Understanding the Mechanisms involved in Age induced alterations in Non-photic regulation of Biological Clock: Effect of Restricted Feeding

A thesis submitted to the University of Hyderabad for the award of a Ph.D. degree in Animal Sciences

By

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UNIVERSITY OF HYDERABAD

(A Central University Established in 1974 By An Act Of Parliament)

HYDERABAD –500 046, INDIA

DECLARATION

I, Dileep Kumar Reddy V, hereby declare that this thesis entitled "Understanding the Mechanisms involved in Age induced alterations in Non-photic regulation of Biological Clock: Effect of Restricted Feeding" submitted by me under the guidance and supervision of Dr. 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 Institution for the award of any degree or diploma.

Date:

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CERTIFICATE

This is to certify that this thesis entitled "Understanding the Mechanisms involved in Age induced alterations in Non-photic regulation of Biological clock: Effect of Restricted Feeding" is a record of bonafide work done by Mr. Dileep Kumar Reddy V, a research scholar for Ph.D. programme in Animal Sciences, School of Life Sciences, University of Hyderabad under my guidance and supervision.

The thesis has not been submitted previously in part or in full to this or any other University or Institution for the award of any degree or diploma.

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Introduction and Review of Literature

Introduction and review of literature

Biological rhythms are the biological events or functions that show cyclic variation over time and serve to align the physiological functions of the body corresponding to the periodic environmental changes. These rhythms increase the ability of an organism to predict, anticipate, and prepare for the upcoming conditions by adjusting its physiology, behavior, and life cycles. This broad spectrum of rhythms that operates simultaneously within an organism's biological processes can be majorly divided into two types, exogenous and endogenous. Exogenous biological rhythms are driven by the environmental cues or any external influence (Zeitgebers) such as a change in the seasons, or the transition from day to night, eating schedules, temperature, social interactions etc. These rhythms include sleep-wake cycle, hibernation, migratory activity and seasonal changes in coat color and behavior, seasonal reproductive activity. Endogenous rhythms are generated from within and are regulated by the organism itself e.g. core body temperature, sleep-wake cycle, and locomotor activity patterns. Apart from these rhythms in the modern world, there are some man-made zeitgebers which can override the natural seasonal changes in day length and can influence the biological rhythms such as artificial light, externally regulated sleep patterns, shift work schedules etc (Kalsbeek *et al.*, 2011).

According to the duration of periodicity biological rhythms are classified as ultradian {<24 hours (h)}, circadian (~24 h), infradian and seasonal or circannual rhythms (Wollnik, 1989). Ultradian rhythms are the recurrent periods that have a period shorter than 24 h, like: respiration, hormonal release, heartbeat, bowel activity, and feeding cycles, etc. Circadian rhythms (derived from Latin phrase *circa diem*, or about a day) are the most significant biological rhythms that govern a very large part of our biology which persist under constant conditions with a period length of approximately 24 h (Liu and Bell-Pedersen, 2006). These circadian oscillations govern a plethora of behavioral and physiological functions such as sleep-wake cycle, and the consumption of food and water (Perreau-Lenz *et al.*, 2007). Infradian rhythms are the rhythms with a period longer than 24 h (> 24 h) such as human menstrual cycle, breeding, tidal or seasonal rhythms. A circannual rhythm spans events that repeat with a period length of approximately one year corresponding to the Earth's seasons. Hibernation, aestivation, reproduction, migration and other seasonal activities of the animals are the examples of circannual rhythms.

Circadian time keeping system and Entrainment of the biological clock:

The earth's rotation around its axis causes changes in the geophysical environment in a cyclic manner like the daily cycles of day and night. These environmental cycles there by exert a profound pressure for adaptation to these predictable daily changes (Green, 1998). Most of the light-sensitive organisms ranging from cyanobacteria to mammals have developed a circadian timing system (CTS) which is set by light to time various daily activities at the biochemical, physiological and behavioral levels. Organisms do measure astronomical time with time measuring devices, known as circadian clocks. These circadian clocks enhance the innate ability of the organisms to survive under ever-changing environment by enabling them to efficiently anticipate and organize their physiological and neurobehavioral variables in a proactive rather than a responsive manner (Dibner, 2010) to the periodic events such as availability of food, light and mates. In this way, precisely timed circadian clock are the outcome of evolutionary natural selection process which confers a greater adaptive advantage of predictive homeostatic regulation to the organisms (Paranjpe and Vijay Kumar Sharma, 2005; Fuller *et al.*, 2008). The mammalian circadian system exhibits a well defined dynamical properties including (i) rhythmicity as such (independent of its frequency) (ii) the circadian range of the period [a period {Tau (τ)} of approximately 24 h in the absence of external environmental cues] (iii) self sustained endogenous oscillations (iv) an amplitude sufficiently robust to drive output rhythms (v) entrainment to the external environmental cues (temperature and light-dark cycles etc.) (vi) temperature compensation over wide range of temperatures (vii) synchronization of multiple cellular clocks despite inevitable molecular noise and (viii) they are under Genetic Control (Roenneberg and Merrow, 2001; Ukai and Ueda, 2010).

Organization of the Circadian Timing System in mammals:

The fundamental property of circadian system is its ability to be entrained to changing environmental parameters and adjust the circadian oscillator period and phase to the external zeitgebers (Caldelas *et al.*, 2005). This external information from outside will reach body structures which can integrate this information and send appropriate signals to various tissues. The mammalian circadian timekeeping system has a complex hierarchical architecture, composed of a central pacemaker in the hypothalamic suprachiasmatic nuclei (SCN) and subsidiary clocks in nearly all types of body tissues/cells (Dibner *et al.*, 2010). The central clock is synchronized to geophysical time by various environmental cues. In turn, the SCN maintain proper phase

alignment of peripheral tissue clocks present in nearly all cells via neuronal and humoral cues (Fig. 1). In mammals the central oscillator resides in cells grouped in two suprachiasmatic nuclei (SCN) in the anteroventral hypothalamus and serves as relay between the external and internal worlds and the body adapts an organism's physiology by exhibiting circadian rhythms of gene expression, metabolic activity, and electrophysiological activity (Jagota *et al.,* 2000; Lowrey and Takahashi, 2000).

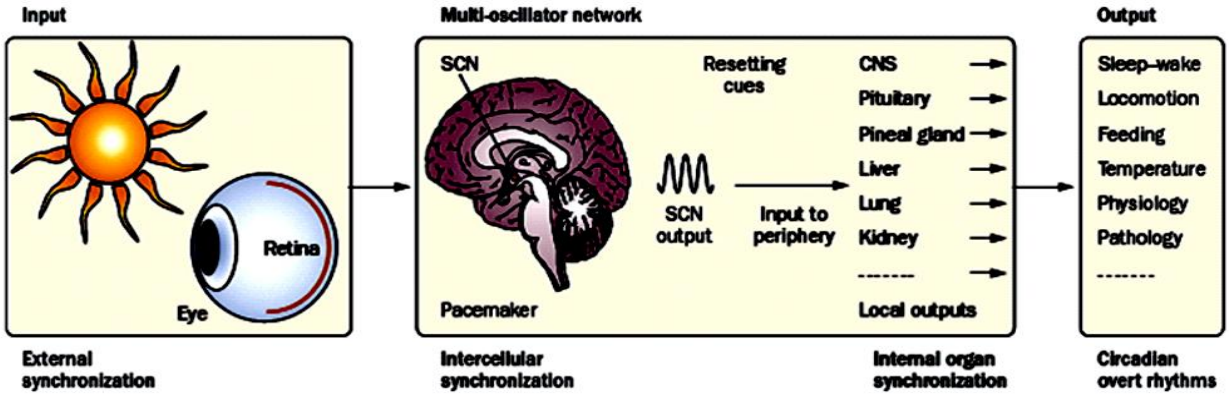


Fig. 1. Hierarchy in the organization of mammalian circadian clock. SCN is the central pacemaker present at the top and is synchronized by external environmental cues. All peripheral oscillators are entrained by the multiple lines of output signals from SCN. Abbreviations: CNS-central nervous system; SCN, suprachiasmatic nucleus (Koch *et al.,* 2009).

SCN is entrained by both photic and non-photic stimuli (Jagota, 2006). The photic stimuli such as the environmental light-dark cycle is the most pervasive and potent entraining stimulus (Jagota *et al.,* 1999; Piccione, 2007). The non-photic stimuli such as socio-professional routines (L´evi *et al.,* 2010), food availability cycles (Damiola *et al*., 2000), relative temperature and humidity cycles, sound, magnetic fields and barometric pressure (Mrosovsky, 1996) also can reset the master circadian oscillator (Sharma and Chandrashekaran, 2005).

Consequences of circadian disruption (CD) in humans:

Any relevant disturbance of the internal temporal order of physiological, biochemical and behavioral circadian rhythms to adapt to environmental and societal time cues leads to circadian disruption of the organism (Garaulet *et al.,* 2010). These alterations impair behaviour, cognition, further disruption of circadian clock genes impairs sleep–wake cycle and social rhythms which lead to profound consequences like emotional behavior and mental disorders (Benca *et al.,* 2009), depressive disorders (Fig. 2), schizophrenia, bipolar disorder, anxiety disorders, seasonal affective disorder (SAD) and a variety of other Central nervous system disorders (Lamont *et al.,* 2007; McClung 2007). This alteration in the circadian

rhythms also alters the metabolic function of the body by affecting peripheral organs (Albrecht, 2012) which leads to chronic circadian misalignment (Fig. 3).

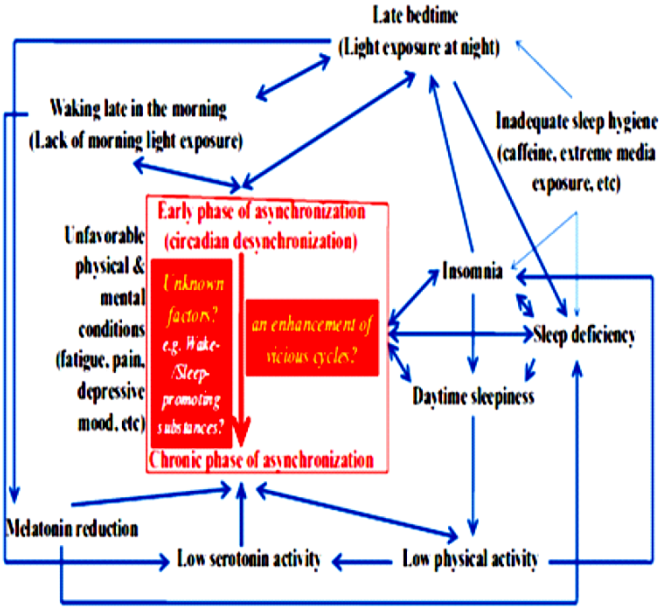


Fig. 2. Development of circadian asynchronization by environmental factors leading to mood and sleep disorders (Kohyama, 2011).

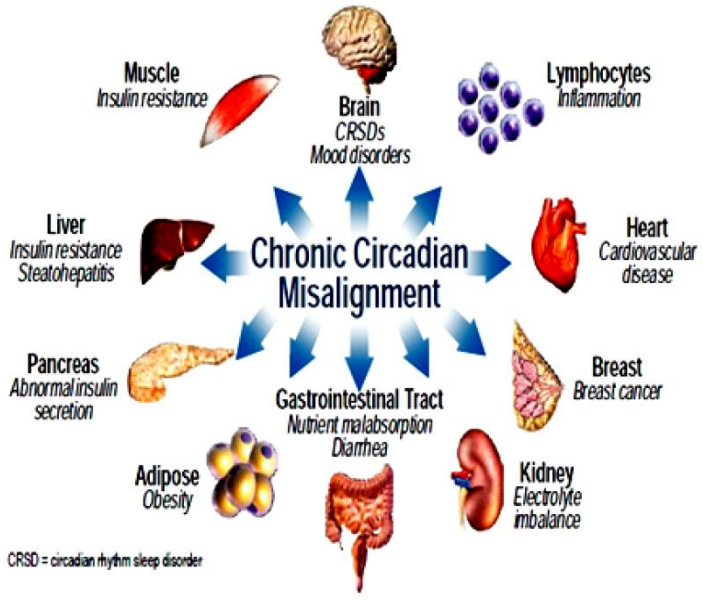


Fig. 3. Interconnection of the circadian and physiological systems: Circadian rhythms alterations leads to peripheral circadian clock desynchrony and circadian rhythm disorders (Young and Bray, 2007).

NEUROANATOMY AND NEUROPHYSIOLOGY OF SUPRACHIASMATIC NUCLEUS:

SCN in mammals is a paired bilateral nucleus containing a distinct group of cells located adjacent to the third ventricle and just above the optic chiasm in the anterior hypothalamus (Fig. 4. A and B) (Jagota *et al.,* 2000; also reviewd in Jagota, 2006). In rat each nucleus contains approximately 10,000 small densely packed neurons (Shirakawa *et al.,* 2001) with simple dendritic trees (Van den Pol, 1991). Though the size of SCN varies with age and gender (Shirakawa *et al.* 2001) the average size of single SCN in adult male rat is approximately with a width of 425 µm and a height of 400 µm with about 300 µm in diameter (Van den Pol 1991, Morre *et al.,* 2002). SCN neurons are the smallest neurons in the brain, with a soma diameter of 7-11 µm.

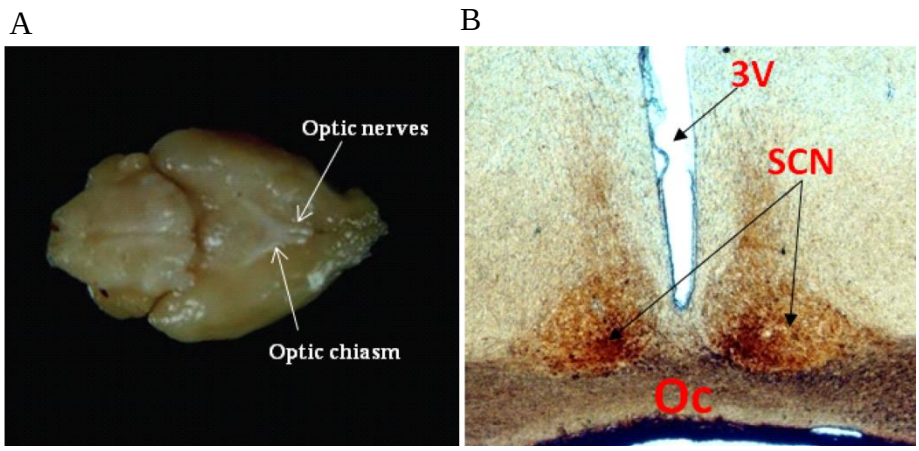


Fig.4. A. Ventral view of the rodent brain, showing the optic nerve and optic chiasm. B. Coronal section of rat brain showing the SCN region. 3V-3rd ventricle; SCN-Suprachiasmatic nucleus; Oc-Optic chiasm (Golombek and Rosenstein, 2010).

SCN: a small-world network:

The SCN contains heterogeneous neuronal population. Based on the peptidergic content, the anatomical location, and the circadian parameters of the component cells SCN can be subdivided into dorsomedial 'shell' (dmSCN) and ventrolateral 'core' (vlSCN) (Jagota, 2006; Mohawk and Takahashi, 2011). The cells in dmSCN are smaller, elongated, more tightly packed. These cells are clustered along the walls of the blood capillaries that course through the SCN. These cells may release substances into the blood which might rhythmically drive target tissues or that they may act as receptors, sensing hormonal signals from elsewhere. The cells present mostly in vlSCN are spherical with multiple invaginations of the nucleus and cytoplasm rich in organelles and rough endoplasmic reticulum, larger and loosely packed.

Arginine vasopressin (AVP) containing cells are rich in dmSCN whereas vlSCN is characterized by expression of vasoactive intestinal polypeptide (VIP), gastrin-releasing peptide (GRP) (Abrahamson and Moore, 2001) and neuromedin S (Miyazato *et al.*, 2008) (Fig. 5.A, B).

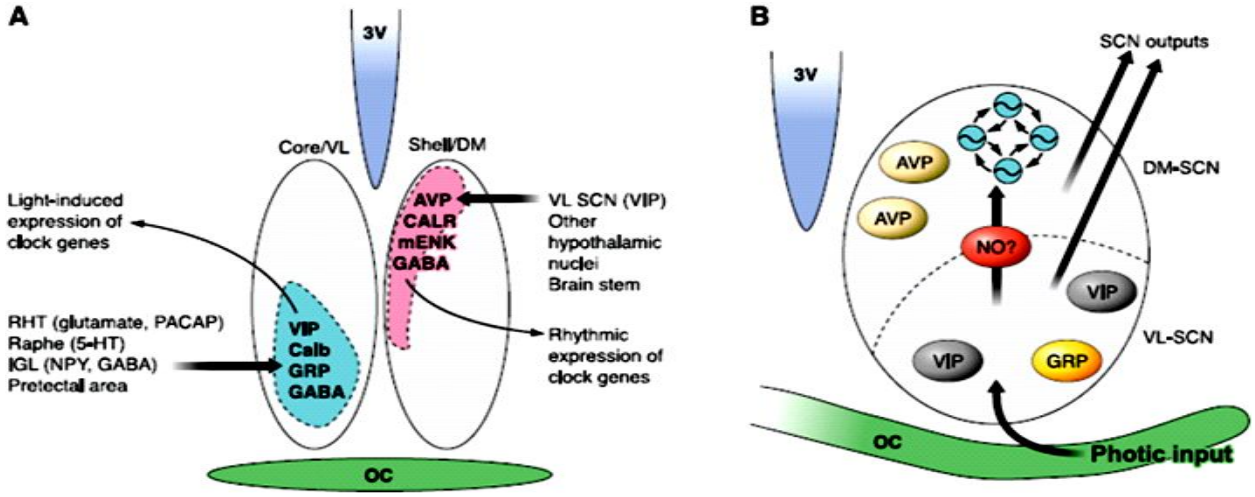


Fig. 5. A. Functional subdivisions and Neurochemicals involved in clock gene expression.
B. Coordination between ventrolateral and dorsomedial regions of SCN in response to light. RHT- Retinohypothalamic tract; 3V-Third ventricle; OC-Optic chiasm; IGL-Intergeniculate leaflet; VL- Ventrolateral; DM-Dorsomedial; VIP-Vasoactive intestinal polypeptide; Calb-Calbindin; GRP- Gastrin-releasing peptide; AVP-Arginine vasopressin; CALR-Calretinin; mENK-Met-enkephalin (Golombek and Rosenstein, 2010).

Distribution of afferents to the SCN core includes retinal and thalamic terminals and serotonergic afferents from the midbrain raphe, whereas inputs from cortex, basal forebrain and hypothalamus are largely segregated to the shell. These core and shell regions of SCN are morphological and functional subdivisions which function as a single unit. The SCN have the capacity to serve as a relay between the external and internal worlds of the body. Information from outside will reach the SCN via different body structures. SCN can integrate this information and send appropriate signals to various tissues.

Afferent or input pathways of SCN:

The SCN can be influenced via three major afferent pathways: the retinohypothalamic tract (RHT), the geniculohypothalamic tract (GHT) and serotonergic (5-HT) input from the dorsal raphe nucleus (DR) and median raphe nucleus (MRN) (Fig. 6). Out of these three input pathways the RHT mediates photic information, whereas the GHT (via intergeniculate leaflet cells) and the raphe nuclei provide non-photic information to the SCN (Dibner *et al.*, 2010).

(i) Retinohypothalamic tract (RHT): In mammals, environmental photic signal is received by the retinal ganglion cells in the retina and transmitted to the SCN via the retino-hypothalamic tract. The neurotransmitters involved in this signal transduction pathway are glutamate, substance P (SP) and PACAP (Pitutary adenylate cyclase activating polypeptide).

(ii) Geniculohypothalamic tract (GHT): GHT is an indirect photic pathway to the SCN which first enters the ventral lateral geniculate nucleus (VLGN) and then the intergeniculate leaflet (IGL) (Card Moore, 1991). GHT also mediates the non-photic stimuli such as motor activity. NPY (Neuropeptide Y), GABA (γ-amino butric acid) and ENK (Enkephalin) are the neurotransmitters involved in this pathway (Moore and Spheh, 1993).

(iii) Retino-raphe pathway: It originates in the median and dorsal raphe nuclei (Moore *et al*., 1978). Serotonin (5-HT) is the neurotransmitter present in raphe terminals, which acts on 5-HT_{2c} receptors of the excitatory interneurons of SCN.

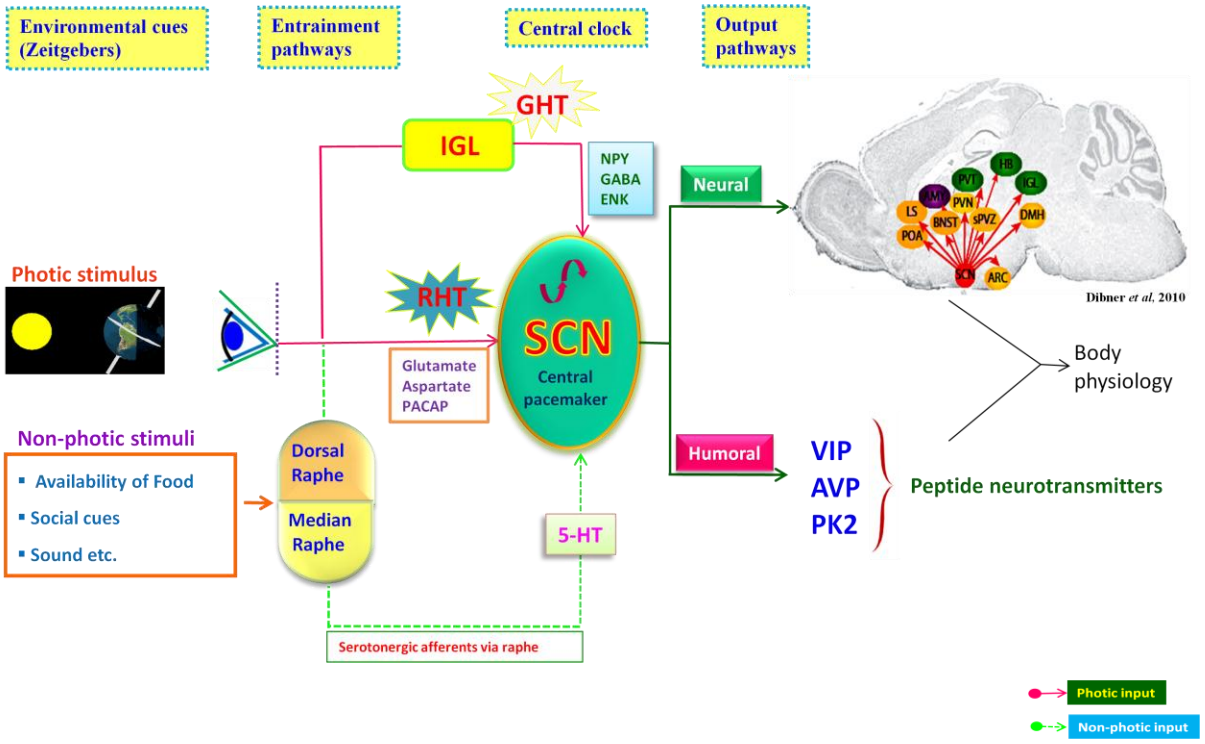


Fig.6. Afferent pathways convey the extrinsic stimuli information to the SCN, which will then, synchronize this information and sends signal to the periphery via efferent pathways. RHT-Retinohypothalamic tract, GHT-Geniculohypothalamic tract, IGL- Intergeniculate leaflet, PACAP-Pituitary adenylate cyclase-activating polypeptide, 5-HT- (5-Hydroxy tryptanine), NPY-Neuropeptide Y, GABA- Gamma amino butyric acid, ENK- Enkephalin; VIP-Vaoactive intestinal peptide, AVP-Arginine vasopressin, PK2-prokineticin 2 (Jagota, 2006).

Efferent or output pathways of SCN:

The communication within SCN neurons as well as other brain centers is achieved through both neural and humoral signals which act as output pathways (Fig. 6), to control the circadian and seasonal behavior of the animal.

i) Neural pathway: The axonal projections from the SCN project to nearby hypothalamic regions (Saper *et al*., 2005). These efferent's affects many body functions such as body temperature, locomotor activity and hormonal levels.

ii) Humoral pathway: This is a non-neural pathway which mediates the circadian signal via diffusible output from the SCN into the cerebrospinal fluid (CSF) and/or extracellular spaces.

Neurochemistry involved in SCN signaling:

External stimuli modulate the SCN at different phases and periods of its circadian cycle and communicate via different pathways. Photosensitive retinal ganglion cells that express melanopsin will receive the photic information. These retinal photoreceptor cells convey the light signals to SCN clock by direct projections into the SCN, some of these neurons also innervate a subdivision of the lateral geniculate complex, the IGL. The retinal ganglion cells projected into the SCN release glutamate and PACAP at synaptic contacts with SCN neurons. Neuropeptide PACAP has a modulatory role and enhances the effect of glutamate and there by these two neurotransmitters convey the photic information to the SCN via CREB (*cAMP response element-binding* protein) mediated pathway (reviewed by Welsh *et al.,* 2010). Within the SCN, the retinohypothalamic terminals project to the neurons synthesizing VIP and/or GRP. Under a light-dark cycle, the levels of both peptides displayed daily variations in the SCN, with a peak of VIP and GRP observed during the night time and at the light-dark transition, respectively. These variations disappeared in the SCN of animals transferred to constant darkness, demonstrating involvement of VIP and GRP in the photic synchronization of the SCN. The IGL relay photic information to the SCN through the GHT. These fibers release NPY, GABA, and ENK into the ventrolateral region of the SCN. IGL activation is thought to induce NPY release from the geniculohypothalamic terminals into the vlSCN. There are two main pathways that communicate non-photic information to the SCN. The first is the GHT, which conveys neural signals from the IGL of the thalamus. The second pathway originates in the MR (median raphe) of the brain stem. NPY and GABA are key neurochemicals of the GHT and 5-HT is the major neurotransmitter of the MR. Serotonergic modulation comes from a direct projection to the SCN originating in the median raphe nucleus and an indirect projection originating in the dorsal raphe nucleus

to the IGL. Glutamate is the brain's main excitatory transmitter, whereas NPY and 5-HT are known to be predominantly inhibitory in the mammalian CNS. There are at least six candidate molecules which are rhythmically synthesized and released by the SCN, such as VIP, prokineticin 2 *(*PK2), TGFα (transforming growth factor α), cardiotropin-like cytokine, GABA and glutamate (Piggins and Guilding, 2011). Secretion of these neurochemical molecules into the CSF of the ventricles or via passive diffusion in the extracellular space between neurons are postulated to alter the activity of brain centers controlling behaviour and physiology.

Role of Serotonin and Melatonin in circadian function:

Serotonin (5 -HT)

Serotonin (or 5-hydroxytryptamine [5-HT]) is a small molecule of the indoleamine family, which acts as a neurochemical messenger to communicate information among nerve cells (Lovinger, 1997). 5-HT is synthesised from the essential amino acid L-tryptophan that is ingested in the diet. The ingested tryptophan will be absorbed via the intestinal walls and (80–90%) binds to albumin from where it is transported through the blood into the brain, and transformed to 5-HT, mainly in serotonergic neurons of the raphe nucleus located in the brain stem and in pinealocytes of the pineal gland. Smaller amounts of serotonin are also synthesized in the retina, in the enterochromaffin cells of the gut, in the neuroepithelial bodies of the lung, and in the parafollicular cells of thyroid. Serotonin can also be found in platelets (Sa´nchez *et al.*, 2008). Moreover, most of the drugs currently in use for the treatment of psychiatric disorders (e.g., depression, mania, schizophrenia, autism, obsessive compulsive disorder and anxiety disorders) are thought to act, at least partially, through serotoninergic mechanisms (Aghajanian and Sanders-Bush, 2002). Serotonin released from the signal-emitting neuron acts on the signal-receiving neurons and alters the function in a process called neuromodulation (Lovinger, 1997). 5-HT has been implicated in the circadian phase resetting by modulating the photic signaling in the SCN. Serotonergic agonists inhibit photically related responses, including light-induced phase shifts in free-running locomotor activity (Rea *et al.*, 1994; Pickard *et al.*, 1996).

Serotonin and Feeding behavior

Central 5-HT is involved in different physiological and behavioral functions, such as waking and sleeping, motor activity, aggression, appetite, cognition, emesis, endocrine function, gastrointestinal function (Lam and Heisler, 2007). Some behaviors such as food consumption and sexual behavior are also regulated by

5-HT (reviewed in Sashihara *et al.*, 2002). Serotonergic system's special role in the central regulation of food intake and dietary selection has been shown by numerous evidences. An inverse relationship exists between brain serotonin levels and food intake (Lam and Heisler, 2007). Serotonin influences both brainstem reflex centers and hypothalamic integratory centers involved in controlling food intake. Selective depletion of central serotonin induces hyperphagia and obesity. Administration of serotonin by central or peripheral routes reduces the food intake and also affects food selection and the pattern of feeding behavior (Baranyiova, 1991; Reviewd in Lam and Heisler, 2007). Serotonin also reduces the rate of food consumption and brings about changes in intra-meal and inter-meal intervals (Reviewed in Baranyiova, 1991). Increased output of serotonergic system is thought to be responsible for some of the characteristic nutritional effects on certain brain functions such as elevated mood, increased sleepiness, reduced pain sensitivity, loss of hunger and reduced appetite.

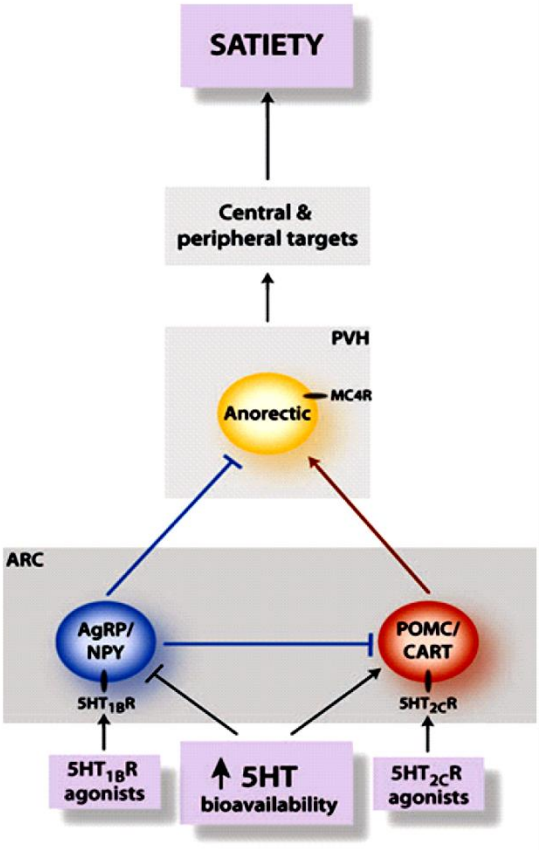


Fig.7. Serotonin causes anorectic effect by down regulating orexigenic peptides (AgRP/NPY) and upregulating anorexigenic peptide (POMC/CART) (Garfield and Heisler, 2009).

Recent studies have shown that serotonergic compounds reduce food intake by manipulating the first order hypothalamic Pro-opiomelanocortin (POMC)/cocaine and amphetamine regulated transcript (CART) and agouti-related protein (AgRP)/NPY neurons (Fig. 7.) (Garfield and Heisler, 2009). Serotonin system is more sensitive to peripheral episodic signals such as those produced by a number of gut peptides released in response to the ingestion of food (Halford *et al.*, 2007) and controls the satiety by acting on 5-HT1 and 5-HT2 receptor subtypes mainly (Feijó *et al.*, 2011).

Melatonin:

Melatonin (N-acetyl-5-methoxytryptamine) is an indolic hormone. It is the principal product of the vertebrate pineal gland and is synthesized from its precursor serotonin. Apart from pineal gland, to a lower extent it is also synthesized in extra cranial sites such as, the gastrointestinal tract, retina of eye, extraorbital lacrimal gland, skin, lymphocytes, Harderian gland, bone marrow and ovary (Karasek, 2007; Pandi-Perumal *et al.*, 2008). Depending upon the production site and target organ, melatonin can act as a hormone, neurotransmitter, cytokine, or biological modifier (Slominski *et al.*, 1996).

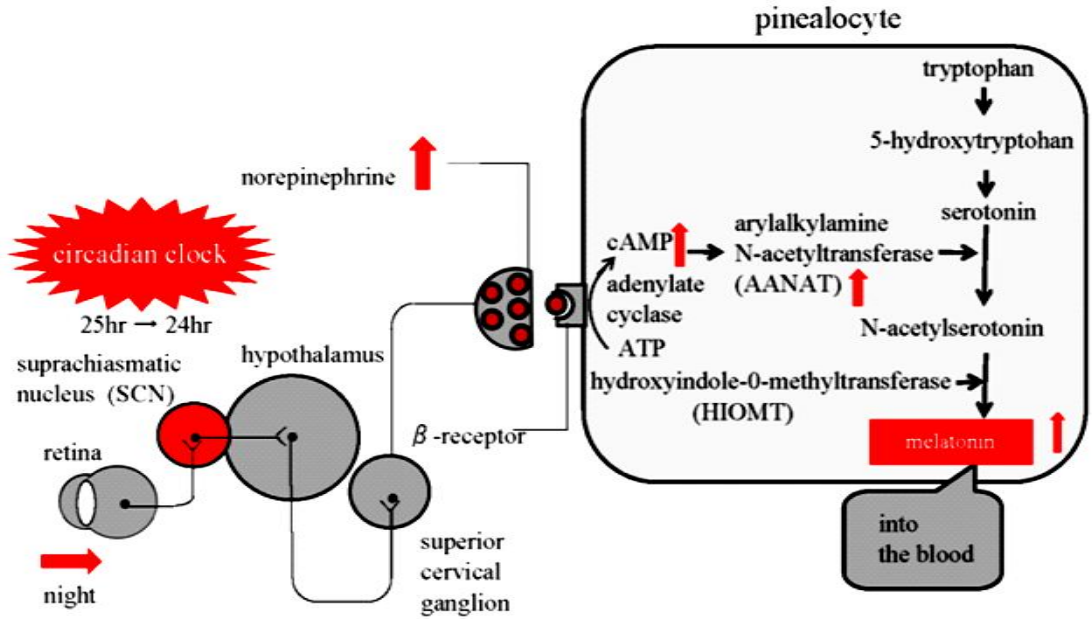


Fig. 8. A multisynaptic pathway involving hypothalamic paraventricular nucleus-intermediolateral column-superior cervical ganglion, SCN stimulates β- adrenergic receptors to synthesize melatonin from the pinealocytes (Yonei *et al.*, 2010).

The rhythmic synthesis and release of melatonin in the pineal gland occurs in a light-dependent rhythmic fashion with high levels always occurring at night and low levels during the light phase, regulated by the SCN via a multisynaptic pathway (Zawilska and Sadowska, 2002). Cells from the SCN project to the paraventricular hypothalamic nucleus. Fibers from this nucleus descend to synapse in the intermediolateral column of the spinal cord, the superior cervical ganglion and postganglionic adrenergic innervations (Fig. 8) (Klante *et al.,* 1999).

Melatonin and circadian clock:

Melatonin provides the organism with the night information and acts as an endogenous synchronizer there by regulates the phase of circadian rhythms and maintains their mutual phase-relationship (reviewed in Pandi-Perumal, 2008). The day-night amplitude of the melatonin rhythm secretion transduces both daily and seasonal time throughout the body of an organism and there by regulates its biological clock (Johnston *et al.*, 2006). Melatonin transduces the photoperiodic information, defining the length of the night to SCN via its two high-affinity melatonin receptors, MT1 and MT2. These receptors, which show distinct molecular structures, chromosomal localization and pharmacological characteristics, are co-localized within the SCN (Dubocovich, 2007). In turn SCN uses the daily melatonin signal to distribute the circadian message to any melatonin receptors possessing structure/organ (reviewed in Pévet and Challet, 2011). The complexity of melatonin's role in modulating a diverse number of physiological processes is achieved by melatonin receptors belonging to two distinct classes of proteins, that is, the G-protein coupled receptor superfamily (MT1, MT2) and the quinine reductase enzyme family (MT3) (Witt-Enderby *et al.,* 2003). Melatonin shows two distinct effects on SCN neurons: acute inhibition of electrical activity and phase-shifting of the clock (Turek and Gillette, 2004).

From a medicinal point of view circadian disruption occurs in three possible ways: (i) reduced amplitudes of single oscillators, as occurs during aging or under weak zeitgeber exposure because of the decreased neuronal activity in the SCN and decreased melatonin biosynthesis and nocturnal plasma melatonin levels (Zee and Vitiello, 2009); (ii) phase shifts that uncouple by differences in the resetting of single oscillators (e.g., rotating shift work, light at night); (iii) a deviating, poorly synchronized period of the central pacemaker, as occurring in blind people, which may show relative coordination with the environmental cycle (Fig. 9) (Hardeland *et al.,* 2012).

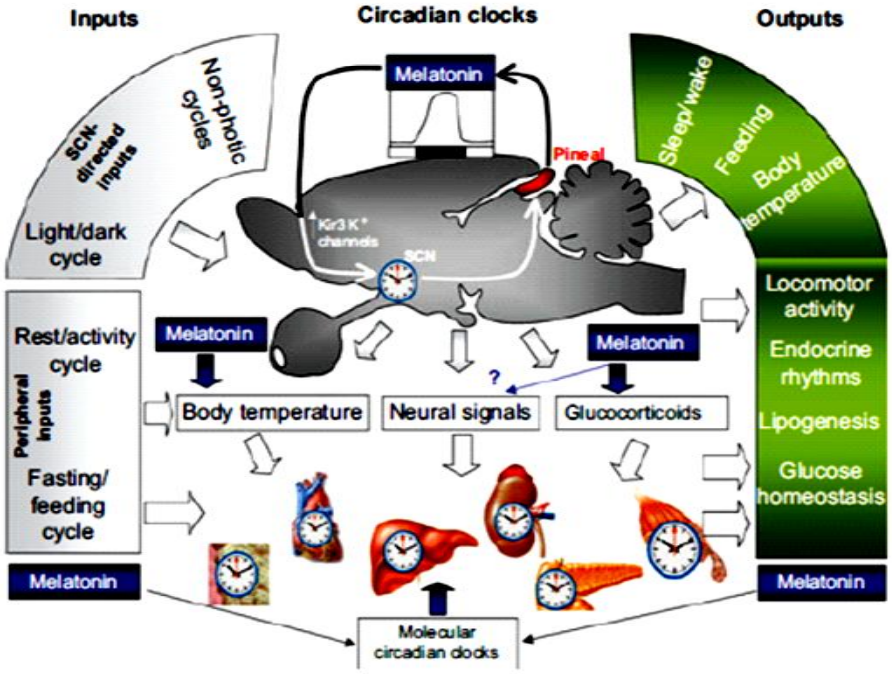


Fig. 9. Melatonin regulates various physiological and behavioral functions by the modulation of suprachiasmatic nucleus (SCN). (Hardeland *et al.*, 2012).

This decreased circulating melatonin levels may lead to a variety of physiologic changes. The therapeutic potential of melatonin with its phase-shifting effects can be used in treating circadian rhythm disorders (Alvarez *et al.*, 2000) like circadian rhythm sleep disorders (CRSD), jet lag, and shift work maladaptation, entraining blind subjects to the 24h rhythm (Borjigin *et al.*, 1999). All of these disorders and syndromes are related to the circadian timing system and can be phase typed, according to whether they are phase delayed or phase advanced (Fig.10) (reviewed in Lewy, 2003; Jagota, 2005).

• Phase-advanced type	• Phase-delayed type
Advanced sleep phase syndrome (ASPS)	Delayed sleep phase syndrome (DSPS)
East-to-west jet lag	West-to-east jet lag
Night work maladaptation (work days)	Night work maladaptation (weekends)
	Typical seasonal affective disorder (SAD)

Fig. 10. Phase advanced and phase delayed circadian rhythm sleep disorders (Lewy, 2003).

In addition, melatonin is involved in the regulation of a variety of important physiological and pathophysiological processes (Fig.11) such as visual and vasoactive, oncostatic, neuroimmunological regulatory, neuroprotective, sexual development, immune system, blood pressure regulation, retinal

physiology, seasonal reproduction, ovarian physiology, osteoblast differentiation and mental disorders and is a potent free radical scavenger (Witt-Enderby *et al.,* 2003; Abdel-Wanis and Tsuchiya, 2002; Rasmussen *et al.,* 2003). Melatonin also reinforces the nocturnal decrease of central temperature, an event which facilitates sleep propensity which is probably via its vasodilatation effects. Melatonin acts as a soporific agent rather than a hypnotic, as it increases sleepiness.

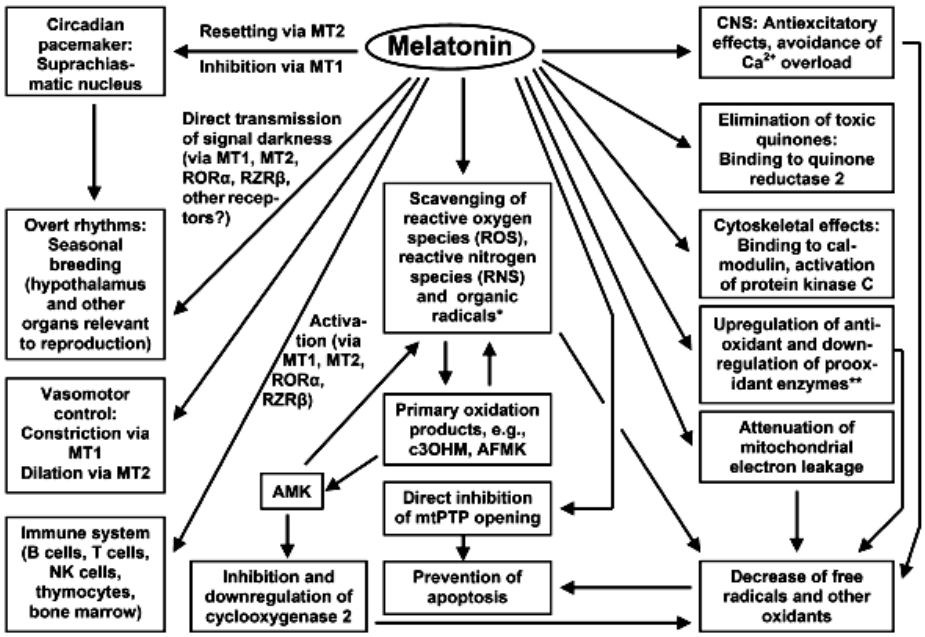


Fig. 11. Pleiotropic actions of melatonin via its MT1 and MT2 receptors (Pandi-Perumal *et al.*, 2006).

