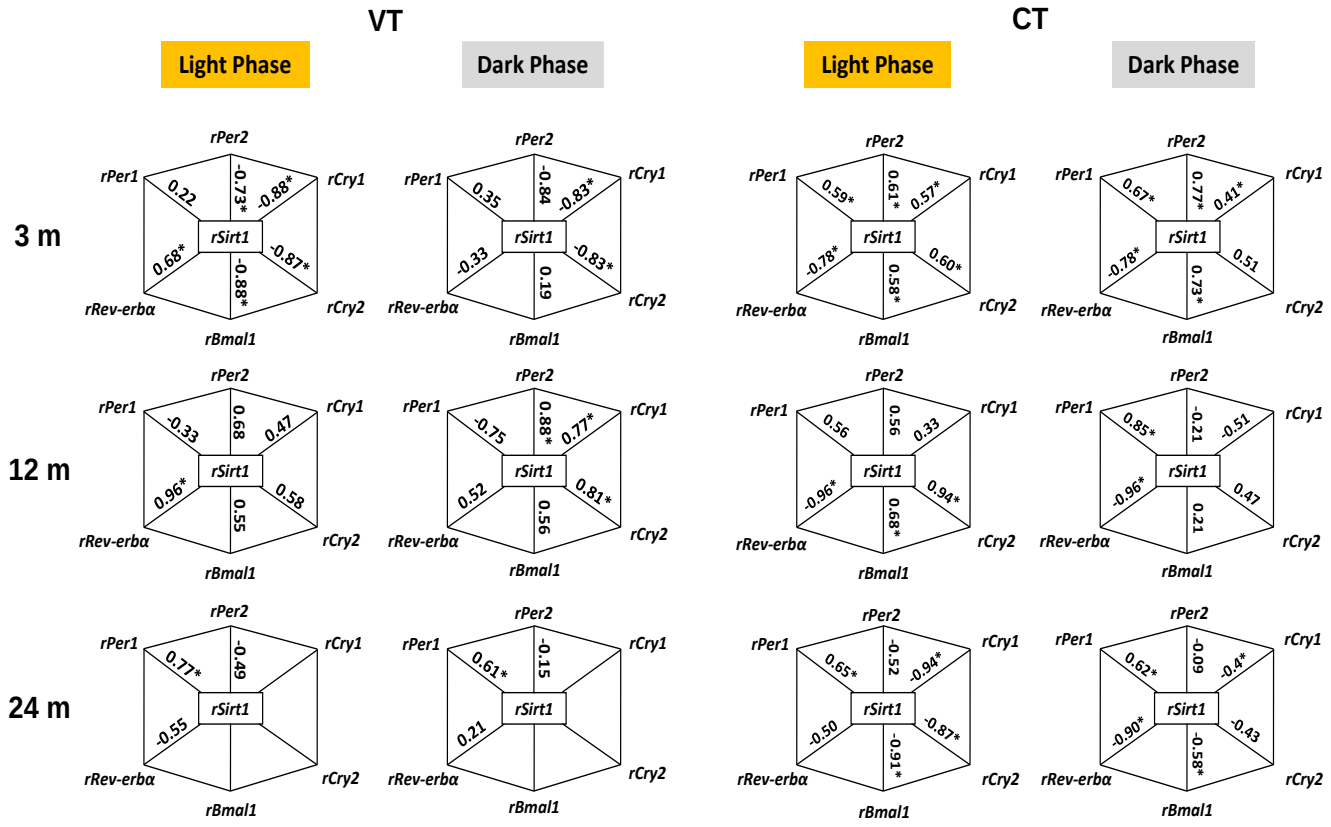


Fig. 31 Effect of curcumin treatment on pair wise correlation between mean light (ZT-0, 6, 12) and mean dark (ZT-12, 18, 24/0) phase values of clock modulator *rSirt1* and all other clock genes studied in SCN of 3, 12 and 24 months (m) aged rats. Each correlation coefficient value represents the positive or negative correlations between *rSirt1* and clock genes. '*' Indicates statistically significant correlations ($p < 0.05$).

Further, the positive correlation between *rSirt1* and *rPer1* and the negative correlation between *rSirt1* and *rRev-erbα* observed in 3 m VT dark phase was restored with significance in 12 m CT (Fig. 31). In 24 m CT light phase, restoration of strong negative correlations between *rSirt1* and *rCry1*, *rCry2*, *rBmal1* with respect to 3 m VT was observed. Other correlations with *rPer1*, *rPer2* and *rRev-erbα* were similar to age matched VT light phase. Similarly, in the dark phase of 24 m CT, negative correlations between *rSirt1* and *rCry1*, *rCry2* were established restoring the correlations in comparison to dark phase of 3 m vehicle group ($p < 0.05$) (Fig. 31).

Table 2 Effect of curcumin treatment on age induced alterations in daily rhythms, mean 24 h levels and daily pulse of clock genes, *Sirt1* and *Nrf2* transcript expression

Gene	Age & Treatment	ZT-0	ZT-6	ZT-12	ZT-18	Mean 24 h levels	Max/Min ratio
rPer1	3m VT	0.81±0.12	3.58±0.2	1.39±0.17	1.91±0.16	1.92±0.28	4.72±0.76
	3m CT	3±0.11	6.56±0.55	5.74±0.59	4.87±0.22	5.04±0.7 ^{w}	2.17±0.12 ^{w}
	12m VT	1.36±0.11	0.99±0.09	1.77±0.25	2.22±0.11	1.58±0.14	2.32±0.29 ^{x}
	12m CT	1.35±0.14	3.12±0.25	2.9±0.28	2.89±0.31	2.57±0.41 ^{x}	2.42±0.37 ^{w}
	24m VT	6.96±0.57	1.24±0.05	0.56±0.06	0.86±0.18	2.41±0.7	12.51±0.74 ^{xy}
	24m CT	1.51±0.13	4.79±0.45	4.7±0.35	3.69±0.23	3.67±0.7 ^{w}	3.26±0.43 ^{x}
rPer2	3m VT	1.63±0.32	1.83±0.33	5.19±0.55	0.37±0.15	2.25±0.49	3.5±0.64
	3m CT	0.65±0.03	0.83±0.12	6.34±0.54	6.2±0.41	3.5±1.6	9.75±0.43 ^{w}
	12m VT	2.13±0.11	5.17±0.13	1.3±0.13	12.5±0.48	5.27±1.14	9.76±0.58 ^{x}
	12m CT	8.07±0.37	4.74±0.15	9.87±0.41	6.42±0.25	7.27±1.1 ^{w}	2.09±0.1 ^{xy}
	24m VT	0.31±0.09	0.46±0.09	1.16±0.09	0.5±0.17	0.61±0.1 ^{x}	5.23±2.09
	24m CT	6.34±0.76	13.4±1.49	2.19±0.29	6.02±0.25	6.99±2.3 ^{w}	6.52±1.34
rCry1	3m VT	0.025±0.003	0.07±0.02	3.23±0.26	0.26±0.04	0.9±0.35	132.98±15.3
	3m CT	0.94±0.03	1±0.1	2.57±0.28	0.77±0.04	1.32±0.42	3.4±0.49 ^{w}
	12m VT	3.57±0.2	6.38±0.29	1.16±0.07	10.4±0.31	5.38±0.9 ^{x}	9.04±0.38 ^{x}
	12m CT	10.3±0.39	6.18±0.33	10.7±0.19	8.32±0.22	8.88±1 ^{xw}	1.75±0.08 ^{w}
	24m VT	?	?	1.52±0.17	?	0.76±0.3	-
	24m CT	11.2±0.55	9.91±0.45	2.46±0.19	7.72±0.45	7.8±1.9 ^{xw}	4.61±0.36 ^{w}
rCry2	3m VT	0.0003±0.00 004	0.05±0.01	3.21±0.33	0.04±0.01	0.82±0.36	147.75±72.5
	3m CT	0.32±0.01	0.43±0.04	10.45±0.9	0.31±0.02	2.88±2.52	33.76±2.87
	12m VT	2.9±0.29	6.92±0.21	0.99±0.1	10.64±0.6	5.4±0.97 ^{x}	10.9±0.64 ^{x}
	12m CT	4.6±0.33	6.68±0.12	9.13±0.34	5.71±0.1	6.53±0.9 ^{w}	2.02±0.16 ^{xw}
	24m VT	?	?	1.9±0.17	?	1.9±0.17	-
	24m CT	5.27±0.17	4.71±0.22	3.4±0.26	4.53±0.23	4.48±0.4 ^{w}	1.58±0.14 ^{xw}
rBmal1	3m VT	0.003±0.001	0.12±0.02	2.59±0.22	4.45±0.79	1.79±0.51	1461.03±26
	3m CT	0.26±0.02	0.16±0.03	1.71±0.1	1.83±0.36	0.99±0.45	11.85±2.07
	12m VT	1.34±0.04	3.1±0.36	0.39±0.03	2.22±0.23	1.76±0.28	8.16±1.23 ^{x}
	12m CT	5.3±0.2	5.62±0.26	6.51±0.41	4.24±0.23	5.4±0.5 ^{xw}	1.54±0.11 ^{xw}
	24m VT	?	?	0.52±0.03	?	0.5±0.03 ^{x}	-
	24m CT	7.01±0.33	4.76±0.29	2.17±0.2	4.06±0.35	4.5±1 ^{xw}	3.35±0.45 ^{xw}
rRev-erbα	3m VT	12.24±0.58	17.29±0.3	9.34±0.32	5.49±1.05	11.09±2.5	3.47±0.57
	3m CT	21.43±0.96	11.76±0.2	4.62±0.12	4.8±0.52	10.65±4.0	4.64±0.15
	12m VT	2.69±0.03	7.58±0.15	5.92±0.14	4.76±0.08	5.24±1.03	2.82±0.07
	12m CT	8.4±0.39	7.92±0.58	2.76±0.13	2.65±0.09	5.43±1.58	3.17±0.09
	24m VT	6.67±0.09	9.89±0.11	6.4±0.03	4.17±0.54	6.78±1.18	2.48±0.28
	24m CT	6.19±0.25	9.07±0.17	4.93±0.04	2.07±0.13	5.57±1.45	4.44±0.25
rSirt1	3m VT	4.14±0.49	3.77±0.16	1.48±0.11	5.69±0.45	3.77±0.87	3.93±0.45

	3m CT	9.66±2.33	15.53±0.8	18.87±1.0	16.94±0.8	15.25±2.0	2.37±0.6
	12m VT	1.45±0.12	3±0.23	0±0	3.64±0.49	2.61±0.47	2.55±0.2 ^{x}
	12m CT	1.46±0.03	2.78±0.16	6.38±0.14	8.07±0.22	4.67±1.5 ^{x}	5.52±0.23 ^{xw}
	24m VT	3.27±0.13	2.39±0.24	2.58±0.11	2.54±0.39	2.7±0.2	1.38±0.06 ^{xy}
	24m CT	2.44±0.09	3.05±0.18	4.73±0.09	6.72±0.6	4.24±1 ^{x}	2.74±0.14 ^{yw}
rNrf2	3m VT	2.89±0.11	3.87±0.12	8.38±0.6	3.27±0.48	4.6±1.28	2.9±0.18
	3m CT	3.06±0.03	4.16±0.52	4.87±0.16	3.14±0.15	3.81±0.43	1.59±0.06
	12m VT	1.64±0.06	3.43±0.13	3.8±0.1	4.02±0.1	3.22±0.54	2.45±0.07
	12m CT	1.86±0.17	3.85±0.25	3.89±0.16	2.46±0.07	3.01±0.51	2.12±0.12 ^{xw}
	24m VT	4.06±0.07	2.96±0.07	3.14±0.2	2.15±0.12	3.08±0.39	1.91±0.14 ^{x}
	24m CT	1.63±0.06	4.2±0.12	4.48±0.17	2.18±0.08	3.12±0.71	2.75±0.08 ^{xy}

Each value is mean ± SEM (n = 4), $p < 0.05$ and expressed as relative gene expression. $p_x < 0.05$; $p_y < 0.05$, $p_z < 0.05$ (where 'x', 'y', 'z' refers to comparison with 3 m, 12 m, 24 m respectively). $p_w < 0.05$ (where 'w' refers to comparison with 3 m vehicle treated group).

Table 3 Effect of curcumin on age induced alterations in daily rhythms, mean 24 h levels and daily pulse of SIRT1 and NRF2 protein expression

Protein	Age & Treatment	ZT-0	ZT-6	ZT-12	ZT-18	Mean 24 h levels	Max/Min ratio
SIRT1	3m VT	83±9.2	79.8±10.8	115±4.6	58.7±4	84.1±6.2	2±0.2
	3m CT	47.2±9.5	84.5±22.1	89.9±11.8	70.4±10.3	73±7.7	2.1±0.5
	12m VT	123±6.1	150.6±9.3	61.2±1	82±3.3	104.2±9.4	2.5±0.1
	12m CT	73.9±6.2	108.3±5.6	149.1±20.6	59.6±4.9	97.7±10.3	2.6±0.5
	24m VT	136.5±12.5	93.1±7.3	111.6±9.8	130±20.4	118±7.4 ^{x}	1.5±0.1 ^{xy}
	24m CT	134.5±19.2	98.4±8.1	119.1±15.5	137.6±19.6	122±8 ^{xw}	1.4±0.2 ^{yw}
NRF2	3m VT	99.3±17.9	50.5±3.8	113±2.8	42.5±10.2	76.3±9.1	3.5±1.3
	3m CT	42.7±5.3	114.7±17.3	96.5±2.8	84.2±15.3	84.5±8.7	2.8±0.6
	12m VT	101.8±24.7	83±11.2	64.1±17.9	46.6±13.5	73.9±9.5	2.3±0.2
	12m CT	85.8±10.4	80.3±17.8	123.3±10.8	141.8±2.2	108±8.4^{xw}	2±0.4
	24m VT	138.3±44.4	89±9.1	68.1±13.2	49.3±5.7	86.2±13.7	2.9±1
	24m CT	119.5±24.4	98.4±24.1	127.1±3.3	92.4±12.5	109.3±9^{xw}	1.4±0.2

Each value is mean ± SEM (n = 4), $p < 0.05$ and expressed as normalized mean density. $p_x < 0.05$; $p_y < 0.05$, $p_z < 0.05$ (where 'x', 'y', 'z' refers to comparison with 3 m, 12 m, 24 m respectively). $p_w < 0.05$ (where 'w' refers to comparison with 3 m vehicle treated group).

b. Effect of leaf extract of *Withania somnifera* on age induced alterations in chronomics of clock gene expression

Effect of hydro-alcoholic leaf extract of WS was studied on *rBmal1, rPer1, rPer2, rCry1, rCry2* and *rRev-erbα* mRNA expression levels and daily rhythms in SCN of 3, 12 and 24 m old rats. There were no significant difference between control and vehicle treated groups.

All the clock genes studied showed rhythmic expression pattern in the SCN of WST animals (Fig. 32). In the SCN of 3 m animals treated with WS *rBmal1*, *rCry2* and *rPer2*, *rCry1* showed maximum expression at ZT-18 and minimum expression at ZT-12 and ZT-6 respectively. *rPer1* showed peak and nadir at ZT-6 and ZT-0 respectively. *rRev-erbα* expression peaked at ZT-6 and nadir was at ZT-12. Compared to 3 m SCN, *rBmal1*, *rPer1*, *and rRev-erbα* showed no change in phase upon WS treatment (Fig. 32). Mean 24 h levels of *rPer1, rBmal1 and rRev-erbα* did not show any significant change upon WS treatment compared to 3 m VT. However, *rPer2, rCry1* and *rCry2* in WST SCN of young rats showed an increase of 7.2, 3.7 and 5.8 folds respectively ($p < 0.05$) (Fig. 33). Daily pulse of *rBmal1, rCry1* and *rCry2* showed significant decrease in 3 m WST group ($p < 0.05$). Whereas *rPer1, rPer2* and *rRev-erbα* showed no significant change in WST group when compared to 3 m control group (Fig. 34).

In the SCN of 12 m WST group *rBmal1* expression was maximum at ZT-18 displaying a phase delay of 12 h compared to 12 m vehicle group, thus restoring the phase when compared to young vehicle controls. Similarly, *rPer1* phase was advanced by 12 h in comparison to 12 m controls and thus restoring the phase in comparison to 3 m. *rPer2*, peaked at ZT-6 showing a phase advance of 12 h and 6 h in comparison to 12 m and 3 m vehicle group respectively. *rCry1* mRNA expression was peaked at ZT-12 showing a phase advance of 6 h in comparison to 12 m control, restoring the phase in comparison to 3 m vehicle group. *rCry2* mRNA expression was maximum at ZT-18 and minimum at ZT-12 which was similar to 12 m control group, and 6 h delay in phase in comparison to 3 m control. Interestingly, *rRev-erbα* showed peak expression at ZT-6 and nadir at ZT-12 with no significant change when compared to age matched as well as 3 m controls (Fig. 32). Mean 24 h levels of *rBmal1, rPer1 and rRev-erbα* did not show significant difference in middle aged WST group in relation to 12 m and 3 m VT. However, *rCry1* and *rCry2* in middle aged WST group showed 8.8 folds and 4.0 folds increase respectively when

compared to 3 m ($p < 0.05$), but the change was not significant with respect to age matched controls. Interestingly, WS treatment significantly decreased *rPer2* mean 24 h levels by 2.0 folds in comparison to 12 m ($p < 0.05$), restoring it when compared to 3 m animals (Fig. 33). Daily pulse of *rPer2* showed a significant decrease by 4.2 folds upon WST in comparison to 12 m vehicle control ($p < 0.05$) thereby bringing a restoration. However, *rBmal1, rCry1, rCry2* and *rRev-erbα* showed a significant attenuation in daily pulse in comparison to both 3 and 12 m VT ($p < 0.05$). On the other hand, *rPer1* showed 4.9 fold and 2.4 fold increase in WST comparison to 12 m and 3 m control group respectively ($p < 0.05$) (Fig. 34).

In 24 m WST SCN, *rBmal1* showed maximum expression at ZT-0 and minimum at ZT-6, showing 12 h phase advance when compared to age matched controls and 6 h phase delay with reference to young controls. WS treatment restored the phase of *rPer1* with maximum expression at ZT-6 and showed a phase delay of 6 h when compared to old age controls. However, *rPer2, rCry1 and rCry2* mRNA showed peak expression at mid-subjective night though with phase delay of 6 h compared to both 24 m as well as 3 m control group. *rRev-erbα* showed peak levels at ZT-6 and nadir at ZT-18 which is similar to young and old aged rats treated with vehicle (Fig. 32). In 24 m WST, mean 24 h levels of *rBmal1* and *rPer2* increased by a significant 2.8 and 3.4 folds respectively in comparison to the age matched control animals ($p < 0.05$), thus resulting in restoration. *rCry1* and *rCry2* showed 4.6 folds and 1.4 folds increase in comparison to 24 m VT and 7.3 folds and 3.4 folds increase in reference to 3 m VT respectively ($p < 0.05$) (Fig. 33). Interestingly, WS treatment to 24 m animals restored daily pulse of *rPer1* by attenuating the pulse by 1.6 folds when compared to age matched vehicle group. However the difference in daily pulse of *rPer2* and *rRev-erbα* compared to both 3 m and 24 m control group were insignificant. *rBmal1, rCry1* and *rCry2* daily pulse were observed to be increased significantly compared to their respective age matched control groups, but decreased significantly when compared to 3 m animals (Fig. 34).

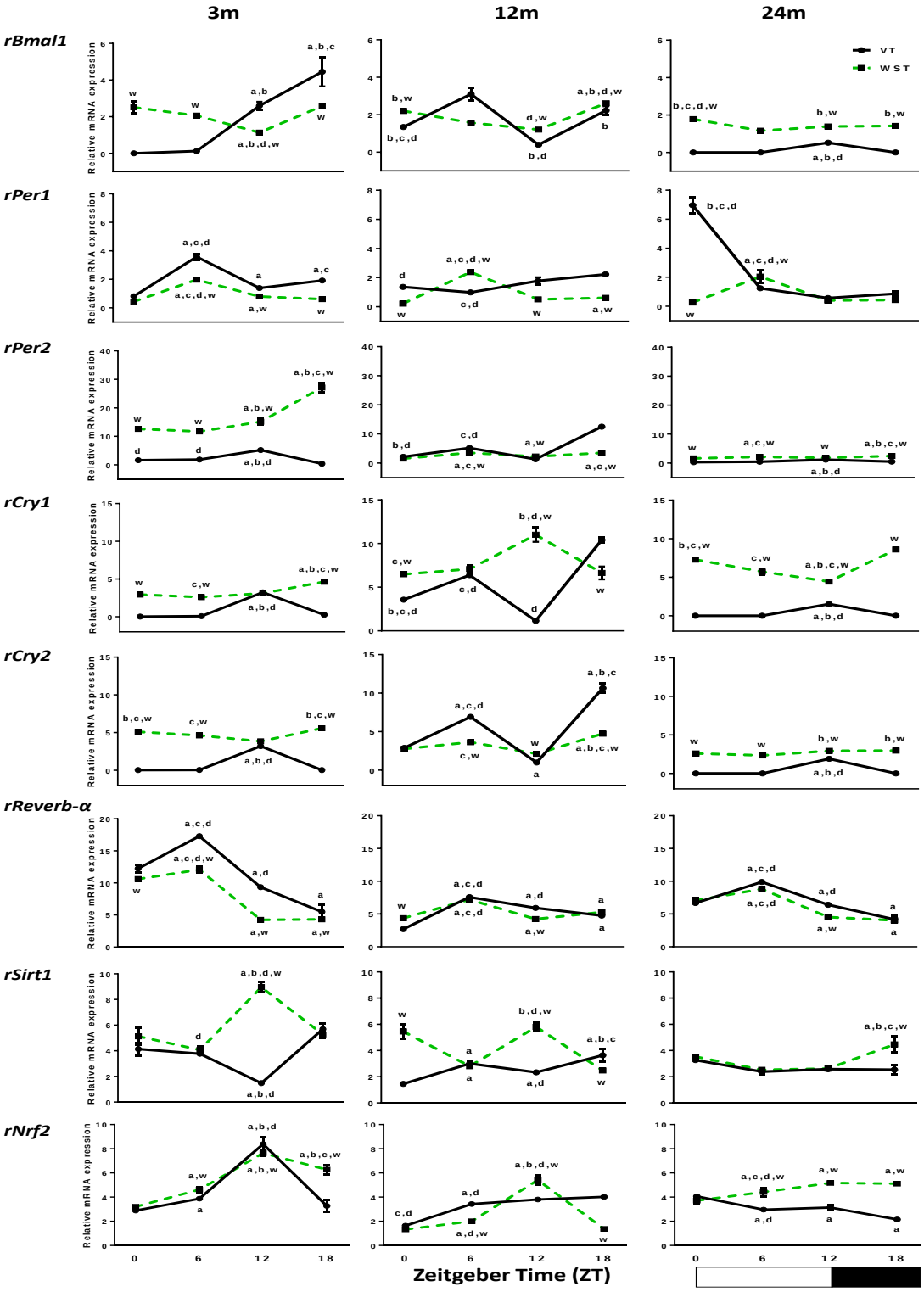


Fig. 32 Effect of *W. somnifera* treatment on daily rhythms of clock genes, *rSirt1* and *rNrf2* mRNA expression in the aging rat SCN in 3, 12 and 24 months (m). Each value is mean ± SEM (n = 4), $p < 0.05$ and expressed as relative gene expression. $p_a < 0.05$; $p_b < 0.05$, $p_c < 0.05$ and $p_d < 0.05$ (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).

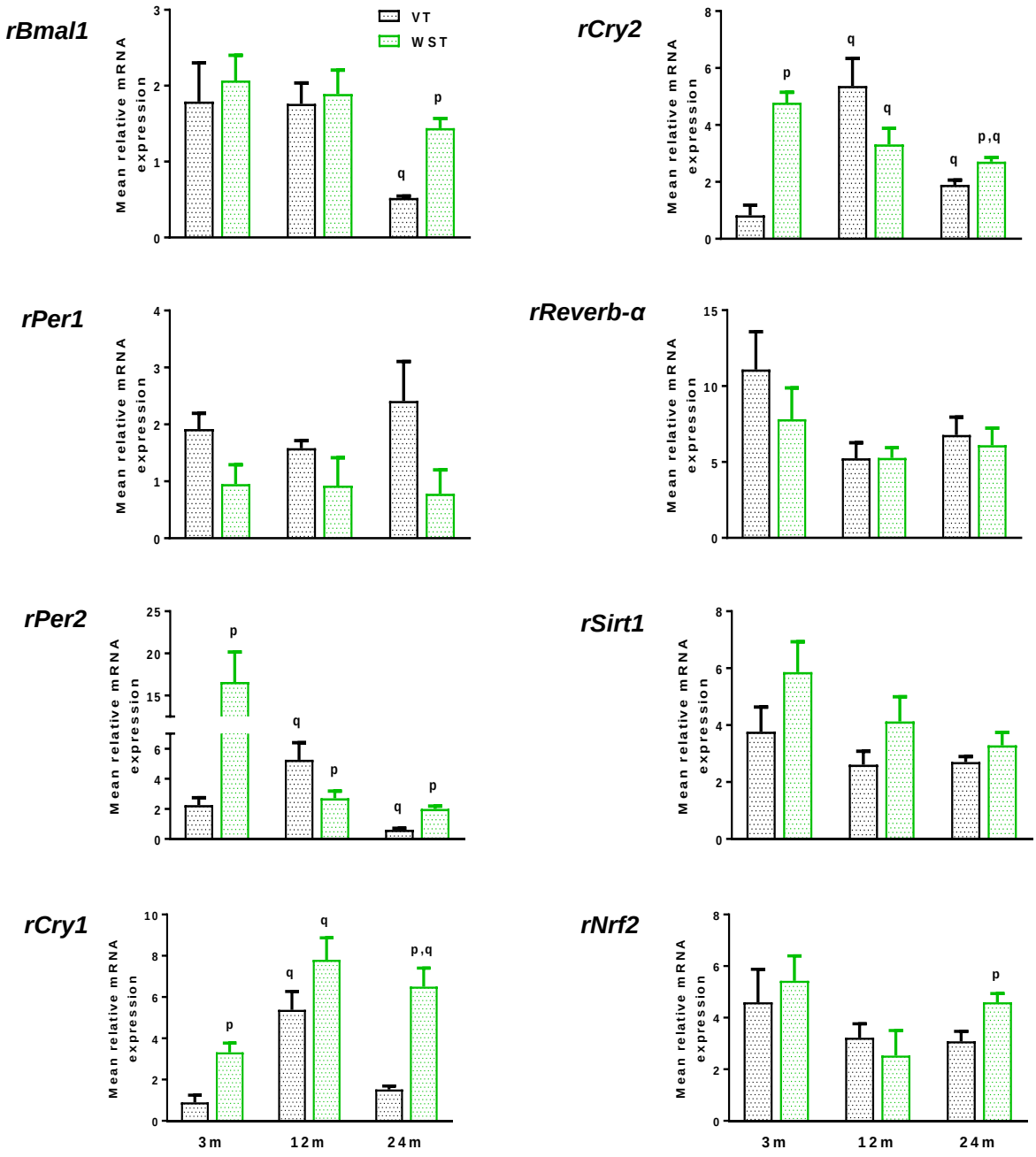


Fig. 33 Effect of *W. somnifera* administration on mean 24 h levels of *rBmal1*, *rPer1*, *rPer2*, *rCry1*, *rCry2*, *rRev-erbα, rSirt1* and *rNrf2* genes in the aging rat SCN in 3, 12 and 24 months (m) rat SCN. Each value is mean ± SEM, $p < 0.05$ and expressed as mean relative gene expression. $p_p < 0.05$ (where 'p' refers to comparison with age matched vehicle group). $p_q < 0.05$ (where 'q' refers to comparison with 3 m vehicle group).

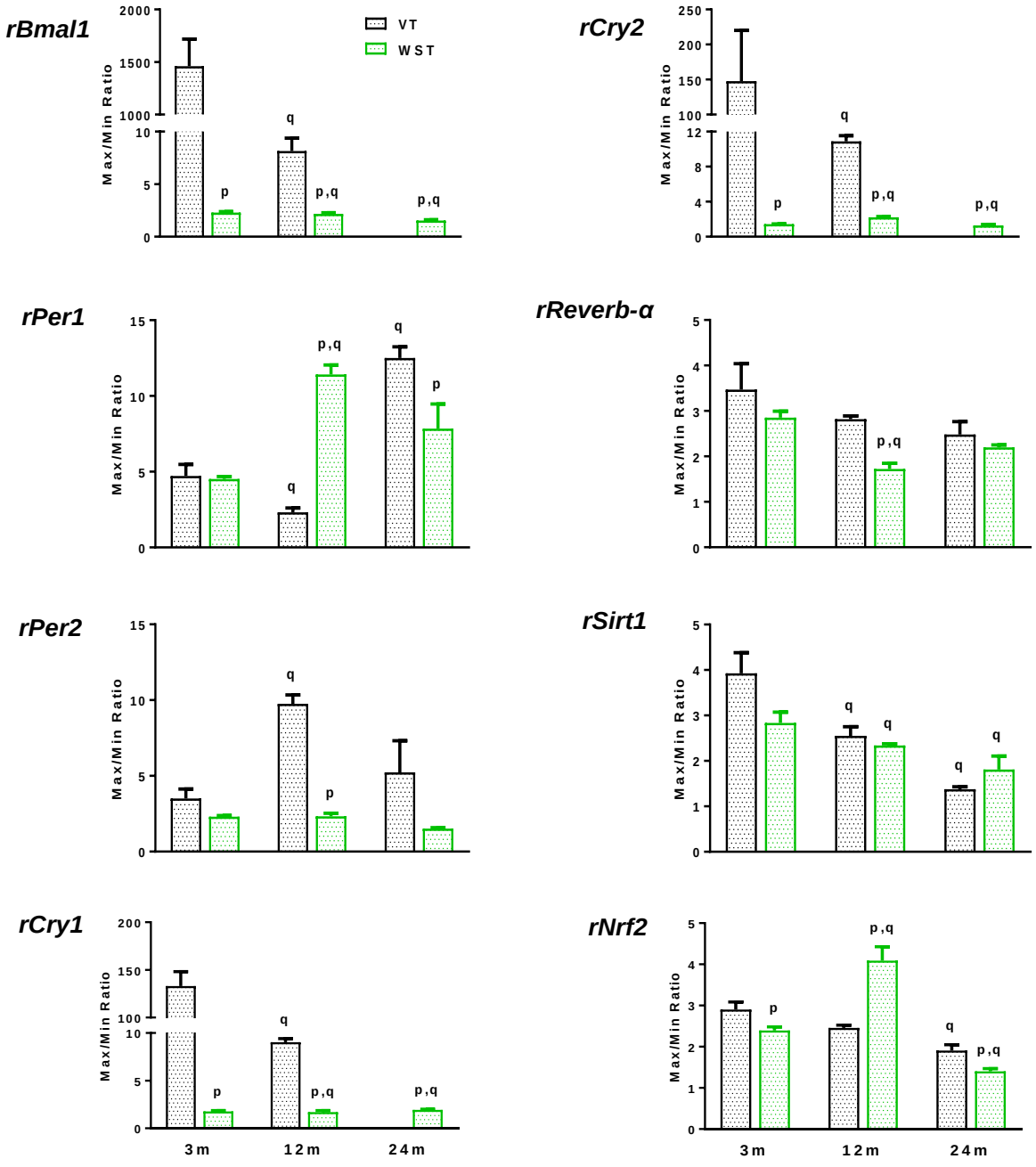


Fig. 34 Effect of *W. somnifera* administration on daily pulse of *rBmal1*, *rPer1*, *rPer2*, *rCry1*, *rCry2*, *rRev-erbα, rSirt1* and *rNrf2* genes in the aging rat SCN in 3, 12 and 24 months rat SCN. Each value is maximum/minimum ratio of relative gene expression. $p_p < 0.05$ (where 'p' refers to comparison with age matched vehicle group). $p_q < 0.05$ (where 'q' refers to comparison with 3 m vehicle group).

Effect of *Withania somnifera* leaf extract on age induced alterations in chronomics of SIRT1 and NRF2 expression

With WS administration to young animals there was phase advance of 6 h in *rSirt1* daily rhythm in comparison to 3 m controls, with significantly higher expression at ZT-12 ($p < 0.05$). WS administration to middle aged rats resulted in 6 h advance in phase in reference 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. 32). 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. 33). Similarly, daily pulse of *rSirt1* in all the three age groups were comparable to respective controls with no significant difference (Fig. 34).

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 peak levels at mid-subjective day and nadir at ZT-12. WS administration to 12 m animals lead to 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 in reference to young controls (Fig. 35A and 35B). 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 young controls ($p < 0.05$) (Fig. 35C).

With WS treatment, *rNrf2* transcripts showed rhythmic expression in 3 m SCN similar to the age matched VT with peak and nadir at ZT-12 and ZT-0 respectively. Interestingly, it advanced the peak expression by 6 h in 12 m animals thus restoring the phase. Similarly in old aged animals, WST delayed the peak *rNrf2* expression by 12 h resulting in phase restoration (Fig. 32). Further, WS resulted in a significant 1.5 fold increase in mean 24 h levels of *rNrf2* in 24 m compared to control group indicative of a restoration when compared to 3 m (Fig. 33). WS administration decreased the daily pulse by 1.3 folds in both 3 and 24 m animals, but elevated it by 1.7 folds in middle aged animals in comparison to respective VT groups ($p < 0.05$) (Fig. 34).

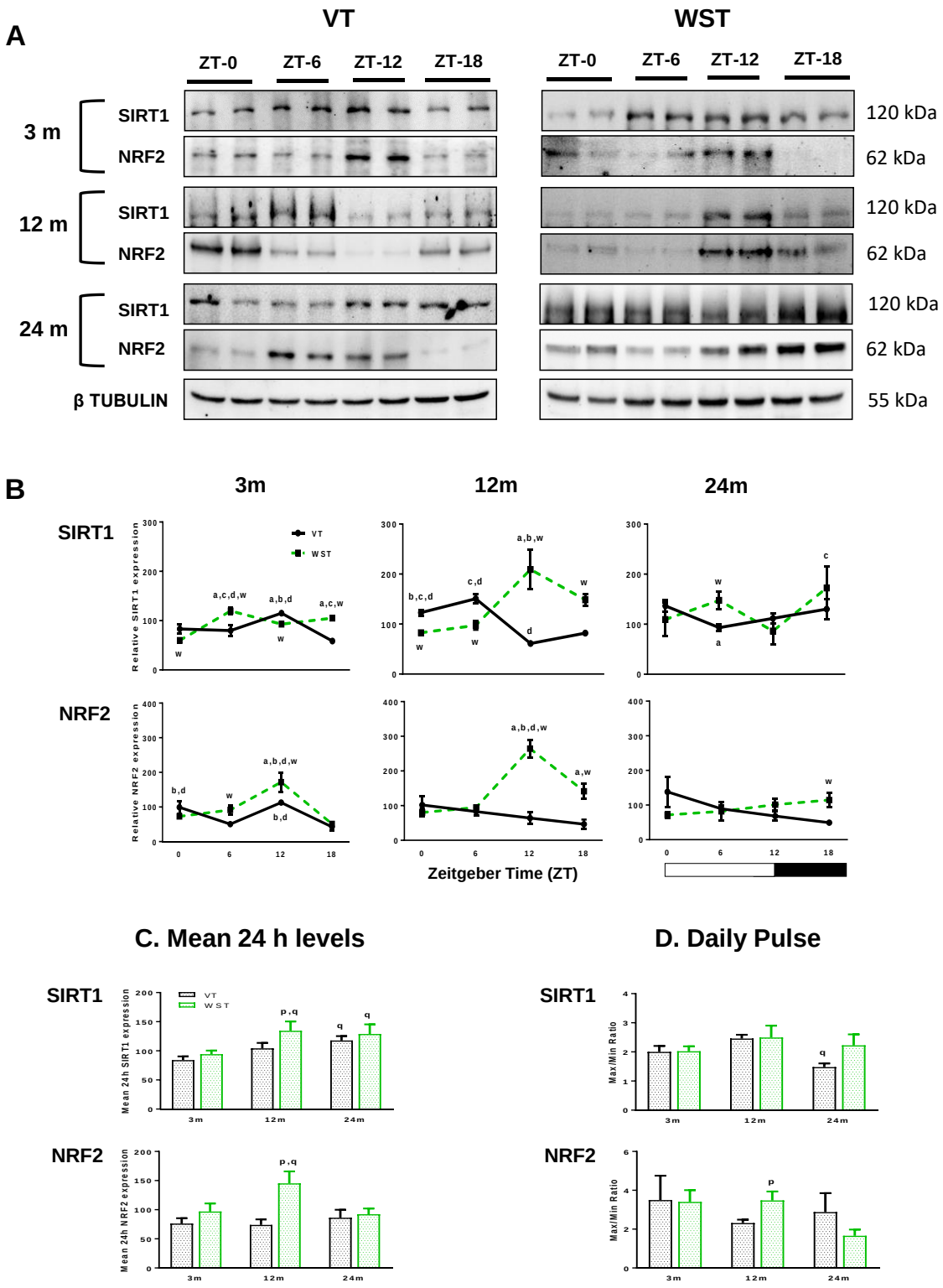


Fig. 35 (A) Immunoblots showing daily rhythms in SIRT1 and NRF2 expression in SCN of 3, 12 and 24 months (m) aged rats at ZT-0, 6, 12 and 18. β-TUBULIN was probed as loading control. VT- vehicle treated; WST- *Withania somnifera* treated. **(B)** Daily rhythms of SIRT1 and NRF2 expression in the aging rat SCN in 3, 12 and 24 months. 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). **(C)** Effect of WST on mean 24 h levels and **(D)** Daily Pulse of SIRT1 and NRF2 in 3, 12 and 24 m rat SCN. 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).

NRF2 protein expression rhythm upon WST in 3 m was similar to the age matched vehicle controls with peak at ZT-12 and nadir 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 old aged animals, WST lead to restoration of NRF2 rhythmicity although there was a phase delay of 6 h in comparison to young VT rats (Fig. 35A and 35B). A significant 2.0 fold and 1.9 fold increase in mean 24 h levels of NRF2 was observed in 12 m WST when matched to 12 and 3 m VT groups respectively (Fig. 35C). WS administration increased the daily pulse by 1.5 folds in middle aged animals in comparison to 12 m VT ($p < 0.05$). However, the change observed in other age groups were not statistically significant (Fig. 35D). Individual values of genes and protein expression are tabulated in Table 4 and 5.

Correlation analysis

i. Clock genes

Pair wise correlation analysis in 3 m WST light phase (LP) revealed maintenance of statistically significant positive correlations between *rBmal1* - *rCry2*; *rPer2* - *rCry1* and negative correlations between *rPer2 – rRev-erbα*; *rCry1* - *rRev-erbα* compared to the LP of 3 m VT. In dark phase (DP), positive correlations between *rPer2 – rCry1*; *rPer2 – rCry2*; *rCry1 – rCry2* and the significant negative correlation between *rPer1 – rRev-erbα* were similar to dark phase of 3 m VT group ($p < 0.05$) (Fig. 36).

In 12 m LP, WS treatment resulted in a significant positive correlation between *rPer1 – rRev-erbα* which was an insignificant negative correlation in LP of 12 m vehicle animals, thus restoring the correlation in comparison to young controls. Similarly, WS abolished the significant negative correlation found between *rPer1 – rCry1* in 12 m LP to restore the correlation similar to LP of 3 m VT. Further, appearance of a negative correlation between *rCry1 – rRev-erbα* in 12 m WST LP as opposed to the positive correlation in 12 m VT LP

indicated restoration when compared with LP of 3 m vehicle group. However in 12 m WST DP, persistence of significant positive correlations between *rBmal1 - rCry2*; *rPer1 – rPer2*; *rPer1 – rRev-erbα* and *rPer2 - rCry2* were observed similar to 12 m control group in DP (Fig. 36).

In 24 m WST LP, we observed a strong and significant negative correlation between *rBmal1 - rPer1*, which is similar to LP of 12 m indicating a partial restoration in correlation. Similarly, appearance of a positive correlation between *rBmal1 - rCry1* and *rBmal1 – rCry2* found to be similar to 3 m controls in light phase. Interestingly, the significant negative correlation observed between *rPer1 – rPer2* in 24 m LP became a significant positive correlation upon WS treatment. Further, WS administration resulted in a positive correlation between *rPer1 – rRev-erbα* in 24 m LP indicating a restoration of correlation compared to 3 m LP. Positive correlations between *rRev-erbα – rPer2* and *rRev-erbα – rCry1* in 24 m WST LP were similar to LP of 12 m VT implying partial restoration. Moreover, there was a restoration of negative correlation between *rRev-erbα – rCry2* in 24 m WST LP when related to 3 m VT group in LP (Fig. 36). Even in DP of old age group, WST established positive correlation among *rPer1 – rPer2* and *rPer1 – rCry2* which was similar to 12 m DP. The establishment of significant positive correlations between *rPer2 - rCry1* and *rPer2 – rCry2* were similar to DP of 3 m suggesting restoration in correlations. The negative correlation that existed between *rPer1 – rRev-erbα* in DP of 3 m vehicle reappeared in 24 m DP upon WS treatment ($p < 0.05$) (Fig. 36).

ii. Clock genes, rSirt1 and rNrf2

The clock modulator *rSirt1* showed a strong negative correlation with most of the clock transcripts studied except with *rPer1, rRev-erbα* in LP of 3 m and except *rBmal1, rPer1, rRev-erbα* in DP of 3 m animals. There existed a significant negative correlation between *rSirt1 – rNrf2* in both LP as well as DP ($p < 0.05$) (Fig. 36). Further, *rNrf2* showed a positive correlation with all the clock genes except *rPer1, rRev-erbα* in LP and *rBmal1, rPer1, rRev-erbα* in DP of 3 m VT animals. However, with WS administration, the negative correlation between *rSirt1 - rBmal1*; *rSirt1 – rCry2* sustained in 3 m LP whereas significant negative correlations became positive in 3 m DP (Fig. 36). And there appeared negative correlations between *rNrf2 – rBmal1* and *rNrf2 - rRev-erbα* in both LP and DP of 3 m WST ($p < 0.05$).

