Studies of circadian profile of a diurnal rodent *Funambulus palmarum* (South Indian Palm Squirrel): Neural regulation of Suprachiasmatic Nucleus Entrainment

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

By

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DECLARATION

I, Anumodh Pazhoor Mammen, hereby declare that this thesis entitled “Studies of circadian profile of a diurnal rodent *Funambulus palmarum* (South Indian Palm Squirrel): Neural regulation of Suprachiasmatic Nucleus Entrainment” 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.

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CERTIFICATE

This is to certify that this thesis entitled "Studies of circadian profile of a diurnal rodent *Funambulus palmarum* (South Indian Palm Squirrel): Neural regulation of Suprachiasmatic Nucleus Entrainment" is a record of bonafide work done by Mr. Anumodh Pazhoor Mammen, a research scholar for Ph.D. programme in Animal Sciences, School of Life Sciences, University of Hyderabad under my guidance and supervision.

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Introduction

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Review of Literature

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Introduction

Biological Rhythms

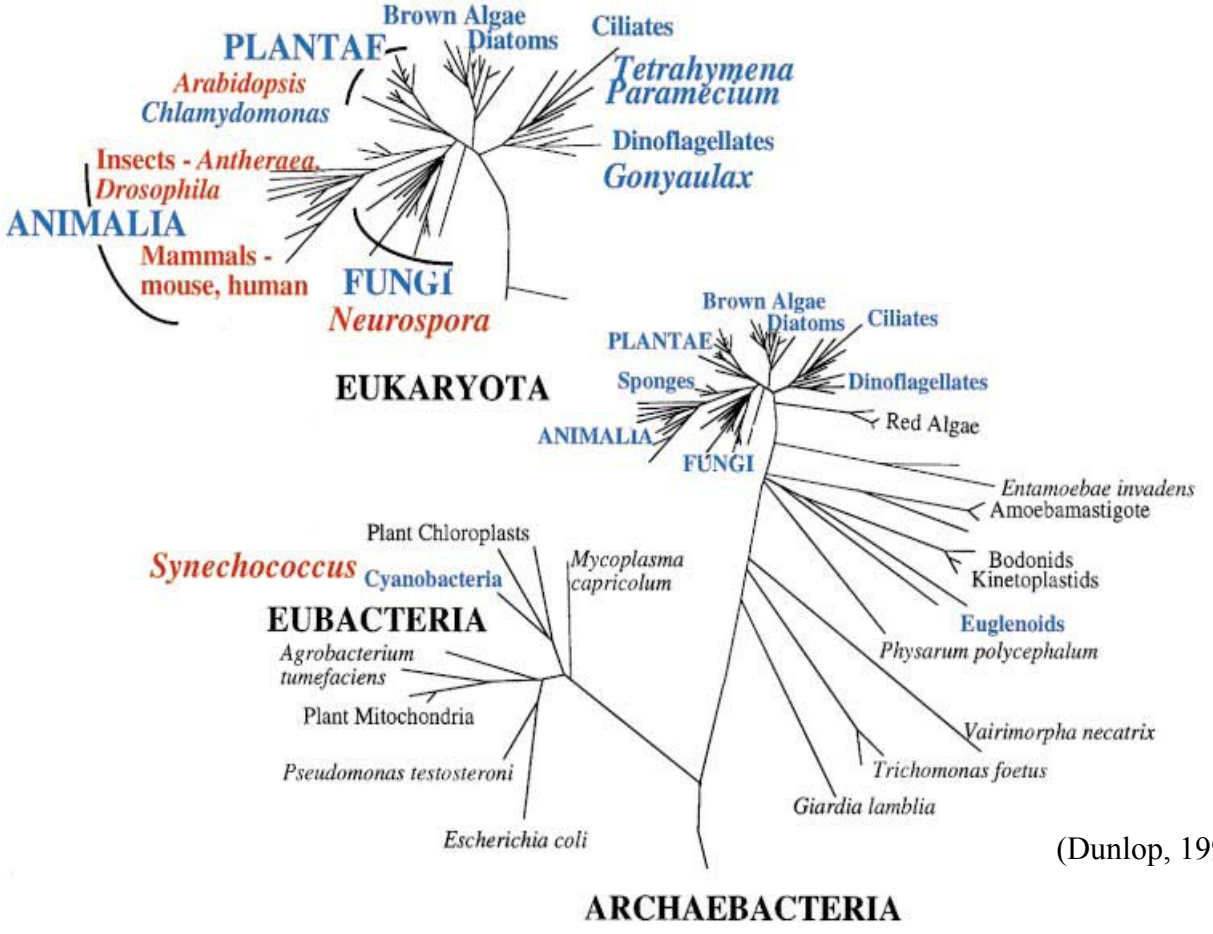
Biological rhythms are periodic phenomenon that plays an important role in the physiology of all living organisms. Biological rhythms are of two types, exogenous and endogenous. Exogenous rhythms are entrained to external cues or Zeitgeber, such as daily day night cycle or a change in the seasons, for example, sleep-wake cycle, feeding pattern, reproduction, hibernation, etc. Endogenous rhythms are those that persist even in the absence of external cues, for example body temperature cycle, etc. Biological rhythms are characterized in that they are free running in the absence of external cues, entrain to the external cues, are temperature compensated and are under genetic control (Klein *et al*., 1991).

Based on the periodicity of these oscillations, biological rhythms can be classified into different types such as ultradian, infradian, circadian, circannual, etc. Ultradian rhythms are those rhythms with a time period of less than 24 hours, as seen in several biological processes such as heart beat, breathing, intestinal mobility, etc. Infradian rhythms, have time periods of more than 24 hours such as the circaseptan rhythms which have a periodicity of approximately 7 days, circavigintin rhythms of 20 days, circalunar rhythms of 29.5 days, etc. (Halberg, 1969). These rhythms are also seen in the occurrence of disorders such as myocardial infarction, stroke, etc. The most important of all is circadian (Latin: circa-about; dies-day) rhythms having a periodicity of 24 hours and are manifested in a plethora of biological functions such as the daily sleep-wake cycle, feeding pattern, reproduction, etc. Circannual rhythms are annual rhythms that follow yearly pattern that are synchronized to seasonal changes such as hibernation, aestivation, egg laying pattern, etc. (Reinberg and Smolensky, 1983).

Circadian Rhythms and its properties

Circadian rhythms are ubiquitous and are seen in all organisms from single celled prokaryotes to multicellular organisms including humans (Figure 1). Based on the organism and the niche they occupy, the circadian rhythms are evolutionarily adapted to the continuous

changes in the environment and hence organisms have acquired an endogenous mechanism to generate and maintain a specific rhythmic pattern that suits best for its survival. This endogenously generated oscillatory mechanism exhibits the characteristics of self-sustaining oscillations, which persists even in the absence of external cues, are entrained by external or environmental cues such as the light-dark cycles, sound, etc., and are capable to drive the biological system with a period approximately equal to 24 hours, even in the absence of external cues. These rhythms are temperature compensated and proceed at the same rate within a range of temperatures. Circadian rhythms are also under genetic control, mediated by a transcriptional-translational feedback loop of several clock genes and their protein products (Klein *et al*., 1991).



(Dunlop, 1999)

Figure 1. The universal tree of life showing the diversity of the circadian timing system (Dunlop, 1999). Shown in blue are phylogenetic groups where circadian rhythms have been well-studied and in red are of those systems where the genetic and molecular analysis of clock mechanism has progressed significantly.

Origin and significance of circadian rhythms

Light is one of the main factors in the sustenance of biological life on Earth. Light influences directly the physiology of all species through the length of the daily photoperiods to which the species are exposed. Phase changes of this photoperiod have a profound influence on the physiology of most animals and especially on the reproduction in lower vertebrates. The periodic phenomena of changes from day to night and the seasonal changes in day lengths are generated by movements of the earth. Earth revolves around the sun with a time of 365 days, 48 min, and 45 sec (Figure 2). Because the axis of the Earth is tilted from perpendicular to the plane of the ecliptic by 23.458°, results in one part of the hemisphere being tilted towards the sun and the other part away from the sun. This tilting is what gives the Earth the four seasons of the year-spring, summer, autumn (fall) and winter. The rotation of earth on its axis results in the daily changes between day and night (Figure 2). The length of the daily photoperiod provides the most reliable external signal in nature that indicates the time of the year (Gwinner, 1989). Timing of events and regulation of activities according to the different seasons of the year are crucial for many organisms in temperate or higher latitudes, where conditions for feeding, survival, and reproduction vary through the year (Moore-Ede *et al*., 1982; Edery, 2000). Many organisms, therefore, have rhythms which are synchronized by the length of the photoperiod to ensure proper timing of physiological events (Sharp, 2005). Thus, in temperate regions, the change in photoperiods triggers various processes such as hibernation in mammals, migration in birds and breeding cycles (Gwinner, 2003).

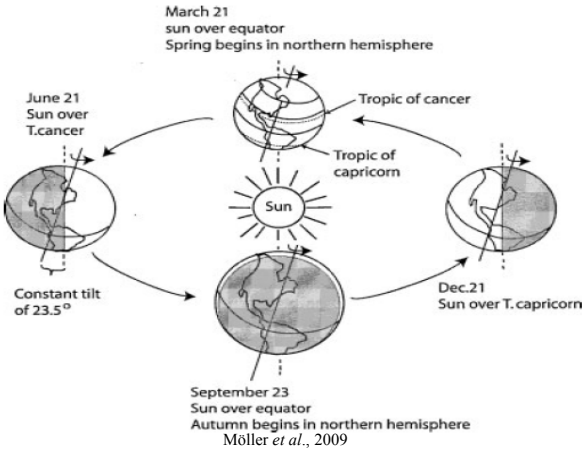


Figure 2. Illustration of movements of the earth, rotation on its own axis, and revolution around the sun (Möller *et al.,* 2009).

Animals are adapted to entrain their behavior to environmental zeitgeber, mainly the external light dark (LD) cycle and other challenges. Based on the proportion of their activity during the day, night or twilight, animals are diurnal, nocturnal or crepuscular respectively (Schumann *et al*., 2005). Diurnal animals are those that are active during the day time when they perform most of their activities such as feeding, nest preparation, etc. such as humans, degus, squirrels, etc. Nocturnal animals are active during the night phase when they forge for food, nest preparation, etc. such as rat, mouse, hamsters, etc. Crepuscular animals are active during the twilight period (i.e. during dusk and dawn) such as owls, bats, etc. Laboratories studies also show these patterns of locomotor activity with distinct difference between them (Refinetti 2006; Smale *et al*., 2008).

Entrainment to environmental time cues

Circadian rhythms in the physiology of an organism are synchronized or entrained to the external time giving cues or zeitgeber (a germen word coined by Aschoff, meaning "time-giver") (Moore-Ede *et al*., 1982) so as to ensure that behaviors and internal metabolic functions are appropriately timed to the daily events in the environment. Environmental cues that include photic, such as daily light-dark cycle, and non-photic cues, such as availability of food, water, temperature, social cues, sound cues, electromagnetic field, atmospheric pressure, etc., play an important role in entraining the daily rhythms in organisms. These zeitgebers are used under laboratory conditions such as daily LD cycle, restricted feeding (RF) schedules, etc. to understand the circadian profiles of locomotor activity rhythms and other biochemical rhythms in several animals and under such conditions the time period are referred to as Zeitgeber time (ZT). For e.g., animals that are subjected to LD 12:12 cycle, and show most of their locomotor activity during dark period and least during light period are considered as nocturnal as seen in the case of rats, mice, hamsters, etc. (Figure 3). These nocturnal animals show on-set of activity corresponding to lights off (ZT-12, which could be same or have several minutes of difference with real or clock time) when they go about their routine activities such as feeding, foraging for food, etc. The reverse is the case for diurnal animals such as humans, most domestic animals, etc.