Serotonin biosynthesis and metabolism:

The amino acid L-tryptophan is an indispensable and essential amino acid required by every living cell (Floc'h *et al.,* 2011). The metabolism of L-tryptophan is a highly regulated physiological process. In the cell, tryptophan has three major activities i.e. protein, kynurenine and serotonin synthesis (Richard *et al.,* 2009). The major part of tryptophan is utilized for protein synthesis whereas; the major catabolic route of tryptophan is via kynurenine and 3-hydroxyanthranilic acid pathway. In the human body only a minor metabolic route for tryptophan represents biosynthesis of serotonin (Kema *et al.,* 2000; Richard *et al.,* 2009) (3% of dietary tryptophan is used for the synthesis of serotonin throughout the body and only 1% of dietary tryptophan is used for serotonin synthesis in the brain). Serotonin synthesis is one of the most important tryptophan pathways which leads to the generation of other neuroactive compounds such as melatonin within the central nervous system and is topic of intense research.

The synthesis of serotonin begins with the action of the enzyme tryptophan hydroxylase, the rate limiting enzyme in this process, which converts L-tryptophan to 5-hydroxytryptophan which is then decarboxylated by 5-hydroxy tryptophan decarboxylase a vitamin B6 dependent step resulting in the formation of 5-hydroxytryptamine (5-HT) (Sa´nchez, 2008; Floc'h *et al.*, 2011). Two isoforms of TPH exists in mammals: TPH1 in enterochromaffin cells and TPH2 in neurons (Floc'h *et al.*, 2011). A majority of the serotonin is metabolized via oxidative deamination by the flavoprotein monoamine oxidase (MAO) (Kema *et al.*, 2000). MAO is mainly located in mitochondria and catalyses oxidative deamination of several monoamines. 5-Hydroxyindoleacetaldehyde then can either be oxidised to 5-hydroxyindoleacetic acid (5-HIAA) by aldehyde dehydrogenase with NAD as coenzyme (Garattini and Valzelli, 1965; Kema *et al.*, 2000) or reduced to 5-hydroxytryptophol (5-HTOH) by aldehyde reductase in the presence of NADH as coenzyme. 5-HTOH is further converted to 5-methoxytryptophol (5-MTOH) by hydroxy indole O-methyl transferase (HIOMT). A subsequent secondary pathway catalyzes serotonin to produce N-acetyl serotonin (NAS) and melatonin in mammals. Serotonin is first acetylated by arylalkylamine N-acetyltransferase (AANAT) to form N-acetyl serotonin (NAS). NAS is then methylated to melatonin (MEL) by HIOMT (Yonei *et al.*, 2009). 5-HT also forms 5-methoxy indole acetic acid (5-MIAA) by the action of HIOMT, MAO and aldehyde dehydrogenase. Tryptophan undergoes decarboxylation to form tryptamine which will be then converted to N-acetyl tryptamine (NAT) by the action of serotonin N-acetyltransferase (Richard *et al.*, 2009) (Fig.12).

In the liver serotonin will be converted to sulfuric acid (formation of serotonin-*O*-sulfate) or glucuronic acid (serotonin-*O*-glucuronide) conjugates by the enzymatic action of serotonin sulfotransferase and uridine diphosphate glucuronyltransferase respectively and excreted out in urine (Tyce, 1990; Kema *et al.*, 2000). Serotonin sulfotransferase is present in liver, lungs, kidney and brain. 5-HIAA is excreted predominantly in the free form, whereas 5-hydroxytryptophol is mainly excreted as conjugate in urine (Kema *et al.*, 2000). Tryptophan is an important neuromediator. Inadequate tryptophan availability leads to changes in various psychological, behavioral and physical processes. In addition, it is a contributing factor in motion sickness, sleep, mood, visual discrimination, cognition, social information processing, memory processes anxiety, aggression, stress, appetite, hemodynamics and others (Richard *et al.*, 2009; Floc'h *et al.*, 2011). In alcoholism and (other) liver pathology, 5-hydroxytryptophol levels considerably increases because of the metabolic shift from 5-HIAA to 5-hydroxytryptophol (Kema *et al.*, 2000).

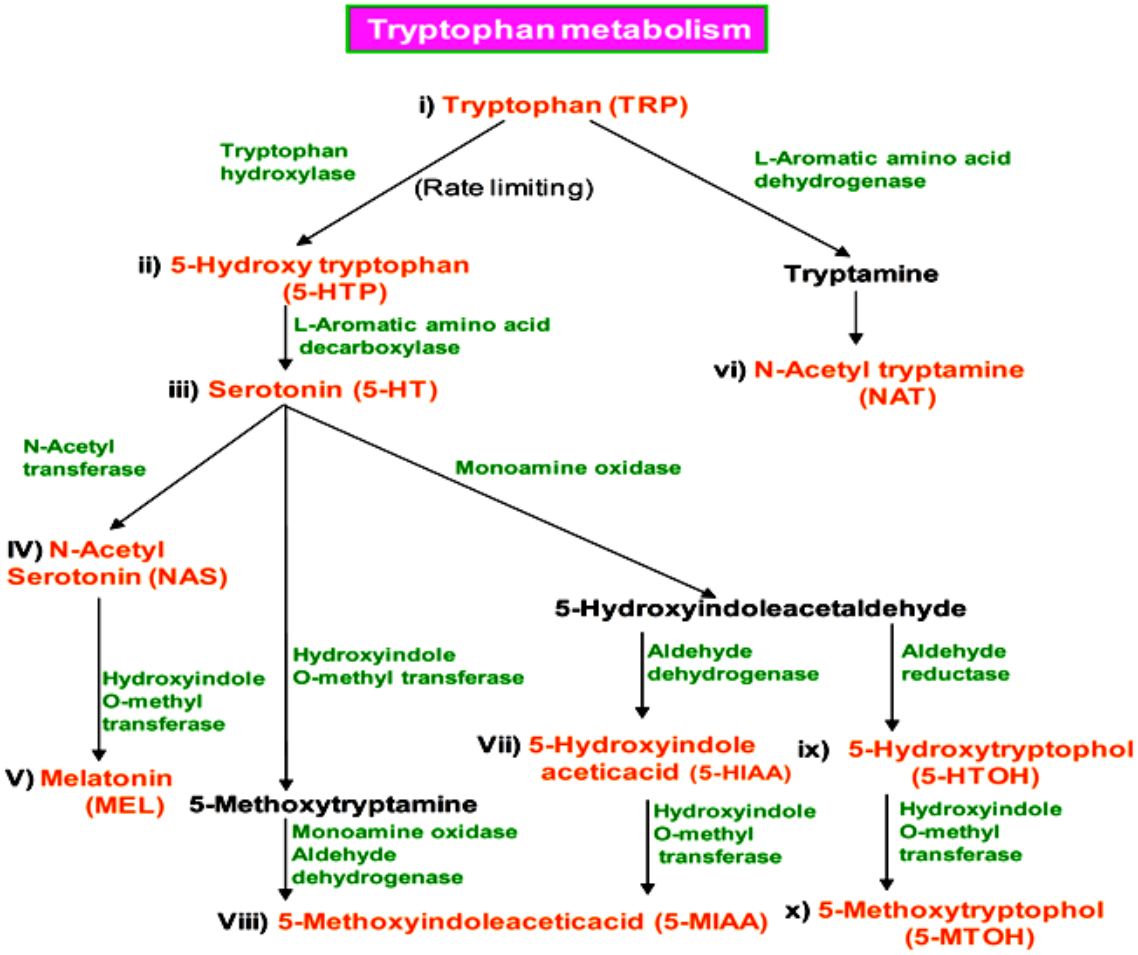


Fig. 12. Tryptophan metabolism (Garattini and Valzelli, 1965; Kema *et al.*, 2000).

Reduced 5-HIAA levels were observed in the CSF of depressed and manic patients. Brain 5-HT and 5-HIAA levels reported to be lower in the brains of suicide subjects (reviewed in Coppen, 1972). 5-HTP acts as an antidepressant. 5-HTP supplementation has shown to decrease the symptoms in patients suffering from anxiety and panic disorder because of the decreased serotonin levels (Shaw *et al.*, 2009). *N*-acetylserotonin has been reported to be an antioxidant. It preserves the optimal levels of fluidity of the biological membranes by reducing lipid peroxidation (Garc´ıa *et al.*, 2001). The pineal hormone 5-MTOH is involved in the developmental process in humans. 5-MTOH also exerts biological actions in fish, birds and mammals, as both pro- and antigonadotropic, depending on the species, age, sex, and photoperiod. In cultured chick retinal pigment epithelium cells 5-MTOH acts as an inhibitor of phagocytic activity (reviewed in Zawilska *et al.*, 1998), whereas in chicks it stimulates both testicular and ovarian growth and development and in adult it shows anti-gonadal activity. Similarly it inhibits oestrus cycle and decreases

spermatogenesis in rats (reviewed in Smith and Francis, 1980). 5-MTOH contributes in the seasonal change in reproductive activity in most species of animals (Saxena *et al.*, 1991).

Role of leptin in circadian system:

Circadian clock functions are reciprocally modulated by certain input signals which relay information about the nutritional status (adiposity, glucose homeostasis, sympathetic and parasympathetic balance) or behavioral status (feeding, sleep etc.) of the body (Laposky *et al.*, 2008). One such input relaying signals of energy and metabolism is leptin, which can reciprocally act as a bridge between energy homeostasis and circadian clock by its circadian oscillation (Froy, 2010). Leptin secretion is regulated in part through the daily feeding rhythm. Plasma leptin in humans shows a strong diurnal rhythm with a peak at 24 h and nadir between 9 and 12 h. When the meal intake timing was altered by 6.5 h without changing the light or sleep cycles, the rhythm of plasma leptin was shifted by 5–7 h (Schoeller et al, 1997). Leptin exerts its prominent effects in various parts in the hypothalamus via its receptors (Guan *et al.*, 1997). In these regions, leptin acts on glucose-sensitive neurons, through modulation of K_{ATP} channels whereas SCN controls the timing of feeding by regulating different hypothalamic regions involved in energy homeostasis including median preoptic area (MPOA) via subparaventricular zone (SPZ) and dorsomedial hypothalamus (DMH). MPOA is the site which regulates the adipose tissue activity and leptin production. Expression of leptin receptors in several hypothalamic regions including SCN has been reported previously (Guan *et al.*, 1997; also reviewed in Prosser and Bergeron, 2003). Leptin has been also reported to phase advance the SCN circadian clock in a dose-dependent manner in isolated *in vitro* brain slices (Prosser and Bergeron, 2003).

The molecular mechanism of the mammalian circadian oscillation:

The intracellular time keeping mechanism which generates the circadian rhythms via a cascade of processes leading from the circadian clock through molecular and cellular rhythms to the temporal organization of physiology and behavior according to the environmental changes represents a basic feature of life of the most organisms living on earth (Giebultowicz, 2000; Zhang *et al.*, 2004). Any alterations in this natural synchrony between cycles of day/night, activity/rest and hormonal or feeding behavior etc. can induce modifications in this highly complex mechanism of anticipation (Zanquetta *et al.*, 2010). Since the internal clock mechanism can count time only approximately it must be adjusted every day in order to be in harmony with the outside world. Circadian oscillators are highly conserved throughout evolution

(Akashi *et al.,* 2006). In mammals the self-sustained circadian oscillations are driven and dynamically regulated by clock proteins through a cell autonomous, auto-regulatory network of interlocking positive and negative transcriptional/translational based core feedback loops, containing a core set of genes which are highly conserved among animals (Zhang *et al.,* 2004). These autoregulatory feedback loops take ~24 h to complete a cycle and constitute a circadian molecular clock. The "delayed negative feedback loops" plays an important role in circadian oscillations generation processes (Harmer *et al.,* 2001).

The elements of these feedback loops consist of at least 10 main proteins in which many of them act as transcription factors since they have PAS (Per-ARNT-Sim; involved in protein-protein interactions) and bHLH (basic helixloop-helix; involved in protein-DNA interaction) domains: CLOCK (circadian locomotor output cycles kaput), BMAL1 (brain and muscle ARNT-like protein 1), NPAS2 (neuronal PAS domain protein 2) {a paralogue of CLOCK and an obligate dimeric partner of BMAL1}, PER1 (period 1), PER2 (period 2), PER3 (period 3), CRY1 (cryptochrome 1), CRY2 (cryptochrome 2), REVERBα (reverse erythroblastosis virus α) and RORα (retinoid-related orphan receptor-α) (Zanquetta *et al.,* 2010). Among these transcription factors the positive feedback loop elements include members of the basic helix-loop-helix (bHLH)-PAS (Period-Arnt-Single-minded) transcription factor family, CLOCK and BMAL1 (Ko and Takahashi, 2006). The intracellular levels of CLOCK remain steady throughout the 24 h period in SCN neurons, whereas BMAL1 levels are high at the beginning of a subjective day and low at the beginning of a subjective night. During the light period, the (bHLH)-PAS containing transcription factor CLOCK (or NPAS2) interact with BMAL1 to form a heterodimer and initiate transcription of target genes containing CACGTG E-box cis-regulatory enhancer sequences, including Period (in mice, *Per1*, *Per2* and *Per3*) and Cryptochrome (*Cry1* and *Cry2*) and a transcription factor and nuclear hormone receptor Reverbα, and one promoting gene Rorα and many output genes that are controlled by the clock (CCGs: clock-controlled genes) results at high levels of these transcripts in the cytosol (Ko and Takahashi, 2006; Garaulet *et al.,* 2010). The resulting protein products undergo post-translational modifications such as phosphorylation and ubiquitination. These processes precisely regulate the generation of the ~24-h mammalian molecular clock by affecting the stability and nuclear translocation of core clock proteins (Ko and Takahashi, 2006). When the PER and CRY proteins in the cytoplasm reaches a critical concentration, they oligomerize and translocate into the nucleus and interact with the CLOCK–BMAL1 complex to inhibit the transcriptional activity of the CLOCK/ BMAL1 heterodimer. The negative arm of the feedback loop is achieved when the CRY1 disrupts the CLOCK/BMAL1-associated transcriptional complex, resulting in the inhibition of *Cry, Per* and *Reverb*-α transcription, and decreases *Bmal1* transcription (Sukumaran *et al.,* 2010). In this way the PER:CRY heterodimers repress their own transcription by acting

on the CLOCK: BMAL1 complex. Casein kinase 1 epsilon (CK1ξ) and Casein kinase 1 delta (CK1 δ) are the critical factors which play a significant role in regulation of the core circadian protein turnover and in maintaining circadian periodicity in mammals by phosphorylating the clock proteins like PER and tags them for degradation, thereby reducing their stability (Ko and Takahashi, 2006). Phosphorylation leads to target the clock proteins for polyubiquitination and degradation by the 26S proteosomal pathway. Phosphorylated proteins will be directly targeted by ubiquitin ligase complexes such as β-TrCP1 and FbXL3 e3 ubiquitin ligase for degradation (Mohawk *et al.*, 2012). As part of the cyclical process, the stability of PER and CRY proteins is tightly controlled by CKIε (casein kinase Iε) and the F-box protein FBXL3 respectively (Fig. 13) (Froy, 2011). However in the late subjective day CRY accumulates in the cytoplasm and promotes the formation of stable CK1ξ /Per/Cry complexes, which enter the nucleus at the beginning of a subjective night. Regardless of whether the animal is diurnal or nocturnal the

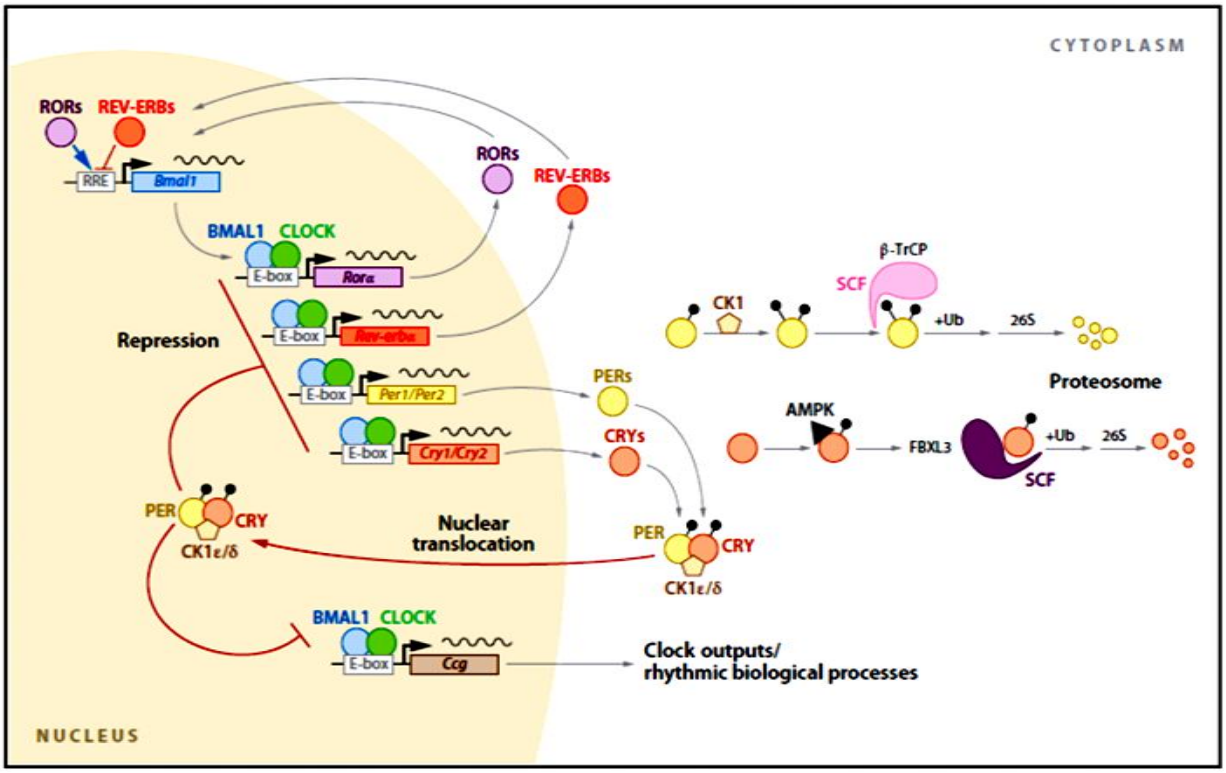


Fig. 13. Molecular machinery of the circadian oscillator constitutes an autoregulatory transcriptional feedback loop containing CLOCK and BMAL1 as activators and *Per1, Per2, Cry1,* and *Cry2* as the target genes of the loop whose protein products form a negative-feedback repressor complex. Another feedback containing *Reverb*-α and *Rorα* represses *Bmal1* transcription. CLOCK: BMAL1complex also promotes the transcription of clock-controlled genes (Ccg). The PER and CRY proteins stability is regulated by SCF (Skp1-Cullin-F-box protein) E3 ubiquitin ligase complexes involving β-TrCP and FBXL3. Casein kinase 1ε/δ (CK1ε/δ) and AMP kinase (AMPK), phosphorylate the PER and CRY proteins, and promotes polyubiquitination by their respective E3 ubiquitin ligases which in turn directs the PER and CRY proteins for degradation by the 26S proteasome complex (Mohawk *et al.*, 2012).

CLOCK:BMAL levels are high during the light period and PER:CRY in the dark period (Sukumaran *et al.,* 2010). During the late subjective night, the PER–CRY repressor complex is degraded, and CLOCK–BMAL1 complex can then activate a new cycle of transcription (Takahashi *et al.,* 2008).

In addition to the primary feedback loops there is a secondary negative feedback loop called the 'stabilizing loop' which is achieved by both positive and negative regulation of the orphan nuclear receptors REV-ERBα and RORα which will cyclically regulates the expression of *Bmal1*. REV-ERBα competes with RORα for binding to the ROR-responsive element (RORE) to the *Bmal1* promoter and negatively regulates the *Bmal1* gene expression in the nucleus where as RORα positively regulate the expression and activates transcription of *Bmal1* (Kwon *et al.,* 2011). REV-ERBα and RORα are involved in the gene expression during circadian night, which is in phase with BMAL1 and in antiphase with Per2 oscillations. Although REV-ERBα and RORα are not involved in rhythm generation, they play a crucial role in regulating the phase and the amplitude of gene expression (Sukumaran *et al.,* 2010).

Aging and circadian rhythms:

Aging is a progressive, more or less random, accumulation of diverse, deleterious changes with time (Harman, 2006). In mammals, aging is associated with changes in many aspects of circadian function including changes in phase relationship of rhythms to the environmental time signals, reduced sensitivity of the central pacemaker to time cues, decreased precision in onset of daily activity, erratic neural firing of SCN, decrement in the amplitude of many overt rhythms (Jagota and Kalyani, 2008 and 2010) including the rhythms of locomotor activity, drinking, body temperature, and reduction in the duration of sleep bouts and increased sleep fragmentation, alterations in the response to the phase-shifting effects of light and shortening in the period of body temperature, reduced tolerance to shift work. In addition to this, species-specific changes in the free-running period {tau (τ)} in constant darkness are also often observed (Kolker *et al.,* 2004; Garau *et al.,* 2006).

In humans upon aging: 1) the amplitude of circadian rhythms reduces, 2) the phase of the circadian rhythms becomes advanced, 3) the natural free-running period shortens and 4) the ability of the individual to tolerate abrupt phase shifts worsens (Monk, 2005). Apart from this most other physiological manifestations of the circadian clock become less pronounced like as age progresses, a phase advancement of nocturnal pineal melatonin secretion and body temperature rhythms have been observed with a reduced number of melatonin binding sites in the hypothalamus (Mirmiran *et al.,* 1989; Brown *et al.,* 2011).

Another important trait of the aging is the strong reduction in the amplitude of the diurnal rhythms of serotonin and melatonin since melatonin is synthesized from serotonin. The temperature rhythm amplitude also decreases in aging condition. In addition to this aged rodents exhibit reduced SCN responsiveness to melatonin. In addition to this aged rodents exhibit reduced SCN responsiveness to melatonin (Jagota and Kalyani, 2010; Froy, 2011; Manikonda and Jagota, 2012).

Under aging condition the pulse amplitude of the neuroendocrine system changes and an irregularity in the periodicity of hormone and neurotransmitter release occurs. Circadian endocrine rhythms are associated with altered carbohydrate and lipid metabolism during aging. These changes lead to reduced glucose tolerance, obesity and peripheral insulin resistance (Smith *et al.,* 2005).

The aging related alterations are paralleled by changes in the regulatory components of the SCN like changes in the neurochemical and electrophysiological output of the SCN with no change in its cell number or size. During aging the rhythmic expression of vasoactive intestinal peptide (VIP), arginine vasopressin (AVP) and transforming growth factor beta (TGF-β) alters in the SCN with a reduction in the amplitude of rhythmic electrical activity (Smith *et al.*, 2005; Froy, 2011).

In aged mice, the *Per1* expression markedly reduces with a significantly longer delay to resynchronization and the expression of *Per2* impairs in the SCN. In addition to this *Clock* mRNA expression reduces in the SCN and non-SCN brain areas of aged animals. Similarly the amplitude of *Bmal1* mRNA expression decreases in aged hamsters, with lower expression during the subjective night, when *Bmal1* expression is normally high. All these changes in the SCN could contribute to age related circadian dysfunction (Froy, 2011).

Role of food availability in regulation of biological clock:

Limiting the time and duration of food availability without calorie reduction is termed as restricted feeding (RF). To an *ad libitum* food receiving animal, if the food is restricted to a particular time of day has profound effects on the physiology and behavior of animal (Froy and Miskin, 2010). Nocturnal animals like rats and mice are fed exclusively during the day time; the phase of circadian gene expression in the SCN of these animals is unaltered, whereas the phase of circadian gene expression in peripheral organs like liver is uncoupled from the SCN pacemaker (reviewed in Sheward *et al.,* 2007). Previous reports have

shown that the persistence of food-entrainable daily rhythms like food anticipatory activity,

gastrointestinal and metabolic functions in SCN-lesioned restricted fed rodents led to the proposal of a food entrainnable oscillator (FEO) which is indipendent from the oscillator in the SCN (light entrainable oscillator) (reviewed in Dibner, 2010). In night-shift workers, the activity period is reversed in relation to the light-dark cycle and the normal synchrony between the light-dark, sleep-wake and fasting-feeding cycles is disturbed (Froy, 2010). In the pre-modern world the temporal feeding and fasting cycles were matching the patterns of sleep-wake cycles which occur according to the daily periods of light and darkness and thereby balancing the biological clock. In today's industrialized society the central pacemaker is unbalanced due to the insufficient or contradictory input signals and there by hampers the synchronization to the outside world and also fails in the entrainment of its output signals (Kalsbeek et al, 2011). Among the non photic stimuli, food restriction is one of the strongest non-photic stimulus that can entrain the clock (Caldelas *et al.,* 2005). When a nocturnal animal like wistar rat fed during the day time, such protocol provides a condition very much similar to the circadian misalignment in many night shift workers. The symptoms are very similar to jet-lag and termed as social jetlag (Kalsbeek et al, 2011). Much light has not been shed on the alterations in the serotonin metabolism, clock gene expression, protein profile of SCN under reversal of feeding time to a nocturnal rodent model under aging condition. The daily leptin production rhythm shows a direct control of adipose tissue activity by the biological clock. Viral tracing studies using injection of the pseudorabies virus into white adipose tissue (WAT) labeled the neurons in SCN, paraventricular nucleus (PVN) and MPOA, indicating the role of SCN in regulation of lipid metabolism via the MPOA (Froy, 2010). Hence we hypothesized that alterations in the adipose tissue activity may alter the leptin production rhythm and there by an altered levels in the SCN region.

With this literature background the following objective were framed:

Objectives

Objectives:

Understanding the Mechanisms involved in Age induced alterations in Non-photic regulation of Biological Clock: Effect of reversal of feeding regimen due to restricted availability of food.

1. **Studies of gross locomotor activity rhythms.**
2. **Chronomics of Serotonin metabolism in SCN and Pineal**
3. **Daily Leptin rhythms in SCN**
4. **Daily rhythms of clock gene *(Per1, Per2, Cry1 and Cry2)* expression in SCN and Pineal**
5. **Daily rhythms of protein profile in SCN and Pineal**

Materials and Methods

Experimental Animals:

Male wistar rats of 3, 12 and 24 months (m) were acclimatized at LD; 12:12 conditions (Zeitgeber time) 06:30 h (ZT-0) – 18:30 h (ZT-12) light phase; 18:30 h (ZT-12)–06:30 h (ZT-24) dark phase for 2 weeks prior to the experiments. All rats were kept individually in polypropylene cages at 23 ± 1˚C with relative humidity (55 ± 6%) contained within well ventilated light proof environmental cabinets isolated in animal facility. During this period food and water were provided *ad libitum*. In the light phase, animals were exposed to 300 lux, automatically controlled by a 24 h timer (Legrad, Germany). Dim red light (Kodak 1A filter) was used for handling the animals in dark. Cage changing was done at random intervals. All experiments were performed as per Institutional Animal Ethics.

Animals were divided into two groups: Group 1 (Control) and Group 2 (Restricted fed).

Group 1A (n=48 animals): All animals in this group were provided with *ad libitum* food. This group was further divided into 3 sub-groups.

1A i. 3 m animals used for the study of daily leptin rhythms in SCN (objective-III) n=16.
1A ii. 12 m animals used for the study of daily leptin rhythms in SCN (objective-III) n=16.
1A iii. 24 m animals used for the study of daily leptin rhythms in SCN (objective-III) n=16.

Group 1B (n=228 animals): All animals in this group were provided with *ad libitum* food. This group was further divided into 3 sub-groups i.e. 1B i (3 m), 1B ii (12 m), 1B iii (24 m)

1B i (3 m) had been subdivided into 5 groups:

1B i.a. (n=76); n=4 animals were used for study of gross locomotor activity rhythms (objective I),

1B i.b. n=24 used for the study of chronomics of serotonin metabolism in SCN and Pineal (objective-II),

1B i.c. n=16 animals were used for the study of daily leptin rhythms in SCN (objective-III),

1B i.d. n=16 animals were used for the study of daily rhythms of clock gene (*Per1, Per2, Cry1 and Cry2)* expression in SCN and Pineal (objective IV) and

1B i.e. n=16 animals were used for the study of Daily rhythms of protein profile in SCN and Pineal (objective V).

1B ii (12 m) had been subdivided into 5 groups:

1B ii.a. (n=76); n=4 animals were used for study of gross locomotor activity rhythms (objective I);

1Bii.b. n=24 used for the study of chronomics of Serotonin metabolism in SCN and Pineal (objective-II),

1B ii.c. n=16 animals were used for the study of daily leptin rhythms in SCN (objective-III),

1B ii.d. n=16 animals were used for the study of daily rhythms of clock gene (*Per1, Per2, Cry1 and Cry2)* expression in SCN and Pineal (objective IV) and

1B ii.e. n=16 animals were used for the study of Daily rhythms of protein profile in SCN and Pineal (objective V).

1B iii (24 m) had been subdivided into 5 groups:

1B iii.a. (n=76); n=4 animals were used for study of gross locomotor activity rhythms (objective I);

1B iii.b. n=24 used for the study of chronomics of serotonin metabolism in SCN and Pineal (objective-II),

1B iii.c. n=16 animals were used for the study of daily leptin rhythms in SCN (objective-III),

1B iii.d. n=16 animals were used for the study of daily rhythms of clock gene (*Per1, Per2, Cry1 and Cry2)* expression in SCN and Pineal (objective IV) and

1B iii.e. n=16 animals were used for the study of Daily rhythms of protein profile in SCN and Pineal (objective V).

Restricted feeding (RF):

After the acclimatization period, the animals were subjected to restricted feeding (RF), where food was provided only during the subjective day from 6:30 A.M. {Zeitgeber Time -0 (ZT-0)} to 6:30 P.M. (ZT-12) (12 h day time feeding) for 3 weeks. (Bodosi *et al.,* 2004).

SCN and pineal tissue preparation

Animals of 3, 12 and 24 m old were sacrificed by decapitation and the brains were dissected out carefully at various Zeitgeber time points (ZT-0, 6, 12 and 18). Using brain slicer (Zivic Instruments; Pittsburg USA), 500µ brain slices were made and SCN tissue was carefully punched out with the help of a sharp scalpel (Jagota and Kalyani, 2010). Superficial Pineal gland was separated. Both SCN and pineal tissues were snap freezed with liquid nitrogen for further use.

Behavioral studies:

Gross locomotor activity

The individual cages for gross locomotor studies were equipped with infra-red (I.R) motion detector sensors (IR 28 kit, INT, India). The output terminals of the detector were connected to data acquisition interface board (DIO-3264) which was attached to computer. The data of the gross locomotor activity of animals of all the three age groups under LD 12:12 conditions was collected, recorded, analyzed and double plotted as an actogram in the computer using Chronobiology Kit (Fig.14.) (Stanford Software Systems, USA) (Mammen and Jagota, 2011).

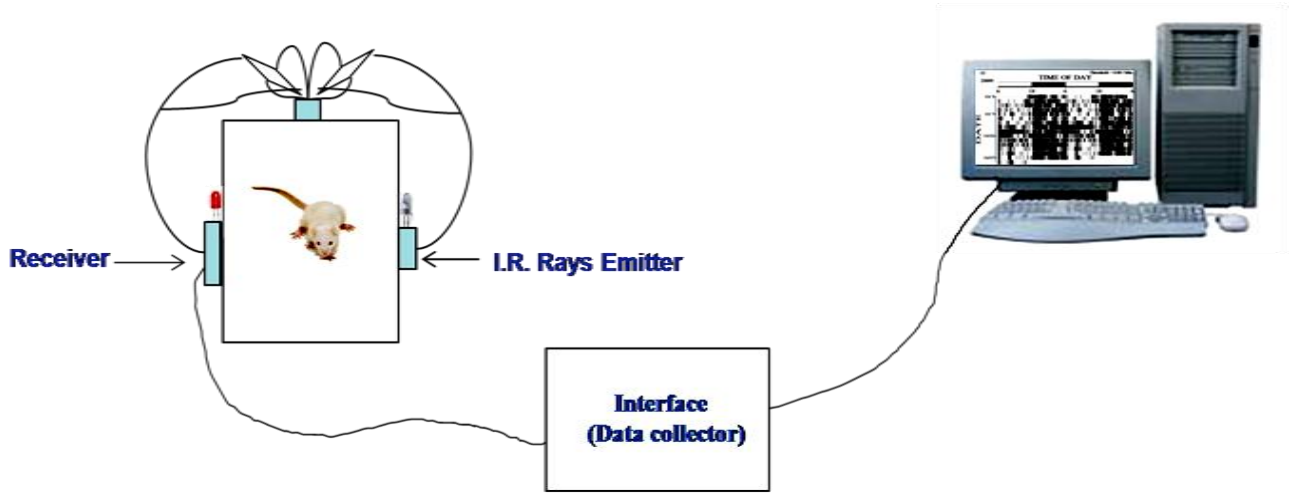


Fig. 14. Set-up for recording gross locomotor activity.

Assay of various components of serotonin metabolism with reversed-phase high performance liquid chromatography using electrochemical detector (RP-HPLC-ECD):

5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), N-acetyl serotonin (NAS), melatonin (MEL), tryptophan (TRP), 5-hydroxytryptophan (5-HTP), 5-hydroxytryptophol (5-HTOH), 5-methoxy tryptophol (5-MTOH), 5-methoxyindoleacetic acid (5-MIAA) and N-acetyltryptamine (NAT) levels were assayed in SCN and pineal tissue using HPLC-ECD method (Mefford *et al*., 1980; Grady *et al*., 1984; Jagota and Reddy, 2007).

Sample preparation for RP-HPLC assays

Tissue samples of SCN and pineal were homogenized in 100 µl of 0.1N Perchloric acid (Qualigens) containing sodium bisulfate (1mM) (Sd-fine Chemicals). The homogenates were then sonicated for 10 seconds and centrifuged at 12,800 x g for 10 minutes at 4ºC to remove tissue debris. Supernatant was filtered through 0.22 µ syringe filters (MDI membrane technologies). The pellet fraction was used for protein estimation.

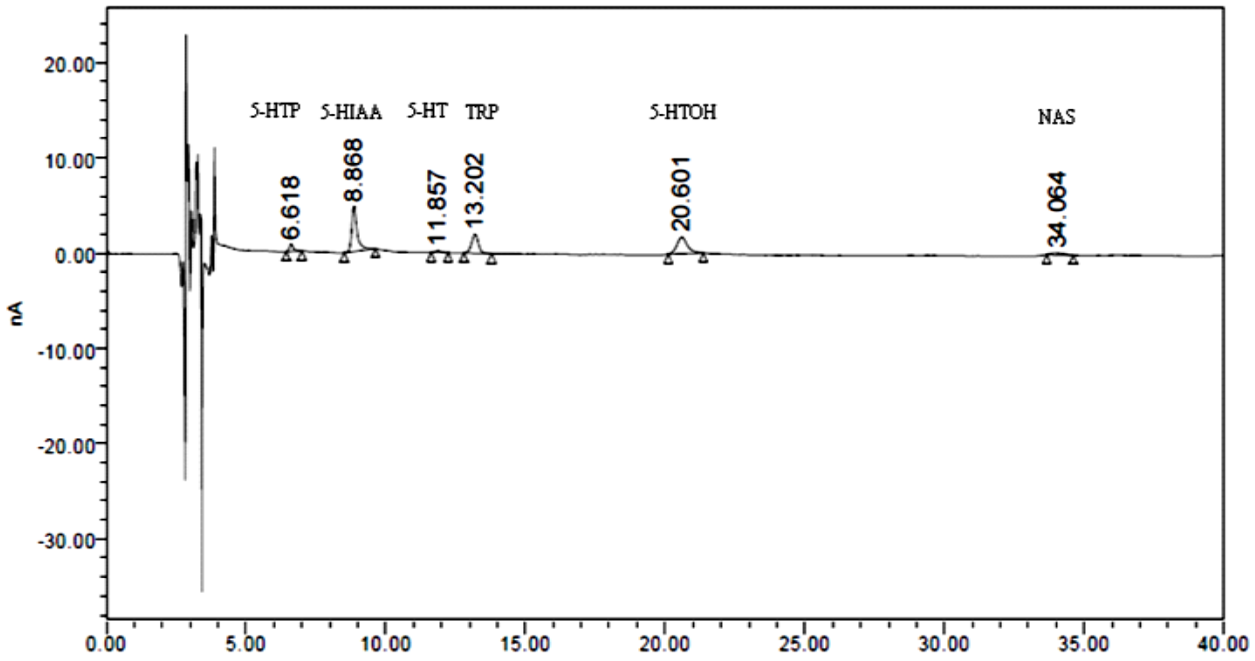
Analysis of the compounds using RP-HPLC-ECD

Serotonin and its related metabolites were assayed by RP-HPLC-ECD (Aliance, Waters, USA) system using C_{18} reversed phase silica column (25 cm X 4.7 mm, 5 µ pore size) coupled with electrochemical detector (2465, Waters) containing carbon working electrode, ISAAC reference electrode, stainless steel auxiliary electrode maintained at potential of 0.71V Flow rate was maintained at 1ml/min. All chromatographic experiments were performed at ambient temperature (25°C) in electrically shielded room.

50 µl of clear filtered supernatant was applied to the chromatographic system and compounds were eluted using a mobile phase containing sodium acetate-citrate buffer system-A {10% methanol; 0.1M citric acid; 0.1M sodium acetate, 50 mg/litre EDTA (pH- 4.1) (HI-MEDIA), NaCl (Qualigens) (0.12 g/L } to estimate TRP, 5-HTP, 5-HT, 5-HTOH, 5-HIAA and NAS and Sodium acetate-citrate buffer system-B {25% methanol; 0.1M citric acid, 0.1M sodium acetate; 50 mg/litre EDTA, NaCl (Qualigens) (0.12 g/L (pH–4.1)} to estimate MEL, 5-MTOH, 5-MIAA and NAT. Before using both the mobile phases were

filtered through 0.22 µ Supor®200 membrane filters (PALL life sciences) and then degassed under vacuum. HPLC grade chemicals were used for the preparation of mobile phases.

A.



B.

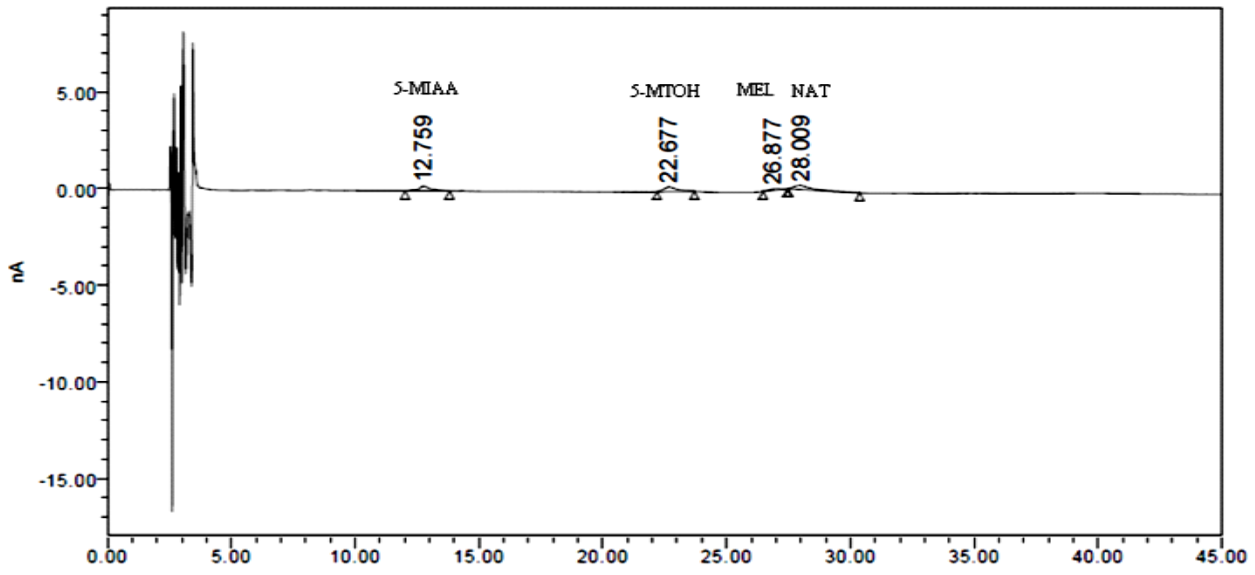


Fig. 15. Standard representative peaks of HPLC for serotonin and its related compounds. (A) 10% methanol; 0.1N Sodium acetate; 0.1N citric acid, 50 mg/litre EDTA was used for detection of compounds- 5-HTP, 5-HT, 5-HIAA, NAS, TRP and 5-HTOH. (B) 25% methanol; 0.1N sodium acetate; 0.1N citric acid, 50 mg/litre EDTA was used for detection of compounds- 5-MIAA, 5-MTOH, MEL and NAT. nA refers to nanoampere (Unit of strength of electric current).

Estimation of the compounds

Known concentrations (2-100 ng) of all standard compounds {TRP, 5-HTP, 5-HT, NAS, 5-HIAA, MEL, 5-MTOH, 5-MIAA, NAT (Sigma); and 5-HTOH (MP Biomed, USA)} were dissolved in 0.1N perchloric acid and applied to the HPLC system. Standard curves were prepared and linearity was established for all the compounds (Fig. 15 A and B). The concentration of each compound in the sample was estimated by comparing the peak area with their respective standard peak (Mefford *et al*., 1980; Reddy, 2010).

Immunohistochemistry:

Daily leptin profile in rat SCN

Sample preparation for IHC studies

For studying daily rhythms of leptin in SCN, animals were anesthetized at variable time points ZT-0, 6, 12 and 18 and transcardial perfusion was done with heparinized 0.9% saline followed by 4% paraformaldehyde in 0.1M phosphate buffer (PB) (pH-7.4). The brains were rapidly removed and immediately post-fixed in 4% paraformaldehyde in 0.1 M PB (pH-7.4) for 2-4 h. The brains were then transferred into a gradient of sucrose solutions {20% sucrose and 30% sucrose prepared with 0.1 M phosphate buffer (pH -7.4)} subsequently and stored at 4˚C till it sinks the bottom in both the solutions. 20 µ sections from these brains were taken using cryostat (Leica CM 1850). The sections were transferred into PBS and left for 1 h at room temperature to remove sucrose. These sections were then incubated in 3% H_2O_2 for 30 min followed by blocking using blocking serum (1% normal goat serum and 1% BSA dissolved in PBS containing 0.3% Triton-x -100) for 20-30 min. The sections were incubated in primary antibody (dilution 1:250) (Rabbit polyclonal IgG primary antibody (Ob (Y-20) Sc-843, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 37˚C for 4 h or overnight at 4˚C and then with corresponding secondary antibody (dilution 1:350) {Horseradish Peroxidase-Conjugated Goat Anti- rabbit IgG (IgG-HRP):HP03, Bangalore Genei, India}. Finally the sections were incubated in substrate solution containing 0.1% DAB (Diamino Benzedene) and 3% H_2O_2 till color develops. The reaction was stopped adding three drops of 2M HCl for 30 seconds and washed immediately with PBS (Caston-Balderrama *et al*., 1998). Sections were dehydrated using absolute alcohol and cleared the sections using Methyl benzoate and mounted using D.P.X (Morash *et al.,* 2001). Images were taken using DP-12 digital camera attached to Olympus microscope (BX-41). For comparing the results densitometric analysis was done using Image Pro AMS software (Fig.16.) (Media Cybernetics, USA) (Petersen *et al*., 2006; Mammen and Jagota, 2011).