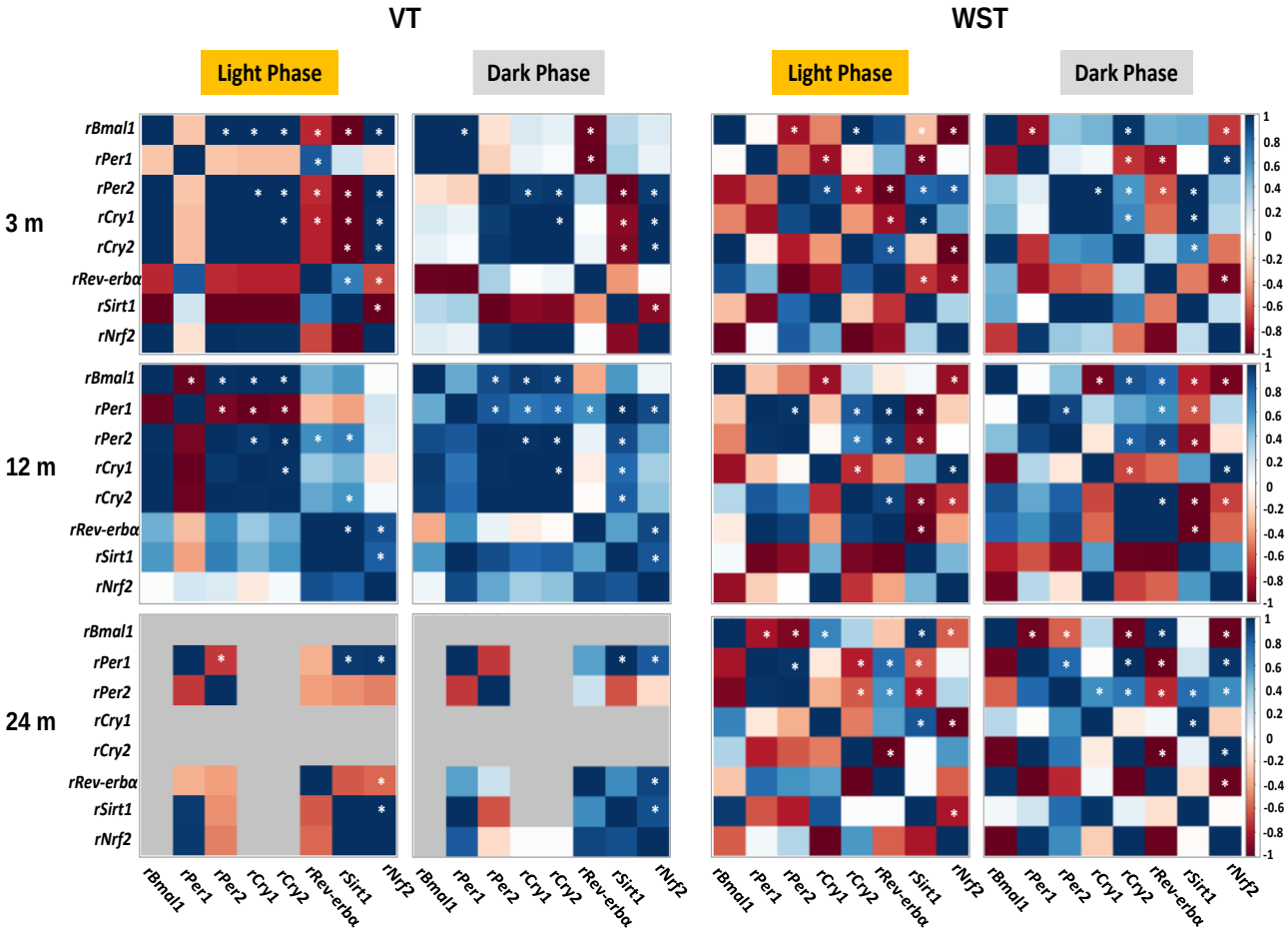


Fig. 36 Effect of *W. somnifera* treatment on pair wise correlation between mean light (ZT-0, 6, 12) and mean dark (ZT-12, 18, 24/0) phase values of *rBmal1*, *rPer1*, *rPer2*, *rCry1*, *rCry2*, *rRev-erbα, rSirt1* and *rNrf2* in SCN of 3, 12 and 24 months (m) aged rats. Every colored square represents correlation coefficient values between the parameters. Where, a positive correlation is indicated by shades of blue, negative correlation by shades of red and white indicates no correlation. Grey squares indicate that the values were not considered for correlation analysis. '*' Indicates statistically significant value between parameters ($p < 0.05$).

Most of the negative correlations existed between *rSirt1* and other clock genes in 3 m turned as positive correlations in 12 m in both the phases. Similarly, significant positive interactions between *rNrf2* and other clock genes became weak interactions in both the phases of 12 m vehicle group (Fig. 36). WS treatment to 12 m animals restored the negative correlations between *rSirt1 – rPer1*; *rSirt1 – rPer2*; *rSirt1 – rCry2* in LP and *rSirt1 – rBmal1*; *rSirt1 – rPer1* in DP. Similarly, positive correlation between *rNrf2 – rCry1* was

restored in both photo and scotophase of 12 m WST. Interestingly, the negative correlation which was lost between *rNrf2* - *rRev-erbα* in 12 m VT animals was re-established upon WS treatment in LP though did not reach significant level.

In 24 m SCN, there was a strong positive correlation between *rSirt1 – rPer1* and *rNrf2 – rPer1* in both LP and DP which were found to be weak interactions in both the phases of 3 m SCN. 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. 36). Interestingly, with WS administration, the negative correlation between *rSirt1 – rPer2* and *rSirt1 – rNrf2* was restored in LP of 24 m SCN. However in dark phase, WS resulted in positive correlations between *rSirt1 – rPer2* and *rSirt1 – rCry1* which were similar to DP of 12 m suggesting a partial restoration. Whereas the significant positive correlation between *rNrf2 – rPer1* and *rNrf2 – rPer2* were similar to 3 m SCN in DP indicating restoration upon WST ($p < 0.05$) (Fig. 36).

Table 4 Effect of *Withania somnifera* leaf extract on age induced alterations in daily rhythms, mean 24 h levels and daily pulse of clock genes, *Sirt1* and *Nrf2* transcript expression

Gene	Age & Treatment	ZT-0	ZT-6	ZT-12	ZT-18	Mean 24 h levels	Max/Min ratio
rPer1	3m VT	0.81±0.12	3.58±0.2	1.39±0.17	1.91±0.16	1.92±0.28	4.72±0.76
	3m WST	0.44±0.01	1.97±0.05	0.8±0.02	0.61±0.03	0.95±0.35	4.52±0.16
	12m VT	1.36±0.11	0.99±0.09	1.77±0.25	2.22±0.11	1.6±0.14	2.32±0.29 ^{x}
	12m WST	0.21±0.01	2.38±0.05	0.51±0.01	0.61±0.01	0.93±0.49	11.42±0.63 ^{xw}
	24m VT	6.96±0.57	1.24±0.05	0.56±0.06	0.86±0.18	2.41±0.7	12.51±0.74 ^{xy}
	24m WST	0.26±0.01	2.04±0.45	0.4±0.01	0.43±0.01	0.78±0.42	7.84±1.63 ^{xw}
rPer2	3m VT	1.63±0.32	1.83±0.33	5.19±0.55	0.37±0.15	2.25±0.49	3.5±0.64
	3m WST	12.6±0.51	11.7±0.5	15.1±1.01	27.1±1.68	16.6±3.57	2.31±0.08
	12m VT	2.13±0.11	5.17±0.13	1.3±0.13	12.5±0.48	5.3±1.14 ^{x}	9.76±0.58 ^{x}
	12m WST	1.53±0.08	3.53±0.13	2.25±0.04	3.49±0.11	2.7±0.49 ^{x}	2.33±0.19
	24m VT	0.31±0.09	0.46±0.09	1.16±0.09	0.5±0.17	0.61±0.1 ^{xy}	5.23±2.09
	24m WST	1.63±0.09	2.14±0.14	1.79±0.04	2.46±0.07	2.0±0.18 ^{x}	1.51±0.07
rCry1	3m VT	0.025±0.003	0.07±0.02	3.23±0.26	0.26±0.04	0.9±0.35	133±15.27
	3m WST	2.94±0.04	2.6±0.07	3.1±0.12	4.63±0.24	3.3±0.45	1.78±0.08 ^{w}
	12m VT	3.57±0.2	6.38±0.29	1.16±0.07	10.4±0.31	5.4±0.89 ^{x}	9.04±0.38 ^{x}

	12m WST	6.48±0.21	7.08±0.41	11±0.82	6.63±0.74	7.8±1.1 ^{xw}	1.71±0.15 ^{w}
	24m VT	?	?	1.52±0.17	?	0.76±0.3 ^{y}	-
	24m WST	7.28±0.31	5.73±0.37	4.44±0.02	8.6±0.25	6.5±0.9 ^{xw}	1.94±0.06 ^{w}
rCry2	3m VT	0.0003±0.00004	0.05±0.01	3.21±0.33	0.04±0.01	0.82±0.36	147.75±72.5
	3m WST	5.1±0.08	4.62±0.16	3.84±0.13	5.57±0.24	4.78±0.37	1.45±0.02 ^{w}
	12m VT	2.9±0.29	6.92±0.21	0.99±0.1	10.64±0.6	5.4±0.97 ^{x}	10.9±0.64 ^{x}
	12m WST	2.77±0.1	3.63±0.2	2.13±0.11	4.73±0.34	3.3±0.56 ^{w}	2.22±0.09 ^{w}
	24m VT	?	?	1.9±0.17	?	1.9±0.2 ^{xy}	-
	24m WST	2.6±0.15	2.34±0.19	2.93±0.05	2.97±0.18	2.7±0.2 ^{xw}	1.28±0.12 ^{w}
rBmal1	3m VT	0.003±0.001	0.12±0.02	2.59±0.22	4.45±0.79	1.79±0.51	1461±256.7
	3m WST	2.51±0.33	2.06±0.04	1.13±0.02	2.58±0.08	2.07±0.33	2.3±0.09 ^{w}
	12m VT	1.34±0.04	3.1±0.36	0.39±0.03	2.22±0.23	1.76±0.28	8.16±1.23 ^{x}
	12m WST	2.2±0.11	1.57±0.02	1.2±0.01	2.61±0.12	1.89±0.32	2.18±0.1 ^{w}
	24m VT	?	?	0.52±0.03	?	0.5±0.03 ^{x}	-
	24m WST	1.78±0.08	1.16±0.09	1.39±0.08	1.42±0.05	1.44±0.13	1.54±0.07 ^{w}
rRev-erbα	3m VT	12.24±0.58	17.29±0.3	9.34±0.32	5.49±1.05	11.09±2.5	3.47±0.57
	3m WST	10.61±0.17	12.08±0.5	4.24±0.09	4.33±0.26	7.82±2.06	2.85±0.14
	12m VT	2.69±0.03	7.58±0.15	5.92±0.14	4.76±0.08	5.24±1.03	2.82±0.07
	12m WST	4.36±0.19	7.19±0.14	4.23±0.27	5.28±0.31	5.27±0.68	1.72±0.13 ^{x}
	24m VT	6.67±0.09	9.89±0.11	6.4±0.03	4.17±0.54	6.78±1.18	2.48±0.28
	24m WST	7.06±0.42	8.83±0.13	4.53±0.09	4.03±0.13	6.11±1.12	2.2±0.06
rSirt1	3m VT	4.14±0.49	3.77±0.16	1.48±0.11	5.69±0.45	3.77±0.87	3.93±0.45
	3m WST	5.13±0.65	4.07±0.27	0±0	8.95±0.39	5.86±1.07	2.83±0.24 ^{w}
	12m VT	1.45±0.12	3±0.23	0±0	3.64±0.49	2.6±0.47	2.55±0.2 ^{x}
	12m WST	5.45±0.57	2.8±0.1	5.8±0.35	2.48±0.16	4.13±0.87	2.34±0.04 ^{w}
	24m VT	3.27±0.13	2.39±0.24	2.58±0.11	2.54±0.39	2.7±0.2	1.38±0.06 ^{xy}
	24m WST	3.53±0.05	2.53±0.19	2.62±0.08	4.47±0.63	3.29±0.45	1.81±0.3 ^{w}
rNrf2	3m VT	2.89±0.11	3.87±0.12	8.38±0.6	3.27±0.48	4.6±1.28	2.9±0.18
	3m WST	3.2±0.04	4.62±0.23	7.64±0.25	6.27±0.37	5.43±0.97	2.39±0.09
	12m VT	1.64±0.06	3.43±0.13	3.8±0.1	4.02±0.1	3.22±0.54	2.45±0.07
	12m WST	1.33±0.02	2±0.09	5.41±0.39	1.37±0.02	2.53±0.97	4.09±0.34 ^{xw}
	24m VT	4.06±0.07	2.96±0.07	3.14±0.2	2.15±0.12	3.08±0.39	1.91±0.14 ^{x}
	24m WST	3.71±0.2	4.42±0.36	5.17±0.16	5.11±0.13	4.6±0.34	1.4±0.07 ^{xyw}

Each value is mean ± SEM (n = 4), $p < 0.05$ and expressed as relative gene expression. $p_x < 0.05$; $p_y < 0.05$, $p_z < 0.05$ (where 'x', 'y', 'z' refers to comparison with 3 m, 12 m, 24 m respectively). $p_w < 0.05$ (where 'w' refers to comparison with 3 m vehicle treated group).

Table 5 Effect of WS on age induced alterations in daily rhythms, mean 24 h levels and daily pulse of SIRT1 and NRF2 protein expression

Protein	Age & Treatment	ZT-0	ZT-6	ZT-12	ZT-18	Mean 24 h levels	Max/Min ratio
SIRT1	3m VT	83±9.2	79.8±10.8	115±4.6	58.7±4	84.1±6.2	2±0.2
	3m WST	59.8±3.4	119.5±6.3	92.8±5	105±3.5	94.3±6.1	2±0.2
	12m VT	123±6.1	150.6±9.3	61.2±1	82±3.3	104.2±9.4	2.5±0.1
	12m WST	82.7±5.7	97.4±8.9	209.2±39.3	148.3±12.3	134±16 ^{xw}	2.5±0.4
	24m VT	136.5±12.5	93.1±7.3	111.6±9.8	130±20.4	118±7.4 ^{x}	1.5±0.1 ^{x}
	24m WST	109.3±33.7	147.7±17.4	86.3±26.4	172.3±42.7	129±17 ^{xw}	2.2±0.4
NRF2	3m VT	99.3±17.9	50.5±3.8	113±2.8	42.5±10.2	76.3±9.1	3.5±1.3
	3m WST	72.9±5.3	91.5±13.9	172±28	51.2±4.4	96.9±13.8	3.4±0.6
	12m VT	101.8±24.7	83±11.2	64.1±17.9	46.6±13.5	73.9±9.5	2.3±0.2
	12m WST	80.1±12.1	95±6.1	264.5±25.2	141.4±22.6	145±20 ^{xw}	3.5±0.5
	24m VT	138.3±44.4	89±9.1	68.1±13.2	49.3±5.7	86.2±13.7	2.9±1
	24m WST	71.5±8.1	81.7±27.3	101.2±16.9	114.4±21.4	92.2±9.8 ^{y}	1.7±0.3 ^{y}

Each value is mean ± SEM (n = 4), $p < 0.05$ and expressed as normalized mean density. $p_x < 0.05$; $p_y < 0.05$, $p_z < 0.05$ (where 'x', 'y', 'z' refers to comparison with 3 m, 12 m, 24 m respectively). $p_w < 0.05$ (where 'w' refers to comparison with 3 m vehicle treated group).

OBJECTIVE 2

a. Understanding the role of SIRT1 in restoratory effects of curcumin using its inhibitor Nicotinamide (NAM) *ex vivo*

Initially SIRT1 activity in the SCN of young rats of 3 m was studied. SIRT1 showed daily oscillations in its activity with peak activity at ZT-18 and minimum activity at ZT-6 (Fig. 37A). Based on this observation, the assay was carried out using the SCN samples collected at ZT-18 from middle and old aged rats. SIRT1 activity in the SCN significantly decreased with advance in age. In 12 m animals SIRT1 activity reduced by 29.6 % whereas in 24 m it was decreased by 55.3 % in comparison to the activity in 3 m at ZT-18 ($p < 0.05$) (Fig. 37B).

Further, to understand the involvement of SIRT1 in age related alterations in clock genes and also to probe the role of SIRT1 in restoratory effects of curcumin, SIRT1 activity in the SCN (from 3 m rats) was inhibited using nicotinamide (NAM) *ex vivo*. Upon NAM treatment SIRT1 activity decreased by a significant 67 % in comparison to control ($p < 0.05$) (Fig. 37C). The expression of clock genes was studied in the SCN explants maintained under various treatment conditions such as control, NAM treated, CT and CT+NAM co-treated.

In control SCN, all the clock genes showed rhythmic expression pattern comparable to expression in the SCN of 3 m rats *in vivo*. *rBmal1* showed maximum expression at ZT-18 and minimum at ZT-6 (Fig. 38). When treated with curcumin alone, *rBmal1* expression was in coherence with control conditions with peak and minimum expressions at ZT-18 and ZT-6 respectively. Upon NAM treatment, there was 12 h phase advance as the *rBmal1* expression peak was at mid-subjective day. Interestingly, in SCN co-treated with NAM and curcumin, expression of *rBmal1* was arrhythmic (Fig. 38). Further, mean 24 h levels and daily pulse of *rBmal1* did not show significant variations in any of the treatment conditions in comparison to control (Fig. 39A and 39B).

rPer1 mRNA also followed a rhythmic pattern of expression both in control and CT SCN explants with maximum and minimum expression at ZT-12 and ZT-0 respectively (Fig. 38). With NAM treatment, rhythm of *rPer1* expression was phase delayed by 6 h as the peak shifted to ZT-18. In addition *rPer1* expression significantly increased in this group in all the four time points compared to control (Fig 38). In CT+NAM group, *rPer1* maximum

expression was at ZT-12 however, elevated expression at all the four time points were similar to the pattern in NAM treatment (Fig. 38). Mean 24 h *rPer1* levels increased by 2.2 folds in NAM treated SCN and in CT+NAM it was 1.8 fold increase compared to control ($p < 0.05$). However, in CT SCN mean levels were similar to control group (Fig. 39A). Interestingly, daily pulse was not affected by any of the treatments compared to control (Fig. 39B).

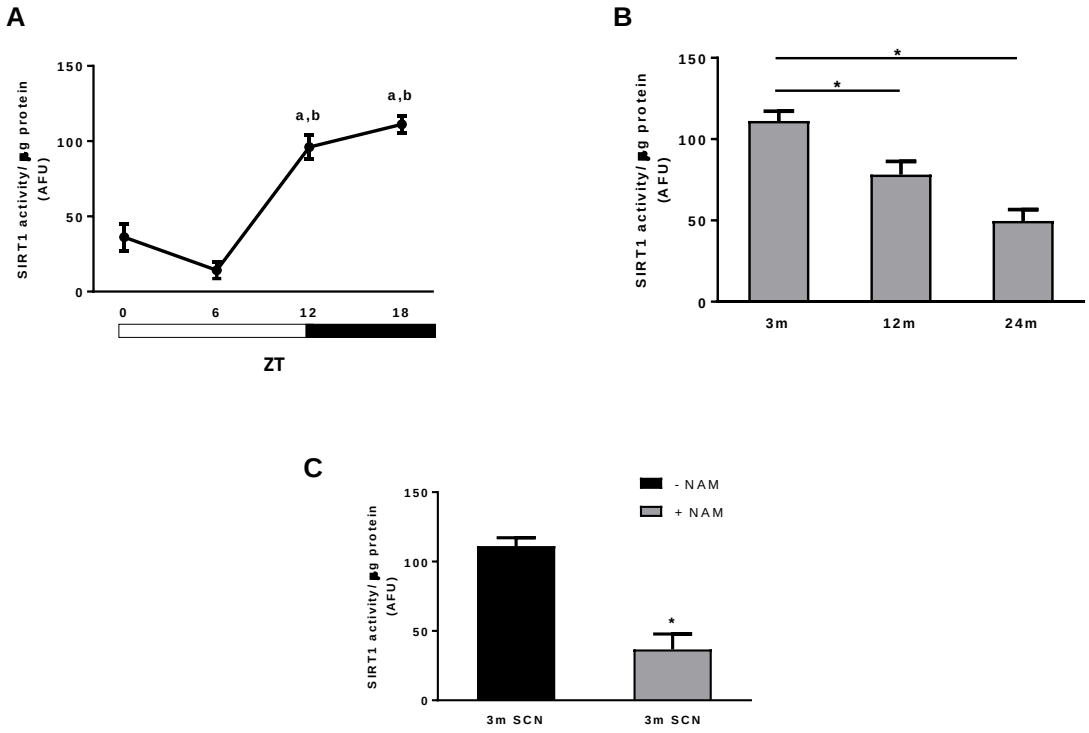


Fig. 37 (A) Daily rhythmicity in SIRT1 activity in 3m SCN. Each value is mean ± SEM (n = 4), $p < 0.05$ and expressed as arbitrary fluorescent units. $p_a < 0.05$; $p_b < 0.05$, $p_c < 0.05$ and $p_d < 0.05$ (where 'a', 'b', 'c' and 'd' refers to comparison with ZT-0, ZT-6, ZT- 12 and ZT-18 respectively within the group). **(B)** SIRT1 activity in SCN reduces with aging (Activity measured at ZT-18). **(C)** 10 nM Nicotinamide (NAM) significantly inhibited the SIRT1 activity ($p < 0.05$). '*' Indicates statistically significant difference ($p < 0.05$).

Similarly *rPer2* expression showed rhythmicity in control and CT SCN as ZT-12 and ZT-18 were the respective time points of peak and nadir. NAM attenuated rhythmic *rPer2* expression (Fig. 38). In CT+NAM group, though *rPer2* expression was rhythmic, 6 h phase advance was observed in comparison to controls as the maximum level was at ZT-6 and nadir was at ZT-0. Further, 24 h *rPer2* mean levels were increased by 2.2 folds in NAM

group and 1.8 folds in CT+NAM groups in comparison to control SCN ($p < 0.05$). CT alone did not affect mean 24 h *rPer2* expression (Fig. 39A). NAM decreased the daily pulse of *rPer2* by 1.5 folds in comparison to control SCN ($p < 0.05$) (Fig. 39B).

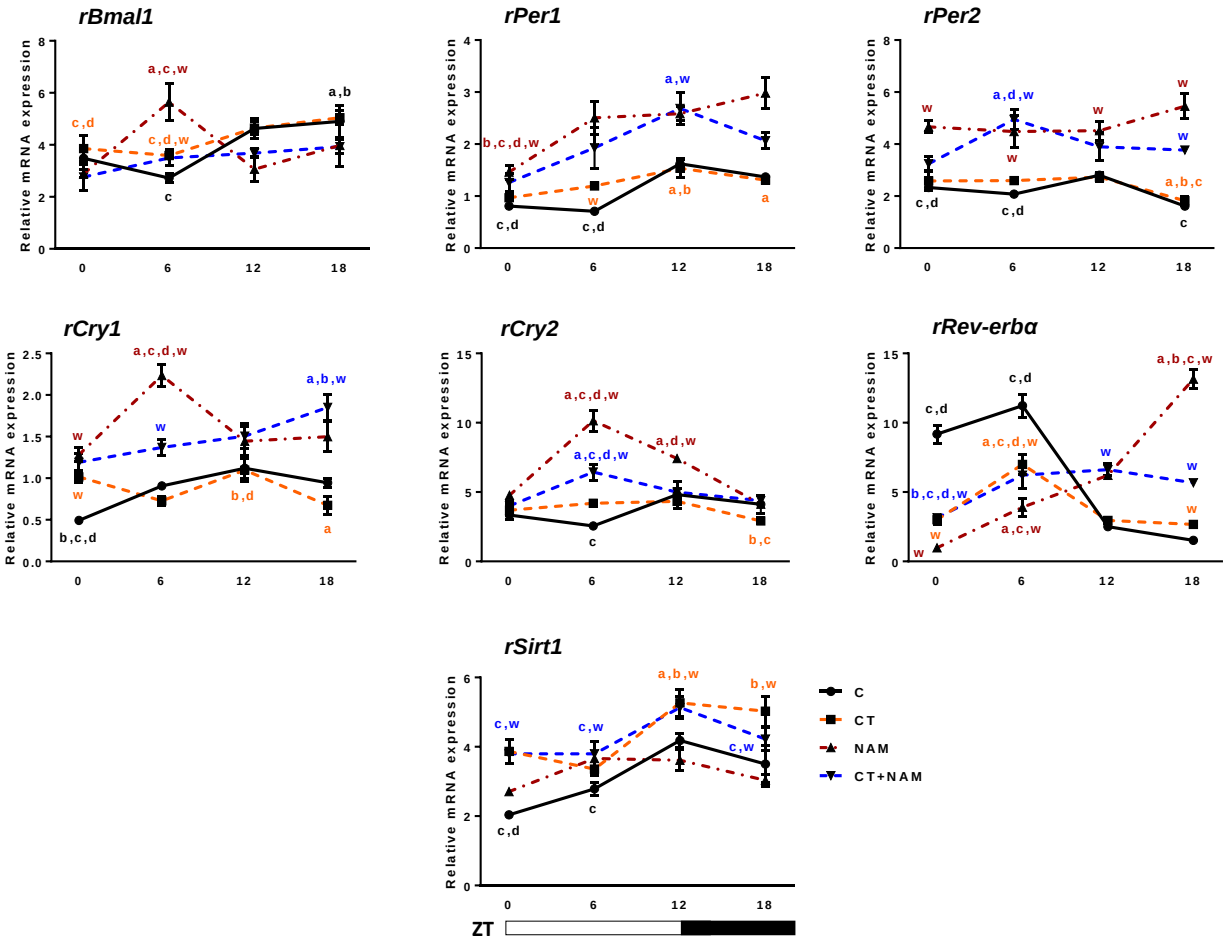


Fig. 38 Expression of *rBmal1*, *rPer1*, *rPer2*, *rCry1*, *rCry2*, *rRev-erbα* and *rSirt1* mRNA in the SCN explants. Each value is mean ± SEM (n = 4), $p < 0.05$ and expressed as relative gene expression. $p_a < 0.05$; $p_b < 0.05$, $p_c < 0.05$ and $p_d < 0.05$ (where 'a', 'b', 'c' and 'd' refers 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 control group). C – control; CT – curcumin treated; NAM – nicotinamide treated; CT+NAM – co-treated with curcumin and nicotinamide. Time points are in reference to the rats from which SCN were harvested.

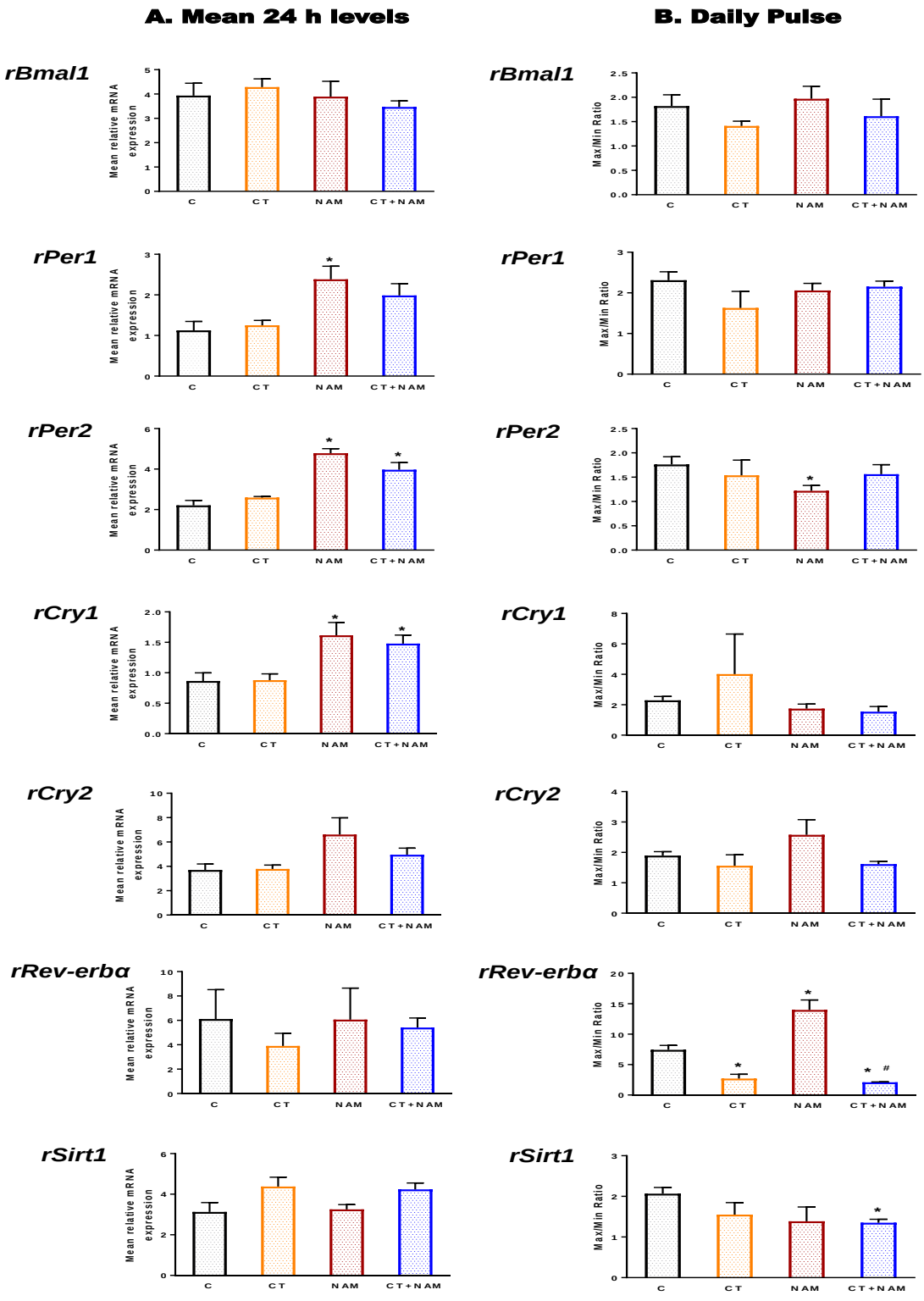


Fig. 39 (A) Mean 24 h levels and **(B)** daily pulse of *rBmal1*, *rPer1*, *rPer2*, *rCry1*, *rCry2*, *rRev-erbα* and *rSirt1* mRNA in the SCN explants. Each value is mean ± SEM, $p < 0.05$ and expressed as mean relative gene expression. '*' Indicates statistically significant difference ($p < 0.05$). C – control; CT – curcumin treated; NAM – nicotinamide treated; CT+NAM – co-treated with curcumin and nicotinamide.

rCry1 mRNA expression was rhythmic in control SCN with respective peak and nadir at ZT-12 and ZT-0. In CT SCN, similar pattern of expression was seen except at ZT-0 (Fig. 38). With NAM, there was a 6 h advance in phase of *rCry1* as the peak shifted to ZT-6. CT+NAM co-treatment delayed the phase by 6 h in comparison to control and by 12 h in comparison to NAM group (Fig. 38). Moreover, 24 h *rCry1* mean levels were elevated by 1.9 folds and 1.7 folds in SCN treated with NAM and co-treated with CT+NAM respectively ($p < 0.05$) (Fig. 39A). However, variations in daily pulse did not reach significance in any of the treatments (Fig. 39B).

rCry2 transcript expression was rhythmic in control SCN with highest expression at ZT-12. Upon CT, similar pattern of expression was seen except at ZT-6 (Fig. 38). NAM advanced the *rCry2* phase by 6 h as the peak levels were at ZT-6. In SCN co-treated with CT+NAM, the expression was in phase with that of NAM group (Fig. 38). Increase in *rCry2* mean levels did not reach significance in SCN treated with NAM and co-treated with CT+NAM in comparison to control ($p > 0.05$) (Fig. 39A). Similarly, daily pulse did not significantly alter in any of the treatments (Fig. 39B).

rRev-erbα expression in control and CT SCN followed rhythmic pattern with maximum and minimum levels in both the groups observed at ZT-6 and ZT-18 respectively (Fig. 38). NAM treatment phase delayed this rhythm by 12 h as the peak expression shifted to mid-subjective night and nadir to night-day transition. However, in CT+NAM SCN the expression of *rRev-erbα* was rhythmic but with altered phase in comparison to both control and NAM group (Fig. 38). Variations in mean 24 h expression of *rRev-erbα* was not significant among different treatments and control (Fig. 39A). Whereas the daily pulse increased by 2.0 folds upon NAM treatment, decreased by 2.7 folds and 3.7 folds in CT and CT+NAM respectively in comparison to control SCN ($p < 0.05$) (Fig. 39B).

Further, *rSirt1* expression also showed rhythmic pattern with expression peaking at ZT-12. This pattern of expression was also observed in CT and CT+NAM SCN, while treatment with NAM alone abolished *rSirt1* rhythmicity (Fig. 38). Mean 24 h levels and daily pulse of *rSirt1* did not change significantly in any of the experimental condition (Fig. 39A and 39B).

Overall, CT did not show restorations of rhythms and phases of clock genes upon SIRT1 inhibition with exception to *rPer1*. Individual values of gene expressions are tabulated in Table 6.

Table 6 Expression of *rBmal1*, *rPer1, rPer2, rCry1, rCry2, rRev-erbα* and *rSirt1* mRNA in the SCN explants. Role of SIRT1 in beneficial effect of curcumin.

Gene	Experimental condition	ZT-0	ZT-6	ZT-12	ZT-18	Mean 24 h levels	Max/Min Ratio
rBmal1	Control	3.48±0.1	2.72±0.19	4.63±0.4	4.91±0.62	3.93±0.51	1.82±0.23
	CT	3.86±0.5	3.59±0.09	4.65±0.3	5.05±0.26	4.28±0.34	1.41±0.1
	NAM	2.89±0.2	5.64±0.71	3.06±0.5	3.98±0.8	3.89±0.63	1.97±0.25
	CT+NAM	2.76±0.5	3.5±0.31	3.68±0.2	3.93±0.27	3.47±0.25	1.61±0.35
rPer1	Control	0.81±0.1	0.71±0.03	1.62±0.1	1.37±0.07	1.13±0.22	2.31±0.21
	CT	0.97±0.1	1.2±0.08	1.54±0.3	1.31±0.08	1.25±0.12	1.63±0.41
	NAM	1.46±0.1	2.5±0.32	2.58±0.1	2.98±0.29	2.4±0.32 ^{wx}	2.06±0.18
	CT+NAM	1.26±0.2	1.93±0.39	2.68±0.3	2.06±0.15	1.99±0.29	2.15±0.14
rPer2	Control	2.33±0.1	2.08±0.06	2.81±0.1	1.61±0.08	2.21±0.25	1.76±0.16
	CT	2.58±0.4	2.6±0.08	2.73±0.5	1.82±0.17	2.43±0.21	1.54±0.31
	NAM	4.66±0.2	4.48±1.63	4.52±0.4	5.46±0.49	4.8±0.23 ^{wx}	1.22±0.11 ^{w}
	CT+NAM	3.24±0.3	4.95±0.39	3.9±0.54	3.77±0.12	3.97±0.4 ^{wx}	1.56±0.19
rCry1	Control	0.49±0.1	0.91±0.03	1.12±0.1	0.94±0.05	0.87±0.13	2.29±0.25
	CT	1.02±0.1	0.73±0.06	1.1±0.14	0.67±0.21	0.88±0.1	4.01±2.64
	NAM	1.28±0.1	2.23±0.43	1.44±0.3	1.5±0.38	1.6±0.21 ^{wx}	1.74±0.3
	CT+NAM	1.19±0.1	1.37±0.09	1.51±0.2	1.85±0.46	1.5±0.14 ^{wx}	1.55±0.34
rCry2	Control	3.34±0.3	2.56±0.09	4.82±0.2	4.12±0.97	3.71±0.49	1.89±0.13
	CT	3.7±0.4	4.19±0.22	4.34±0.8	2.92±0.25	3.79±0.32	1.56±0.36
	NAM	4.76±0.3	10.14±1.8	7.42±0.2	4.13±0.65	6.61±1.37	2.58±0.5
	CT+NAM	4±0.34	6.45±0.59	4.99±0.3	4.38±0.22	4.96±0.54	1.62±0.09
rRev-erbα	Control	9.18±0.7	11.23±1.1	2.51±0.1	1.51±0.05	6.11±2.41	7.42±0.73
	CT	3±0.33	2.67±0.3	7±1.7	2.95±0.21	3.91±1.03	2.7±0.71 ^{w}
	NAM	0.98±0.1	3.91±0.64	6.21±0.3	13.14±0.7	6.06±2.59	14±1.64 ^{wx}
	CT+NAM	3.15±0.1	6.22±0.96	6.62±0.4	5.67±0.12	5.41±0.78	2.1±0.06 ^{wy}
r Sirt1	Control	2.04±0.1	2.79±0.19	4.18±0.2	3.5±0.54	3.13±0.46	2.07±0.15
	CT	3.87±0.6	3.36±0.12	5.27±1.1	5.03±0.44	4.38±0.46	1.55±0.3
	NAM	2.71±0.1	3.66±0.9	3.61±0.3	3.03±0.47	3.25±0.23	1.39±0.35
	CT+NAM	3.8±0.04	3.8±0.11	5.13±0.3	4.22±0.34	4.24±0.31	1.35±0.08 ^{w}

Each value is mean ± SEM (n = 4), $p < 0.05$ and expressed as relative gene expression. $p_w < 0.05$; $p_x < 0.05$; $p_y < 0.05$, $p_z < 0.05$ (where 'w', 'x', 'y', 'z' refers to comparison with C, CT, NAM, CT+NAM respectively). C – control; CT – curcumin treated; NAM – nicotinamide treated; CT+NAM – co-treated with curcumin and nicotinamide. Time points are in reference to the rats from which SCN were harvested.

b. Understanding role of SIRT1 in restoratory effects of *Withania somnifera* leaf extract using its inhibitor Nicotinamide (NAM) *ex vivo*

Similar to the previous set of experiments, clock gene expression studies in SCN explants upon SIRT1 inhibition were carried out to understand the involvement of SIRT1 in restoratory effects of WS.

Rhythmic *rBmal1* expression in control and WST SCN were observed as ZT-18 and ZT-6 were the respective time points of peak and nadir (Fig. 40). NAM treatment phase advanced this rhythm by 12 h. But upon co-treatment of WST+NAM, the phase of *rBmal1* rhythm was similar to that of control with highest expression at ZT-18. However, mean 24 h levels and daily pulse of *rBmal1* did not show significant variations in any of the treatment conditions in comparison to control (Fig. 41A and 41B).

rPer1 mRNA expression was rhythmic in control as well as WST SCN explants with maximum and minimum expression at ZT-12 and ZT-6 respectively (Fig. 40). With NAM treatment, rhythm of *rPer1* expression was phase delayed by 6 h as the peak shifted to ZT-18. In addition *rPer1* expression significantly increased in this group in all the four time points compared to control (Fig. 40). In WST+NAM group, *rPer1* expression was arrhythmic. Mean 24 h *rPer1* levels increased by 2.2 folds in NAM treated SCN, whereas WST+NAM co-treatment restored these levels comparable to control ($p < 0.05$) (Fig. 41A). However, daily pulse of *rPer1* showed no significant difference (Fig. 41B).

rPer2 expression also followed rhythmic pattern in control and WST SCN with respective highest and lowest expression at ZT-12 and ZT-6. NAM treatment abolished the rhythmicity of *rPer2* expression. Whereas, similar to control SCN, rhythmic *rPer2* expression persisted in WST+NAM group with maximum expression at ZT-12 (Fig. 40). Moreover, the 2.2 fold increase in mean 24 h *rPer2* levels observed upon NAM treatment was reduced by significant 1.4 folds in WST+NAM group, indicating partial restoration in comparison to control SCN ($p < 0.05$) (Fig. 41A).