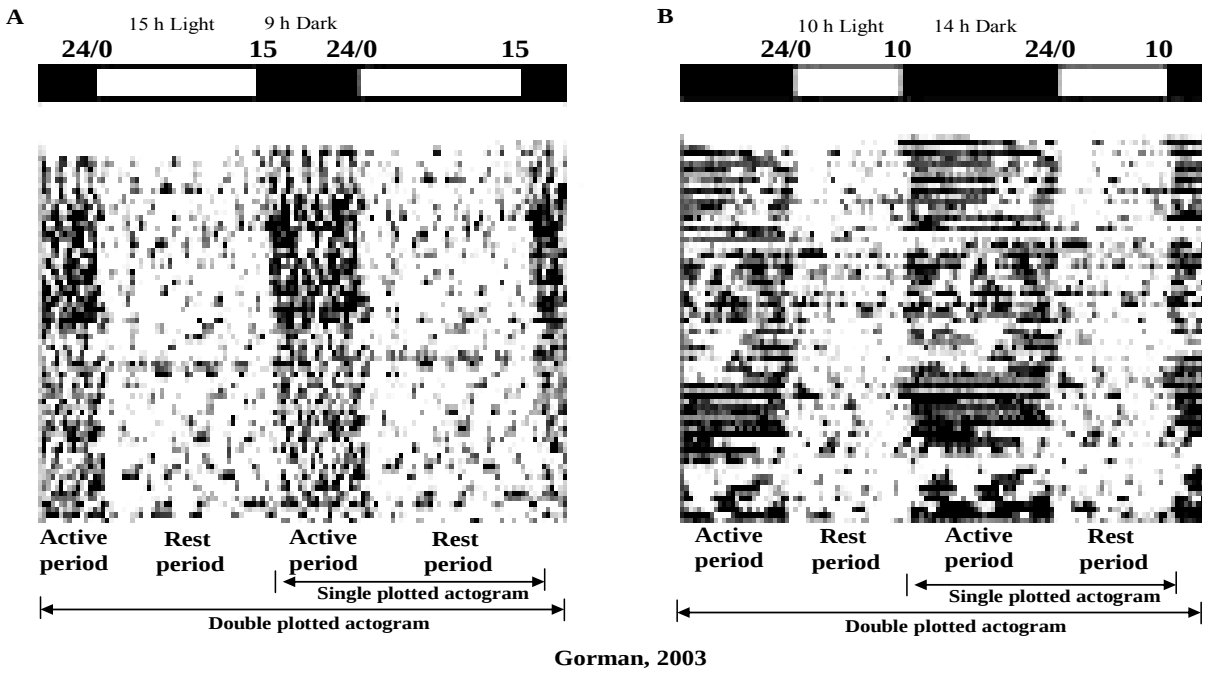


Figure 3. Double plotted actograms showing the nocturnal pattern of locomotor activity in Siberian hamsters under laboratory condition of LD cycle of 15L: 9D (A) and 10L:14D (B) Activity onset and offset corresponds to lights off and on respectively in these nocturnal rodents (Gorman, 2003).

Free-running clock

When animals are isolated from environmental time cues i.e., when placed in constant darkness (DD) or light conditions (LL) they show persisting circadian rhythms. Under these constant conditions the activity-rest cycle progressively drifts with respect to clock or real time (RT) and thus free-runs with a period either a little longer or shorter than 24 hours (Figure 4A). Free running rhythms are independent of RT and under constant conditions (DD or LL) are called circadian time (CT). These free-running rhythms are an indication that circadian rhythms are endogenous and persist even in the absence of entraining signals. Each species show a characteristic free running circadian time period Tau (τ) that may not be exactly 24 hours. For example the τ for humans is 24.2 hours which is more than 24 hours (Czeisler and Gooley, 2007). τ shows the extend of entrainment limits of the near-24-h circadian pacemaker and in humans have important implications for the entrainment of people who are exposed to longer work scheduled such as shift workers, transmeridian travelers, navy personnel, astronauts, etc. (Wright *et al*., 2001).

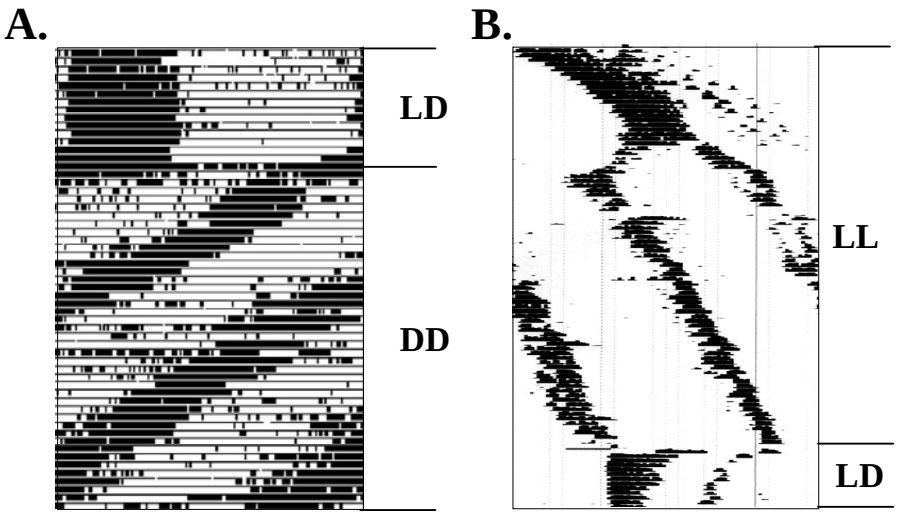


Figure 4. Actograms showing free running and splitting pattern of activity. A. The entrained pattern of temperature rhythms under LD became free-running under DD condition (Moore-Ede *et al*., 1982). B. The running wheel activity in constant LL showing splitting which entrained to LD cycle (Indic *et al*., 2008).

Splitting of rhythms

The free running and entrained rhythms that are produced by the master oscillator indicate it to be a single functional component. However, under certain conditions such as seasonal changes of day length (photoperiod), the circadian rhythm of locomotor activity dissociates into two distinct components and has been termed as splitting (Figure 4B) (Bittman *et al*., 2007). Splitting of activity rhythm has been reported in both diurnal and nocturnal rodents influenced by changes in photoperiodic condition such as lighting intensity and access to running wheel (RW) (Pittendrigh and Daan, 1976; Rosenthal *et al*., 2005). The mechanism of splitting has been studied extensively in the nocturnal hamsters (Zlomanczuk *et al*., 1991; Jagota *et al*., 2000; de la Iglesia *et al*., 2000) and has been attributed to the uncoupling of two activity components, intrinsic to the SCN, that initially free run with different periods under constant light conditions and then stabilize out of phase with each other until they become stably coupled 180° apart (Gorman and Steele, 2006). Splitting indicates a complex system of the mammalian oscillator. The circadian clock consists of two

mutually coupled oscillators differentially affected by light, one that is synchronized to the sunrise at dawn (M, for morning) and the other to the sunset at dusk (E, for evening) (Pittendrigh and Daan, 1976; Daan and Berde, 1978; Jagota *et al*., 2000; de la Iglesia *et al*., 2000). Splitting has also been reported to be seen in the rhythmicity of pineal *N*-acetyltransferase activity and electrophysiological activity in the SCN (Illnerová and Vaněček, 1982; Zlomanczuk *et al*., 1991).

Relationship between zeitgeber and locomotor activity phase

Most animals confine their activity to certain time of the day so as to maximize survival in their respective temporal niches where food availability and predator activity are cyclic. This is achieved only if the circadian activity rhythms are synchronized to environmental time cues. Under certain conditions (such as change in photoperiodic conditions or administration of a drug) the phase relationship (ψ) between a zeitgeber and a rhythm could differ either in timing (hours) or in terms of the phase angle difference (degrees). The phase angle can vary from 0º, i.e. indicating no effect of a zeitgeber, to 360º where the effect is drastic. (Moore-Ede *et al*., 1982).

The effect of a zeitgeber on the circadian timing system (CTS) depends on the sensitivity of an organism to a time cue. The sensitivity of the CTS can be studied using a phase response curve (PRC), which is a graphical representation of the relationship between the time in the animals subjective day when a light pulse is given and the phase shift obtained is plotted (Moore-Ede *et al*., 1982). PRC shows difference in phase shift that could be either an advance (+) or a delay (-) as compared to a reference phase. For e.g. if the reference phase chosen is the on-set of light in a LD cycle and the on-set of activity, and if an animals daily bout of activity began an average 2 hours before the on-set of light, then ψ has advanced + 2 hours or +30º (1 hour = 15 minutes) respectively. Photic cues being a major resetting cue induces a phase delay, a phase advance, or no phase shift at all; depending on when in its subjective day or night (time corresponding to real time when the animal is under constant conditions) is exposed to light. A representative PRC for light of a nocturnal mammal shown in Figure 5 gives the animal's responsiveness to light. Light pulse could induce phase shifts only during the subjective night with no effect during the time when it is normally exposed to

light. The PRC also documents phase advances during dawn so that the animal would start activity earlier and phase delay at dusk so that the animal would continue activity longer. The PRC shows that the natural daily light-dark cycle is constantly forcing the circadian clocks in animals forward in the morning and backward in the evening each 24-hour day, thus fine tuning of the CTS's phase each day at dawn and dusk (Moore-Ede *et al*., 1982).

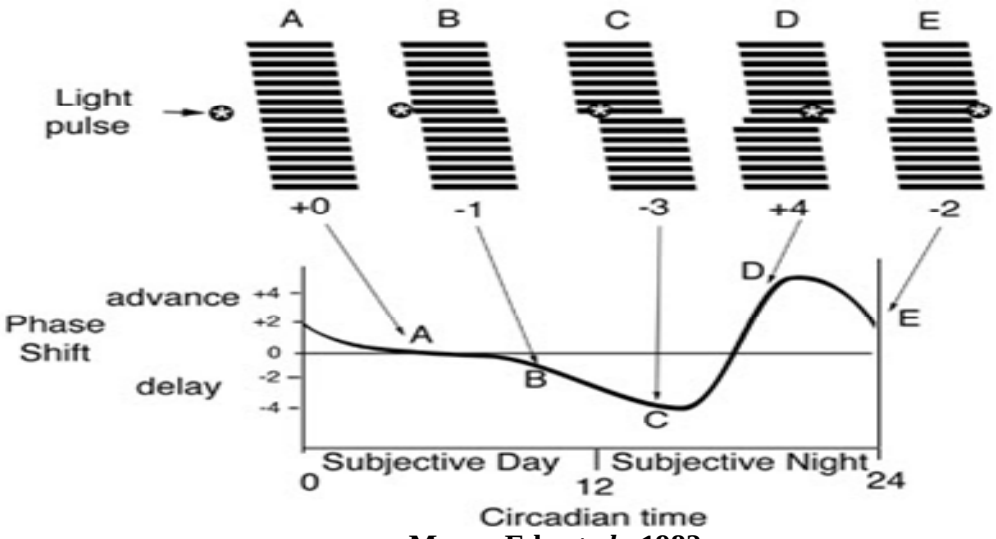


Figure 5. Illustration for the derivation of a phase response curve for a nocturnal mammal. The animal is allowed to free-run under constant darkness. Light pulses were given at various circadian times (A-E). The phase shift in hours is plotted as a function of circadian time (Moore-Ede *et al*., 1982).

Chronobiology and its relevance to humans

In mammals, the CTS influences nearly all aspects of physiology and behavior, such as sleep-wake cycles, cardiovascular activity, endocrine system, body temperature, renal activity, physiology of the gastrointestinal tract, hepatic metabolism, etc. (Reppert and Weaver, 2002). A failure of body to synchronize with external cues leads to alteration in circadian rhythm. This alteration if persists for a longer time, leads to circadian clock disorders (Figure 6). Incidence of myocardial infarction, pulmonary edema, hypertensive crisis, asthma and allergic rhinitis attacks, peak at certain times during the day (Maron *et al*., 1994; Staels, 2006; Burioka *et al*., 2007). Disruption of circadian coordination in humans is

manifested by hormone imbalance resulting in the onset of disease and leading to reduced life span (Reppert and Weaver, 2002; Penev *et al*., 1998; Kondratov *et al*., 2009).