Relative Quantitation of gene expression (*per1, per 2, Cry1 and Cry2*) in rat SCN and Pineal by Reverse transcription quantitative PCR (RT-qPCR):

Total RNA extraction

Total RNA was extracted from SCN and pineal tissue by phenol-chloroform/guanidine HCl method. Samples were homogenized in TriReagent™ (Sigma) (1 ml TRI Reagent/100 mg tissue) and incubated at room temperature for 5 minutes and then 0.1 ml of chloroform was added and mixed vigorously. The homogenate was allowed to stand at room temperature for 5 minutes and then centrifuged at 12,000 x g for 15 minutes at 4˚C. After centrifugation the upper aqueous layer of the homogenate containing the RNA was transferred into a fresh tube. To this 0.5 ml of isopropanol per ml of TRI reagent was added and allowed to stand for 10 min at room temperature to precipitate RNA. These tubes were then centrifuged at 12,000 x g for 10 minutes at 4˚C. RNA will precipitate on the side/bottom of the tube. Supernatant is discarded and the RNA pellet is reconstituted in 75% ethanol and centrifuged at 7,500 x g for 5 minutes at 4˚C to wash the pellet fraction. Same step was repeated twice and then the supernatant was decanted and the RNA pellet was briefly dried and reconstituted in 100 µl of RNase free water and then the concentration and purity of the RNA were determined using A260 and A280 measurements taken with nano-drop spectrophotometer (Thermo Fischer 1000) (Chomczynski and Sacchi, 2006).

cDNA synthesis

The basic reaction mixture for the catalytic conversion of an RNA template to cDNA contains murine moloney leukemia virus (M-MLV) reverse transcriptase, forward and reverse primers at a concentration of approximately 1 mg/ml and dNTP's (each 5mM). In the kit based method we carried out the reverse transcription (RT) was with total RNA using iScript cDNA Synthesis Kit (Bio Rad). Reverse transcription (RT) reactions comprised of 2µg of total RNA, 4.0 µl of 5x iScript reaction mix**,** and 1µl iScript reverse transcriptase (containing M-MuLV Reverse Transcriptase) and the total volume was made up to 20 µl with RNase free water. Cycle parameters for the RT procedure were 25˚C for 5 min, 42˚C for 30 min and 85˚C for 5 min. Assuming the 100% reverse transcription, input RNA (2 µg) is equal to amount of cDNA synthesized and aliquoted as 8 µl vials for further analysis (Kamphuis *et al*., 2005). The cDNA was then diluted in 1:20 ratio with RNase free water and stored at -20˚C prior to relative quantification of gene expression (Kamphuis *et al*., 2005).

Real time PCR:

Real time (qRT-PCR) assays were performed in duplicate using the Fast 7500 real time PCR System (Applied Biosystems; Warrington, UK) in 96 well plates. Reaction volume in each well was 20 µl (10 µl of 2X Power SYBR® Green (Applied biosystems), 2 µl Forward primer (10 pM), 2 µl Reverse primer (10 pM), and 8 µl (40 ng) of cDNA. Cycle parameters for the Real time (qRT-PCR) assay were: denaturation for 3min at 95˚C, followed by 40 cycles of 30s at 95˚C, 20s at 60˚C, and 20s at 72˚C. Dissociation curves showed a single amplified product and the absence of primer-dimer formation (Fig. 25.). PCR data were obtained as Ct values (the point within the exponential phase of the amplification in which the fluorescent signal is first recorded as being statistically significant above background) for the respective target genes with SDS software v. 2.1 (Applied Biosystems). Relative gene expression of target genes was normalized with *β-actin.* The expression levels were quantified by using comparative Delta Ct method (Livak and Schmittgen, 2001).

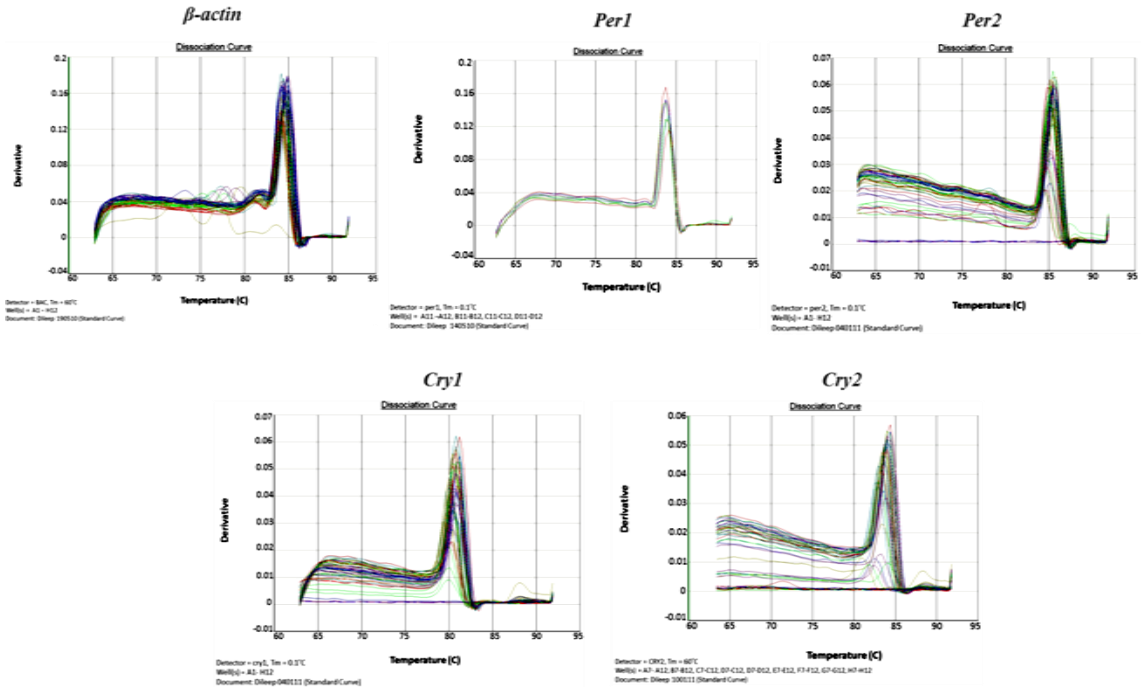


Fig.16. Representative dissociation curves for *β-actin, Per1, Per2, Cry1* and *Cry2.*

Primers used:

β-actin

Forward- AGCCATGTACGTAGCCATCC

Reverse- CTCTCAGCTGTGGTGGTGAA

Per1: Forward- CTGGTTCGGGATCCACGAA

Reverse- GAAGAGTCGATGCTGCCAAAG

Per2: Forward- CACCCTGAAAAGAAAGTGCGA

Reverse- CAACGCCAAGGAGCTCAAGT

Cry1: Forward-AAGTCATCGTGCGCATTTCA
Reverse-TCATCATGGTCGTCGGACAGA

Cry2: Forward-GGATAAGCACTTGGAACGGAA

Reverse-ACAAGTCCCACAGGCGGT (Kamphuis *et al*., 2005).

Protein estimation

Protein concentrations in the samples were estimated by Bradford's method by using crystalline bovine serum albumin (BSA) (Sigma) as standard (Bradford, 1976). Tissue samples collected at various time points (ZT-0, 6, 12 and 18) were homogenized and prepared a 5% homogenate and the volume of each tissue sample was adjusted to 100 µl with double distilled water. 1 ml of Bradford's reagent was added to each sample and mixed thoroughly. The absorbance was measured at 595 nm after 2 minutes against a reagent blank containing of 100 µl of double distilled water and 1 ml of Bradford's reagent. The standard graph was prepared using BSA of concentrations ranging from 1 µg to 10 µg. The concentration of total protein in the tissue samples were determined by comparing the values with the standards.

Protein profiling of SCN and Pineal using 2-D Gel electrophoresis:

Tissue samples were then homogenized in lysis buffer (7M Urea, 2M Thiourea, 2% Ampholines, 20mM DTT, 1 mM EDTA,1 mM PMSF) (O'Farrell, 1975). Protein fraction was precipitated using ice cold acetone. Then the homogenate was centrifuged at 10,000 rpm for 20 min at 4˚C. Supernatant was decanted and the pellet fraction was dissolved in rehydration buffer. Protein estimation was done by amido black method.

Amido Black method of protein estimation:

In the sample preparation step, amido black method was opted for protein estimation instead of Bradford's method. Because the presence of thiourea in the rehydration buffer could react with Bradford's reagent giving false positive result (Bradford, 1975). 2 µl of protein sample was spotted on nitrocellulose membrane and allowed the sample to dry. Then the dried membrane was incubated in Amido black solution (containing 1% amido black, 10: 45: 45 vol% of acetic acid, methanol, and Milli q water respectively) with occasional shaking. Membrane was desatined by washing it in destaining solution (containing 90:2:8 vol % methanol: glacial acetic acid: distilled water). The membrane is then blotted and

the blue color stained areas were cut out of the strip and each disk was transferred to a poly propelene tube containing 1 ml of eluant solution (containing 25mM NaOH, 0.05mM EDTA in 50% aqueous ethanol). These were incubated for 10 min at room temperature with vortexing in between. After the blue dye was completely extracted from the membrane disks in to the eluant solution absorbance of the eluant was measured at 630 nm against eluant solution as blank (Schaffner and Weissmann, 1973).

300 µg of protein was loaded on to the IEF (Isoelectric Focusing) strip (pH 3-10) and allowed for overnight passive rehydration and then for focusing at 10,000 V h/24 h. After focusing, the IEF strip was equilibrated in 25mM DTT containing equilibration buffer subsequently in 50mM Iodoacetamide containing equilibration buffer. After equilibration step focused IEF strips were placed on a SDS-PAGE (12% SDS-PAGE) gel and a second dimension (2D) gel was run. After the second dimension run gels were transferred into fixing solution (containing 50:12:0.018:38 vol% methanol, acetic acid, formaldehyde and Milli q water) for overnight incubation (Mortz *et al.,* 2001). After fixation, the gels were stained using improved silver staining method.

Protocol for the improved silver staining

Soon after the fixation step the gels were washed with 35% ethanol (3x 20 min.) and subsequently washed with sensitization solution 0.02 % $Na_2 S_2O_3$ (w/v). Then the gels were washed Milli q water (2x 3 min) and incubated in staining solution {containing 0.2% $AgNO_3$ (w/v) and 0.028 % formaldehyde (v/v)} for 30 min. Again the gels were washed with Milli q water (2x 20 s.) and then treated with developing solution {containing 6% Na_2CO_3 (w/v), 0.018% formaldehyde (v/v) and 0.0004% $Na_2 S_2O_3$ (w/v}. The reaction was terminated using stopping solution (containing 50% formaldehyde (v/v), 12% acetic acid) (Mortz *et al.,* 2001). The developed gels were scanned and analyzed with Image Platinum software (GE Health care).

Statistical analysis

All comparisons were done by using Jandel Scientific Sigma stat software by Student's t-test and one way-ANOVA followed by Post hoc Duncan's multiple tests (Sigma Stat) for multiple comparisons. Mean activity profiles were prepared using the Chronobiology Kit and analyzed using the Kit Analyze software (Stanford Software systems, USA). Correlation analysis was done between various parameters using pair wise correlation (Manikonda and jagota, 2012).

Age induced alteration in serotonin metabolism, clock genes expression has been studied parallel in the laboratory served as control for R.F. studies.

Results

OBJECTIVE-I

Studies of gross locomotor activity rhythms:

Under *ad libitum* fed condition, all the 3, 12 and 24 m old age animals showed a nocturnal pattern of activity with a robust activity with 82.49 ± 0.27, 92.46 ± 0.18, 88.88 ± 0.56 % respectively during the night time and with a circadian period (τ) of ~24 h (Fig. 17. A.i, ii, iii) (Table.1). Upon R.F. for 21 days, the animals showed bouts of activity mostly after the food was provided and shortly before (~3-4 h) the normal time due to the food anticipatory activity in all the three age groups studied (Fig. 17. A.i, ii, iii.).

When food was provided *ad libitum*, the duration of active period (α) of 3, 12 and 24 m old animals was approximately 12 h. Under R.F. regimen, the night percentage nocturnality was decreased to 68.53 ± 0.21, 49.42 ± 0.22, and 18.42 ± 0.39 in 3, 12 and 24 m old rats respectively. The diurnality percentage was also increased significantly ($p \leq 0.05$) from 17.48 ± 0.24, 7.92 ± 0.26 and 11.44 ± 0.17 to 31.8 ± 0.31, 29.16 ± 0.13 and 72.75 ± 0.21 in 3, 12 and 24 m old rats. The nocturnality percentage was decreased significantly ($p \leq 0.05$) from 3 m to 24 m under R.F. However, diurnality percentage diurnality was more in 24 m old rats when compared to 3 and 24 m old rats (Table 1.).

Under R.F. condition in 12 m old rats, α was increased significantly ($p \leq 0.05$) (18.09 ± 0.45 h) when compared in 3 m (15.37 ± 0.62). In 24 m old rats, α further decreased significantly ($p \leq 0.05$) (13.03 ± 0.3) (Table1.). In 24 m old rats the activity was shifted more towards the light phase whereas the night time activity was decreased (Fig. 17. A.ii, iii.). Under *ad libitum* fed condition, the τ was~24 h in 3, 12 and 24 m old rats i.e. 24.16 ± 0.16, 23.97 ± 0.22 and 23.95 ± 0.02 h respectively. But under R.F. the τ significantly ($p \leq 0.05$) increased from 23.97 ± 0.22 to 24.72 ± 0.06 h in 12 m old rats whereas in 24 m old rats τ was significantly ($p \leq 0.05$) decreased from 23.95 ± 0.02 to 23.55 ± 0.1 under R.F. (Table 1.).

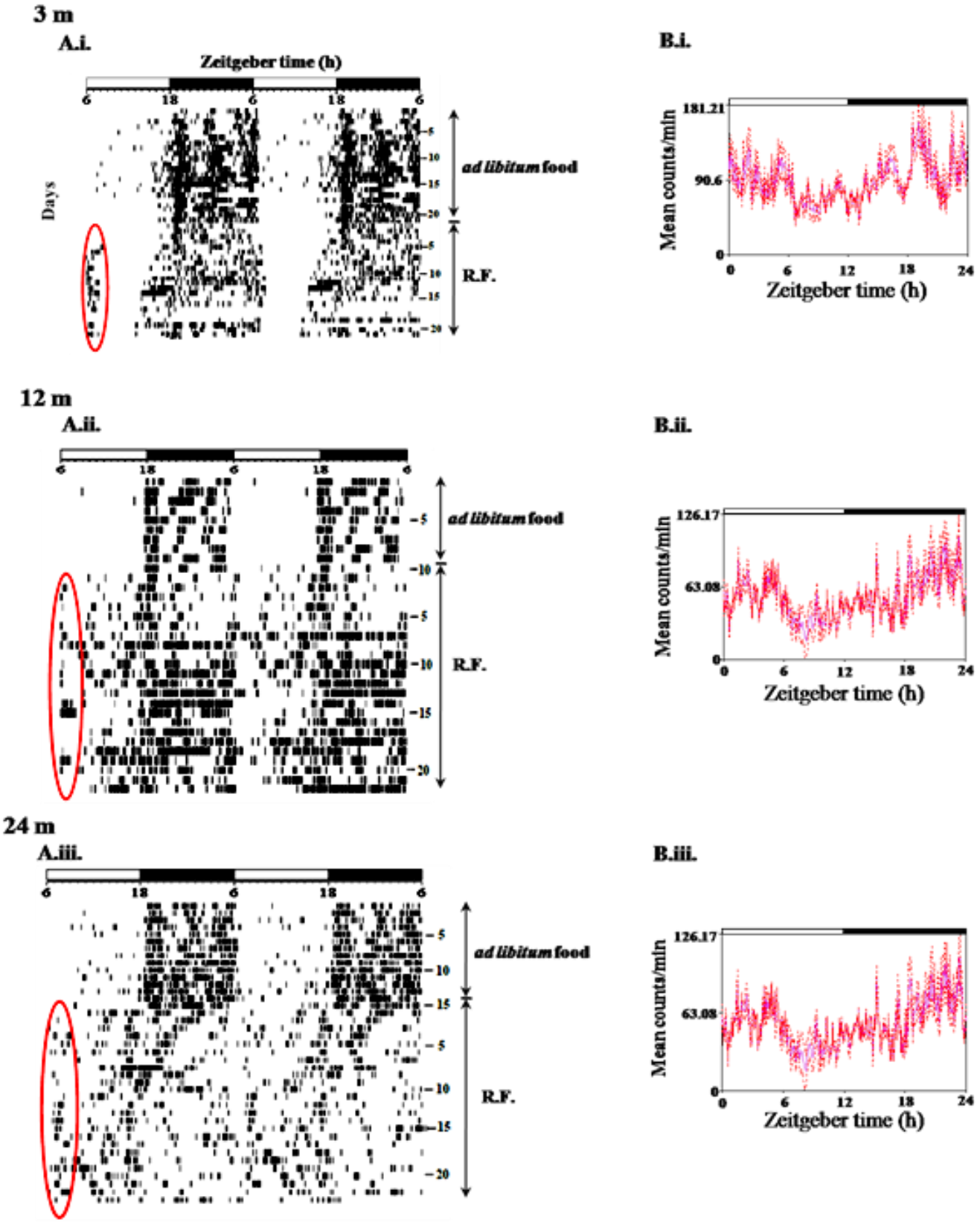


Fig.17. A. Double plotted actograms of gross locomotor activity under *ad libitum* and food restricted condition in 3, 12 and 24 m male wistar rats. The dashed lines on the activity profile indicates lights on and off for animals in LD (12:12). B. Mean activity profile of 3, 12 and 24 m rats.

Table 1. Effect of restricted feeding on various circadian parameters.

Age Group	Experimental condition	Activity parameters			
		Circadian period (τ) (hours)	Duration of active period (α) (hours)	% diurnality	% nocturnality
3 m	*ad libitum* fed	24.16 ± 0.16	12.66 ± 0.14 *	17.48 ± 0.24	82.49 ± 0.27
	R.F.	24.29 ± 0.08 ǂ	15.37 ± 0.62 ǂ	31.8 ± 0.31 *	68.53 ± 0.21 *
12 m	*ad libitum* fed	23.97 ± 0.22 *	12.52 ± 0.27 *	7.92 ± 0.26 ǂ	92.46 ± 0.18 ǂ
	R.F.	24.72 ± 0.06 ǂ	18.09 ± 0.45 ǂ	29.16 ± 0.13 *, ǂǂ	49.42 ± 0.22 *, ǂǂ
24 m	*ad libitum* fed	23.95 ± 0.02 *	12.37 ± 0.16	11.44 ± 0.17	88.88 ± 0.56
	R.F.	23.55 ± 0.1	13.03 ± 0.3	72.75 ± 0.21 *	18.42 ± 0.39 *

Each value is mean ± S.E. (n=4). P ≤0.05, (where * represents comparison between *ad libitum* and R.F. in the same age group; ǂ represents comparison between 3, 12 and 24 m under *ad libitum* fed or R.F. condition across the age groups; ǂǂ represents comparison between 3, 12 and 24 m under *ad libitum* fed or R.F. condition across the age groups. m-months, R.F.-Restricted feeding.

OBJECTIVE-II

Effect of restricted feeding on serotonin metabolism in various age groups

SCN

3 months

Control animals showed rhythmicity for all the ten compounds estimated at four different time points (ZT-0, ZT-6, ZT-12, ZT-18) (Fig. 18.). The 5-HT, 5-MIAA, 5-MTOH and NAT levels were high at ZT-6 with 39.29 ± 2.58, 2.8 ± 0.98, 22.89 ± 1.4, 0.24 ± 0.09 µmol/g protein respectively, whereas TRP was higher at ZT-12 with 0.92 ± 0.05 µmol/g protein (Table 2.).

Upon food restriction the rhythmicity of 5-HT, TRP and 5-HTOH abolished and the rhythmicity of 5-HTP, 5-HIAA, NAS, 5-MIAA, 5-MTOH, MEL and NAT rhythms were persisted in SCN (Fig. 18).

The ratio of 5-HT/5-HIAA was maximum at ZT-0 (4.64 ± 0.53) (onset of light) and minimum at ZT-12 (2.3 ± 0.56) (onset of darkness) in controls. Upon food restriction, 5-HT/5-HIAA ratio maximum occurred at ZT-6 (0.86 ± 0.27) and minimum occurred at ZT-0 (0.23 ± 0.05).

In controls the maximum levels for TRP and NAT occurred at ZT-12 and ZT-6 with 0.91 ± 0.05 and 0.24 ± 0.09 µmol/g protein respectively. In food restricted animals the peak levels for TRP and NAT occurred at ZT-6 and ZT-0 with 0.3 ± 0.22 and 0.04 ± 0.02 µmol/g protein respectively and the levels were phase advanced by 6 h for both the compounds upon food restriction.

TRP did not show any significant change in the daily pulse ratio between controls (6.29 ± 3.63) and food restricted (2.75 ± 1.23) animals. In controls the 5-MTOH levels increased from ZT-0 to ZT-6 and then decreased gradually from ZT-6 to ZT-18. In food restricted animals the 5-MTOH levels decreased from ZT-0 to ZT-6 and gradually increased from ZT-6 to ZT-18. NAT levels upon food restriction did not shown any significant difference between ZT-0 (0.04 ± 0.007), 12 (0.04 ± 0.001) and 18 (0.04 ± 0.002) time points but at ZT-6 (0.02 ± 0.005) the levels were slightly decreased. The mean 24 h levels of NAT decreased by 2.84 folds upon food restriction, and no significant difference was observed in the daily pulse ratio between controls and food restricted animals.

Under restricted feeding regimen the levels of 5-HTOH at ZT-0, 6 and 18 time points were 0.51 ± 0.17, 0.51 ± 0.11 and 0.86 ± 0.41 µmol/g protein respectively no significant difference was observed in the levels between these time points. But at ZT-12, the levels were increased to 0.73 ± 0.06 µ moles/g protein. The change in the daily pulse ratio and in mean 24 h levels between control and food restricted animals was not significant for both these compounds (Table 3.).

In the case of 5-HTP and NAS, even after food restriction the levels showed the same rhythmic pattern as in controls with increased levels at all the four time points. NAS levels in both controls and food restricted animals decreased from ZT-0 to 6 and then gradually increased from ZT-6 to 18. Upon food restriction the mean 24 h levels of 5-HTP and NAS increased by 7 and 9.5 fold approximately (Fig. 19). There was no change in the daily pulse ratio of 5-HTP observed between controls and food restricted animals, whereas the NAS levels decreased by 5 folds approximately. The daily pulse ratio of NAS, 5-MIAA and NAT showed a significant decrease with 5, 9 and 7 folds respectively. However, no significant difference was observed in the daily pulses of other compounds upon food restriction (Table 3.).

Under food restriction the mean 24h levels of serotonin decreased approximately by 2.12 folds (Fig. 19). When compared to controls, 5-HT levels were decreased at all the time points except at ZT-18 (Fig. 18, Table 2.).

The mean 24h levels of 5-MIAA and 5-MTOH decreased by 16.27, 4.83 folds respectively upon food restriction (Fig. 19). Though the rhythmicity of 5-MIAA persisted upon food restriction the levels decreased at all the four time points, and the daily pulse ratio was also decreased. In the case of melatonin upon food restriction the levels started decreasing from ZT-0 to ZT-6 and slowly increased from ZT-12 to ZT-18 as in controls. When compared to controls, melatonin levels were decreased in food restricted animals at ZT-0, 12 and 18 and the decrement was approximately 2.58, 1.81 and 6.24 folds respectively, whereas at ZT-6 the levels were slightly increased by 0.24 folds upon food restriction. NAT levels also decreased at all the time points except at ZT-18 where the levels were increased upon food restriction (Fig. 18, Table 2.).

Table 2. Effect of restricted feeding on serotonin and its related compounds in SCN of 3 m old rats.

Compound	Experimental group	Zeitgeber time (h) (ZT)			
		0	6	12	18
5-HTP	Control	0.67 ± 0.18	1.89 ± 0.16 ^{c}	2.132 ± 0.02 ^{b}	0.196 ± 0.03
	R.F.	4.66 ± 0.06 ^{c,d,w}	3.68 ± 0.94 ^{c,d,w}	7.05 ± 0.14 ^{a,b,w}	7 ± 1.04 ^{a,b,w}
5-HT	Control	19.37 ± 1.05 ^{b,d,w}	39.29 ± 2.58 ^{a,c,d,w}	24.26 ± 5.26 ^{b,d,w}	8.65 ± 0.9
	R.F.	6.29 ± 2.37	11.28 ± 3.65	11.86 ± 3.07	11.38 ± 4.1
5-HIAA	Control	2.83 ± 0.67	8.76 ± 1.66 ^{c,d}	12.05 ± 4.32 ^{a,d}	2.79 ± 0.46
	R.F.	25.79 ± 0.98 ^{c,w}	15.71 ± 6.57 ^{c,d}	47.11 ± 3.95 ^{a,b,c,w}	40.21 ± 8.1 ^{b,w}
NAS	Control	0.16 ± 0.05 ^{d}	0.07 ± 0.0007 ^{d}	0.32 ± 0.08 ^{d}	0.91 ± 0.18 ^{a,b,c}
	R.F.	3.15 ± 0.29 ^{c,d,w}	2.23 ± 0.62 ^{c,d,w}	6.33 ± 0.7 ^{a,b,w}	7.29 ± 1.35 ^{a,b,w}
TRP	Control	0.28 ± 0.08 ^{b,c}	0.61 ± 0.08 ^{a,c,d}	0.91 ± 0.05 ^{a,b,d,w}	0.14 ± 0.09 ^{b,c}
	R.F.	0.11 ± 0.02	0.3 ± 0.22	0.14 ± 0.02	0.15 ± 0.002
5-HTOH	Control	0.49 ± 0.12 ^{b}	0.66 ± 0.14 ^{a,c,d}	0.41 ± 0.14 ^{b,w}	0.39 ± 0.07 ^{b}
	R.F.	0.51 ± 0.17	0.51 ± 0.11	0.73 ± 0.06	0.86 ± 0.41
5-MIAA	Control	0.84 ± 0.16 ^{b,d,w}	2.8 ± 0.98 ^{a,c,d,w}	1.2 ± 0.4 ^{b,d,w}	0.16 ± 0.03 ^{b,c}
	R.F.	0.05 ± 0.006	0.05 ± 0.006 ^{c,d}	0.09 ± 0.01 ^{a,b}	0.1 ± 0.004 ^{a,b}
5-MTOH	Control	20.99 ± 3.2 ^{d,w}	22.89 ± 1.4 ^{c,d,w}	15.15 ± 2.07 ^{b,w}	12.27 ± 0.55 ^{a,b,w}
	R.F.	0.11 ± 0.02 ^{a,b,d}	0.09 ± 0.03 ^{a}	0.12 ± 0.01 ^{a}	0.12 ± 0.01 ^{a}
MEL	Control	0.64 ± 0.04 ^{d,w}	0.04 ± 0.03 ^{d}	0.68 ± 0.02 ^{d,w}	2.93 ± 0.57 ^{a,b,c,w}
	R.F.	0.25 ± 0.07 ^{d}	0.19 ± 0.01 ^{c,d,w}	0.37 ± 0.06 ^{b}	0.46 ± 0.04 ^{a,b,c}
NAT	Control	0.11 ± 0.02 ^{b,w}	0.24 ± 0.09 ^{a,c,d,w}	0.06 ± 0.001 ^{b,w}	0.02 ± 0.002 ^{b}
	R.F.	0.04 ± 0.007 ^{b}	0.02 ± 0.005 ^{a,d}	0.04 ± 0.001	0.04 ± 0.002 ^{b}

All values are expressed as µmol/g protein. Each value is mean ± S.E (n=6). Zeitgeber Time (ZT): ZT-0 = 6:30 h (Lights on); ZT-12 = 18:30 h (Lights off). $p \leq 0.05$ (a, b, c and d refers to comparison between ZT-0, 6, 12 and 18 respectively; w, refers to significant difference in the levels of the compound at same time point between control and R.F. group in same age group).

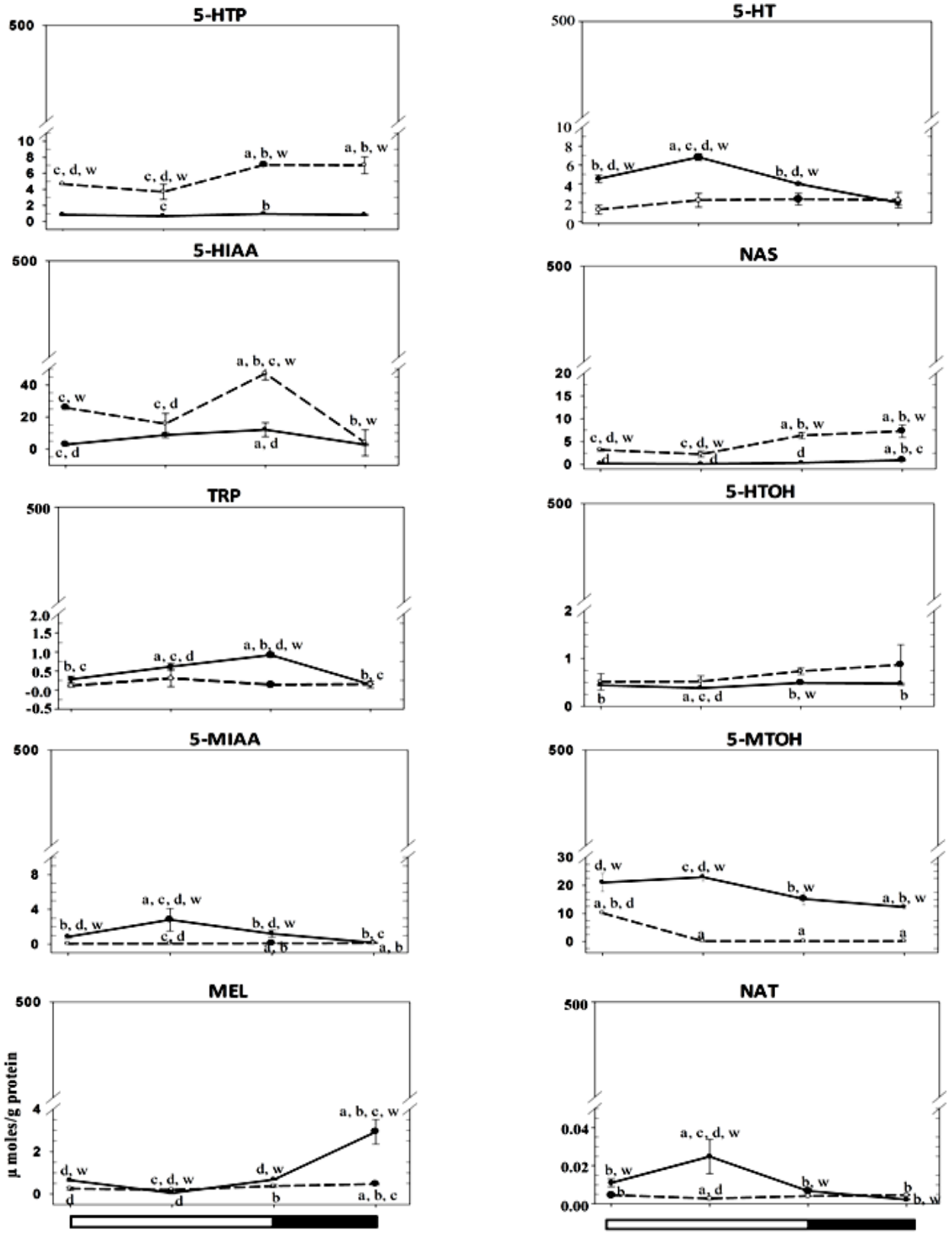


Fig. 18. Comparison of daily rhythms of various components of serotonin metabolism in control and food restricted groups in the SCN of 3 m old rats. Each value is mean ± S.E (n=6). $p \leq 0.05$ (a, b, c and d refers to comparison between ZT-0, 6, 12 and 18 respectively; w, refers to significant difference in the levels of the compound at same time point between control and R.F. group in same age group). ——— Line indicates controls; - - - - - Line indicates R.F

Table 3. Effect of restricted feeding on serotonin and its related compounds in the SCN of 3 m old rats.

Compound	Experimental group	Max : Min ratio	Mean 24 h (µ moles/g protein)	Rhythmicity
5-HTP	Control	10.87 ± 3.77	1.22 ± 0.04	Rhythmic
	R.F.	1.91 ± 0.3	5.6 ± 0.53	Persisted
5-HT	Control	1.88 ± 0.32	22.89 ± 6.36	Rhythmic
	R.F.	4.54 ± 0.59 *	10.2 ± 1.59	Abolished
5-HIAA	Control	4.31 ± 1.02	6.61 ± 0.37	Rhythmic
	R.F.	2.9 ± 0.82	32.21 ± 4.39	Persisted
NAS	Control	11.54 ± 1.34	0.37 ± 0.18	Rhythmic
	R.F.	2.3 ± 0.69 *	4.75 ± 0.73	Persisted
TRP	Control	6.29 ± 3.63	0.49 ± 0.17	Rhythmic
	R.F.	2.75 ± 1.23	0.17 ± 0.05	Abolished
5-HTOH	Control	1.65 ± 0.24	0.49 ± 0.006	Rhythmic
	R.F.	1.7 ± 0.66	0.65 ± 0.11	Abolished
5-MIAA	Control	17.3 ± 5.24	1.25 ± 0.05	Rhythmic
	R.F.	2.01 ± 0.46	0.07 ± 0.007	Persisted
5-MTOH	Control	1.86 ± 0.09	17.82 ± 2.47	Rhythmic
	R.F.	1.38 ± 0.29	0.11 ± 0.01	Abolished
MEL	Control	61.04 ± 42.38	1.07 ± 0.63	Rhythmic
	R.F.	2.36 ± 0.13	0.32 ± 0.03	Persisted
NAT	Control	11.27 ± 2.47	0.11 ± 0.04	Rhythmic
	R.F.	1.66 ± 0.23 *	0.039 ± 0.03	Abolished

* indicate significant difference between control and food restricted animals ($p \leq 0.05$) determined by one way-ANOVA;

Each value is mean ± S.E. (n = 6).

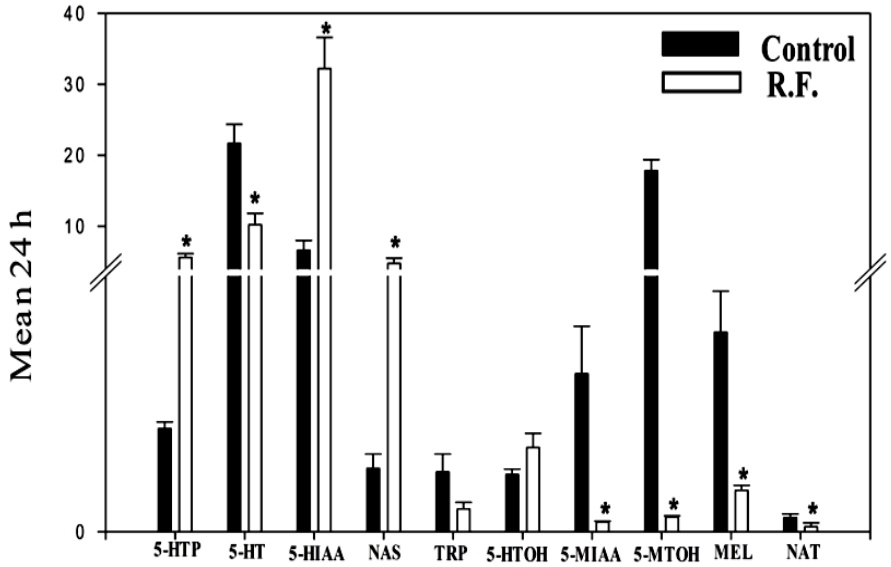


Fig. 19**.** Effect of restricted feeding on mean 24 h. levels of serotonin and its related compounds in the SCN of 3 m old rats. * indicate significant difference between control and food restricted animals ($p \leq 0.05$) (n=24) determined by one way-ANOVA.

12 months

In the SCN of 12 m old rats, 5-HTP, 5-HT, NAS, MEL and NAT have shown daily rhythmicity whereas 5-HIAA, TRP, 5-HTOH, 5-MIAA and 5-MTOH did not show daily rhythm (Fig. 20 and Table. 4). A significant decrease in the daily pulse of 5-HT, 5-HIAA, and NAT levels by 1.63, 1.68, and 1.48 folds respectively and a significant increase in the daily pulse of 5-HTP and 5-HTOH by 0.72 and 2 folds was observed between controls to food restricted animals respectively (Table 5.).

The mean 24 h levels of 5-HTP, TRP, 5-MTOH and NAT levels were 1.47 ± 0.24, 0.03 ± 0.03, 0.19 ± 0.03, 0.06 ± 0.01 µmoles/g protein which were increased to 3.61 ± 0.43, 0.16 ± 0.007, 0.34 ± 0.008, 0.18 ± 0.007 µmoles/g protein respectively upon food restriction with a fold increase of 2.45, 4.28, 1.78 and 2.64. However the mean 24 h levels of 5-HT, 5-HIAA, NAS, 5-HTOH, 5-MIAA and MEL levels were decreased from 35.66 ± 7.16, 24.9 ± 4.62, 5.46 ± 0.63, 1.09 ± 0.19, 0.24 ± 0.08, 0.61 ± 0.1 µmoles/g protein to 0.37 ± 0.02, 9.66 ± 0.79, 1.23 ± 0.07, 0.55 ± 0.07, 0.04 ± 0.004, 0.22 ± 0.01 µmoles/g protein respectively and the fold decrease was 95.6, 2.57, 4.43, 1.98, 5.14 and 2.73 (Table 5. and Fig. 21).

Under food restricted condition 5-HT, 5-HIAA, TRP, 5-HTOH, NAS, 5-MIAA and MEL levels were decreased and the levels of 5-HTP, 5-MTOH and NAT were increased at all the four time points studied when compared to controls (Table 4. and Fig. 20).

Table 4. Effect of restricted feeding on serotonin and its related compounds in the SCN of 12 m old rats.

Compound	Experimental group	Zeitgeber time (h) (ZT)			
		0	6	12	18
5-HTP	Control	1.58 ± 0.22 [c]	2.02 ± 0.09 [b, c, w]	0.77 ± 0.1 [a, b, w]	1.02 ± 0.36 [b]
	R.F.	6.94 ± 0.33 [b, c, d, w]	3.6 ± 0.14 [a, c, d]	1.97 ± 0.08 [a, b]	1.93 ± 0.22 [a, b]
5-HT	Control	46.08 ± 2.89 [b, c, d, w]	57.88 ± 3.51 [a, c, d, w]	25.54 ± 1.39 [a, b, w]	18.6 ± 0.92 [a, b, w]
	R.F.	0.27 ± 0.03 [b, d]	0.51± 0.02 [a, c, d]	0.28 ± 0.01 [b, d]	0.41 ± 0.02 [a, b, c]
5-HIAA	Control	20.98 ± 6.07	29.74 ± 6.55	28.39 ± 7.66	36.44 ± 8.03
	R.F.	15.24 ± 0.73 [b, c, d]	10.61 ± 0.43 [a, c, d, w]	6.25 ± 0.26 [a, b, w]	6.55 ± 0.29 [a, b, w]
NAS	Control	4.99 ± 1.34 [d, w]	4.33 ± 0.49 [d]	4.98 ± 0.986 [d, w]	7.92 ± 0.53 [a, b, c, w]
	R.F.	1.03 ± 0.05 [d]	1.1 ± 0.07 [d]	1.04 ± 0.02 [d]	1.74 ± 0.11
TRP	Control	0.43 ± 0.15	0.29 ± 0.02	0.3 ± 0.02	0.23 ± 0.03
	R.F.	0.17 ± 0.008 [b, c, d]	0.14 ± 0.01 [a, d, w]	0.13 ± 0.004 [a, d, w]	0.21 ± 0.01 [a, b, c]
5-HTOH	Control	1.66 ± 0.83	1.41 ± 0.26	0.81 ± 0.17	0.9 ± 0.13
	R.F.	0.98 ± 0.04 [b, c, d]	0.83 ± 0.02 [a, c, d]	0.19 ± 0.006 [a, b, w]	0.19 ± 0.009 [a, b, w]
5-MIAA	Control	0.53 ± 0.2	0.11 ± 0.03	0.16 ± 0.08	0.3 ± 0.15
	R.F.	0.03 ± 0.001 [b, c, d, w]	0.03 ± 0.002 [a, d]	0.03 ± 0.001 [a, d]	0.08 ± 0.003 [a, b, c]
5-MTOH	Control	0.25 ± 0.12	0.11 ± 0.17	0.3 ± 0.19	0.18 ± 0.08
	R.F.	0.3 ± 0.01 [d]	0.33 ± 0.01 [d]	0.34 ± 0.01	0.38 ± 0.01 [a, b, w]
MEL	Control	0.63 ± 0.05 [d, w]	0.4 ± 0.11 [d]	0.56 ± 0.22 [d]	1.01 ± 0.08 [b, c, w]
	R.F.	0.25 ± 0.01 [b, c, d]	0.12 ± 0.006 [a, c, d, w]	0.17 ± 0.007 [a, b, d]	0.34 ± 0.01 [a, b, c]
NAT	Control	0.11 ± 0.02 [b, c, d, w]	0.05 ± 0.01 [a, w]	0.04 ± 0.0005 [a, w]	0.058 ± 0.01 [a, w]
	R.F.	0.17 ± 0.008 [b]	0.23 ± 0.01 [a, c, d]	0.16 ± 0.005 [b]	0.14 ± 0.007 [b]

All values are expressed as µmol/g protein. Each value is mean ± S.E (n=6). Zeitgeber Time (ZT): ZT-0 = 6:30 h (Lights on); ZT-12 = 18:30 h (Lights off). $p \leq 0.05$ (a, b, c and d refers to comparison between ZT-0, 6, 12 and 18 respectively; w, refers to significant difference in the levels of the compound at same time point between control and R.F. group in same age group).