Similarly, rhythmic *rCry1* mRNA expression was observed in control and WST SCN with peak at ZT-12. NAM treatment advanced the *rCry1* phase by 6 h as the peak shifted to ZT-6. Whereas in WST+NAM co-treated group, *rCry1* rhythm was in phase with that of control (Fig. 40). The 1.9 folds increase in mean 24 h levels of *rCry1* upon NAM was significantly decreased in WST+NAM indicating levels similar to control (Fig. 41A). Daily pulse of *rCry1* were similar in all the groups (Fig. 41B).

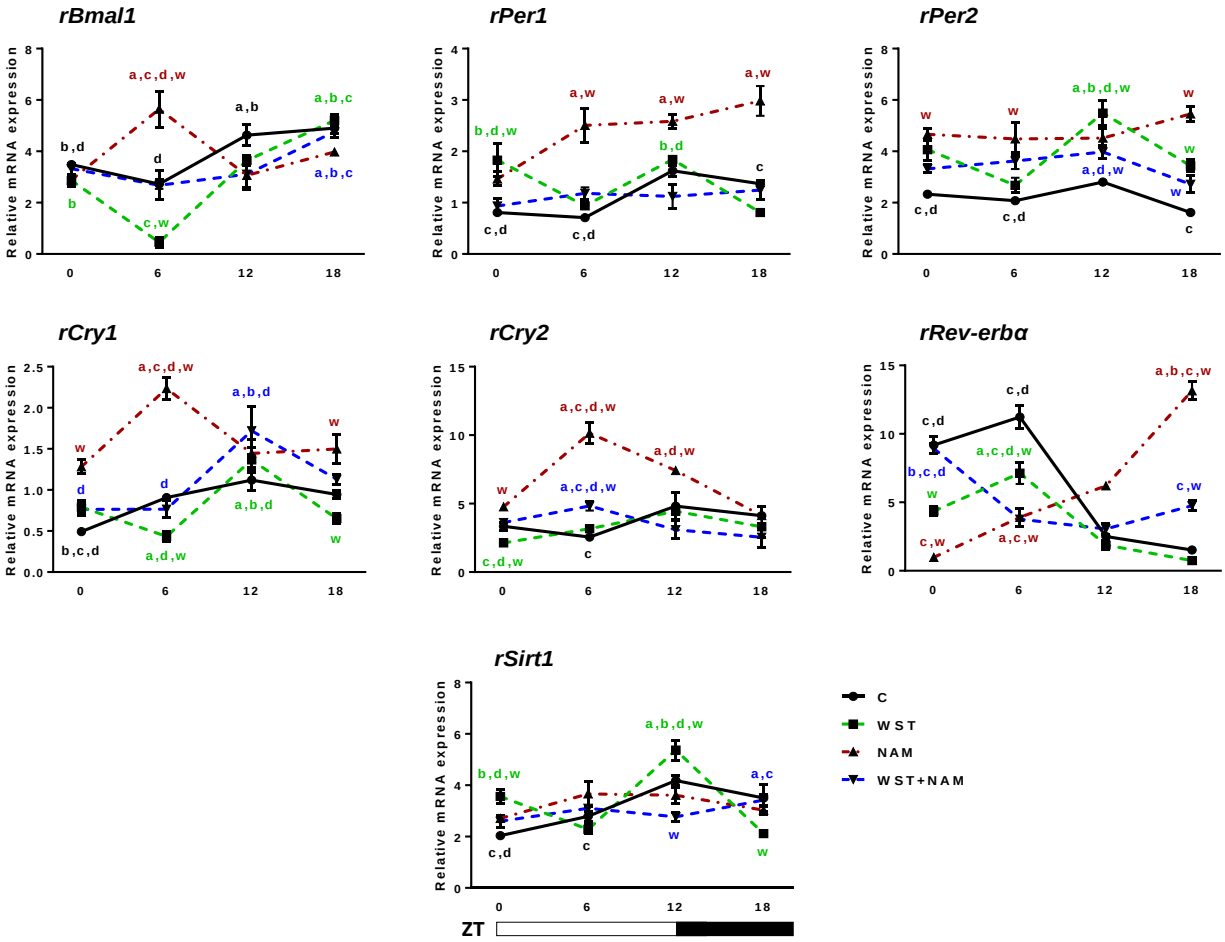


Fig. 40 Expression of *rBmal1*, *rPer1*, *rPer2*, *rCry1*, *rCry2*, *rRev-erbα* and *rSirt1* mRNA in the SCN explants. Each value is mean ± SEM (n = 4), $p < 0.05$ and expressed as relative gene expression. $p_a < 0.05$; $p_b < 0.05$, $p_c < 0.05$ and $p_d < 0.05$ (where 'a', 'b', 'c' and 'd' refers 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 control group). C – control; WST – *Withania somnifera* treated; NAM – nicotinamide treated; WST+NAM – co-treated with *Withania somnifera* and nicotinamide. Time points are in reference to the rats from which SCN were harvested.

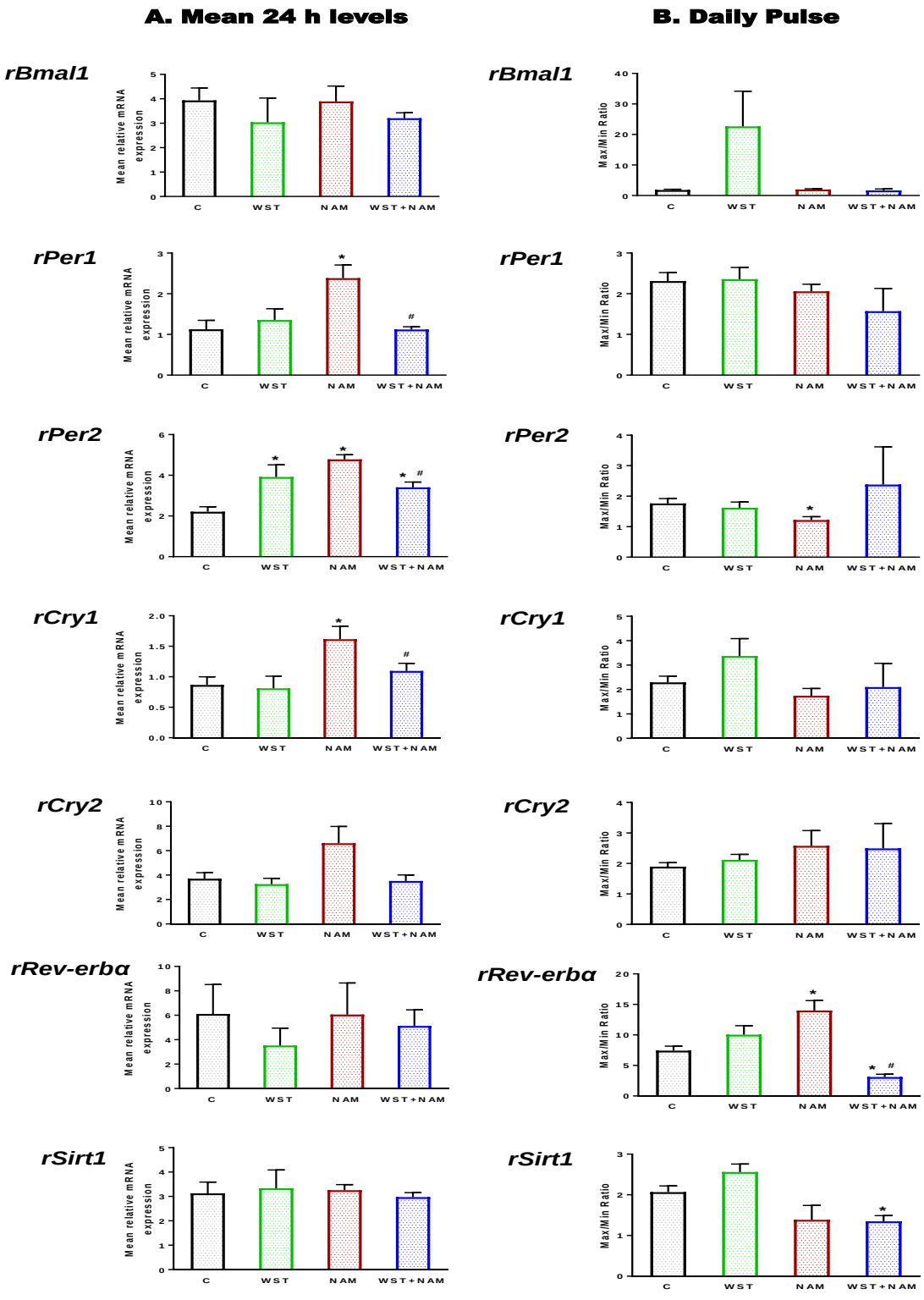


Fig. 41 (A) Mean 24 h levels and **(B)** daily pulse of *rBmal1*, *rPer1*, *rPer2*, *rCry1*, *rCry2*, *rRev-erbα* and *rSirt1* mRNA in the SCN explants. Each value is mean ± SEM, $p < 0.05$ and expressed as mean relative gene expression. '*' Indicates statistically significant difference ($p < 0.05$). C – control; WST – *Withania somnifera* treated; NAM – nicotinamide treated; WST+NAM – co-treated with *Withania somnifera* and nicotinamide.

rCry2 transcript expression was rhythmic with peak at ZT-12 and nadir at ZT-6 in both control and WST SCN. NAM resulted in phase advancement by 6 h. However, in SCN co-treated with WST+NAM, the expression was in phase with that of NAM group but the amplitude of the rhythm was similar to control (Fig. 40). Further, daily pulse and mean levels of *rCry2* did not significantly alter in any of the treatments (Fig. 41A and 41B).

rRev-erbα expression in control and WST SCN followed rhythmic pattern with maximum and minimum levels in both the groups observed at ZT-6 and ZT-18 respectively (Fig. 40). NAM treatment phase delayed this rhythm by 12 h as the peak expression shifted to ZT-18 and minimum expression to ZT-0. Interestingly, WST+NAM co-treatment phase advanced the rhythmic expression of *rRev-erbα* with peak expression at ZT-0 (Fig. 40). Though mean 24 h expression of *rRev-erbα* were not significant among different treatments and control (Fig. 41A), the 2.0 fold increase in daily pulse observed upon NAM treatment, was decreased by 4.5 folds in WST+NAM indicating partial restoration ($p < 0.05$) (Fig. 41B).

Similar to clock genes, *rSirt1* expression in control and WST SCN were in coherence with peak expression at ZT-12. Though NAM treatment abolished *rSirt1* rhythmicity, co-treatment of WST+NAM resulted in persistence of rhythm with highest expression at subjective midnight (Fig. 40). Similar to the previous observation with curcumin, mean 24 h levels and daily pulse of *rSirt1* did not change significantly in any of the experimental condition (Fig. 41A and 41B).

Overall, WST showed restoration of rhythms and phases of clock genes upon SIRT1 inhibition.

Individual values of gene expression, mean 24 h levels as well as daily pulse are tabulated in Table 7.

Table 7 Expression of *rBmal1, rPer1, rPer2, rCry1, rCry2, rRev-erbα* and *rSirt1* mRNA in the SCN explants. Role of SIRT1 in beneficial effect of *Withania somnifera*.

Gene	Experimental condition	ZT-0	ZT-6	ZT-12	ZT-18	Mean 24 h levels	Max/Min Ratio
rBmal1	Control	3.48±0.1	2.72±0.19	4.63±0.4	4.91±0.62	3.93±0.51	1.82±0.23
	WST	2.86±0.3	0.46±0.19	3.64±0.2	5.2±0.23	3.04±0.99	22.65±11.5
	NAM	2.89±0.2	5.64±0.71	3.06±0.5	3.98±0.8	3.89±0.63	1.97±0.25
	NAM+WST	3.32±0.2	2.68±0.56	3.11±0.6	4.77±0.23	3.47±0.45	1.68±0.47
rPer1	Control	0.81±0.1	0.71±0.03	1.62±0.1	1.37±0.07	1.13±0.22	2.31±0.21
	WST	1.83±0.3	0.94±0.07	1.84±0.1	0.81±0.08	1.35±0.28	2.36±0.29
	NAM	1.46±0.1	2.5±0.32	2.58±0.1	2.98±0.29	2.4±0.3 ^{wx}	2.06±0.18
	NAM+WST	0.94±0.1	1.18±0.12	1.12±0.2	1.24±0.18	1.1±0.07 ^{y}	1.57±0.56
rPer2	Control	2.33±0.1	2.08±0.06	2.81±0.1	1.61±0.08	2.21±0.25	1.76±0.16
	WST	4.07±0.4	2.67±0.3	5.49±0.5	3.44±0.23	3.92±0.6 ^{w}	1.62±0.19
	NAM	4.66±0.2	4.48±1.63	4.52±0.4	5.46±0.49	4.8±0.23 ^{w}	1.22±0.1 ^{wx}
	NAM+WST	3.32±0.2	3.82±0.32	3.78±0.6	2.72±0.73	3.4±0.3 ^{wy}	2.38±1.24
rCry1	Control	0.49±0.1	0.91±0.03	1.12±0.1	0.94±0.05	0.87±0.13	2.29±0.25
	WST	0.79±0.1	0.43±0.06	1.37±0.2	0.65±0.06	0.81±0.2	3.37±0.71
	NAM	1.28±0.1	2.23±0.43	1.44±0.3	1.5±0.38	1.6±0.2 ^{wx}	1.74±0.3
	NAM+WST	0.76±0.1	0.77±0.11	1.72±0.9	1.13±0.06	1.1±0.23 ^{y}	2.1±0.97
rCry2	Control	3.34±0.3	2.56±0.09	4.82±0.2	4.12±0.97	3.71±0.49	1.89±0.13
	WST	4.44±0.2	2.13±0.15	3.31±0.8	3.17±0.14	3.26±0.47	2.12±0.18
	NAM	4.76±0.3	10.14±1.8	7.42±0.2	4.13±0.65	6.61±1.37	2.58±0.5
	NAM+WST	3.62±0.2	4.83±0.34	3.1±0.65	2.55±0.71	3.52±0.49	2.5±0.81
rRev-erbα	Control	9.18±0.7	11.23±1.1	2.51±0.1	1.51±0.05	6.11±2.41	7.42±0.73
	WST	4.35±0.3	7.13±0.78	1.89±0.3	0.74±0.08	3.53±1.42	10.04±1.47
	NAM	0.98±0.1	3.91±0.64	6.21±0.3	13.14±0.7	6.06±2.59	14±1.64 ^{w}
	NAM+WST	8.93±0.4	3.77±0.19	3.05±0.4	4.76±0.35	5.13±1.31	3.1±0.5 ^{wxy}
rSirt1	Control	2.04±0.1	2.79±0.19	4.18±0.2	3.5±0.54	3.13±0.46	2.07±0.15
	WST	3.56±0.7	2.29±0.19	5.37±0.4	2.11±0.15	3.33±0.75	2.56±0.19
	NAM	2.71±0.1	3.66±0.9	3.61±0.3	3.03±0.47	3.25±0.23	1.4±0.35 ^{wx}
	NAM+WST	2.6±0.23	3.11±0.32	2.77±0.4	3.42±0.2	2.97±0.18	1.4±0.14 ^{wx}

Each value is mean ± SEM (n = 4), $p < 0.05$ and expressed as relative gene expression. $p_w < 0.05$; $p_x < 0.05$; $p_y < 0.05$, $p_z < 0.05$ (where 'w', 'x', 'y', 'z' refers to comparison with C, WST, NAM, WST+NAM respectively). C – control; WST – *Withania somnifera* treated; NAM – nicotinamide treated; WST+NAM – co-treated with *Withania somnifera* and nicotinamide. Time points are in reference to the rats from which SCN were harvested.

OBJECTIVE 3

a. Effect of curcumin on age induced alterations in chronomics of components of serotonin metabolism

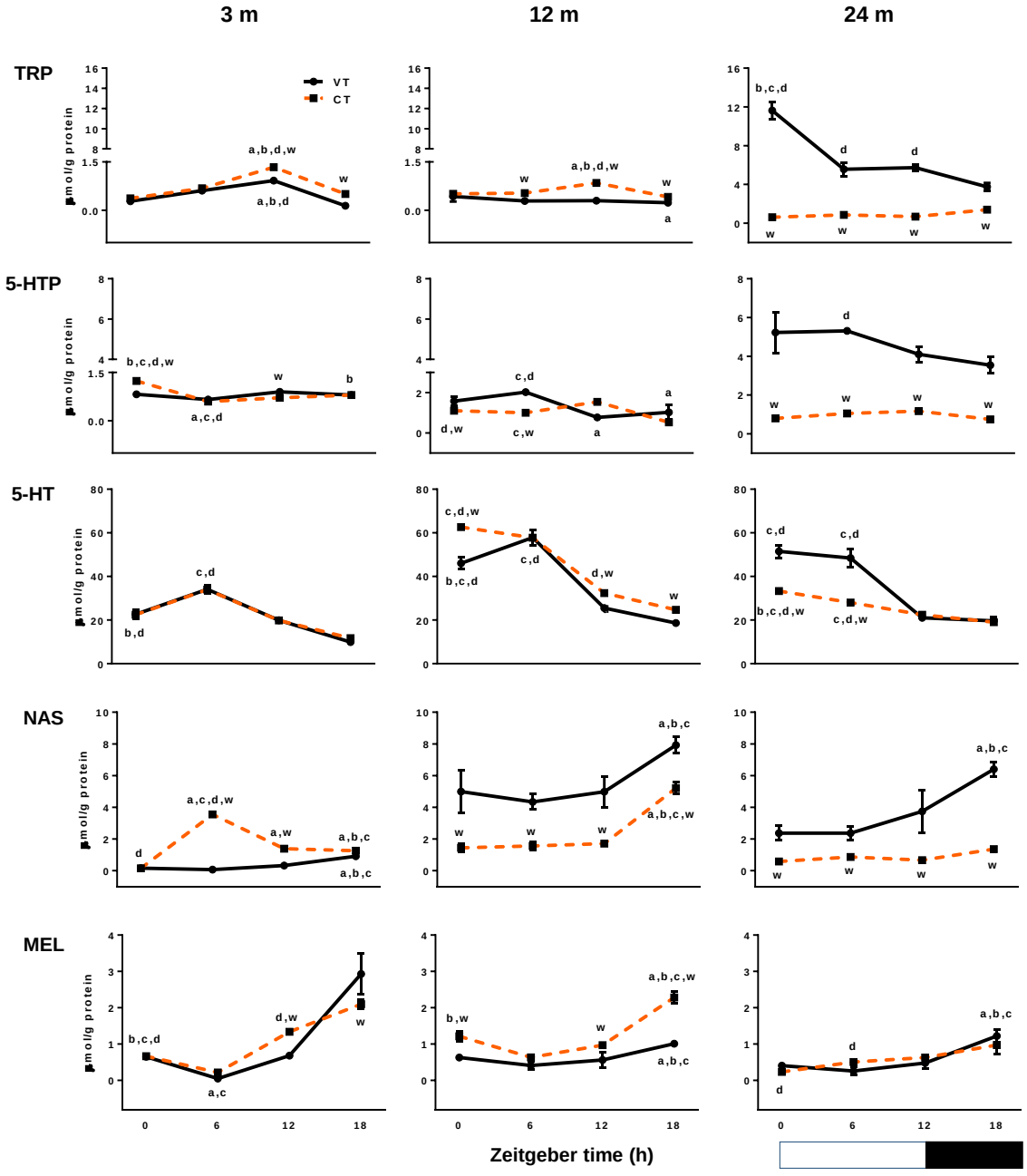
The daily rhythms and levels of components of serotonin metabolism were studied in the SCN of 3, 12 and 24 m aged rats at various time points such as ZT-0, 6, 12 and 18.

Upon curcumin treatment, **TRP** showed daily rhythmicity in young age with maximum and minimum levels at ZT-12 and ZT-0 respectively, which is similar to 3 m vehicle group. In 12 m SCN, TRP rhythms were restored upon CT with maximum levels at ZT-12. TRP daily rhythms were abolished in middle aged control group. However, in 24 m animals treated with curcumin, TRP levels significantly decreased with abolished rhythmicity (Fig. 42). The mean 24 h TRP levels were comparable to control group in the SCN of young rats administered with curcumin. Interestingly, in 12 m, curcumin significantly increased the mean levels by 14.8 folds thus bringing restoration ($p < 0.05$). On the other hand, in old aged animals, curcumin significantly decreased the mean 24 h TRP levels by 6.9 folds thereby bringing partial restoration ($p < 0.05$) (Fig. 43A). However, daily pulse of TRP remained similar to age matched VT in both 12 and 24 m, whereas decreased by 3.0 folds in 3 m with curcumin administration ($p < 0.05$) (Fig. 43B).

5-HTP daily rhythm persisted in 3 m SCN upon CT with maximum and minimum levels at ZT-0 and ZT-6 respectively, indicating 12 h advance in phase when compared to young VT group. However, curcumin restored the phase in middle age by resetting the 5-HTP peak to ZT-12. In 24 m SCN, 5-HTP rhythmicity was attenuated with significant reduction in levels at all the four time points studied in comparison to old age control group ($p < 0.05$) (Fig. 42). Mean 24 h 5-HTP levels did not vary in young and middle aged groups, however in old aged animals, curcumin restored the mean 5-HTP levels with 4.8 folds reduction in comparison to age matched vehicle controls (Fig. 43A). Similarly, daily pulse of 5-HTP in 12 and 24 m CT remained comparable to respective age matched controls. Whereas, it increased by 1.5 folds in 3 m with curcumin administration ($p < 0.05$) (Fig. 43B).

5-HT levels were rhythmic in 3 m rats treated with curcumin and the daily rhythm was in coherence with age matched vehicle controls with maximum and minimum levels at ZT-6 and ZT-18 respectively. In middle age, curcumin phase advanced serotonin rhythm by 6 h in comparison to 12 m VT with maximum levels at ZT-0. In 24 m CT SCN, 5-HT daily

rhythm was similar to age matched controls with maximum and minimum levels at ZT-0 and ZT-18 respectively (Fig. 42). 5-HT mean 24 h levels did not vary with age, curcumin increased it in middle age by 2.0 folds compared to young adults ($p < 0.05$) (Fig. 43A). Daily pulse of serotonin decreased in all the three age groups upon CT (Fig. 43B).



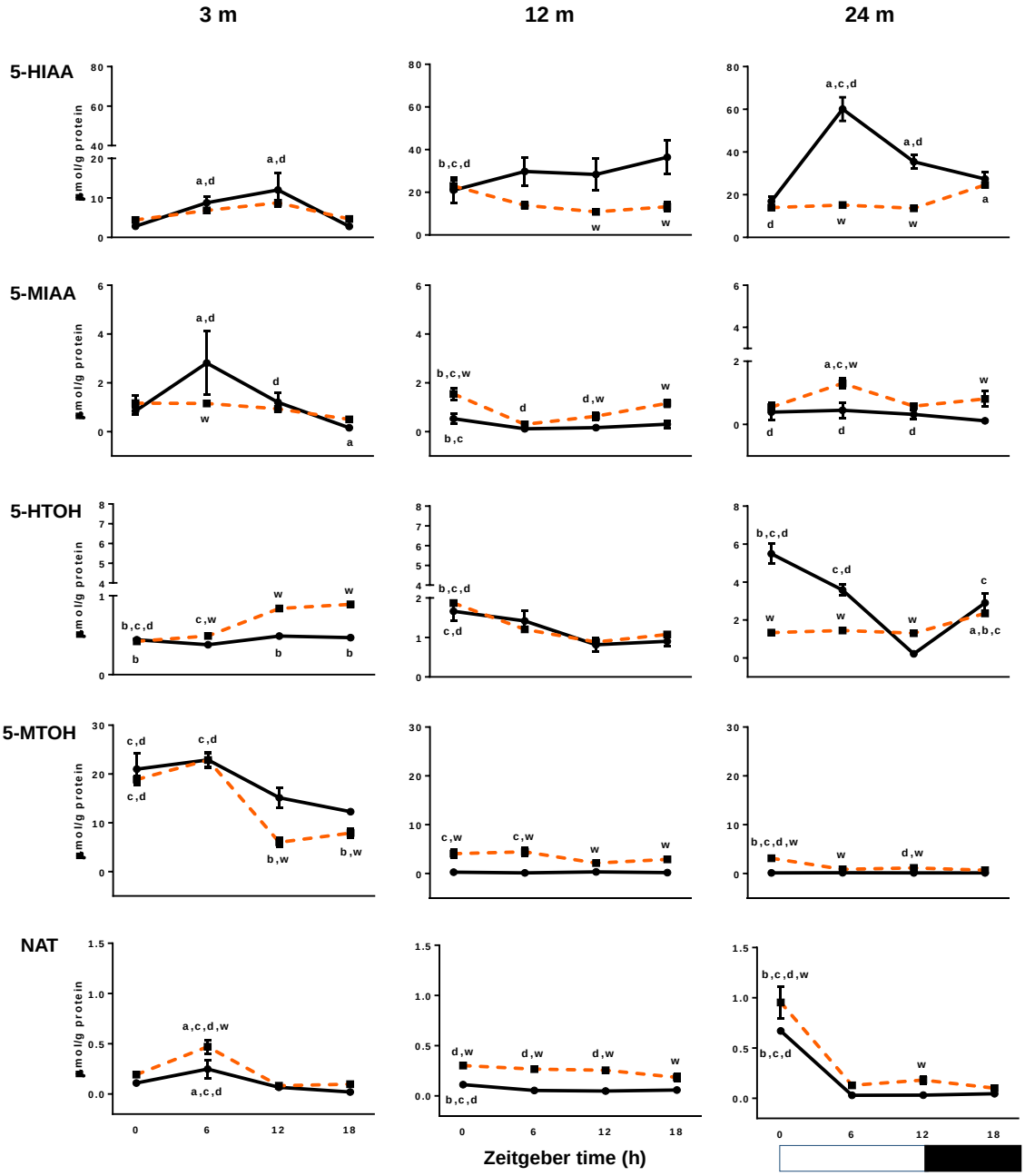


Fig. 42 Effect of curcumin administration on daily rhythms of various components of serotonin metabolism in the SCN of in 3, 12 and 24 months (m) rats. Each value is mean ± SEM (n = 6). $p_a < 0.05$; $p_b < 0.05$, $p_c < 0.05$ and $p_d < 0.05$ (where 'a', 'b', 'c' and 'd' refers to comparison with ZT-0, ZT-6, ZT- 12 and ZT-18 respectively within the group). $p_w < 0.05$ (where 'w' refers to comparison of levels at same time point in the age matched vehicle treated group).

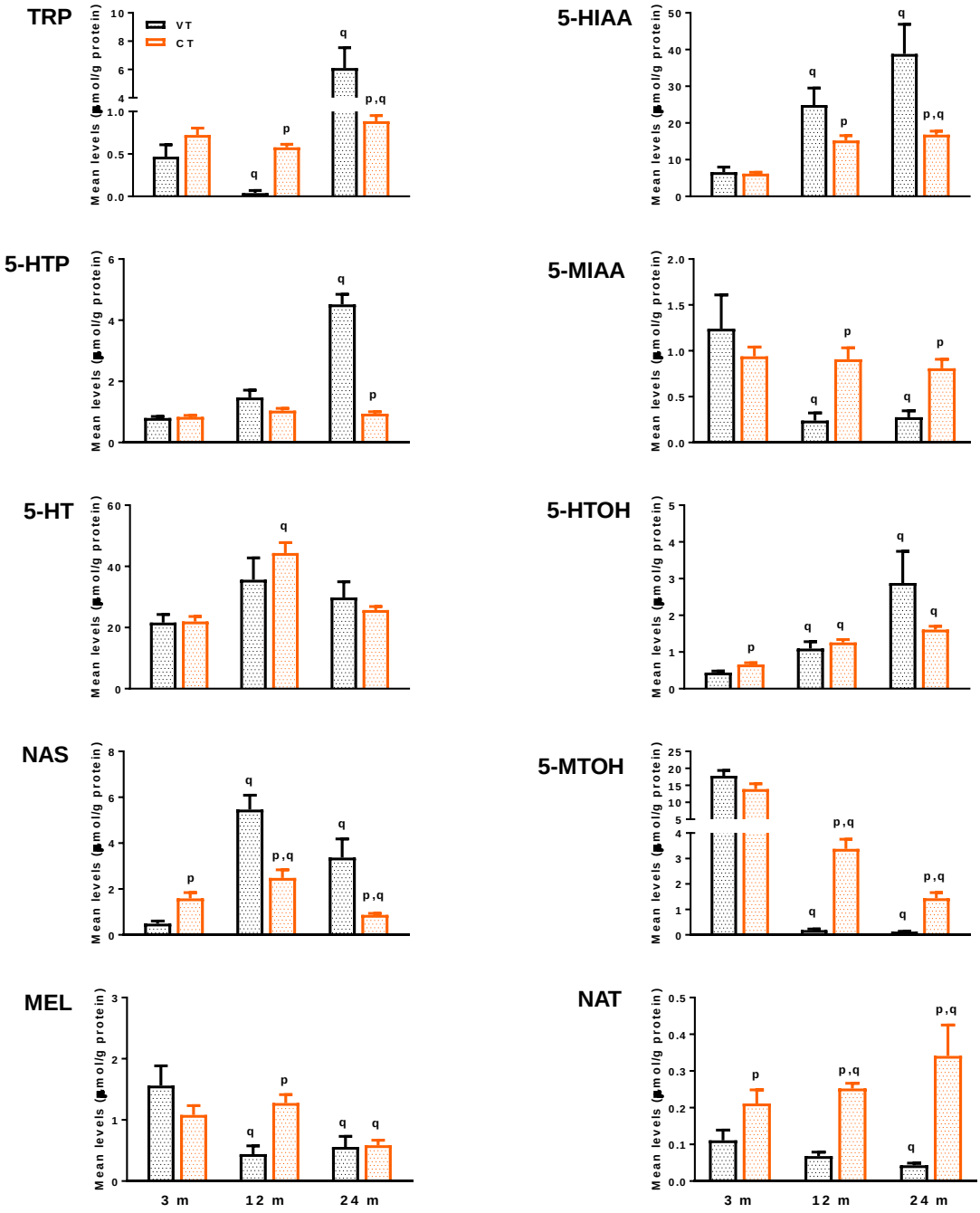


Fig. 43 (A) Effect of curcumin administration on mean 24 h levels of various components of serotonin metabolism in 3, 12 and 24 months (m) rat SCN. Each value is mean ± SEM (n = 6) $p < 0.05$. $p_p < 0.05$ (where 'p' refers to comparison with age matched vehicle group). $p_q < 0.05$ (where 'q' refers to comparison with 3 m vehicle treated group).

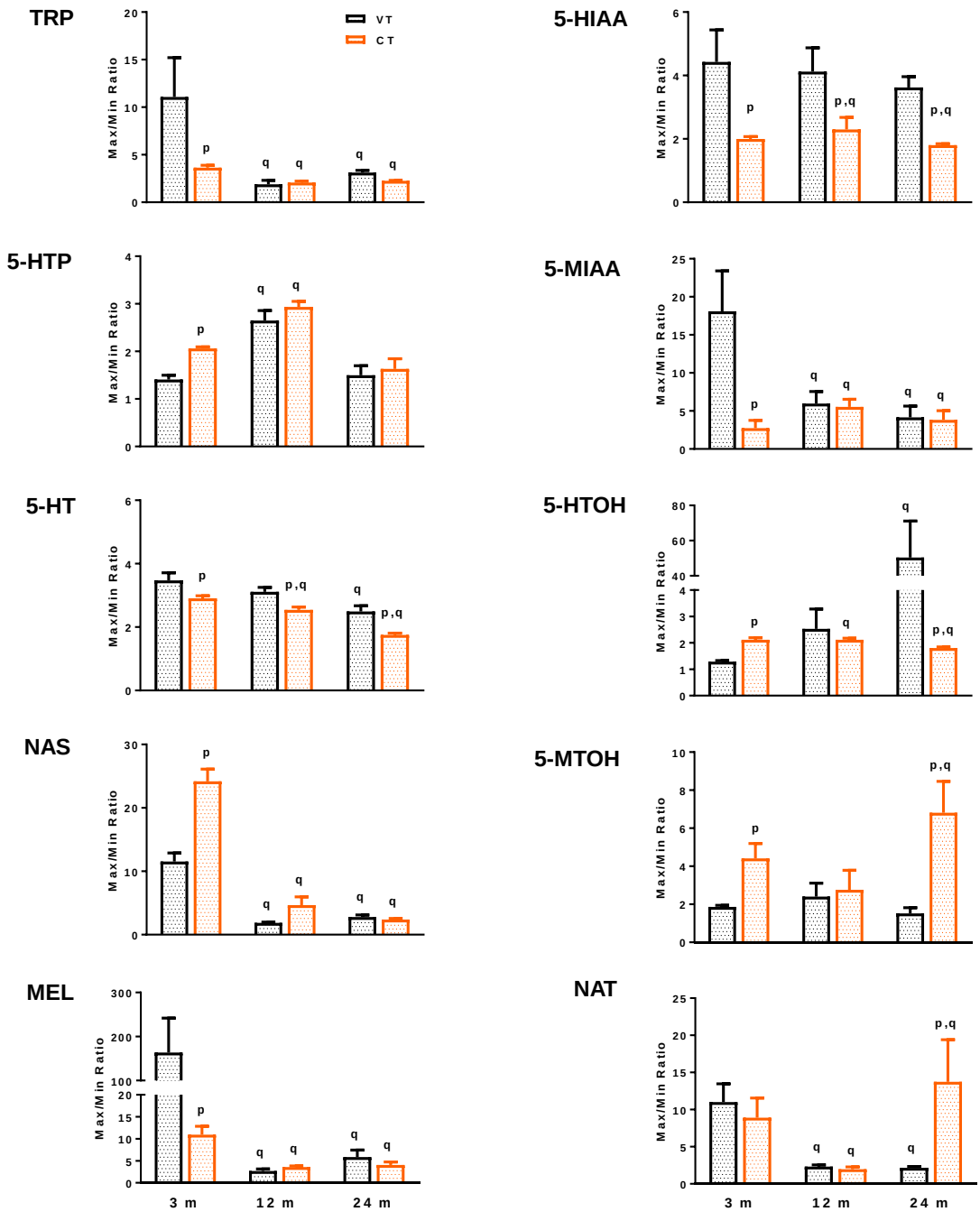


Fig. 43 (B) Effect of curcumin administration on daily pulse of various components of serotonin metabolism in 3, 12 and 24 months (m) rat SCN. Each value is maximum/minimum ratio (n = 6) $p < 0.05$. $p_p < 0.05$ (where 'p' refers to comparison with age matched vehicle group). $p_q < 0.05$ (where 'q' refers to comparison with 3 m vehicle treated group).

Daily rhythmicity of **NAS** levels in the SCN of 3 m CT rats was phase advanced by 12 h with maximum and minimum levels at ZT-6 and ZT-0 respectively. In 12 m CT, NAS daily rhythm was similar to controls with maximum levels at ZT-18. However in old age, NAS rhythmicity was attenuated with CT (Fig. 42). Aging significantly altered the mean 24 h NAS levels and curcumin increased it by 3.2 folds in young age whereas, decreased it by 2.2 folds in middle age and 3.8 folds in old aged rats ($p < 0.05$) (Fig. 43A). Daily pulse of NAS diminished with aging and curcumin did not show significant effect in 12 and 24 m rats (Fig. 43B).

Melatonin daily rhythm in 3 m rats treated with curcumin was in coherence with young control group with maximum levels at ZT-18 and minimum at ZT-6. In middle age, curcumin restored amplitude of melatonin rhythm in comparison to 12 m control with maximum levels at ZT-18. However in old age it was similar to age matched control group (Fig. 42). Mean 24 h melatonin levels decreased with age and curcumin showed restoratory effect in 12 m by bringing 2.8 fold increase. In old age however, it was similar to 24 m control (Fig. 43A). Daily pulse of melatonin also decreased with advance in age and curcumin had no significant effect in middle or old age (Fig. 43B).

5-HIAA also exhibited daily rhythmicity in its levels in the SCN of 3 m rats treated with curcumin similar to control group with maximum and minimum levels at ZT-12 and ZT-0. Curcumin restored the rhythmicity in 12 m with 12 h phase advance in comparison to young age. In old age, 5-HIAA rhythm was phase delayed by 12 h and 6 h in comparison to 24 m and 3 m vehicle groups respectively (Fig. 42). Mean 24 h 5-HIAA levels increased with advance in age and curcumin decreased it by 1.6 and 2.3 folds in middle and old age groups respectively ($p < 0.05$) (Fig. 43A). Daily pulse of 5-HIAA did not vary significantly with age however decreased in all the three age groups with CT (Fig. 43B).

5-MIAA levels did not show daily rhythmicity in 3 m CT group. With advance in age 5-MIAA rhythms were attenuated in 12 and 24 m. Curcumin restored the rhythm in 12 m and in 24 m it restored the phase of 5-MIAA daily rhythm with maximum level at ZT-6 (Fig. 42). Mean 24 h level of 5-MIAA decreased with age and curcumin restored it in both 12 and 24 m with an increase of 4.5 and 4 folds respectively ($p < 0.05$) (Fig. 43A). Daily pulse also significantly decreased with age and with curcumin administration it was similar to age matched controls in middle and old age groups (Fig. 43B).

Daily rhythmicity in **5-HTOH** levels persisted in 3 m CT rats with maximum and minimum levels at ZT-18 and ZT-0. Aging phase advanced 5-HTOH rhythm by 12 h both in middle

and old age. In 12 m CT, 5-HTOH rhythm was in coherence with vehicle control where as in 24 m, curcumin delayed the phase by 18 h and 6 h in comparison to old age and young control group respectively (Fig. 42). Mean 24 h 5-HTOH levels increased in middle and old age compared to 3 m controls and upon CT it was similar to age matched vehicle groups in both 12 and 24 m (Fig. 43A). Daily pulse of 5-HTOH increased in 24 m and curcumin decreased it by 27.8 folds indicative of a partial restoration in comparison to 3 m ($p < 0.05$) (Fig. 43B).

5-MTOH daily rhythmicity in 3 m CT group was similar to young controls with maximum and minimum levels at ZT-6 and ZT-12 respectively. With advance in age, the rhythmicity was abolished and curcumin restored the daily rhythms in both middle and old age with maximum levels at ZT-0 (Fig. 42). Aging resulted in decrease in the mean 24 h levels of 5-MTOH however, curcumin increased it by 17.0 and 10.8 folds in 12 and 24 m groups respectively ($p < 0.05$) (Fig. 43A). Though daily pulse did not alter with age, curcumin resulted in 4.5 folds increase in 24 m ($p < 0.05$) (Fig. 43B).

Daily rhythmicity of **NAT** levels in 3 m CT group was in coherence with controls with maximum and minimum at ZT-6 and ZT-18. In middle and old age also NAT daily rhythms in CT groups were similar to respective control groups with maximum and minimum levels at ZT-0 and ZT-18 (Fig. 42). Curcumin increased NAT mean levels in SCN of all three ages by 2.0, 4.2 and 8.5 folds respectively ($p < 0.05$) (Fig. 43A). NAT daily pulse decreased with age and curcumin increased it by 6.3 folds in 24 m indicating partial restoration ($p < 0.05$) (Fig. 43B).

Age induced alterations in pairwise correlations among components of serotonin metabolism

In light phase (LP) of 3 m vehicle controls there were significant ($p < 0.05$) positive correlation between TRP:NAS, TRP:5-HIAA, 5-HTP:NAS, 5-HTP:MEL, 5-HTP:5-HTOH, 5-HT:5-MIAA, 5-HT:5-MTOH, 5-HT:NAT, NAS:MEL, NAS:5-HTOH, MEL:5-HTOH, 5-MIAA:5-MTOH and 5-MIAA:NAT. Similarly, negative correlations between TRP:5-MTOH, 5-HTP:5-HT, 5-HTP:5-HTOH, 5-HTP:5-MTOH, 5-HTP:NAT, 5-HT:NAS, 5-HT:MEL, 5-HT:5-HTOH, NAS:5-MIAA, NAS:5-MTOH, NAS:NAT, MEL:5-MIAA, MEL:5-MTOH, MEL:NAT, 5-MIAA:5-HTOH, 5-HTOH:5-MTOH and 5-HTOH:NAT (Fig. 44). Whereas in dark phase (DP), positive correlations between TRP:5-HTP, TRP:5-HIAA, TRP:5-MIAA, 5-HTP:5-HIAA, 5-HTP:5-MIAA, 5-HT:5-MIAA, 5-

HT:5-MTOH, 5-HT:NAT, NAS:MEL, 5-HIAA:5-MIAA, 5-HIAA:5-HTOH, 5-MIAA:5-MTOH, 5-MIAA:NAT and 5-MTOH and NAT were observed. Negative correlations between TRP:MEL, 5-HTP:MEL, 5-HT:NAS, 5-HT:MEL, NAS:5-MIAA, NAS:5-MTOH, NAS:NAT, MEL:5-MIAA, MEL:5-MTOH, MEL:NAT and 5-HTOH:5-MTOH were significant and similar to LP (Fig. 44).