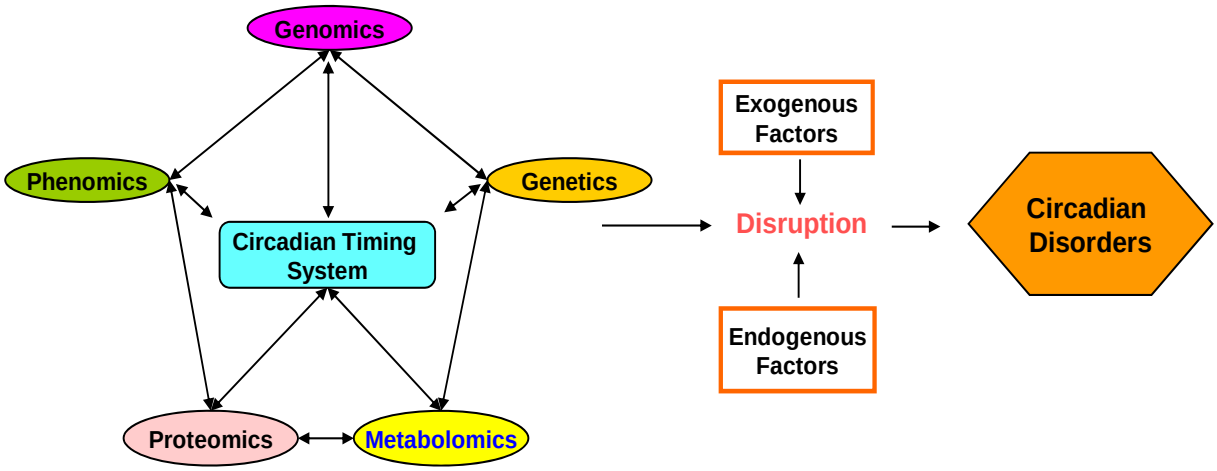


Figure 6. Circadian timing system and disorders. The CTS regulates the physiology at various levels such as phonemics, genomic, genetic, metabolomics and proteomic level. Disruption in the functioning of any one level either due to exogenous or endogenous factors results in several circadian disorders in humans.

Circadian disorders

Circadian disorders are mostly manifested as sleep disorders (Reppert and Weaver, 2002), cardiovascular diseases (Montagnana *et al*., 2009), cancer proneness and malignant growth (Filipski *et al*., 2003; Pelicano *et al*., 2005; Davis and Mirick, 2006), fatigue, disorientation, insomnia, altered hormone profiles and morbidity (Davis and Mirick, 2006; Hofman and Swaab, 2006; Gibson *et al*., 2009; Froy and Miskin, 2010). Some of the disorders seen in humans are described below:

Rapid Time-Zone Change Syndrome (Jet Lag)

The jet lag syndrome is a disorder due to modern life style changes in humans being subjected to time zone change. The typical symptoms of jet lag include sleep disruption, fatigue, difficulty concentrating, gastrointestinal distress, impaired psychomotor coordination, reduced cognitive skills, and alterations of mood (Sack, 2009).

Shift work Syndrome

With rapid industrialization and advancement in global economy, many occupations require unusual work hours that include professions such as health care workers, police and security guards, truck drivers, workers in heavy industry, etc. The major symptom of shift work disorder is impaired sleep, due to altered nighttime melatonin levels and reproductive hormone profiles that could increase the risk of hormone-related diseases, including cancers (Davis and Mirick, 2006).

Delayed Sleep Phase Syndrome (DSPS)

DSPS is characterized by a persistent inability to fall asleep and arise at conventional clock times i.e., the phase of the sleep-wake cycle is delayed. Sleep onset is usually delayed until early morning, with a consequent delay in rising. This syndrome has customarily been viewed as a consequence of choice, or lifestyle changes in some individuals while in others it is clearly a disorder of entrainment, a failure of pacemaker sensitivity to light in the phase-advance portion of the light PRC (Moore, 1997).

Advanced Sleep Phase Syndrome (ASPS)

Individuals with ASPS have persistent early onset of sleep with consequent awakening. Attempts to delay sleep onset in these individual results in severe evening fatigue and are mostly found in the elderly population (Chang, 2009).

Non–24-Hour Sleep-Wake Syndrome

This syndrome is usually associated with congenital and acquired blindness resulting in failure of entrainment of the SCN to external environmental cues (Skene and Arendt, 2007). This syndrome is also manifested in normal subjects with symptoms similar to ASPS or DSPS, with reports of an abnormal timing of melatonin production (Czeisler *et al*., 1995).

Irregular Sleep-Wake Pattern Syndrome

Individuals affected with irregular sleep-wake pattern syndrome have an irregular distribution of sleep and wake with numerous interruptions. These individuals complain both of their difficult sleep-wake schedule, insomnia and of daytime fatigue (Cohen and Albers, 1991).

Seasonal Affective Disorders (SAD)

Seasonal affective disorder (SAD) is characterized by recurrent depressions in fall and winter (Roecklein *et al*., 2009). SAD is manifested as disturbances of mood associated with disturbances of sleep, appetite, libido, cognition and memory (Figure 7) (Moore, 1997; Hajak and Landgrebe, 2010).

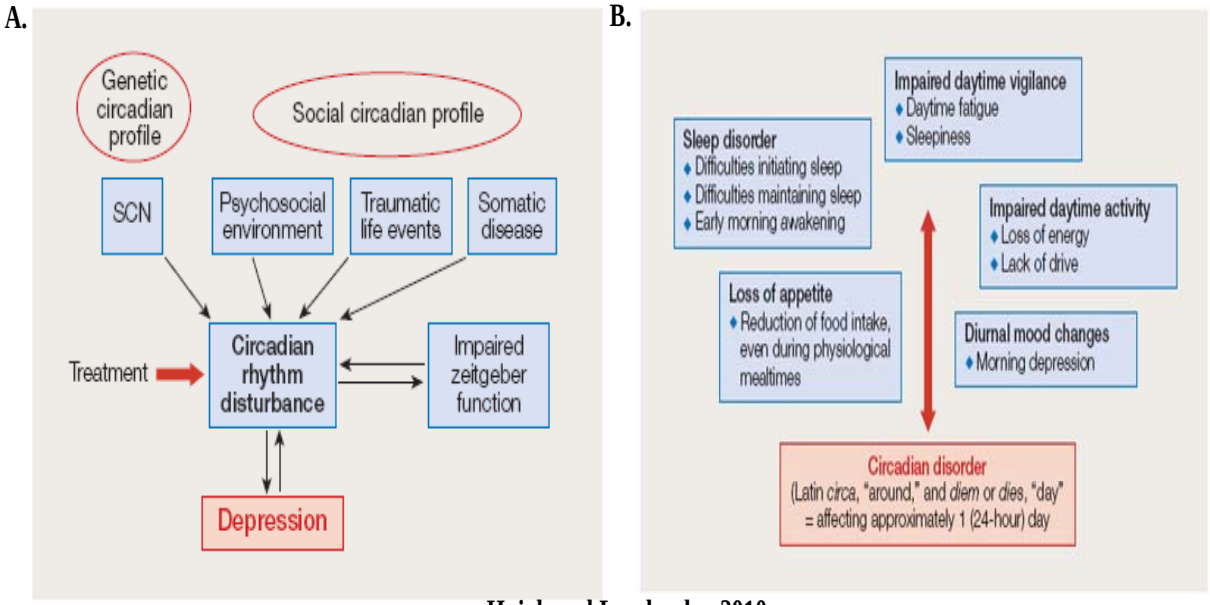


Figure 7. Depression and circadian rhythms A. causes of depression. B. Clinical symptoms of depression (Hajak and Landgrebe, 2010).

With several reports showing the apparent role of the circadian systems diverse and significant role and its implications for human health and disease, an understanding on the underlining mechanism of circadian disruption is more widely recognized. Several drugs exhibit circadian rhythm in their toxicity and therapeutic effect and this in turn have been used to determine the effective dosage and least toxicity timing regimes.

The Circadian Timing System in mammals

For an organism to adopt its physiology to changing environmental conditions, information from outside has to reach body structures that can integrate this information and send appropriate signals to various tissues (Jagota *et al*., 2000; Jagota, 2006; Dibner *et al*,

2010). This function is mediated by the CTS in all organisms, playing an important role in regulating and coordinating internal metabolic processes with the environment. The function of the CTS can be described by three elements: (i) the input or afferent pathways that relay external cues to the (ii), master oscillator which generates entrained daily rhythms that are conveyed through the (iii) output or efferent pathways and are manifested in the physiology and behavior of the organism (Figure 8) (Jagota, 2006).

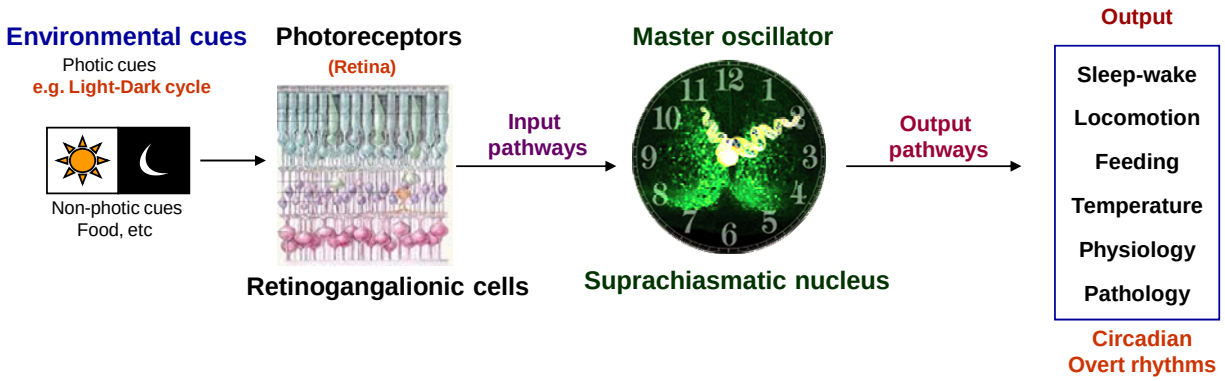


Figure 8. The mammalian circadian timing system. It consists of three components: the input pathway that receives the external cues such as the retinoganglionic cells in the retina, the master oscillator (suprachiasmatic nucleus) that generates entrained circadian rhythms and the output pathways that convey the generated rhythms to the entire system and are manifested as the physiology and behavior of the mammal.

Suprachiasmatic nucleus

The SCN is the master oscillator in mammals and consists of a bilaterally symmetrical group of neurons, located in the anteroventral hypothalamus immediately above the optic chiasm on either side of the third ventricle (Figure 9) (Klein *et al*., 1991).

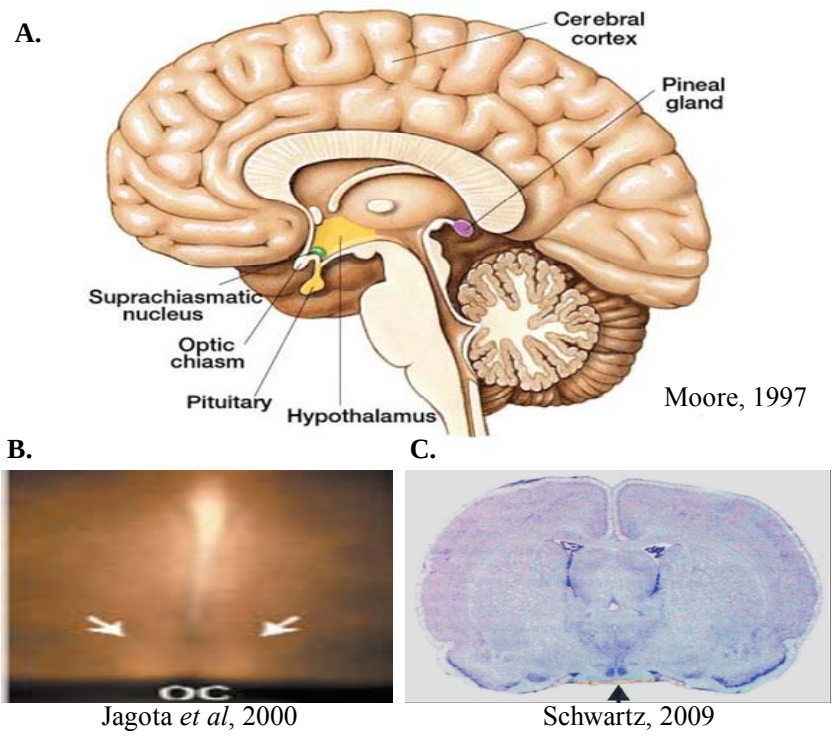


Figure 9. The suprachiasmatic nucleus. A. Location (**Moore, 1977**) B. Thick coronal section (Jagota *et al*., 2000) and C. Cresyl violet staining of the coronal section (Schwartz, 2009). The SCN is a bilateral nuclei located above the optic chiasm (OC) on either side of the third ventricle.