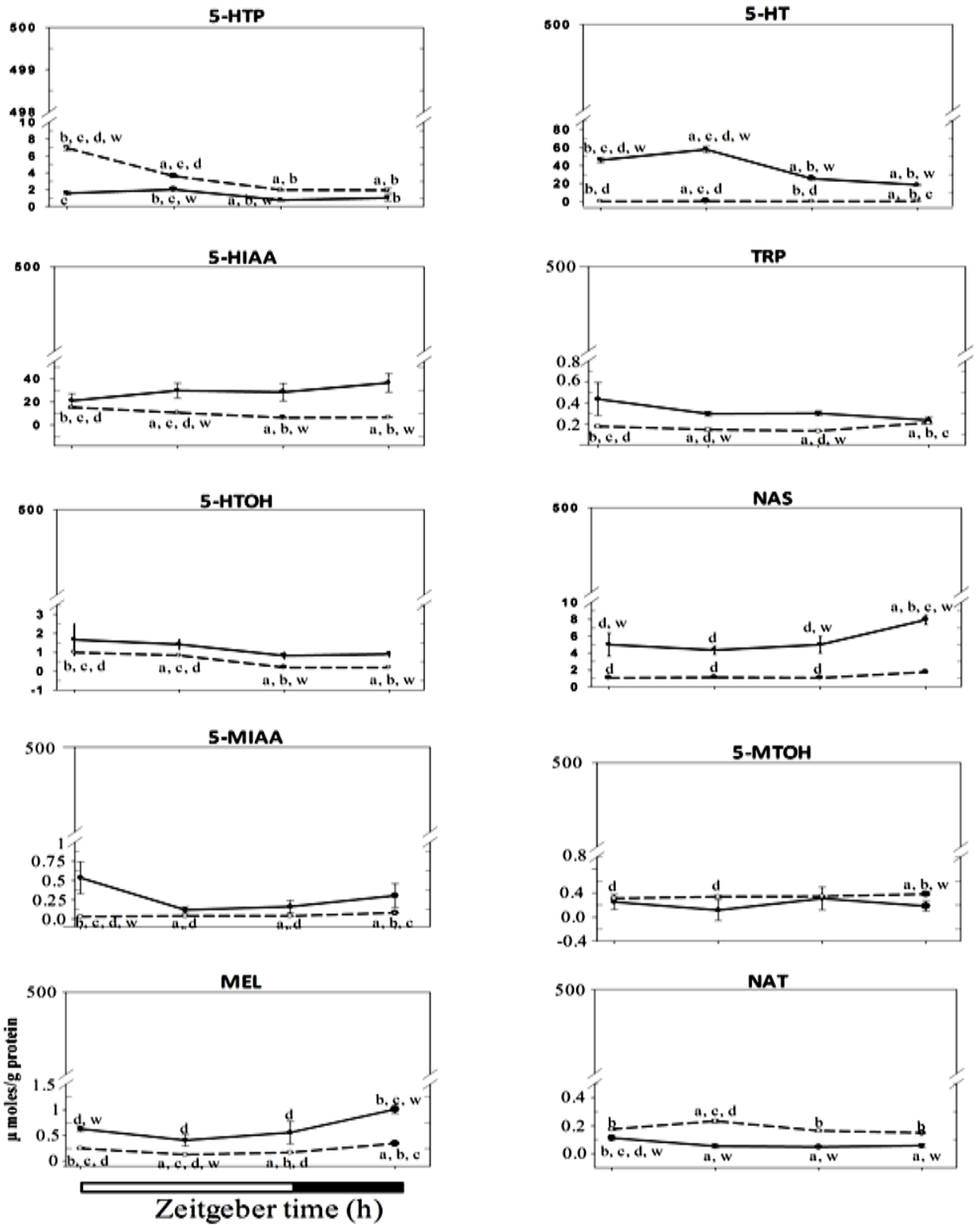


Fig. 20. Comparison of daily rhythms of various components of serotonin metabolism in control and food restricted groups in the SCN of 12 m old rats. Each value is mean ± S.E (n=6). $p \leq 0.05$ (a, b, c and d refers to comparison between ZT-0, 6, 12 and 18 respectively; w, refers to significant difference in the levels of the compound at same time point between control and R.F. group in same age group). ——— Line indicates controls; - - - - - Line indicates R.F.

Table 5. Effect of restricted feeding on serotonin and its related compounds in the SCN of 12 m old rats.

Compound	Experimental group	Max : Min ratio	Mean 24 h (µ moles/g protein)	Rhythmicity
5-HTP	Control	2.65 ± 0.21	1.47 ± 0.24	Rhythmic
	R.F.	3.63 ± 0.26 *	3.61 ± 0.43	Persisted
5-HT	Control	3.11 ± 0.14	35.66 ± 7.16	Rhythmic
	R.F.	1.9 ± 0.15 *	0.37 ± 0.02	Persisted
5-HIAA	Control	4.12 ± 0.74	24.9 ± 4.62	Arhythmic
	R.F.	2.44 ± 0.08 *	9.66 ± 0.79	Rhythmic
NAS	Control	1.85 ± 0.14	5.46± 0.63	Rhythmic
	R.F.	1.68 ± 0.07	1.23 ± 0.07	Persisted
TRP	Control	1.89 ± 0.41	0.03 ± 0.03	Arhythmic
	R.F.	1.59 ± 0.05	0.16 ± 0.007	Rhythmic
5-HTOH	Control	2.53 ± 0.74	1.09 ± 0.19	Arhythmic
	R.F.	5.21 ± 0.19 *	0.55 ± 0.07	Rhythmic
5-MIAA	Control	5.97 ± 1.57	0.24 ± 0.08	Arhythmic
	R.F.	2.57 ± 0.09	0.04 ± 0.004	Rhythmic
5-MTOH	Control	2.4 ± 0.71	0.19 ± 0.03	Arhythmic
	R.F.	1.23 ± 0.04	0.34 ± 0.008	Rhythmic
MEL	Control	2.68 ± 0.45	0.61± 0.1	Rhythmic
	R.F.	2.67 ± 0.11	0.22 ±0.01	Persisted
NAT	Control	2.32 ± 0.23	0.06 ± 0.01	Rhythmic
	R.F.	1.56 ± 0.06 *	0.18 ± 0.007	Persisted

* indicate significant difference between control and food restricted animals ($p \leq 0.05$) determined by one way-ANOVA.

Each value is mean ± S.E. (n = 6).

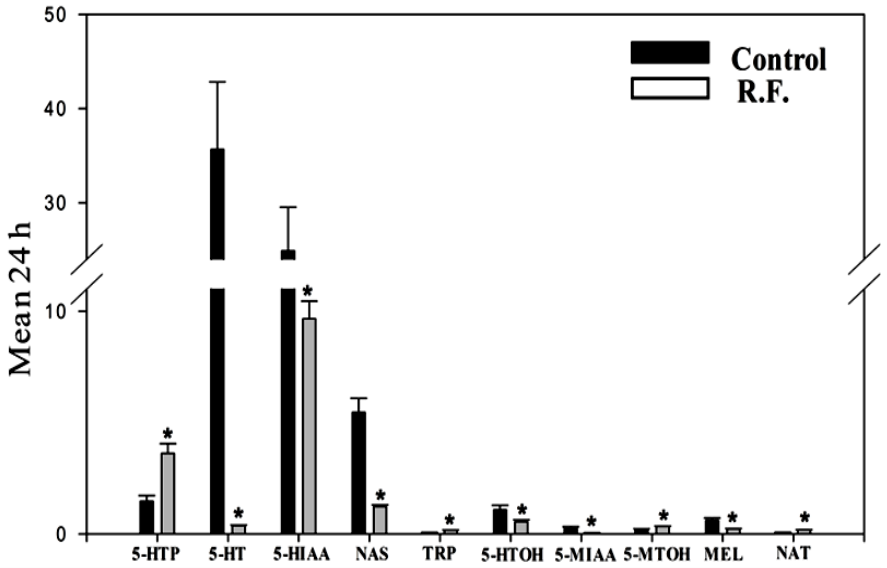


Fig. 21. Effect of restricted feeding on mean 24 h. levels of serotonin and its related compounds in the SCN of 12 m old rats. * indicate significant difference between control and food restricted animals ($p \leq 0.05$) (n=24) determined by one way-ANOVA.

24 months

In 24 m old rats SCN, 5-HT, 5-HIAA, NAS, TRP, 5-HTOH, MEL and NAT have shown daily rhythmicity whereas, 5-HTP, 5-MIAA and 5-MTOH did not show daily rhythm in controls (Fig. 22, Table 6.). A significant decrease in the daily pulse between controls to food restricted animals was observed in the case of 5-HIAA, TRP, and MEL from 3.62 ± 0.3, 3.12 ± 0.24 and 5.86 ± 1.56 to 2.44 ± 0.08, 1.28 ± 0.04, 2.06 ± 0.07 with a fold difference of 1.48, 2.43 and 2.83 respectively. A significant increase in the daily pulse of 5-HTP and 5-HT was observed between control to food restricted animals from 1.5 ± 0.2, 2.49 ± 0.17 to 2.77 ± 0.07, 3.38 ± 0.13 with a fold change by 1.85 and 1.36 folds respectively (Table 7.).

The mean 24 h levels of 5-HTP, 5-HT, 5-HIAA, NAS, TRP, 5-HTOH, 5-MIAA and MEL were decreased by 1.29, 240.46, 5.6, 4.71, 14.85, 3.47, 9.2 and 3.72 folds respectively whereas, the mean 24 h levels of 5-MTOH and NAT were decreased by 1.58 and 2.67 folds respectively under R.F. (Fig. 23. and Table 7.).

In 24 m old food restricted animals the levels of 5-HT, 5-HIAA, TRP, 5-HTOH, NAS, 5-MIAA, MEL were decreased and 5-MTOH, NAT levels were increased in all the four time points (Fig. 22. and Table 6.).

Correlation analysis data shows that in light phase there was a positive significant correlation between 5-HT and TRP in the SCN of 3 and 24 m old rats whereas, in dark phase this correlation was abolished in these two age groups. Only in 12 m old rats SCN there was a positive correlation between 5-HT and TRP

in dark phase. A positive significant correlation was observed between 5-HT and 5-MIAA at dark phase in 12 m old rats and at light phase in 24 m old rats. 5-HT and 5-MTOH were in a significant positive correlation at both light and dark phases in the SCN of 12 m old rats, and only in dark phase of the SCN of 24 m old rats 5-HT was in significant negative correlation with MEL at light phase and in positive correlation at dark phase in 12 m old rats SCN. In 24 m old rats though 5-HT was in negative correlation with MEL, it was not significant in light phase whereas, in dark phase 5-HT has a significant negative correlation with MEL. 5-HT and 5-HTP were in significant correlation, 5-HT and NAT were in negative correlation at dark phase in 3 and 24 m old rats SCN respectively (Fig. 24**)**.

Table 6. Effect of restricted feeding on serotonin and its related compounds in the SCN of 24 m old rats.

Compound	Experimental group	Zeitgeber time (h) (ZT)			
		0	6	12	18
5-HTP	Control	5.23 ± 1.05	5.31 ± 0.05	4.1 ± 0.41	3.54 ± 0.43
	R.F.	4.45 ± 0.32 [b, d]	2.87 ± 0.16 [a, c, d, w]	4.86 ± 0.18 [b, d]	1.75 ± 0.04 [a, b ,c, w]
5-HT	Control	51.5 ± 2.87 [c, d, w]	48.47 ± 4.18 [c, d, w]	21.06 ± 0.61 [a, b, w]	19.63 ± 1.75 [a,b,w]
	R.F.	0.06 ± 0.003 [b, d]	0.21 ± 0.01 [a, c, d]	0.08 ± 0.01 [b, d]	0.13 ± 0.003 [a, b, c]
5-HIAA	Control	16.88 ± 2.22 [b,c]	60.11 ± 5.56 [a, c, d, w]	35.48 ± 3.39 [a, b, w]	27.37 ± 3.35 [b,w]
	R.F.	8.82 ± 0.42 [b, d]	6.4 ± 0.33 [a, c, d]	8.86 ± 0.49 [b, d]	3.62 ± 0.06 [a, b, c]
NAS	Control	2.37 ± 0.46 [d, w]	2.37 ± 0.43 [d, w]	3.74 ± 1.35 [d]	6.4 ± 0.45 [a, b, c, w]
	R.F.	0.41 ± 0.02	0.43 ± 0.02	1.24 ± 0.04	0.76 ± 0.04
TRP	Control	11.62 ± 0.9 [b, c, d, w]	5.56 ± 0.69 [a, w]	5.73 ± 0.36 [a, d, w]	3.75 ± 0.4 [a, c, w]
	R.F.	0.41 ± 0.01 [d]	0.44 ± 0.02 [d]	0.43 ± 0.03 [d]	0.34 ± 0.01 [a, b, c]
5-HTOH	Control	5.49 ± 0.53 [b, c, d, w]	3.59 ± 0.29 [a, c, w]	0.21 ± 0.15 [a, b, d]	2.9 ± 0.51 [a, c, w]
	R.F.	1.26 ± 0.03 [b, c, d]	0.94 ± 0.05 [a, c]	0.15 ± 0.005 [a, b, d]	0.95 ± 0.04 [a, c]
5-MIAA	Control	0.39 ± 0.26	0.45 ± 0.24	0.31 ± 0.15	0.11 ± 0.03
	R.F.	0.02 ± 0.001 [b]	0.03 ± 0.001 [a, c, d]	0.03 ± 0.001 [b, d]	0.02 ± 0.006 [b, c, w]
5-MTOH	Control	0.11 ± 0.02	0.15 ± 0.04	0.11 ± 0.03	0.13 ± 0.01
	R.F.	0.24 ± 0.01 [d, w]	0.24 ± 0.01 [d]	0.21 ± 0.005 [c, w]	0.17 ± 0.003 [a, b ,c, w]
MEL	Control	0.4 ± 0.03 [d, w]	0.25 ± 0.11 [d]	0.47 ± 0.14 [d, w]	1.22 ± 0.18 [a, b, c, w]
	R.F.	0.2 ± 0.009 [b, c, d]	0.15 ± 0.008 [a, c, d]	0.13 ± 0.006 [a, b, d]	0.1 ± 0.004 [a, b, c]
NAT	Control	0.06 ± 0.01 [b, c, w]	0.03 ± 0.004 [a, w]	0.03 ± 0.004 [a,w]	0.04 ± 0.01
	R.F.	0.15 ± 0.008 [b, c, d]	0.12 ± 0.005 [a, c, d]	0.1 ± 0.004 [a, b, d]	0.08 ± 0.002 [a, b, c, w]

All values are expressed as µmol/g protein. Each value is mean ± S.E (n=6). Zeitgeber Time (ZT): ZT-0 = 6:30 h (Lights on); ZT-12 = 18:30 h (Lights off). $p \leq 0.05$ (a, b, c and d refers to comparison between ZT-0, 6, 12 and 18 respectively; w, refers to significant difference in the levels of the compound at same time point between control and R.F. group in same age group).

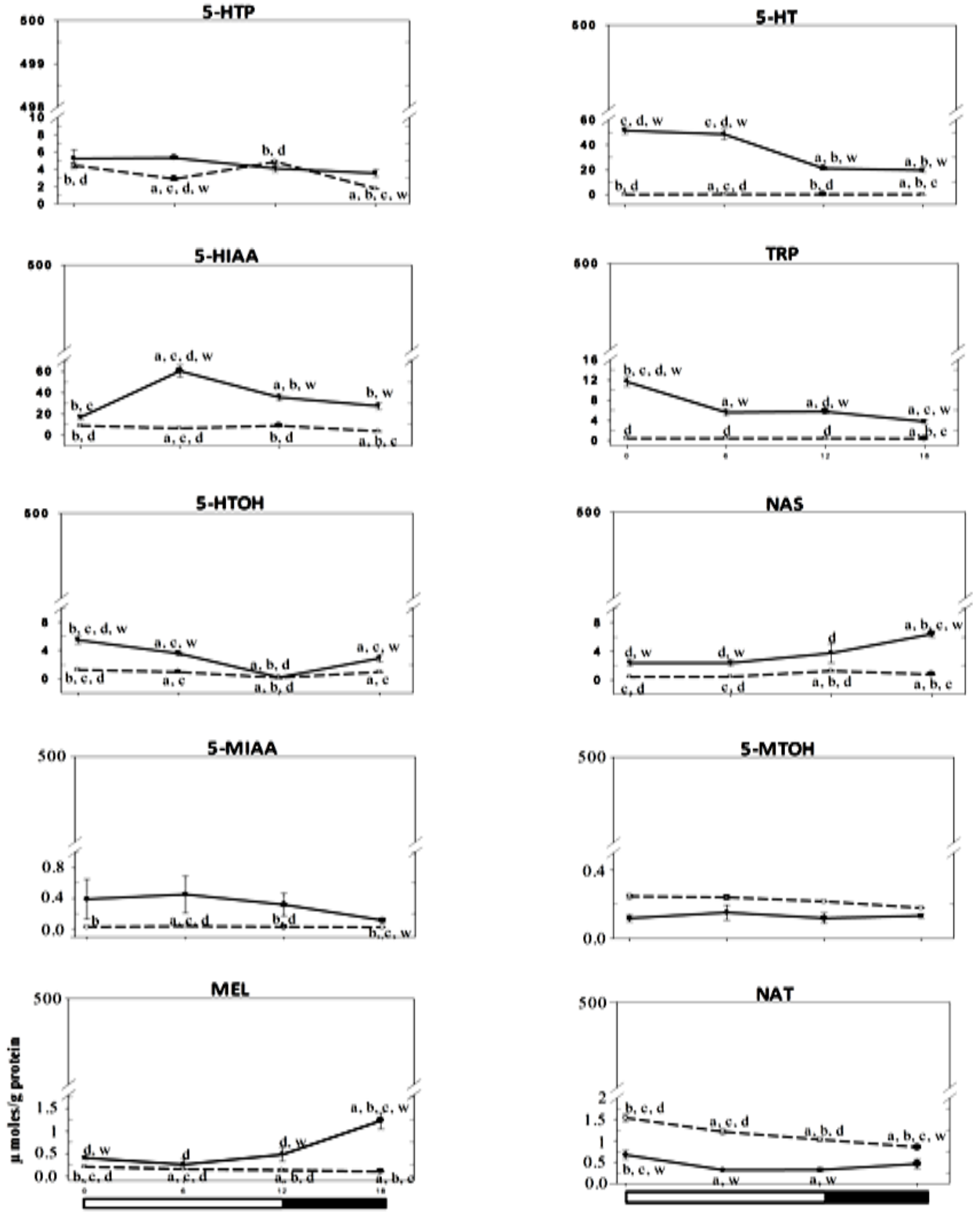


Fig. 22**.** Comparison of daily rhythms of various components of serotonin metabolism in control and food restricted groups in the SCN of 24 m old rats. Each value is mean ± S.E (n=6). $p \leq 0.05$ (a, b, c and d refers to comparison between ZT-0, 6, 12 and 18 respectively; w, refers to significant difference in the levels of the compound at same time point between control and R.F. group in same age group). ——— Line indicates controls; - - - - - Line indicates R.F.

Table 7. Effect of restricted feeding on serotonin and its related compounds in the SCN of 24 m old rat SCN.

Compound	Experimental group	Max : Min ratio	Mean 24 h (µ moles/g protein)	Rhythmicity
5-HTP	Control	1.5 ± 0.2	4.52 ± 0.33	Arhythmic
	R.F.	2.77 ± 0.07 *	3.48 ± 0.27	Rhythmic
5-HT	Control	2.49 ± 0.17	29.81 ± 5.16	Rhythmic
	R.F.	3.38 ± 0.13 *	0.12 ± 0.01	Persisted
5-HIAA	Control	3.62 ± 0.33	38.81 ± 8.1	Rhythmic
	R.F.	2.44 ± 0.08 *	6.92 ± 0.48	Persisted
NAS	Control	2.79 ± 0.31	3.37 ± 0.81	Rhythmic
	R.F.	3 ± 0.11	0.71 ± 0.07	Persisted
TRP	Control	3.12 ± 0.24	6.1 ± 1.44	Rhythmic
	R.F.	1.28 ± 0.04 *	0.41 ± 0.01	Persisted
5-HTOH	Control	50.42 ± 20.7	2.88 ± 0.86	Rhythmic
	R.F.	8.32 ± 0.21	0.82 ± 0.08	Persisted
5-MIAA	Control	4.15 ± 1.48	0.27 ± 0.07	Arhythmic
	R.F.	1.5 ± 0.04	0.03 ± 0.001	Rhythmic
5-MTOH	Control	1.52 ± 0.29	0.13 ± 0.01	Arhythmic
	R.F.	1.38 ± 0.04	0.22 ± 0.007	Rhythmic
MEL	Control	5.86 ± 1.56	0.55 ± 0.17	Rhythmic
	R.F.	2.06 ± 0.07 *	0.15 ± 0.008	Persisted
NAT	Control	2.16 ± 0.18	0.04 ± 0.006	Rhythmic
	R.F.	1.8 ± 0.06	0.11 ± 0.005	Persisted

* indicate significant difference between control and food restricted animals ($p \leq 0.05$) determined by one way-ANOVA. Each value is mean ± S.E. (n = 6).

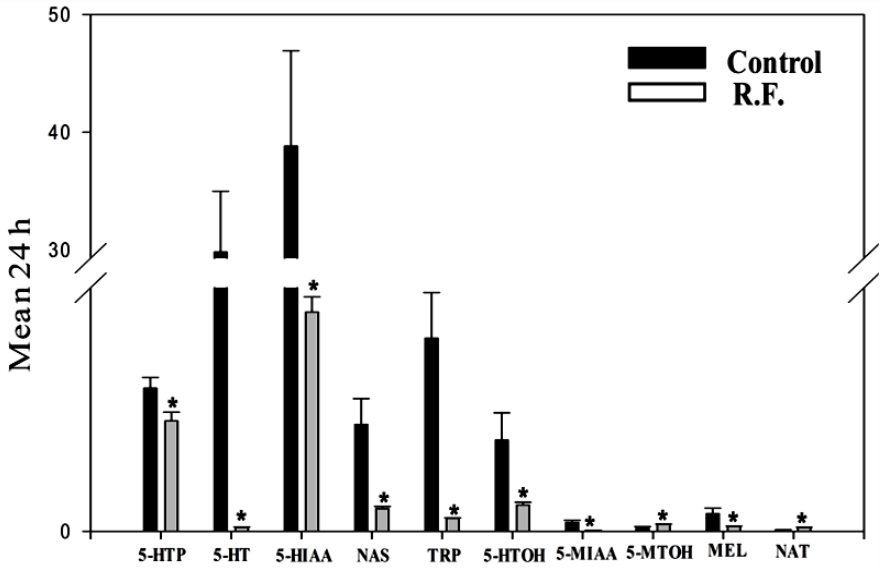


Fig. 23**.** Effect of restricted feeding on mean 24 h. levels of serotonin and its related compounds in the SCN of 24 m old rats. * indicate significant difference between control and food restricted animals ($p \leq 0.05$) (n=24) determined by one way-ANOVA.

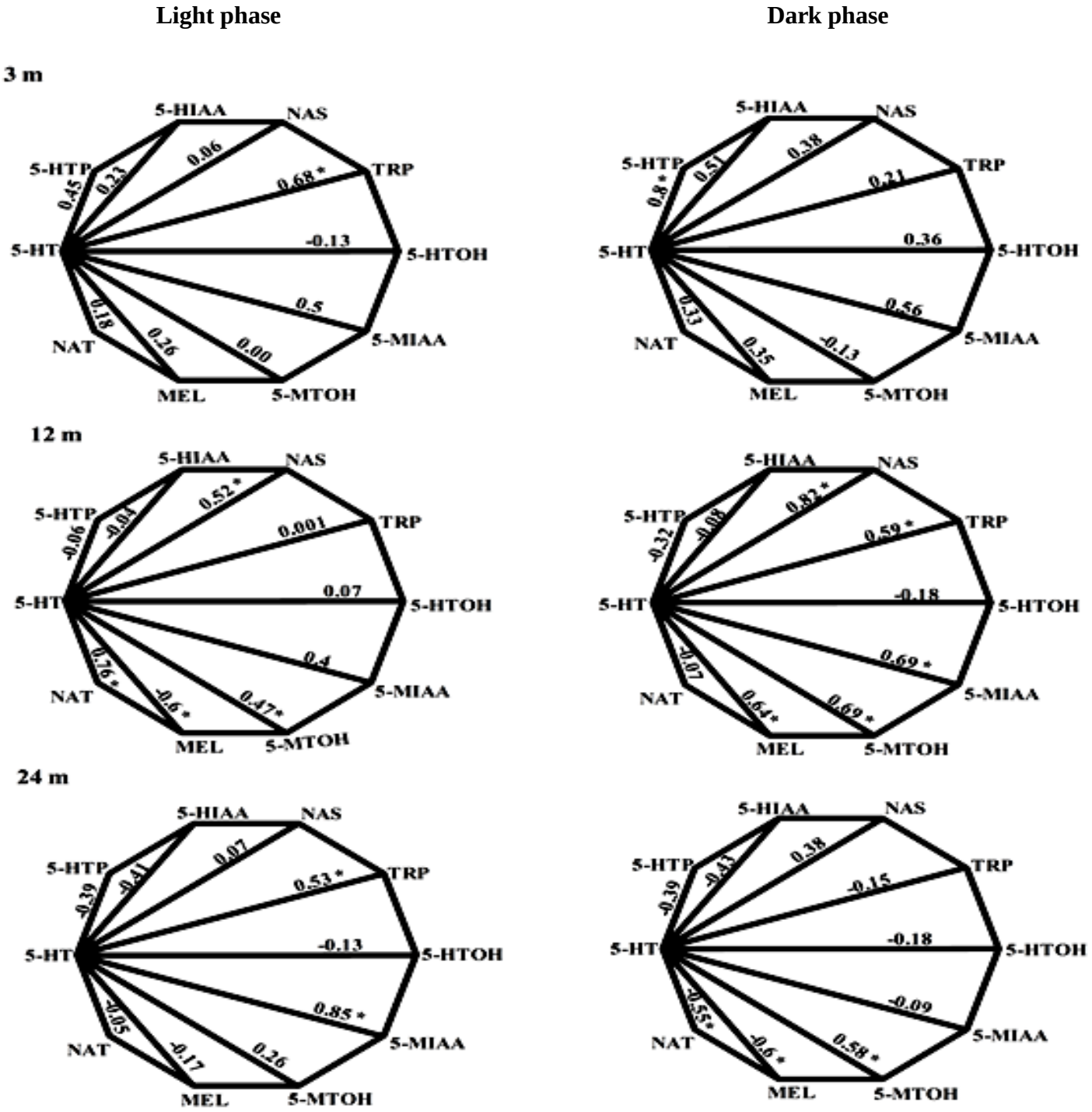


Fig. 24**.** Effect of restricted feeding on pair wise correlation between mean light (ZT-0, 6, 12) and mean dark (ZT-12, 18, 0) phase values in various age groups 3, 12 and 24 old rats SCN. Each value is a correlation coefficient value between parameters. * indicates statistically significant value between parameters ($p \leq 0.05$). Negative value indicates negative correlation between parameters.

Effect of restricted feeding on serotonin metabolism in various age groups

PINEAL

3 months

In 3 m old rats pineal also all the ten compounds have shown rhythmicity in controls. Upon food restriction the rhythmicity of 5-HIAA, NAS, 5-HTOH, 5-MIAA, 5-MTOH, MEL and NAT was abolished and the mean 24 h levels were also decreased from 10.88 ± 3.12, 3.84 ± 1.23, 2.09 ± 0.71, 1.74 ± 0.63, 4.17 ± 1.56 and 0.38 ± 0.09 µmoles/g protein to 7.7 ± 0.97, 2.26 ± 0.72, 0.85 ± 0.17, 0.07 ± 0.005, 7.72 ± 6.82, 0.45 ± 0.07, 0.02 ± 0.04 µmoles/g protein. But the rhythmicity of 5-HTP, 5-HT and TRP persisted upon restricted feeding (Fig. 25 and Table. 8). When compared to controls 5-HTP levels were high at ZT-0 (27.3 ± 7.05 µmoles/g protein) under food restricted condition but from ZT-0 onwards as in controls the levels started coming down and reached to its minimum at ZT-18 (2.36 ± 0.47 µ moles/g protein).

In pineal also both serotonin and melatonin have shown the inverse relation but, upon food restriction the serotonin levels were increased from ZT-0 (47.84 ± 4.21 µmoles/g protein) to ZT-12 (408.69 ± 35.34 µmoles/g protein) and then decreased at ZT-18 (138.35 ± 20.51 µmoles/g protein) with a phase delay of 6 h whereas, upon food restriction melatonin levels were significantly decreased in all the four time points when compared to its control (Fig. 25.).

Under food restriction serotonin didn't show any significant change in the mean 24 h levels whereas, the daily pulse ratio between control and food restricted animals was increased by 2.3 folds approximately (Fig. 26 and Table 9.).

TRP showed 12 h phase advancement after food restriction. In food restricted animals the levels started increasing from ZT-0 (0.11 ± 0.02 µmoles/g protein) and reached its maximum at ZT-6 (7.94 ± 1.17 µmoles/g protein). From ZT-6 to ZT-12 (7.52 ± 1.73 µmoles/g protein) the levels were slightly decreased and suddenly the levels decreased from ZT-12 to ZT-18 (0.62 ± 0.29 µmoles/g protein) and no significant difference was observed in the levels between ZT-0 and ZT-18 time points whereas, in controls the levels gradually increased from ZT-0 (0.975 ± 0.014 µmoles/g protein) to ZT-18 (0.766 ± 0.11 µmoles/g protein) (Fig. 25.).

Table 8. Effect of restricted feeding on serotonin and its related compounds in 3 m old rat Pineal.

Compound	Experimental group	Zeitgeber time (h) (ZT)			
		0	6	12	18
5-HTP	Control	2.55 ± 0.42 [b, c, d]	1.76 ± 0.36 [a]	0.78 ± 0.21 [a, w]	1.25 ± 0.51 [a, w]
	R.F.	27.3 ± 7.05 [b, c, d, w]	4.29 ± 0.65 [a]	5.93 ± 0.67 [a]	2.36 ± 0.47 [a]
5-HT	Control	206.03 ± 39.79 [b, d, w]	291.61 ± 58.38 [a, c, d]	179.44 ± 28.13 [b, d, w]	28.87 ± 12.5 [a, b, c, w]
	R.F.	47.84 ± 4.21 [b, c, d]	239.07 ± 33.47 [a, c, d]	408.69 ± 35.34 [a, b, d]	138.35 ± 20.51 [a, b, c]
5-HIAA	Control	11.98 ± 1.54 [b, d]	18 ± 1.81 [a, c, d, w]	10.75 ± 1.12 [b, d, w]	2.79 ± 0.71 [a, b, c]
	R.F.	10.77 ± 2.21	7.22 ± 0.56	6.71 ± 1.06	6.09 ± 2.8
NAS	Control	2.96 ± 0.41 [c, d]	1.46 ± 0.27 [c, d, w]	3.69 ± 0.83 [a, b, d, w]	7.28 ± 0.59 [a, b, c]
	R.F.	0.68 ± 0.18	3.65 ± 0.47	1.48 ± 0.12	3.24 ± 2.89
TRP	Control	0.97 ± 0.01 [d]	1.02 ± 0.32 [d, w]	0.43 ± 0.09 [d, w]	0.76 ± 0.11 [a, b, c]
	R.F.	0.11 ± 0.02 [b, c]	7.94 ± 1.17 [a, d]	7.52 ± 1.73 [a, d]	0.62 ± 0.29 [b, c]
5-HTOH	Control	4.18 ± 1.21 [b, c, d, w]	1.55 ± 0.33 [a, w]	0.99 ± 0.23 [a]	1.64 ± 0.63 [a]
	R.F.	0.6377 ±0.141	0.3979 ±0.164	1.5306 ±0.487	0.8718 ±0.19
5-MIAA	Control	2.02 ±0.31 [b, d, w]	3.40 ±0.69 [a, c, d, w]	1.07 ±0.41 [b, w]	0.479 ±0.13 [a, b, w]
	R.F.	0.08 ± 0.01	0.06 ± 0.007	0.08 ± 0.008	0.07 ± 0.01
5-MTOH	Control	25.63 ± 4.09 [c, d]	29.86 ± 2.32 [c, d, w]	13.7 ± 1.87 [a, b, w]	10.16 ± 0.35 [a, b, w]
	R.F.	28.16 ± 27.29	1.15 ± 0.24	0.57 ± 0.01	1.02 ± 0.11
MEL	Control	3.27 ± 0.66 [d, w]	1.1 ± 0.16 [c, d, w]	3.79 ± 0.85 [b, d, w]	8.53 ± 1.1 [a, b ,c, w]
	R.F.	0.63 ± 0.25	0.41 ± 0.1	0.24 ± 0.06	0.53 ± 0.1
NAT	Control	0.25 ± 0.07 [c, w]	0.39 ± 0.02 [c, d, w]	0.65 ± 0.01 [a, b, d, w]	0.22 ± 0.06 [b, c, w]
	R.F.	0.03 ± 0.009	0.02 ± 0.004	0.01 ± 0.009	0.03 ± 0.01

All values are expressed as µmol/g protein. Each value is mean ± S.E (n=6). Zeitgeber Time (ZT): ZT-0 = 6:30 h (Lights on); ZT-12 = 18:30 h (Lights off). $p \leq 0.05$ (a, b, c and d refers to comparison between ZT-0, 6, 12 and 18 respectively; w, refers to significant difference in the levels of the compound at same time point between control and R.F. group in same age group).

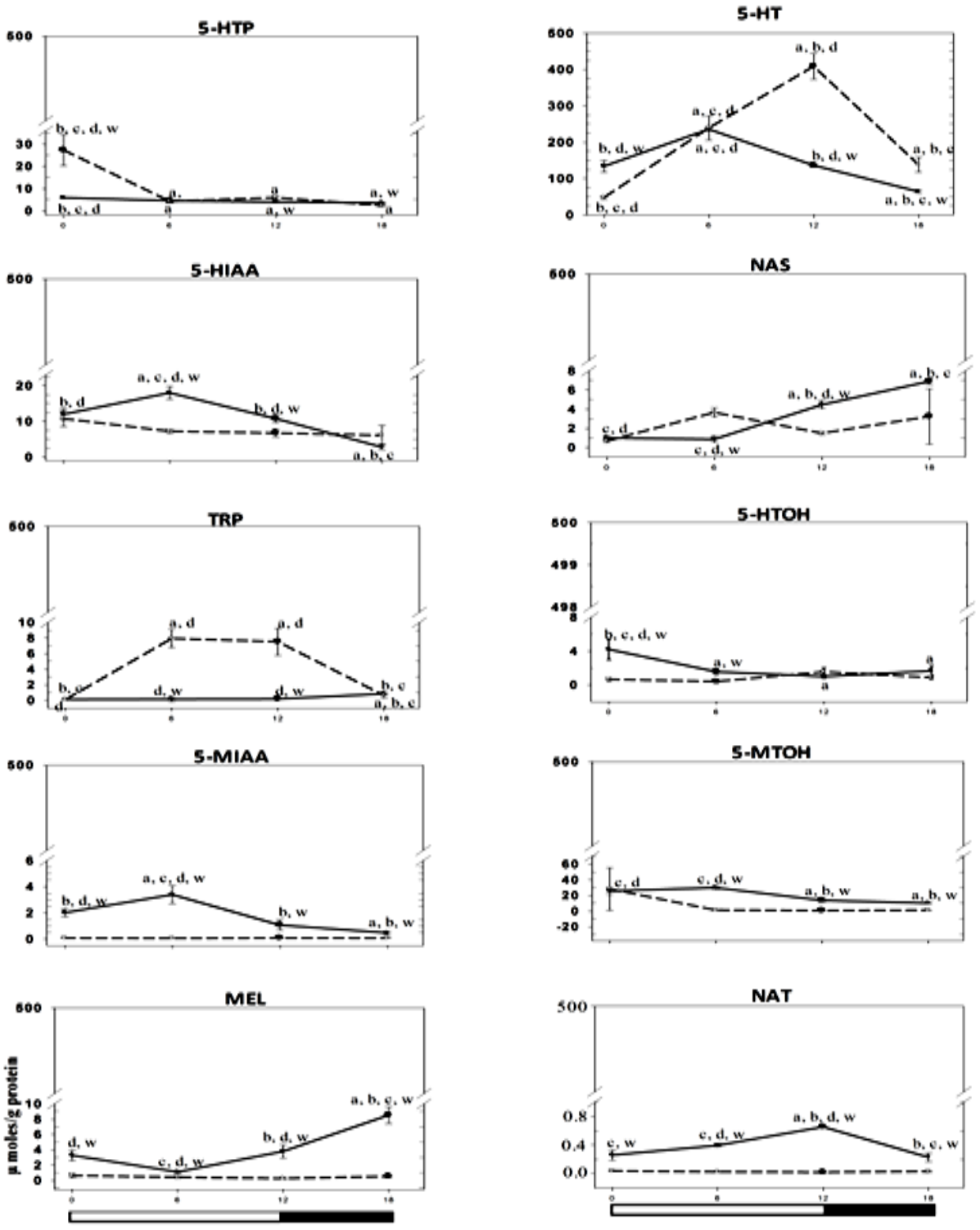


Fig. 25**.** Comparison of daily rhythms of various components of serotonin metabolism in control and food restricted groups in the pineal of 3 m old rats. Each value is mean ± S.E (n=6). $p \leq 0.05$ (a, b, c and d refers to comparison between ZT-0, 6, 12 and 18 respectively; w, refers to significant difference in the levels of the compound at same time point between control and R.F. group in same age group). ——— Line indicates controls; - - - - - Line indicates R.F.

Table 9. Effect of restricted feeding on serotonin and its related compounds in 3 m old rat Pineal.

Compound	Experimental group	Max : Min ratio	Mean 24 h (µ moles/g protein)	Rhythmicity
5-HTP	Control	3.24 ± 0.48 *	1.59 ± 0.03	Rhythmic
	R.F.	11.54 ± 2.28	9.97 ± 3.39	Persisted
5-HT	Control	10.1 ± 3.47	176.48 ± 54.71	Rhythmic
	R.F.	8.54 ± 0.61	208.49 ± 41.93	Persisted
5-HIAA	Control	6.45 ± 1.09 *	10.88 ± 3.12	Rhythmic
	R.F.	1.76 ± 0.66	7.7 ± 0.97	Abolished
NAS	Control	4.98 ± 0.6	3.84 ± 1.23	Rhythmic
	R.F.	5.32 ± 2.83	2.26 ± 0.72	Abolished
TRP	Control	2.33 ± 0.53 *	0.8 ± 0.01	Rhythmic
	R.F.	72.2 ± 10.16	4.04 ± 1.2	Persisted
5-HTOH	Control	4.22 ± 0.95	2.09 ± 0.71	Rhythmic
	R.F.	3.84 ± 1.45	0.85 ± 0.17	Abolished
5-MIAA	Control	7.09 ± 1.13*	1.74 ± 0.63	Rhythmic
	R.F.	1.13 ± 0.06	0.07 ± 0.005	Abolished
5-MTOH	Control	2.93 ± 0.12 *	19.84 ± 4.7	Rhythmic
	R.F.	49.3 ± 27.65	7.72 ± 6.82	Abolished
MEL	Control	7.71 ± 0.91 *	4.17 ± 1.56	Rhythmic
	R.F.	2.6 ± 0.79	0.45 ± 0.07	Abolished
NAT	Control	2.86 ± 0.43	0.38 ± 0.09	Rhythmic
	R.F.	1.9 ± 0.79	0.02 ± 0.04	Abolished

* indicate significant difference between control and food restricted animals ($p \leq 0.05$) determined by one way-ANOVA.

Each value is mean ± S.E. (n = 6).

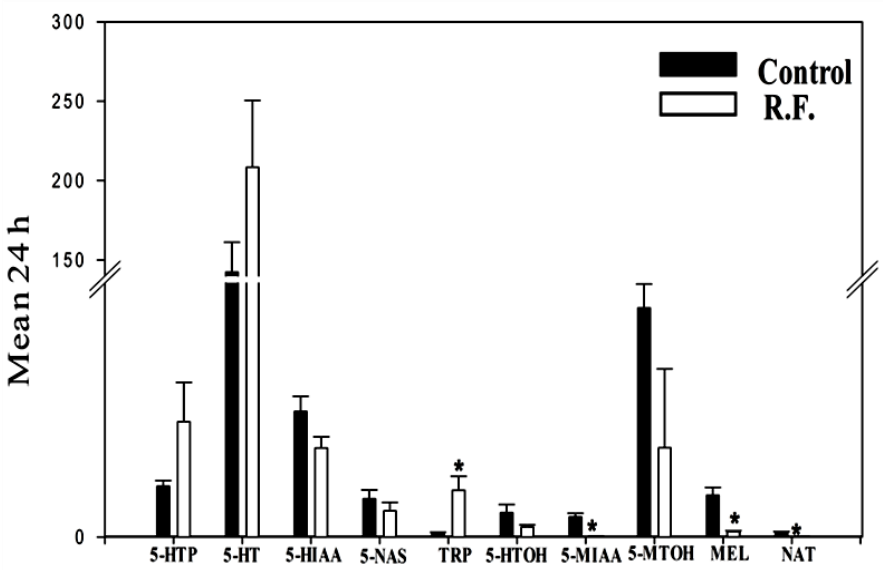


Fig. 26. Effect of restricted feeding on the mean 24 h levels of serotonin and its related compounds in the pineal of 3 m old rats. * indicate significant difference between control and food restricted animals ($p \leq 0.05$) (n=24) determined by one way-ANOVA.

The daily pulse ratio of 5-HTP, 5-HT, TRP and 5-MTOH between control and food restricted animals was increased by approximately 6.48, 2.3, 10 and 16.82 folds respectively (Table 9.). Whereas the mean 24 h levels of TRP increased by 12.53 folds and there was no significant difference in the mean 24 h levels of 5-HTP, 5-HT, 5-HIAA, NAS, 5-HTOH and 5-MTOH was observed between controls and food restricted animals (Fig. 26 and Table 9.).

Upon food restriction the daily pulses of 5-HIAA and 5-MIAA showed 4.05 and 6.45 folds decrease respectively between controls and food restricted animals (Table 9.).

The ratio of 5-HT/5-HIAA was maximum at ZT-0 (17.48 ± 2.35) in control animals and upon food restriction 5-HT/5-HIAA ratio maximum occurred at ZT-12 (62.46 ± 6.51) with a 6 h phase delay. Whereas the 5-HT/5-HIAA minimum levels occurred at ZT-18 (11.33 ± 3.43) and after food restriction the minimum levels were observed at ZT-0 (4.63 ± 0.59). In 3 months old rat pineal also the mean 24h levels of 5-MIAA, 5-MTOH and MEL were decreased (Table 8. and Fig. 25.).

12 months

In 12 m old control rats pineal, 5-HTP, 5-HT, 5-HIAA, TRP, 5-MIAA, and MEL have shown daily rhythmicity whereas; NAS, 5-HTOH, 5-MTOH and NAT did not show daily rhythm (Fig. 27 and Table. 10). In these rats the pineal daily pulse of 5-HTP, 5-HTOH, 5-MIAA, 5-MTOH, MEL and NAT were decreased significantly from 3.27 ± 0.32, 1.97 ± 0.26, 2.72 ± 0.39, 6.16 ± 0.41, 2.57 ± 0.21 and 6.35 ± 1.16 to 1.28 ± 0.05, 1.32 ± 0.06, 1.73 ± 0.05, 1.64 ± 0.04, 1.78 ± 0.05 and 1.81 ± 0.05 and this decrease was 2.53, 1.48, 1.56, 3.73, 1.44 and 3.5 folds respectively. However the daily pulse of 5-HT, 5-HIAA, NAS and TRP have increased significantly from 3.26 ± 0.06, 2.84 ± 0.33, 1.6 ± 0.19, 2.81 ± 0.19 to 5.32 ± 0.16, 4.82 ± 0.17, 2.38 ± 0.09, 6.53 ± 0.21 and the increase was 1.63, 0.58, 0.67 and 0.43 fold respectively between controls to food restricted animals (Table. 11).