With advance in age the correlations among components of serotonin metabolism altered. In 12 m LP, significant positive correlations between TRP:MEL, TRP:5-MIAA and TRP:NAT appeared which were not present in 3 m LP. Further, 5-HTP:5-HT showed positive correlation which was significantly negative in 3 m LP. Whereas 5-HTP:NAS, 5-HTP:MEL showed negative correlations which were positive in LP of young rats. Negative correlation between 5-HT:NAS, 5-HT:MEL persisted in 12 m LP, however correlations between 5-HT:5-HTOH, 5-HT:5-MTOH became opposite in comparison to 3 m. In addition, except positive correlation between NAS:MEL, all significant correlations with NAS and other components were altered in 12 m LP. Similarly, negative correlation between MEL:5-MIAA, MEL:5-MTOH, MEL:NAT turned positive in middle age. There appeared a significant negative correlation between 5-HIAA:5-MIAA and 5-HIAA:NAT in this age group. Furthermore, significant negative correlation between 5-MIAA:5-HTOH, 5-HTOH:5-MTOH, 5-HTOH:NAT and positive correlation between 5-MIAA:5-MTOH, 5-MTOH:NAT were altered in 12 m LP (Fig. 44). Similarly in DP of 12 m, strong positive correlations between TRP:5-HT, TRP:5-HTOH, TRP:NAT, 5-HTP:5-HT, 5-HTP:5-HTOH and 5-HTP:NAT appeared which were not significant in 3 m DP. Whereas TRP:5-HIAA, TRP:5-MTOH, 5-HTP:5-HIAA, 5-HTP:5-MTOH showed significant negative correlation in 12 m DP. Though the negative correlation between 5-HT:NAS and 5-HT:MEL persisted, significant negative correlations appeared between 5-HT:5-HIAA and 5-HT:5-MTOH. Further, negative correlation between NAS:5-MTOH and MEL:5-MTOH which existed in 3 m DP became strongly positive by middle age. Similarly strong negative correlations appeared between 5-HIAA:5-HTOH, 5-HIAA:NAT, 5-MIAA:5-MTOH and 5-MTOH:NAT which were positive in 3 m DP (Fig. 44).

In LP of 24 m old rats, correlations between TRP:NAS, TRP:5-HIAA, 5-HTP:NAS, 5-HTP:MEL, NAS:5-HTOH, MEL:5-HTOH, 5-HIAA:NAT and 5-MTOH:NAT altered to negative. And correlations between TRP:NAT, 5-HTP:5-HT, 5-HTP:5-MIAA, 5-HTP:5-MTOH, 5-HTP:NAT, 5-HT:5-HTOH, 5-HIAA:5-MTOH, 5-MIAA-5-HTOH and 5-HTOH:NAT turned to positive (Fig. 44). Similarly in DP, TRP:5-HIAA, 5-HTP:5-HIAA,

NAS:5-HTOH, MEL:5-HTOH, 5-HIAA:5-MIAA, 5-HIAA:5-HTOH, 5-HIAA:5-MTOH and 5-HIAA:NAT showed strong negative correlations. Whereas TRP:5-MTOH, TRP:NAT, 5-HTP:NAT, 5-HT:5-HTOH, NAS:5-HIAA, MEL:5-HIAA and 5-HTOH:NAT showed strong positive correlations (Fig. 44).

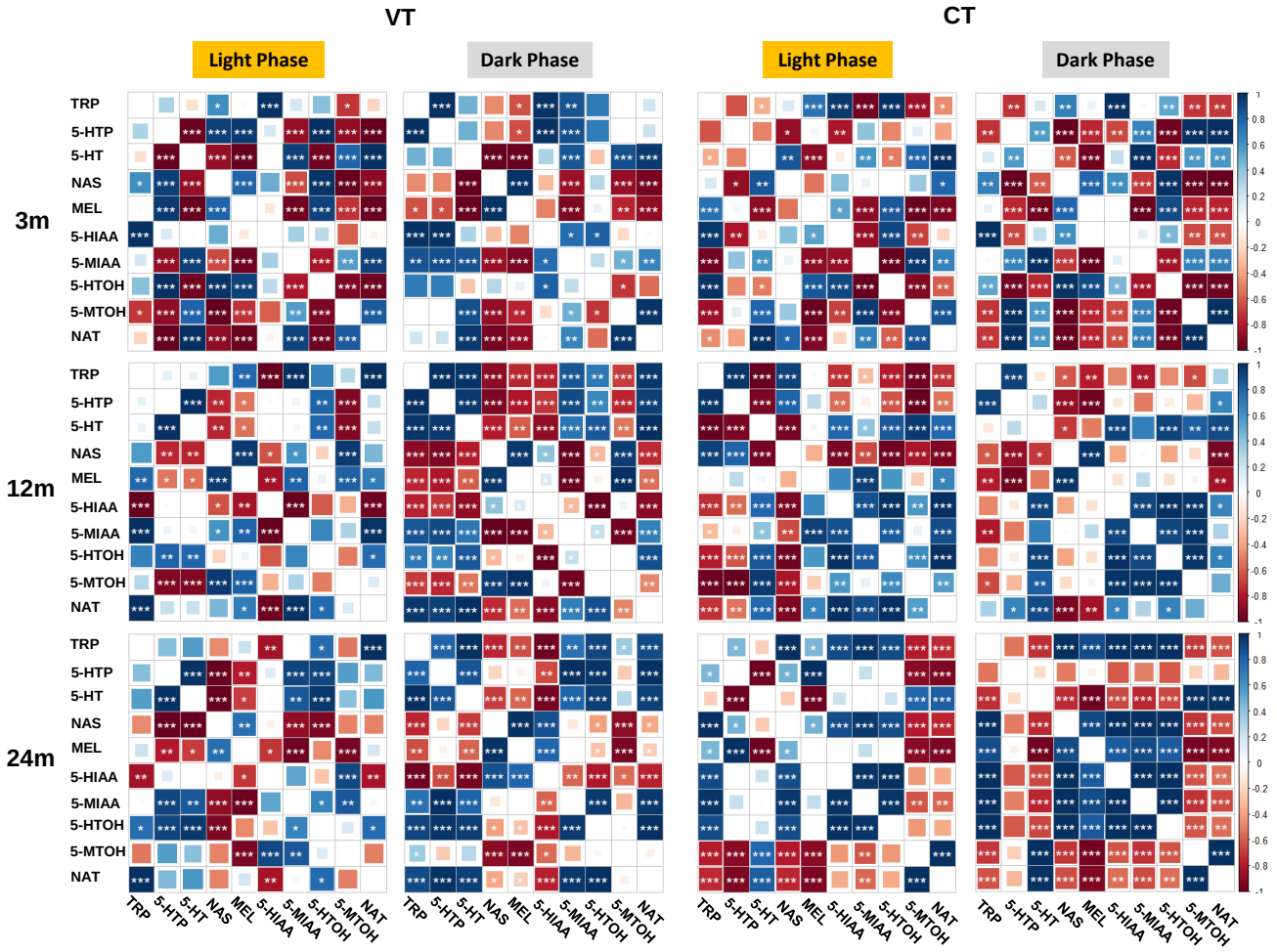


Fig. 44 Effect of curcumin administration on pair wise correlation between mean light (ZT-0, 6, 12) and mean dark (ZT-12, 18, 24/0) phase values of various components of serotonin metabolism in SCN of 3, 12 and 24 months (m) aged rats. Every colored square represents correlation coefficient values between the parameters. Where, positive correlations are indicated by shades of blue, negative correlations by shades of red and white indicates no correlation. '*' Indicates statistically significant correlations ($p < 0.05$).

Effect of curcumin on the age induced alterations in pairwise correlations among components of serotonin metabolism

Pairwise correlations in LP of 3 m CT were comparable to respective vehicle control group where, positive correlations between TRP:NAS, TRP:5-HIAA, 5-HT:5-MIAA, 5-HT:5-MTOH, 5-HT:NAT, MEL:5-HTOH, 5-MIAA:5-MTOH, 5-MIAA:NAT and 5-MTOH:NAT persisted. Similarly, negative correlations between TRP:5-MTOH, TRP:NAT, 5-HTP:5-HT, 5-HTP:5-HTOH, 5-HTP:NAT, 5-HT:MEL, 5-HT:5-HTOH, MEL:5-MIAA, MEL:5-MTOH, MEL:NAT, 5-MIAA:5-HTOH, 5-HTOH:5-MTOH and 5-HTOH:NAT were observed (Fig. 44). In DP, positive correlations between TRP:5-HIAA, TRP:5-HTOH, 5-HTP:5-HT, 5-HTP:5-HIAA, 5-HT:5-MIAA, 5-HT:5-MTOH, 5-HT:NAT, NAS:MEL, 5-HIAA:5-HTOH, 5-MIAA:5-MTOH, 5-MIAA:NAT and 5-MTOH and NAT were similar to 3 m VT LP. And negative correlations between 5-HTP:MEL, 5-HT:MEL, 5-HT:5-HTOH, NAS:5-MIAA, NAS:5-MTOH, NAS:NAT, MEL:5-MIAA, MEL:5-MTOH, MEL:NAT, 5-HTOH:5-MTOH and 5-HTOH:NAT were similar to 3 m VT DP (Fig. 44).

With curcumin administration, 12 m LP showed restorations in positive correlations between TRP:5-HTP, TRP:NAS, 5-HTP:NAS, 5-HT:5-MIAA, 5-HT:5-MTOH, 5-HT:NAT, MEL:5-HTOH, 5-HIAA:5-MIAA, 5-HIAA:5-HTOH and 5-MTOH:NAT. Similarly restorations in negative correlations between TRP:5-MTOH, TRP:NAT, 5-HTP:5-HT, 5-HTP:NAT, NAS:5-MIAA, NAS:5-MTOH and NAS:NAT were observed in LP of middle age (Fig. 44). In 12 m CT DP, positive correlations between 5-HT:5-HIAA, 5-HT:5-MTOH, 5-HIAA:5-MIAA, 5-HIAA:5-HTOH, 5-MIAA:5-MTOH and 5-MTOH:NAT were restored. Though restorations in negative correlations were limited, a weak negative correlation between NAS:5-MTOH appeared indicating correlation similar to 3 m DP (Fig. 44).

In 24 m CT LP, positive correlations between TRP:NAS, TRP:5-HIAA, 5-HTP:NAS, 5-HTP:MEL, 5-HT:5-MTOH, 5-HT:NAT, NAS:5-HIAA and NAS:5-HTOH were restored. Whereas, negative correlation between TRP:5-MTOH, TRP:NAT, 5-HTP:5-MTOH, 5-HTP:NAT, NAS:5-MTOH, NAS:NAT, MEL:NAT, 5-HIAA:5-MTOH, 5-HTOH:5-MTOH and 5-HTOH:NAT were restored and found to be similar to 3 m vehicle group in LP (Fig. 44). In 24 m DP however, CT restored positive correlations between TRP:5-HIAA, 5-HT:5-MTOH, 5-HIAA:5-MIAA, 5-HIAA:5-HTOH, 5-MTOH:NAT and

negative correlation between 5-HTP:NAS, 5-HT:5-HTOH, NAS:NAT, MEL:NAT, 5-HTOH:5-MTOH, 5-HTOH:NAT (Fig. 44).

b. Effect of *Withania somnifera* leaf extract on age induced alterations in chronomics of components of serotonin metabolism

With WS administration, **TRP** daily rhythmicity in young age was similar to 3 m VT with maximum and minimum levels at ZT-12 and ZT-0 respectively. In 12 m SCN, TRP levels were arrhythmic similar to middle aged vehicle group. In 24 m animals treated with WS, TRP daily rhythmicity was in coherence with age matched controls with maximum and minimum levels at ZT-0 and ZT-18 respectively, indicating 12 h advance in phase compared to 3 m (Fig. 45). The mean 24 h TRP levels were similar to control group in 3 m WST SCN. In 12 m, WS significantly increased the mean levels by 8.7 folds thereby restoring it in comparison to 3 m ($p < 0.05$). In old age treated with WS, mean 24 h TRP levels were similar to 24 m vehicle group (Fig. 46A). Daily pulse of TRP were similar to age matched VT in both 12 and 24 m, whereas decreased by 3.0 folds in 3 m WST group ($p < 0.05$) (Fig. 46B).

5-HTP daily rhythm persisted in 3 m SCN upon WST with maximum and minimum levels at ZT-0 and ZT-18 respectively, indicating 12 h advance in phase when compared to young controls. WS restored the phase in middle age by resetting the 5-HTP peak to ZT-12. In 24 m WST, 5-HTP rhythmicity was attenuated with significant reduction in levels at all the four time points studied in comparison to old age control group ($p < 0.05$) (Fig. 45). Mean 24 h 5-HTP levels were restored in old age with 7.7 folds reduction in comparison to 24 m VT ($p < 0.05$) (Fig. 46A). Daily pulse of 5-HTP increased in 3 in 12 m WST by 2.2 and 4.8 folds respectively ($p < 0.05$) (Fig. 46B).

5-HT daily rhythmicity was in coherence with age matched vehicle controls with maximum and minimum levels at ZT-6 and ZT-18 respectively. In middle age, WS restored the amplitude of 5-HT rhythm whereas in old age, WS restored the phase (Fig. 45). 5-HT mean 24 h levels were similar to respective vehicle treated groups upon WST (Fig. 46A). Daily pulse of serotonin decreased by 2.1 folds in 3 m and increased by 1.8 folds in 24 m with WS administration ($p < 0.05$) (Fig. 46B).

NAS daily rhythmicity was in coherence with 3 m vehicle group in young animals treated with WS with maximum levels at ZT-18. WS restored the amplitude of NAS rhythms in

both 12 and 24 m without altering the phase (Fig. 45). WS decreased the mean 24 h NAS levels in middle and old age by 12.4 and 6.5 folds respectively, thus restoring it in comparison to 3 m vehicle group ($p < 0.05$) (Fig. 46A). Daily pulse of NAS increased significantly in 12 and 24 m by 10.5 and 3.0 folds respectively with WST (Fig. 46B).

Daily rhythmicity of **Melatonin** in 3 m and 24 m WST groups were in coherence with respective age matched control groups with maximum and minimum levels at ZT-18 and ZT-6. In middle age, WS restored the amplitude of melatonin rhythm (Fig. 45). Mean 24 h melatonin levels were similar to respective age matched controls in all the age groups (Fig. 46A). Daily pulse of melatonin increased in middle age by 4.1 folds compared to 12 m control animals, indicative of partial restoration (Fig. 46B).

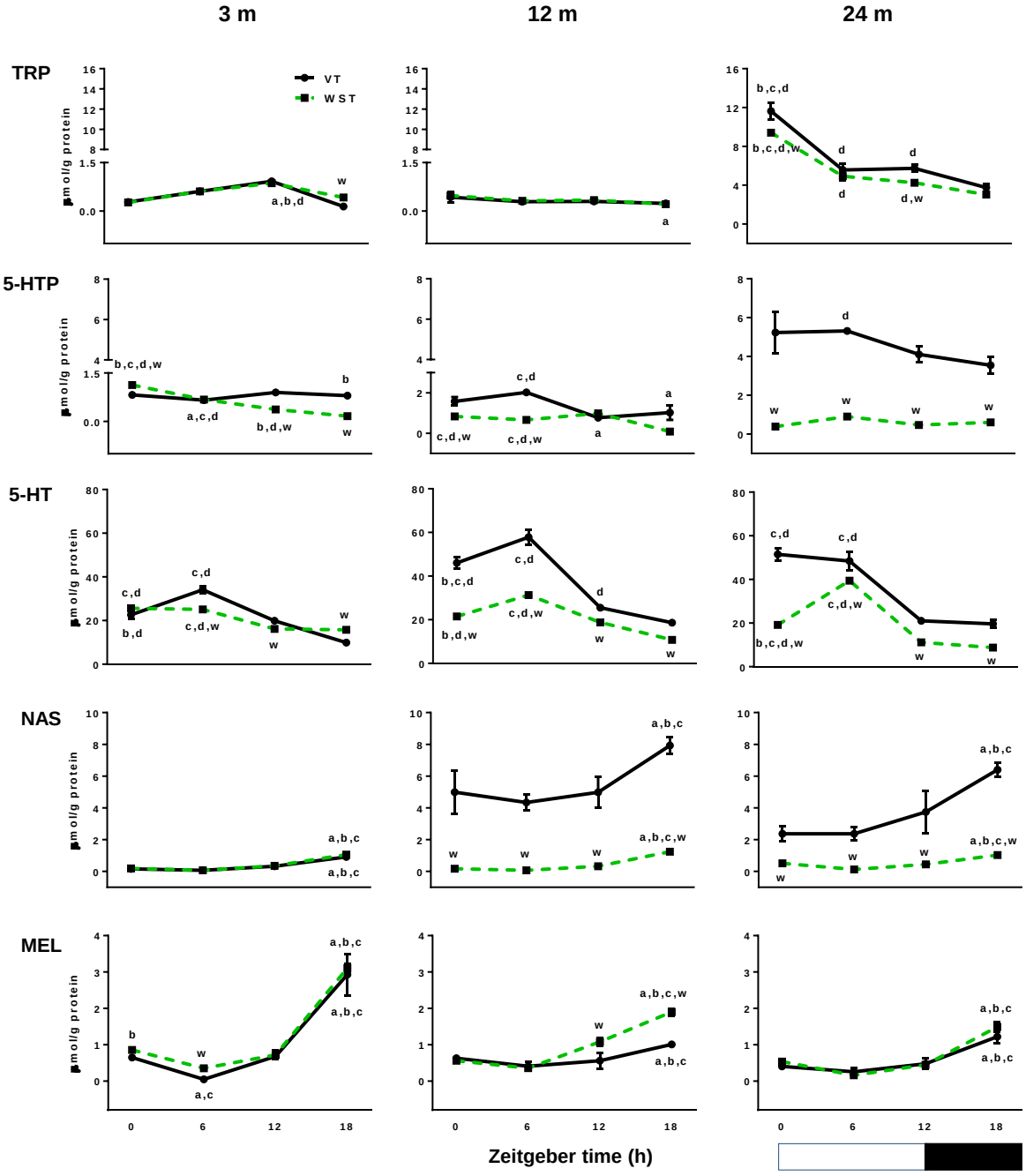
5-HIAA daily rhythm in young rats treated with WS was similar to 3 m control group with maximum and minimum levels at ZT-12 and ZT-0. WS restored the phase and amplitude in middle age with 6 h phase advance in comparison to 12 m control group. In old age, 5-HIAA rhythm was phase delayed by 12 h and 6 h in comparison to 24 m and 3 m vehicle groups respectively (Fig. 45). Mean 24 h 5-HIAA levels were restored in 12 m and in 24 m it was similar to old age vehicle group (Fig. 46A). Daily pulse of 5-HIAA did not vary significantly with age however decreased in old age with WST (Fig. 46B).

5-MIAA showed daily rhythmicity in 3 m WS group with maximum and minimum levels at ZT-0 and ZT-18 respectively. WS restored the daily rhythm in 12 m and in 24 m it restored the phase of 5-MIAA daily rhythm with maximum level at ZT-6 (Fig. 45). Mean 24 h level of 5-MIAA was restored upon WST in both 12 and 24 m with an increase of 4.4 and 3.8 folds respectively ($p < 0.05$) (Fig. 46A). Daily pulse also significantly decreased with age and with WS administration it was similar to age matched controls in middle and old age groups (Fig. 46B).

5-HTOH daily rhythm in 3 m WST was similar to control group with maximum and minimum levels at ZT-12 and ZT-6. However both in middle and old age, 5-HTOH rhythms were attenuated with WS administration (Fig. 45). Interestingly, mean 24 h levels were restored in both 12 and 24 m with WST (Fig. 46A). Daily pulse of 5-HTOH was restored in 24 m upon WS treatment ($p < 0.05$) (Fig. 46B).

5-MTOH levels in 3 m WST group was rhythmic with maximum and minimum levels at ZT-0 and ZT-12 respectively which is 6 h phase advance in comparison to 3 m VT. WS restored the daily rhythms in both middle and old age with maximum and minimum levels at ZT-0 and ZT-18 respectively (Fig. 45). Mean 24 h levels of 5-MTOH were increased by

74.6 and 84.4 folds in 12 and 24 m WST groups respectively which is indicative of restoration ($p < 0.05$) (Fig. 46A). Daily pulse increased in 12 and 24 m by 1.9 and 5.1 folds respectively in WS groups ($p < 0.05$) (Fig. 46B).



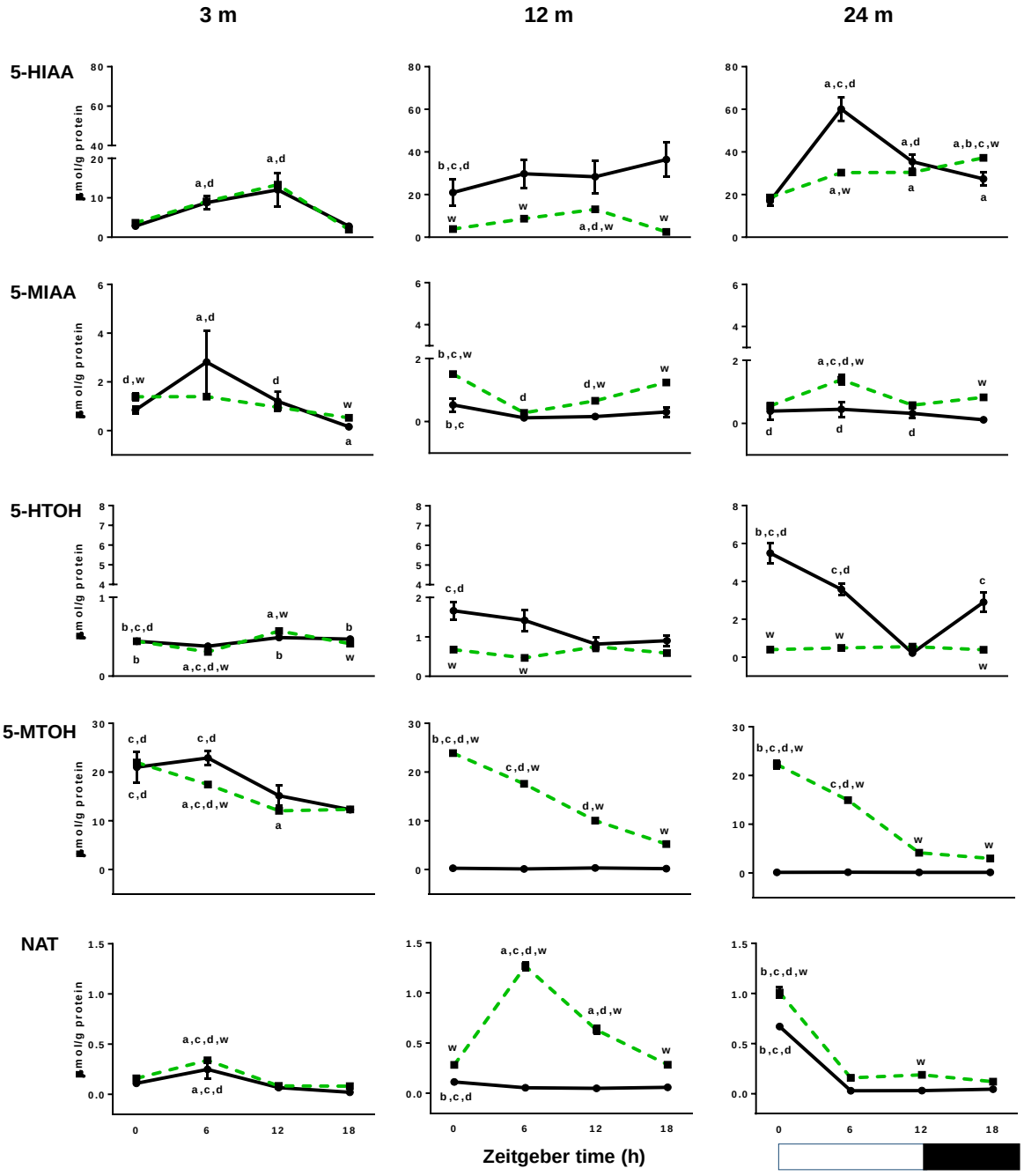


Fig. 45 Effect of *Withania Somnifera* administration on daily rhythms of various components of serotonin metabolism in the SCN of in 3, 12 and 24 months (m) rats. Each value is mean ± SEM (n = 6). $p_a < 0.05$; $p_b < 0.05$, $p_c < 0.05$ and $p_d < 0.05$ (where 'a', 'b', 'c' and 'd' refers to comparison with ZT-0, ZT-6, ZT- 12 and ZT-18 respectively within the group). $p_w < 0.05$ (where 'w' refers to comparison of levels at same time point in the age matched vehicle treated group).

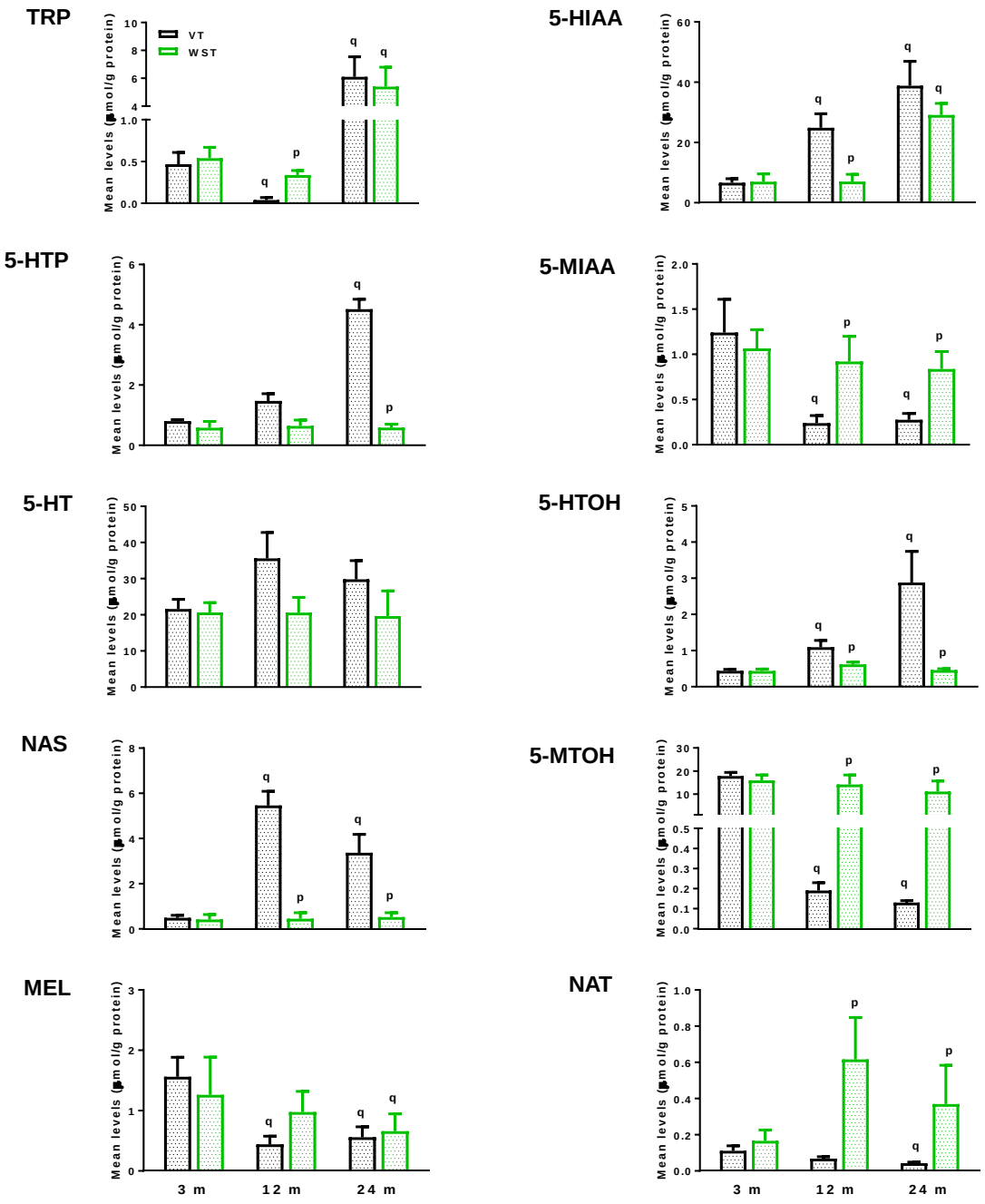


Fig. 46 (A) Effect of *Withania somnifera* administration on mean 24 h levels of the various components of serotonin metabolism in 3, 12 and 24 months (m) rat SCN. Each value is mean ± SEM (n = 6) $p < 0.05$. $p_p < 0.05$ (where 'p' refers to comparison with age matched vehicle group). $p_q < 0.05$ (where 'q' refers to comparison with 3 m vehicle treated group).

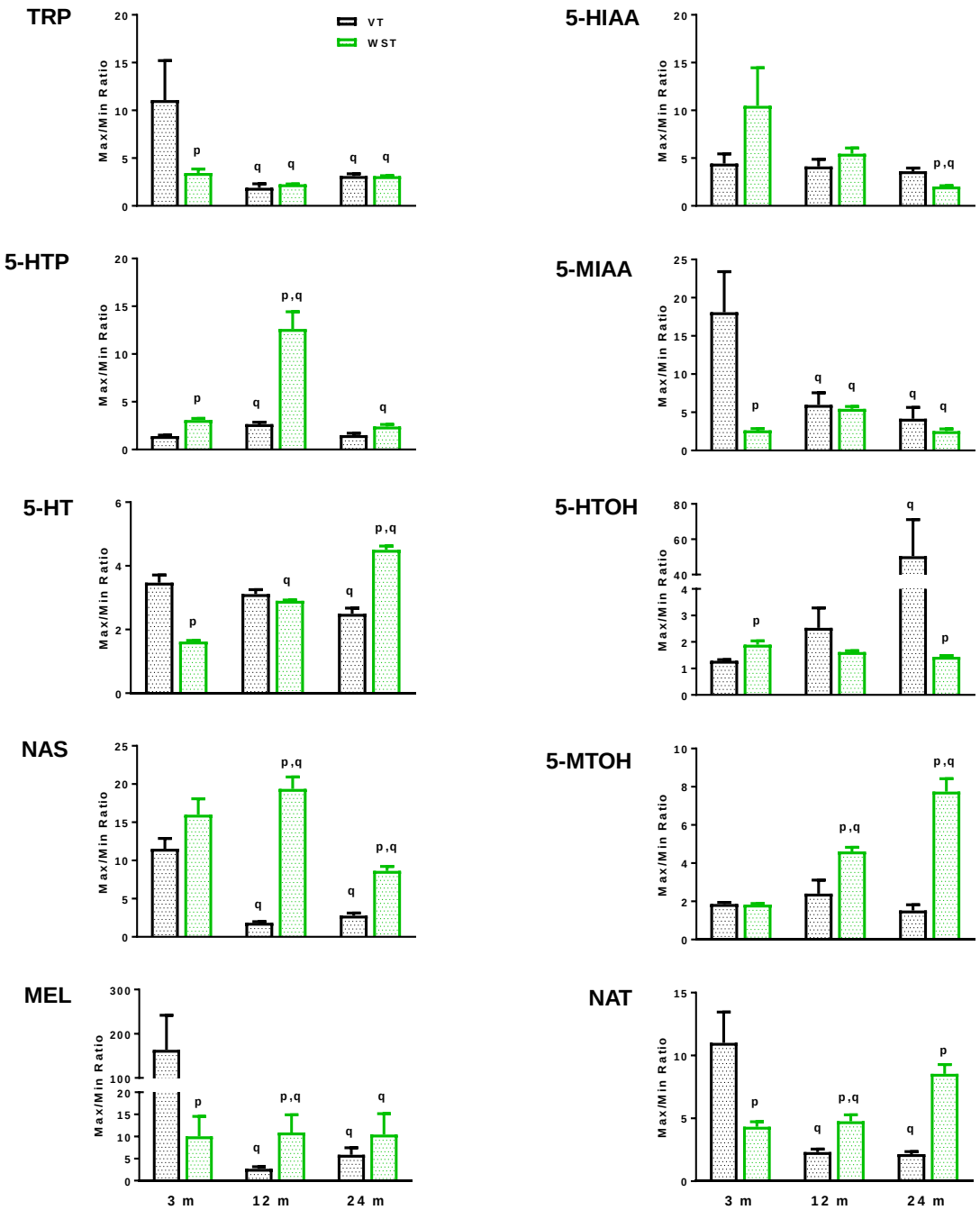


Fig. 46 (B) Effect of *Withania somnifera* administration on daily pulse of the various components of serotonin metabolism in 3, 12 and 24 months (m) rat SCN. Each value is maximum/minimum ratio (n = 6) $p < 0.05$. $p_p < 0.05$ (where 'p' refers to comparison with age matched vehicle group). $p_q < 0.05$ (where 'q' refers to comparison with 3 m vehicle treated group).

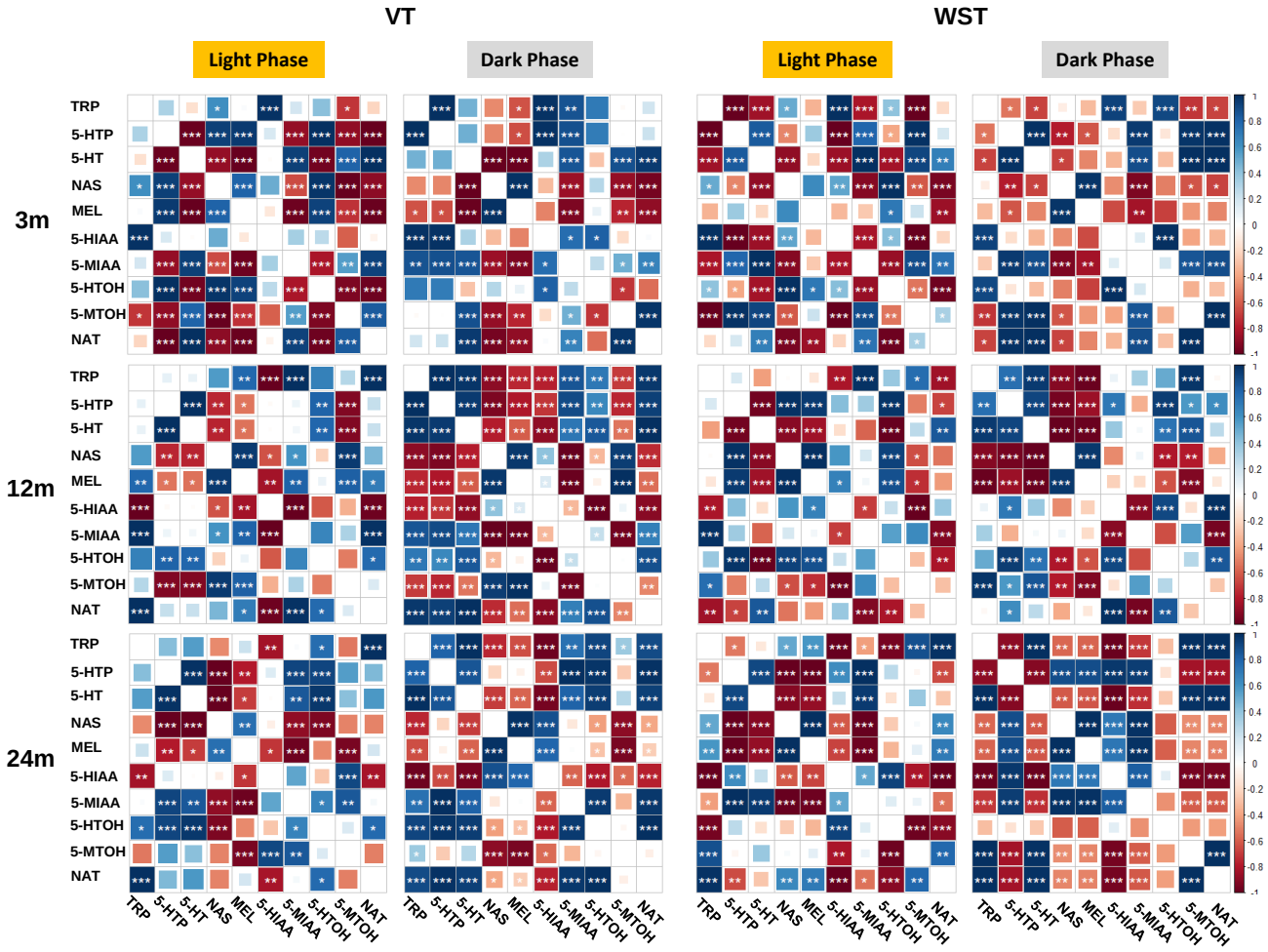


Fig. 47 Effect of *Withania somnifera* administration on pair wise correlation between mean light (ZT-0, 6, 12) and mean dark (ZT-12, 18, 24/0) phase values of various components of serotonin metabolism in SCN of 3, 12 and 24 months (m) aged rats. Every colored square represents correlation coefficient values between the parameters. Where, positive correlations are indicated by shades of blue, negative correlations by shades of red and white indicates no correlation. '*' Indicates statistically significant correlations ($p < 0.05$).

Daily rhythmicity of **NAT** in 3 m and 24 m WST groups were in coherence with age matched controls with maximum and minimum levels at ZT-6 and ZT-18 in young age. In middle age, phase of NAT daily rhythm was restored in WST (Fig. 45). WS increased the mean 24 h levels in middle and old aged rats by 10 and 7.5 folds respectively ($p < 0.05$) (Fig. 46A). NAT daily pulse was restored in 24 m upon WS administration ($p < 0.05$) (Fig. 46B).

Effect of *Withania somnifera* leaf extract on the age induced alterations in pairwise correlations among components of serotonin metabolism

With WS administration to 3 m, correlations in LP were comparable to respective vehicle control group wherein, positive correlations between TRP:NAS, TRP:5-HIAA, 5-HT:5-MIAA, 5-HT:5-MTOH, 5-HT:NAT, MEL:5-HTOH, 5-MIAA:5-MTOH, 5-MIAA:NAT and 5-MTOH:NAT persisted. Similarly, negative correlations between TRP:5-MTOH, TRP:NAT, 5-HT-NAS, 5-HT:MEL, 5-HT:5-HTOH, MEL:NAT, 5-HIAA:5-MTOH, 5-MIAA:5-HTOH, 5-HTOH:5-MTOH and 5-HTOH:NAT were observed (Fig. 47). In DP, positive correlations between TRP:5-HIAA, TRP:5-HTOH, 5-HTP:5-HT, 5-HTP:5-HIAA, 5-HT:5-MIAA, 5-HT:5-MTOH, 5-HT:NAT, NAS:MEL, 5-HIAA:5-HTOH, 5-MIAA:5-MTOH, 5-MIAA:NAT and 5-MTOH and NAT were similar to 3 m VT LP. Negative correlations between 5-HTP:NAS, 5-HTP:MEL, 5-HT:NAS, 5-HT:MEL, 5-HT:5-HTOH, NAS:5-MIAA, NAS:5-MTOH, NAS:NAT, MEL:5-MIAA, MEL:5-MTOH, MEL:NAT, 5-HTOH:5-MTOH and 5-HTOH:NAT were similar to 3 m VT DP (Fig. 47).
With WS administration, 12 m LP showed restorations in positive correlations between 5-HTP:NAS, 5-HTTP:MEL, 5-HT:NAT and 5-HIAA:5-HTOH. Similarly restorations in negative correlations between TRP:NAT, 5-HTP:5-HT, 5-HTP:NAT, 5-HT:5-HTOH, NAS:5-MTOH, NAS:NAT, MEL:5-MTOH, MEL:NAT and 5-HTOH:NAT were observed in LP of middle age (Fig. 47). In 12 m WST DP, positive correlations between 5-HTP:5-HIAA, 5-HT:5-HIAA, 5-HT:5-MTOH, 5-HIAA:5-HTOH and 5-MIAA:5-MTOH were restored. Restorations in negative correlations were observed between NAS:5-MTOH and MEL:5-MTOH in 12 m DP (Fig. 47).
In 24 m WST LP, positive correlations between TRP:NAS, 5-MTOH:NAT were restored. Whereas, negative correlation between 5-HTP:NAT, 5-HIAA:5-MTOH, 5-HTOH:5-MTOH and 5-HTOH:NAT were restored and found to be similar to 3 m vehicle group in LP (Fig. 47). In 24 m DP however, WST restored positive correlations between 5-HTP:5-HIAA, 5-HT:5-MTOH, 5-HIAA:5-MIAA, 5-MTOH:NAT and negative correlation between 5-HT:5-HTOH, 5-HTOH:5-MTOH and 5-HTOH:NAT (Fig. 47).

Levels of each component of serotonin metabolism at four time points, and their mean 24 h levels as well as daily pulse values are tabulated in Table 8 - 10.

Table 8 Age induced alterations in daily rhythms, mean 24 h levels and daily pulse of components of serotonin metabolism in the SCN.