Histological studies using serial sections of the brain show that the SCN of male rat is about 0.13 to 0.16 mm^3 in volume. The longest dimension of the SCN is 950 μm in the rostro-caudal direction, with a width of 425 μm and a height of 400 μm. Based on Kluver-Barrera stain for nissel substances, the neuronal population of the rat SCN is about 8000 in each nucleus or 16000 in the paired SCN (van den Pol, 1980). In the mouse, each unilateral SCN contains ~10,000 neurons with cell bodies of fairly small (~10 μm) size with simple dendritic arbors that are closely apposed (Leak *et al*., 1999). The SCN neurons are distinguished by their neurochemical content and are differentiated in two anatomic subdivisions; a ventral "core" region, which receives the optic chiasm and retinal input, and a dorsal "shell" region, which partially envelops and receives input from the core (Shigeyoshi *et al*., 1997; Abrahamson and Moore, 2001; Jagota, 2006) (Figure 10).

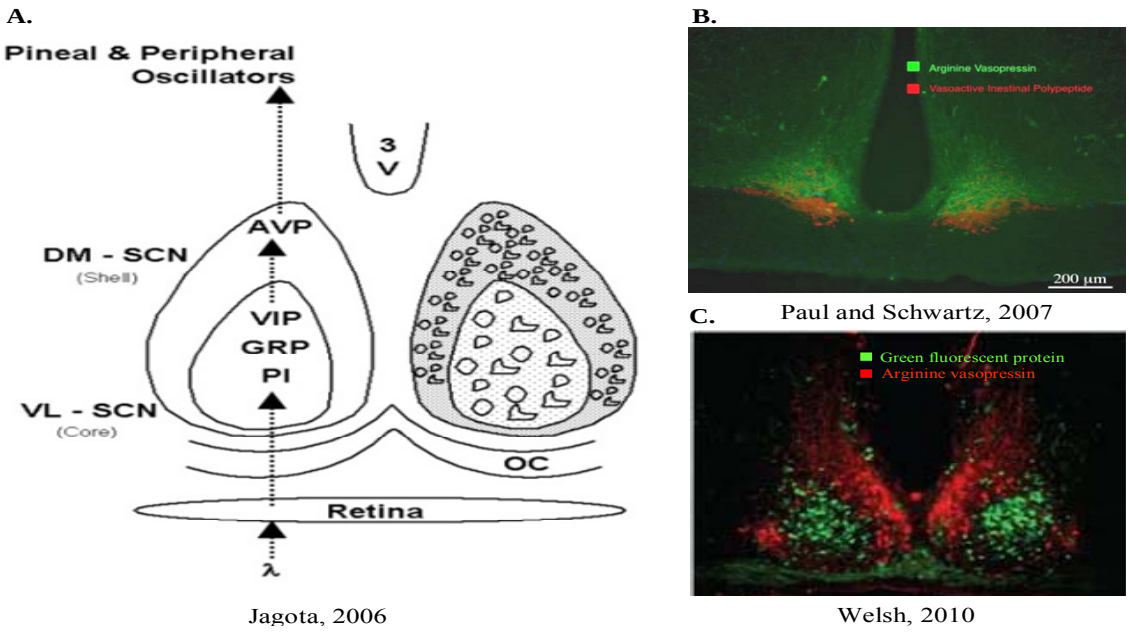


Figure 10. Core shell differentiation of the SCN. A. Diagrammatic representation of the neuropeptides in the core (ventrolateral) and shell (dorsomedial) regions of the SCN (Jagota, 2006). B. Double-label immunofluorescence of the coronal section of the SCN showing the ventral or core neurons expressing vasoactive intestinal polypeptide (VIP) (*red*) and the dorsal or shell region neurons showing arginine vasopressin (AVP) (*green*) (Paul and Schwartz, 2007). C. Coronal section of mouse SCN, showing the ventral region delineated by green fluorescent protein (GFP) expressed in gastrin releasing peptide (GRP) neurons (*green*) and the dorsal shell region delineated by immunofluorescent labeling for AVP (*red*) (Welsh *et al*., 2010).

The SCN is also composed of glial cells that contribute to pacemaker functions. Glial astrocytes exhibit circadian rhythms of clock gene expression (Prolo *et al*., 2005), and immunolabeling for glial fibrillary acidic protein (GFAP) exhibit remarkable circadian variations (Lavialle and Servi`ere, 1993). Studies show that GFAP mutant mice have altered locomotor activity rhythms in constant light (Moriya *et al*., 2000). Drugs that specifically inhibit glial cell metabolism such as Fluorocitrate, disrupts neuronal firing rhythms in SCN slices (Prosser *et al*., 1994). Disruption of glial cell signaling affects neuronal function in other brain areas as well (Nedergaard, 1994; Fellin and Carmignoto, 2004; Pascual *et al*., 2005; Welsh *et al*., 2010).

Input or afferent pathway

The SCN receives photic and non-photic information via three major input pathways: the retinohypothalamic tract (RHT), the geniculohypothalamic tract (GHT), and retino-raphe pathway (Figure 13) (reviewed in Jagota, 2006). Light information is perceived by the retina of the eye, where non-image forming photoreceptors termed photosensitive retinal ganglion cells (pRGC) (Berson *et al*., 2002; Moore *et al*., 1995; Provencio *et al*., 1998), which express the photopigment melanopsin (a homolog of a photoreceptor in amphibian skin) (Provencio *et al*., 2000; Gooley *et al*., 2001; Hattar *et al*., 2002), send photic information directly to the SCN via the RHT. From these three input pathways the RHT mediates photic information whereas the GHT and the raphe nuclei SCN pathway provide non-photic information to the SCN (Figure 11) (Jagota, 2006; Dibner *et al*., 2010).

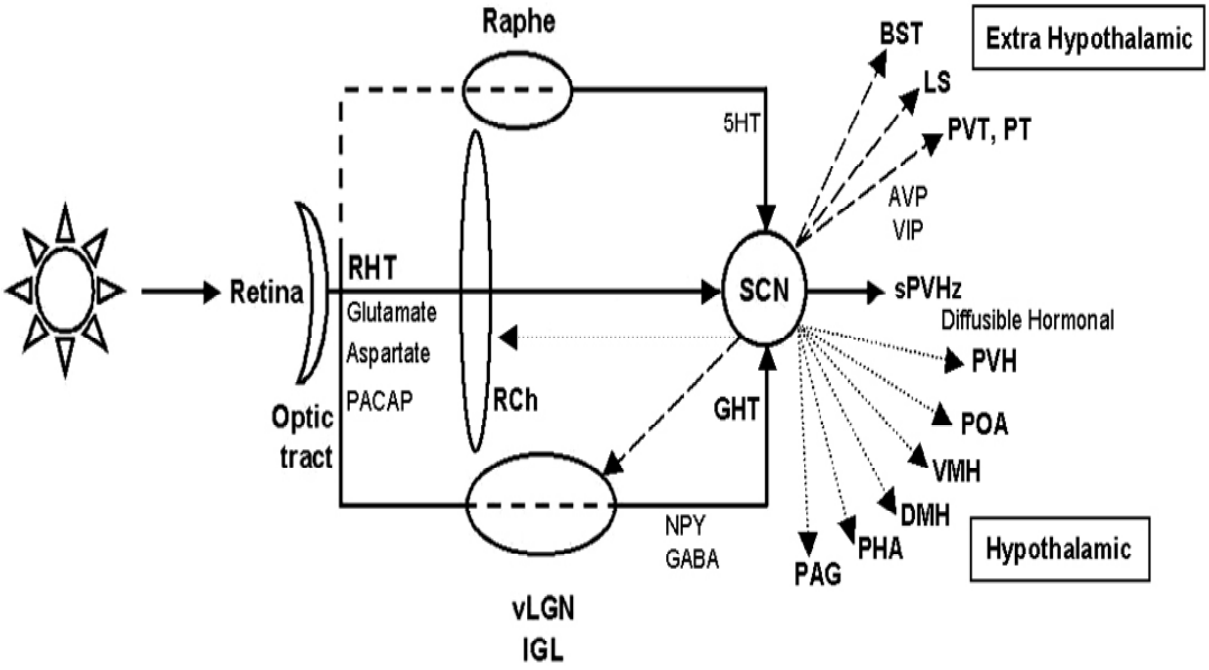


Figure 11. Input and output pathways and neurotransmitters. (i) Afferents of SCN: RHT - retinohypothalamic tract; GHT – geniculohypothalamic tract via ventral lateral geniculate nucleus (vLGN) and the intergeniculate nucleus (IGL); from median and dorsal raphe (ii) Efferents of the SCN towards various extra hypothalamic and hypothalamic targets: BST, bed nuclei of the stria terminalis; LS, lateral septal nucleus; PVT/PT, paraventricular nucleus of the thalamus, paratanial nucleus; IGL, intergeniculate leaflet; AHA anterior hypothalamic area; PVH, paraventricular nucleus of the hypothalamus; MPQ and POA, preoptic area nuclei; RCh, retrochiasmatic area; VMH, ventromedial nucleus of the hypothalamus; DMH, dorsomedial nucleus of the hypothalamus; ZI, zona incerta; PHA, posterior hypothalamic area; PAG, periaqueductal gray (Jagota, 2006).

Output or efferent pathways

The SCN mediates its function as a pacemaker and synchronizer to other brain regions and peripheral oscillators either via neural connections or by chemical messengers. Antero- and retrograde tracers studies show SCN afferents to terminate to different regions of the brain that are responsible for complex functions such as emotion, learning, memory, etc. (Figure 12) (Watts and Swanson, 1987). Within the hypothalamus, SCN afferents terminate most densely in the subparaventricular zone (sPVZ) (Morin *et al*., 1994) and rostrally in the preoptic area (POA), the bed nucleus of the stria terminalis (BNST), and the lateral septum (LS). Dorsally, they terminate in the dorsomedial hypothalamus (DMH) and the arcuate nucleus (ARC). In the thalamus, axons from the SCN innervate the paraventricular nucleus (PVN) and possibly the IGL. Inter species variation in the specific subdivisions within the SCN that project to designated areas of the brain as well as the patterns of projections can also differ especially between diurnal and nocturnal animals (reviewed in Jagota 2006; Morin and Allen, 2006; Morin, 2007).