In the pineal of 12 m old control rats the mean 24 h levels of 5-HTP, 5-HIAA, TRP, 5-MIAA, 5-MTOH, MEL and NAT were 0.56 ± 0.11, 2.09 ± 0.47, 0.008 ± 0.001, 0.03 ± 0.007, 0.02 ± 0.01, 0.16 ± 0.02 and 0.01 ± 0.005 µmoles/g protein. Upon food restriction the levels were increased to 0.91 ± 0.02, 147.26 ± 15.74, 0.16 ± 0.01, 0.08 ± 0.004, 0.55 ± 0.02, 0.18 ± 0.008, 0.15 ± 0.008 µmoles/g protein and the increment was 1.63, 70.15, 20.12, 2.51, 21.3, 1.13 and 8.5 folds respectively under food restriction. The mean 24 h levels of 5-HT, NAS and 5-HTOH were decreased upon food restriction from 3.26 ± 0.06, 1.6 ± 0.199, 1.97 ± 0.26 to 40.61 ± 4.11, 1.83 ± 0.12, 0.2 ± 0.006 by 2.88, 3.9 and 1.16 folds respectively (Fig. 28. and Table 11.).

In the pineal of 12 m old animals the levels of 5-HT and NAS were decreased and the levels of 5-HIAA, TRP, 5-MTOH and NAT were increased at all the four time points. 5-MIAA levels also increased at all the time points except at ZT-0 where the levels were slightly increased upon food restriction (Fig. 27. and Table 10.).

Table 10. Effect of restricted feeding on serotonin and its related compounds in the pineal of 12 m old rats.

Compound	Experimental group	Zeitgeber time (h) (ZT)			
		0	6	12	18
5-HTP	Control	0.49 ± 0.06 [b, w]	0.98 ± 0.07 [a, c, d, w]	0.3 ± 0.04 [b, d, w]	0.59 ± 0.07 [b, c, w]
	R.F.	0.88 ± 0.04 [d]	0.79 ± 0.03 [c, d]	0.97 ± 0.03 [b]	1.02 ± 0.05 [a, b]
5-HT	Control	108.39 ± 3.17 [b, c, d, w]	191.9 ± 3.56 [a, c, d, w]	134.61 ± 2.32 [a, b, d, w]	58.77 ± 1.54 [a, b, c, w]
	R.F.	63.53 ± 2.68 [b, c, d]	35.86 ± 1.61 [a, c , d]	51.11 ± 2.28 [a, b, d]	11.93 ± 0.38 [a, b, c]
5-HIAA	Control	1.43 ± 0.28 [c, w]	1.54 ± 0.09 [c, w]	3.94 ± 0.15 [a, b, d, w]	2 ± 0.27 [c, w]
	R.F.	146.39 ± 8.04 [c, d]	129.84 ± 5.28 [c, d]	259.03± 12.44 [a, b, d]	53.77 ± 2.3 [a, b, c]
NAS	Control	7.53 ± 0.26	5.12 ± 0.52	8.14 ± 1.53	7.87 ± 0.69
	R.F.	1.09 ± 0.05 [b, c, d, w]	2.59 ± 0.11 [a, c, d, w]	1.52 ± 0.06 [a, b, d, w]	2.1 ± 0.06 [a, b, c, w]
TRP	Control	0.004 ± 0.0007 [c, w]	0.006 ±0.0001 [c, w]	0.01 ± 0.002 [a, b, d, w]	0.005 ± 0.0009 [c, w]
	R.F.	0.19 ± 0.009 [b, d]	0.21 ± 0.006 [a, d]	0.2 ± 0.009 [d]	0.03 ± 0.001 [a, b, c]
5-HTOH	Control	0.3 ± 0.05	0.25 ± 0.04	0.19 ± 0.02	0.16 ± 0.02
	R.F.	0.17 ± 0.01 [b, d, w]	0.2 ± 0.004 [a]	0.2 ± 0.009	0.22 ± 0.01 [a, w]
5-MIAA	Control	0.06 ± 0.01 [b, c, d]	0.02 ± 0.004 [a, w]	0.02 ± 0.002 [a]	0.02 ± 0.004 [a]
	R.F.	0.06 ± 0.002 [b, c, d]	0.1 ± 0.003 [a, c, d]	0.07 ± 0.003 [a, b, d, w]	0.09 ± 0.002 [a, b, c, w]
5-MTOH	Control	0.1 ± 0.02	0.04 ± 0.005	0.09 ± 0.05	0.02 ± 0.002
	R.F.	0.44 ± 0.01 [b, d, w]	0.72 ± 0.02 [a, c, d, w]	0.45 ± 0.01 [b, d, w]	0.59 ± 0.02 [a, b, c, w]
MEL	Control	0.2 ± 0.03 [b]	0.11 ± 0.008 [a, d, w]	0.14 ± 0.01 [d]	0.24 ± 0.02 [c, d]
	R.F.	0.12 ± 0.005 [b, c, d]	0.22 ± 0.006 [a, c]	0.17 ± 0.01 [a, b, d]	0.2 ± 0.006 [a, c]
NAT	Control	0.01 ± 0.001	0.03 ± 0.02	0.01 ± 0.001	0.01 ± 0.003
	R.F.	0.11 ± 0.004 [b, d, w]	0.2 ± 0.007 [a, c, d, w]	0.13 ± 0.005 [b, d, w]	0.16 ± 0.009 [a, b, c, w]

All values are expressed as µmol/g protein. Each value is mean ± S.E (n=6). Zeitgeber Time (ZT): ZT-0 = 6:30 h (Lights on); ZT-12 = 18:30 h (Lights off). $p \leq 0.05$ (a, b, c and d refers to comparison between ZT-0, 6, 12 and 18 respectively; w, refers to significant difference in the levels of the compound at same time point between control and R.F. group in same age group).

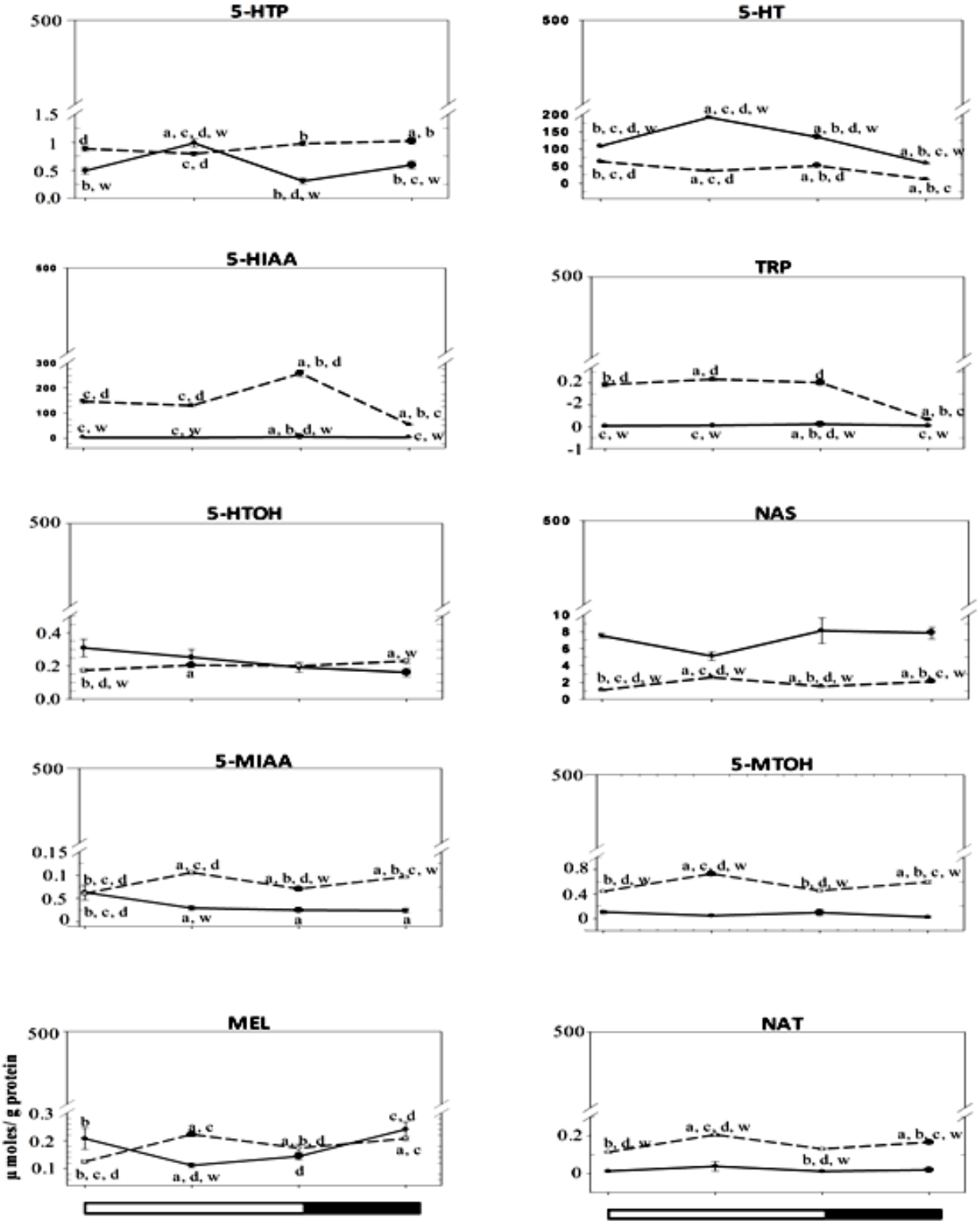


Fig. 27**.** Comparison of daily rhythms of various components of serotonin metabolism in control and food restricted groups in the pineal of 12 m old rats. Each value is mean ± S.E (n=6). $p \leq 0.05$ (a, b, c and d refers to comparison between ZT-0, 6, 12 and 18 respectively; w, refers to significant difference in the levels of the compound at same time point between control and R.F. group in same age group). ——— Line indicates controls; - - - - - Line indicates R.F.

Table 11. Effect of restricted feeding on serotonin and its related compounds in 12 m old rat Pineal.

Compound	Experimental group	Max : Min ratio	Mean 24 h (µ moles/g protein)	Rhythmicity
5-HTP	Control	3.27 ± 0.32	0.56 ± 0.11	Rhythmic
	R.F.	1.28 ± 0.05 *	0.91 ± 0.02	Persisted
5-HT	Control	3.26 ± 0.06	117 ± 22.4	Rhythmic
	R.F.	5.32 ± 0.16 *	40.61 ± 4.11	Persisted
5-HIAA	Control	2.84 ± 0.33	2.09 ± 0.47	Rhythmic
	R.F.	4.82 ± 0.17 *	147.26 ± 15.74	Persisted
NAS	Control	1.6 ± 0.19	7.15 ± 0.53	Arhythmic
	R.F.	2.38 ± 0.09 *	1.83 ± 0.12	Rhythmic
TRP	Control	2.81 ± 0.19	0.008 ± 0.001	Rhythmic
	R.F.	6.53 ± 0.21 *	0.16 ± 0.01	Persisted
5-HTOH	Control	1.97 ± 0.26	0.23 ± 0.02	Arhythmic
	R.F.	1.32 ± 0.06 *	0.2 ± 0.006	Rhythmic
5-MIAA	Control	2.72 ± 0.39	0.03 ± 0.007	Rhythmic
	R.F.	1.73 ± 0.05 *	0.08 ± 0.004	Persisted
5-MTOH	Control	6.16 ± 0.41	0.02 ± 0.01	Arhythmic
	R.F.	1.64 ± 0.04 *	0.55 ± 0.02	Rhythmic
MEL	Control	2.57 ± 0.21	0.16 ± 0.02	Rhythmic
	R.F.	1.78 ± 0.05 *	0.18 ± 0.008	Persisted
NAT	Control	6.35 ± 1.16	0.01 ± 0.005	Arhythmic
	R.F.	1.81 ± 0.05 *	0.15 ± 0.008	Rhythmic

* indicate significant difference between control and food restricted animals ($p \leq 0.05$) determined by one way-ANOVA. Each value is mean ± S.E (n=6).

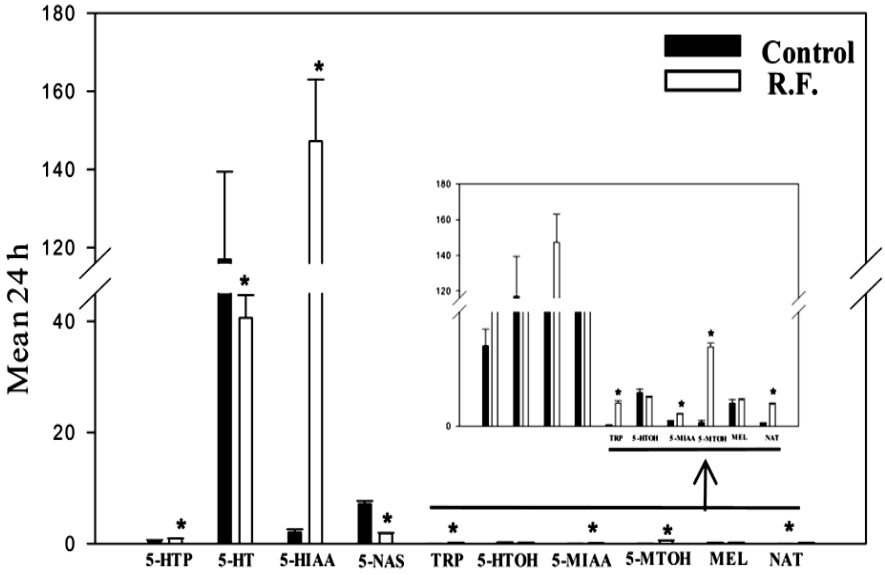


Fig. 28. Effect of restricted feeding on the mean 24 h levels of serotonin and its related compounds in the pineal of 12 m old rats. * indicate significant difference between control and food restricted animals ($p \leq 0.05$) (n=24) determined by one way-ANOVA.

24 months

In the pineal of 24 m old rats, 5-HTP, 5-HT, 5-HIAA, NAS, TRP, 5-HTOH, 5-MIAA, and MEL have shown daily rhythmicity whereas, 5-MTOH and NAT did not show daily rhythm (Fig. 29. and Table. 12.). A significant decrease in the daily pulse of 5-HT by 1.4 folds, and a significant increase in NAS, 5-HTOH, and 5-MIAA levels by 1.5, 2, and 89 folds were observed respectively between controls and food restricted animals (Table. 13.).

In 24 m old food restricted animals, pineal 5-HT, 5-HTOH, NAS levels were decreased at all the time points whereas, the TRP levels were increased slightly at ZT-0 and then decreased at the remaining time points like the other compounds. The levels of 5-HTP, 5-HIAA, 5-MTOH and NAT levels were increased upon food restriction at all the time points, but in the case of 5-MIAA, the levels were decreased at ZT-0 and increased at the remaining three time points (Table. 12. and Fig. 29.).

In the pineal of 24 m old rats also the mean 24 h levels of 5-MIAA, 5-MTOH and NAT were increased as in the pineal of 12 m old rats, but the levels were decreased in the case of TRP and 5-HTOH under R.F. (Table. 13. and Fig. 30.).

Table 12. Effect of restricted feeding on serotonin and its related compounds in the pineal of 24 m old rats.

Compound	Experimental group	Zeitgeber time (h) (ZT)			
		0	6	12	18
5-HTP	Control	0.24 ± 0.007 d, w	0.3 ± 0.03 d, w	0.23 ± 0.02 d, w	0.12 ± 0.01 a, b, c, w
	R.F.	1.86 ± 0.1 b, c	3.72 ± 0.1 a, c, d	4.43 ± 0.19 a, b, d	1.95 ± 0.17 b, c
5-HT	Control	58.09 ± 0.62 **b, c, d, w**	113.37 ± 8.15 a, c, d, w	97.79 ± 6.52 a, b, d, w	31.2 ± 0.65 a, b, c, w
	R.F.	24.9 ± 1.14 c, d	27.28 ± 0.57 c, d	17.29 ± 0.83 a, b, d	10.22 ± 0.5 a, b, c
5-HIAA	Control	4.96 ± 1.08 c, w	4.78 ± 0.4 c, w	1.53 ± 0.34 a, b, d, w	6.16 ± 0.41 c, w
	R.F.	200.01 ± 10.37 b, d	305.96 ± 9.77 a, c, d	229.29 ± 8.48 b, d	146.46 ± 14.67 a, b, c
NAS	Control	0.42 ± 0.02 c, d, w	0.74 ± 0.11 c, d, w	1.52 ± 0.03 **a, b, d, w**	3.13 ± 0.2 a, b, c, w
	R.F.	0.1 ± 0.004	0.39 ± 0.03	0.47 ± 0.02	1.15 ± 0.06
TRP	Control	0.42 ± 0.26 b, c, d, w	2.28 ± 0.2 a, c, d, w	3.36 ± 0.03 a, b, d, w	1.62 ± 0.02 a, b, c, w
	R.F.	1.65 ± 0.09 b, c, d	1.11 ± 0.01 a, c, d	0.4 ± 0.01 a, b, d	0.09 ± 0.007 a, b, c
5-HTOH	Control	0.51 ± 0.04 b, d, w	0.32 ± 0.04 a, d, w	0.43 ± 0.02 d, w	0.63 ± 0.04
	R.F.	0.22 ± 0.005 b, d	0.14 ± 0.01 a, d	0.18 ± 0.01 d	0.57 ± 0.03 a, b, c
5-MIAA	Control	0.01 ± 0.001 b, c, w	0.04 ± 0.01 **a, c, d, w**	0.09 ± 0.0001 a, b, d, w	0.01 ± 0.003 b, c, w
	R.F.	0.83 ± 0.03 **b , c, d**	1.34 ± 0.03 a, c, d	0.03 ± 0.001 a, b	0.06 ± 0.002 a, b
5-MTOH	Control	0.04 ± 0.01	0.03 ± 0.009	0.01 ± 0.001	0.03 ± 0.007
	R.F.	0.09 ± 0.004 **b, c, d, w**	0.08 ± 0.002 a, c, d, w	0.04 ± 0.002 a, b, d	0.1 ± 0.003 a, b, c, w
MEL	Control	0.09 ± 0.01	0.04 ± 0.003	0.22 ± 0.13	0.29 ± 0.12
	R.F.	0.19 ± 0.006 b, d, w	0.09 ± 0.002 a, c, d, w	0.16 ± 0.007 b, d	0.23 ± 0.02 **a, b, c**
NAT	Control	0.01 ± 0.001	0.01 ± 0.003 c	0.006 ± 0.001 b, d, w	0.01 ± 0.002 d, w
	R.F.	0.07 ± 0.002 **b, c, d, w**	0.08 ± 0.001 a, c, d, w	0.04 ± 0.001 a, b, d	0.1 ± 0.004 a, b, c

All values are expressed as µmol/g protein. Each value is mean ± S.E (n=6). Zeitgeber Time (ZT): ZT-0 = 6:30 h (Lights on); ZT-12 = 18:30 h (Lights off). $p \leq 0.05$ (a, b, c and d refers to comparison between ZT-0, 6,12 and 18 respectively; w, refers to significant difference in the levels of the compound at same time point between control and R.F. group in same age group).

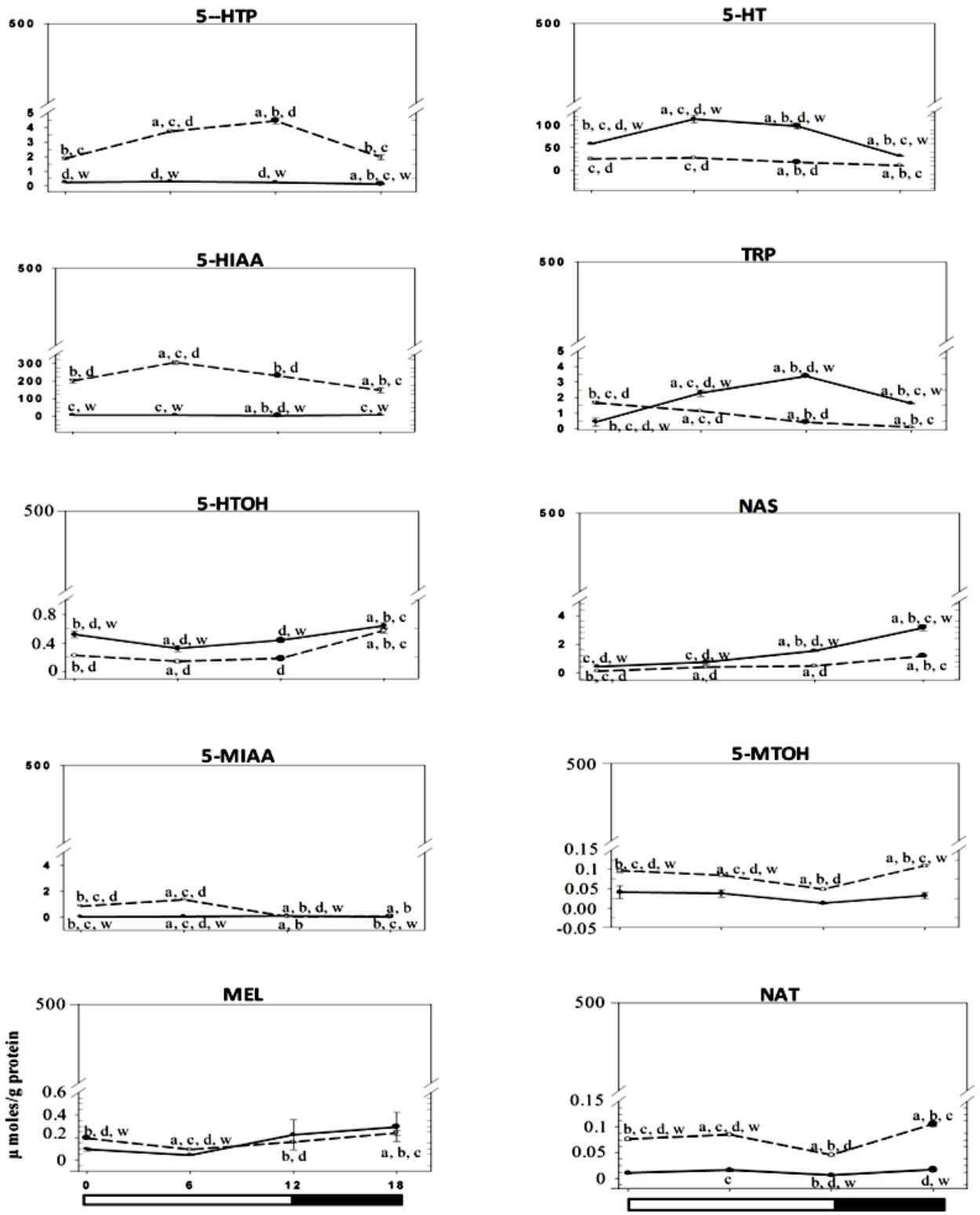


Fig. 29. Comparison of daily rhythms of various components of serotonin metabolism in control and food restricted groups in the pineal of 24 m old rats. Each value is mean ± S.E (n=6). $p \leq 0.05$ (a, b, c and d refers to comparison between ZT-0, 6, 12 and 18 respectively; w, refers to significant difference in the levels of the compound at same time point between control and R.F. group in same age group). ——— Line indicates controls; - - - - - Line indicates R.F.

Table 13. Effect of restricted feeding on serotonin and its related compounds in the pineal of 24 m old rats:

Compound	Experimental group	Max : Min ratio	Mean 24 h (µ moles/g protein)	Rhythmicity
5-HTP	Control	2.49 ± 0.2	0.21 ± 0.02	Rhythmic
	R.F.	2.38 ± 0.09	2.99 ± 0.24	Persisted
5-HT	Control	3. 66 ± 0.17 *	75.23 ± 14.48	Rhythmic
	R.F.	2.67 ± 0.08	19.92 ± 1.44	Persisted
5-HIAA	Control	3.02 ± 0.54	3.93 ± 0.87	Rhythmic
	R.F.	2.1 ± 0.12	220.43 ± 13.07	Persisted
NAS	Control	7.37 ± 0.39 *	1.49 ± 0.5	Rhythmic
	R.F.	10.6 ± 0.45	0.53 ± 0.08	Persisted
TRP	Control	13.15 ± 4.75	2.03 ± 0.49	Rhythmic
	R.F.	17.52 ± 0.94	0.81 ± 0.12	Persisted
5-HTOH	Control	2.01 ± 0.16 *	0.47 ± 0.05	Rhythmic
	R.F.	4.07 ± 0.3	0.28 ± 0.03	Persisted
5-MIAA	Control	0.45 ± 0.06 *	0.07 ± 0.05	Rhythmic
	R.F.	40.2 ± 1.3	0.57 ± 0.11	Persisted
5-MTOH	Control	3.45 ± 0.53	0.03 ± 0.004	Arhythmic
	R.F.	2.25 ± 0.07	0.08 ± 0.004	Rhythmic
MEL	Control	4.59 ± 1.29	0.14 ± 0.04	Arhythmic
	R.F.	2.58 ± 0.13	0.17 ± 0.01	Rhythmic
NAT	Control	2.74 ± 0.39	0.01 ± 0.001	Rhythmic
	R.F.	2.31 ± 0.06	0.07 ± 0.004	Persisted

* indicate significant difference between control and food restricted animals ($p \leq 0.05$) determined by one way-ANOVA. Each value is mean ± S.E. (n = 6).

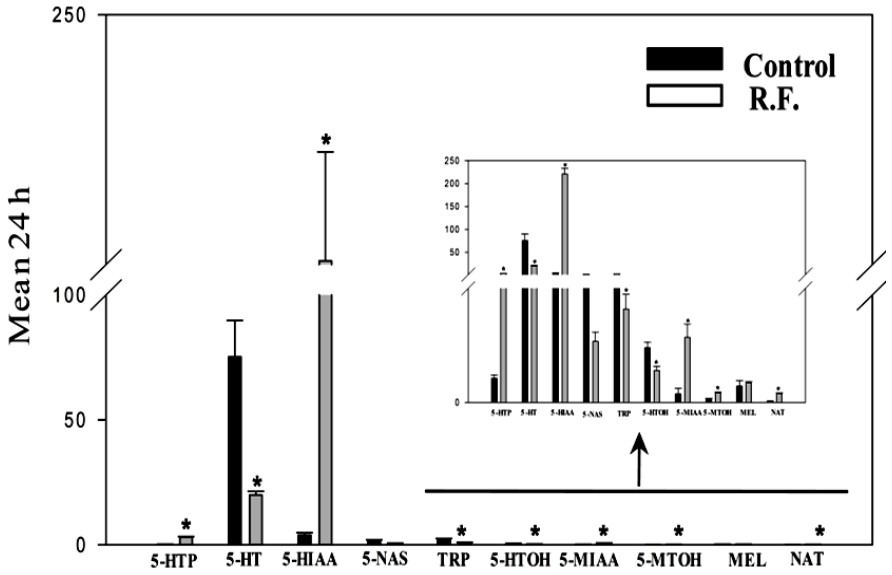


Fig. 30**.** Effect of restricted feeding on the mean 24 h levels of serotonin and its related compounds in the pineal of 24 m old rats. * indicate significant difference between control and food restricted animals ($p \leq 0.05$) (n=24) determined by one way-ANOVA.

5-HT has a significant positive correlation with TRP in the pineal of 3 and 24 m old rats at both light and dark phases and only at dark phase in 12 m old rats. 5-HT was also in positive correlation with 5-HIAA in 12 and 24 m old rats at both light and dark phases. 5-HT and 5-HTOH have a positive correlation at dark phase, whereas with 5-HTP, 5-HT has a positive correlation at the light phase in the pineal of 3 m old rats. 5-HT has a positive correlation with NAS in the pineal of 3 m old rats and a negative correlation in 24 m old rats pineal at dark phase whereas, both these compounds were in negative correlation in 24 m old rats at both light and dark phases. 5-HT has a negative correlation with 5-MIAA at both light and dark phases in the pineal of 3 and 12 m old rats whereas, it has a positive correlation with 5-MIAA at both the phases. 5-HT and 5-MTOH have negative correlation at both light and dark phases in the pineal of 3 and 12 m old rats whereas, 5-MTOH has a positive correlation only at light phase in 24 m old rats. 5-HT and NAT were in positive correlation at both light and dark phases in the pineal of 12 m old rats whereas they were in positive correlation only at light phase in 24 m old rats (Fig. 31).

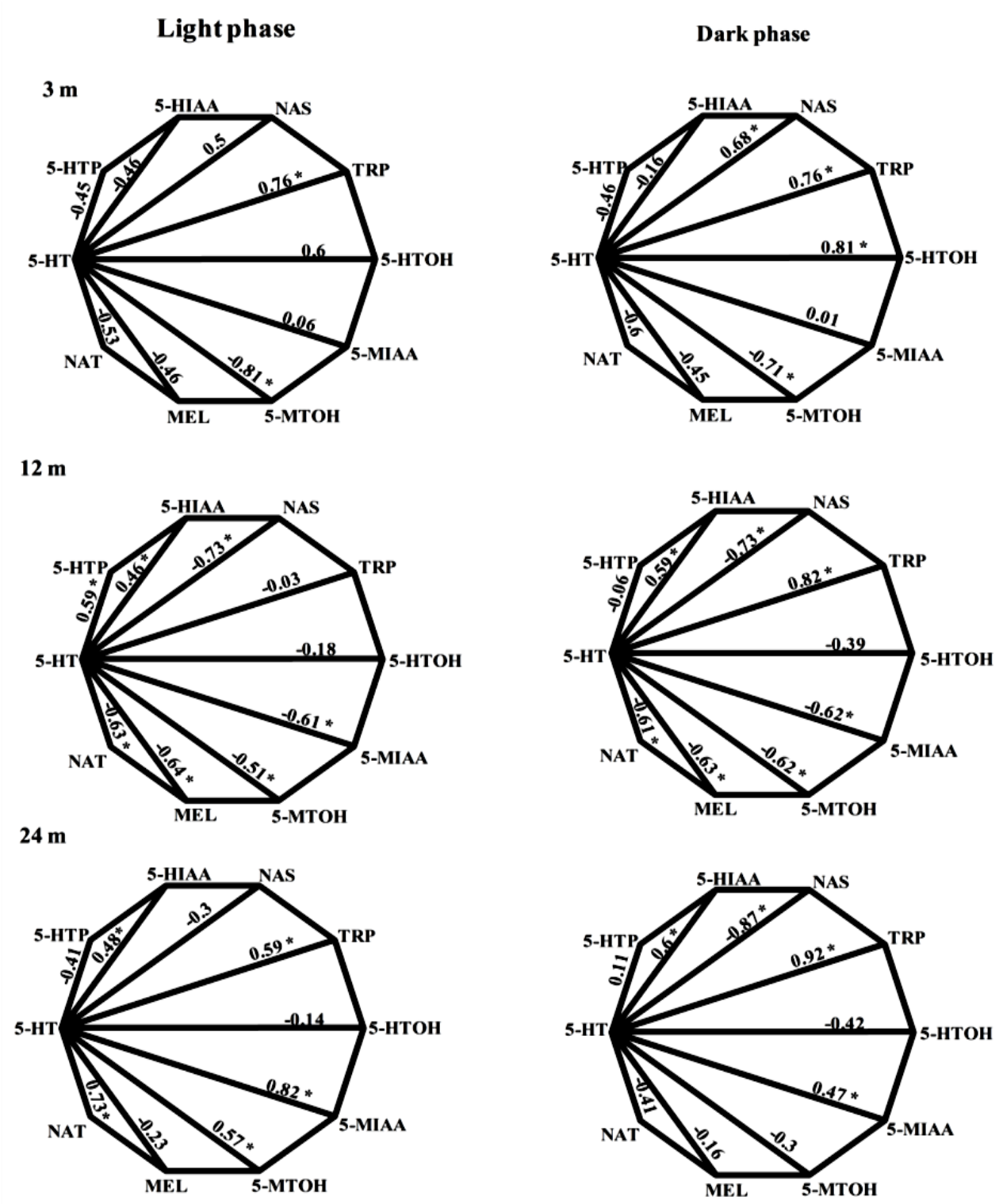


Fig. 31**.** Effect of restricted feeding on pair wise correlation between mean light (ZT-0, 6, 12) and mean dark (ZT-12,18, 0) phase values in various age groups 3, 12 and 24 old rats pineal. Each value is a correlation coefficient value between parameters. * indicates statistically significant value between parameters ($p \leq 0.05$). Negative value indicates negative correlation between parameters.

OBJECTIVE – III

Daily Leptin rhythms in SCN

We screened all the coronal sections of SCN for the leptin immune reactivity (-ir). But we could not find any leptin-ir in this region in both control and R.F. group animals. (Fig. 32A). However we scanned all the coronal sections serially from SCN to optic chiasm and interestingly we could observe leptin-ir in MPOA (medial preoptic area) region of 3 m old rats.

Leptin-ir in the MPOA region of 3, 12 and 24 m old control rats

Leptin-ir was observed in the medial preoptic area of all the three age groups (3, 12 and 24 m). In all these three age groups the peak and nadir for leptin levels were observed at ZT-0 and ZT-18 respectively in control rats (Fig. 32Bi, Ci, Di). Though the levels were low at ZT-18 in both 12 and 24 m old rats, there was no statistically significant difference found in the immunoreactivity levels between ZT-12 and 18 in these age groups. When compared to 3 m old controls the leptin-ir peak and nadir levels gradually decreased in 12 and 24 m old rats (Fig. 32Ci, Di).

In controls the mean 24 h levels of leptin-ir was high in 3 m old rats, and there was no significant difference in the levels between 12 and 24 m old rats (Fig. 33).

Leptin-ir in the MPOA region of 3, 12 and 24 m old food restricted rats

In 3 m old food restricted rats the maximum levels were observed at ZT-6 which was a 6 h phase delay when compared to its control group. The minimum value for the 3 m old R.F. animals was observed at ZT-18 as its control group. No significant difference was observed in the leptin-ir levels between ZT-0 and 12 time points (Fig. 32Bi).

In 12 m old food restricted animals maximum and minimum leptin-ir levels were observed at ZT-18 and ZT-0 respectively, although there was no significant difference observed between ZT-0 and ZT-6. An 18 h phase delay was observed in the leptin-ir maximum upon R.F. when compared to its controls in this age group (Fig. 32Ci).

In 24 m old rats maximum leptin-ir levels were observed at ZT-0 as in its control group. The minimum levels were observed at ZT-12 with 6 h phase advancement, though there was no significant difference in the levels between ZT-12 and ZT-18 time points (Fig. 32Di).

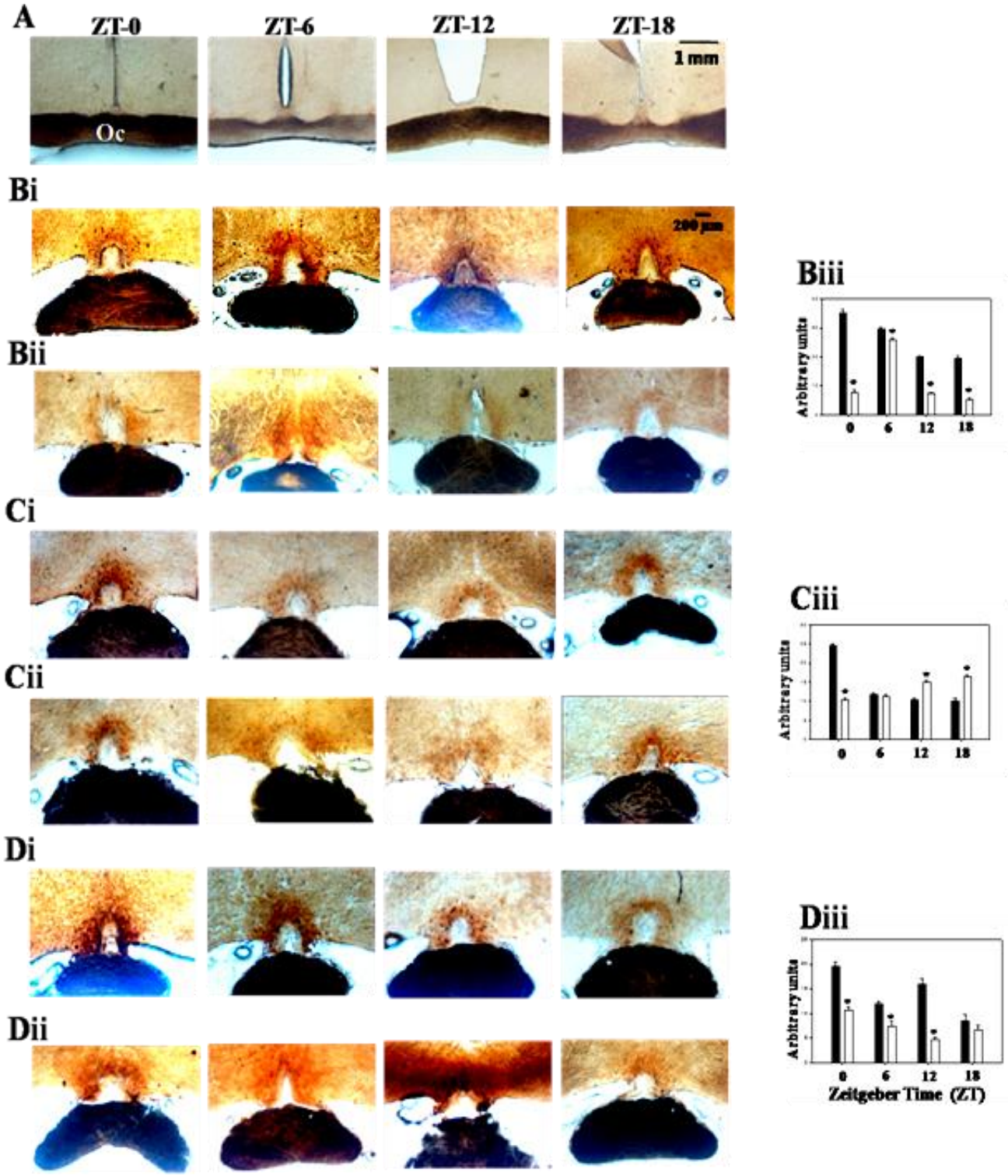


Fig. 32. A. Leptin-ir in the coronal brain sections (25 µm) of 3 m old male wistar rats SCN , no leptin-ir was observed in any of these time points studied in the SCN region. Bi, Ci, Di. Leptin-ir in the MPOA region of 3, 12 and 24 m old control rats. Bii, Cii, Dii. Leptin-ir in the MPOA region of 3, 12 and 24 m old R.F. rats sacrificed at various zeitgeber times (ZT) 0, 6, 12 and 18. Biii, Ciii, Diii. Histograms of densitometric analysis of the Leptin-ir between control R.F. rats in corresponding age group. Each value is mean ± S.E (n=4). * indicates significant ($p \leq 0.05$) difference between control and food restricted rats. Scale bar=1mm for panel A and 200 µm for panel B, C, D.

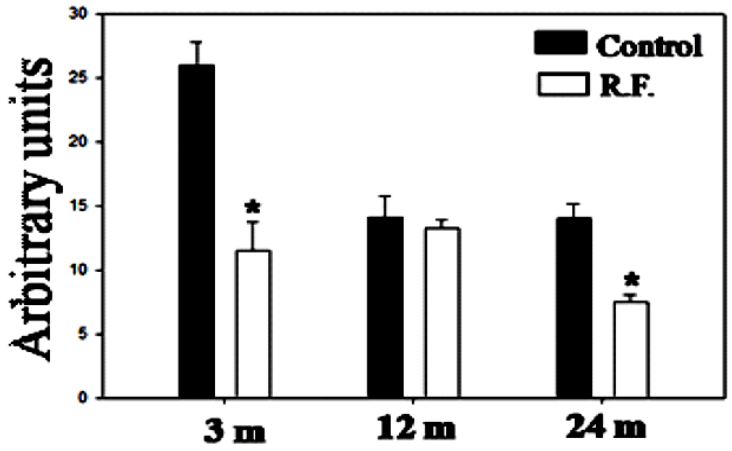


Fig. 33. Mean 24 h levels of leptin-ir in the MPOA region of 3, 12 and 24 m rats. Each value is mean ± S.E. (n=16). * indicates significant difference between control and food restricted animals. ($p \leq 0.05$).

In food restricted animals although the mean 24 h levels were highest in 12 m old rats, there was no significant difference exists between 3 and 12 m old rats. The mean 24 h levels were lowest in 24 m old rats (Fig. 33).

Upon restricted feeding the leptin-ir levels were significantly decreased in both 3 and 24 m old rats at all the time points except at ZT-18 in 24 m old rats ($p \leq 0.05$) (Fig. 32Biii. and Diii). In 12 m old rats leptin-ir levels were significantly decreased at ZT-0 and increased at ZT-12 and ZT-18 ($p \leq 0.05$) (Fig. 32Ciii). Whereas at ZT-6 in 12 m old rats there was no significant change in the leptin-ir levels between controls and food restricted groups.

OBJECTIVE-IV

Daily rhythms of clock gene (*Per1, Per2, Cry1 and Cry2)* expression in SCN

Under food restriction condition the *Per1* expression levels significantly declined in the SCN and Pineal of all the three age groups of rats and the mean 24h levels also decreased in both SCN and pineal of all the three age groups except in 3 m old rats pineal (Fig. 35 and 37).

3 months

In the SCN of 3 m old rats upon food restriction the rhythmicity of all the four genes studied persisted. The phase of *Per2* and *Cry2* genes under food restriction didn't alter under R.F in this age group and the relative expression levels were increased at ZT-12 for both the genes to $0.6 \times 10^{-2} \pm 0.03 \times 10^{-2}$ and $3 \times 10^{-2} \pm 1 \times 10^{-2}$ from $0.5 \times 10^{-2} \pm 0.05 \times 10^{-2}$, $3 \times 10^{-2} \pm 3 \times 0.10^{-2}$. However the relative *Cry1* gene expression levels were decreased to $0.08 \times 10^{-2} \pm 0.05 \times 10^{-2}$ from $0.3 \times 10^{-2} \pm 0.02 \times 10^{-2}$ at ZT-12 under R.F (Fig. 34).

The mean 24 h of *Per1* gene expression drastically decreased by 100 folds in food restricted rats and no significant change in *Per2*, *Cry1* and *Cry2* was observed upon R.F (Fig. 35.Ai). The max:min ratio of *Per 2* was increased from 3.5 ± 0.63 to 85.4 ± 11.86 but, no significant change in the *Per 1*, *Cry 1* and *Cry 2* was observed under R.F (Fig. 35.Aii).

12 months

In 12 m old rats SCN, food restriction caused a significant decrease in the relative expression levels of *Per2, Cry1* and *Cry2* and also all these three genes were in the same phase of expression (Fig. 34). The mean 24h levels of *Per1, Per2, Cry1* and *Cry2* genes were significantly decreased by 142, 25, 16.6 and 8.3 folds respectively under R.F (Fig. 35.Bi) whereas the daily pulse ratios of *Cry1* and *Cry2* increased upon food restriction (478.27 ± 93.7 and 1066.59 ± 181.9) when compared to their respective controls (9.03 ± 0.38 and 10.89 ± 0.63) (Fig. 35.Bii).