Component	Age	ZT 0	ZT 6	ZT 12	ZT 18	Mean 24 h	Max/Min
TRP	3m	0.28±0.08	0.61±0.08	0.92±0.05	0.14±0.09	0.47±0.14	11.07±4.13
	12m	0.43±0.16	0.29±0.02	0.3±0.02	0.24±0.03	0.04±0.03 ^{x}	1.89±0.41 ^{x}
	24m	11.62±0.9	5.56±0.69	5.73±0.36	3.75±0.40	6.1±1.44 ^{xy}	3.12±0.24 ^{xy}
5-HTP	3m	0.82±0.02	0.66±0.05	0.9±0.07	0.8±0.07	0.8±0.05	1.41±0.09
	12m	1.58±0.22	2.02±0.09	0.77±0.1	1.02±0.36	1.47±0.24	2.65±0.21 ^{xz}
	24m	5.23±1.05	5.31±0.05	4.1±0.41	3.54±0.43	4.5±0.33 ^{xy}	1.5±0.2
5-HT	3m	22.6±2.04	34.1±1.73	19.9±0.5	9.9±1.13	21.6±2.67	3.47±0.24
	12m	46±2.8	57.8±3.51	25.5±1.39	18.6±0.92	35.6±7.16	3.11±0.14
	24m	51.5±2.87	48.4±4.18	21±0.61	19.6±1.75	29.81±5.16	2.49±0.18 ^{xy}
NAS	3m	0.16±0.06	0.07±0.001	0.32±0.08	0.91±0.18	0.49±0.11	11.54±1.34
	12m	4.99±1.34	4.33±0.49	4.98±0.98	7.92±0.53	5.46±0.63 ^{x}	1.85±0.14 ^{x}
	24m	2.37±0.46	2.37±0.43	3.74±1.35	6.4±0.45	3.37±0.81 ^{x}	2.79±0.32 ^{x}
MEL	3m	0.648±0.04	0.05±0.04	0.68±0.02	2.93±0.57	1.56±0.32	163.5±78.4
	12m	0.63±0.05	0.41±0.12	0.56±0.22	1.01±0.08	0.44±0.14 ^{x}	2.68±0.45 ^{x}
	24m	0.41±0.04	0.26±0.11	0.48±0.15	1.22±0.18	0.56±0.17 ^{x}	5.86±1.57 ^{x}
5-HIAA	3m	2.83±0.67	8.76±1.66	12±4.3	2.79±0.46	6.61±1.37	4.43±1.01
	12m	20.98±6.07	29.74±6.55	28.39±7.6	36.4±8.03	24.9±4.62 ^{x}	4.12±0.75
	24m	16.88±2.22	60.1±5.56	35.4±3.3	27.37±3.3	38.81±8.1 ^{x}	3.62±0.34
5-MIAA	3m	0.845±0.16	2.81±1.3	1.2±0.4	0.16±0.03	1.24±0.37	18.09±5.31
	12m	0.53±0.21	0.12±0.03	0.16±0.08	0.3±0.15	0.24±0.08 ^{x}	5.97±1.58 ^{x}
	24m	0.39±0.26	0.45±0.24	0.32±0.15	0.12±0.03	0.28±0.07 ^{x}	4.15±1.48 ^{x}
5-HTOH	3m	0.44±0.01	0.38±0.02	0.49±0.02	0.47±0.02	0.44±0.04	1.29±0.04
	12m	1.66±0.83	1.42±0.27	0.82±0.17	0.9±0.13	1.09±0.19 ^{x}	2.53±0.75
	24m	5.49±0.54	3.58±0.3	0.22±0.15	2.9±0.51	2.88±0.86 ^{x}	50.4±20.7 ^{xy}
5-MTOH	3m	20.99±3.2	22.89±1.4	15.15±2.07	12.27±0.5	17.82±1.55	1.87±0.08
	12m	0.25±0.12	0.12±0.17	0.31±0.19	0.18±0.09	0.19±0.04 ^{x}	2.4±0.71
	24m	0.12±0.02	0.15±0.04	0.12±0.03	0.13±0.01	0.13±0.01 ^{x}	1.52±0.3
NAT	3m	0.11±0.02	0.25±0.09	0.07±0.001	0.02±0.002	0.11±0.03	11±2.46
	12m	0.11±0.02	0.05±0.01	0.05±0.001	0.06±0.01	0.07±0.01	2.32±0.24 ^{x}
	24m	0.67±0.01	0.03±0.001	0.03±0.01	0.05±0.01	0.04±0.01 ^{x}	2.16±0.19 ^{x}

Each value is mean ± SEM (n = 6). $p_x < 0.05$; $p_y < 0.05$, $p_z < 0.05$ (where 'x', 'y', 'z' refers to comparison with 3 m, 12 m, 24 m respectively).

Table 9 Effect of curcumin on age induced alterations in daily rhythms, mean 24 h levels and daily pulse of components of serotonin metabolism in the SCN.

Component	Age	ZT 0	ZT 6	ZT 12	ZT 18	Mean 24h	Max/Min
TRP	3m	0.37±0.01	0.68±0.02	1.34±0.08	0.51±0.02	0.72±0.08	3.64±0.26
	12m	0.51±0.025	0.53±0.02	0.85±0.02	0.42±0.03	0.58±0.04	2.08±0.14 ^{x}
	24m	0.62±0.02	0.85±0.03	0.68±0.05	1.39±0.02	0.89±0.07 ^{xy}	2.27±0.04 ^{x}
5-HTP	3m	1.23±0.02	0.59±0.003	0.71±0.01	0.79±0.01	0.84±0.05	2.06±0.03
	12m	1.10±0.03	0.99±0.04	1.54±0.06	0.52±0.01	1.04±0.08	2.93±0.12
	24m	0.79±0.05	1.05±0.16	1.17±0.12	0.75±0.06	0.94±0.06	1.63±0.21 ^{y}
5-HT	3m	22.41±0.22	33.94±0.51	19.85±0.23	11.69±0.23	21.97±1.67	2.91±0.08
	12m	62.62±0.88	57.74±0.67	32.39±1	24.72±0.61	44.4±3.4 ^{xz}	2.54±0.09
	24m	33.3±0.82	28.04±0.64	22.43±0.45	19.06±0.65	25.71±1.17	1.8±0.05 ^{xy}
NAS	3m	0.15±0.02	3.55±0.07	1.4±0.07	1.26±0.05	1.58±0.26	24.19±1.91
	12m	1.44±0.25	1.56±0.25	1.71±0.19	5.21±0.36	2.5±0.35 ^{x}	4.65±1.3 ^{x}
	24m	0.58±0.05	0.86±0.02	0.66±0.03	1.36±0.08	0.87±0.07 ^{xy}	2.38±0.2 ^{xy}
MEL	3m	0.67±0.05	0.22±0.04	1.34±0.02	2.09±0.13	1.08±0.15	10.95±1.91
	12m	1.21±0.13	0.63±0.02	0.97±0.06	2.28±0.16	1.28±0.14	3.6±0.23 ^{x}
	24m	0.23±0.05	0.51±0.05	0.63±0.06	0.97±0.24	0.59±0.08 ^{xy}	4.1±0.69 ^{x}
5-HIAA	3m	4.41±0.13	6.85±0.21	8.77±0.21	4.65±0.14	6.17±0.38	1.99±0.08
	12m	22.98±2.58	13.92±1.62	10.81±1.42	13.25±2.14	15.24±1.3 ^{x}	2.3±0.38
	24m	13.97±0.14	15.15±0.19	13.67±0.6	24.54±0.65	16.8±0.96 ^{x}	1.8±0.04
5-MIAA	3m	1.17±0.33	1.15±0.1	0.93±0.12	0.5±0.07	0.94±0.1	2.74±1.03
	12m	1.54±0.23	0.3±0.04	0.63±0.16	1.16±0.15	0.91±0.12	5.53±1.01 ^{x}
	24m	0.55±0.14	1.3±0.16	0.58±0.08	0.81±0.24	0.81±0.1 ^{xy}	3.81±1.21
5-HTOH	3m	0.42±0.01	0.49±0.01	0.84±0.02	0.89±0.03	0.66±0.04	2.11±0.08
	12m	1.87±0.04	1.2±0.05	0.88±0.01	1.08±0.06	1.26±0.08 ^{x}	2.12±0.06
	24m	1.34±0.01	1.45±0.02	1.31±0.06	2.35±0.06	1.61±0.09 ^{x}	1.8±0.04
5-MTOH	3m	18.86±0.74	22.88±1.6	6.01±0.99	7.91±0.96	13.92±1.58	4.41±0.78
	12m	4.04±0.91	4.42±0.81	2.14±0.39	2.9±0.61	3.38±0.38 ^{x}	2.76±1.03 ^{x}
	24m	3.12±0.28	0.84±0.13	1.13±0.1	0.67±0.19	1.44±0.22 ^{xy}	6.8±1.65 ^{xy}
NAT	3m	0.19±0.02	0.47±0.07	0.08±0.03	0.1±0.02	0.21±0.04	8.9±2.66
	12m	0.3±0.003	0.3±0.03	0.26±0.01	0.18±0.04	0.25±0.01	1.96±0.3 ^{x}
	24m	0.95±0.16	0.13±0.03	0.18±0.04	0.1±0.02	0.34±0.08	13.7±5.7 ^{xy}

Each value is mean ± SEM (n = 6). $p_x < 0.05$; $p_y < 0.05$, $p_z < 0.05$ (where 'x', 'y', 'z' refers to comparison with 3 m, 12 m, 24 m respectively).

Table 10 Effect of *Withania somnifera* on age induced alterations in daily rhythms, mean 24 h levels and daily pulse of components of serotonin metabolism in the SCN.

Component	Age	ZT 0	ZT 6	ZT 12	ZT 18	Mean 24h	Max/Min
TRP	3m	0.27±0.02	0.61±0.03	0.87±0.01	0.42±0.002	0.54±0.13	3.43±0.43
	12m	0.48±0.01	0.32±0.005	0.34±0.002	0.21±0.001	0.34±0.05	2.25±0.03 ^{x}
	24m	9.41±0.13	4.93±0.39	4.25±0.04	3.03±0.05	5.4±1.39 ^{xy}	3.11±0.05
5-HTP	3m	1.13±0.10	0.68±0.06	0.37±0.03	0.16±0.01	0.58±0.21	3.08±0.17
	12m	0.84±0.06	0.66±0.04	0.99±0.04	0.09±0.01	0.64±0.19	12.63±1.8 ^{xz}
	24m	0.38±0.02	0.90±0.05	0.47±0.03	0.59±0.07	0.59±0.11	2.40±0.22
5-HT	3m	25.60±0.61	25.07±0.36	16.20±0.30	15.79±0.25	20.67±2.69	1.62±0.04
	12m	21.52±0.37	31.31±0.5	18.74±0.19	10.78±0.15	20.59±4.24	2.90±0.02 ^{xz}
	24m	19.19±0.44	39.41±1.22	11.2±0.19	8.75±0.1	19.64±6.9	4.5±0.12 ^{x}
NAS	3m	0.19±0.01	0.07±0.01	0.35±0.02	1.06±0.06	0.42±0.22	15.98±2.08
	12m	0.17±0.01	0.06±0.01	0.33±0.02	1.24±0.09	0.45±0.27	19.4±1.6 ^{xz}
	24m	0.51±0.05	0.12±0.01	0.45±0.02	1.03±0.05	0.53±0.19	8.64±0.58 ^{x}
MEL	3m	0.86±0.09	0.35±0.05	0.72±0.12	3.11±0.12	1.26±0.62	10.02±4.51
	12m	0.56±0.07	0.36±0.07	1.08±0.12	1.9±0.1	0.98±0.34	10.89±3.99
	24m	0.54±0.05	0.16±0.02	0.45±0.02	1.49±0.13	0.66±0.29 ^{x}	10.43±4.7
5-HIAA	3m	3.65±0.34	9.06±0.59	13.32±0.5	1.91±0.4	6.99±2.6	10.47±3.99
	12m	3.83±0.54	8.71±0.55	13.08±0.71	2.51±0.23	7.03±2.42	5.45±0.61 ^{x}
	24m	18.6±0.67	30.29±1.28	30.41±0.76	37.22±0.6	29.1±3.9 ^{xy}	2.02±0.09 ^{xy}
5-MIAA	3m	1.39±0.15	1.39±0.13	0.95±0.03	0.53±0.02	1.06±0.21	2.63±0.23
	12m	1.51±0.08	0.28±0.01	0.66±0.01	1.24±0.06	0.92±0.28	5.45±0.32 ^{x}
	24m	0.56±0.03	1.39±0.14	0.58±0.04	0.83±0.04	0.84±0.19	2.53±0.3
5-HTOH	3m	0.44±0.02	0.31±0.02	0.57±0.01	0.41±0.01	0.43±0.05	1.9±0.14
	12m	0.68±0.01	0.47±0.02	0.75±0.02	0.59±0.01	0.62±0.06 ^{x}	1.62±0.05
	24m	0.39±0.01	0.49±0.01	0.56±0.01	0.39±0.01	0.46±0.04	1.44±0.05 ^{x}
5-MTOH	3m	21.97±0.23	17.43±0.38	12.04±0.38	12.34±0.33	15.94±2.36	1.83±0.06
	12m	23.89±0.68	17.58±0.41	10.03±0.59	5.24±0.31	14.18±4.11	4.61±0.21 ^{x}
	24m	22.24±0.84	14.94±0.22	4.16±0.19	2.96±0.23	11.08±4.59	7.7±0.68 ^{xy}
NAT	3m	0.16±0.01	0.34±0.01	0.08±0.01	0.08±0.01	0.17±0.06	4.33±0.41
	12m	0.28±0.03	1.27±0.04	0.63±0.04	0.28±0.03	0.62±0.23 ^{x}	4.77±0.51
	24m	1.01±0.05	0.16±0.01	0.19±0.01	0.12±0.01	0.37±0.21	8.5±0.74 ^{xy}

Each value is mean ± SEM (n = 6). $p_x < 0.05$; $p_y < 0.05$, $p_z < 0.05$ (where 'x', 'y', 'z' refers to comparison with 3 m, 12 m, 24 m respectively).

OBJECTIVE 4

a. Age induced alterations in Melatonin Receptor expression in the SCN of male Wistar rats

***Melatonin receptor transcript expression*:** MT1 receptor transcripts have shown robust rhythmic expression in the SCN of 3 m animals with peak expression at ZT-18 and nadir at ZT-0. Similar to 3 m, in middle aged animals MT1 expression was maximum at ZT-18 however, minimum was at ZT-6. In 24 m animals, MT1 showed daily rhythm with peak expression at ZT-0 and nadir at ZT-12 thus showing a phase advance of 18 h in comparison to 3 m control group (Fig. 48A). Mean 24 h levels of MT1 mRNA showed a significant decrease of 1.9 folds in 12 m SCN in comparison to 3 m ($p < 0.05$). Interestingly, in old aged rats the mean 24 h levels were similar to that of young animals (Fig. 48B). The daily pulse of MT1 decreased by 2.5 folds in middle age and 1.6 folds in old age respectively in comparison to young adults ($p < 0.05$) (Fig. 48C).

Similarly, MT2 receptor transcripts showed daily rhythmicity in expression levels in 3 m SCN. The maximum and minimum expression time points in 3 m were at ZT-12 and ZT-0 respectively. In 12 m animals, in addition to decreased robustness of the daily rhythm, there was a 6 h phase delay in MT2 expression in comparison to young group with peak at ZT-18 and nadir at ZT-6. In old aged animals, MT2 showed peak expression at ZT-0 and nadir at ZT-18 thus showing a phase advance of 18 h in comparison to 3 m (Fig. 48A). Mean 24 h levels of MT2 mRNA decreased by significant 2.0 folds from 3 m to 12 m ($p < 0.05$). But change in mean 24 h levels in 24 m group was not significant compared to young animals (Fig. 48B). Aging resulted in altered daily pulse of MT2 as it decreased by 2.8 folds and 1.5 folds in 12 m and 24 m respectively when compared to 3 m group ($p < 0.05$) (Fig. 48C).

Melatonin Receptor protein expression: Western blot analysis revealed that melatonin receptor MT1 shows rhythmic expression pattern in the SCN of 3 m aged rats. The maximum and minimum expression were found at ZT-12 and ZT-6 respectively. Upon aging daily rhythms of MT1 receptor expression were found to be abolished both in 12 and 24 m rat SCN (Fig. 49A, 49B). However, mean 24 h levels of MT1 did not vary upon aging (in 12 and 24 m) when compared to young adults (3m) (Fig. 49C). Similarly, the daily pulse of MT1 expression remained same across the age groups (Fig. 49D).

Interestingly, MT2 expression also followed a rhythmic pattern very similar to MT1 with respective maximum and minimum expression at ZT-12 and ZT-6. Aging significantly affected the MT2 expression resulting in abolition of daily rhythms both in 12 and 24 m groups (Fig. 49A, 49B). The mean 24 h levels of MT2 did not vary significantly from 3 m to 12 m, but reduced by significant 1.5 folds in 24 m rats compared to young controls ($p < 0.05$) (Fig. 49C). However, the daily pulse of MT2 was observed to be consistent in all the three age groups similar to MT1 (Fig. 49D).

b. Effect of curcumin on age induced alterations in Melatonin Receptor expression in the SCN of male Wistar rats

***Melatonin receptor transcript expression*:** Curcumin administration to 3 m animals resulted in decreased robustness in MT1 expression, however the maximum and minimum expressions were at ZT-18 and ZT-6 respectively which is in phase with 3 m control group. In 12 m animals the maximum expression was at ZT-18 and minimum was at ZT-6 which is similar to age matched and 3 m control groups. Interestingly in 24 m, curcumin resulted in 12 h phase delay in MT1 expression thus showing partial restoration (Fig. 48A). Administration of curcumin significantly reduced the mean 24 h levels by 3.3 folds in 3 m. In 12 m, there was 2.3 and 4.5 folds decrease in comparison to 3 m and age matched control animals respectively. But in 24 m animals, curcumin increased the mean 24 h levels by a significant 2.3 folds in comparison to both age matched as well as 3 m controls ($p < 0.05$) (Fig. 48B). Daily pulse of MT1 upon curcumin administration to 3 m showed 2.2 folds decrease. But in 12 m showed a partial restoration as it increased by 1.5 folds compared to age matched VT. Interestingly, in old age, curcumin increased the daily pulse by a significant 6.7 and 4.2 folds in comparison to age matched as well as 3 m control group respectively ($p < 0.05$) (Fig. 48C).

In 3 m animals treated with curcumin, though robustness of MT2 daily rhythm was decreased, respective maximum and minimum expressions were found at ZT-18 and ZT-6, indicating 6 h phase delay in comparison to 3 m controls. Curcumin advanced the phase of MT2 in 12 m by 6 h and 12 h respectively in comparison to young and middle aged control groups (Fig. 48A).

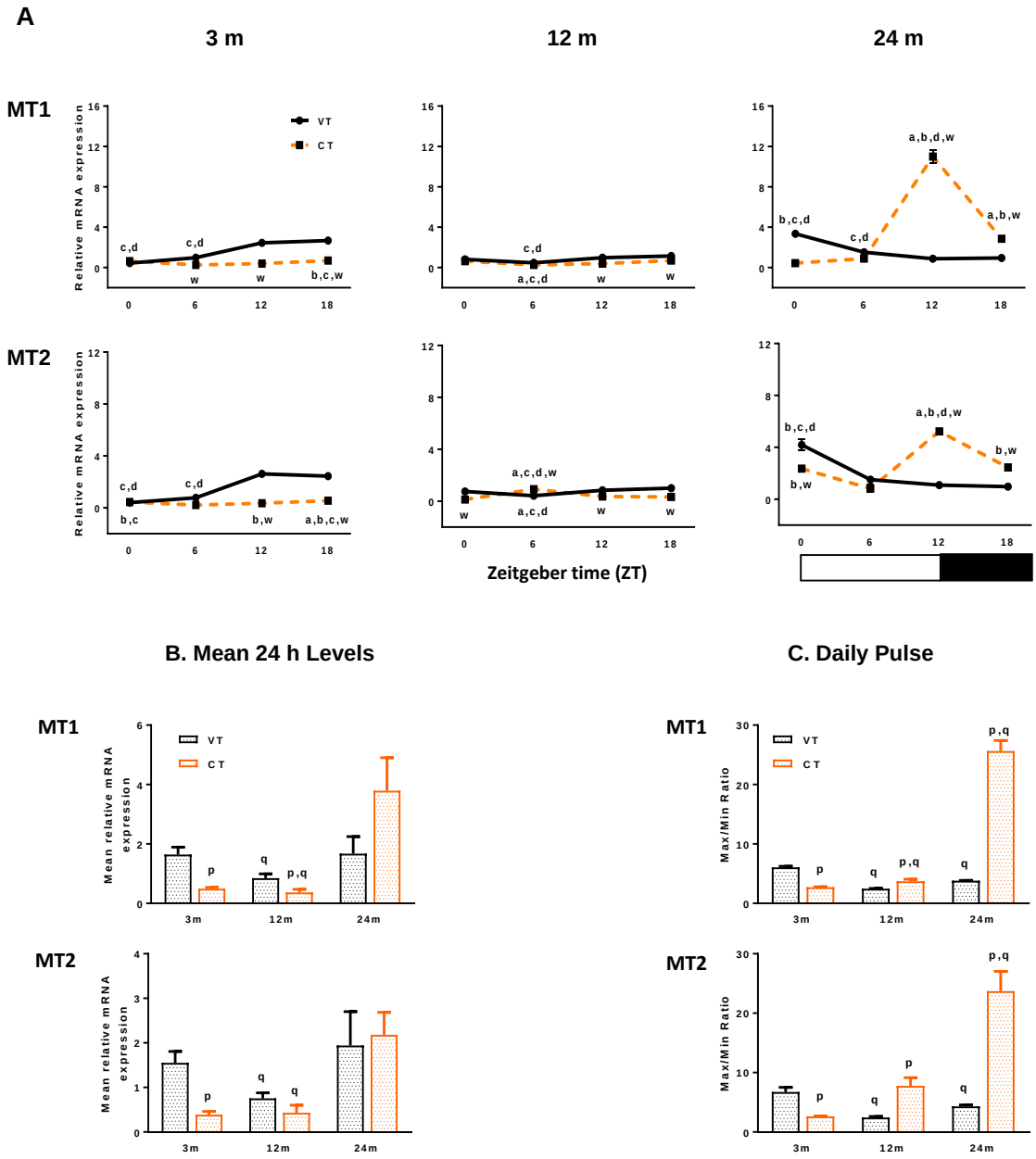


Fig. 48 Effect of curcumin treatment on **(A)** Daily rhythms of MT1 and MT2 mRNA expression in the SCN of 3, 12 and 24 months (m) old rats. Each value is mean ± SEM (n = 4), $p < 0.05$ and expressed as relative mRNA expression. $p_a < 0.05$; $p_b < 0.05$, $p_c < 0.05$ and $p_d < 0.05$ (where 'a', 'b', 'c' and 'd' refers to comparison with ZT-0, ZT-6, ZT- 12 and ZT-18 respectively within the group). $p_w < 0.05$ (where 'w' refers to comparison of mRNA expression levels at same time point in the age matched vehicle group). **(B)** Mean 24 h levels and **(C)** Daily Pulse of MT1 and MT2 mRNA expression in 3, 12 and 24 m old rat SCN. $p_p < 0.05$ (where 'p' refers to comparison with age matched vehicle group). $p_q < 0.05$ (where 'q' refers to comparison with 3 m vehicle group).

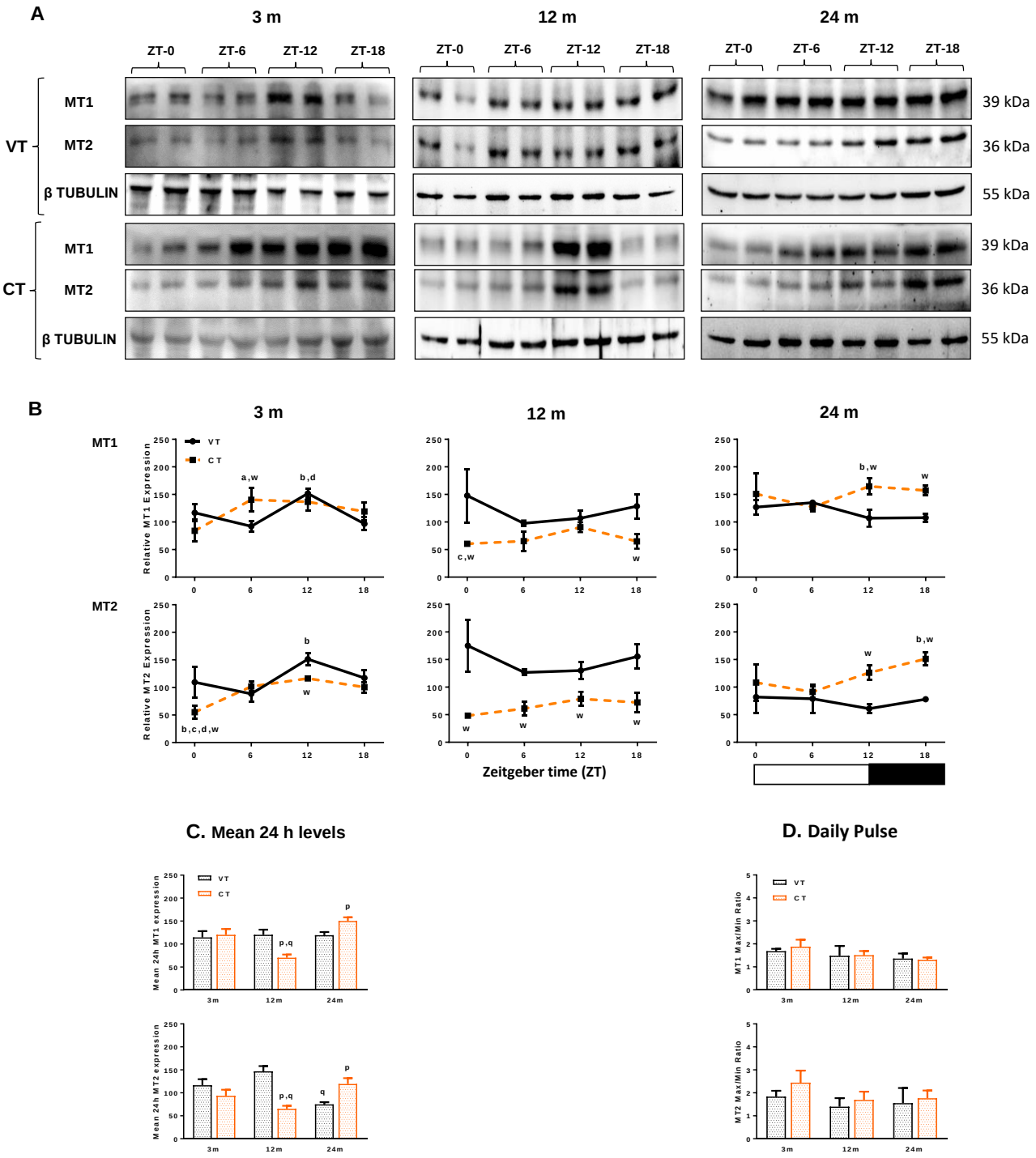


Fig. 49 (A) Immunoblots showing daily rhythms in MT1 and MT2 expression in SCN of 3, 12 and 24 m aged rats at ZT-0, 6, 12 and 18. β-TUBULIN was probed as loading control. VT- vehicle treated; CT - curcumin treated. **(B)** Daily rhythms of MT1 and MT2 protein expression in 3, 12 and 24 m aged rats. Each 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). **(C)** Mean 24 h levels and **(D)** Daily

Pulse of MT1 and MT2 protein expression in 3, 12 and 24 m aged rat SCN. $p_p < 0.05$ (where 'p' refers to comparison with age matched vehicle group). $p_q < 0.05$ (where 'q' refers to comparison with 3 m vehicle group).

Interestingly, curcumin restored the phase of MT2 daily rhythm in old aged rats by bringing a 12 h delay, thus resetting the maximum and minimum expression to ZT-12 and ZT-0 respectively (Fig. 48A). Mean 24 h levels decreased by 4.0 and 3.5 folds respectively in 3 m and 12 m rats upon curcumin administration ($p < 0.05$). However in old age, mean 24 h levels were similar to age matched VT group and were not significantly different in comparison to young control animals (Fig. 48B). Daily pulse of MT2 transcripts decreased by a significant 2.5 folds in 3 m upon curcumin treatment ($p < 0.05$). However, curcumin increased the daily pulse in 12 m by 3.2 folds in comparison to age matched control group thus resulting in restoration with respect to young animals. However, it resulted in significant 3.5 and 5.5 folds increase in the pulse in old aged animals when compared to 3 m and 24 m controls respectively ($p < 0.05$) (Fig. 48C).

Melatonin Receptor protein expression: Curcumin treatment to 3 m rats resulted in 6 h phase advance in the daily rhythms of MT1 with maximum and minimum expression at ZT-6 and ZT-0 respectively which is similar to melatonin treatment (Fig. 49A and 49B). Interestingly, curcumin treatment also resulted in restoration of daily rhythms and phase of MT1 receptor expression both in 12 and 24 m rat SCN which were found to be abolished in age matched vehicle groups. The maximum and minimum expression in 12 and 24 m CT animals were at ZT-12 and ZT-6 which is similar to 3 m vehicle group (Fig. 49B). Curcumin treatment did not alter the mean 24 h levels of MT1 in 3 m SCN, however, in 12 m it was decreased by 1.6 folds and in 24 m it was increased by 1.3 folds compared to young controls ($p < 0.05$) (Fig. 49C). The daily pulse of MT1 upon curcumin administration was however found to be similar to that of 3 m as well as age matched vehicle groups with no significant difference (Fig. 49D).

MT2 daily rhythms in 3 m curcumin treated rat SCN were in phase with young VT rats with maximum expression at ZT-12 and minimum at ZT-0 (Fig. 49A and 49B). In 12 m CT rats, MT2 expression was found to be arrhythmic which was similar to 12 m VT. In this group, MT2 expression significantly decreased in all the four time points studied. However in 24 m rats, CT resulted in restoration of daily rhythms with maximum

expression at ZT-18 and minimum at ZT-6 which is 6 h phase delay in comparison to 3 m VT (Fig. 49B). MT2 mean levels in 3 m CT animals were similar to 3 m VT. However, in 12 m rat SCN, curcumin decreased the MT2 mean 24 h levels by 1.7 and 2.2 folds in comparison to 3 m and 12 m vehicle groups respectively ($p < 0.05$). Interestingly, curcumin increased the mean levels in 24 m rats by 1.6 folds resulting in restoration (Fig. 49C). The daily pulse of MT2 upon curcumin administration did not vary significantly (Fig. 49D).

c. Effect of *Withania somnifera* leaf extract on age induced alterations in Melatonin Receptor expression in the SCN of male Wistar rats

***Melatonin receptor transcript expression*:** In 3 m rats administered with *Withania somnifera*, the daily rhythmicity of MT1 mRNA expression persisted with maximum and minimum levels at ZT-18 and ZT-0 respectively, which is in coherence with the rhythmicity in SCN of young control animals. In 12 m WST, MT1 peak expression was at ZT-18 and minimum was at ZT-0 which is similar to age matched and 3 m control. WS treatment resulted in 12 h phase delay in 24 m old rats with peak expression at ZT-12, indicating partial restoration in comparison to young rats (Fig. 50A). The change in mean 24 h levels of MT1 in both 3 and 12 m groups upon WST was not significant compared to respective age matched control groups. Similarly in 24 m WST group, MT1 mean levels did not show significant change compared to old aged controls however, there was 1.7 fold decrease when compared to young controls (Fig. 50B). Daily pulse of MT1 decreased by 3.3 folds in 3 m WST compared to young control group. In 12 m WST the pulse was similar to middle aged controls animals. Interestingly, WS significantly increased the daily pulse in 24 m rats by 5.5 and 3.5 folds in comparison to age matched as well as 3 m control groups respectively ($p < 0.05$) (Fig. 50C).

In 3 m WST, the daily rhythmicity of MT2 mRNA expression persisted with maximum and minimum levels at ZT-12 and ZT-0 respectively, which is in coherence with the rhythmicity in SCN of young control animals, though with decreased robustness. WS advanced the phase of MT2 in 12 m by 6 h thus resulting in phase restoration with peak expression at ZT-12 and nadir at ZT-0. Interestingly, in old aged rats treated with WS, MT2 expression peaked at ZT-18 which is 18 h phase delay in comparison to age matched control group and 6 h phase delay when compared to young control SCN (Fig. 50A). The

mean 24 h levels decreased by 2.0 folds in 3 m upon WS. In middle age, WST did not change the mean levels with respect to 12 m VT, however, there was a significant 3.2 fold decrease when compared to young control group ($p < 0.05$). In 24 m WST, the mean levels were similar to both 3 m and 24 m controls (Fig. 50B). In 3 m WST, MT2 daily pulse decreased by 3.5 folds. However in 12 m, WS increased the daily pulse by 2.5 folds thus resulting in restoration. But in 24 m, WS significantly increased the daily pulse by 7.4 and 4.7 folds respectively in comparison to age matched and 3 m control SCN ($p < 0.05$) (Fig. 50C).

***Melatonin receptor protein expression*:** WS treatment to 3 m rats also resulted in 6 h phase advance in MT1 receptor protein expression with maximum and minimum levels at ZT-6 and ZT-0 respectively (Fig. 51A and 51B). In 12 m animals treated with WS, MT1 receptor expression remained arrhythmic which is similar to 12 m VT. However, it restored the rhythmicity in 24 m with maximum MT1 expression at ZT-18 which is 6 h phase delay in comparison to young controls (Fig. 51B). WS increased the mean 24 h MT1 expression by a significant 1.4 folds in 3 m ($p < 0.05$). Whereas in middle and old age it was not altered when compared to age matched as well as young controls (Fig. 51C). Daily pulse of MT1 did not vary upon WS administration in any of the age groups (Fig. 51D).

In 3 m WS treated rats, MT2 protein expression was rhythmic with peak expression at ZT-18 and minimum at ZT-0 which is indicative of a 6 h delay in phase with respect to 3 m control animals (Fig. 51A and 51B). WS administration to 12 m rats resulted in decreased MT2 expression with abolished rhythm similar to 12 m VT. However in old aged rats, WS resulted in restoration of daily rhythmicity with maximum MT2 expression at ZT-18 and minimum at ZT-0 which is 6 h phase delay in reference to young rats of vehicle group (Fig. 51B). MT2 mean 24 h levels in 3 m WST was similar to young control group. However in 12 m it was decreased by 1.6 folds in comparison age matched vehicle group ($p < 0.05$). Interestingly, WS increased the mean levels in 24 m rats by 1.4 folds resulting in restoration (Fig. 51C). The variations in daily pulse of MT2 upon WS administration were insignificant (Fig. 51D).

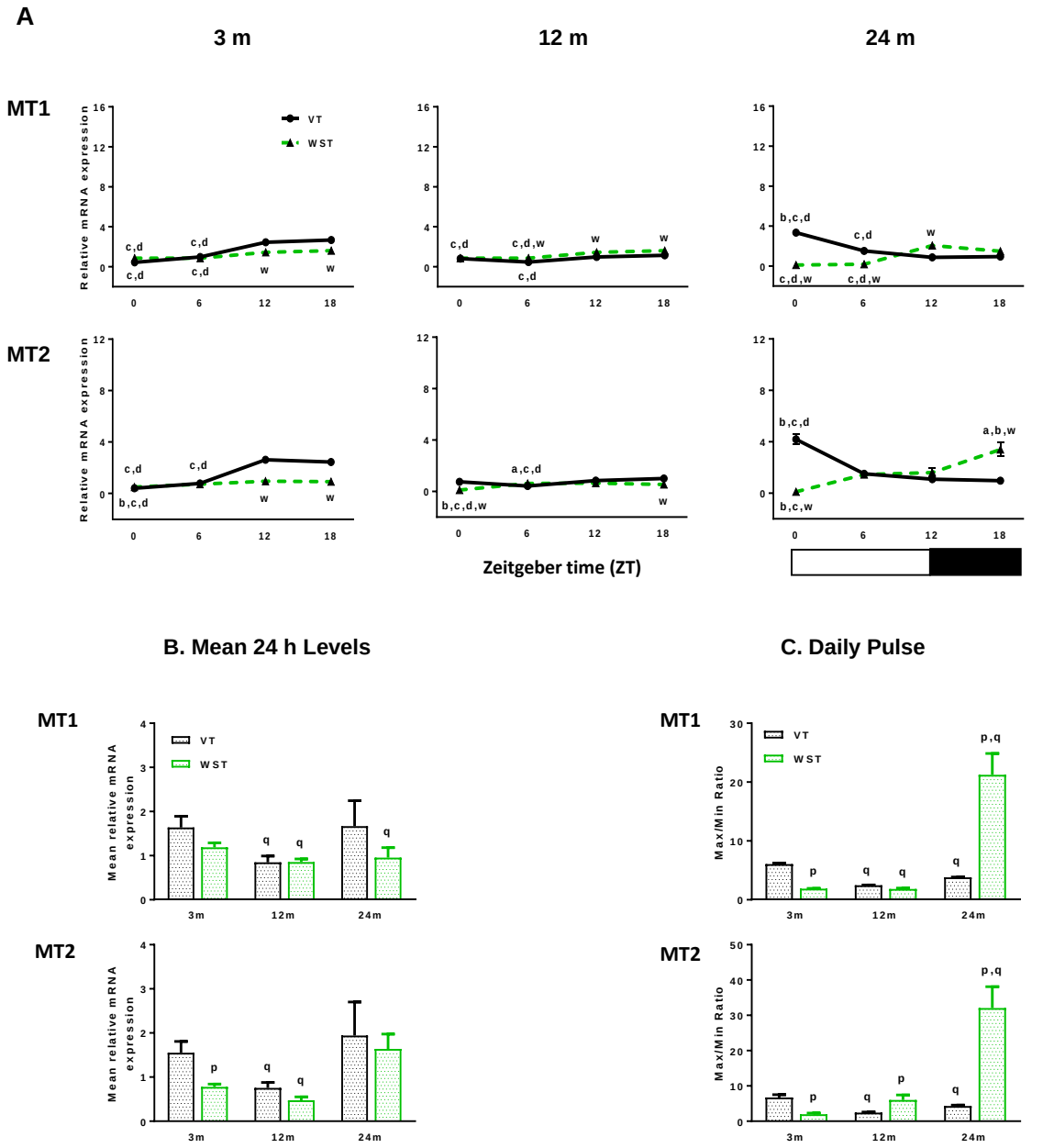


Fig. 50 Effect of *Withania somnifera* treatment on (A) Daily rhythms of MT1 and MT2 mRNA expression in the SCN of 3, 12 and 24 m old rats. Each value is mean ± SEM (n = 4), $p < 0.05$ and expressed as relative mRNA expression. $p_a < 0.05$; $p_b < 0.05$, $p_c < 0.05$ and $p_d < 0.05$ (where 'a', 'b', 'c' and 'd' refers to comparison with ZT-0, ZT-6, ZT- 12 and ZT-18 respectively within the group). $p_w < 0.05$ (where 'w' refers to comparison of mRNA expression levels at same time point in the age matched vehicle group). (B) Mean 24 h levels and (C) Daily Pulse of MT1 and MT2 mRNA expression in 3, 12 and 24 m old rat SCN. $p_p < 0.05$ (where 'p' refers to comparison with age matched vehicle group). $p_q < 0.05$ (where 'q' refers to comparison with 3 m vehicle group).