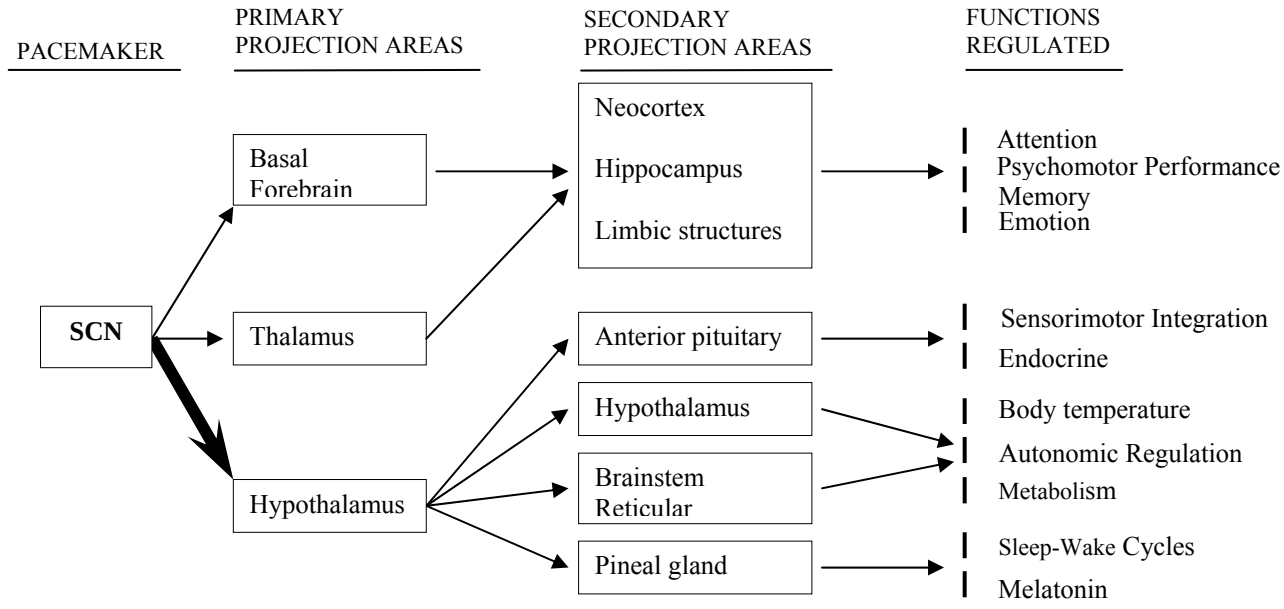


Figure 12. Projections of the SCN pacemaker and functional correlations. The SCN has limited primary projections but widespread secondary projections to areas involved in control of the many complex functions (Morin, 2007).

Neuroactive components of the SCN

The SCN functioning is mediated by several neuroactive compounds that include neuropeptides, such as vasoactive intestinal polypeptide (VIP), arginine vasopressin (AVP), gastrin releasing peptide (GRP), neuropeptive-Y (NPY), neurotensin (NT), etc., neurotransmitters, such as serotonin, γ aminobutric acid, glutamate, etc., and neurohormones e.g. melatonin, etc.

Though anatomically distinct nuclei, the SCN can be demarcated into less defined core and shell regions based on different neuropeptides expressed by the neurons. Within each unilateral core regions are ~1100 neurons containing VIP that comprises 10% of all SCN cells and a smaller numbers containing calretinin (900 cells), neurotensin (NT; 700 cells) and gastrin releasing peptide (GRP; 500 cells). The shell region consists of ~2100 neurons containing AVP that comprises ~20% of all SCN cells, ~1100 containing angiotensin II, and ~500 containing met-enkephalin. Most of the different types of neurons of the SCN are co-localized with GABA as well as glutamenergic neurons with most synapses among SCN neurons being GABAergic (Moore and Speh, 1993; Strecker *et al*., 1997; Cui *et al*., 2001). The neurons of the core region projects densely to the shell region, where as there is only sparse projection back to the core region (Figure 10) (Leak *et al*., 1999). The intrinsic anatomy of the SCN and neuropeptides vary considerably across species (Morin *et al*., 2006) and are more complex in its function (Jagota, 2006; Morin, 2007).

The SCN mediates its oscillatory functions, despite of heterogeneity in functional and neurochemical organization, by coordinated activity of all the cells. Photic information received from retino-recipient cells (Ohta *et al*., 2005) causes the expression of GABA in most of the SCN neurons that result in expression of VIP, GRP and prokineticin 2. Neurotransmitters released by neurons of the ventral part of the SCN have been shown to be necessary for maintaining synchrony of the whole SCN (Aton and Herzog, 2005). Output message from the SCN is mediated mainly by vasopressin (AVP) expressed by the neurons of the shell region (Figure 11) (reviewed in Jagota, 2006).

Vasoactive intestinal polypeptide (VIP)

VIP, initially identified as a gut polypeptide, has also been identified as one of the main functional neuropeptide of SCN neurons that is responsive to photic stimulation. In

the ventrolateral SCN, VIP expressing neurons receive direct retinal inputs and response to photic stimulation (Figure 10) (reviewed in Ibata *et al*., 1997; Jagota, 2006). Exogenous application of VIP also resets the circadian clock in a manner similar to that of photic resetting, both *in-vitro* and *in-vivo* conditions (Piggins and Cutler, 2003). In rats there are two types of VIP neuronal components in the SCN, a medial GRP-free group that do not co-express GRP and a lateral group that co-express GRP (Kawamoto *et al*., 2003). The lateral group also expresses the clock gene *period* (*per*) *1* following a light pulse stimulation, however, few VIP-containing cells rhythmically express both *per1* and *per2* (reviewed in Dardente *et al*., 2002; 2004; Kawamoto *et al*., 2003).

VIP is synthesized as a pre-pro VIP and is cleaved to produce VIP and peptide histidine isoleucine (PHI). PHI is co-localized to VIP neurons in the SCN (Stopa *et al*., 1986; Card *et al*., 1988). VIP and PHI are structurally related to PACAP. VIP mediates its function via its receptor VPAC2, also known as VIPR-2, and is expressed in about 60% of the SCN neurons. These neurons respond to VIP with changes in firing rate (Reed *et al*., 2002; Cutler *et al*., 2003). VIP act through VPAC2 and participate in both resetting by light and maintenance of ongoing rhythmicity in the SCN (Piggins and Cutler, 2003). VIP along with GRP and AVP show circadian variation in the level of mRNA in constant environmental conditions (Dardente *et al*., 2004). In DD condition both VIP and GRP do not exhibit rhythmicity but show rhythmicity only in LD condition (Takeuchi *et al*., 1992; Shinohara *et al*., 1993; Ban *et al*., 1997). Light affects changes in the synthesis and release of peptides that shows circadian variation (Shinohara *et al.,* 1993; 2000). It has been reported that treatment of SCN slices with VIP produces phase shifts similar to those induced by light pulses (Reed *et al*., 2001). Nielsen and co-workers (Nielsen *et al*., 2002) showed that VIP induces *per1* and *per2* gene expression in rat SCN in a phase dependent manner. More recently, VIP has been shown to be necessary for the coordination of daily rhythms in behavior and physiology at the level of biological clock in mice (Aton *et al*., 2005). Loss of internal de-synchronization and its subsequent restoration were achieved by adding VIP into the mice cells. Hence, VIP signaling through its receptor serves two important functions in the SCN, namely, circadian rhythmicity in a subset of neurons and maintenance of synchrony between intrinsically rhythmic neurons. VIP-expressing neurons themselves are circadian pacemakers

in the SCN for establishing and synchronizing rhythmic activity (reviewed in Reghunandanan and Reghunandanan, 2006).

Arginine Vasopressin (AVP)

AVP is a major neuropeptide identified in the SCN and is expressed by neurons in the dorsomedial part of the SCN (Castel *et al*., 1990; van den Pol and Gorcs, 1986). AVP expressing neurons receive information the ventrolateral region of the SCN and relay it to near by brain regions that regulate several physiological functions (Figure 10) (reviewed in Ibata *et al*., 1997; Jagota, 2006). Nearly one third of the SCN neurons in rats synthesize AVP (van den Pol and Tsujimoto, 1985; Ingram *et al*., 1996; 1998). AVP synthesis and secretion by the SCN neurons exhibits a circadian pattern. AVP exhibits an excitatory role by activating V1a receptors resulting in increased amplitude of firing rates in the SCN during subjective day and enhanced SCN output (reviewed in Mihai *et al*., 1994; Ingram *et al*., 1996; 1998).

Abnormalities in AVP expression have been shown to affect expressed rhythms as reported from studies on AVP-deficient Brattleboro rats. Local application of AVP into the SCN does not affect the free running circadian wheel running rhythm or entrained circadian food intake and water intake in hamsters (Albers *et al*., 1984; Reghunandanan *et al*., 1987; Reghunandanan *et al*., 1992). Transplantation studies in rats show vasopressin to have a supportive role in SCN circadian functions (De Coursey and Buggy, 1986; Lehman *et al*., 1987). Antagonist to V1 receptor has been reported to produce no significant effect on the wheel running activity in rats, thereby indicating no role for AVP in the generation of circadian rhythms (Stoynev and Nagai, 1996). In depressed patients, both synthesis and release of AVP in the SCN is reduced, which leads to an impaired functional activity of the circadian clock, although there was an increase in the number of AVP-ir neurons (Zhou *et al*., 2001). *In- vitro* studies show long-term AVP transcription in the SCN that indicates circadian rhythmicity and is dependent on the ongoing electrical and synaptic transmission in the cultures (Arima *et al*., 2002).

AVP mediates its excitatory actions via V1 receptors, however the role of V1a or V1b subtypes in the SCN is not known. A decrease in the AVP neurons and AVP content in the

SCN has been associated with decreased amplitude of activity rhythms, increased rhythm fragmentation and disruption of the normal sleep/wake cycle (Hofman and Swaab, 1994; 1995; Lucassen *et al*., 1995). However, in the study by Hochstetler *et al* (2004) no difference in activity level and circadian expression were associated with differences in the number of AVP-ir cells in the SCN. Studies in aged male rats show a decrease in the amplitude of the daily rhythm in the expression of V1a receptor mRNA along with persistently elevated level for V1b mRNA as compared to young adult ones (Kalamatianos *et al*., 2004). SCN-AVP expression and circadian organization of locomotor behavior has been shown across species including rats (Isobe and Nishino, 1998) and hamsters (van der Zee *et al*., 2002). However, transplantation studies indicate some other diffusible factor other than AVP to play a role in the regulation of circadian rhythmicity (reviewed in Boer *et al*., 1999).

Serotonin (5 -HT)

5-HT (5-Hydroxytryptamine (5-HT) is a monoamine neurotransmitter. It is derived from the essential amino acid L-tryptophan. Serotonin is primarily found in the gastrointestinal (GI) tract, platelets and in the central nervous system (CNS) of animals including humans (Berger *et al*., 2009). Serotonin and its receptors are important in the regulation of virtually all brain functions, and dysregulation of the serotonergic system has been implicated in the pathogenesis of many psychiatric and neurological disorders (Roth, 1994; Roth and Xia, 2004). The behavioral and neuropsychological processes modulated by serotonin include mood, perception, reward, anger, aggression, appetite, memory, sexuality, attention, etc. In the SCN, serotonin modulates photic response through its receptors 5-HT_{1a}, 5-HT_{1b}, 5-HT_{2C}, 5-HT_{5a} and 5-HT_{7} (Moyer and Kennayway, 1999; Gannon, 2001; Viyoch *et al.,* 2005).

5-HT is the major neurotransmitter involved in the retino-raphe SCN pathway with dense serotonergic projection from midbrain raphe nuclei terminating predominantly in the retino-recipient region of the SCN (Moore *et al*., 1978). *In-vitro* and *in-vivo* studies using 5-HT agonists induces phase shifts in the SCN rhythmicity when administered at times in the circadian cycle during which light does not cause phase shifts (Medanic and Gillette, 1992; Edgar *et al*., 1993). In rat, lesion of raphe nuclei reduces the amplitudes of circadian activity rhythm with persistent rhythmicity (Block and Zucker, 1976). Serotonergic projections from

the input pathways terminates to the VIP containing neurons in the ventrolateral part of the SCN (Okamura *et al*., 1987) and plays a major role in the modulation of the pacemaker responses to light. In *in-vitro* studies, serotonin along with GABA advances the phase of the circadian pacemaker during the day and delays it at night (Prosser, 2000). Serotonin has also been shown to regulate both the pre- and post synaptic inhibitory mechanisms of neuronal excitation in SCN neurons (Jiang *et al*., 2000). 5-HT and 5-HT agonists inhibit optic nerve-induced field potentials in the SCN slice preparation and also light induced Fos expression and phase shifts of the circadian rhythm of wheel-running activity (Rea *et al*., 1994). 5-HT antagonists increases light-induced firing rates of SCN neurons (Ying and Rusak, 1994) and light induced phase shifts (Rea *et al*., 1995). Serotonin also plays a role in setting overall metabolic rate and temperature control. Serotonin-deficient mice show a rapid hypothermic response when placed in a cold environment (Hodges *et al*., 2008), a response that is mediated by hypothalamic 5-HT1A and 5-HT7 receptors (reviewed in Hedlund *et al*., 2004).