24 months

In the SCN of 24 m old rats, at ZT-0, 6 and 18 the relative expression levels of *Per2, Cry1* and *Cry2* were increased and at ZT-12 the levels were decreased when compared to controls. And also these three genes were in the same phase under R.F. (Fig. 34).

The mean 24h levels of *Per1* was decreased drastically whereas, *Per2* levels were increased by 2.3 folds upon R.F. in 24 m old rats (Fig. 33.Ci). The daily pulse ratios of *Per1* and *Cry2* were decreased from 12.51 ± 0.73, 11748.81 ± 2026.78 to 3.374 ± 1.22, 57.93 ± 52.86 under R.F whereas, no significant change was observed in the case of *Per2* and *Cry1* (Fig. 35.Cii).

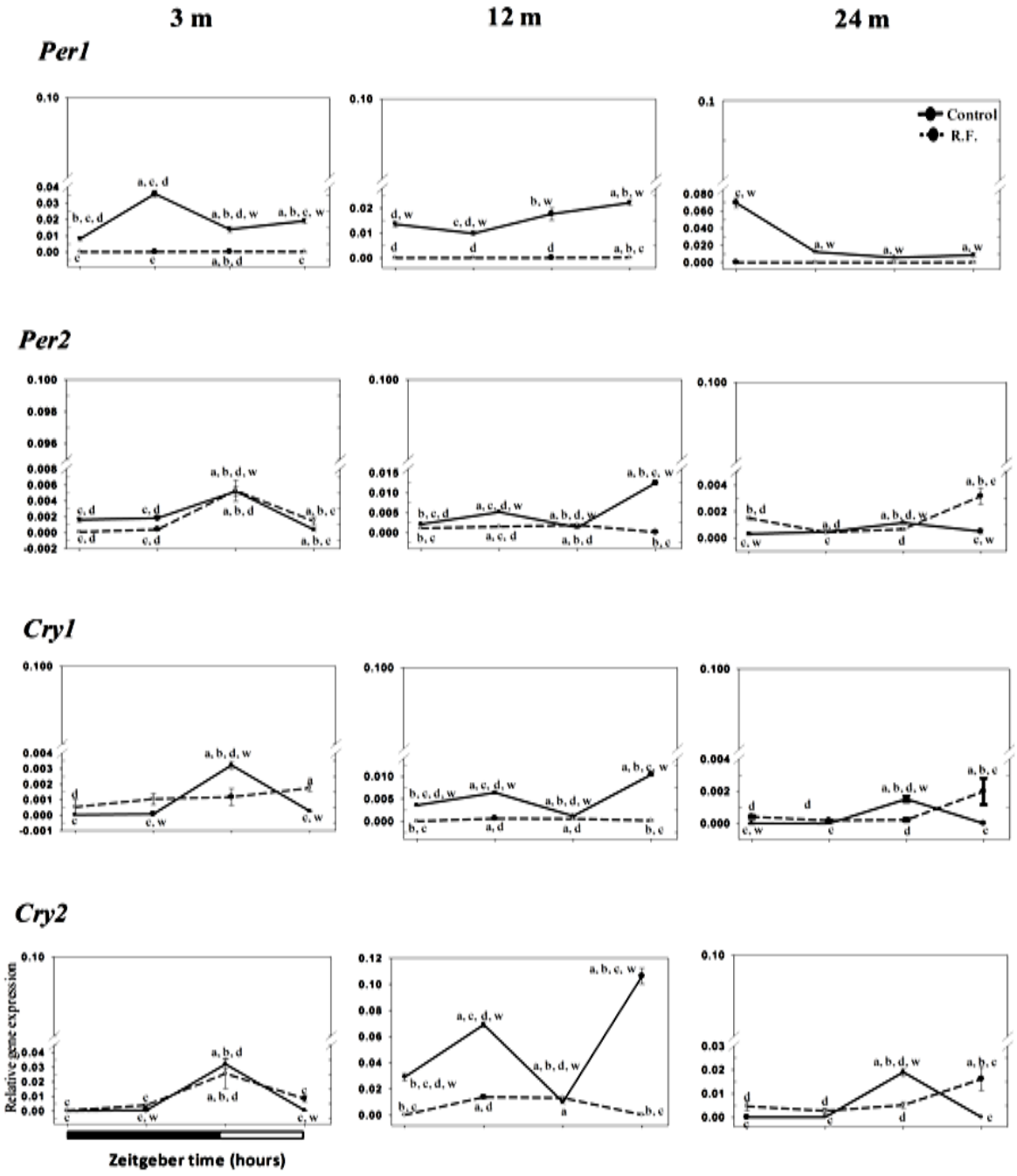


Fig. 34. Comparison of daily expression rhythms of *Per1*, *Per2*, *Cry1* and *Cry2* in the SCN region in control and food restricted condition upon aging. A. 3 months B. 12 months C. 24 months. Each value is mean ± S.E (n=4). ZT-Zeitgeber time. $p \leq 0.05$ (where a, b, c and d refers to comparison between ZT-0, 6,12 and 18 respectively; w, refers to significant difference in the levels of the compound at same time point between control and R.F. group in same age group).

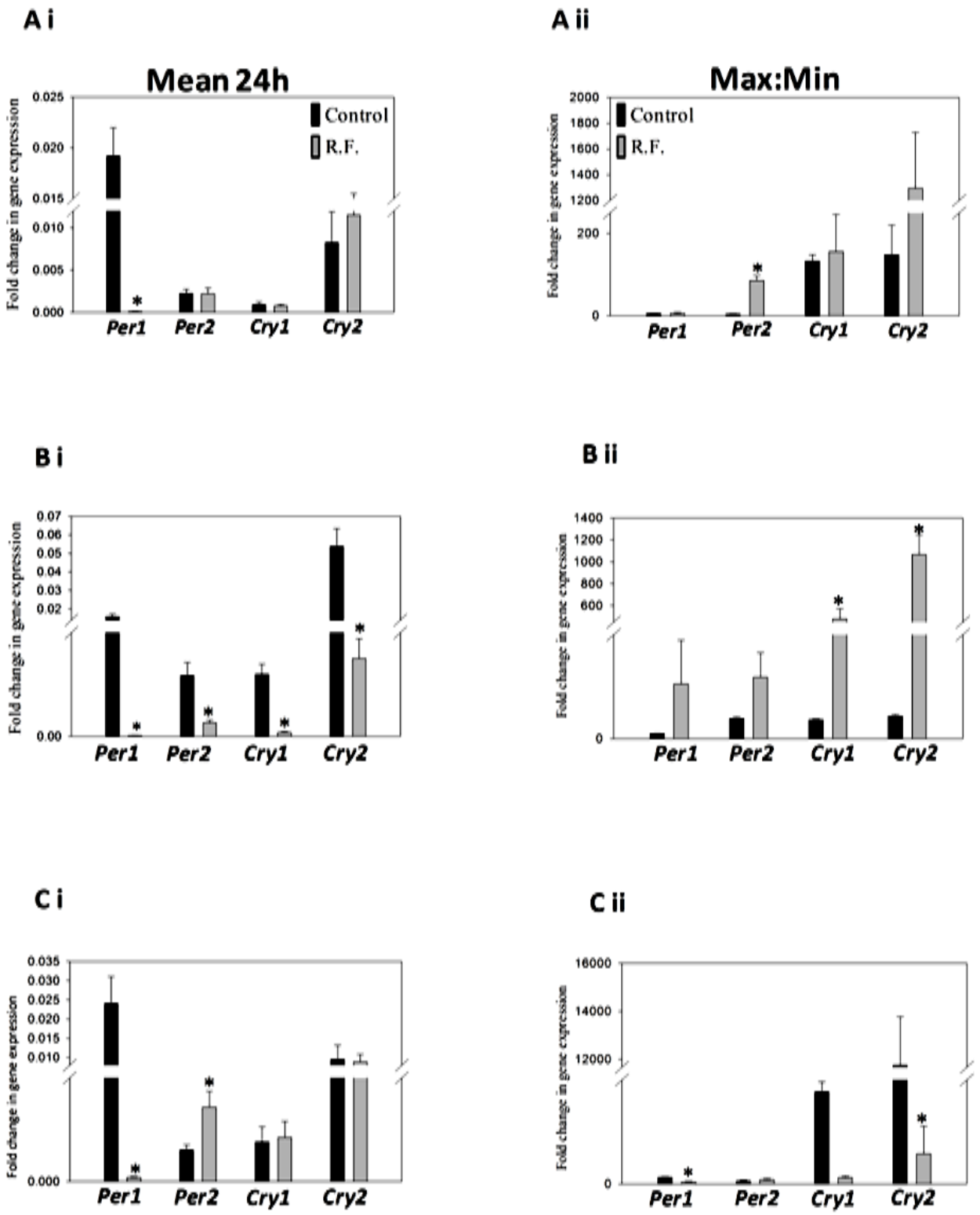


Fig. 35. Effect of restricted feeding on the mean 24 h levels (Ai, Bi and Ci) and Max: Min ratios (Aii, Bii and Cii) on *Per1*, *Per2*, *Cry1* and *Cry2* gene expression levels in the SCN region of control and food restricted animals upon aging. * indicate significant difference between control and food restricted animals ($p \leq 0.05$) determined by one way-ANOVA. ZT-Zeitgeber time.

Daily rhythms of clock gene (*Per1, Per2, Cry1 and Cry2)* expression in Pineal

3 months

In the pineal of 3 m old rats upon food restriction, the rhythmicity of *Per1*, *Per2*, *Cry1* and *Cry2* was abolished (Fig.36). But no significant change was observed in the mean 24 h levels between the control and food restricted groups of all the four genes studied (Fig. 37.Ai). The daily pulse of *Cry2* was significantly decreased from 2362.1 ± 593.2 to 11.15 ± 3.17 upon R.F. in the pineal of 3 m old rats, but no significant difference was observed in the daily pulses of *Per1*, *Per2* and *Cry1* between control and food restricted groups (Fig. 37.Aii).

12 months

In the pineal of 12 m old rats the rhythmicity persisted for all these four genes and the relative expression levels of *Per2*, *Cry1*and *Cry2* were increased at ZT-0, 12 and 18 and decreased at ZT-6 (Fig. 36). *Per2*, *Cry1* and *Cry2* genes were in the same phase under food restriction in this group also (Fig.36). The mean 24 h levels of *Per1* decreased by 144 folds and *Per2*, *Cry2* were increased by 7.5 and 3 folds respectively upon food restriction. Whereas no significant change in the max:min ratio was observed for all the genes between control and food restricted groups in 12 m old rats (Fig. 37.Bi and Bii).

24 months

In 24 m old rats the rhythmicity of all these genes studied was persisted and also the expression levels were increased for *Per2*, *Cry1* and *Cry2* under food restriction at all the four time points studied and they were in same phase in the pineal of 24 m old rats (Fig. 36). The mean 24h levels of *Per1* and *Cry1* decreased approximately by 0.01 fold whereas, *Per2* and *Cry2* was increased by 0.3 and 0.02 folds respectively upon food restriction (Fig. 37.Ci). The max:min ratio of *Cry1* was decreased and *Cry2* was increased (Fig. 37.Cii).

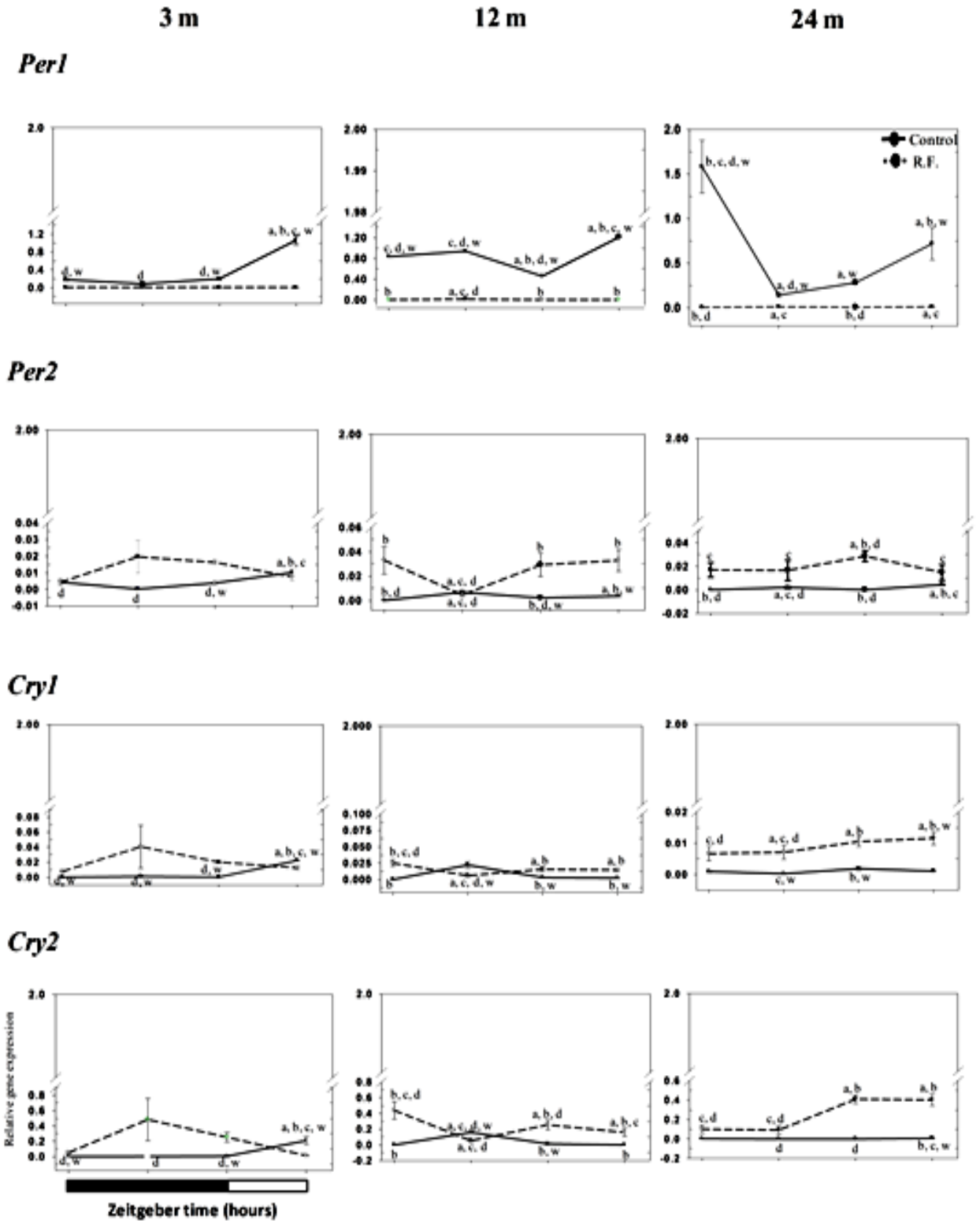


Fig. 36. Comparison of daily expression rhythms of *Per1*, *Per2*, C*ry1* and *Cry2* in the pineal of control and food restricted animals under aging. A. 3 months B. 12 months C. 24 months. Each value is mean ± S.E (n=4). ZT-Zeitgeber time. $p \leq 0.05$ (where a, b, c and d refers to comparison between ZT-0, 6, 12 and 18 respectively; w, refers to significant difference in the levels of the compound at same time point between control and R.F. group in same age group).

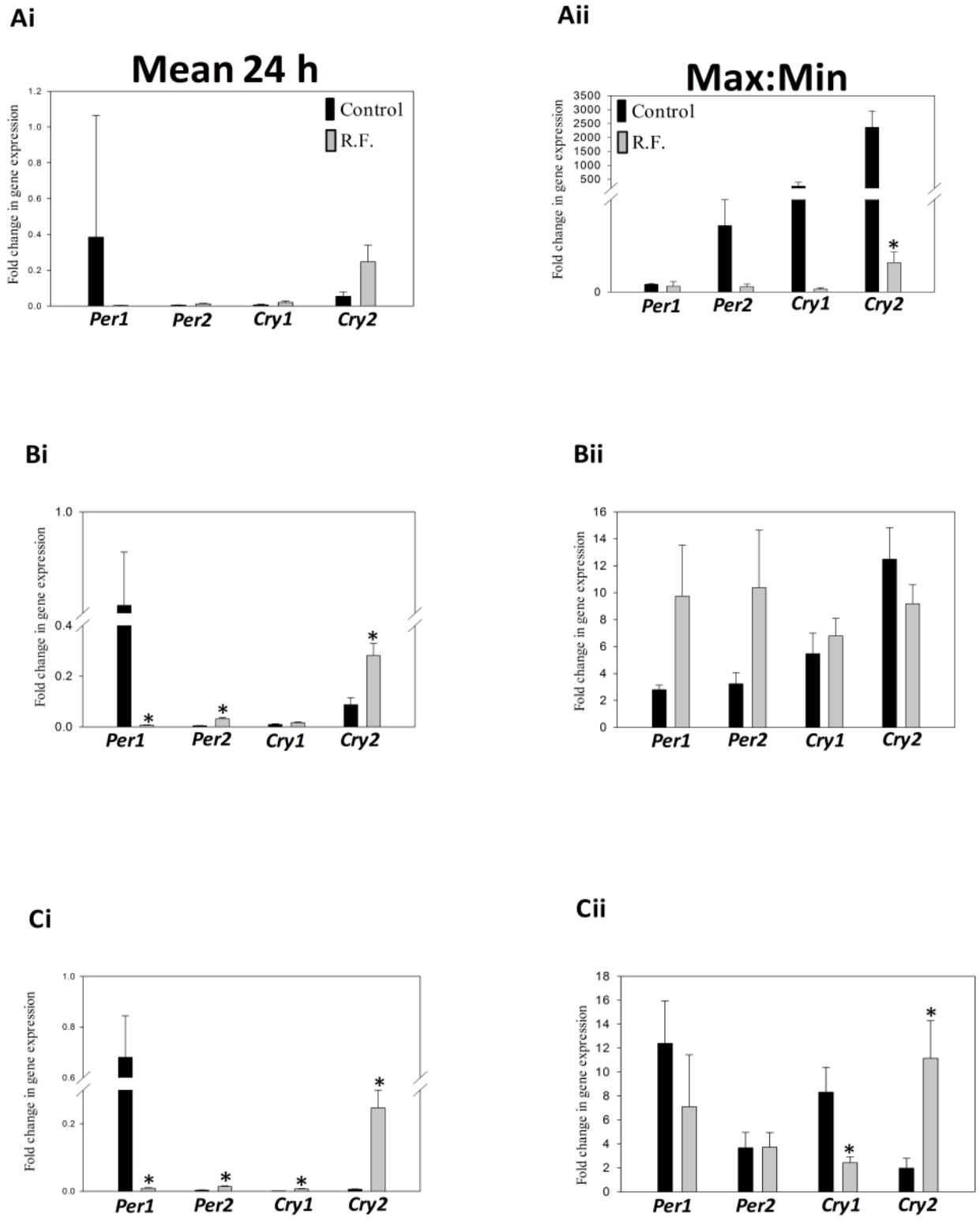


Fig. 37. Effect of restricted feeding on the mean 24 h levels (Ai, Bi and Ci) and Max: Min ratios (Aii, Bii and Cii) on *Per1*, *Per2*, *Cry1* and *Cry2* gene expression levels in the pineal of control and food restricted animals upon aging. * indicate significant difference between control and food restricted animals ($p \leq 0.05$) determined by one way-ANOVA. ZT-Zeitgeber time.

Effect of restricted feeding on pair wise correlation between light-dark phases of *Per1, Per2, Cry1 and Cry2* in various age groups in rat SCN and pineal:

In the light phase a positive correlation was existed between *Per2* and *Cry2* in both SCN and pineal of 3 m old rats and under food restriction and also this positive correlation was present in the dark phase of 12 m old rats SCN and in the light phase of 12 m rats pineal. A positive correlation between these genes was also present in the SCN of 24 m old rats and in the pineal of 3 m old rats at the dark phase (Fig. 38 and 39).

Per 2 and *Cry1* had a positive correlation in the SCN in the light phase of 24 m old rats and in the dark phase of 12 m old rats. These genes also have a positive correlation in 3 and 12 m old rats pineal both light and dark phases (Fig. 38 and 39).

A statistically significant positive correlation was observed between *Per1* and *Per2* at the dark phase of 3 and 12 m old rats SCN and also at the light phase of 3 old rats pineal. But a negative correlation existed in the SCN in the dark phase and in the light phase in the pineal of 12 m old rats (Fig. 38 and 39).

There was a significant positive correlation between *Per1* and *Cry1* at the dark phase in the pineal of 3 m old rats. Also a negative correlation was existed at the light phase in the pineal of 12 m old rats. Between *Per1* and *Cry2* a positive correlation was existed at the light phase in the pineal of 3 m old rats but in 12 m old rats the correlation became negative (Fig. 39).

A positive correlation exists between *Cry1* and *Cry2* in the pineal of restricted fed animals at both light and dark phases in all the three age groups studied except in the dark phase of 24 m old rats pineal. There was also a positive correlation existed between *Cry1* and *Cry 2* in the SCN at the dark phase of 12 and 24 m old rats (Fig. 38 and 39).

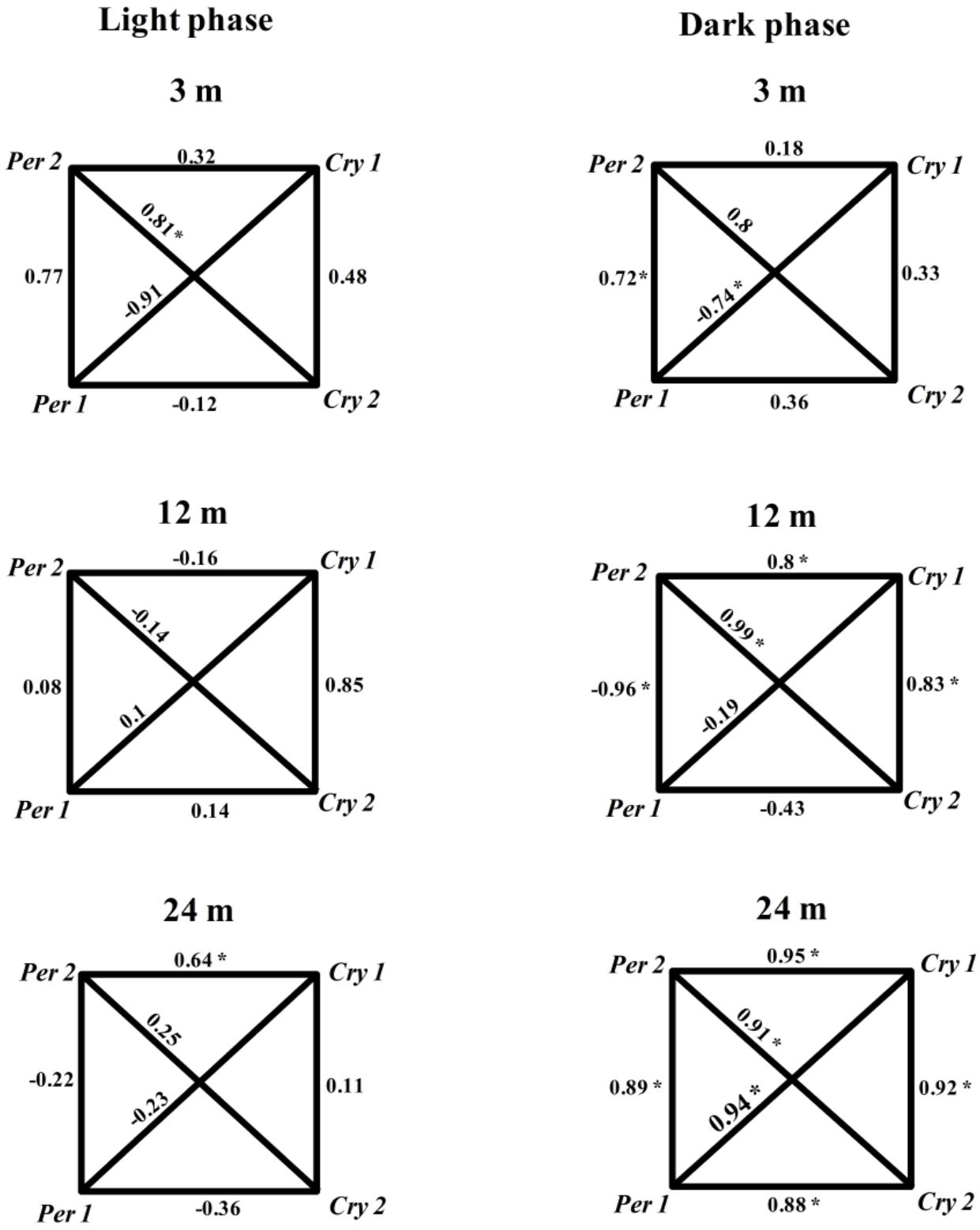


Fig. 38. Effect of restricted feeding on pair wise correlation between mean light (ZT 0, 6,12) and mean dark (ZT 12,18, 24/0) phase values of *Per 1*, *Per 2*, *Cry 1 and Cry 2* in various age groups 3, 12 and 24 months in rat SCN. Each value is a correlation coefficient value between parameters. * indicates statistically significant value between parameters ($p \leq 0.05$). Negative value indicates negative correlation between parameters.

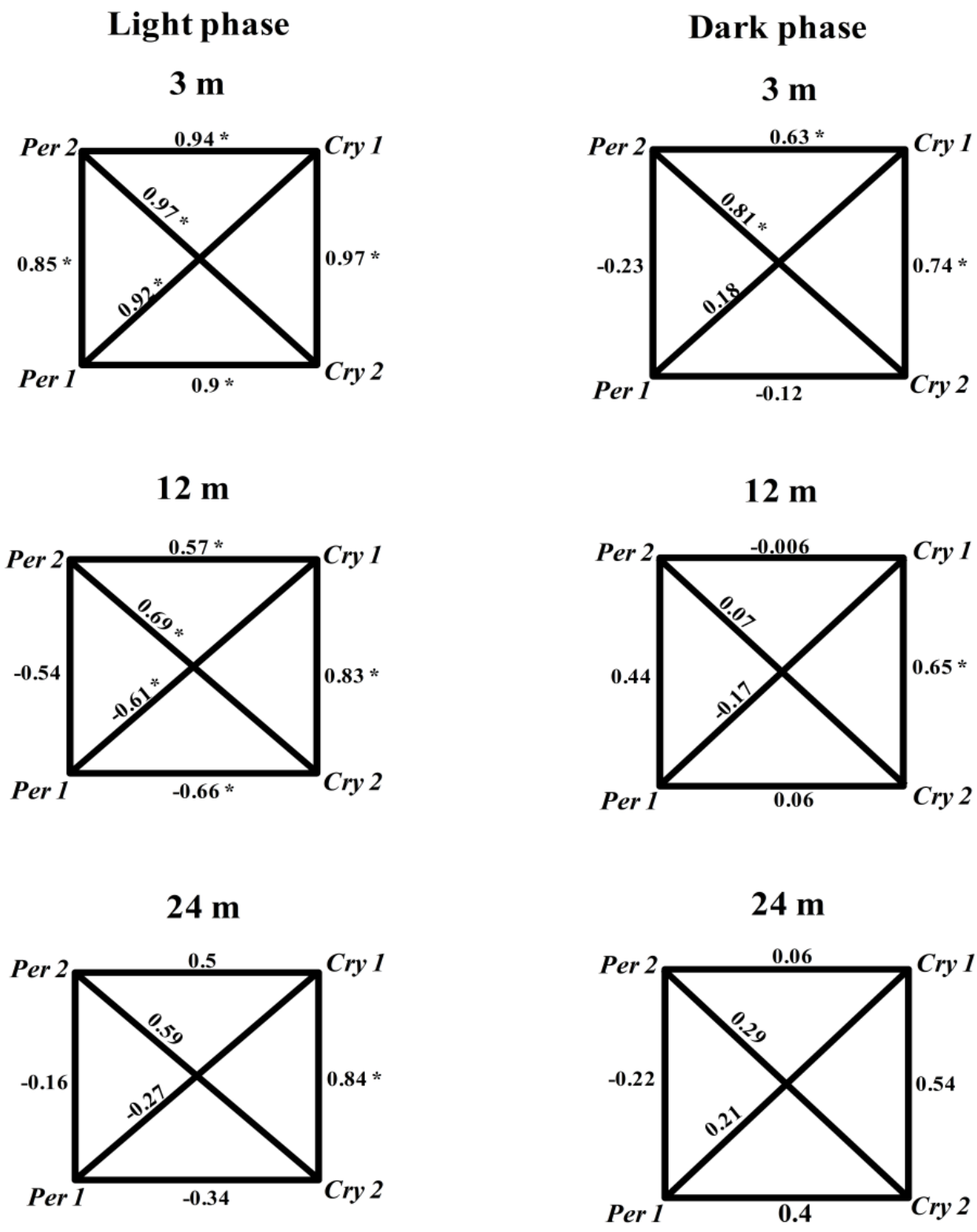


Fig. 39. Effect of restricted feeding on pair wise correlation between mean light (ZT 0, 6,12) and mean dark (ZT 12,18, 24/0) phase values of *Per1*, *Per2*, *Cry1 and Cry2* in various age groups 3, 12 and 24 months in rat pineal. Each value is a correlation coefficient value between parameters. * indicates statistically significant value between parameters ($p \leq 0.05$). Negative value indicates negative correlation between parameters.

OBJECTIVE – V

Daily rhythms of protein profile in SCN of R.F. rats

Under R.F., in the SCN of 3 m old rats a total of 489 and 240 spots were identified at ZT-6 and ZT-18 respectively. The number of protein spots which were showing daily rhythm and a differential pattern of expression between these two time points were 33 ($p \leq 0.05$; Fig. 40). In the SCN of 12 m old rats 519 protein spots were identified at ZT-6 and 306 spots were identified at ZT-18. The total number of protein spots that were differentially expressed and showing daily rhythm between ZT-6 and ZT-18 in the SCN of 12 m old rats were 24 ($p \leq 0.05$; Fig. 40). In 24 m old rats under R.F. regimen, 357 spots were identified at ZT-6 and 231 spots were identified at ZT-18. The differentially expressed protein spots with a daily rhythm between ZT-6 and ZT-18 in this age group was 38 ($p \leq 0.05$; Fig. 40, 46. A).

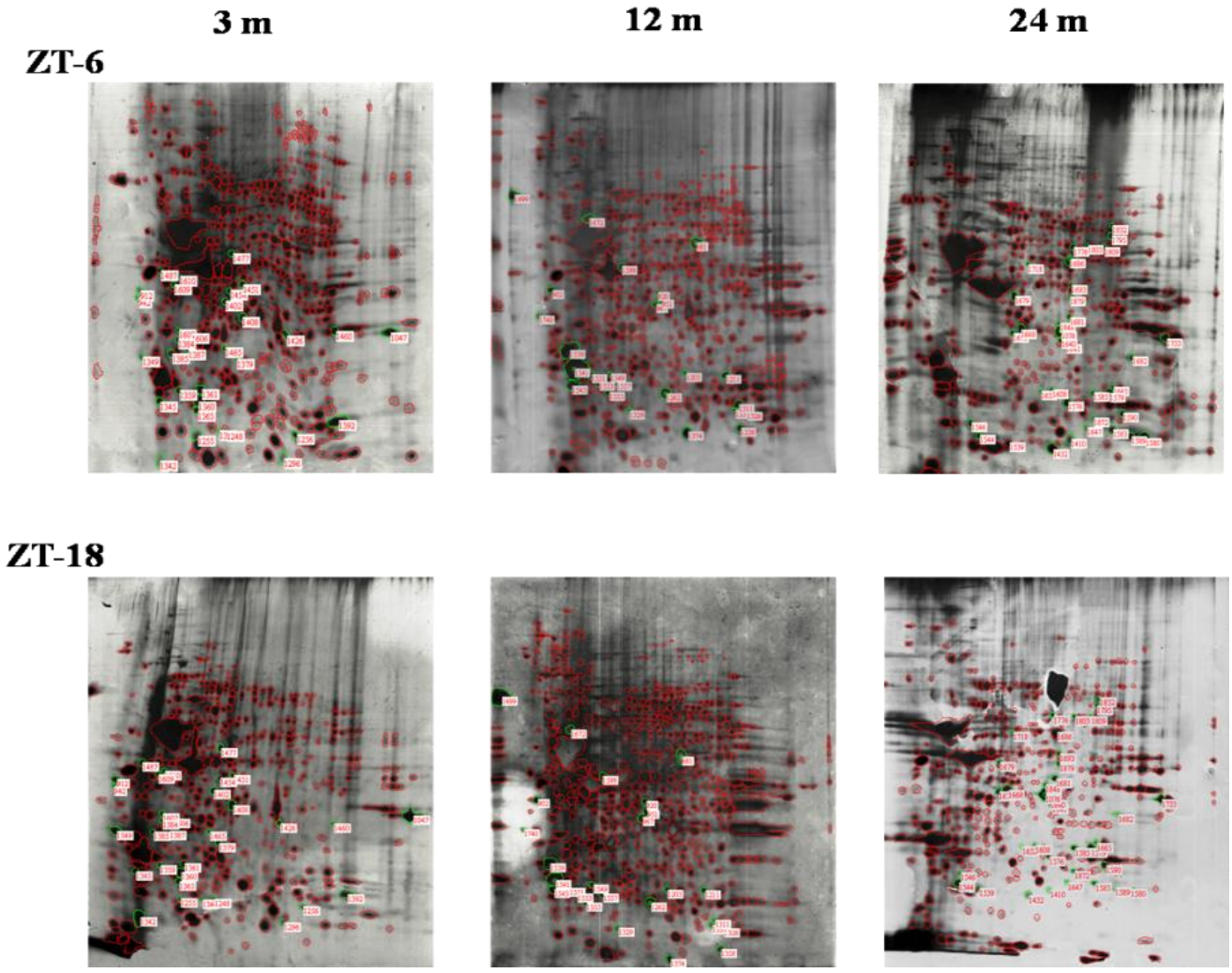


Fig. 40. Daily rhythms of protein profiles in (i) 3, (ii) 12 and (iii) 24 m SCN gels. Protein spots which were differentially expressed between ZT6 and ZT18 of SCN are highlighted in green.

In a comparison of ZT-6 time point of 3, 12 and 24 m old age group gels of SCN, a total of 33 spots were differentially expressed showing daily rhythmicity whereas at ZT-18 17 spots were differentially expressed showing daily rhythmicity between these three age groups ($p \leq 0.05$; Fig. 41, 46 B). When all the gels of SCN at ZT-6 and ZT-18 time points of 3, 12 and 24 m old were compared, 32 protein spots were differentially expressed between these two time points within a day (Fig. 42; $p \leq 0.05$).

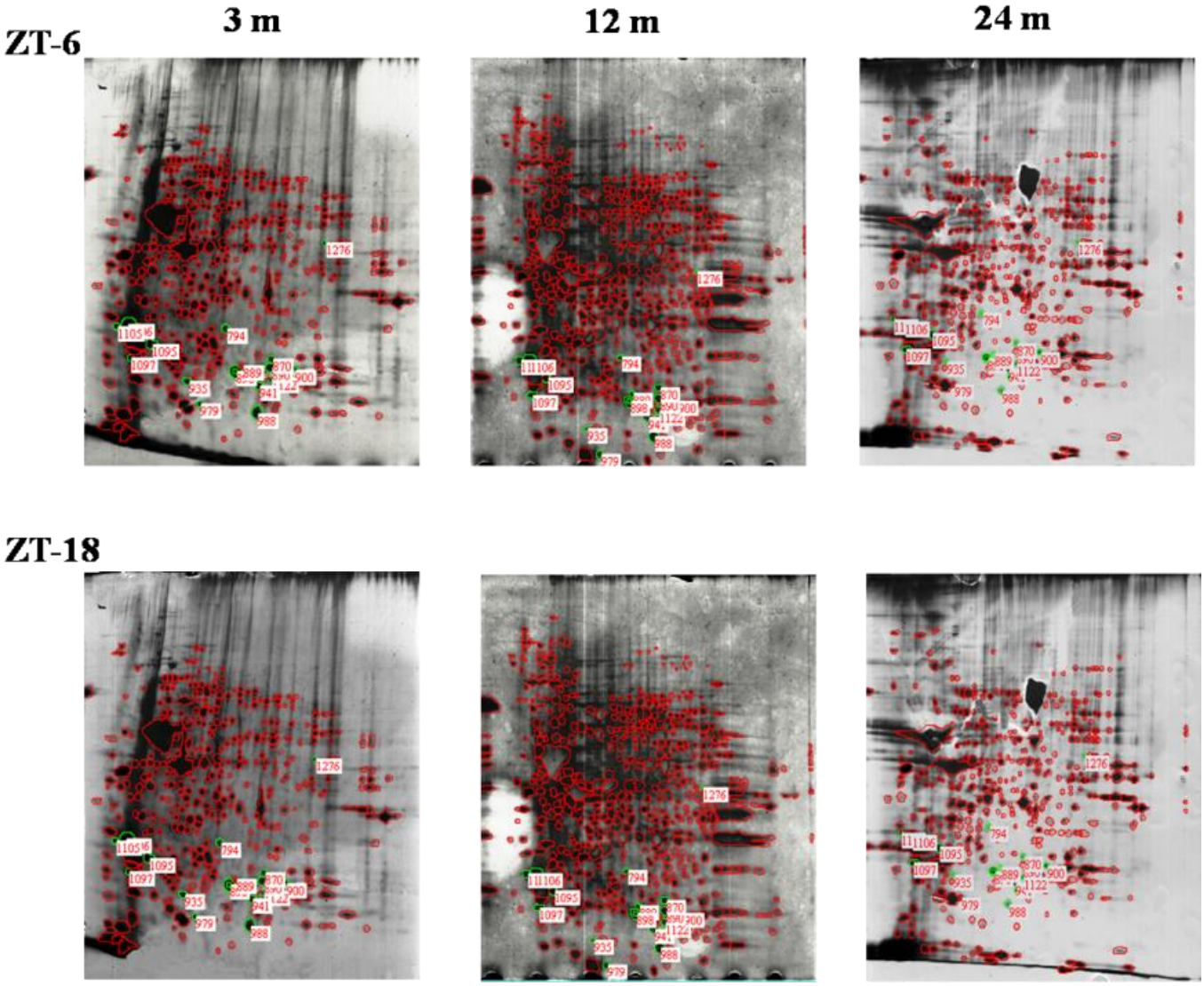


Fig. 41. Protein spots which were differentially expressed between 3, 12 and 24 m SCN at ZT6 and ZT18 are highlighted in green.

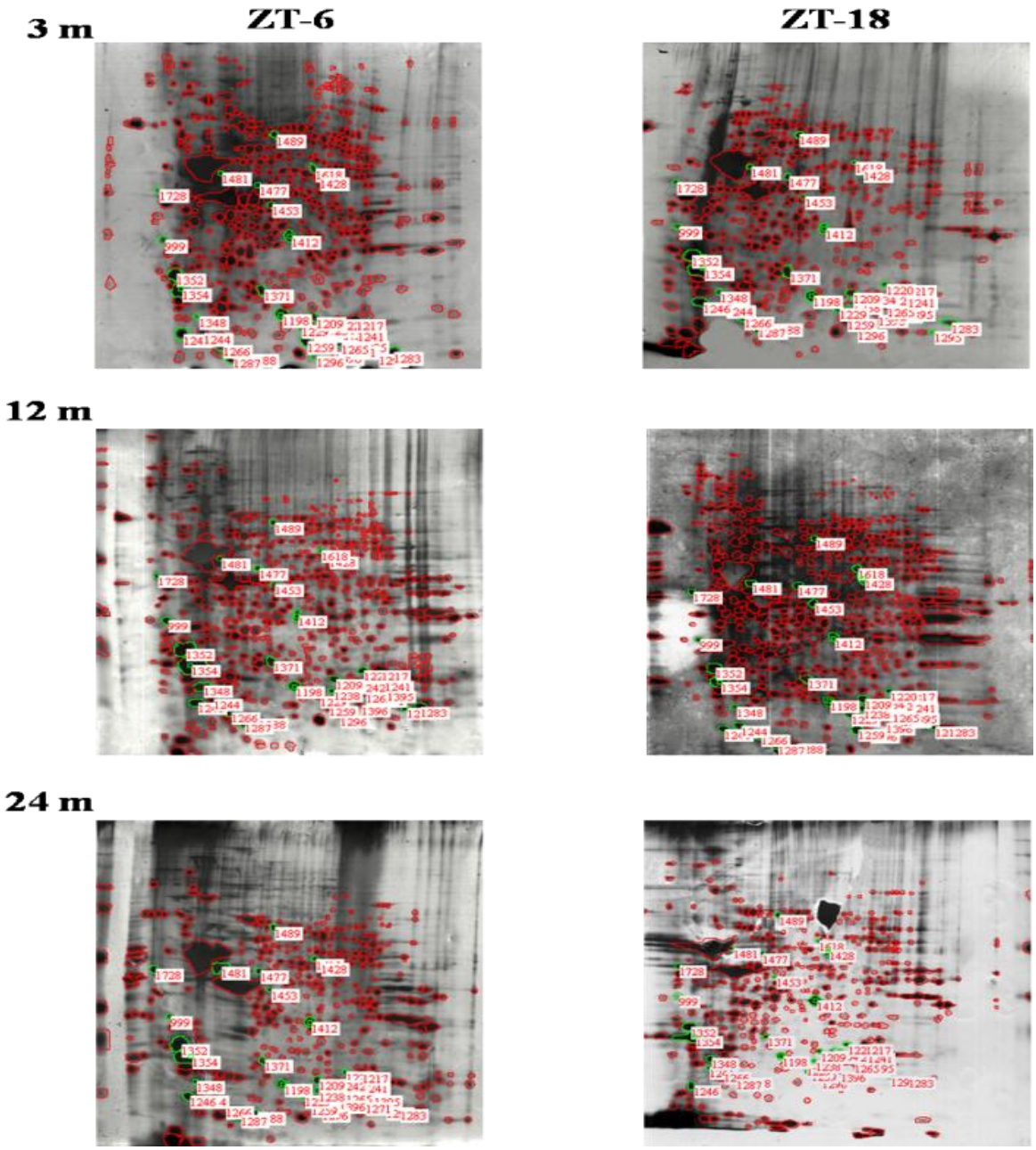


Fig. 42. Protein spots which were differentially expressed daily in 3, 12, and 24 m old rat SCN were highlighted.

The daily pulse (maximum:minimum ratio) between ZT-6 and 18 time points was calculated in 3, 12 and 24 m old rats, by taking a cut off value of 3 the values greater than 3 were considered to express the daily pulse. The daily pulse between ZT-6 and 18 time points in the SCN of 3, 12 and 24 m old rats was 8, 7 and 14 respectively (Table 14, 15 and 16.).