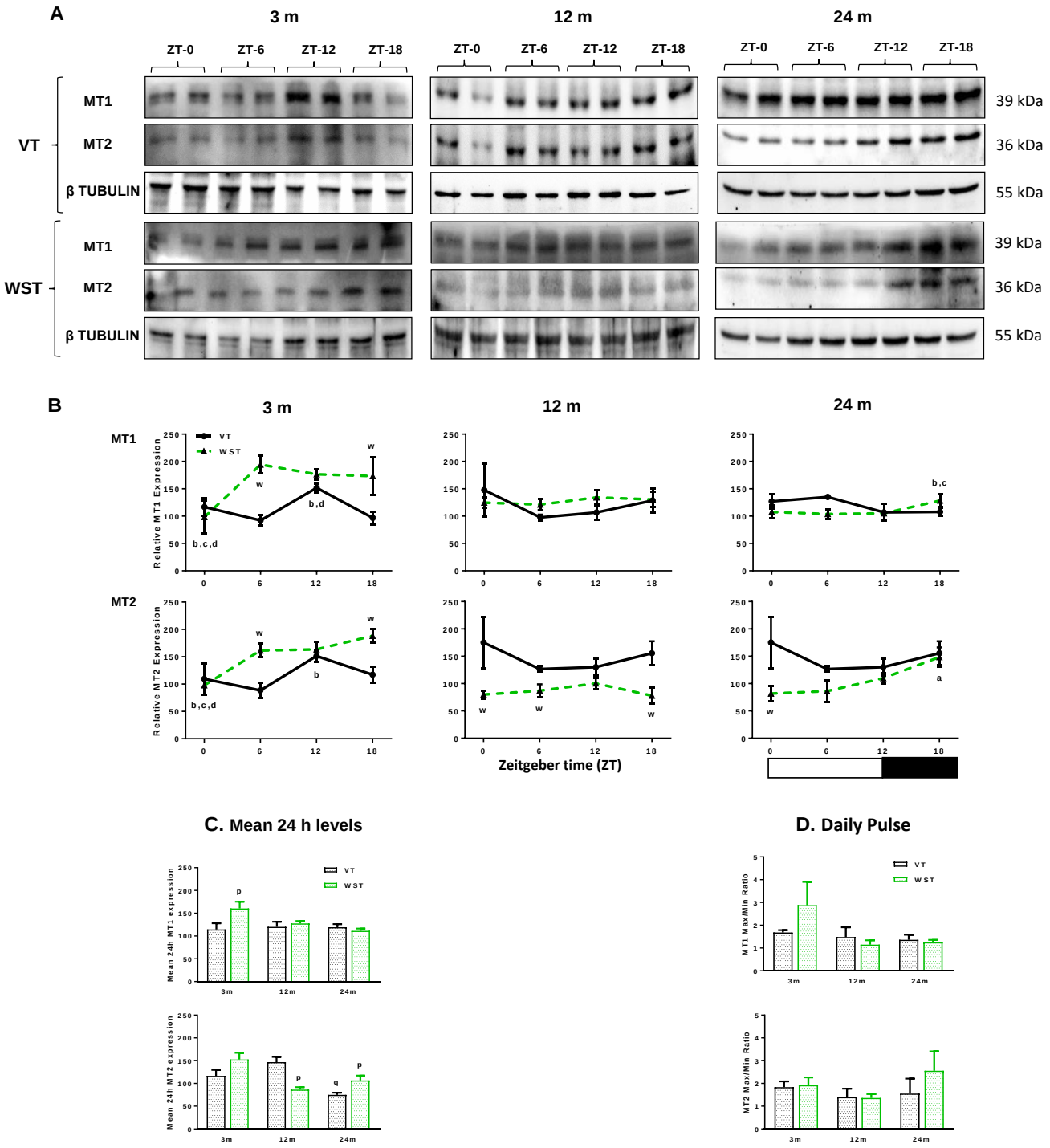


Fig. 51 (A) Immunoblots showing daily rhythms in MT1 and MT2 expression in SCN of 3, 12 and 24 m aged rats at ZT-0, 6, 12 and 18. β-TUBULIN was probed as loading control. VT- vehicle treated; WST – *Withania somnifera* treated. **(B)** Daily rhythms of MT1 and MT2 protein expression in 3, 12 and 24 m aged 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). **(C)** Mean 24 h

levels and (**D)** Daily Pulse of MT1 and MT2 protein expression in 3, 12 and 24 m aged rat SCN. $p_p < 0.05$ (where 'p' refers to comparison with age matched vehicle group). $p_q < 0.05$ (where 'q' refers to comparison with 3 m vehicle group).

d. Effect of Melatonin on age induced alterations in Melatonin Receptor expression in the SCN of male Wistar rats

***Melatonin receptor transcript expression*:** With melatonin treatment, MT1 transcripts showed increased robustness of daily rhythms in 3 m SCN with significant increase in the relative mRNA levels in all the time points studied except ZT-18. The peak expression was at ZT-12 which is a 6 h phase advance in comparison to the young control group. In 12 m, MT1 expression was arrhythmic though there was a significant increase at mid-subjective day (ZT-6). In 24 m animals, the MT1 peak was at ZT-6 and minimum was at ZT-0 which is indicative of a partial restoration in phase as melatonin delayed the peak by 6 h in this age group compared to age matched control animals (Fig. 52A). Mean 24h levels of MT1 increased by 2.4 folds in the SCN of young rats upon melatonin treatment. In 12 m, the mean 24 h levels restored with respect to 3 m control group. However, in 24 m SCN melatonin treatment augmented the mean levels by 5.6 folds in comparison to both young and age matched controls ($p < 0.05$) (Fig. 52B). The daily pulse of MT1 transcripts in 3 m decreased by a significant 1.7 folds upon melatonin administration ($p < 0.05$). However, in both 12 and 24 m animals the daily pulse were similar to the respective age matched controls and decreased by a significant 2.8 and 1.8 folds respectively in comparison to 3 m vehicle controls ($p < 0.05$) (Fig. 52C).

Upon melatonin administration to 3 m animals, MT2 expression was in coherence with the 3 m vehicle group with expression maximum at ZT-12. In middle aged animals, peak expression was at ZT-18 and nadir was at ZT-0 which is also similar to age matched vehicle group. However, in 24 m animals, melatonin resulted in robust MT2 daily rhythm with maximum and minimum expression at ZT-6 and ZT-0 respectively. This is indicative of partial restoration in phase of MT2 expression as the peak was delayed by 6 h in comparison to old age control group and advanced by 6 h in comparison to young control animals (Fig. 52A). Mean 24 h level expression of MT2 did not alter significantly in young age, but restored in middle aged animals treated with melatonin.

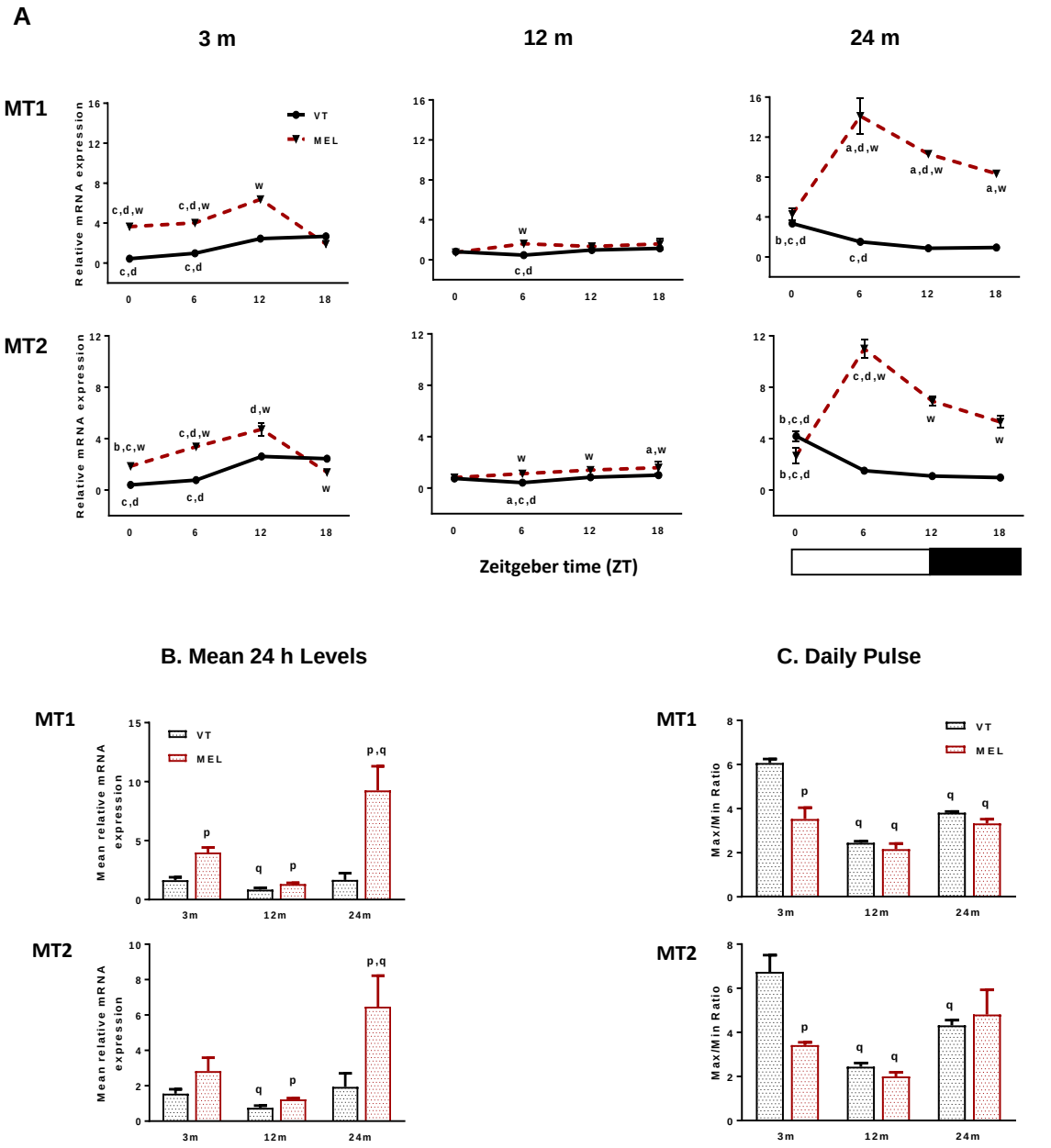


Fig. 52 Effect of melatonin treatment on (**A)** Daily rhythms of MT1 and MT2 mRNA expression in the SCN of 3, 12 and 24 m old rats. Each value is mean ± SEM (n = 4), $p < 0.05$ and expressed as relative mRNA expression. $p_a < 0.05$; $p_b < 0.05$, $p_c < 0.05$ and $p_d < 0.05$ (where 'a', 'b', 'c' and 'd' refers to comparison with ZT-0, ZT-6, ZT- 12 and ZT-18 respectively within the group). $p_w < 0.05$ (where 'w' refers to comparison of mRNA expression levels at same time point in the age matched vehicle group). (**B)** Mean 24 h levels and (**C)** Daily Pulse of MT1 and MT2 mRNA expression in 3, 12 and 24 m old rat SCN. $p_p < 0.05$ (where 'p' refers to comparison with age matched vehicle group). $p_q < 0.05$ (where 'q' refers to comparison with 3 m vehicle group).

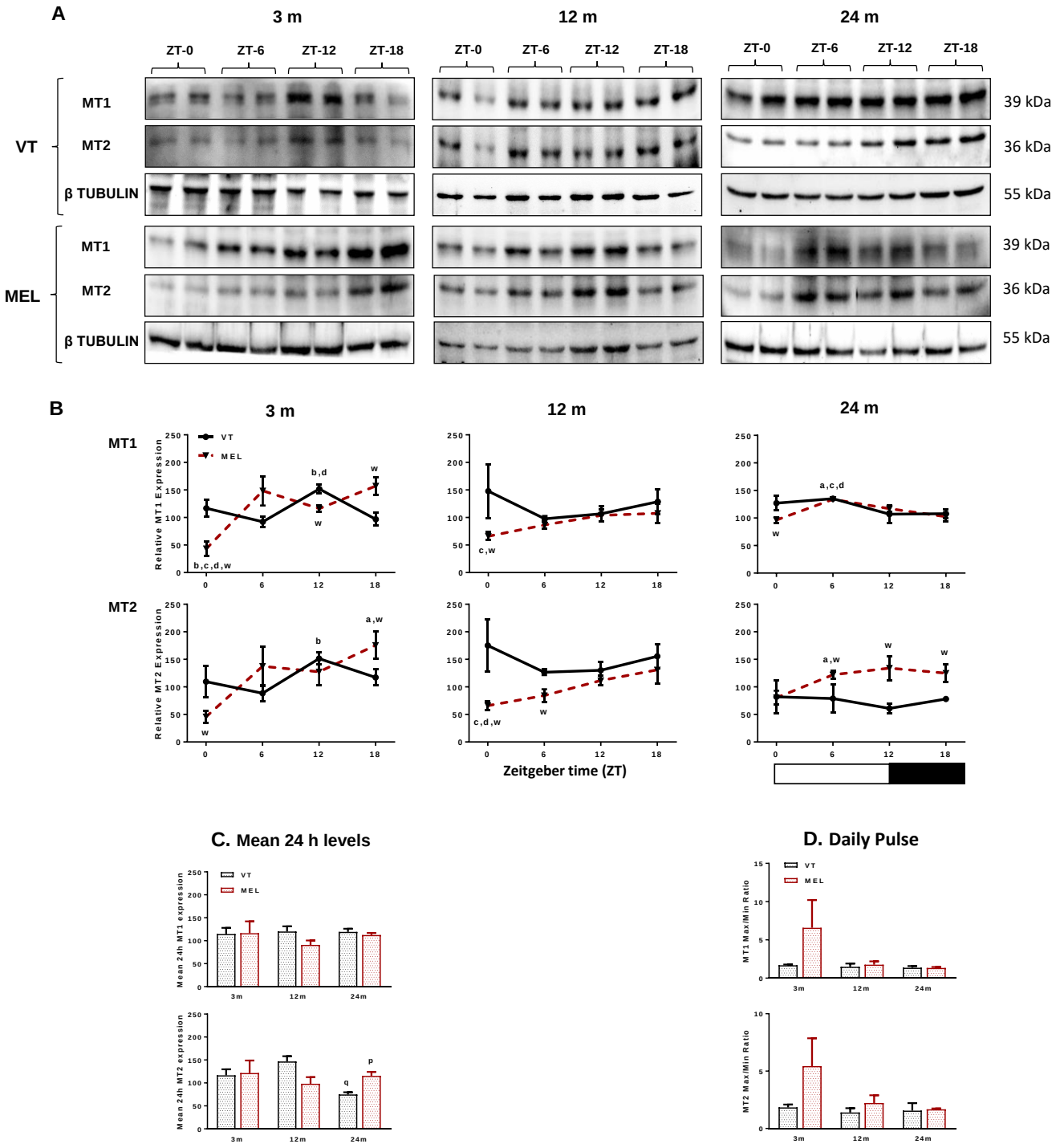


Fig. 53 (A) Immunoblots showing daily rhythms in MT1 and MT2 expression in SCN of 3, 12 and 24 m aged rats at ZT-0, 6, 12 and 18. β-TUBULIN was probed as loading control. VT- vehicle treated; MEL- melatonin treated. **(B)** Daily rhythms of MT1 and MT2 protein expression in 3, 12 and 24 m aged rats. 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). **(C)** Mean 24 h levels and **(D)** Daily

pulse of MT1 and MT2 protein expression in 3, 12 and 24 m aged rat SCN. $p_p < 0.05$ (where 'p' refers to comparison with age matched vehicle group). $p_q < 0.05$ (where 'q' refers to comparison with 3 m vehicle group).

However, it showed significant 3.3 and 4.2 fold increase in 24 m group in comparison to age matched and 3 m control animals respectively ($p < 0.05$) (Fig. 52B). Melatonin decreased the daily pulse in 3 m by 2.0 folds ($p < 0.05$). However, in both middle and old aged animals it was similar to age matched control groups (Fig. 52C).

***Melatonin receptor protein expression*:** Melatonin administration to 3 m rats resulted in 6 h phase advance in MT1 expression, where maximum and minimum expressions were at ZT-6 and ZT-0 respectively (Fig. 53A and 53B). Interestingly, melatonin restored both rhythmicity and phase of MT1 in 12 m rat SCN with maximum expression at ZT-12 which were found to be abolished in 12 m vehicle group. Daily rhythms of MT1 expression were restored in 24 m old rat SCN as well upon melatonin treatment, with maximum expression at ZT-6, which is a 6 h phase advancement in comparison to 3 m vehicle group (Fig. 53B). Melatonin did not affect the mean 24 h MT1 levels in any of the age group (Fig. 53B). Similarly, the alterations in daily pulse of MT1 in all the three age groups treated with melatonin were statistically insignificant (Fig. 53C).

The daily rhythms of MT2 expression was persistent in 3 m rat SCN treated with melatonin with maximum and minimum expression at ZT-18 and ZT-0 respectively, which is a 6 h phase delay in comparison to 3 m VT (Fig. 53A and 53B). Interestingly, melatonin restored the daily rhythms of MT2 expression in both middle and old aged rat SCN with peak expression at ZT-18 and ZT-12 respectively (Fig. 53B). Further, the mean 24 h levels of MT2 in 3 and 12 m melatonin group were found to be comparable to that of 3 m VT. However, melatonin significantly increased the mean 24 h levels of MT2 in 24 m rats by 1.5 folds in reference to 24 m VT thus resulting in restoration ($p < 0.05$) (Fig. 53C). Similar to MT1, melatonin did not significantly alter the daily pulse of MT2 in any of the three age groups studied (Fig. 53D).

Individual values of melatonin receptor mRNA and protein expression at different time points and under various conditions are tabulated in Table 11 and 12.

Table 11 Age induced alterations in MT1 and MT2 mRNA expression in rat SCN: effect of curcumin, *Withania somnifera* leaf extract and melatonin.

Gene	Age & Treatment	ZT-0	ZT-6	ZT-12	ZT-18	Mean 24 h levels	Max/Min Ratio
MT1	3m VT	4.39±0.19	9.8±0.53	24.55±1.7	26.7±1.91	16.36±2.52	6.07±0.18
	3m CT	6.26±0.11	2.56±0.14	3.91±0.14	6.89±0.3	4.9±0.46 ^{w}	2.7±0.1 ^{w}
	3m WST	8.53±0.15	8.54±0.64	14.38±1.9	16.07±0.7	11.88±1 ^{w}	1.88±0.1 ^{w}
	3m MEL	36.51±2.0	40.25±1.4	63.72±2.8	19.12±2.5	39.9±4.2 ^{w}	3.53±0.5 ^{w}
	12m VT	8.18±0.83	4.67±0.26	9.7±0.58	11.39±0.3	8.48±1.4 ^{xw}	2.5±0.07 ^{xw}
	12m CT	1.75±0.21	6.67±1.28	3.24±0.39	2.95±0.49	3.65±1.06 ^{w}	3.72±0.3 ^{xw}
	12m WST	6.75±0.17	7.35±1.24	12.29±0.9	7.8±0.98	8.55±0.7 ^{xw}	1.83±0.2 ^{w}
	12m MEL	7.38±0.87	15.97±2.9	13.34±1.9	15.93±5.6	13.15±2 ^{x}	2.2±0.26 ^{xw}
	24m VT	33.39±0.9	15.24±0.7	8.75±0.23	9.43±0.43	16.7±5.75 ^{x}	3.8±0.05 ^{xw}
	24m CT	4.39±0.55	8.82±1.84	110±6.06	28.49±2.8	37.9±11.1 ^{xyw}	26±1.8 ^{xyw}
	24m WST	1.04±0.22	1.78±0.18	20.54±2.6	14.89±1.0	9.56±2.26 ^{w}	21±3.6 ^{xyw}
	24m MEL	42.74±6.1	141±18.26	103±2.06	83.37±1.5	92.5±20 ^{xyw}	3.3±0.2 ^{yw}
MT2	3m VT	3.93±0.17	7.73±0.29	26.12±1.73	24.4±1.1	15.54±2.58	6.75±0.76
	3m CT	4.47±0.26	2.11±0.2	3.47±0.21	5.5±0.55	3.89±0.72 ^{w}	2.6±0.09 ^{w}
	3m WST	4.93±0.44	7.13±1.11	9.46±1.04	9.08±1.18	7.79±0.64 ^{w}	2±0.33 ^{w}
	3m MEL	18.61±1.19	33.74±2.1	47.23±5.13	13.75±1.15	28.34±7.6	3.4±0.13 ^{w}
	12m VT	7.5±0.63	4.2±0.47	8.41±0.52	10.04±0.49	7.54±1.23 ^{x}	2.44±0.2 ^{x}
	12m CT	1.28±0.25	9.17±1.55	3.59±0.44	3.27±0.83	4.33±1.7 ^{w}	7.76±1.4 ^{x}
	12m WST	1.09±0.06	6.19±1.64	6.38±1.33	5.4±0.97	4.77±0.76 ^{xw}	6.03±1.4 ^{x}
	12m MEL	8.12±1.3	15.95±2.08	11.18±0.49	14.03±4.64	12.32±1.7 ^{x}	2±0.2 ^{xw}
	24m VT	41.92±4.1	15.05±0.92	10.89±1.81	9.65±0.4	19.38±7.6 ^{x}	4.3±0.24 ^{xw}
	24m CT	2.36±0.39	8.19±2.81	52.28±0.98	24.45±1.97	21.8±5.1 ^{xyw}	24±3.3 ^{xyw}
	24m WST	1.11±0.13	14.42±2.14	15.96±3.54	33.99±5.28	16.37±3.4 ^{xy}	32±6.06 ^{xyw}
	24m MEL	26.62±5.98	110±7.5	69.18±3.53	53±4.53	65±17.5 ^{xyw}	4.8±1.1 ^{yw}

Each value is mean ± SEM (n = 4), $p < 0.05$ and expressed as relative mRNA expression. $p_x < 0.05$; $p_y < 0.05$, $p_z < 0.05$ (where 'x', 'y', 'z' refers to comparison with 3 m, 12 m, 24 m respectively). $p_w < 0.05$ (where 'w' refers to comparison with 3 m vehicle group).

Table 12 Age induced alterations in MT1 and MT2 protein expression in rat SCN: effect of curcumin, *Withania somnifera* leaf extract and melatonin.

Protein	Age & Treatment	ZT-0	ZT-6	ZT-12	ZT-18	Mean 24 h levels	Max/Min Ratio
MT1	3m VT	116.8±15.8	92.2±9.6	151.8±8	96.8±11.9	114.4±13.6	1.7±0.1
	3m CT	84.1±19.9	140.4±21.1	136.6±15.6	119.1±16.7	120.1±12.9	1.9±0.3
	3m WST	98.5±29.9	194.3±16	176.5±9.8	173.2±35	160.6±15 ^{w}	2.9±1 ^{w}
	3m MEL	43.5±13.9	148.5±26.7	116.2±6	157.2±16.3	116.3±25.8	6.6±3.6 ^{w}
	12m VT	147.7±48.7	97.6±5.2	106.7±13.6	128.6±22.1	120.2±11.3	1.5±0.4
	12m CT	60.5±1.6	65.2±17.3	90.6±9.1	64.8±13.5	70.3±6.9 ^{xw}	1.5±0.2
	12m WST	124.6±9.7	121.1±9.8	134.3±13.3	130.5±14.2	127.6±5.5 ^{x}	1.2±0.2 ^{xw}
	12m MEL	66±7.4	86.6±7.6	103.8±10.5	107.6±17.3	91±9.5 ^{x}	1.8±0.4 ^{x}
	24m VT	126.9±13.2	135.1±3.1	107±15.6	107.8±7.6	119.2±7	1.4±0.2
	24m CT	151±37.9	127.1±8.3	164.7±14.5	157.3±9.4	150±8.2 ^{xyw}	1.3±0.1 ^{xw}
	24m WST	107.5±12	103.9±9.3	105.2±3.4	128.4±11.6	111±5.1 ^{xy}	1.3±0.1 ^{xw}
	24m MEL	96.4±5.4	133.8±4.7	117±4.9	102.4±8	112.4±4.6	1.3±0.1 ^{xyw}
MT2	3m VT	109.4±28.6	88.5±14.7	151.3±10.9	117.1±14.4	116.6±13.1	1.8±0.3
	3m CT	55.1±11.7	101.8±9.1	116.4±4.8	100.5±9.7	93.4±13.3	2.4±0.5 ^{w}
	3m WST	97.8±4.3	161.3±12.5	163.4±13.4	188.2±12.7	153±14.5 ^{w}	1.9±0.3
	3m MEL	45.7±11.2	137.4±35.2	127.3±25.1	175.6±24.7	121.5±27.3	5.4±2.4 ^{w}
	12m VT	175.2±47.5	126.6±5.2	130.1±15.2	155.5±21.9	146.8±11	1.4±0.4 ^{w}
	12m CT	48.3±4.6	61.1±12	78.6±12.6	72.2±17.3	65.1±6.7 ^{xw}	1.7±0.4
	12m WST	80±7.4	87±11.7	100.3±10.5	77.8±14.3	86.3±5.5 ^{xw}	1.4±0.2
	12m MEL	65.9±7.8	84.2±11.6	111.7±9.2	130.9±25.3	98.2±14.4	2.2±0.7 ^{w}
	24m VT	126.9±13.2	135.1±3.1	107±15.6	107.8±7.6	75±4.7 ^{xy}	1.6±0.7
	24m CT	151±37.9	127.1±8.3	164.7±14.5	157.3±9.3	119.2±13 ^{y}	1.8±0.3
	24m WST	81.8±13.9	86.1±19.3	109.9±9.6	148.4±17.8	106.5±11 ^{xy}	2.6±0.9
	24m MEL	96.3±5.4	133.8±4.6	117±4.9	102.4±8	115.3±8.8	1.7±0.1 ^{xy}

Each value is mean ± SEM (n = 4), $p < 0.05$ and expressed as normalized mean density. $p_x < 0.05$; $p_y < 0.05$, $p_z < 0.05$ (where 'x', 'y', 'z' refers to comparison with 3 m, 12 m, 24 m respectively). $p_w < 0.05$ (where 'w' refers to comparison with 3 m vehicle group).

OBJECTIVE 5

a. Effect of curcumin on age induced alterations in gross locomotor activity rhythms

The gross locomotor activity recordings (Fig. 54) in young adult rats of 3 m age showed nocturnal pattern with 88.7 ± 0.67 % night time activity and 11.3 ± 0.68 % activity during the light phase (Fig. 55). Aging resulted in significant alterations in gross locomotor activity of rats. In 12 m old middle aged rats, the dark phase and light phase activity were 85.8 ± 0.9 % and 14.2 ± 1.0 % respectively indicating significant decrease in percentage night activity and significant increase in percentage day activity in comparison to 3 m rats ($p < 0.05$). In old aged rats (24 m), night activity further reduced to 81.8 ± 0.8 % and day activity significantly increased to 18.8 ± 0.8 % ($p < 0.05$) (Fig. 55).

With curcumin administration, the gross locomotor activities in all the three age groups were found to be similar to respective age matched control groups with no significant difference (Fig. 54 and Fig. 55, Table 13).

b. Effect of *Withania somnifera* leaf extract on age induced alterations in gross locomotor activity rhythms

Gross locomotor activity of rats of all the three age groups under WS treatment was recorded (Fig. 56). In 3 m rats treated with WS, 88.31 ± 1.59 % activity in the night and 11.69 ± 1.59 % activity during the day was recorded which is similar to the percentage activity in control animals of 3 m age. However, with WS treatment the dark phase and light phase activity of middle aged rats were 86.6 ± 0.2 % and 13.4 ± 0.1 % respectively indicating restoration of percentage activities in comparison to young control rats. Whereas in 24 m rats administered with WS, the percentage activity was similar to age matched control group (Fig. 57, Table 14).

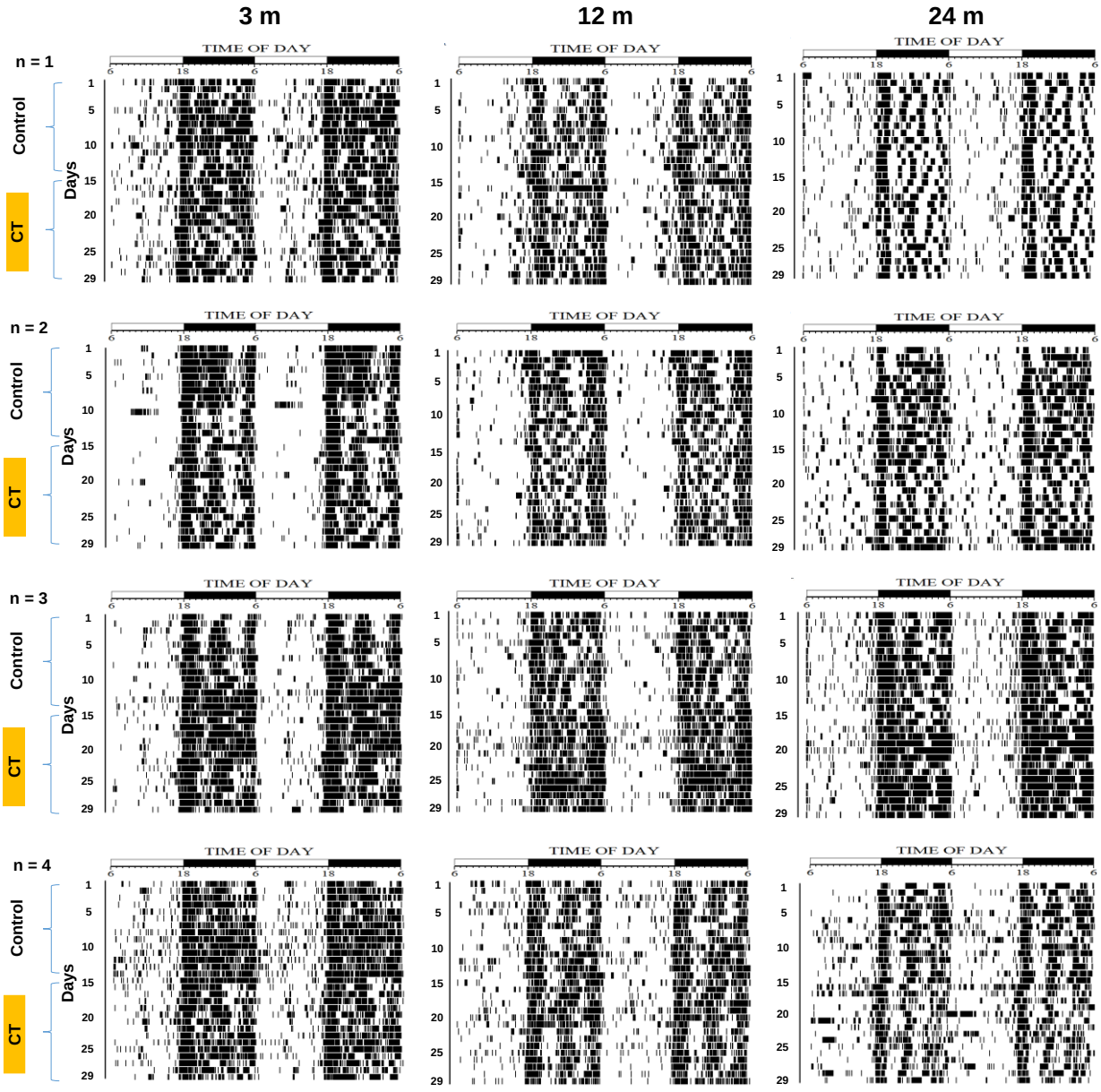


Fig. 54 Double plotted actograms of gross locomotor activity under control (C) and curcumin treated (CT) conditions in 3, 12 and 24 months (m) aged rats.

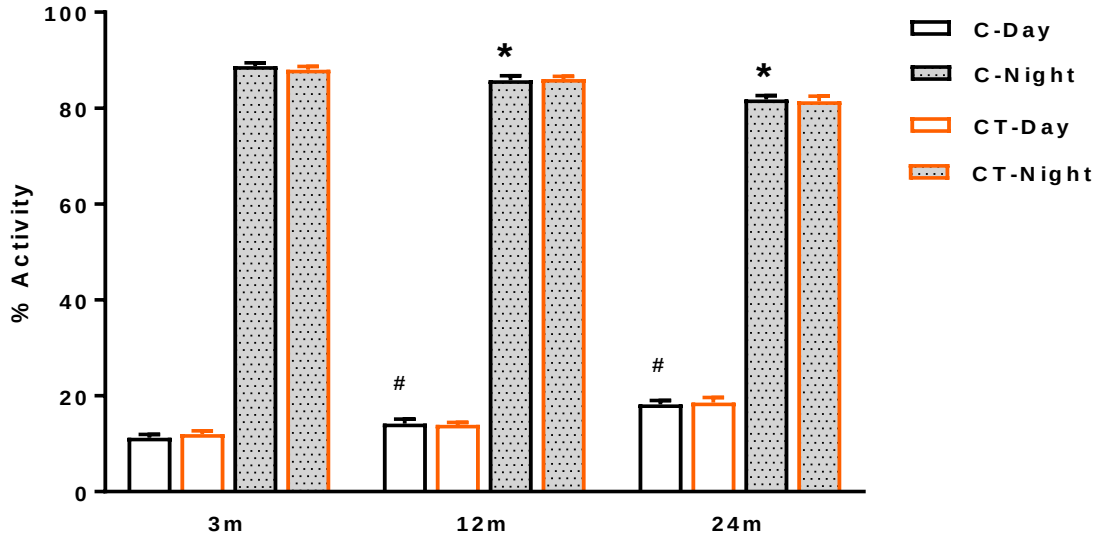


Fig. 55 Effect of curcumin on gross locomotor activity of 3, 12 and 24 m aged rats. C – Control; CT - curcumin treated. Each value is mean ± SEM (n = 4), and expressed as percentage activity. '*' indicates significant difference in comparison to night activity in 3 m controls and '#' indicates significant difference in comparison to day activity in 3 m controls ($p < 0.05$).

Table 13 Effect of curcumin on age induced alterations in gross locomotor activity

Age	Treatment	Nocturnal activity (%)	Diurnal activity (%)
3m	C	88.7 ± 0.68	11.26 ± 0.68
	CT	88.01 ± 0.70	11.99 ± 0.7
12m	C	85.8 ± 0.96*	14.19 ± 0.96#
	CT	86.08 ± 0.53	13.92 ± 0.53
24m	C	81.8 ± 0.83*	18.22 ± 0.83#
	CT	81.43 ± 1.08	18.57 ± 1.08

Each value is mean ± SEM (n = 4), and expressed as percentage activity. '*' indicates significant difference in comparison to night activity in 3 m controls and '#' indicates significant difference in comparison to day activity in 3 m controls ($p < 0.05$). C – Control; CT - curcumin treated.

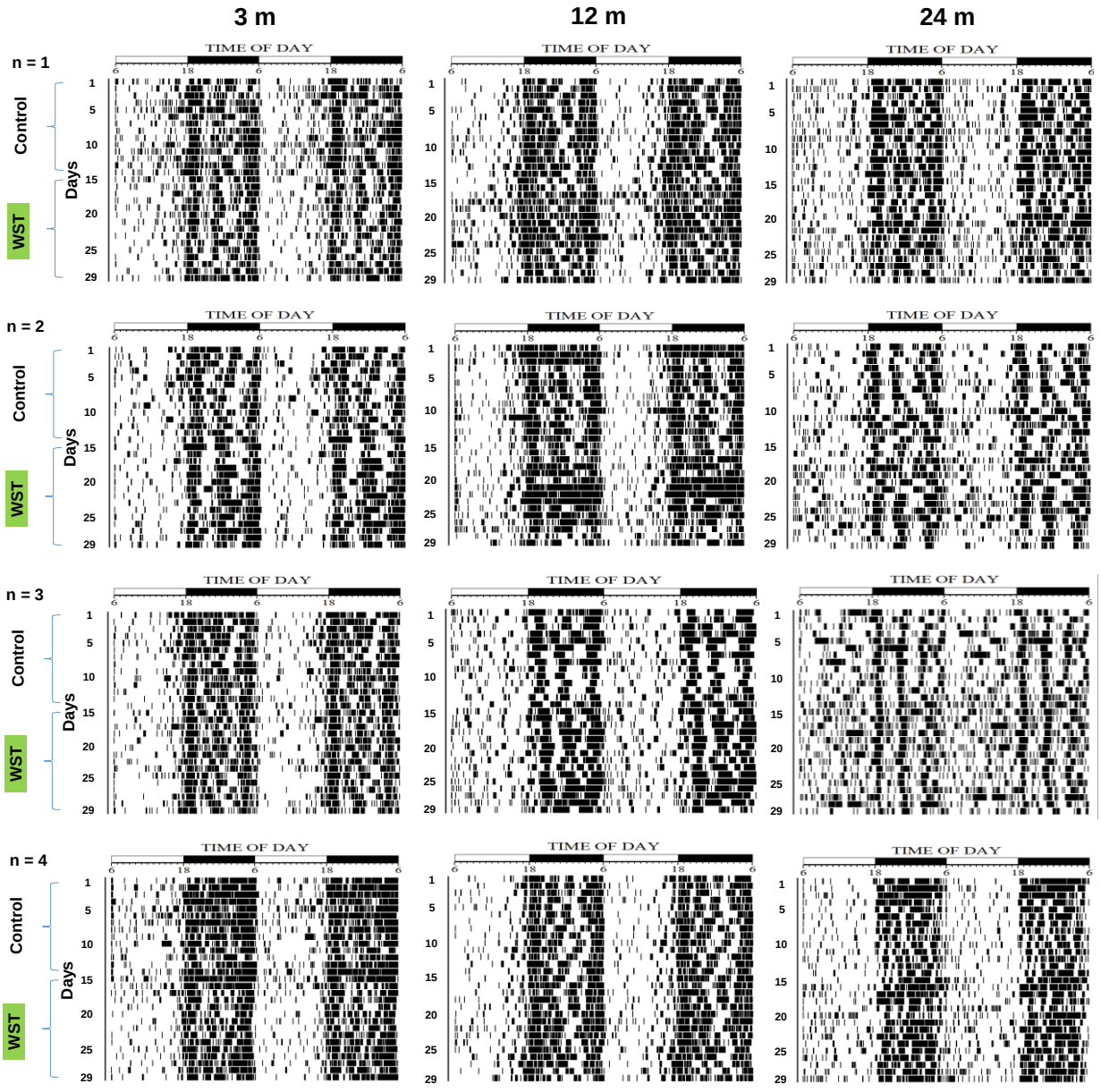


Fig. 56 Double plotted actograms of gross locomotor activity under control (C) and *Withania somnifera* treated (WST) conditions in 3, 12 and 24 months (m) aged rats.

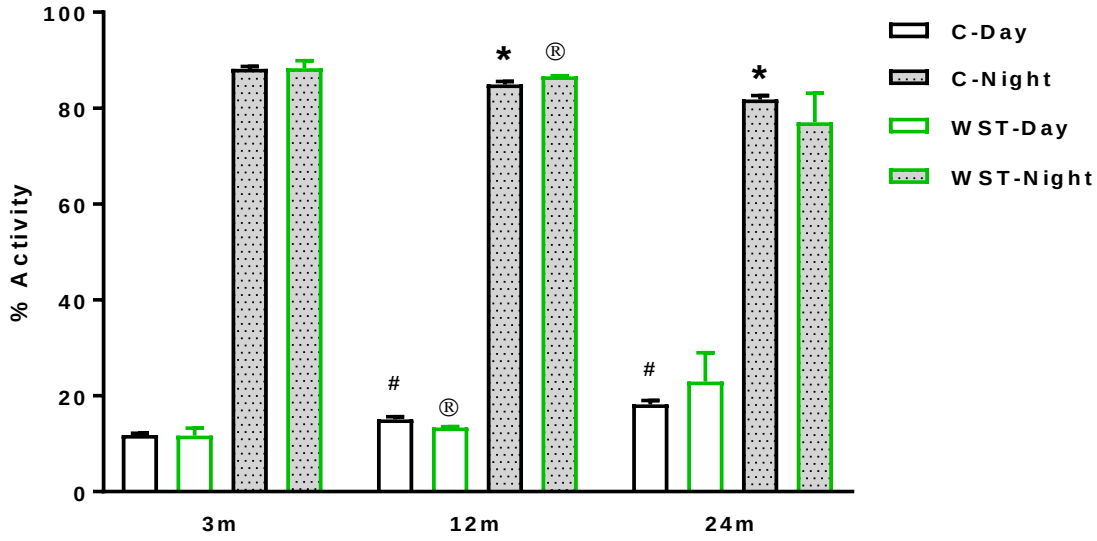


Fig. 57 Effect of *Withania somnifera* on gross locomotor activity of 3, 12 and 24 m aged rats. C – Control; WST - *Withania somnifera* treated. Each value is mean ± SEM (n = 4), and expressed as percentage activity. '*' indicates significant difference in comparison to night activity in 3 m controls and '#' indicates significant difference in comparison to day activity in 3 m controls. ® indicates significant difference in percentage activity in comparison to 12 m control ($p < 0.05$).

Table 14 Effect of *Withania somnifera* leaf extract on age induced alterations in gross locomotor activity.

Age	Treatment	Nocturnal activity (%)	Diurnal activity (%)
3m	C	88.22 ± 0.48	11.78 ± 0.4
	WST	88.31 ± 1.59	11.69 ± 1.59
12m	C	84.94 ± 0.59*	15.05 ± 0.59#
	WST	86.59 ± 0.15®	13.40 ± 0.15®
24m	C	81.78 ± 0.83*	18.22 ± 0.83#
	WST	77.06 ± 6.05	22.93 ± 6.05

Each value is mean ± SEM (n = 4), and expressed as percentage activity. '*' indicates significant difference in comparison to night activity in 3 m controls and '#' indicates significant difference in comparison to day activity in 3 m controls. ® indicates significant difference in percentage activity in comparison to 12 m control ($p < 0.05$). C – Control; WST - *Withania somnifera* treated.

CHAPTER - IV

DISCUSSION

Objective 1

Effect of curcumin and *Withania somnifera* leaf extract on age induced alterations in chronomics of clock genes, SIRT1 and NRF2

With **curcumin** administration, clock genes *rPer1*, *rPer2*, *rCry1*, *rCry2* and *rBmal1* showed a robust rhythmicity similar to 3 m vehicle treated groups (Mattam and Jagota 2014). We observed rhythmic *rRev-erbα* transcript expression in 3 m vehicle treated group which is similar to previous reports (Preitner et al. 2002) but upon CT it showed a 6 h phase advance.

Curcumin administration in middle aged animals (12 m) restored the phase of *rPer1*, *rPer2*, *rCry1*, *rCry2* compared to the young adults (3 m VT). These results were similar to melatonin administration in rats of same age group (Mattam and Jagota 2014). Curcumin's role in resetting altered phases of *rCry1* and *rCry2* with maximum expression back to ZT-12 in SCN of 12 m aged animals could be of great significance. Earlier, genetic complementation studies in *Cry1 and 2* double knockout cells showed that prolonged delay in *Cry1* expression slows down circadian oscillation whereas, an optimal delay (evening time expression) is pivotal to restoration of circadian rhythmicity (Ukai-Tadenuma et al. 2011).