Melatonin

Melatonin, a derivative of serotonin is a neuro-hormone synthesized mainly by the pineal gland an important component of the CTS, is an effector follower system for SCN (Figure 13). It plays a major role in the regulation of various physiological, endocrine and behavioral functions (Malpaux *et al*., 2001). The efferent signals from the SCN relay the external light–dark information to pineal gland via the intermediolateral cell column and superior cervical ganglion (SCG) (Figure 13) (Foulkes *et al*., 1997). In response to the onset of darkness noradrenaline secreted from SCG nerve terminals binds to β-receptors on pinealocytes. This activates adenylate cyclase and accelerates cAMP production. Arylalkylamine N-acetyltransferase activity (AANAT), a rate-limiting enzyme involved in melatonin synthesis from serotonin is activated downstream of cAMP and the synthesized melatonin is then released into capillaries for extracellular actions (Jagota *et al*., 1999; reviewed in Jagota, 2006; Chattoraj *et al*., 2009).

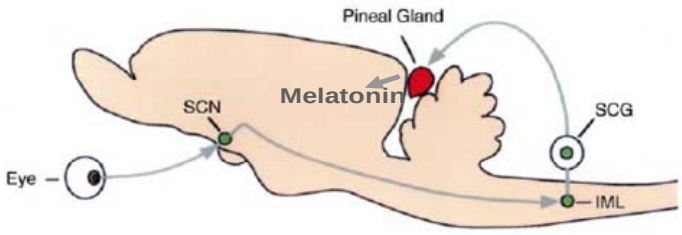


Figure 13. Diagrammatic representation of the location of pineal gland and the neuronal pathway regulating pineal melatonin synthesis in the rodents. Photic information is transmitted to the pineal through the suprachiasmatic nucleus (SCN) and the superior cervical ganglion (SCG). IML: intermediolateral cell column (Foulkes *et al*., 1997).

Melatonin acts as the messenger of darkness exhibiting marked daily rhythm in its concentration with higher night time levels (von Gall and Weaver, 2008).The primary roles of melatonin in mammals is to entrain the SCN clock to ensure synchronicity with the environment, to regulate shedding of photopigment disc, phagocytosis and the inhibition of retinal dopamine release and to regulate the seasonal changes in reproductive activity in response to changes in daylength (Foulkes *et al*., 1997). Melatonin mediates its biological effects by binding to high affinity receptors belonging to the G-protein coupled receptor superfamily such as Mel_{1a}, Mel_{1b} and ML_2 (von Gall and Weaver, 2008; Berra and Rizzo, 2009). The mammalian Mel_{1a} receptor is selectively concentrated in the SCN and pars tuberalis (Reppert *et al*., 1994) while Mel_{1b} is expressed in the retina and, at low levels, in the whole brain and hippocampus (Reppert *et al*., 1995).

A change or decrease in levels of melatonin has been implicated in various circadian disorders. Exogenous melatonin has been shown to suppress neuronal SCN activity towards night time (van den Top *et al*., 2001), lowers VP secretion from SCN neurons of rats (Watanabe *et al*., 1998) and also accelerates sleep initiation in humans at circadian phases when the SCN would normally stimulate waking (Dollins *et al*., 1994), as an immediate effect. The long term effect of melatonin includes phase shifting and amplification of circadian rhythmicity of the SCN. Melatonin application has been found to be useful in synchronizing the endogenous circadian rhythms in people affected from jet lag, blind individuals (Lockley *et al*., 2000; Sack *et al*., 2000; Jagota and Kalyani, 2010), patients with dementia (Mishima *et al*., 2000), and shift workers (Sack and Lewy, 1997). Melatonin thus is a promising chronobiotic agent for treatment of circadian disorders. Revell and co-workers

reported that administration of a combination of morning intermittent bright light and afternoon melatonin along with a gradually advancing sleep schedule can advance circadian rhythms almost an hour a day, with very little circadian misalignment. These combination protocols have been applied for jet travel or for delayed sleep phase syndrome to evoke a phase advance of the circadian clock (Revell *et al.*, 2006).

Indole metabolism: Serotonin and Melatonin biosynthesis and degradation

L-tryptophan, an essential amino acid, is mainly absorbed from the diet. Tryptophan is hydroxylated to 5-hydroxytryptophan (5-HTP) by *tryptophan 5-hydroxylase* and then decarboxylated to 5-HT by *5-hydroxytryptophan decarboxylase*. 5-HT is further oxidized by *monoamine oxidase* (MAO) to form 5-hydroxytryptophan acetaldehyde which is then dehydrogenated to 5-hydroxy-indoleacetic acid (5-HIAA) by *aldehyde dehydrogenase* (Garattini and Valzelli, 1965). 5-HIAA is further reduced to 5-hydroxytryptophol (5HTOH) by *aldehyde reductase* that is further acted upon by *hydroxyindole O-methyltransferase* (HIOMT) to form 5-methoxytryptophol (5MTOH). 5-HT is also acetylated to N-acetyl serotonin (NAS) by *arylalkylamine N-acetyltransferase* (AANAT). NAS is further methylated to melatonin (MEL) by HIOMT. 5-HT is also converted to 5-methoxyindole acetic acid (5-MIAA) by HIOMT, MAO and aldehyde dehydrogenase. Tryptophan also undergoes decarboxylation to form tryptamine which is then converted to N-acetyl tryptamine (NAT) by *serotonin N-acetyltransferase* (Reiter *et al*., 2007). In the liver the hydroxyl groups of indoles is conjugated with glucoronate or sulphate and are excreted in the urine (Garattini and Valzelli, 1965) (Figure 14).

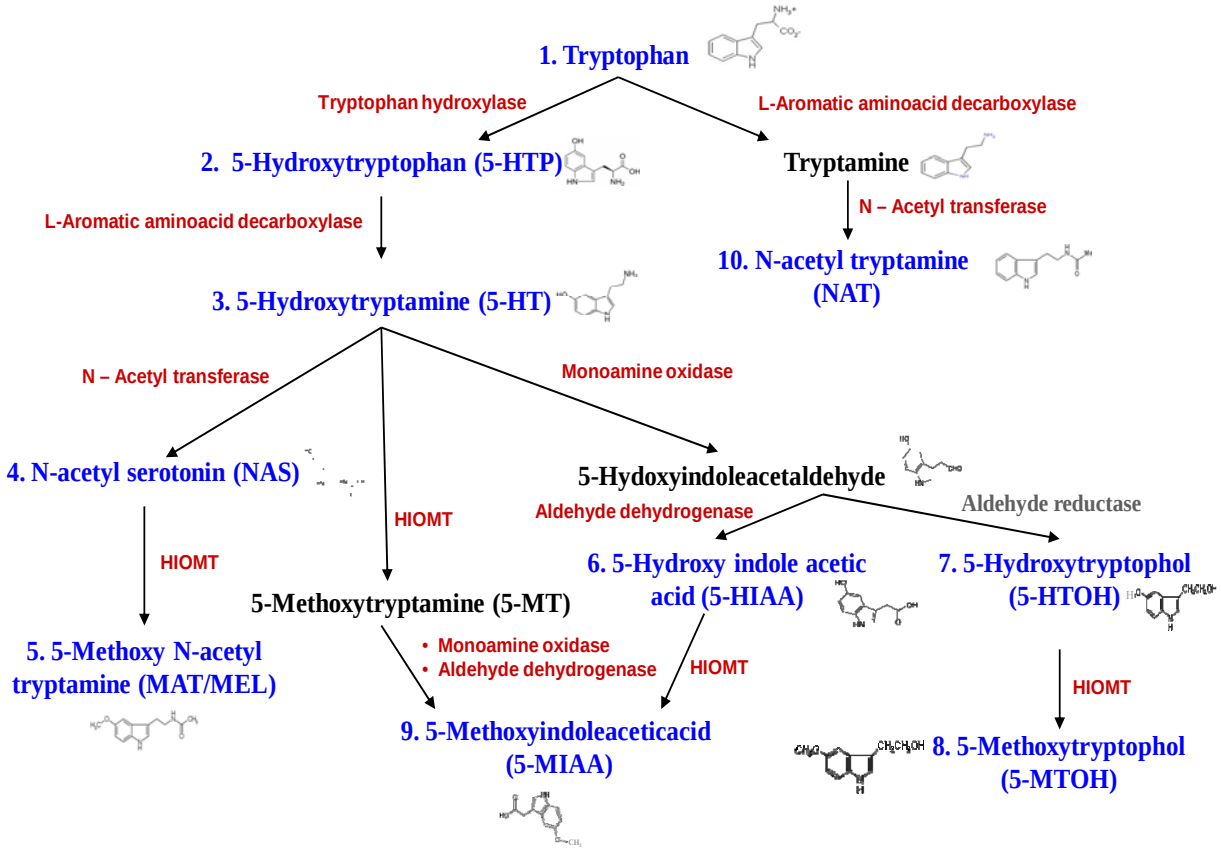


Figure 14. Metabolic pathways of indoles showing the biosynthesis of serotonin and melatonin and their catabolism (Garattini and Valzelli, 1965).

Indole metabolites play a significant role in the physiology of organisms. 5-HT acts as a neurotransmitter and neuromodulator in both the central and entric nervous system of mammals with circadian rhythmicity in its levels (Squires *et al*., 2006). In the pineal, melatonin acts as a messenger of darkness and its levels mediate the daily sleep-wake cycle. A change in the level of melatonin has been implicated in several sleep disorders (Abbas *et al*., 2010). Melatonin also acts as an antioxidant in the brain and peripheral tissues (Reiter *et al*., 2007). L-tryptophan, an essential indole amine, its levels in the plasma play a determining role as it effects the production of 5-HT and melatonin and thus consequently increase susceptibility for depressive mood and other neuropsychiatric symptoms (Maes *et al*., 1994). Decrease in levels of plasma L-tryptophan levels upon aging have been shown to impair of T-cell responsiveness and thus implicated in age associated development of immunodeficiency (Frick *et al*., 2004). 5-HTP and 5-MTOH have been shown to induce

sleep in mice (Feldstein *et al*., 1970). Studies of 5-MTOH in pineal have been shown to have seasonal variation and play a role in hibernation in rodents (Lakhdar-Ghazal *et al*., 1992). In humans, 5-MTOH has been shown to modulate sexual development and reproductive cycles. Studies on human plasma levels of 5-MTOH show major variations with menstrual cycle and highest values were found in the first 9 days of the cycle and dropping to the lowest in the final third of the cycle (Hooper *et al*., 1979). Age related decrease in levels of pineal 5-MTOH have been reported in humans (Skene *et al*., 1990). The plasma levels of 5-MTOH levels showed a drastic drop upon hypoglycemic stress that recovered as the blood glucose levels returned to normal, thus playing a role in glucose metabolism (Hooper *et al*., 1979). It has been reported by some workers that 5-MTOH also has antioxidant activity as it enhances the activities of superoxide dismutase and glutathione reductase, lowers the ratio of oxidized to reduced glutathione, and preserved membrane fluidity during oxidative stress (Zawilska *et al*., 2002).