Table 14. Daily rhythms of Protein Profiles in 3 month SCN

S.No.	Match ID	ZT-6 of 3 m SCN	ZT-18 of 3 m SCN	Max:Min ratio	Mean 24 h
1	912	0.19	0.47	2.38	0.33 ± 0.14
2	942	0.1	0.05	2.1	0.07 ± 0.02
3	1047	1.08	2.31	2.13	1.69 ± 0.61
4	1248	0.24	0.1	2.45	0.17 ± 0.07
5	1255	0.44	0.06	7.07	0.25 ± 0.19
6	1256	0.33	0.13	2.41	0.23 ± 0.1
7	1296	0.3	0.1	2.94	0.2 ± 0.1
8	1342	0.38	0.91	2.39	0.64 ± 0.26
9	1344	0.02	0.07	2.91	0.04 ± 0.02
10	1345	0.49	0.18	2.63	0.33 ± 0.15
11	1349	0.09	0.66	6.91	0.37 ± 0.28
12	1359	0.17	0.06	2.61	0.11 ± 0.05
13	1360	0.51	0.17	2.87	0.34 ± 0.17
14	1361	0.51	0.17	2.87	0.34 ± 0.17
15	1365	0.45	0.19	2.35	0.32 ± 0.13
16	1379	0.03	0.07	2.08	0.05 ± 0.02
17	1384	0.06	0.02	3.08	0.04 ± 0.02
18	1385	0.18	0.05	3.28	0.11 ± 0.06
19	1387	0.07	0.03	2.17	0.05 ± 0.02
20	1392	1.91	0.73	2.6	1.32 ± 0.59
21	1402	0.11	0.28	2.52	0.19 ± 0.08
22	1408	0.11	0.24	2.12	0.17 ± 0.06
23	1426	0.37	0.15	2.45	0.26 ± 0.11
24	1451	0.19	0.09	2.01	0.14 ± 0.05
25	1454	0.07	0.15	2.16	0.11 ± 0.04
26	1455	0.3	0.06	4.47	0.18 ± 0.12
27	1465	0.13	0.06	2.15	0.09 ± 0.03
28	1477	0.07	0.21	3.12	0.14 ± 0.07
29	1487	0.08	0.21	2.44	0.14 ± 0.06
30	1606	0.01	0.04	3.43	0.02 ± 0.01
31	1607	0.02	0.07	3.55	0.04 ± 0.02
32	1609	0.03	0.08	2.68	0.05 ± 0.02
33	1610	0.01	0.05	2.8	0.03 ± 0.02

Each value in column 2 refers to particular protein spot ID given by the software, column 3 and 4 refers to mean percent volume of particular spot in ZT6 and ZT18, column 5 refers daily pulse (max/min ratio) and column 6 refers mean 24 h levels.

Table 15. Daily rhythms of Protein Profiles in 12 month SCN

S.No.	Match ID	ZT-6 of 12 m SCN	ZT-18 of 12 m SCN	Max:Min ratio	Mean 24 h
1	902	0.27	0.7	2.52	0.48 ± 0.21
2	920	0.03	0.16	5.27	0.09 ± 0.06
3	951	0.1	0.25	2.37	0.17 ± 0.07
4	967	0.13	0.29	2.24	0.21 ± 0.08
5	1203	0.07	0.14	2.07	0.1 ± 0.03
6	1211	0.37	0.17	2.18	0.27 ± 0.1
7	1262	0.78	0.21	3.73	0.49 ± 0.28
8	1311	0.44	0.12	3.52	0.28 ± 0.16
9	1326	0.32	0.13	2.42	0.22 ± 0.09
10	1329	0.08	0.24	3.04	0.16 ± 0.08
11	1331	0.07	0.19	2.58	0.13 ± 0.06
12	1358	0.47	0.22	2.06	0.34 ± 0.12
13	1374	1.39	0.24	5.62	0.81 ± 0.57
14	1499	1.99	4.89	2.45	3.44 ± 1.45
15	1539	1.59	0.56	2.81	1.07 ± 0.51
16	1541	4.04	0.61	6.61	2.32 ± 1.71
17	1543	0.92	0.42	2.15	0.67 ± 0.25
18	1549	0.06	0.15	2.34	0.1 ± 0.04
19	1551	0.21	0.07	2.97	0.14 ± 0.07
20	1553	0.37	0.14	2.64	0.25 ± 0.11
21	1555	0.15	0.33	2.12	0.24 ± 0.09
22	1557	0.11	0.53	4.66	0.32 ± 0.21
23	1598	0.13	0.3	2.35	0.21 ± 0.08
24	1672	0.13	0.06	2.09	0.09 ± 0.03
25	902	0.27	0.7	2.52	0.48 ± 0.21
26	920	0.03	0.16	5.27	0.09 ± 0.06
27	951	0.1	0.25	2.37	0.17 ± 0.07
28	967	0.13	0.29	2.24	0.21 ± 0.08
29	1203	0.07	0.14	2.07	0.1 ± 0.03
30	1211	0.37	0.17	2.18	0.27 ± 0.1
31	1262	0.78	0.21	3.73	0.49 ± 0.28
32	1311	0.44	0.12	3.52	0.28 ± 0.16
33	1326	0.32	0.13	2.42	0.22 ± 0.09
35	1329	0.08	0.24	3.04	0.16 ± 0.08
36	1331	0.07	0.19	2.58	0.13 ± 0.06
37	1358	0.47	0.22	2.06	0.34 ± 0.12
38	1374	1.39	0.24	5.62	0.81 ± 0.57
39	1499	1.99	4.89	2.45	3.44 ± 1.45
40	1539	1.59	0.56	2.81	1.07 ± 0.51
41	1541	4.04	0.61	6.61	2.32 ± 1.71
42	1543	0.92	0.42	2.15	0.67 ± 0.25
43	1549	0.06	0.15	2.34	0.1 ± 0.04
44	1551	0.21	0.07	2.97	0.14 ± 0.07
45	1553	0.37	0.14	2.64	0.25 ± 0.11
46	1555	0.15	0.33	2.12	0.24 ± 0.09
47	1557	0.11	0.53	4.66	0.32 ± 0.21
48	1598	0.13	0.3	2.35	0.21 ± 0.08
49	1672	0.13	0.06	2.09	0.09 ± 0.03

Each value in column 2 refers to particular protein spot ID given by the software, column 3 and 4 refers to mean percent volume of particular spot in ZT6 and ZT18, column 5 refers daily pulse (max/min ratio) and column 6 refers mean 24 h levels.

Table 16. Daily rhythms of Protein Profiles in 24 month SCN

S.No.	Match ID	ZT-6 of 24 m SCN	ZT-18 of 24 m SCN	Max:Min ratio	Mean 24 h
1	1410	0.3	0.04	6.91	0.17 ± 0.13
2	1432	0.81	0.16	4.9	0.48 ± 0.32
3	1539	0.16	0.07	2.26	0.11 ± 0.04
4	1544	0.13	0.48	3.51	0.3 ± 0.17
5	1546	0.13	0.05	2.36	0.09 ± 0.04
6	1576	0.31	0.14	2.18	0.22 ± 0.08
7	1579	0.26	0.62	2.34	0.44 ± 0.18
8	1580	1.05	0.07	14.73	0.56 ± 0.49
9	1583	0.37	0.14	2.61	0.25 ± 0.11
10	1585	0.1	0.21	2.00	0.15 ± 0.05
11	1586	0.11	0.31	2.75	0.21 ± 0.1
12	1589	0.11	0.04	2.42	0.07 ± 0.03
13	1590	0.15	0.46	3.1	0.3 ± 0.15
14	1608	0.11	0.04	2.48	0.07 ± 0.03
15	1640	0.11	0.23	2.06	0.17 ± 0.06
16	1641	0.05	0.15	2.65	0.1 ± 0.05
17	1647	0.09	0.04	2.12	0.06 ± 0.02
18	1652	0.04	0.01	2.48	0.02 ± 0.01
19	1665	0.03	0.1	3.00	0.06 ± 0.03
20	1668	0.2	0.81	4.07	0.5 ± 0.3
21	1673	0.1	0.29	2.81	0.19 ± 0.09
22	1679	0.23	0.1	2.26	0.16 ± 0.06
23	1681	0.12	0.27	2.19	0.19 ± 0.07
24	1682	0.17	0.07	2.28	0.12 ± 0.05
25	1686	0.33	1.15	3.47	0.74 ± 0.41
26	1693	0.06	0.15	2.56	0.1 ± 0.04
27	1718	0.08	0.03	2.14	0.05 ± 0.02
28	1733	0.25	0.62	2.4	0.43 ± 0.18
29	1776	0.11	0.24	2.06	0.17 ± 0.06
30	1795	0.04	0.15	3.77	0.09 ± 0.05
31	1803	0.02	0.13	5.22	0.07 ± 0.05
32	1809	0.05	0.13	2.59	0.09 ± 0.04
33	1810	0.05	0.25	4.21	0.15 ± 0.1
35	1832	0.02	0.13	4.92	0.07 ± 0.05
36	1848	0.04	0.14	3.03	0.09 ± 0.05
37	1872	0.03	0.09	2.95	0.06 ± 0.03
38	1879	0.02	0.17	5.94	0.09 ± 0.07

Each value in column 2 refers to particular protein spot ID given by the software, column 3 and 4 refers to mean percent volume of particular spot in ZT6 and ZT18, column 5 refers daily pulse (max/min ratio) and column 6 refers mean 24 h levels.

The protein spots that were up regulated between ZT-6 and ZT-18 time points in the SCN of 3, 12 and 24 m old rats were 89, 104 and 86 respectively. The protein spots that were down regulated between these two time points were 90 in 3 m, 103 in 12 m and 86 in 24 m old rat pineal.

Daily rhythms of protein profile in Pineal of R.F. rats

In the pineal of 3 m old rats under R.F. regimen, a total of 605 and 560 spots were identified at ZT-6 and ZT-18 respectively. 14 spots have shown daily rhythm with a differential pattern of expression when the gels of ZT-6 and ZT-18 time points were compared (Fig. 43). In the pineal of 12 m old rats, 343 spots were identified at ZT-6 and 477 spots were identified at ZT-18. 5 spots have shown differential pattern of expression between ZT-6 and ZT-18 time points in 12 m old rats (Fig. 43). In 24 m old rats, a total of 337 spots were identified at both ZT-6 and 18. 42 spots were shown differential pattern of expression with a daily rhythm in the pineal of 24 m old rats ($p \leq 0.05$*;* Fig 43, 46. A).

The gels of ZT-6 and18 were compared for the commonly but differentially expressed spots in the pineal across the age groups, the number of differentially expressed spots were 3 at both ZT-6 and18 time points ($p \leq 0.05$; Fig 44). When all the gels of pineal at ZT-6 and ZT-18 time points of 3, 12 and 24 m old were compared, 2 protein spots were differentially expressed between these two time points within a day ($p \leq 0.05$; Fig. 45, 46. B;).

The number of protein spots that have shown daily pulse (maximum: minimum ratio) greater than 3 (cut off value was considered as 3) were 11 in 3 m, 2 in 12 m and 18 in 24 m (Table 17, 18 and 19.).

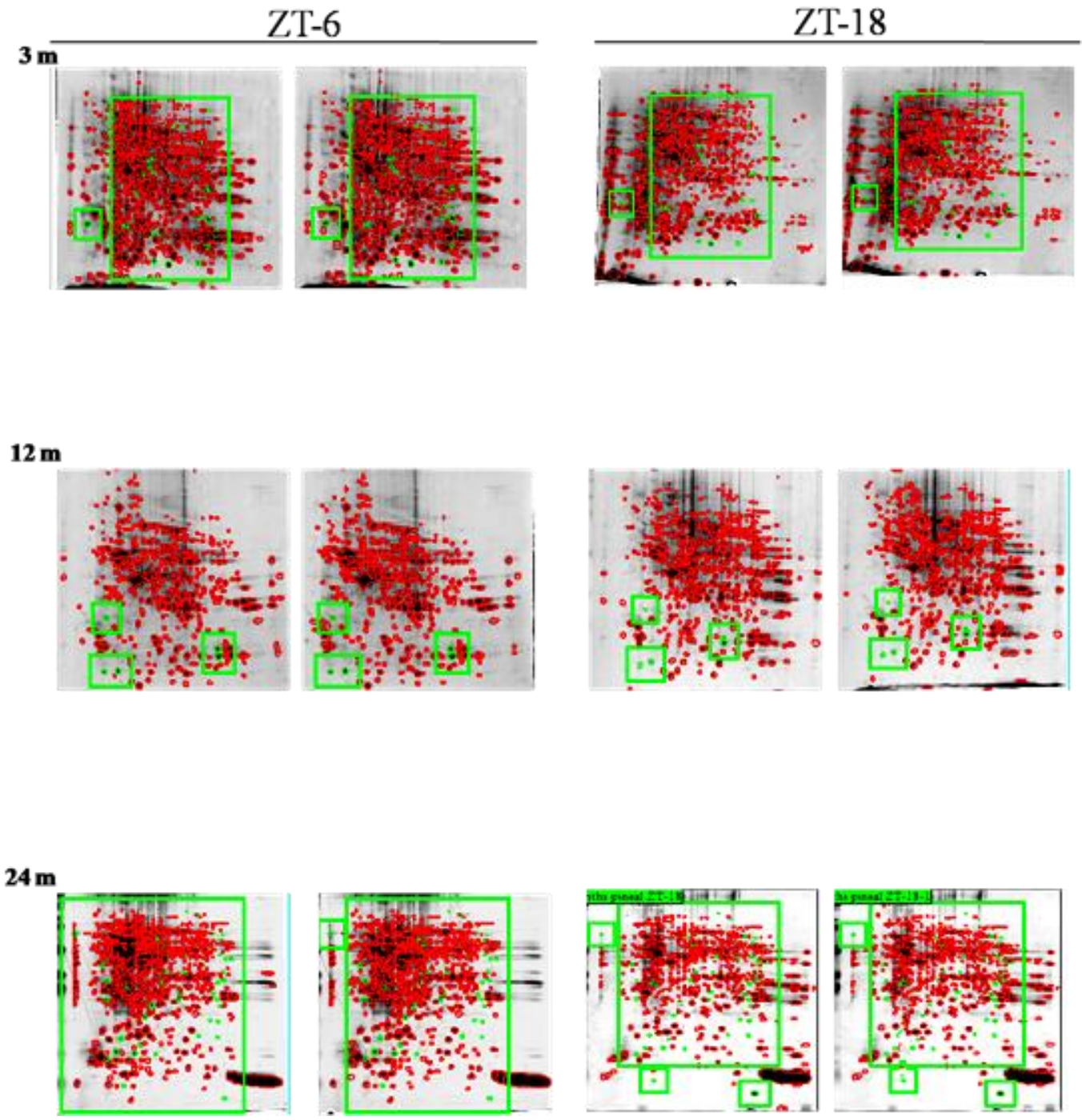


Fig. 43. Daily rhythms of protein profiles in (i) 3, (ii) 12 and (iii) 24 m pineal gels. Protein spots which were differentially expressed between ZT6 and ZT18 of pineal are highlighted in green (n=2).

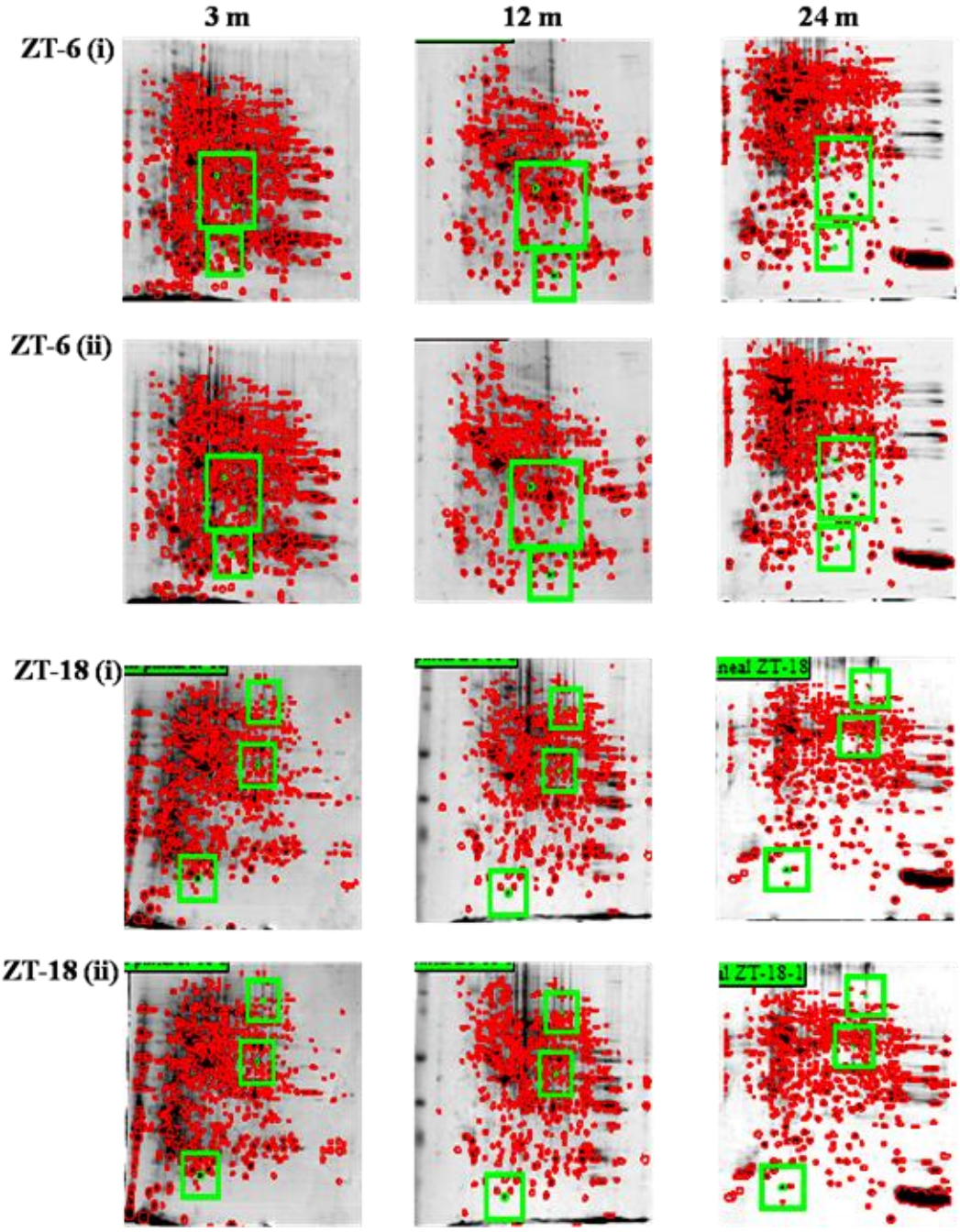


Fig. 44. Protein spots which were differentially expressed between 3, 12 and 24 m pineal at ZT6 and ZT18 were highlighted.

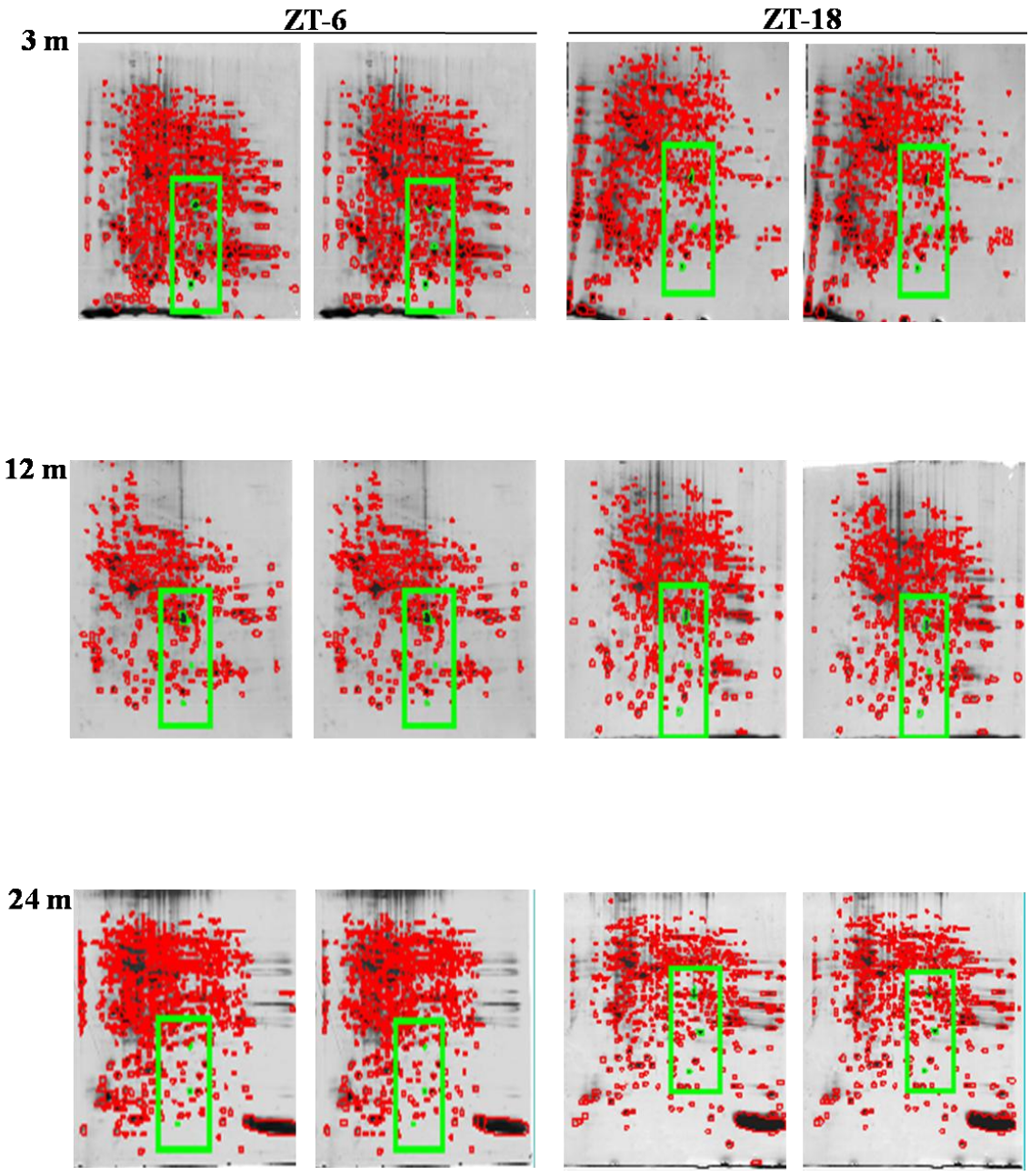


Fig. 45. Protein spots which were differentially expressed daily in 3, 12, and 24 m old rat pineal were highlighted.

Table 17. Daily rhythms of Protein Profiles in the pineal of 3 m old rats

S.No.	Match ID	ZT-6 of 12 m SCN	ZT-18 of 12 m SCN	Max:Min ratio	Mean 24 h
1	26	13.15	0.07	187.85	6.61 ± 6.54
2	28	4.85	0.2	24.25	2.5 2± 2.32
3	30	2.22	0.44	5.04	1.33 ± 0.89
4	33	2.57	0.38	6.76	1.47 ± 1.09
5	43	2.04	0.49	4.16	1.26 ± 0.77
6	44	3.8	0.26	14.61	2.03 ± 1.77
7	46	3.25	0.3	10.8	1.77 ± 1.47
8	61	4.45	0.22	20.22	2.33 ± 2.11
9	81	7.99	0.12	66.58	4.05 ± 3.93
10	83	3.53	0.28	12.6	1.90 ± 1.62
11	86	2.05	0.48	4.27	1.26 ± 0.78
12	100	3.69	0.27	13.66	1.98 ± 1.71
13	103	4.77	0.2	23.85	2.48 ± 2.28
14	107	7.83	0.12	65.25	3.97 ± 3.85
15	110	2.14	0.46	4.65	1.30 ± 0.84
16	136	2.42	0.41	5.9	1.41 ± 1.00
17	155	0.43	2.3	0.18	1.36 ± 0.93
18	158	2.49	0.4	6.22	1.44 ± 1.04
19	166	2.01	0.49	4.1	1.25 ± 0.76
20	177	2.58	0.38	6.78	1.48 ± 1.10
21	183	0.37	2.65	0.13	1.51 ± 1.14
22	208	2.17	0.46	4.71	1.31 ± 0.85
23	229	4.7	0.21	22.38	2.45 ± 2.24
24	231	2.13	0.46	4.63	1.29 ± 0.83
25	239	3.43	0.29	11.82	1.86 ± 1.57

Each value in column 2 refers to particular protein spot ID given by the software, column 3 and 4 refers to mean percent volume of particular spot in ZT6 and ZT18, column 5 refers daily pulse (max / min ratio) and column 6 refers mean 24 h levels.

Table 18. Daily rhythms of Protein Profiles in the pineal of 12 m old rats

S.No.	Match ID	ZT-6 of 12 m SCN	ZT-18 of 12 m SCN	Max:Min ratio	Mean 24 h
1	9	2.03	0.34	5.97	1.18 ± 0.84
2	10	2.91	1.72	1.69	2.31 ± 0.59
3	14	2.06	0.84	2.45	1.45 ± 0.61
4	18	3.23	0.64	5.05	1.93 ± 1.29
5	26	3.37	0.2	16.85	1.78 ± 1.58

Each value in column 2 refers to particular protein spot ID given by the software, column 3 and 4 refers to mean percent volume of particular spot in ZT6 and ZT18, column 5 refers daily pulse (max / min ratio) and column 6 refers mean 24 h levels.

Table 19. Daily rhythms of Protein Profiles in the pineal of 24 m old rats

S.No.	Match ID	ZT-6 of 12 m SCN	ZT-18 of 12 m SCN	Max:Min ratio	Mean 24 h
1	0	2.05	0.48	4.27	1.26 ± 0.78
2	16	0.48	2.07	0.23	1.27 ± 0.79
3	29	0.36	2.76	0.13	1.56 ± 1.20
4	37	0.28	3.47	0.08	1.87 ± 1.59
5	39	2.01	0.49	4.10	1.25 ± 0.76
6	46	0.11	8.45	0.01	4.28 ± 4.17
7	48	3.55	0.28	12.68	1.91 ± 1.63
8	61	5.6	0.17	32.94	2.94 ± 2.66
9	64	2.97	0.33	9.00	1.65 ± 1.32
10	65	2.1	0.47	4.47	1.28 ± 0.81
11	70	4.04	0.24	16.83	2.14 ± 1.90
12	74	4.44	0.22	20.18	2.33 ± 2.11
13	78	2.7	0.36	7.50	1.53 ± 1.17
14	80	0.37	2.63	0.14	1.5 ± 1.13
15	92	0.34	2.91	0.12	1.62 ± 1.28
16	93	0.45	2.21	0.20	1.33 ± 0.88
17	96	2.17	0.45	4.82	1.31 ± 0.86
18	107	0.38	2.6	0.15	1.49 ± 1.11
19	110	15.49	0.06	258.17	7.77 ± 7.71
20	117	3.2	0.31	10.32	1.75 ± 1.44
21	119	2.03	0.49	4.14	1.26 ± 0.77
22	121	0.47	2.1	0.22	1.28 ± 0.81
23	134	0.32	3.03	0.11	1.67 ± 1.35
24	136	2.39	0.41	5.83	1.40 ± 0.99
25	168	0.4	2.46	0.16	1.43 ± 1.03
26	173	0.44	2.24	0.20	1.34 ± 0.90
27	174	9.84	0.1	98.40	4.97 ± 4.87
28	188	3.35	0.29	11.55	2.11 ± 1.86
29	190	0.25	3.98	0.06	2.11 ± 1.86
30	202	2.9	0.34	8.53	1.62 ± 1.28
31	209	0.28	3.51	0.08	1.89 ± 1.61
32	210	0.38	2.62	0.15	1.50 ± 1.12
33	217	0.17	5.61	0.03	2.89 ± 2.72
34	223	0.33	2.95	0.11	1.64 ± 1.31
35	224	0.45	2.2	0.20	1.32 ± 0.87
36	259	0.49	2	0.25	1.24 ± 0.75
37	284	3.44	0.29	11.86	1.86 ± 1.57
38	296	0.41	2.4	0.17	1.40 ± 0.99
39	300	0.35	2.7	0.13	1.52 ± 1.17
40	306	4.66	0.21	22.19	2.43 ± 2.22
41	342	2.71	0.36	7.53	1.53 ± 1.17
42	343	4.1	0.24	17.08	2.17 ± 1.93

Each value in column 2 refers to particular protein spot ID given by the software, column 3 and 4 refers to mean percent volume of particular spot in ZT6 and ZT18, column 5 refers daily pulse (max / min ratio) and column 6 refers mean 24 h levels.

In the pineal, under R.F. 51 spots were up regulated in 3 m, 8 in 12 m, and 20 in 24 m between ZT-6 and ZT-18 time points. 7 spots were down regulated in 3 m, 3 in 12 m and 21 in the pineal of 24 m old rats under R.F.

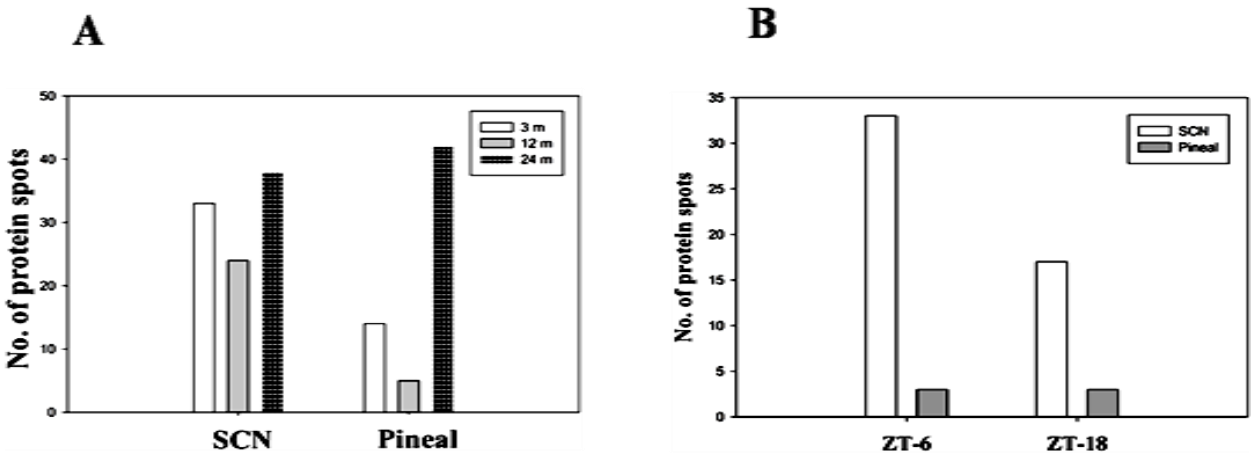


Fig. 46. A. Number of differentially expressed protein spots in the SCN and pineal of 3, 12 and 24 m old rats. The number is high in 24 m old rats in both the tissues. B. Graph showing the number of differentially expressed protein spots in the gels of ZT-6 and 18 time points which were compared across the age groups (3, 12 and 24 m old rats) were plotted.

Discussion

I. Studies of gross locomotor activity rhythms

Under R.F the robustness of night time activity decreased in 3 m old rats and it gradually deteriorated in 12 and 24 m old rats. Altered meal timing made the animal partially diurnal with an increased day time activity and decreased night time activity which is, in agreement with that food deprivation and caloric restriction makes the nocturnal animals partially diurnal (Challet, 2010). When compared to 3 and 24 m old rats the τ and α were increased in 12 m old rats under R.F. A significant increase in the τ in 12 m old rats and a significant decrease in 24 m old rats was observed under R.F. condition when compared to its *ad libitum* fed condition. Whereas the duration of α was increased upon R.F. when compared to *ad libitum* fed condition in both 3 and 12 m old rats. Under R.F. α was highest (18.09 ± 0.45) in 12 m old rats. The increased day time and decreased night time activity in 24 m old rats indicates that food availability can act as zeitgebers for entrainment of locomotor activity.

II. Chronomics of Serotonin metabolism in SCN and Pineal

Mammalian aging is associated with the impairment of the circadian function (Jagota, 2005; Kolker *et al.,* 2004; Garau *et al.,* 2006). In such aged individuals nonvisual zeitgebers like regular meal intake, work and social schedules and awareness of time will play a major role in the entrainment of the clock. Altered meal timings in rats lead to many physiological changes including decreased serotonin levels in different brain regions (Haleem and Haider, 1996).

Here we report that in 3 m old rats under R.F. regimen the rhythmicity of 5-HT, TRP, 5-HTOH were abolished in SCN, with the serotonin levels significantly decreasing at all the time points except at ZT-18. Whereas the daily pulses of NAS, 5-MIAA and NAT were decreased and no significant change in other compounds was observed under R.F. Serum TRP shows a diurnal rhythm with a sharp increase after the dark onset (Chik *et al.,* 1986), but in the SCN of 3 m old controls rats, TRP levels were decreased from ZT-12 to 18 whereas no significant difference was observed in the levels between ZT-12 to 18 in the SCN of R.F. rats. Also no significant difference was observed in the daily pulse as well as in the mean 24 h levels between controls and food restricted animals.

In the SCN of 3 m control group rats, the peak 5-HT levels were observed at ZT-6 and then the levels started decreasing from ZT-6 to 18. In food restricted animals no significant change was observed in the levels throughout the day, unlike the serum 5-HT levels increased after 10-13 h when the food availability restricted to a 3 h period (Ho *et al.,* 1985). Decreased serotonin levels and no significant change in TRP and 5-HIAA levels in the SCN of 3 m old rats are in correlation with the decreased levels of serotonin and

no significant change in TRP and 5-HIAA levels in the hypothalamus of 4 h caloric restricted rat (Haleem and Haider, 1996).

Upon food restriction in 3 m old rats, the NAS levels showed an anticipatory rise before feeding time in SCN which is in agreement with the anticipatory rise of NAS before feeding (Ho *et al.*, 1985). But in SCN, NAS peak occurred at ZT-18 and ZT-18 onwards the levels started decreasing. After feeding the levels further decreased and the trough occurred 6 h after feeding.

Food restriction had a significant effect on serotonin metabolism in pineal as well. Upon food restriction all the compounds rhythmicity was abolished except 5-HTP, 5-HT and TRP in 3 m old rats. Though the rhythmicity of 5-HT was not affected in these animals, it had shown a phase delay by approximately 6 h. The decreased melatonin levels in the dark phase suggesting an altered pattern of rhythmicity in melatonin secretion from the pineal.

The decreased levels of 5-MIAA, 5-MTOH and MEL in 3 m old rats upon food restriction in both SCN and pineal clearly indicating that the hydroxy indole o-methyl transferase activity was decreased in these tissues.

In SCN of 3 m old rats, the increased levels of 5-HTP, NAS and 5-HIAA suggesting that the activities of both rate limiting enzymes tryptophan hydroxylase and N-acetyl transferase along with monoamine oxidase increased. The decreased NAT levels upon food restriction in both SCN and pineal of 3 m old rats could be due to the decreased activity of L-aromatic amino acid dehydrogenase. Upon food restriction TRP levels in 3 m old rats greatly decreased in SCN and increased in pineal. In these animals, the 5-MIAA rhythmicity was abolished and the mean 24 h levels decreased drastically in both SCN and pineal. The 5-MTOH followed the same pattern of rhythmicity in SCN and pineal in both controls and food restricted animals.

In pineal of 12 and 24 m old animals, under food restriction the 5-HT levels were decreased and the 5-HIAA levels were increased at all the time points. The 5-HIAA levels were in correlation with 5-HT under food restriction in middle aged and old aged animals, indicating that whatever the 5-HT that was synthesized getting converted into 5-HIAA immediately. There was a gradual dip in the melatonin levels from ZT-0 to 12 and an increment from ZT-12 to 18 which was in correlation with in both 12 and 24 m

old rats. After food restriction melatonin levels were increased during the mid subjective day and the levels were slightly decreased at but statistically insignificant in the mid subjective night.

In the pineal of 24 m old rats, 5-HTOH levels were decreased from ZT-0 to 6 and then increased from ZT-6 to 18. In food restricted animals also, though the levels were decreased at all the time points it followed the same pattern as in controls. The NAS levels in 24 m old rat pineal were gradually increased from ZT-0 to 18 in both controls and food restricted animals although the levels were significantly decreased in food restricted animals.

In the SCN of 12 and 24 m old control animals, the melatonin levels were decreased from ZT-0 to 6 and then increased from ZT-6 to 18. Under food restriction, in 12 m old animals SCN, melatonin levels were significantly decreased in all the time points but, it followed the same pattern as in controls. In 24 m old animals, the levels were gradually decreased from ZT-0 to 18 upon food restriction.

The levels of most of the compounds were decreased in both 12 and 24 m old animals but, NAT levels were increased in both the age groups indicating that the most of the TRP may be converting in to NAT rather than converting into serotonin and its metabolites.

In 12 m old food restricted rats SCN, the positive correlation between 5-HT and 5-MTOH at both light and dark phases revealed that the magnitude of correlation increased by 1.4 folds. In the SCN of the same age group, the magnitudes of negative correlation in light phase and positive correlation in dark phase between 5-HT and MEL are almost same (Fig. 24).

The positive correlation between 5-HT and TRP in 3 m old R.F. rats and between 5-HT and NAS in 12 m old rats has equal magnitude at both light and dark phases. The negative correlation between 5-HT-5-HTP, 5-HT-5-HIAA, 5-HT-5-MTOH, 5-HT-5-MEL and 5-HT-NAT which was seen at the light phase was also maintained at dark phase in the pineal of 3 m old rats. A negative correlation between 5-HT-5-HTP, 5-HT-NAS, 5-HT-5-HTOH, 5-HT-5-MIAA, 5-HT-5-MTOH, 5-HT-5-MEL and 5-HT-NAT was maintained in both the phases in the pineal of 12 m old rats under R.F. Also such negative correlation was observed in both the phases between the compounds 5-HT-NAS, 5-HT-5-HTOH, 5-HT-MEL and 5-HT-NAT in the pineal of 24 m old rats (Fig. 30).

III. Effect of restricted feeding on daily Leptin rhythms in SCN

Prosser and Bergeron, 2003 have shown that coronal brain slices containing SCN were sensitive to phase modulation by the hormone, leptin. So we hypothesized that leptin is directly acting on SCN and wanted to see the circadian profile of leptin in SCN of control rats. We also studied the leptin-ir levels in SCN for any alterations under R.F. interestingly we could not find any leptin-ir in the SCN region of hypothalamus neither in control nor in R.F. animals. However, we scanned all the coronal sections serially for any leptin-ir up to the end of the optic chiasm and interestingly we localized leptin-ir in MPOA region which has been known to play an important role in thermogenesis (Curley *et al.,* 2005). MPOA region receive signals from SCN via subparaventricular zone (SPZ) (Froy, 2010) and involved in the regulation of thermogenesis. The leptin-ir levels were highest in MPOA region of 3 m old rats. Upon aging the leptin-ir levels gradually decreased though there was no significant difference in the levels between 12 and 24 m old rats.

These results are indicating that leptin is not directly acting on the SCN to modulate the phase of the SCN neurons in both control and food restricted animals. Whereas leptin is directly acting in the MPOA region, with a peak and nadir at ZT-0 and 18 respectively in all the three control age groups studied.

Our data suggests that aging caused a significant decrease in the leptin levels from 3 to 24 m, although there was no significant difference between 12 and 24 m.

Restricted feeding altered the phase of the leptin levels in all the three age groups. This data indicates that restricted feeding is acting as a non-photic cue to entrain the leptin levels in the MPOA region.

IV. Daily rhythms of clock gene (*Per1, Per2, Cry1 and Cry2)* expression in SCN and pineal

From the previous reports it is known that the feeding of nocturnal rodents exclusively during the day time has little if any effect on the SCN of these animals (Damiola, *et al.,* 2000). But whether aging has any effect on the SCN in response to temporal food restriction is not known.

Under R.F. the *Per1* levels were drastically decreased at all the four time points in all the three age groups studied. There is no significant change in the expression levels of *Per2* between control and R.F. animals in the SCN region of 3 m old rats in ZT-0, 6 and 18 time points. The phase of expression of *Per2* also did not alter upon R.F. which is correlating with unaltered expression of *Per2* in the SCN of 8 d restricted fed

mice (Damiola *et al.,* 2000). The *Per2* gene expression levels were decreased in 12 m old rats and whereas the levels were increased in 24 m old rats at ZT-0 and 18 when compared to controls. Except at ZT-0, the *Per2* gene expression is in the same phase in the SCN of 12 and 24 m old rats under R.F. The decreased *Per2* gene expression levels in 3 m old SCN is in agreement with the results of decreased *Per2* expression in the intestine of the rats fed under light phase (Balakrishnan *et al.,* 2010) and also decreased *Per2* expression when glucose administration in to the culture medium of rat-1 fibroblasts (Hirota *et al.,* 2002). The glucose suppression of *Per* gene expression may be the molecular basis for resetting the clocks by nutrient availability (Balakrishnan *et al.,* 2010). This is giving evidence that food availability can act as Zeitgeber for entraining the biological clock. Under food restriction the increased *Per2* gene expression in SCN of 3 m old rats at ZT-12 is in agreement with the increased *Per2* gene expression at ZT-12 in the SCN of 2 h and 6 h food restricted rats (Verwey and Amir, 2011). The altered *Per* gene expression is also in agreement with the abolished locomotor activity of the animals under food restriction in previous objective.

The *Cry1* and *Cry2* levels were decreased in the SCN of 12 m old rats upon R.F at all the four time points studied except at ZT-12 of *Cry2* where the levels were slightly increased when compared to its control group. In 24 m old rats both *Cry1* and *Cry2* genes were in same phase in controls and upon R.F. though there is a phase delay in the expression of these genes by 6 h, and both the genes are in the same phase.

In pineal also the *Per1* levels were decreased at all the four time points studied in all the three age groups when compared to their respective controls. The *Per2* levels were increased in 12 and 24 m old rats at all the time points except at ZT-6 in the pineal of 12 m old rats where the levels were slightly increased.

In the pineal of 3 m old rats the peak expression levels for *Cry1* and *Cry2* occurred at ZT-18 and also both the genes are in same phase of expression in controls. Under R.F. there is phase advancement by 12 h was observed for both the genes and the peak occurred at ZT-6. Despite of this alteration in the expression, the phase shift followed the same pattern in both the genes. The relative expression levels of *Cry 1* and *Cry 2* were increased in the pineal of 12 and 24 m old rats when compared to controls at all the time points studied except at ZT-6 where the levels were slightly decreased for both the genes.

In the SCN of 3 m old rats at the light phase *Per2* and *Cry2, Per2* and *Cry1,* a positive correlation was observed and also these genes were in the same phase of expression upon R.F.
In the SCN of 12 and 24 m old rats at the dark phase there was a positive correlation observed between *Per2* and *Cry1, Cry1* and *Cry2* and these correlating genes expressions were in the same phase upon R.F.