Aging drastically altered the phases and the daily pulses of all the clock genes studied, except *rRev-erbα* in 24 m animals. Curcumin treatment restored the phase and daily pulse of *rPer1* and partially restored the daily pulse of *rBmal1*, *rCry1* and *rCry2* in 24 m. A very similar result was observed with melatonin treatment, where a complete restoration of *rPer1* daily pulse and partial restoration of *rBmal1*, *rCry1* and *rCry2* daily pulse in SCN of 24 m rats was reported (Mattam and Jagota 2014). Curcumin treatment elevated *rPer2*, *rCry1*, *rCry2* and *rBmal1* levels in 12 and 24 m rats. Melatonin also showed a similar effect (Mattam and Jagota 2014) possibly due to its inhibitory action on proteasomes (Vriend and Reiter 2015) which was also recently reported for curcumin (Banerjee et al. 2018). The increased transcript levels of *Bmal1*, *Per1* and *Per2* are also in agreement with the earlier studies of treatment of polyphenol resveratrol in rat fibroblasts (Oike and Kobori 2008). Aging showed a decline in the gene expression levels of *rPer2* and *rBmal1* which is shown to be partially restored with the curcumin treatment. The upregulation in expression of *rBmal1*, *rPer1,2* and *rCry1,2* transcript levels by curcumin suggests that it could possibly be considered as a clock enhancing small molecule (CEM) (Gloston et al. 2017). In

agreement with our results, there are reports demonstrating the role of curcumin in altering the molecular circadian timing of clock. *Bmal1* is reported to be activated by curcumin through stimulation of PPAR-γ (Wang et al. 2008, 2010).

The restoration in the correlation between *rPer1* and *rBmal1*; *rPer1* and *rPer2*; *rPer1* and *rCry1* in the light phase of 12 m CT animals compared to 3 m vehicle group could be due to increased expression of these transcripts in the light phase. Similarly, increase in expression levels of *rPer1, rPer2* and *rCry2* would have restored the correlations between *rPer1* and *rPer2*; *rPer1* and *rCry2* in dark phase of 12 m CT animals. However such restoration of correlations were unique to curcumin administration (Mattam and Jagota 2014). Further, in 12 m CT group the restoration of negative correlations between *rRev-erbα* and *rBmal1* was achieved in the light phase. Earlier as REV-ERBα had been reported as a negative regulator of *Bmal1* (Preitner et al. 2002), the restoration of this negative correlation with CT could have therapeutic advantage in restoring circadian clock function. Similarly, anti-phasic expression of *Period* and *Bmal1* had been reported by many researchers (Abe et al. 1998; Yan et al. 1999), and restoration of the significant negative correlation between *rPer1* and *rBmal1* in the light phase of 24 m animals upon CT could be important. Interestingly these restorative effects were similar to that of melatonin (Mattam and Jagota 2014). Aging drastically affected the correlation between the clock genes in the dark phase by diminishing the transcript levels. CT restored the correlations between *Per* and *Cry* similar to melatonin (Mattam and Jagota 2014) suggesting curcumin as a potent candidate in the management of age related dysfunction of biological clock.

Sirt1 has been reported as a circadian modulator (Liu et al. 2007; Asher et al. 2008; Nakahata et al. 2008) and SIRT1/PGC-1α mediates circadian control by regulating *Bmal1* expression in aging (Chang and Guarente 2013). Interestingly, curcumin is known to activate SIRT1 and results in neuroprotection (Ayissi et al. 2014; Sun et al. 2014; Jia et al. 2016; Grabowska et al. 2017). Hence we further studied effects of aging on *Sirt1* followed by effects of curcumin on age induced alterations in daily rhythms of *Sirt1*.

The *rSirt1* transcript levels did not show significant change with aging in the SCN. Absence of any significant difference in *Sirt1* mRNA levels between young and old aged rats is consistent with an earlier report in the hippocampus and cortex of male Wistar rats (Quintas et al. 2012). In 24 m, though *rSirt1* levels did not alter, *rBmal1* expression levels were attenuated which may be due to the decline in SIRT1 activity in the old age (Braidy et al.

2015). The curcumin treatment has not shown a significant effect on *rBmal1* expression levels via *rSirt1*. This may be due to the dose and age dependent mechanism of curcumin on SIRT1.

The correlation analysis between *rSirt1* and clock genes in 3 m age group shows a tightly interlocked clock interactions, where in the dark phase *rSirt1* showed a positive correlation with *rBmal1* and a negative correlation with *rRev-erbα*. These correlations are consistent with the SIRT1 mediated circadian gene activation model (Chang and Guarente 2013). The observed negative correlations between *rSirt1* and *rPer2, rCry1* in 3 m vehicle control groups is interesting as SIRT1 enhances PER2 degradation and PER2 in turn modulates CRY1 stability (Asher et al. 2008). However, in an earlier study, it was observed that the temporal pattern of SIRT1 accumulation and its interaction with core clock binding partners such as PER2 does not correlate (Asher et al. 2008), thus corroborating these negative correlations observed in our study. In 12 and 24 m old groups, the correlation analysis showed insignificant interactions of clock components with *rSirt1* where, the critical positive and negative correlations observed in 3 m were found to be altered. Interestingly, with the curcumin administration, the lost correlations were restored between *rSirt1* and *rCry1, rCry2, rBmal1* in light phase and *rCry1, rCry2, rRev-erbα* in dark phase of 24 m animals, thus indicating curcumin's role in resetting the stoichiometric balance between the clock genes and its modulator.

Moreover, 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). And 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 young 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 middle aged and 12 h phase delay in old aged vehicle groups in comparison to young control group. Further, at protein level NRF2 expression was found to be arrhythmic both in 12 and 24 m rats.

Interestingly, curcumin had significant influence on age related alterations in nrf2 mRNA as well as protein expression rhythms. Both in middle and old age, curcumin restored the phase of *Nrf2* transcript rhythm in addition to restoring the daily pulse in old age. Further, curcumin restored the daily rhythmicity in NRF2 protein expression in middle age and old age. In old age curcumin had also resulted in its phase restoration. These results suggest one of the most important beneficial effects of curcumin. In addition to its inherent antioxidant activity, curcumin appears to modulate the expression of NRF2 and hence may influence the endogenous antioxidant enzymes which are known to decrease with advance in age.

Overall, curcumin could be influencing the circadian clock in aging SCN mainly through following possible mechanisms; (a) Curcumin being a strong antioxidant may restore clock genes via ROS elimination and by modulating NRF2 pathway (b) Curcumin's differential restoration of clock gene expression may be SIRT1 mediated, (c) Proteasome inhibiting effect of curcumin may have led to elevated clock gene expression, and (d) Curcumin could be acting through all the above mentioned mechanisms synergistically resulting in restorative effects. We have recently reported curcumin's differential restoratory effect on age associated variations in daily rhythms of clock genes and *Sirt1* expression in the SCN (Kukkemane and Jagota 2019). Our group has also reported the chronobiotic effect of curcumin in synchronizing the clock and immune genes in peripheral clock kidney (Thummadi and Jagota 2019).

In another set of experiments, in 3 m rats treated with leaf extract of ***Withania somnifera***, except *rPer2, rCry1* and *rCry2*, phases of rest of the clock transcripts were in coherence with that of 3 m vehicle group. In middle-aged animals, the WS treatment resulted in restorations in the phase of *rBmal1*, *rPer1* and *rCry1* compared to 3 m animals. Similarly, WS restored the phase of *rPer1* completely and that of *rCry1* partially in old-aged rats. These restorations are similar to the results observed with administration of melatonin and curcumin (Mattam and Jagota 2014; Kukkemane and Jagota 2019) and could be of great significance as the timing of peak expression is pivotal in mammalian clock function (Ukai-

Tadenuma et al. 2011). 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.

rRev-erbα, a negative component of auxiliary loop regulating transcriptional repression of *rBmal1,* peaked at ZT-6 and was in antiphase with the latter which is in agreement with earlier reports (Preitner et al. 2002). BMAL1-CLOCK (by binding to E-box elements) regulates the transcriptional activation of *rRev-erbα* (Preitner et al. 2002). Therefore this decrease in amplitude of *rRev-erbα* could be due to the decreased levels of *rBmal1* in SCN of old aged rats (Mattam and Jagota 2014) and levels of BMAL1 in SCN and other brain regions reported earlier (Wyse and Coogan 2010). Interestingly, WS did not alter the phase of *rRev-erbα* in any of the age groups studied, however with curcumin, 6 h phase advance in both young and middle aged animals was observed (Kukkemane and Jagota 2019).

WS restored the mean 24 h levels of *rPer2* in both 12 m and 24 m in addition to restoration of *rBmal1* mean levels in 24 m rat SCN. These restorations in mean 24 h levels are of particular interest as the earlier study with melatonin had shown significantly increased mean levels of all clock genes in all age groups with a single exception of *rPer1* in 24 m (Mattam and Jagota 2014). Curcumin had also resulted in elevated mean levels of *rPer2*, *rCry1, rCry2* and *rBmal1* levels in both 12 and 24 m rats (Kukkemane and Jagota 2019). Considering the importance of ubiquitin–proteasome system in turnover of clock proteins and their availability towards feedback (reviewed in Vriend and Reiter 2015), the age related impairment in proteasome activity (Vernace et al. 2007) would have resulted in altered mean 24 h levels of several clock transcripts including *rPer2, rCry2* in 12 m and *rPer2*, *rBmal1* in 24 m vehicle groups. Further, the restoratory effects of WS leaf extract could be attributed to the proteasome inducing activity of active constituents like Withanone in Ashwagandha leaves (Widodo et al. 2009).

The interactions among various clock transcripts in photo phase and scotophase were analyzed by Pearson pairwise correlation. The analysis revealed a strong negative correlation between *rBmal1* and *rRev-erbα* in both LP and DP of young rats which

corroborates the earlier reports on their anti-phasic nature (Preitner et al. 2002). Aging abolished these correlations in both photo and scotophase of middle and old aged animals. Interestingly, WS established a weak negative correlation between *rBmal1* and *rRev-erbα* in LP of both mid and old aged rat SCN. However a significant restoration in these correlations were observed in middle aged animals treated with curcumin (Kukkemane and Jagota 2019). Moreover, significant positive correlation between *rPer1* and *rRev-erbα* which was present in LP of 3 m SCN was found to be negative in DP. These correlations were lost upon aging in 12 m and 24 m vehicle groups. WS leaf extract re-established the significant positive correlation in LP and negative correlation in DP between *rPer1* and *rRev*-erbα. The negative correlation between *rPer1* and *rPer2* in LP of both mid and old aged vehicle treated animals were abolished by WS, thus establishing a positive correlation. Further, the role of WS in resetting the significant negative correlation between *rPer1* and *rBmal1* in both LP and DP of 24 m animals is 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 show daily rhythmicity in transcript levels of *rSirt1* with peak expression at mid subjective night and nadir at day-night transition in the SCN of young rats. This is similar to a previous report where the maximum *Sirt1* mRNA levels were at dark phase in SCN of young mice (Chang and Guarente 2013). 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 substantially 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, WST resulted in its restoration (Kukkemane and Jagota 2019). The declined mean 24 h levels and attenuated rhythm of *rBmal1* in the SCN of 24 m old rat could be due to the abolished *rSirt1* daily rhythm in the same age group. 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 expression in the same group. 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).

Interestingly, WS resulted in restoration of phase of *rNrf2* in middle age and restored the mean 24 h levels in old age 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, 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. 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).

Objective 2

Understanding role of SIRT1 in restoratory effects of curcumin and *Withania somnifera* leaf extract using its inhibitor Nicotinamide (NAM) *ex vivo*

The age induced dysregulation of circadian clock mechanism has been well known and previous studies from our group as well as other researchers have shown alterations in clock genes expression in the SCN (Bonaconsa et al. 2014; Mattam and Jagota 2014). Interestingly, several reports have linked the histone deacetylase SIRT1 to the molecular mechanism of clock regulation (Asher et al. 2008; Nakahata et al. 2008; Ramsey and Bass 2011; Masri and Sassone-corsi 2014). SIRT1 activity in several tissues and various brain regions were known to decrease with advance in age (Quintas et al. 2012; Braidy et al. 2015) and this decrease was demonstrated to have a significant role in age associated circadian dysfunction (Chang and Guarente 2013). In agreement, our results suggested that SIRT1 activity shows temporal pattern of expression in SCN with maximum activity during the mid-subjective night and the activity significantly reduces with advance in age. As our results from objective 1 indicated a differential restoratory effect of both curcumin and WS on age linked variations in temporal expression of canonical clock genes (Jagota and Kowshik 2017; Kukkemane and Jagota 2019), we hypothesized the involvement of SIRT1 in these restoratory effects. To test the hypothesis, SIRT1 was selectively inhibited in SCN explants (harvested from 3 m rats) using nicotinamide and expression of clock genes were measured. In control SCN all the clock genes showed rhythmicity in mRNA expression wherein *rBmal1* peak was at mid-subjective night, *rPer1*, *rPer2*, *rCry1*, *rCry2* maximum expressions were at day-night transition and *rRev-erbα* peak at mid-subjective day. These pattern of expressions were similar to the daily rhythms of respective genes in the SCN of 3 m rats (Objective 1). Interestingly, when SCN cultures were treated with curcumin (CT) or *Withania somnifera* (WST) the expression of these clock genes were not affected and the phase of the rhythms of all the clock genes were in coherence with that of controls. Similarly, mean 24 h levels and daily pulse of majority of clock genes studied were not affected upon CT or WST in comparison to controls.

However, inhibition of SIRT1 in SCN explants resulted in significant alterations in mRNA expression of these clock genes. SIRT1 inhibition was associated with phase advances in rhythms of *rBmal1*, *rCry1* and *rCry2*, phase delays in *rPer1* and *rRev-erbα*, in addition to arrhythmic expression of *rPer2*. Moreover, mean 24 h levels of *rPer1*, *rPer2* and *rCry1*

expression significantly increased upon SIRT1 inhibition. These results indicate a dysregulated clock mechanism represented by alterations/abolitions in rhythmic expression of clock genes as an outcome of reduced SIRT1 activity, a condition similar to aging (Chang and Guarente 2013). Curcumin is known to elicit neuroprotective effects via SIRT1 activation (Jia et al. 2016; Grabowska et al. 2017). However, the underlying mechanism in its clock restoratory role has been elusive. Thus, to understand the involvement of SIRT1 in curcumin's rhythm restoratory effect on clock genes, we co-treated SCN explants with curcumin and NAM (CT+NAM). Our results showed that when SIRT1 was inhibited in presence of curcumin, the rhythmicity of all the clock genes were altered in comparison to control and CT group, with an exception to *rPer1*. Moreover, the increased mean 24 h levels of *rPer1*, *rPer2* and *rCry1* observed in NAM treated SCN persisted in CT+NAM group as well. These results suggest that curcumin is unable to protect/restore the phase and rhythms of majority of clock genes upon SIRT1 inhibition.

In a similar set of experiments, SIRT1 was inhibited in SCN explants in the presence of WS (WST+NAM). Interestingly, the rhythms and phases of *rBmal1*, *rPer2* and *rCry1* transcripts were protected from major alterations and were similar to control and WST group. Further, restoration in the amplitude of *rCry2* rhythms and partial restoration in the phase of *rRev-erbα* were observed. In addition mean 24 h levels of *rPer1*, *rPer2* and *rCry1* were significantly reduced and these levels were comparable to the respective mean levels in control and WST. These results suggest that WS can show protective/restoratory effects on majority of clock gene expression rhythms even when SIRT1 activity is suppressed.

Overall, our *ex vivo* studies suggested that, curcumin may be majorly acting via SIRT1 towards restoratory effects on clock genes observed in our study whereas, WS leaf extract may involve SIRT1 independent mechanisms.

Objective 3

Effect of curcumin and *Withania somnifera* leaf extract on age induced alterations in chronomics of components of serotonin metabolism

All the components of 5-HT metabolism exhibited daily rhythmicity in their levels in the SCN of 3 m rats which were similar to earlier report from our laboratory (Reddy and Jagota 2015). With curcumin treatment, daily rhythms of all the components except NAS were in coherence with controls in 3 m. Similarly with WS treatment, daily rhythms of all the ten components were similar to young control group. Mean 24 h levels of NAS, 5-HTOH and NAT increased in 3 m CT group compared to young controls, whereas in 3 m WST group, mean 24 h levels of all the metabolites were similar to 3 m controls. Persistence of daily rhythms and mean levels in both curcumin and WS administered young animals with minimum alterations confirms the pharmacological safety of both natural compounds (Lao et al. 2015; Patel et al. 2016).

Aging differentially altered the daily rhythmicity and 24 h mean levels of most of the components analyzed. The age induced changes in rhythms and levels of TRP and all other metabolites has been reported (Reddy and Jagota 2015). Similarly, in the SCN of RIPD rat model 5-HTP and MEL rhythms were found to be abolished in addition to phase alterations in rest of the components (Mattam and Jagota 2015). Both curcumin and WS had shown beneficial effects in resetting clock genes expression in SCN of aged rats (Jagota and Kowshik 2017; Kukkemane and Jagota 2019), similarly they exhibited differential restoratory effects on components of 5-HT metabolism.

TRP as an important essential amino acid in addition to being precursor of 5-HT and MEL plays key role in several sleep disorders (Kalużna-czaplińska et al. 2017; Bravo et al. 2018). TRP levels are known to significantly decrease in PD and AD patients (Kalużna-czaplińska et al. 2017). The diminished mean 24 h TRP levels were restored in middle age by WST whereas CT restored it in old age. Interestingly, TRP daily rhythm was abolished in middle age which was restored with CT. Alterations in TRP metabolism in aging and associated pathologies has been reported (van der Goot and Nollen 2013).

5-HTP, an immediate metabolite of TRP plays important role in suppressing the oxidative damage (Kayacan et al. 2019). TPH, the enzyme converting TRP to 5-HTP shows maximum concentration during the subjective day in rat SCN (Barassin et al. 2002), and in agreement our results show 5-HTP maximum levels at day-night transition in young age.

With advance in age, 5-HTP showed 6 h phase advance in both middle and old age in addition to considerable surge in mean 24 h levels in old age. CT restored 5-HTP phase in middle age whereas both CT and WST restored the mean levels in old age. TPH activity is known to decline with aging (Hussain and Mitra 2004) and exogenous melatonin administration was shown to increase TPH activity in rats (Moranta et al. 2014). In agreement, exogenous melatonin had also restored 5-HTP mean levels in old age (Reddy and Jagota 2015). Polyphenol antioxidants such as Silymarin, Quercitin and Naringenin were reported to increase TPH activity in aged rat brain in addition to increasing SIRT1 levels (Sarubbo et al. 2018). Curcumin had restored the daily rhythms and daily pulse of Sirt1 mRNA expression in aged rat SCN (Kukkemane and Jagota 2019).

5-HT is important in clock functioning and it is known to have phase advancing effect on neuronal firing rhythms in SCN (Prosser 2003). Daily variations in brain 5-HT levels were found to be arrhythmic middle age onwards and mean 24 h levels declined significantly (Jagota and Kalyani 2008). However, in SCN daily rhythmicity of 5-HT persisted with advance in age though 6 h phase advanced in old age and mean levels were unaltered (Reddy and Jagota 2015). WS restored 5-HT amplitude in 12 m and phase in 24 m by resetting the maximum levels to mid-subjective day. In addition it increased 5-HT daily pulse in 24 m. Moreover, the increase in 5-HT mean 24 h levels in middle age upon CT is consistent with increase in serotonin levels observed with various polyphenols in aged rats (Sarubbo et al. 2018).

AANAT, the enzyme that converts 5-HT to NAS follows temporally gated expression pattern with maximum levels at night time and is known to decline in old age (Kim et al. 2007; Sarlak et al. 2013). NAS daily rhythms persisted in SCN of middle and old aged rats, however, mean 24 h NAS levels increased whereas daily pulse decreased with advance in age. Both CT and WST decreased mean NAS levels suggesting restoration. Interestingly, melatonin treatment had also resulted in similar effect on NAS mean levels (Reddy and Jagota 2015). These restorations could be crucial as NAS plays many important roles ranging from free radical scavenging to melatonin signaling (Alvarez-Diduk et al. 2015; Jockers et al. 2016).

Daily rhythmicity in MEL levels persevered in middle and old age albeit with reduced robustness. Both curcumin and WS increased the MEL levels during the dark phase particularly in middle age. Although NAS mean levels elevated upon aging, MEL mean levels were found to be declining which corroborates earlier reports (Liu and Borjigin

2005). Moreover, activity and expression of HIOMT is known to reduce with aging (Sarlak et al. 2013). CT in middle age, restored mean 24 h MEL levels whereas WST increased the daily pulse of MEL.

5-HT and 5-HIAA levels alter in region specific manner in aging brain. In hippocampus and striatum the levels declined in old age (Sarubbo et al. 2015) whereas in SCN 5-HT levels were unaltered but 5-HIAA mean levels increased. Both curcumin and WS had resulted in decreased mean 5-HIAA levels similar to polyphenol resveratrol (Sarubbo et al. 2015). In addition, altered phase of 5-HIAA and NAT were restored in 12 m WST rats. Daily rhythm of 5-MTOH was restored in middle age by both curcumin and WS.

In old age, further phase alterations were observed in daily rhythms of 5-HTOH and NAT in addition to attenuated rhythms of 5-MIAA and 5-MTOH. Curcumin exhibited minimum effect in 24 m however, WS restored the phase of 5-MIAA and rhythmicity of 5-MTOH in old age. Earlier study with exogenous melatonin administration had resulted in restoration in daily rhythmicity of 5-MTOH in SCN of 12 and 24 m old rats (Reddy and Jagota 2015).

The pairwise correlation analysis showed strong interactions among various components of serotonin metabolism. Aging differentially altered the correlations indicating impairment in the stoichiometric balance and such impairments are linked to decreased buffering capacity and clock dysfunction (Jagota et al. 2019). Interestingly, a significant negative correlation between 5-HT and MEL persisted in LP and DP of all the age groups. Both curcumin and WS showed differential restorations on correlations. CT restored the negative correlation between 5-HTP:5-HT and TRP:NAT in LP of middle and old aged rats. However, WS restored these negative correlations only in middle age. Exogenous melatonin had also showed differential restorations in correlations between 5-HT and other components mainly in old age (Reddy and Jagota 2015).

Objective 4

Effect of curcumin and *Withania somnifera* leaf extract on age induced alterations in chronomics of melatonin receptor expression

Melatonin membrane receptors have been previously reported to be located in SCN of mammals (Dubocovich and Markowska 2005). In agreement with previous reports, our results show the expression of both MT1 and MT2 mRNA as well as protein in the hypothalamic SCN of Wistar rats. In young rats, both the receptors exhibited typical daily rhythmicity in mRNA and protein expression with remarkable similarity in their pattern which was also observed in an earlier study in the rat liver (Venegas et al. 2013). MT1 mRNA showed maximum expression at mid-subjective night (ZT-18) and minimum expression at the beginning of light phase (ZT-0). At protein level however, maximum MT1 expression was at day-night transition (ZT-12) and minimum at mid-subjective day (ZT-6). These expression pattern were in agreement with previous report where the maximum expression of MT1 receptor was seen at night phase (Masana et al. 2000). Interestingly, MT2 mRNA expression peaked at ZT-12 which is 6 h in advance compared to MT1 and minimum expression was at ZT-0. MT2 protein expression was in coherence with that of MT1 with maximum and minimum expressions at ZT-12 and ZT-6 respectively. Rhythmic expression of these receptors could be important as melatonin secreted from pineal gives temporal feedback to circadian oscillators in the SCN via these receptors thus influencing clock gene expression (Vriend and Reiter 2015). It has been shown that MT1 receptor is essential for mediating melatonin's effect on clock gene expression in other regions of the brain and rhythmic expression of clock proteins in pituitary gland (Jilg et al. 2005; Imbesi et al. 2009).

Our results show that the maximum expression of these receptors coincide with elevated serum melatonin levels (Masana et al. 2000; Karasek 2004) and also with peaking time of *Bmal1,* which constitutes the positive limb of transcriptional translational feedback loops (TTFL) (Abe et al. 1998; Mattam and Jagota 2014). Melatonin's inhibitory action on proteasome is proposed to have a role in stabilizing BMAL1 protein in the SCN (Vriend and Reiter 2015). Further, *Rev-erbα,* the suppressor of *Bmal1* is reported to show peak expression at mid-subjective day which is phase opposed to MT receptors expression (Preitner et al. 2002; Kukkemane and Jagota 2019). Considering the reported role of MT1 in inhibition of CREB phosphorylation and clock gene regulation (Dubocovich and

Markowska 2005), *Period* genes which are CRE dependent negative TTFL elements are expected to be in antiphase with MT1 (Travnickova-Bendova et al. 2002). Our results corroborate this anti-phasic expression as melatonin receptors show maximum expression in the dark phase as opposed to the light phase maximum expression of *Period* genes in SCN (Yan et al. 1999; Mattam and Jagota 2014). These results are consistent with reported suppressive role of melatonin on the expression of *Per1* (von Gall et al. 2002). Further, the interplay between melatonin and NAD^+ dependent histone deacetylase SIRT1 in modulating circadian clock has been evident (Jung-Hynes et al. 2010; Mayo et al. 2017). In agreement with this, daily rhythms of SIRT1 mRNA and protein expression in the SCN (Chang and Guarente 2013; Kukkemane and Jagota 2019) were in coherence with MT1 and MT2 receptor expression pattern in young age thus suggesting the possible interaction between melatonin and SIRT1 in governing robust circadian function (Hardeland 2017).

Aging significantly altered the expression of both the receptors in SCN at mRNA as well as protein level. Though MT1 mRNA expression in middle age did not show phase alterations in daily rhythmicity, the mean 24 h levels and daily pulse significantly decreased. In old aged animals we found delay in phase as the maximum expression was at ZT-0 as opposed to ZT-18 in SCN of young animals. Daily pulse of MT1 mRNA also showed significant decline in old age. Daily rhythmicity in MT1 protein expression was abolished in both 12 and 24 m indicating age induced loss of temporal regulation in receptor turnover. These results are consistent with an earlier report where aging SCN was shown to be associated with loss of sensitivity to melatonin (von Gall and Weaver 2008). Similarly, daily rhythmicity of MT2 mRNA persisted in middle age but with attenuated robustness and there was a 6 h phase delay in comparison to young age. In old age, similar to MT1, MT2 mRNA peak expression was at ZT-0 indicating 12 h delay in phase. Daily pulse of MT2 transcripts reduced in both middle and old age whereas, mean levels declined in middle age. However, MT2 protein expression was arrhythmic in SCN of both 12 and 24 m SCN indicating age related abolition of day night variation in receptor expression. These age induced alterations in expression of both the receptors in SCN is interesting as an earlier study in several peripheral clocks of male Wistar rats had shown significant decrease in mRNA (MT1, MT2) and protein (MT1) expression by middle age with an exception to thymus (Sanchez-Hidalgo et al. 2009). However, our results indicate decline in mean 24 h protein levels of MT2 receptors in old age suggesting age and tissue specific

alteration of melatonin receptor abundance. Moreover, the loss of rhythmicity in MT1 and MT2 protein expression upon aging as observed in our study can be linked to age associated decrease in melatonin levels (Pandi-Perumal et al. 2005). Corroborative data for the role of melatonin in rhythmic receptor expression originates from an earlier report where both the receptors showed attenuated rhythms upon pinealectomy (Venegas et al. 2013). Further, previous studies in aging SCN has shown severe variations in temporal oscillations in clock genes expression (Wyse and Coogan 2010; Mattam and Jagota 2014). The age associated phase delays in MT1, MT2 transcripts and abolished protein expression rhythms observed in the present study correlates with similar phase alterations and attenuated rhythms of *Periods*, *Cryptochromes* and *Bmal1* in aged SCN (Mattam and Jagota 2014) signifying the interlinking mechanisms of clock disruption.

Curcumin, a pharmacologically safe antioxidant with enormous health benefits (Tsai et al. 2011; Eckert et al. 2013; Grabowska et al. 2017; Vidal et al. 2017) has been reported to activate BMAL1 and also show clock restoratory effects (Jagota and Reddy, 2007; Wang et al. 2008, 2010; Kukkemane and Jagota 2019). In our study involving melatonin receptors, curcumin showed robust restoratory effects in old aged animals in comparison to middle age. The daily rhythms of MT1 and MT2 mRNA in 12 m animals treated with curcumin were found to be attenuated similar to respective age matched controls. However at protein level, curcumin restored both rhythmicity and phase of MT1 receptors in middle age. Whereas, curcumin did not show significant restoratory effect on daily rhythms of MT2 protein expression in middle age. Interestingly, we have observed phase restorations of *Period* and *Cryptochrome* genes in the SCN of 12 m old rats treated with curcumin in objective 1 of our study (Kukkemane and Jagota 2019). These results indicate that similar to melatonin, curcumin may also influence gene expression mainly via MT1 receptors (Imbesi et al. 2009). In old aged animals, the phase of MT1 mRNA daily rhythm was partially restored as curcumin treatment delayed the peak expression to day-night transition (ZT-12). Further, the phase of MT2 mRNA daily rhythm was restored completely as its maximum expression in young controls was at ZT-12. In addition to these phase restorations, curcumin increased the amplitude of the rhythms of both MT1 and MT2 transcripts in old age. Further, daily rhythms of both MT1 and MT2 receptor proteins showed restoration with dark phase maximum expression in old age animals treated with curcumin. Curcumin also restored the mean 24 h levels of MT2 protein in old age. These

restorations in MT receptor expression is in agreement with curcumin-induced restorations in clock genes expression in old aged rats (Kukkemane and Jagota 2019). Moreover, curcumin is reported to elicit neuroprotective effects via SIRT1 (Jia et al. 2016; Grabowska et al. 2017) and in our earlier study we have seen restorations in *Sirt1* mRNA expression in the SCN of 24 m old rats with curcumin administration (Kukkemane and Jagota 2019). As melatonin and SIRT1 are known to synergistically affect circadian clock function (Jung-Hynes et al. 2010; Hardeland et al. 2012), our results cumulatively indicate a potent therapeutic role for curcumin in old age associated clock dysfunction.

Withania somnifera leaf extract with several biologically active constituents has been reported to show anti-aging and neuroprotective effects along with strong free radical scavenging activity (Widodo et al. 2009; Manchanda et al. 2016; Jagota and Kowshik 2017). We have vividly discussed the therapeutic role of WS in brain aging and clock dysfunction (Jagota and Kowshik 2017). In the present study, WS treatment showed differential restoratory effects on age induced alterations in melatonin receptor expression. In middle age, attenuated rhythms of MT1 and MT2 transcripts persisted with WS administration, but the daily pulse of MT2 mRNA was restored in this age group. Similarly, the abolished daily rhythms of MT receptor proteins in the SCN of 12 m animals persevered with WS treatment. However, WS had shown phase restorations in daily rhythms of *Per1*, *Cry1* and *Bmal1* in SCN of 12 m old rats (Jagota and Kowshik 2017), suggesting an independent clock restoratory mechanism of WS (Das et al. 2002; Konar et al. 2011). However, similar to curcumin, WS also showed significant restoratory effects in old age. The maximum expression of MT1 and MT2 mRNA was reset to night phase in 24 m old rats treated with WS indicating restoration. Similarly, the abolished rhythms of MT receptor proteins in old age were restored with both the receptors showing maximum expression at ZT-18. In addition, the mean 24 h MT2 protein expression also restored in old aged animals upon WS administration. These results are consistent with WS induced restoration in phase and daily pulse of *Per1* mRNA expression and Mean 24 h levels of *Per2* and *Bmal1* in 24 m old rats (Jagota and Kowshik 2017). The observed restorations in melatonin receptor expression upon WS administration could be of great therapeutic significance as WS is a well-known neuro-stimulant and reported to show sleep promoting effects (Wadhwa et al. 2016; Kaushik et al. 2017).

Melatonin administration has been shown to exhibit beneficial effects in various aging and associated neurodegenerative diseases (Jagota 2012; Jagota et al. 2019). In addition to its chronobiotic property, melatonin is known to possess strong free radical scavenging activity which makes it a potential therapeutic candidate against age related ailments including circadian dysfunction (Tan et al. 2015). In our study, exogenous melatonin administration resulted in significant restorations in melatonin receptor expression in aging SCN. Interestingly, both the receptors displayed very similar pattern in daily rhythms of mRNA expression across the age group upon melatonin treatment. In middle aged animals, the mean 24 h levels of both MT1 and MT2 receptor transcripts were restored, though daily rhythms remained attenuated. Interestingly, at protein level there was restoration in the phase and daily rhythms of both the receptors in this age group. These restorations in receptor expression rhythms are consistent with the restorations in clock genes expression in the SCN of 12 m old rodents treated with melatonin (Mattam and Jagota 2014). However, an earlier study in striatal neurons had demonstrated melatonin's differential effect on clock gene expression via MT1. *Clock* and *Per1* expression decreased whereas *Npas2* increased while *Bmal1* was unaltered (Imbesi et al. 2009). In old aged animals, melatonin partially restored the phase of MT1 and MT2 mRNA in addition to increasing the robustness of the daily rhythms. Also mean 24 h levels of both the transcripts considerably elevated in old age. This increase in mean 24 h mRNA expression is similar to our earlier study where melatonin with its proteasome inhibitory effects had resulted in elevated mean 24 h levels of *Bmal1, Cry1, Cry2* and *Per2* in old aged rats (Mattam and Jagota 2014; Vriend and Reiter 2015). Further, the daily rhythm of MT1 receptor protein was restored in old aged animals. Interestingly, melatonin restored both phase and daily rhythm of MT2 in addition to restoring its mean 24 h levels in old age. Melatonin had similar rhythm restoratory effect on *Period* genes in the SCN of old age (Mattam and Jagota 2014) which further signifies the MT receptor mediated regulation of *Period* genes in the central clock (Kandalepas et al. 2016). Melatonin's restoratory effect on MT2 is of great significance as MT2 receptors were reported to mediate melatonin-induced defense against redox stress and impaired memory in a rodent model of aging (Shin et al. 2015).

Overall, our results indicate significant alterations in daily rhythms and levels of melatonin receptors (MT1 and MT2) expression at both mRNA and protein level which may contribute to age associated circadian dysfunction. Exogenous melatonin, curcumin and

WS leaf extracts show differential restoratory effects on these receptors suggesting therapeutic role for these antioxidants.

Objective 5

Effect of curcumin and *Withania somnifera* leaf extract on age induced alterations in gross locomotor activity rhythms

Studies on gross locomotor behavior in aging rats indicated an age dependent dysregulation of rest-activity pattern. Robust night time activity observed in young rats progressively decreased with advance in age. On the other hand, activity during subjective day increased in middle and old age compared to young rats. The age associated alterations in locomotor activity rhythms observed in our study is consistent with earlier reports (Reddy and Jagota 2014; Gubin and Weinert 2016). Our results suggest that curcumin administration does not have a significant impact on the gross locomotor activity in any of the age groups studied. However, WS significantly increased the locomotor activity during subjective night and decreased it during the subjective day in middle age, suggesting a beneficial role. Sleep promoting effects of WS leaf extract and its neuroprotective role in sleep disturbed rodents has been demonstrated (Manchanda et al. 2016; Kaushik et al. 2017). In old age, WST did not show significant effects on motor behavior which might be due to the dose dependent effects of active constituents such as triethylene glycol (Kaushik et al. 2017).

Overall, results of our experiments recording the gross locomotor behavior of nocturnal rodents of three age groups indicated an age dependent decline in robust night activity and the beneficial effect of WS in middle age.

SUMMARY AND CONCLUSION

Age associated deterioration in CTS has been an important subject of interest as emerging studies from across the world hints at strong interlinks between circadian disruption and various pathologies of aging including neurodegenerative disorders. Our laboratory has extensively studied the dysregulation in circadian clocks in aging and PD in rat models previously and reported alterations in various parameters of central and peripheral clocks. In addition, the therapeutic properties of melatonin has been investigated on such age linked alterations. Based on the previous observations, the present study was designed to understand the effects of antioxidants from natural sources on age associated Chronodisruption. In the present study, two such candidates: curcumin and hydro-alcoholic leaf extract of *Withania somnifera* with potent antioxidant properties were administered to rats of three different age groups and their effects were studied on chronomics of various parameters in the central clock SCN. Our gene expression studies revealed that canonical clock genes such as *rBmal1, rPer1, rPer2, rCry1, rCry2, rRev-erbα* and clock modulator *rSirt1* as well as clock controlled gene *rNrf2* expression were altered with advance in age. In addition, age associated alterations in daily rhythms of SIRT1 and NRF2 were observed at protein level as well. Moreover, correlation analysis indicated the alterations in the interactions among clock genes and also between clock genes and *Sirt1* upon aging.

Both curcumin and WS showed differential restoratory effects on the daily rhythms, mean levels, daily pulse and correlations of clock genes, *Sirt1* and *Nrf2* suggesting their potential therapeutic properties. Curcumin in middle age restored the phase of *rPer1, rPer2, rCry1, rCry2* and *rSirt1*, while restoring phase of *rPer1* and rhythm of *rSirt1* in old age. Moreover, it restored the daily pulse of *rPer2* in middle age and that of *rPer1, rSirt1* and *rNrf2* in old age. Further, curcumin also resulted in restoration of SIRT1 phase in middle age and NRF2 daily rhythm in old aged rats. Interestingly, the correlation analysis between various clock genes and *Sirt1* indicated the presence of significant interactions in young age which severely alter with aging. Curcumin treatment restored these interactions in age dependent manner suggesting a possible mechanism of curcumin via Sirt1. Similarly, WS administration also showed restorations in *rPer1, rCry1, rBmal1* and *rNrf2* phases in middle age in addition to restoring phase of *rPer1* and rhythm of *rSirt1* in old age. *rPer2* and *rBmal1* mean 24 h levels were restored in old age in addition to age specific restorations in correlations. WS also restored the phase and daily rhythm of SIRT1 and NRF2 protein expression in middle age. Overall curcumin showed robust restoratory effect on clock genes in comparison to WS. Further to understand the possible mechanism of

restoratory effects of curcumin and WS on clock genes, SCN explant cultures were maintained followed by SIRT1 inhibition *ex vivo.* Our results indicated that curcumin may possibly involve SIRT1 for its restoratory effects on clock genes whereas, WS may involve SIRT1 independent mechanisms.

Age associated alterations in the daily rhythms, levels and stoichiometric interactions of components of 5-HT biosynthesis and metabolism in SCN has been previously studied in our laboratory and in the present study the effects of curcumin and WS were probed. While curcumin restored TRP, 5-HTP and Melatonin daily rhythms and mean levels in middle age, in old age it restored mean levels of 5-MIAA and maximum/minimum ratio of 5-HTOH. Whereas, WS showed robust restorations in the daily rhythms of serotonin, NAS, Melatonin, NAT and 5-HIAA in middle age, in addition to restoring mean levels of TRP, NAS, 5-HIAA, 5-MIAA, 5-HTOH and 5-MTOH. In old age, WS restored serotonin and NAS daily rhythms and 5-HTP, 5-HTOH, NAS, 5-MIAA, 5-MTOH mean levels and 5-HTOH, NAT daily pulse. However, both curcumin and WS showed age and component specific effect on correlations among the metabolites. Overall, WS elicited substantially robust restoratory effects on components of serotonin metabolism in comparison to curcumin.

Moreover, our studies on expression of melatonin receptors in the SCN confirmed the temporal expression pattern of MT1 and MT2 transcript as well as protein levels in young rats. Aging significantly affected the daily rhythms in expression of these receptors. MT1 and MT2 mRNA expression were arrhythmic in middle age and phase advanced in old age. The daily rhythms of both the receptor proteins were attenuated in middle as well as old age. Curcumin restored the phase and daily rhythms of MT1, MT2 mRNA as well as protein in old age whereas, in middle age it restored rhythmic MT1 protein expression. Similarly, WS restored the phase and daily rhythms of MT1, MT2 mRNA as well as protein in old age. However, the multitasking molecule melatonin (when exogenously administered) restored the mean levels of both MT1 and MT2 mRNA in middle age in addition to restoring the daily rhythms of MT1, MT2 protein expression in both middle and old age. Our results suggest that the administration of melatonin and curcumin to aged rats have significantly higher restoratory effects on melatonin receptors expression in comparison to WS.