Serotonin Chrono-metabolomics

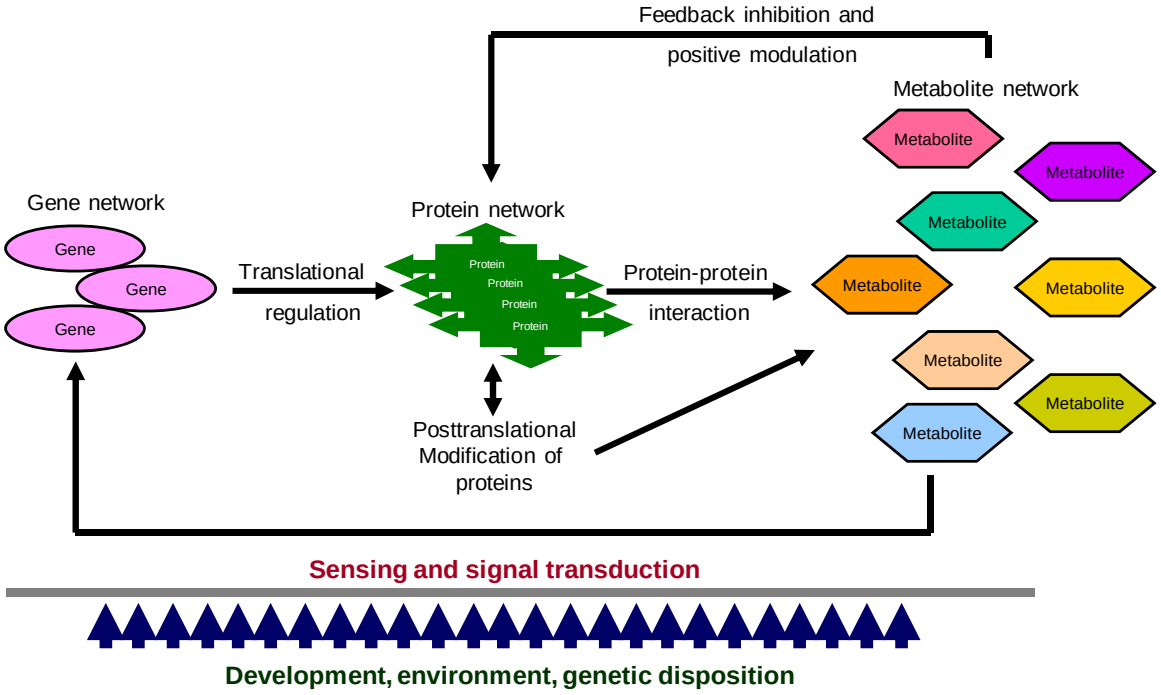


Figure 15. Amplification of a metabolic network and feedback regulation in response to developmental and environmental conditions (Weckworth, 2003).

Changes in the metabolome of an organism are an ultimate validation to genetic alterations, disease, or environmental influences (Weckwerth, 2003) (Figure 15). As metabolome represents the collection of metabolites in a biological sample, which are the end products of its gene expression, metabolic profiling can give an instantaneous snapshot of serotonin metabolism under specific conditions (Kaddurah-Daouk *et al.*, 2008; Mattha¨us *et al.*, 2008; Reddy, 2010). Hence, we choose 10 compounds of the indole pathway (Figure 14), which include the precursors and the breakdown products, to study the daily rhythms in the serotonin chronometabolome.

Molecular Basis for Circadian Oscillation in mammals

The basic function or nature of an oscillatory system is to return the system when it moves away from its regular manner. This is achieved by a process wherein a product feedbacks to slow down the rate of the process itself (a negative element) and a delay in the execution of the feedback. The biological oscillator also requires a positive element which acts as a source of excitation or activation that keeps the oscillator from winding down. All known circadian oscillators use this kind of a loop that consists of a negative element that controls the rate and a positive element that keeps the system thriving. At the gene level, the oscillator relays on these positive and negative elements where in the protein product of the negative elements are transcribed in a rhythmic pattern based on the external cues that acts on the positive elements, whose role is to activate the clock gene (King and Takahashi, 2000; Lowrey and Takahashi, 2000).

In mammals, the circadian clock operates on delayed negative (Sato *et al*., 2006) transcriptional translational feedback loop (Siepka *et al*., 2007; Takahashi *et al*., 2008). The positive regulators include *clock*, *bmal1*, *rorα*, where as the negative elements comprise of *per*, *cry* and *rev-erbα*. All genes are expressed rhythmically except *clock* that is constitutively expressed. When CLOCK and BMAL1 reaches critical concentrations they dimerise. The CLOCK/BMAL1 hetrodimers then act at E-box elements to promote transcription of *Period* (*per1*, *per2*, *per3*) and *Cryptochrome* (*cry1*, *cry2*) genes, leading to increased PER and CRY levels. PER and CRY in the cytoplasm is phosporylated in the presence of casin kinase 1 ε and δ (Lee *et al*., 2009), hetrodimerize and then enters the nucleus and inhibit transcription of their own genes by interacting with CLOCK:BMAL1 heterodimer complex. Delays

associated with transcription, translation, dimerization, nuclear entry of PER/CRY dimers and degradation regulated by the ubiquitin ligase complexes β-TrCP1 and FBXL3, leads to decline in cytoplasmic PER and CRY levels. These cellular events relive the existing inhibition and permit a new cycle to begin. In addition to this core loop, another negative feedback loop, in which REV-ERBα acts at ROR elements to inhibit *Bmal1* transcription, also contributes to clock precision and robustness (Figure 16). Thus, the intracellular circadian timekeeper can be conceptualized as a genetic network, a web of interconnected negative feedback loops regulating transcription of core clock genes and output genes (Ko and Takahashi, 2006; Doi *et al*., 2011).

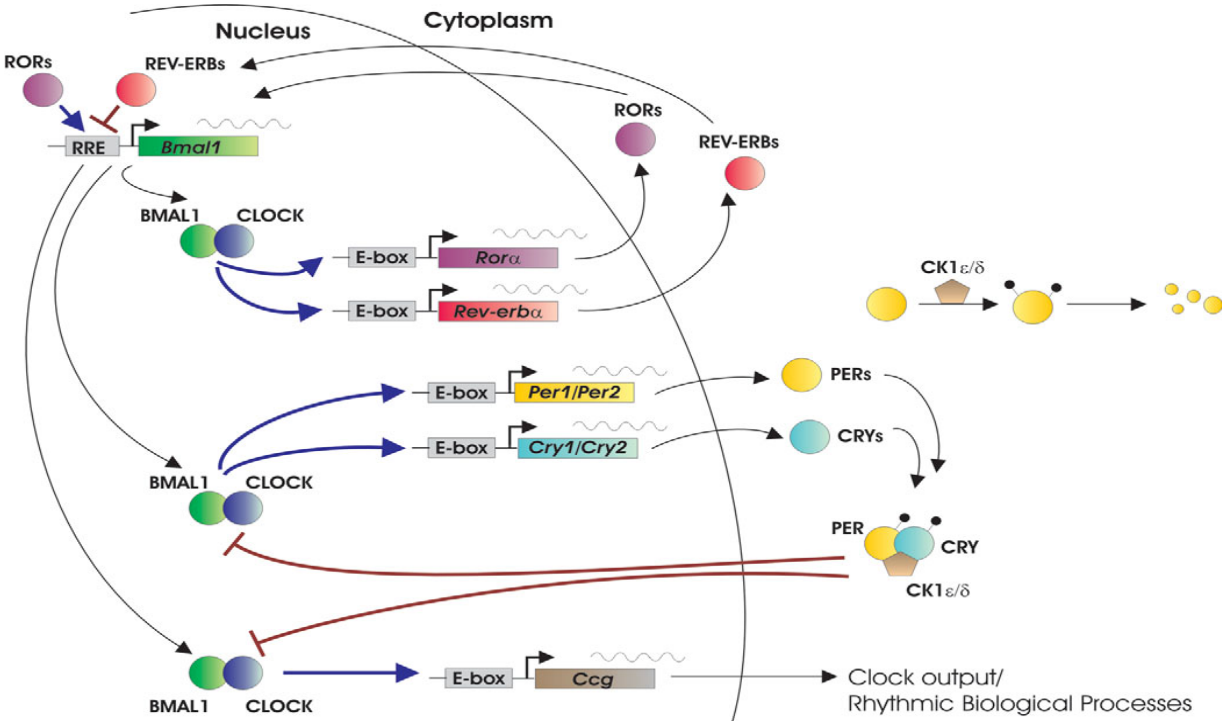


Figure 16. A network of transcriptional–translational feedback loops constitutes the mammalian circadian clock (Ko and Takahashi, 2006).

The negative elements in the feed back loop tend to block the positive element of the clock genes so that the amount of gene products, i.e., the mRNA and eventually the level of clock protein also declines. As the circadian oscillator loop cycles it generates cyclical inhibition of transcription factors (the positive elements), which acts on other clock-

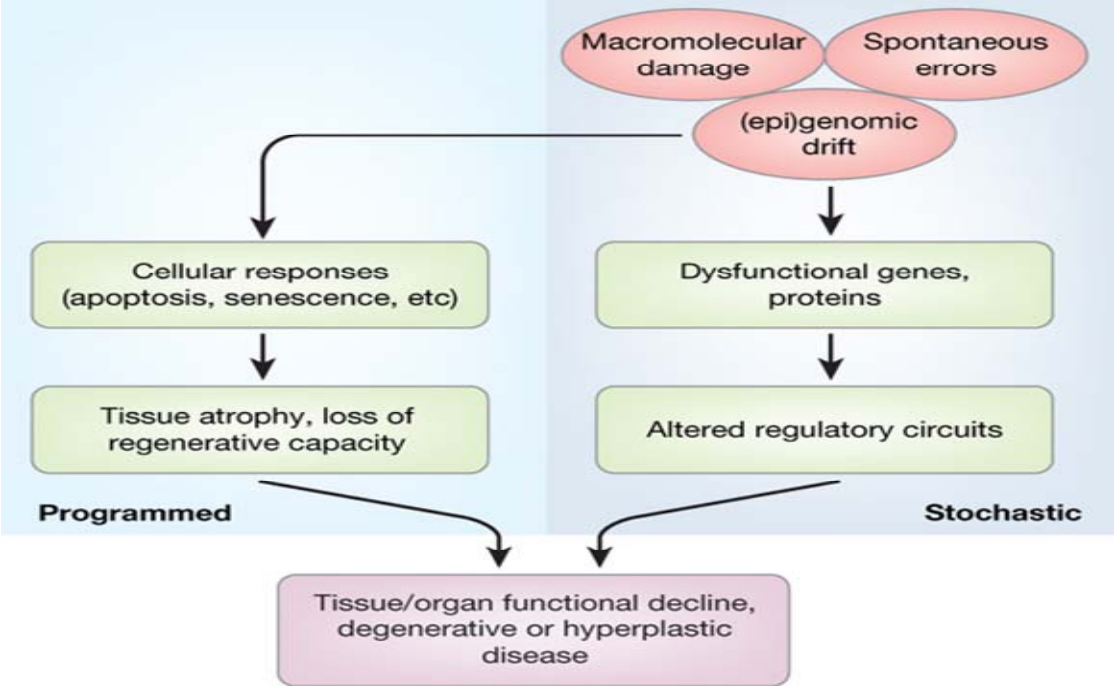
controlled genes (*ccgs*), a mechanism by which time information from the oscillator is driven to the output system that is exhibited in the rhythmic behavior of the organism (Honma *et al*., 1998; Dunlap *et al*., 1999; Jin *et al*., 1999). This robust daily cycling of clock gene mRNA, clock protein and other clock controlled genes and their protein products is characteristic of circadian systems (reviewed in Dunlap, 1999).