Whereas in the 3 and 12 m old rats pineal tissue also at light phase the positive correlations between *Per2* and *Cry1* was existed and as in the SCN tissue of 3 m old rats, and in pineal also these genes showed same phase of expression under R.F. Interestingly at the dark phase also in the pineal of 3 m old rats a positive correlation was observed and in this phase also a phase relationship was observed between these genes.

The *Cry1* and *Cry2* genes were in positive correlation at the light phase in the pineal of 3, 12 and 24 m old rats under R.F. The positive correlation between *Cry1* and *Cry2* genes was also maintained at the dark phase in the pineal of 12 and 24 m old rats upon R.F.

Upon R.F. the *Per2* and *Cry2* genes were also in a positive correlation and in the same phase of expression at light phase in both 3 and 12 m old rats pineal. This positive correlation and phase relationship was also seen at the dark phase in the pineal of 3 m old rats.

V. Daily rhythms of protein profile in SCN and pineal of R.F. rats:

Under R.F. the number of differentially expressed protein spots which have shown daily rhythm was decreased in both SCN and pineal of 12 m old rats when compared to 3 and 12 m old rats whereas the differentially expressed protein spots number was increased from 12 m to 24 m in both the tissues under R.F. When the gels of ZT-6 and ZT-18 were compared across the age groups in 3, 12 and 24 m, the number of protein spots that have shown daily rhythm and are differentially expressed were decreased at ZT-18 when compared to ZT-6 in the SCN. But in pineal there was no difference in the number of differentially expressed protein spots observed between ZT-6 and ZT-18 time points.

In the SCN, the number of up regulated as well as down regulated protein spots were more in the case of 12 m old rats. However in pineal, more up regulated protein spots were seen in 3 m old rats and the number of down regulated protein spots was more in 24 m old rats.

The differentially expressed protein spots among the time points and across the age groups will be analyzed and the spots showing robust changes will be put to MALDI-TOF/TOF mass spectrometry analysis to know the sequence and, the obtained sequences will be compared with the protein database to know the proteins.

Summary and Conclusion

Under forced day time feeding regimen, the active period length and the percentage diurnality were increased in all the three age groups. 24 m old rats seemed to be more entrained to the light phase than 3 and 12 m. Thus with R.F. the nocturnality could be switched to diurnality differentially in variable age groups. Under R.F. in the SCN, the rhythmicity of 5-HT, TRP, 5-MTOH, NAT was abolished whereas in pineal the rhythmicity of 5-HIAA, NAS, 5-HTOH, 5-MIAA, 5-MTOH, MEL and NAT was abolished. However in the SCN and pineal of 12 and 24 m old rats, the levels of most of the compounds were decreased but the entire compound have shown rhythmicity under R.F. We could not find any leptin-ir in SCN but we could see leptin-ir in MPOA region. Under day time feeding regimen the levels of leptin were decreased in MPOA region of all the three age groups. The rhythmicity of all the genes was abolished in SCN under R.F. in 3 m old rats and the *Per1* levels were decreased drastically in the SCN and pineal of all the three age groups. The number of differentially expressed spots between ZT-6 and ZT-18 time points were more in both SCN and pineal of 24 m old rats when compared to 3 and 12 m (Fig. 47).

Thus our work demonstrates that food is acting as a potent non-photic zeitgebers playing an important role in entraining the biological clock.

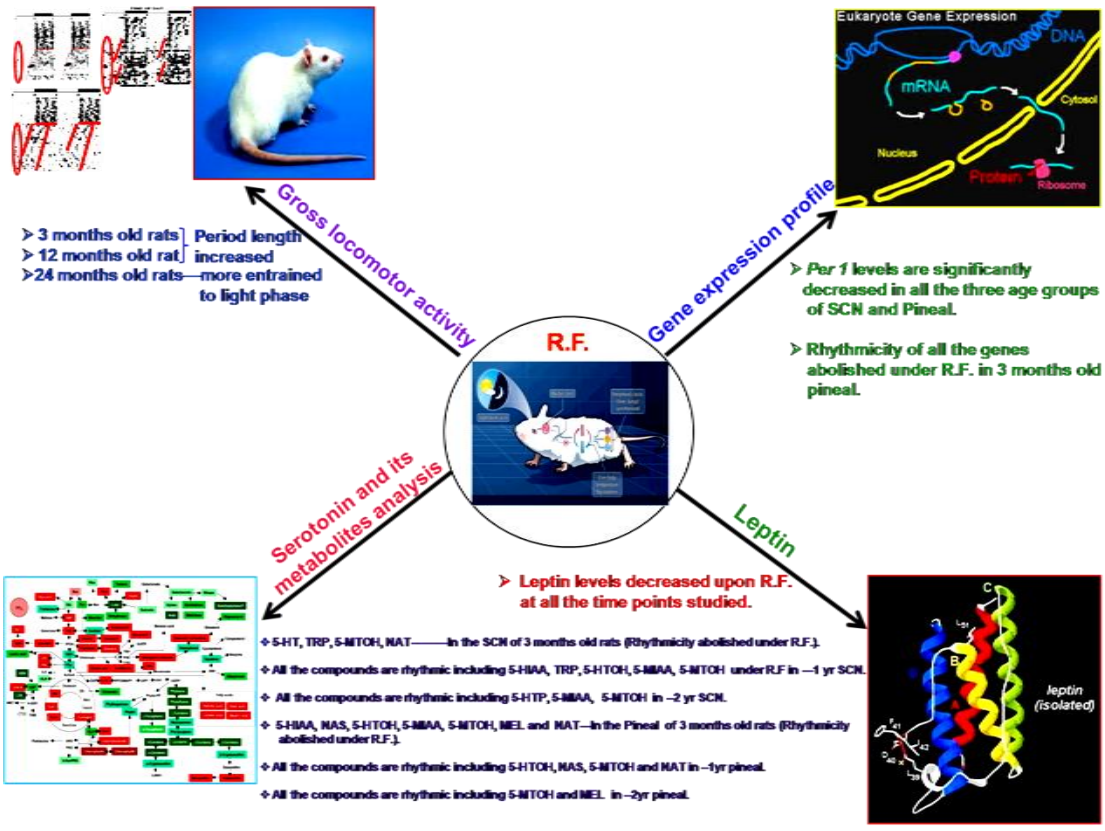


Fig. 47. The alterations observed in the gross locomotor activity, serotonin metabolism, clock gene expression, and protein profile in SCN, pineal along with the alterations in leptin levels in MPOA region under forced day time feeding regimen.

References:

Abdel-Wanis ME, Tsuchiya H (2002) Melatonin deficiency and fibrous dysplasia: might a relation exist? Med Hypotheses. 59:552-554.

Abrahamson EE, Moore RY (2001) Suprachiasmatic nucleus in the mouse: retinal innervation, intrinsic organization and efferent projections. Brain Res. 916:172-191.

Aghajanian GK, Sanders-Bush E (2002) "Serotonin" in "Neuropsychopharmacology-5th Generation of Progress". pp 15-34.

Akashi M, Ichise T, Mamine T, Takumi T (2006) Molecular Mechanism of Cell-autonomous Circadian Gene Expression of *Period 2*, a Crucial Regulator of the Mammalian Circadian Clock. Mol Biol Cell. 17:555-565.

Albrecht U (2012) Timing to Perfection: The Biology of Central and Peripheral Circadian Clocks.Neuron. Cell Press. 74:246-260.

Alvarez CF, Rodrı´guez ED, Vinuesa DP, Parras AIE, Lo´pez BD (2000) Ageing and melatonin influence on *in vitro* gonadotropins and prolactin secretion from pituitary and median eminence. Mech Ageing Dev. 114:173-183.

Balakrishnan A, Stearns AT, Ashley SW, Tavakkolizadeh A, Rhoads DB (2010) Restricted feeding phase shifts clock gene and sodium glucose cotransporter 1 (SGLT1) expression in rats. J Nutr. 140:908-914.

Baranyiova E (1991) Effect of serotonin on food intake by piglets during the early postnatal period. Acta Vet Brno. 60:127-136.

Benca R, Duncan MJ, Frank E, McClung C, Nelson RJ, Vicentic A (2009) Biological rhythms, higher brain function, and behavior: Gaps, opportunities, and challenges. Brain research reviews. 62:57-70.

Bodosi B, Gardi J, Hajdu I, Szentirmai E, Obal F Jr., Krueger JM. (2004) Rhythms of ghrelin, leptin, and sleep in rats: effects of the normal diurnal cycle, restricted feeding, and sleep deprivation. Am J Physiol Regul Integr Comp Physiol. 287:R1071-R1079.

Borjigin J, Li X, Solomon HS (1999) The pineal gland and melatonin: Molecular and Pharmacologic Regulation. Annu Rev Pharmacol Toxicol. 39:53-65.

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle protein-dye binding. Anal Biochem. 72:248-254.

Brown SA, Pagani L, Cajochen C, Eckert A (2011) Systemic and Cellular Reflections on Ageing and the Circadian Oscillator –A Mini-Review. Gerontology. 57: 427-34

Caldelas I, Chimal-monroy J, Marti'nez-go' mez M, Hudson R (2005) Non-photic circadian entrainment in mammals: A brief review and proposal for study during development. Biological Rhythm Research. 36:23-37.

Card JP, Moore RY (1991) "The organisation of visual circuits influencing the circadian activity of the suprachiasmatic nucleus" in "suprachiasmatic nucleus: the mind's clock". Oxford University Press, New York. pp 51-76.

Caston-Balderrama AL, Cameron JL, Hoffman GE (1998) Immunohistochemical localization of Fos in perfused nonhuman primate brain tissue: fixation and antisera selection. J Histochem Cytochem. 46:547-556.

Challet E (2010) Interactions between light, mealtime and calorie restriction to control daily timing in mammals. J Comp Physiol B. 180:631-644.

Chik CL, Ho AK, Brown GM (1986) Effect of food restriction on the circadian rhythms of circulating tryptophan and serotonin in rats. J Neural Transm. 66:261-269.

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

Coppen A (1972) Indoleamines and affective disorders. Journal of Psychiatric Research. 9:163-171.

Curley JP, Pinnock SB, Dickson SL, Thresher R, Miyoshi N, Surani MA, and Keverne EB (2005) Increased body fat in mice with a targeted mutation of the paternally expressed imprinted gene *Peg3*. 19:1302-1304.

Damiola F, Le Minch N, Preitner N, Kornmann B, Fleury-Olela F, Schibler U (2000) Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. Genes Dev. 14:2950-2961.

Dibner C, Schibler U, Albrecht U (2010) The Mammalian Circadian Timing System: Organization and Coordination of Central and Peripheral Clocks. Annu Rev Physiol. 72:517-549.

Dubocovich ML (2007) Melatonin receptors: Role on sleep and circadian rhythm regulation. Sleep Medicine. 8:S34-S42.

Feijó FdM, Bertoluci MC, Cíntia Reis (2011) Serotonin and hypothalamic control of hunger: a review. Rev Assoc Med Bras. 57:74-77.

Floc'h NL, Otten W, Merlot E (2011) Tryptophan metabolism, from nutrition to potential therapeutic applications. Amino Acids. 41:1195–1205.

Froy O. (2010) Metabolism and Circadian Rhythms - Implications for Body Weight. The Open Neuroendocrinology Journal. 3:28-37.

Froy O, Miskin R (2010) Effect of feeding regimens on circadian rhythms: Implications for aging and longevity. Aging. 2:7-27.

Froy O (2011) The circadian clock and metabolism. Clinical Science. 120:65-72.

Froy O (2011) Circadian Rhythms, Aging, and Life Span in Mammals. Physiology. 26:225-235.

Fuller PM, Lu J, Saper CB (2008) Differential Rescue of Light- and Food-Entrainable Circadian Rhythms. Science. 320:1074-1077.

Garattini S, Valzelli L (1965) "Metabolism of serotonin *in vivo*" in "Serotonin". Elsevier Publishing Company, Amsterdam. pp 9-73.

Garau C, Aparicio S, Rial RV, Nicolau MC, Esteban S (2006) Age-related changes in circadian rhythm of serotonin synthesis in ring doves: Effects of increased tryptophan ingestion. Exp Gerontol. 41:40-48.

Garaulet M., Ordova JM, Madrid JA (2010) The chronobiology, etiology and pathophysiology of obesity. Int J Obes. 34:1667-1683.

Garfield AS, Heisler LK (2009) Pharmacological targeting of the serotonergic system for the treatment of obesity. J Physiol. 587:49-60.

Giebultowicz JM (2000) Molecular mechanism and cellular distribution of insect circadian clocks. Annu Rev Entomol. 45:769-793.

Golombek DA, Rosenstein RE (2010) Physiology of Circadian Entrainment. Physiol Rev. 90:1063-1102.

Grady RK Jr, Caliguri A, Mefford IN (1984) Day/night differences in pineal indoles in the adult pigeon (*Columba livia*). Comp Biochem Physiol C. 78:141-143.

Green CB (1998) How cells tell time. Trends Cell Biol. 8:224-230.

Guan XM, Hess JF, Yu H, Hey PJ, van der Ploeg LH (1997) Differential expression of mRNA for leptin receptor isoforms in the rat brain. Mol Cell Endocrinol. 133:1-7.

Haleem DJ, Haider S (1996) Food restriction decreases serotonin and its synthesis rate in the hypothalamus. Neuroreport. 7:1153-1156.

Halford JCG, Harrold JA, Boyland EJ, Lawton CL, Blundell JE (2007) Serotonergic Drugs Effects on Appetite Expression and Use for the Treatment of Obesity. Drugs. 67:27-55.

Hardeland R, Madrid JA, Dun-Xian Tan, Reiter RJ (2012) Melatonin, the circadian multioscillator system and health: the need for detailed analyses of peripheral melatonin signaling. J Pineal Res. 52:139-166.

Harman D (2006) Free Radical Theory of Aging: An Update.Increasing the Functional Life Span. Ann N Y Acad Sci. 1067:10-21.

Harmer SL, Panda S, Kay SA (2001) Molecular bases of circadian rhythms. Annu Rev Cell Dev Biol. 17:215-253.

Hirota T, Okano T, Kokame K, Shirotani-Ikejima H, Miyata T, Fukada Y (2002) Glucose Down-regulates *Per1* and *Per2* mRNA Levels and Induces Circadian Gene Expression in Cultured Rat-1 Fibroblasts. J Biol Chem. 277:44244-44251.

Ho AK, Chik CL, Brown GM (1985) Scheduled feeding versus the light-dark cycle on the rhythms of circulating tryptophan, serotonin and N-acetylserotonin in rats. Life Sci. 37:1619-1626.

Jagota A, Olcese J, Harinarayana Rao S, Gupta PD (1999) Pineal rhythms are synchronized to light-dark cycles in congenitally anophthalmic mutant rats. Brain Res. 825:95-103.

Jagota A, de la Iglesia HO, Schwartz WJ (2000) Morning and evening circadian oscillations in the suprachiasmatic nucleus *in vitro*. Nat Neurosci. 3:372-376.

Jagota A (2005) Aging and sleep disorders. Indian journal of Gerontology. 19:415-424.

Jagota A (2006) Suprachiasmatic Nucleus:Center for Circadian Timing System in Mammals. Proc Indian Natl Sci Acad B. 71:285-300.

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

Jagota A, Kalyani D (2008) Daily serotonin rhythms in rat brain during postnatal development and aging. Biogerontology. 9:229-234.

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

Johnston JD, Tournier BB, Andersson H, Masson-Pe´vet M, Lincoln GA, Hazlerigg DG (2006) Multiple Effects of Melatonin on Rhythmic Clock Gene Expression in the Mammalian Pars Tuberalis. Endocrinology. 147:959-965.

Jun Kohyama J (2011) Neurochemical and Neuropharmacological Aspects of Circadian Disruptions: An Introduction to Asynchronization. Curr Neuropharmacol. 9:330-341.

Kalsbeek A, Scheer FA, Perreau-Lenz S, La Fleur SE, Yi C, Fliers E, Buijs RM (2011) Circadian disruption and SCN control of energy metabolism. FEBS Lett. 585:1412-1426.

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

Karasek M (2007) Does melatonin play a role in aging process. J Physiol Pharmacol. 58:105-113.

Kema IP, de Vries EGE, Muskiet FAJ (2000) Clinical chemistry of serotonin and metabolites. J Chromatogr B Biomed Sci Appl. 747:33-48.

Klante G, Secci K, Masson-pevet M, Paul pe´ vet P, Vivien-roels B, Steinlechner S, Wollnik F, (1999) Interstrain differences in activity pattern, pineal function, and SCN melatonin receptor density of rats. Am J Physiol. 276:1078-1086.

Ko CH, Takahashi JS (2006) Molecular components of the mammalian circadian clock. Human Molecular Genetics. 15:R271-R277.

Koch BCP, Nagtegaal JE, Kerkhof GA, ter Wee PM (2009) Circadian sleep–wake rhythm disturbances in end-stage renal disease. Nat Rev Nephrol. 5:407-416.

Kohyama J (2011) Neurochemical and neuropharmacological aspects of circadian disruptions: an introduction to asynchronization. Curr Neuropharmacol. 9:330-41.

Kolker DE, Vitaterna MH, Fruechte EM, Takahashi JS, Turek FW (2004) Effects of age on circadian rhythms are similar in wild-type and heterozygous *Clock* mutant mice. Neurobiol Aging. 25:517-523.

Kwon I, Choe HK, Son GH, Kim K (2011) Mammalian Molecular Clocks. Exp Neurobiol. 20:18-28.

Lam DD, Heisler LK (2007) Serotonin and energy balance: molecular mechanisms and implications for type 2 diabetes. Expert Rev Mol Med. 9:1-24

Lamont EW, Legault-Coutu D, Cermakian N, Boivin DB (2007) The role of circadian clock genes in mental Disorders. Dialogues Clin Neurosci. 9:333-347**.**

Laposky AD, Bass J, Kohsaka A, Turek FW (2008) Sleep and circadian rhythms: Key components in the regulation of energy metabolism. FEBS Lett. 582:142-151.

L´evi F, Okyar A, Dulong S, Innominato PF, Clairambault J (2010) Circadian Timing in Cancer Treatments. Annu. Rev. Pharmacol. Toxicol. 50:377-421.

Lewy AJ (2003) Clinical applications of melatonin in circadian disorders. Dialogues Clin Neurosci. 5:399-413.

Liu Y, Bell-Pedersen D (2006) Circadian rhythms in *Neurospora crassa* and other filamentous fungi. Eukaryot Cell. 5:1184-93.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔ Ct Method. Methods. 25:402-408.

Lovinger DM (1997) Serotonin's role in alcohol's effects on the brain. Alcohol Health Res World. 21:114-120.

Lowrey PL, Takahashi JS (2000) Genetics of the mammalian circadian system: photic entrainment, circadian pacemaker mechanisms, and posttranslational regulation. Annu Rev Genet. 34:533-562.

Mammen AP, Jagota A (2011) Immunocytochemical evidence for different patterns in daily rhythms of VIP and AVP peptides in the suprachiasmatic nucleus of diurnal *Funambulus palmarum*. Brain Res. 2373:39-47.

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

McClung CA (2007) Circadian Genes, Rhythms and the Biology of Mood Disorders. Pharmacol Ther. 114:222-232.

Mefford IN, Chang P, Klein DC, Namboodiri MAA, Sugden D, Barchas J (1980) Determination of tryptophan and metabolites in rat brain and pineal tissue by reversed-phase high-performance liquid chromatography with electrochemical detection. J Chromatogr. 181:187-193.

Mirmiran M, Swaab DF, Witting W, Honnebier MBOM, van Gool WA, Eikelenboom P (1989) Biological clocks in development, ageing and alzheimer's disease. Brain Dysfunct. 2:57-66.

Miyazato M, Mori K, Ida T, Kojima M, Murakami N, Kangawa K (2008) Identification and functional analysis of a novel ligand for G protein-coupled receptor, Neuromedin S. Regul Pept. 145:37-41.

Mohawk JA, Takahashi JS (2011) Cell autonomy and synchrony of suprachiasmatic nucleus circadian oscillators. Trends in Neurosciences. 34:349-358.

Mohawk JA, Green CB, Takahashi JS (2012) Central and Peripheral Circadian Clocks in Mammals. Annu Rev Neurosci. 35:445-462.

Monk TH (2005) Aging Human Circadian Rhythms: Conventional Wisdom May Not Always Be Right. J Biol Rhythms. 20:366-374.

Moore RY, Halaris AE, Jones BA (1978) Serotonin neurons of the midbrain raphe: ascending projections. J Comp Neurol. 180:417-438.

Moore RY, Speh JC (1993) GABA is the principal neurotransmitter of the circadian system. Neurosci Lett. 150:112-116.

Morre DJ, Chueh PJ, Pletcher J, Tang X, Wu LY, Morre DM (2002) Biochemical basis for the biological clock. Biochemistry. 41:11941-11945.

Ejvind Mortz E, Krogh TN, Vorum H, Görg A (2001) Improved silver staining protocols for high sensitivity protein identification using matrix-assisted laser desorption/ionization-time of flight analysis Proteomics 1:1359–1363.

Mrosovsky N (1996) Locomotor activity and non-photic influences on circadian clocks. Biological Reviews. 71:343-372.

Pandi-Perumal SR, Srinivasan V, Maestroni GJM, Cardinali DP, Poeggeler B, Hardeland R (2006) Melatonin: Nature's most versatile biological signal? FEBS J. 273:2813-2838.

Pandi-Perumal SR, Trakht I, Srinivasan V, Warren Spence D, Maestroni GJM, Zisapel N, Cardinali DP (2008) Physiological effects of melatonin: Role of melatonin receptors and signal transduction pathways. Prog Neurobiol. 85:335-353.

Paranjpe DA, Vijay Kumar Sharma (2005) Evolution of temporal order in living organisms. J Circadian Rhythms. 3:1-13.

Perreau-Lenz S, Zghoul T, Spanagel R (2007) Clock genes running amok. Clock genes and their role in drug addiction and depression. EMBO Rep. 8:S20-S3.

Petersen MS, Petersen CC, Agger R, Hokland M, Gundersen HJG (2006) A simple method for unbiased quantitation of adoptively transferred cells in solid tissues. J Immunol Methods 309:173-181.

Pevet P, Challet E (2011) Melatonin: Both master clock output and internal time-giver in the circadian clocks network. J Physiol Paris. 105:170-182.

Piccione G, Bertolucci C, Caola G, Foa F (2007) Effects of restricted feeding on circadian activity rhythms of sheep - A brief report. Applied Animal Behaviour Science. 107:233-238.

Piggins HD, Guilding C (2011) The neural circadian system of mammals. Essays Biochem. 49:1-17.

Prosser RA, Bergeron HE (2003) Leptin phase-advances the rat suprachiasmatic circadian clock *in vitro*. Neurosci Lett. 336:139-142.

Rasmussen DD, Marck BT, Boldt BM, Yellon SM, Matsumoto AM (2003) Suppression of hypothalamic pro-opiomelanocortin (POMC) gene expression by daily melatonin supplementation in aging rats. J Pineal Res. 34:127-133.

Rea MA, Glass JD, Colwel CS (1994) Serotonin Modulates Photic Responses in the Hamster Suprachiasmatic Nuclei. J Neurosci. 74:3635-3642.

Reddy YM (2010) Effect of Curcumin on Ethanol Induced Changes in Daily Serotonin Chronometabolomics and Gene Expression in SCN and Pineal: Locomotor Rhythms. Ph.D thesis with Jagota A, University of Hyderabad, India

Richard DM, Dawes MA, Mathias CW, Acheson A, Hill-Kapturczak N, Dougherty DM (2009) *L*-Tryptophan: Basic Metabolic Functions, Behavioral Research and Therapeutic Indications. Int J Tryptophan Res. 2:45-60.

Roennberg T, Merrow M (2001) Circadian systems: different levels of complexity. Philos Trans R Soc Lond B Biol Sci. 356:1687-1696.

Sánchez S, Sánchez C, Paredes SD, Cubero J, Rodríguez AB, Barriga C (2008) Circadian variations of serotonin in plasma and different brain regions of rats. Mol Cell Biochem. 317:105-111.

Saper CB, Lu J, Chou TC, Googley J (2005) The hypothalamic integrator for circadian rhythms. Trends Neurosci. 28:152-157.

Sashihara K, Bungo' T, Ando R, Ohgushi A, Kawakami S-I, Denbow DM, Furuse M (2002) Role of Central Serotonergic Systems on the Regulation of Feeding Behavior of Chicks in Two Different Strains. Journal of Applied Animal Research. 21:17-23.

Saxena N, Khan EA, Sinha MP, Mehrotra PN (1991) Effect of pinealectomy and 5-methoxytryptophol administration on the gonads and accessory sex organs of male Indian palm squirrel, *Funambulus pennant*. The Indian Zoologist. 15:197-199.

Schoeller DA, Cella LK, Sinha MK, Caro JF (1997) Entrainment of the Diurnal Rhythm of Plasma Leptin to Meal Timing. J Clin Invest. 100:1882-1887.

Sharma VK, Chandrashekaran MK (2005) Zeitgebers (*time cues*) for biological clocks. Current Science. 89:1136-1146.

Shaw KA, Turner J, Mar CD (2009) Tryptophan and 5-Hydroxytryptophan for depression. Cochrane Database Syst Rev. 3:1-17.

Sheward WJ, Maywood ES, French KL, Horn JM, Hastings MH, Seckl JR, Holmes MC, Harmar AJ (2007) Entrainment to Feeding but Not to Light: Circadian Phenotype of VPAC2 Receptor-Null Mice. J Neurosci. 27:4351-4358.

Shirakawa T, Honma S, Honma K (2001) Multiple oscillators in the suprachiasmatic nucleus. Chronobiol Int. 18:371-387.

Slominski A, Baker J, Rosano TG, Guisti LW, Ermak G, Grande M, Gaudet SJ (1996) Metabolism of Serotonin to *N*-Acetylserotonin, Melatonin, and 5-Methoxytryptamine in Hamster Skin Culture. J Biol Chem. 271:12281-12286.

Smith I, Francis P (1980) Identification of O-Acetyl-5-methoxytryptophenol in the Pineal Gland by Gas Chromatography-Mass Spectrometry. Biochem J. 185:537-540.

Smith RG, Betancourt L, Sun Y (2005) Molecular Endocrinology and Physiology of the Aging Central Nervous System. Endocrine Reviews. 26:203-250.

Sukumaran S, Almon RR, DuBois DC, Jusko WJ. (2010) Circadian rhythms in gene expression: Relationship to physiology, disease, drug disposition and drug action. Adv Drug Deliv Rev. 62:904-917.

Takahashi JS, Hong HK, Ko CH, McDearmon EL (2008) The genetics of mammalian circadian order and disorder: implications for physiology and disease. Nature Reviews Genetics. 9:764-775.

Turek FW, Gillette MU (2004) Melatonin, sleep, and circadian rhythms: rationale for development of specific melatonin agonists. Sleep Med. 5:523-532.

Ukai H, Ueda HR (2010) Systems Biology of Mammalian Circadian Clocks. Annu Rev Physiol. 72:579-603.

van den Pol AN (1991) "The suprachiasmatic nucleus: morphological and cytochemical substrates for cellular interactions" in "Suprachiasmatic nucleus: The Minds clock". Oxford University Press, New York. pp 17-50.

Verwey M, Amir S (2011) Nucleus-specific effects of meal duration on daily profiles of Period1 and Period2 protein expression in rats housed under restricted feeding. Neuroscience. 192:304-311.

Welsh DK, Takahashi JS, Kay SA (2010) Suprachiasmatic Nucleus: Cell Autonomy and Network Properties. Annu Rev Physiol. 72:551-577.

Witt-Enderby PA, Bennett J, Jarzynka MJ, Firestine S, Melan MA (2003) Melatonin receptors and their regulation: biochemical and structural mechanisms. Life Sci. 72:2183-2198.

Wollnik F (1989) Physiology and regulation of biological rhythms in laboratory animals: an overview. Laboratory Animals. 23:107-125.

Yonei Y, Hattori A, Tsutsui K, Okawa M, Ishizuka B (2010) Effects of Melatonin: Basics Studies and Clinical Applications. Anti-Aging Medicine. 7:85-91.

Young ME, Bray MS (2007) Potential role for peripheral circadian clock dyssynchrony in the pathogenesis of cardiovascular dysfunction. Sleep Med. 8:656-667.

Zanquetta MM, Corrêa-Giannella ML, Monteiro MB, Villares SMF (2010) Body weight, metabolism and clock genes. Diabetol Metab Syndr. 2:53.

Zawilska JB, Skene DJ, Nowak JZ (1998) 5-Methoxytryptophol rhythms in the chick pineal gland: effect of environmental lighting conditions. Neurosci Lett. 251:33-36.

Zawilska JB, Sadowska M (2002) Prolonged treatment with glucocorticoid dexamethasone suppresses melatonin production by the chick pineal gland and retina. Pol J Pharmacol. 54:61-66.

Zee PC, Vitiello MV (2009) Circadian Rhythm Sleep Disorder : Irregular Sleep Wake Rhythm. Sleep Med Clin. 4:213-218.

Zhang J, Dong X, Fujimoto Y, Okamura H (2004) Molecular Signals of Mammalian Circadian Clock. Kobe J Med Sci. 50:101-109.

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Appendix-I

List of Figures

Fig.12. Tryptophan metabolism (Garattini and Valzelli, 1965; Kema *et al.*, 2000).

Fig.13. Molecular machinery of the circadian oscillator constitutes an autoregulatory transcriptional feedback loop containing CLOCK and BMAL1 as activators and *Per1, Per2, Cry1,* and *Cry2* as the target genes of the loop whose protein products form a negative-feedback repressor complex. Another feedback containing *Rev-erbα* and *Rorα* represses *Bmal1* transcription. CLOCK: BMAL1complex also promotes the transcription of clock-controlled genes (Ccg). The PER and CRY proteins stability is regulated by SCF (Skp1-Cullin-F-box protein) E3 ubiquitin ligase complexes involving β-TrCP and FBXL3. Casein kinase 1ε/δ (CK1ε/δ) and AMP kinase (AMPK), phosphorylate the PER and CRY proteins, and promotes polyubiquitination by their respective E3 ubiquitin ligases which in turn directs the PER and CRY proteins for degradation by the 26S proteasome complex (Mohawk *et al.*, 2012).

Fig.14. Set-up for recording gross locomotor activity.

Fig.15. Standard representative peaks of HPLC for Serotonin and its related compounds. (A) 10% methanol; 0.1N Sodium acetate; 0.1N citric acid, 50 mg/litre EDTA was used for detection of compounds- 5-HTP, 5-HT, 5-HIAA, NAS, TRP and 5-HTOH. (B) 25% methanol; 0.1N Sodium acetate; 0.1N citric acid, 50 mg/litre EDTA was used for detection of compounds- 5-MIAA, 5-MTOH, MEL and NAT. nA refers to nanoampere (Unit of strength of electric current).

Fig.16. Representative dissociation curves for *β-actin, Per1, Per2, Cry1* and *Cry2*.

Fig.17. A. Double plotted actograms of gross locomotor activity under *ad libitum* and food restricted condition in 3, 12 and 24 m male wistar rats. The dashed lines on the activity profile indicates lights on and off for animals in LD (12:12). B. Mean activity profile of in 3, 12 and 24 m rats.

Fig. 18. Comparison of daily rhythms of various components of serotonin metabolism in control and food restricted groups in the SCN of 3 m old rats. Each value is mean ± S.E (n=6). $p \leq 0.05$ (a, b, c and d refers to comparison between ZT-0, 6,12 and 18 respectively; w, refers to significant difference in the levels of the compound at same time point between control and R.F. group in same age group). —— Line indicates controls; ----- Line indicates R.F.

Fig. 19. Effect of restricted feeding on mean 24 h. levels of serotonin and its related compounds in the SCN of 3 m old rats. * indicate significant difference between control and food restricted animals ($p \leq 0.05$) (n=24) determined by one way-ANOVA.

Fig. 20. Comparison of daily rhythms of various components of serotonin metabolism in control and food restricted groups in the SCN of 12 m old rats. Each value is mean ± S.E (n=6). $p \leq 0.05$ (a, b, c and d refers to comparison between ZT-0, 6,12 and 18 respectively; w, refers to significant difference in the levels of the compound at same time point between control and R.F. group in same age group).

—— Line indicates controls; ----- Line indicates R.F.

Fig. 21. Effect of restricted feeding on mean 24 h. levels of serotonin and its related compounds in the SCN of 12 m old rats. * indicate significant difference between control and food restricted animals ($p \leq 0.05$) (n=24) determined by one way-ANOVA.

Fig. 22. Comparison of daily rhythms of various components of serotonin metabolism in control and food restricted groups in the SCN of 24 m old rats. Each value is mean ± S.E (n=6). $p \leq 0.05$ (a, b, c and d

refers to comparison between ZT-0, 6,12 and 18 respectively; w, refers to significant difference in the levels of the compound at same time point between control and R.F. group in same age group).

—— Line indicates controls; ----- Line indicates R.F.

Fig. 23. Effect of restricted feeding on mean 24 h. levels of serotonin and its related compounds in the SCN of 24 m old rats. * indicate significant difference between control and food restricted animals ($p \leq 0.05$) (n=24) determined by one way-ANOVA.

Fig 24. Effect of restricted feeding on pair wise correlation between mean light (ZT-0, 6,12) and mean dark (ZT-12,18,0) phase values in various age groups 3, 12 and 24 old rats SCN. Each value is a correlation coefficient value between parameters. * indicates statistically significant value between parameters ($p \leq 0.05$). Negative value indicates negative correlation between parameters.

Fig. 25. Comparison of daily rhythms of various components of serotonin metabolism in control and food restricted groups in the pineal of 3 m old rats. Each value is mean ± S.E (n=6). $p \leq 0.05$ (a, b, c and d refers to comparison between ZT-0, 6,12 and 18 respectively; w, refers to significant difference in the levels of the compound at same time point between control and R.F. group in same age group).

—— Line indicates controls; ----- Line indicates R.F.

Fig. 26. Effect of restricted feeding on the mean 24 h levels of serotonin and its related compounds in the pineal of 3 m old rats. * indicate significant difference between control and food restricted animals ($p \leq 0.05$) (n=24) determined by one way-ANOVA.

Fig. 27. Comparison of daily rhythms of various components of serotonin metabolism in control and food restricted groups in the pineal of 12 m old rats. Each value is mean ± S.E (n=6). $p \leq 0.05$ (a, b, c and d refers to comparison between ZT-0, 6,12 and 18 respectively; w, refers to significant difference in the levels of the compound at same time point between control and R.F. group in same age group).

—— Line indicates controls; ----- Line indicates R.F.

Fig. 28. Effect of restricted feeding on the mean 24 h levels of serotonin and its related compounds in the pineal of 12 m old rats. * indicate significant difference between control and food restricted animals ($p \leq 0.05$) (n=24) determined by one way-ANOVA.

Fig. 29. Comparison of daily rhythms of various components of serotonin metabolism in control and food restricted groups in the pineal of 24 m old rats. Each value is mean ± S.E (n=6). $p \leq 0.05$ (a, b, c and d refers to comparison between ZT-0, 6,12 and 18 respectively; w, refers to significant difference in the levels of the compound at same time point between control and R.F. group in same age group).

—— Line indicates controls; ----- Line indicates R.F.

Fig. 30. Effect of restricted feeding on the mean 24 h levels of serotonin and its related compounds in the pineal of 24 m old rats. * indicate significant difference between control and food restricted animals ($p \leq 0.05$) (n=24) determined by one way-ANOVA.

Fig. 31. Effect of restricted feeding on pair wise correlation between mean light (ZT-0, 6,12) and mean dark (ZT-12,18,0) phase values in various age groups 3, 12 and 24 old rats pineal. Each value is a

correlation coefficient value between parameters. * indicates statistically significant value between parameters ($p \leq 0.05$). Negative value indicates negative correlation between parameters.

Fig. 32. A. Leptin-ir in the coronal brain sections (25 µm) of 3 m old male wistar rats SCN , no leptin-ir was observed in any of these time points studied in the SCN region. Bi, Ci, Di. Leptin-ir in the MPOA region of 3, 12 and 24 m old control rats. Bii, Cii, Dii. Leptin-ir in the MPOA region of 3, 12 and 24 m old R.F. rats sacrificed at various zeitgeber times (ZT) 0, 6, 12 and 18. Biii, Ciii, Diii. Histograms of densitometric analysis of the Leptin-ir between control R.F. rats in corresponding age group. Each value is mean ± S.E (n=4). * indicates significant ($p \leq 0.05$) difference between control and food restricted rats. Scale bar=1mm for panel A and 200 µm for panel B, C, D.

Fig. 33. Mean 24 h levels of leptin-ir in the MPOA region of 3, 12 and 24 m rats. Each value is mean ± S.E. (n=16). * indicates significant difference between control and food restricted animals. ($p \leq 0.05$).

Fig. 34. Comparison of daily expression rhythms of *Per1, Per2, Cry1* and *Cry2* in the SCN region in control and food restricted condition upon aging. A. 3 months B. 12 months C. 24 months. Each value is mean ± S.E (n=4). ZT-Zeitgeber time. $p \leq 0.05$ (where a, b, c and d refers to comparison between ZT-0, 6,12 and 18 respectively; w, refers to significant difference in the levels of the compound at same time point between control and R.F. group in same age group).

Fig. 35. Effect of restricted feeding on the mean 24 h levels (Ai, Bi and Ci) and Max: Min ratios (Aii, Bii and Cii) on *Per1, Per2, Cry1* and *Cry2* gene expression levels in the SCN region of control and food restricted animals upon aging. * indicate significant difference between control and food restricted animals ($p \leq 0.05$) determined by one way-ANOVA. ZT-Zeitgeber time.

Fig. 36. Comparison of daily expression rhythms of *Per1, Per2,* C*ry1* and *Cry2* in the pineal of control and food restricted animals under aging. A. 3 months B. 12 months C. 24 months. Each value is mean ± S.E (n=4). ZT-Zeitgeber time. $p \leq 0.05$ (where a, b, c and d refers to comparison between ZT-0, 6,12 and 18 respectively; w, refers to significant difference in the levels of the compound at same time point between control and R.F. group in same age group).

Fig. 37. Effect of restricted feeding on the mean 24 h levels (Ai, Bi and Ci) and Max: Min ratios (Aii, Bii and Cii) on *Per1, Per2, Cry1* and *Cry2* gene expression levels in the pineal of control and food restricted animals upon aging. * indicate significant difference between control and food restricted animals ($p \leq 0.05$) determined by one way-ANOVA. ZT-Zeitgeber time.

Fig. 38. Effect of restricted feeding on pair wise correlation between mean light (ZT 0, 6,12) and mean dark (ZT 12,18, 24/0) phase values of *Per 1, Per 2, Cry 1 and Cry 2* in various age groups 3, 12 and 24 months in rat SCN. Each value is a correlation coefficient value between parameters. * indicates statistically significant value between parameters ($p \leq 0.05$). Negative value indicates negative correlation between parameters.

Fig. 39. Effect of restricted feeding on pair wise correlation between mean light (ZT 0, 6,12) and mean dark (ZT 12,18, 24/0) phase values of *Per 1, Per 2, Cry 1 and Cry 2* in various age groups 3, 12 and 24 months in rat pineal. Each value is a correlation coefficient value between parameters. * indicates statistically significant value between parameters ($p \leq 0.05$). Negative value indicates negative correlation between parameters.

Fig. 40. Daily rhythms of protein profiles in (i) 3, (ii) 12 and (iii) 24 m SCN gels. Protein spots which were differentially expressed between ZT6 and ZT18 of SCN are highlighted in green.

Fig. 41. Protein spots which were differentially expressed between 3, 12 and 24 m SCN at ZT6 and ZT18 are highlighted in green.

Fig. 42. Protein spots which were differentially expressed daily in 3, 12, and 24 m old rat SCN were highlighted.

Fig. 43. Daily rhythms of protein profiles in (i) 3, (ii) 12 and (iii) 24 m pineal gels. Protein spots which were differentially expressed between ZT6 and ZT18 of pineal are highlighted in green (n=2).

Fig. 44. Protein spots which were differentially expressed between 3, 12 and 24 m pineal at ZT6 and ZT18 were highlighted.

Fig. 45. Protein spots which were differentially expressed daily in 3, 12, and 24 m old rat pineal were highlighted.

Fig. 46. A. Number of differentially expressed protein spots in the SCN and pineal of 3, 12 and 24 m old rats. The number is high in 24 m old rats in both the tissues. **B.** Graph showing the number of differentially expressed protein spots in the gels of ZT-6 and 18 time points which were compared across the age groups (3, 12 and 24 m old rats) were plotted.

Fig. 47. The alterations observed in the gross locomotor activity, serotonin metabolism, clock gene expression, and protein profile in SCN, pineal along with the alterations in leptin levels in MPOA region under forced day time feeding regimen.

Abbreviations

5-HIAA	:	5-Hydroxy indole acetic acid
5-HT	:	5-Hydroxytryptamine
5-HTOH	:	5-Hydroxy tryptophol
5-HTP	:	5-Hydroxytryptophan
5-MIAA	:	5-Methoxy indole acetic acid
5-MTOH	:	5-Methoxy indole acetic acid
AA-NAT	:	Arylalkylamine N-acetyl transferase
ANOVA	:	Analysis of Variance
ASPS	:	Advanced Sleep Phase Syndrome
AVP	:	Arginin vasopressin
Bmal1	:	Brain-muscle-Arnt-like-protein1
º C	:	degree centigrade/ degree Celsius
Cal B	:	Calbindin
cAMP	:	Cyclic Adenosine Mono Phosphate
CCG	:	Clock controlled genes
cDNA	:	Complentary DNA
CkIε	:	Casein kinase Iε
CkIδ	:	Casein kinase Iδ
Clock	:	Circadian locomotor output cycles kaput
Cry	:	*Cryptochrome*
DM-SCN	:	Dorsomedial SCN
DSPS	:	Delayed Sleep Phase Syndrome
EC	:	Electrochemical detector
EDTA	:	Ethylene di-amine tetra acetic acid
GABA	:	Gamma amino butyric acid
LD	:	Light Dark cycle
MEL	:	Melatonin

mg	:	milligram
ml	:	milliliter
mM	:	mill molar
mRNA	:	messenger ribonucleic acid
MT1	:	Melatonin receptor subtype1
NAS	:	N-acetyl serotonin
NAT	:	N- Acetyl tryptamine
NPY	:	Neuropeptide Y
PCR	:	Polymerase chain reaction
Per	:	*Period*
PHI	:	Peptide histidine isoleucine
PRC	:	Phase response curve
R.F.	:	Restricted feeding
RHT	:	Retinohypothalamic tract
RP-HPLC	:	Reverse phase high pressure liquid chromatography
PK2	:	Prokineticin 2
pM	:	Pico mole
ror	:	Retinoic acid orphan related
RT	:	Room temperature
SCG	:	Superior cervical ganglion
SCN	:	Suprachiasmatic nucleus
TGFα	:	Transforming growth factor α
TH	:	Tyrosine hydroxylase
TRP	:	Tryptophan
VL-SCN	:	Ventrolateral SCN
VIP	:	Vasoactive intestinal peptide
ZT	:	Zeitgeber time
µl	:	micro litre
µM	:	micro molar