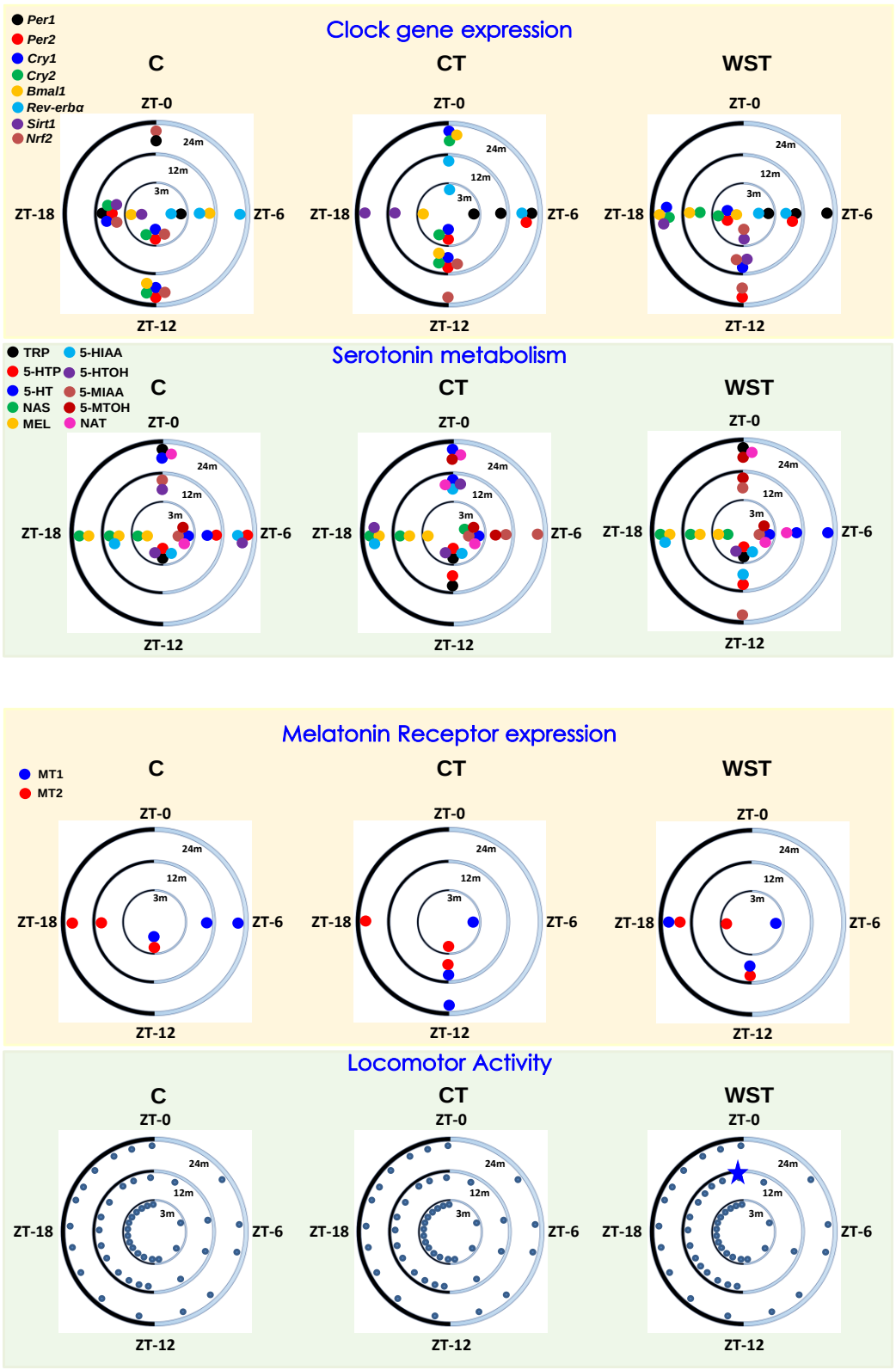


Fig. 58 Diagrammatic summary showing age linked alterations in time points of peak expression/levels of various components studied at gene, protein and metabolite levels in addition to alterations in gross locomotor activity. Differential restorative effects of CT and WST. Blue half of the circle – subjective day; black half of the circle – subjective night.

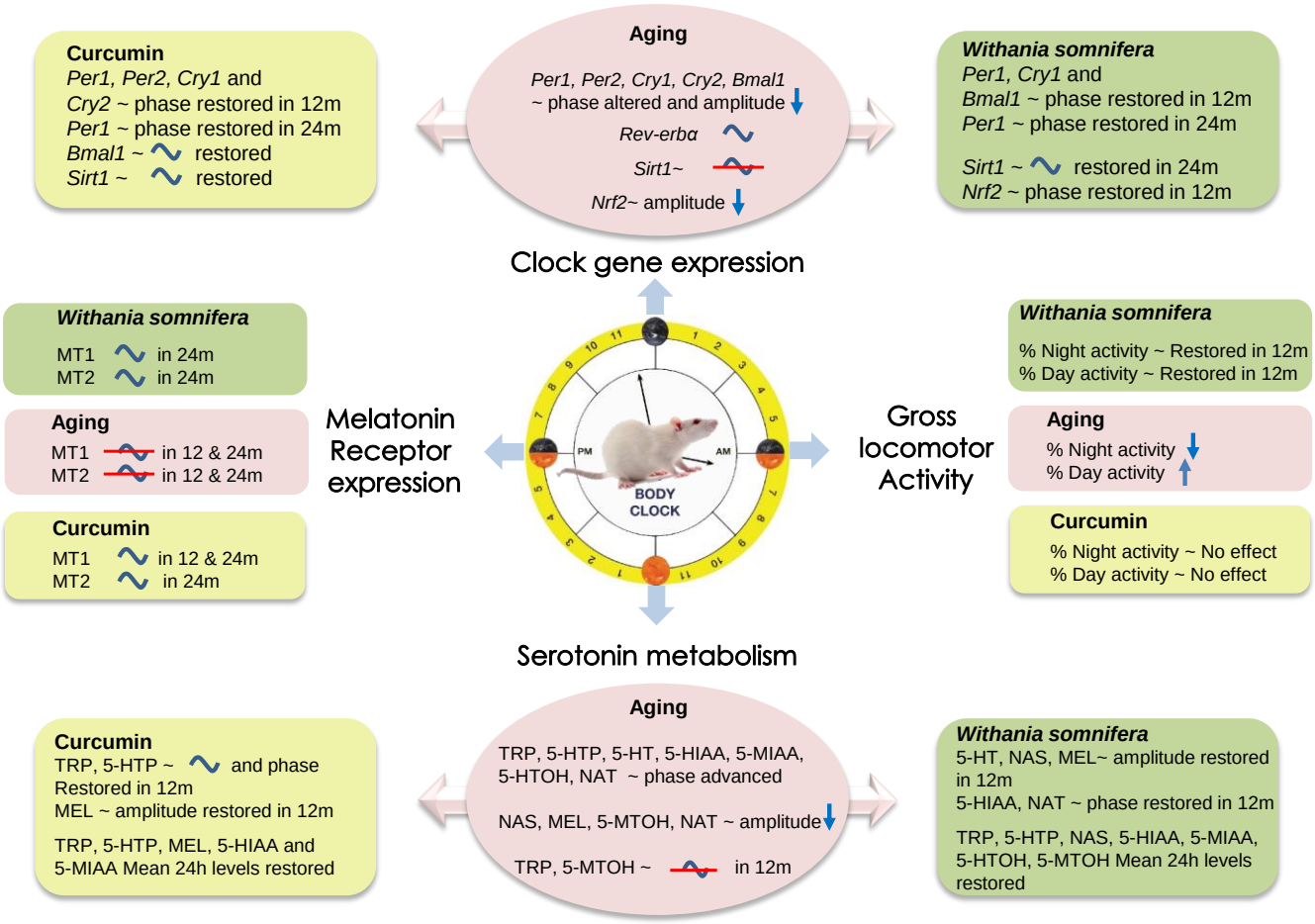


Fig. 59 Schematic representation of overall summary of the present study. Differential restoratory effects of curcumin and leaf extract of *W. somnifera* on chronomics of age induced alterations in clock genes, SIRT1 and NRF2 expression, Serotonin metabolism and Melatonin receptor expression in the SCN of male Wistar rats as well as Gross locomotor activity.

Finally behavioral experiments studying the gross locomotor activity in rats showed an age dependent decline in activity during the night and increase in activity during the day suggesting age dependent dysregulation of activity-rest cycles. Curcumin treatment did not have a significant effect on the gross locomotor activity in any of the age groups studied. Interestingly, WS showed a beneficial effect in middle age. Upon WS treatment night time activity in middle aged rats significantly increased compared to age matched controls and day activity was found to be decreased suggesting restoration. However, in old age WS did not show significant effect on locomotor activity.

In conclusion, age-related circadian dysfunction is a global health challenge which is marked by alterations in various aspects of the central clock including desynchronizations in gene/protein expression, variations in metabolite networks, dysregulated receptor functioning and behavioral rhythms. As most of these ailments arise from failure of repair mechanisms there is a necessity to test affordable and safe prospective drug candidates from natural sources. Pleiotropic drug candidates like curcumin and WS could be highly efficacious in healthy aging considering their enormous beneficial effects and pharmacological safety. In the present study, both curcumin and leaf extract of WS showed differential restoratory effects of on various age induced alterations in the SCN (Fig. 58 and Fig. 59). Our results reveal the potential therapeutic and chronobiotic properties of these natural compounds in managing age associated dysfunction of circadian timing system.

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APPENDICES

In vitro antioxidant assay

The free radical scavenging activity of curcumin, hydro-alcoholic extracts of WS leaf and root were studied using DPPH assay (Brand-Williams et al. 1995; Jagota and Kowshik 2017).

Briefly, 1 mg/ml stock solutions of curcumin, WS leaf extract and root extract in 95 % Methanol were prepared. A range of working stocks from 1 – 1000 µg/ml were used for the assay. 0.1 mM diphenyl-1-picryl hydrazyl (DPPH) (from Sigma) in 95 % Methanol was prepared in dark and stored in amber colored bottle. 0.3 ml from working stocks were added to 1.2 ml of 0.1 mM DPPH solution. Vertexed and incubated at room temperature for 20 minutes in dark. Color changes from deep violet to light yellow. Absorbance was measured at 517 nm. Assay was carried out similarly with a positive control ascorbic acid. The scavenging activity was calculated using the following formulae:

% scavenging activity = $[A_0 – A_1/A_0]$ X 100

Where, A_0 – absorbance of color reaction (DPPH + Methanol);

A_1 – absorbance of test compound

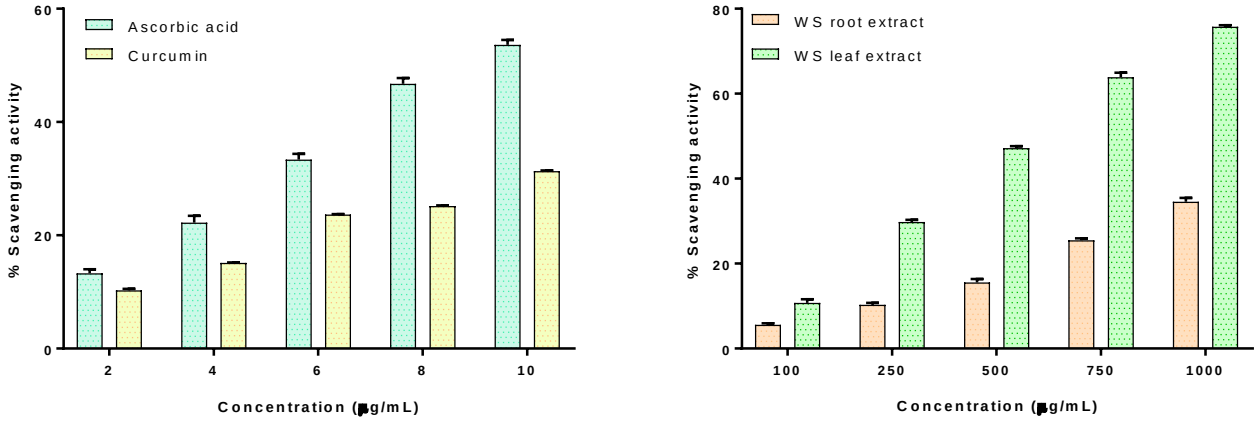

Fig. 60 DPPH free radical scavenging activity assay. Scavenging activities (represented as % scavenging activity at concentration per milliliter of test compound): Curcumin – 36 % at 10 µg/mL; WS leaf extract - 78 % at 1 mg/mL; WS root extract – 38 % at 1mg/mL

APPENDIX – II

List of Figures

Fig. 1 Basic organization of the circadian time keeping system. Input to the clock from the external environment, integration of information by the clock and output from the clock to temporally regulate physiology.

Fig. 2 (a) and **(b)** Location of suprachiasmatic nucleus (SCN) in the mammalian brain. **(c)** Coronal brain section containing the SCN as observed under dissecting microscope. **(d)** Coronal section of the SCN showing ventrolateral core region with gastrin releasing peptide (GRP) neurons (green) and the dorsomedial shell region with arginine vasopressin (AVP) neurons (red).

Fig. 3 (a) Major afferent pathways to the SCN. Photic inputs are represented by orange arrows; non-photic inputs are represented by blue arrows. RHT – retino-hypothalamic tract, GHT – geniculo-hypothalamic tract, IGL – intergeniculate leaflet, DRN – dorsal raphe nuclei, MRN – median raphe nuclei, Glu – glutamate, PACAP – pituitary adenylyl cyclase activating peptide, 5-HT – serotonin, NPY- neuropeptide Y, GABA – γ amino butyric acid. **(b)** Major efferent pathways from the SCN (red arrows). sPVZ – subparaventricular zone, PVN – paraventricular nucleus of hypothalamus, DMH – dorsomedial hypothalamus, POA – preoptic area, BNST – bed nucleus of the stria terminalis, LS – lateral septum, ARC – acuate nucleus, AMY – amygdala, IGL – inter-geniculate leaflet, HB – habenula, PVT – paraventricular nucleus of thalamus.

Fig. 4 Diagrammatic illustration of circadian time keeping system in mammals. Temporal regulation of pineal melatonin synthesis and release. RHT – retino-hypothalamic tract, PACAP – pituitary adenylyl cyclase activating peptide, cAMP – cyclic adenosine monophosphate, PKA – protein kinase A, NO – nitric oxide, CaMK – calmodulin kinase, PVN – paraventricular nucleus, ILCC – inter-mediolateral cell column, SCG – superior cervical ganglion, NE – norepinephrine, AC – adenylate cyclase, AANAT – arylalkylamine N-acetyl transferase, HIOMT – hydroxyindole-*O*-methyl transferase, AP – anterior pituitary, PP – posterior pituitary.

Fig. 5 Molecular architecture of the circadian clock machinery in mammals. Interconnected autoregulatory transcriptional translational feedback loops (TTFL) function together to regulate 24 h endogenous periodicity of the clock. mRNA profiles of clock genes in mouse liver (Top right). AMPK – AMP activated protein kinase, Ccg – clock controlled genes, CRE – cAMP response element, CK1 – casein kinase 1, DBP – D-box binding protein, RRE – ROR responsive element, SRE – serum response element, SCF – SKP1-cullin-F-box protein, FBX – F-box protein, Ub – ubiquitin.

Fig. 6 Diagrammatic representation of networks inter-connecting central and peripheral clocks in mammals.

Fig. 7 (a) Diagrammatic representation of SIRT1-mediated circadian gene activation model in the SCN. **(b)** SIRT1 and SIRT6 mediated nuclear regulation of circadian gene expression. **(c)** Circadian regulation of NAD+ synthesis and SIRTUIN activation.

Fig. 8 Central serotonergic pathways regulating various physiological and behavioral effects.

Fig. 9 Overview of tryptophan metabolism in the central nervous system (CNS) (a) Availability of unbound tryptophan for uptake into the brain. (b) Free tryptophan is transported across the BBB via the L-type amino acid transporter (LAT1), at the luminal and abluminal surfaces of endothelial cells. (c) From CSF, tryptophan is up taken by all cells for protein synthesis, and specialized cells that synthesize neuroactive metabolites of tryptophan, including serotonin (in serotonergic neurons and mast cells), tryptamine (in 'D' neurons that express aromatic amino acid decarboxylase), and kynurenines (in astrocytes, microglia, macrophages and dendritic cells). (d) Melatonin synthesized in the pineal gland has ready access to the brain, where it is involved in neural signaling through G-protein-coupled receptors, but also has antioxidant effects and can serve as a precursor for synthesis of other molecules with antioxidant effects, including kynuramines.

Fig. 10 Diagrammatic representation of the gene-protein-metabolite network.

Fig. 11 Serotonin biosynthesis and metabolism. TPH – Tryptophan hydroxylase, MAO – Monoamine oxidase, AANAT – Aryl alkylamine N-acetyl transferase, HIOMT – Hydroxy indole-O-methyl transferase.

Fig. 12 Overview of the roles of melatonin in the circadian multi-oscillator system. Kir3 K^+ channels - type 3 inward rectifier K^+ channels, SCN - suprachiasmatic nucleus.

Fig. 13 Regulation of melatonin production and receptor function.

Fig. 14 Melatonin receptor signaling pathways. Melatonin activation of MT1 receptors triggers $G\alpha_i$ activation, decreasing the levels of the secondary messenger cAMP, and $G\beta\gamma$-dependent activation of PI3K/Akt, PKC and ERK pathways. MT1 coupling to Gq leads to PLC activation and increase in intracellular Ca^{2+}. Melatonin-induced modulation of neuronal action potential is mediated by MT1-dependent activation of the potassium and calcium ion channels ($K_{ir}3$ and $Ca_v2.2$). The physical interaction of MT1 receptors with $Ca_v2.2$ channels tonically inhibits $Ca_v2.2$-mediated calcium entry through $G\beta\gamma$ subunits. Melatonin activation of MT2 receptors triggers $G\alpha_i$-dependent cAMP and ERK signaling pathways and inhibits cGMP levels. Melatonin induced β-arrestin recruitment to both MT1 and MT2 receptors, but β-arrestin-dependent down-streaming signaling is not yet reported. β-ARR - β-arrestin, $Ca_v2.2$ - voltage-gated calcium channel, *ccgs* - clock-controlled genes, CREB - cAMP-responsive element binding, $K_{ir}3$ - G protein-coupled inwardly rectifying potassium channel, sGC - soluble GC.

Fig. 15 Extended, context-specific melatonin receptor signaling pathways. Depending on the cell type or the presence of cell stressors, melatonin can activate additional melatonin

receptor-dependent signaling cascades. These pathways have been reported mainly for MT1 receptors, but the participation of MT2 receptors cannot be excluded. Melatonin modulation of mitochondrial function is reported under oxidative stress condition and in neurodegenerative diseases. Proposed signaling pathways involve the regulation of the activity and/or translocation of Bcl2/Bax and SIRT proteins. Activation of JAK2, ERK and the Akt/FOXO1 complex are suggested to mediate melatonin-induced cell survival and to modulate pluripotency/differentiation of stem cells, while melatonin-induced inhibition of these pathways is reported in cancer cells. MT1-dependent activation of SIRT1 might underlie melatonin's anti-inflammatory and anti-oxidative effects through regulation of transcription factors like Nrf2, PGC1α and NF-κB. MT1-coupling to G_{16} protein occurs in hematopoietic cells and triggers the JNK pathway.

Fig. 16 Schematic representation that links free radicals and the associated oxidative damage with the progression of the aging phenotype and the onset and development of age-related diseases. The cloud at the top lists many of the iterations of the free radical theory of aging that have been introduced over the last 60 years. ROS = Reactive oxygen species; AD = Alzheimer disease; PD = Parkinson disease; HD = Huntington disease; MS = Multiple sclerosis; ALS = amyotrophic lateral sclerosis.

Fig. 17 Molecular and cellular factors that contribute to the selective vulnerability of neurons to oxidative stress. Vulnerable neurons are characterized by low ATP production and mitochondrial dysfunction, because of the high oxidative stress (OS) and factors such as calcium dysregulation. Low ATP production can affect DNA repair, which, when combined with high DNA oxidation, can cause change of genomic activity and decreased metabolic activity in mitochondria. Chronic inflammatory response exist in vulnerable neurons, which can further elevate OS within them. Calcium dysregulation and glutamate hyperactivity are closely connected to OS generation and underlie many adverse conditions that are characterized by SNV. There is emerging evidence that directly connects these factors, such as low calcium-buffering capacity and glutamate-mediated selective neurodegeneration, to the selective vulnerability of neurons.

Fig. 18 Schematic representation of age associated circadian dysfunction leading to sleep disorders.

Fig. 19 (a) Peak melatonin levels at night tend to decrease with advanced age in humans. **(b)** Age associated SIRT1 mediated decline in circadian clock function.

Fig. 20 Chemical structure of curcumin and its derivatives. **(A–C)** Curcuma longa, its rhizomes and turmeric extract **(D**) Different chemical components of turmeric extract **(E)** Chemical structure of principal ingredients of curcuminoid **(F)** pathway of curcumin biosynthesis **(G)** curcumin metabolism in our body.

Fig. 21 Amelioration of oxidative stress by curcumin in brain. The CNS is vulnerable to oxidative stress due to high metabolic rate, which causes higher O_2 demand. This leads to

an increase in oxidative stress in the brain. Curcumin, as a potent free radical scavenger, ameliorates these effects (Maiti and Dunbar 2018).

Fig. 22 Schematic diagram showing the classification of chemical constituents of Ashwagandha plant and active components of roots and leaves (Wadhwa et al. 2016).

Fig. 23 Neuroprotective mechanisms of *Withania somnifera* (Kumar et al. 2015).

Fig. 24 Representative Dissociation curves showing specific amplification of target genes.

Fig. 25 Standard representative chromatograms showing all components of serotonin metabolism. **(A)** 10% methanol; 0.1N Sodium acetate; 0.1N citric acid, 50 mg/L EDTA was used for detection of compounds- 5-HTP, 5-HT, 5-HIAA, TRP, 5-HTOH and NAS. **(B)** 25% methanol; 0.1N Sodium acetate; 0.1N citric acid, 50mg/L EDTA was used for detection of compounds- 5-MIAA, 5-MTOH, MEL and NAT. nA refers to nanoampere (Unit of electric current).

Fig. 26 Effect of curcumin on daily rhythms of *rPer1, rPer2, rCry1, rCry2, rBmal1, rRev-erbα, rSirt1* and *rNrf2* mRNA expression in 3, 12 and 24 months (m) old rat SCN. 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 treated group).

Fig. 27 Effect of curcumin administration on mean 24 h levels of *rPer1, rPer2, rCry1, rCry2, rBmal1, rRev-erbα, rSirt1* and *rNrf2* expression in 3, 12 and 24 months (m) old rat SCN. Each value is mean ± 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 treated group).

Fig. 28 Effect of curcumin administration on daily pulse of *rPer1, rPer2, rCry1, rCry2, rBmal1, rRev-erbα, rSirt1* and *rNrf2* expression in 3, 12 and 24 months (m) old rat SCN. Each value is maximum/minimum ratio of 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 treated group).

Fig. 29 (A) Immunoblots showing daily rhythms in SIRT1 and NRF2 expression in SCN of 3, 12 and 24 months (m) aged rats at ZT-0, 6, 12 and 18. β-TUBULIN was probed as loading control. VT- vehicle treated; CT- curcumin treated. **(B)** Daily rhythms of SIRT1 and NRF2 expression in the aging rat SCN in 3, 12 and 24 m and effect of curcumin. 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). **(C)** Effect of curcumin on mean 24 h levels and **(D)** Daily Pulse

(maximum/minimum ratio) of SIRT1 and NRF2 in 3, 12 and 24 m rat SCN. $p_p < 0.05$ (where 'p' refers to comparison with age matched vehicle group). $p_q < 0.05$ (where 'q' refers to comparison with 3 m vehicle group).

Fig. 30 Effect of curcumin treatment on pair wise correlation between mean light (ZT-0, 6, 12) and mean dark (ZT-12, 18, 24/0) phase values of *rPer1, rPer2, rCry1, rCry2, rBmal1* and *rRev-erbα* in SCN of 3, 12 and 24 months (m) aged rats. Every colored square represents correlation coefficient values between the parameters. Where, positive correlations are indicated by shades of blue, negative correlations by shades of red and white indicates no correlation. Grey squares indicate that the values were not considered for correlation analysis. '*' Indicates statistically significant correlations ($p < 0.05$).

Fig. 31 Effect of curcumin treatment on pair wise correlation between mean light (ZT-0, 6, 12) and mean dark (ZT-12, 18, 24/0) phase values of clock modulator *rSirt1* and all other clock genes studied in SCN of 3, 12 and 24 months (m) aged rats. Each correlation coefficient value represents the positive or negative correlations between *rSirt1* and clock genes. '*' Indicates statistically significant correlations ($p < 0.05$).

Fig. 32 Effect of *W. somnifera* treatment on daily rhythms of *rBmal1, rPer1, rPer2, rCry1, rCry2, rRev-erbα, rSirt1* and *rNrf2* mRNA expression in the aging rat SCN in 3, 12 and 24 months. 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).

Fig. 33 Effect of *W. somnifera* administration on mean 24 h levels of *rBmal1, rPer1, rPer2, rCry1, rCry2, rRev-erbα, rSirt1* and *rNrf2* genes in the aging rat SCN in 3, 12 and 24 months rat SCN. Each value is mean ± 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).

Fig. 34 Effect of *W. somnifera* administration on daily pulse of *rBmal1, rPer1, rPer2, rCry1, rCry2, rRev-erbα, rSirt1* and *rNrf2* genes in the aging rat SCN in 3, 12 and 24 months rat SCN. Each value is maximum/minimum ratio of 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).

Fig. 35 (A) Immunoblots showing daily rhythms in SIRT1 and NRF2 expression in SCN of 3, 12 and 24 months (m) aged rats at ZT-0,6,12 and 18. β-TUBULIN was probed as loading control. VT- vehicle treated; WST- *Withania somnifera* treated. **(B)** Daily rhythms of SIRT1 and NRF2 expression in the aging rat SCN in 3, 12 and 24 months. 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). **(C)** Effect of WST on mean 24 h levels and **(D)** Daily Pulse of SIRT1 and NRF2 in 3, 12 and 24 m rat SCN. $p_p < 0.05$ (where 'p' refers to comparison with age matched vehicle group). $p_q < 0.05$ (where 'q' refers to comparison with 3 m vehicle group).

Fig. 36 Effect of *W. somnifera* treatment on pair wise correlation between mean light (ZT-0, 6, 12) and mean dark (ZT-12, 18, 24/0) phase values of *rBmal1, rPer1, rPer2, rCry1, rCry2, rRev-erbα, rSirt1* and *rNrf2* in SCN of 3, 12 and 24 months (m) aged rats. Every colored square represents correlation coefficient values between the parameters. Where, a positive correlation is indicated by shades of blue, negative correlation by shades of red and white indicates no correlation. Grey squares indicate that the values were not considered for correlation analysis. '*' Indicates statistically significant value between parameters ($p < 0.05$).

Fig. 37 (A) Daily rhythmicity in SIRT1 activity in 3m SCN. **(B)** SIRT1 activity in SCN reduces with aging (Activity measured at ZT-18). **(C)** 10 nM Nicotinamide (NAM) significantly inhibited the SIRT1 activity ($p < 0.05$). '*' Indicates statistically significant difference ($p < 0.05$).

Fig. 38 Expression of *rBmal1, rPer1, rPer2, rCry1, rCry2, rRev-erbα* and *rSirt1* mRNA in the SCN explants. 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 control group). C – control; CT – curcumin treated; NAM – nicotinamide treated; CT+NAM – co-treated with curcumin and nicotinamide. Time points are in reference to the rats from which SCN were harvested.

Fig. 39 (A) Mean 24 h levels and **(B)** daily pulse of *rBmal1, rPer1, rPer2, rCry1, rCry2, rRev-erbα* and *rSirt1* mRNA in the SCN explants. Each value is mean ± SEM, $p < 0.05$ and expressed as mean relative gene expression. '*' Indicates statistically significant difference ($p < 0.05$). C – control; CT – curcumin treated; NAM – nicotinamide treated; CT+NAM – co-treated with curcumin and nicotinamide.

Fig. 40 Expression of *rBmal1, rPer1, rPer2, rCry1, rCry2, rRev-erbα* and *rSirt1* mRNA in the SCN explants. 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 control group). C – control; WST – *Withania somnifera* treated; NAM – nicotinamide treated; WST+NAM – co-treated with *Withania somnifera* and nicotinamide. Time points are in reference to the rats from which SCN were harvested.

Fig. 41 (A) Mean 24 h levels and **(B)** daily pulse of *rBmal1, rPer1, rPer2, rCry1, rCry2, rRev-erbα* and *rSirt1* mRNA in the SCN explants. Each value is mean ± SEM, $p < 0.05$

and expressed as mean relative gene expression. '*' Indicates statistically significant difference ($p < 0.05$). C – control; WST – *Withania somnifera* treated; NAM – nicotinamide treated; WST+NAM – co-treated with *Withania somnifera* and nicotinamide.

Fig. 42 Effect of curcumin administration on daily rhythms of various components of serotonin metabolism in the SCN of in 3, 12 and 24 months (m) rats. Each value is mean ± SEM (n = 6). $p_a < 0.05$; $p_b < 0.05$, $p_c < 0.05$ 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 levels at same time point in the age matched vehicle treated group).

Fig. 43 Effect of curcumin administration on **(A)** mean 24 h levels **(B)** daily pulse of various components of serotonin metabolism in 3, 12 and 24 months (m) rat SCN. Each value is mean ± SEM (n = 6) $p < 0.05$. $p_p < 0.05$ (where 'p' refers to comparison with age matched vehicle group). $p_q < 0.05$ (where 'q' refers to comparison with 3 m vehicle treated group).

Fig. 44 Effect of curcumin administration on pair wise correlation between mean light (ZT-0, 6, 12) and mean dark (ZT-12, 18, 24/0) phase values of various components of serotonin metabolism in SCN of 3, 12 and 24 months (m) aged rats. Every colored square represents correlation coefficient values between the parameters. Where, positive correlations are indicated by shades of blue, negative correlations by shades of red and white indicates no correlation. '*' Indicates statistically significant correlations ($p < 0.05$).

Fig. 45 Effect of WS administration on daily rhythms of various components of serotonin metabolism in the SCN of in 3, 12 and 24 months (m) rats. Each value is mean ± SEM (n = 6). $p_a < 0.05$; $p_b < 0.05$, $p_c < 0.05$ 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 levels at same time point in the age matched vehicle treated group).

Fig. 46 Effect of *Withania somnifera* administration on **(A)** mean 24 h levels **(B)** daily pulse of the various components of serotonin metabolism in 3, 12 and 24 months (m) rat SCN. Each value is mean ± SEM (n = 6) $p < 0.05$. $p_p < 0.05$ (where 'p' refers to comparison with age matched vehicle group). $p_q < 0.05$ (where 'q' refers to comparison with 3 m vehicle treated group).

Fig. 47 Effect of *Withania somnifera* administration on pair wise correlation between mean light (ZT-0, 6, 12) and mean dark (ZT-12, 18, 24/0) phase values of various components of serotonin metabolism in SCN of 3, 12 and 24 months (m) aged rats. Every colored square represents correlation coefficient values between the parameters. Where, positive correlations are indicated by shades of blue, negative correlations by shades of red and white indicates no correlation. '*' Indicates statistically significant correlations ($p < 0.05$).

Fig. 48 Effect of curcumin treatment on **(A)** Daily rhythms of MT1 and MT2 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 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). $p_w < 0.05$ (where 'w' refers to comparison of mRNA expression levels at same time point in the age matched vehicle group). **(B)** Mean 24 h levels and **(C)** Daily Pulse of MT1 and MT2 mRNA expression in 3, 12 and 24 m old rat SCN. Each value is mean ± SEM, $p < 0.05$ and expressed as mean relative mRNA 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).

Fig. 49 (A) Immunoblots showing daily rhythms in MT1 and MT2 expression in SCN of 3, 12 and 24 m aged rats at ZT-0, 6, 12 and 18. β-TUBULIN was probed as loading control. VT- vehicle treated; CT - curcumin treated. (**B)** Daily rhythms of MT1 and MT2 protein expression in 3, 12 and 24 m aged 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). (**C)** Mean 24 h levels and (**D)** Daily Pulse of MT1 and MT2 protein expression in 3, 12 and 24 m aged rat SCN. 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).

Fig. 50 Effect of *Withania somnifera* treatment on (A) Daily rhythms of MT1 and MT2 mRNA expression in the SCN of 3, 12 and 24 m old rats. 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). $p_w < 0.05$ (where 'w' refers to comparison of mRNA expression levels at same time point in the age matched vehicle group). (B) Mean 24 h levels and (C) Daily Pulse of MT1 and MT2 mRNA expression in 3, 12 and 24 m old rat SCN. Each value is mean ± SEM, $p < 0.05$ and expressed as mean relative mRNA 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).

Fig. 51 (A) Immunoblots showing daily rhythms in MT1 and MT2 expression in SCN of 3, 12 and 24 m aged rats at ZT-0, 6, 12 and 18. β-TUBULIN was probed as loading control. VT- vehicle treated; WST – *Withania somnifera* treated. (**B)** Daily rhythms of MT1 and MT2 protein expression in 3, 12 and 24 m aged 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). (**C)** Mean 24 h levels and (**D)** Daily Pulse of MT1 and MT2 protein expression in 3, 12 and 24 m aged rat SCN. 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).

Fig. 52 Effect of melatonin treatment on (**A)** Daily rhythms of MT1 and MT2 mRNA expression in the SCN of 3, 12 and 24 m old rats. 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). $p_w < 0.05$ (where ‘w’ refers to comparison of mRNA expression levels at same time point in the age matched vehicle group). (**B)** Mean 24 h levels and (**C)** Daily Pulse of MT1 and MT2 mRNA expression in 3, 12 and 24 m old rat SCN. Each value is mean ± SEM, $p < 0.05$ and expressed as mean relative mRNA 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).

Fig. 53 (A) Immunoblots showing daily rhythms in MT1 and MT2 expression in SCN of 3, 12 and 24 m aged rats at ZT-0, 6, 12 and 18. β-TUBULIN was probed as loading control. VT- vehicle treated; MEL- melatonin treated. (**B)** Daily rhythms of MT1 and MT2 protein expression in 3, 12 and 24 m aged 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). (**C)** Mean 24 h levels and (**D)** Daily Pulse of MT1 and MT2 protein expression in 3, 12 and 24 m aged rat SCN. 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).

Fig. 54 Double plotted actograms of gross locomotor activity under control (C) and curcumin treated (CT) conditions in 3, 12 and 24 months (m) aged rats.

Fig. 55 Effect of curcumin on gross locomotor activity of 3, 12 and 24 m aged rats. C – Control; CT - curcumin treated. Each value is mean ± SEM (n = 4), and expressed as percentage activity. ‘*’ indicates significant difference in comparison to night activity in 3 m controls and ‘#’ indicates significant difference in comparison to day activity in 3 m controls ($p < 0.05$).

Fig. 56 Double plotted actograms of gross locomotor activity under control (C) and *Withania somnifera* treated (WST) conditions in 3, 12 and 24 months (m) aged rats.

Fig. 57 Effect of *Withania somnifera* on gross locomotor activity of 3, 12 and 24 m aged rats. C – Control; WST - *Withania somnifera* treated. Each value is mean ± SEM (n = 4), and expressed as percentage activity. ‘*’ indicates significant difference in comparison to night activity in 3 m controls and ‘#’ indicates significant difference in comparison to day activity in 3 m controls. ® indicates significant difference in percentage activity in comparison to 12 m control ($p < 0.05$).

Fig. 58 Diagrammatic summary showing age linked alterations in time points of peak expression/levels of various components studied at gene, protein and metabolite levels in addition to alterations in gross locomotor activity. Differential restorative effects of CT and WST. Blue half of the circle – subjective day; black half of the circle – subjective night.

Fig. 59 Schematic representation of overall summary of the present study. Differential restoratory effects of curcumin and leaf extract of *W. somnifera* on chronomics of age induced alterations in (1) clock genes, SIRT1 and NRF2 expression (2) Serotonin metabolism and (3) Melatonin receptor expression in the SCN of male Wistar rats as well as (4) Gross locomotor activity.

Fig. 60 DPPH free radical scavenging activity assay. Scavenging activities (represented as % scavenging activity at concentration per milliliter of test compound): Curcumin – 36 % at 10 µg/mL; WS leaf extract - 78 % at 1 mg/mL; WS root extract – 38 % at 1mg/mL

APPENDIX – III

List of Tables

APPENDIX – IV

Abbreviations

ºC	:	degree centigrade/ degree Celsius
5-HIAA	:	5-Hydroxy indole acetic acid
5-HT	:	5-Hydroxytryptamine
5-HTOH	:	5-Hydroxy tryptophol
5-MIAA	:	5-Methoxy indole acetic acid
5-MTOH	:	5-Methoxy indole acetic acid
AANAT	:	Arylalkylamine N-acetyl transferase
AC	:	Adenylyl cyclase
AFMK	:	N-acetyl- N-formyl-5-methoxy kynuramine
AMK	:	N-acetyl-5-methoxy kynuramine
ANOVA	:	Analysis of Variance
ARNT	:	Arylhydrocarbon receptor nuclear translocator
Arntl	:	Arylhydrocarbon receptor nuclear translocator like
ASPS	:	Advanced Sleep Phase Syndrome
ATP	:	Adenosine triphosphate
AVP	:	Arginine vasopressin
BBB	:	Blood brain barrier
bHLH	:	basic helix-loop-helix
Bmal1	:	Brain and muscle Arnt-like protein 1
BSA	:	Bovine serum albumin
CaM	:	Calmodulin
CAMK	:	Ca^{2+}/calmodulin-dependent protein kinase
cAMP	:	Cyclic Adenosine Mono Phosphate
CAT	:	Catalase
CCGs	:	Clock controlled genes
cDNA	:	Complentary DNA
CK	:	Casein kinase
CL	:	Cardiolipin
Clock	:	Circadian locomotor output cycles kaput
CRE	:	cAMP response element

CREB	:	cAMP response element binding protein
Cry	:	Cryptochrome
CSF	:	Cerebrospinal fluid
Ct	:	Cycle threshold
CTS	:	Circadian timekeeping system
DBP	:	D-box binding protein
DBPE	:	DBP-binding element
DD	:	constant dark
DNA	:	deoxy-ribo nucleic acid
DP	:	Dark phase
DSPS	:	Delayed Sleep Phase Syndrome
ECD	:	Electrochemical detection
EDTA	:	Ethylene di-amine tetra acetic acid
GABA	:	Gamma amino butyric acid
GHT	:	Geniculo-hypothalamic tract
Gi	:	G Inhibitory
GPCRs	:	G protein-coupled receptors
GRd	:	Glutathione reductase
GRP	:	Gastrin-releasing peptide
Gs	:	G stimulatory
GSH	:	Reduced glutathione
GSSG	:	Oxidized glutathione
HIOMT	:	Hydroxy indole -O-methyl transferase
H3	:	Histone 3
HAT	:	Histone Acetyl Transferase
HDAC	:	Histone Deacetylase
IGL	:	Inter-geniculate leaflet
LD	:	Light Dark cycle
LGICs	:	Ligand-gated ion channels
LP	:	Light Phase
MAO	:	Monoamine oxidase
MAPK	:	Mitogen activated protein kinase
MEK	:	Map ERK kinase

MEL	:	Melatonin
mg	:	milligram
mL	:	milliliter
mM	:	milli molar
MnSOD	:	Magnesium superoxide dismutase
mRNA	:	messenger RNA
miRNA	:	micro RNA
MT1	:	Melatonin receptor subtype1
MT2	:	Melatonin receptor subtype2
NAD^+	:	Nicotinamide adenine dinucleotide
NAMPT	:	Nicotinamide phosphoribosyl transferase
NAS	:	N-acetyl serotonin
NAT	:	N- Acetyl tryptamine
NE	:	Norepinephrine
NO	:	Nitric Oxide
NOS	:	Nitric oxide synthase
Npas2	:	Neuronal PAS Domain Protein 2
NPY	:	Neuropeptide Y
O_2^-	:	Superoxide anion radical
PACAP	:	Pituitary adenylyl cyclase activating peptide
PAS	:	Period-Arnt- Sim
PCD	:	Programmed cell death
PCR	:	Polymerase Chain Reaction
PD	:	Parkinson’s disease
Per	:	Period
PHI	:	peptide histidine-isoleucin
PI3K	:	Phospho ionositol -3 kinase
PK2	:	Prokineticin 2
PKA	:	Protein kinase A
PKC	:	Protein kinase C
PLC	:	Phospholipase C
PLMS	:	periodic leg movements in sleep
pM	:	Pico mole

qRT-PCR	:	Quantitative real time polymerase chain reaction
Rev-Erb α, β	:	Reverse-Erythroblastosis α, β
RHT	:	Retino-hypothalamic tract
RIPD	:	Rotenone induced Parkinson disease
RNA	:	Ribonucleic acid
ROR	:	Retinoic-acid related orphan nuclear receptors
RRE	:	ROR response elements
ROS	:	Reactive oxygen species
RP-HPLC	:	Reverse phase high pressure liquid chromatography
RRP	:	Retino-raphe pathway
RT	:	Room temperature
SCG	:	Superior cervical ganglion
SCN	:	Suprachiasmatic nucleus
SDB	:	Sleep disorder breathing
SDS	:	Sodium Dodecyl Sulfate
SIRT1	:	silent mating type information regulation 2 homolog 1
SIM	:	single minded protein
SN	:	Substantia nigra
SP	:	Substance P
TPH	:	Tryptophan hydroxylase
TRP	:	Tryptophan
TTFL	:	Transcriptional Translational Feed back
UPS	:	Ubiquitin proteasome system
VIP	:	Vasoactive intestinal peptide
ZT	:	Zeitgeber time
μM	:	micro molar

APPENDIX – V

PUBLICATION