Aging and biological clock

Aging is the progressive deterioration of behavior, biochemical and physiological aspects of an organism (Jagota, 2005). Aging can be due to external factors as well as deterioration in cellular mechanisms (Figure 17). Circadian disruptions associated with aging contribute to poor health consequences and hastened senescence in elderly people (Gibson *et al*., 2009). Aging reduces the sensitivity of the SCN to retinal stimulations, causing loss of temporal coordination among bodily systems, leading to deficits in homeostasis and sub-optimal functioning of the physiology that accelerate the aging process and contribute to senescence (Figure 20) (Jagota, 2005; Gibson *et al*., 2009). Age related deterioration in the circadian system is manifested as disruptions in the sleep-wake cycle leading to system wide changes affecting physiology (Jagota and Kalyani, 2008; 2010; Gibson *et al*., 2009). Aging causes decrease robustness in the functioning of the CTS in humans. Though the mechanism is not known it could be caused due to a shorter period of the endogenous oscillator, reduced exposure to synchronizing stimuli such as light, or altered responsiveness to zeitgeber (Benloucif *et al*., 2006). Reports of decreased synthesis of melatonin in various species, including humans have been implicated in the onset and progression of age related diseases and disorders (Figure 18). Disturbed circadian melatonin rhythm have profound effects on the health and well-being of the elderly subjects (Wu and Swaab, 2005; Poeggeler, 2005).

Age associated loss of precision in the functioning of CTS contributes to a variety of age-related pathologies (Weinert and Waterhouse, 1999; Davidson *et al*., 2008). Aging does not decrease cell size or numbers of cells in the SCN (Madeira *et al*., 1995). However, alterations in VIP and AVP peptide expression, a reduction in the amplitude of circadian rhythms of electrical activity and decrease in amplitude and timing of core clock genes have been reported upon aging (Satinoff *et al*., 1993; Kawakami *et al*., 1997). Aging results in decreased synchronization to the environmental cues and decreased sensitivity to the

entraining effects of light compared to young animals (Zhang *et al*., 1998). Aging causes an advance or delay in phase of locomotor activity (Miller *et al*., 2005), and is associated with increased fragmentation of sleep, decreased amplitude of the body temperature rhythm (Prinz *et al*., 2000), and decreased amplitude of the cortisol and melatonin rhythms (Sharma *et al*., 1989). Various studies on aging show a decrease in pacemaker output and that the symptomatic expression of this abnormality is insomnia and sleep disorders (Myers and Badia, 1995). Melatonin therapy have been shown to improve sleep disturbances in the elderly indicating that a complex interaction of decreased pacemaker output as well as responsiveness of effector systems to pacemaker outputs to be playing a major role in aging (Haimov *et al*., 1995).



Vijg and Campisi, 2008

Figure 17. Causes of Aging. Aging is caused by the accumulation of damage to the cells. Some of the causes are unavoidable such as ultraviolet radiation, free radicals, and genetic effects; others involve environmental and behavioral influences (Vijg and Campisi, 2008).

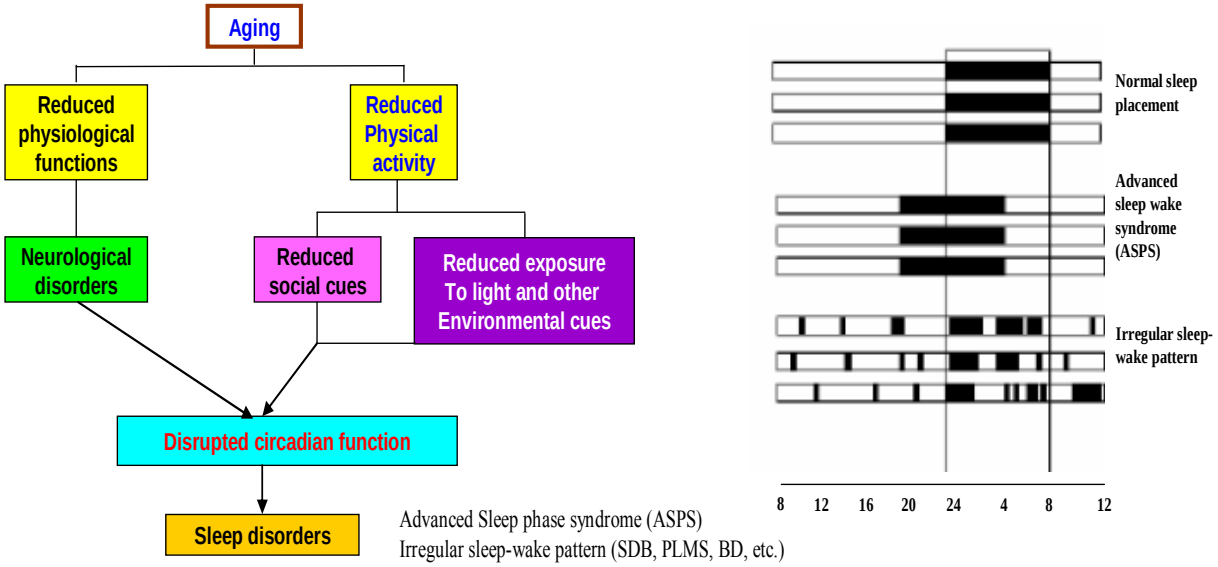


Figure 18. Diagrammatic representation of the causes of age associated circadian disruption associated sleep disorders. The open bars represent intervals during which wakefulness typically occurs, black bars correspond to usual sleep times in normal and pathological conditions and vertical rectangle represents desired sleep time. Individuals with Advanced sleep phase syndrome (ASPS) sleep at earlier than desired time whereas individuals with irregular sleep-wake patterns such as sleep disordered breathing (SDB), periodic leg movements in sleep (PLMS) and rapid eye movement (REM) behavior disorder (RBD) etc. may have disturbed sleep at other than desired times also (Jagota, 2005).

Diurnal circadian physiology

The mammalian CTS though anatomically similar in several species are thus able produce and sustain varying behavioral types such as diurnal, nocturnal and crepuscular (Figure 19) (Kronfeld-Schor and Dayan, 2008). However, the mechanisms responsible for such differentiation are still not known (Refinetti, 2008). Nocturnal and diurnal species differ with respect to the temporal organization of a wide range of physiological and behavioral functions including locomotor activity, copulatory behavior, parturition, body temperature and sleep, which are under the regulation of the circadian timing system (Challet, 2007). The functioning of SCN also shows several degrees of similarity and differences between diurnal and nocturnal species. Differences in the circadian properties of nocturnal and diurnal mammals have been described with diurnal mammals responding poorly to brief, low-

intensity of light pulses (DeCoursey, 1972; 1973), however, much brighter and longer light pulses are effective for generating PRC's in diurnal rodents (Lee, 2004). Similarities in the SCN functioning such as the daily rhythms in multiple-unit activity (MUA) (Schwartz *et al*., 2004), the rates of glucose utilization (Schwartz, 1991), and the expression of immediate early gene product c-Fos (Smale and Boverhof, 1999; Novak *et al*., 2000) have been reported. The mechanisms responsible for the differences in nocturnality and diurnality could be present either within the SCN or downstream of it or both (Smale *et al*., 2008). The recent approach is to understand the functioning of SCN in diurnal species (reviewed in Smale *et al*., 2008; Cuesta *et al*., 2009).

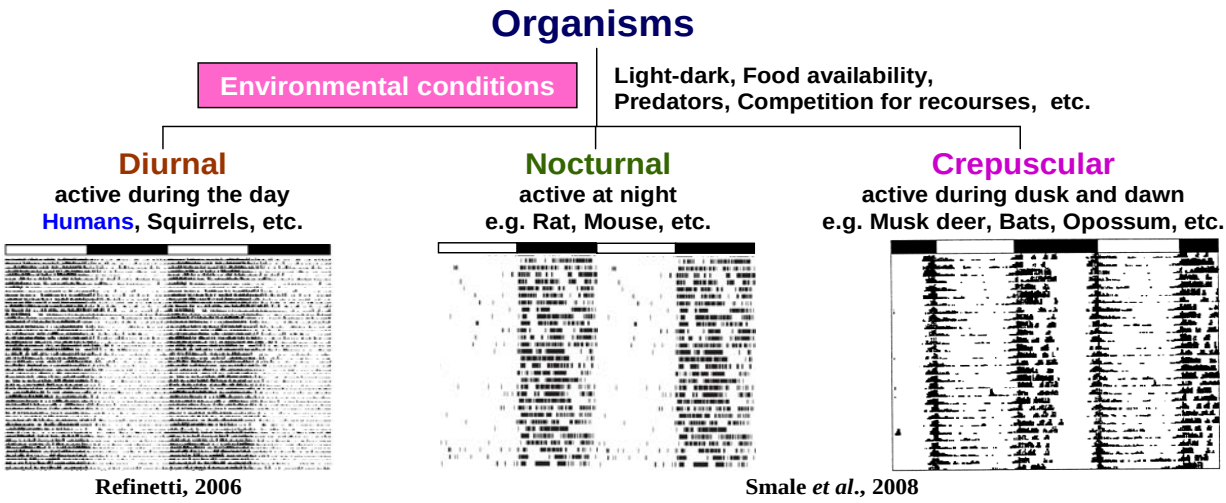


Figure 19. Behavioral types of organisms. Based on the environmental challenges, organisms have evolved into diurnal-day active, nocturnal-night active and crepuscular-active during dusk and dawn (Refinetti, 2006; Smale *et al*., 2008).

Though animals show distinct diurnal and nocturnal pattern of activity, differences in the pattern of locomotor activity within species have been reported (Refinetti, 2008). These difference is based on the diurnality index or percentage diurnality computed from the locomotor activity data (i.e. the ratio the amount of activity during the light phase and the total amount of activity during the whole day), where animals with indices below 0.5 or 50 % are more active during the night than during the day (i.e., they are nocturnal) and animals with indices above 0.5 or 50 % are more active during the day than during the night (i.e., they are diurnal). Mongolian gerbils (*Meriones unguiculatus*) in the laboratory show that 24% of them are predominantly nocturnal whereas 76% are predominantly diurnal (Figure 20)

(Refinetti, 2006). Nocturnal Mongolian gerbils are more active during the night than during the day but exhibit a considerable amount of activity during the light hours, whereas diurnal gerbils initiate activity shortly after lights-on and exhibit little activity during the dark hours. Similar existence of diurnal and nocturnal individuals within the same species have been reported in goldfish (*Carassius auratus*) (Iigo and Tabata, 1996), carpenter ants (*Camponotus compressus*) (Sharma *et al*., 2004), cotton rats (*Sigmodon hispidus*) (Johnston and Zucker, 1983), degus (*Octodon degus*) (Refinetti, 2006) and subterranean mole rats, (Oster *et al*., 2002; Oosthuizen *et al*., 2003), etc.

A switch in the behavioral pattern of diurnal and nocturnal habits in the same individual has also been reported. Degu's (*Octodon degus*), though they are considered to be diurnal in the wild (Kenagy *et al*., 2002) but in the laboratory (Refinetti, 1996) great variability in the time of initiation of locomotor activity has been reported (Labyak *et al*., 1997). The running-wheel activity records of degus maintained under LD, 12:12 conditions in the laboratory showed initially a predominant nocturnal pattern of activity which after few weeks shifted to predominantly diurnal type of activity (Refinetti, 2006). In degus when running wheels were provided a switch in the locomotor activity pattern from diurnal to nocturnal was observed (Kas and Edgar, 1999); however the patterns were not reversed when activity was recorded with infrared motion detectors. Similar spontaneous shifts in the locomotor activity are also reported in cotton rats (*Sigmodon hispidus*) (Johnston and Zucker, 1983). In the natural environment switching has been observed in wolves (*Canis lupus*) that are normally nocturnal; however, when traveling over long distances, they travel during the day (Merrill and Mech, 2003). Similarly migratory birds that are normally diurnal do most of their migratory flights at night (Cochran, 1987; Rattenborg *et al*., 2004).

Most of our understandings of the functioning of the circadian timing system are obtained from nocturnal rodents such as the rat, mouse and hamsters. The extent to which the findings from nocturnal models that can be extrapolated to humans who are predominantly diurnal may be limited (Smale *et al.*, 2003). Studies using diurnal models provide fresh insight into the workings of the circadian system and help to extrapolate these findings to diurnal humans where a rampant increase in disorders due to life style changes are being reported (Toh, 2008). An understanding of the diurnal physiology could help in designing

better treatment approach for those affected, design regimes for adjusting to fast paced life styles and also in the development of more potent chronobiotic agents.

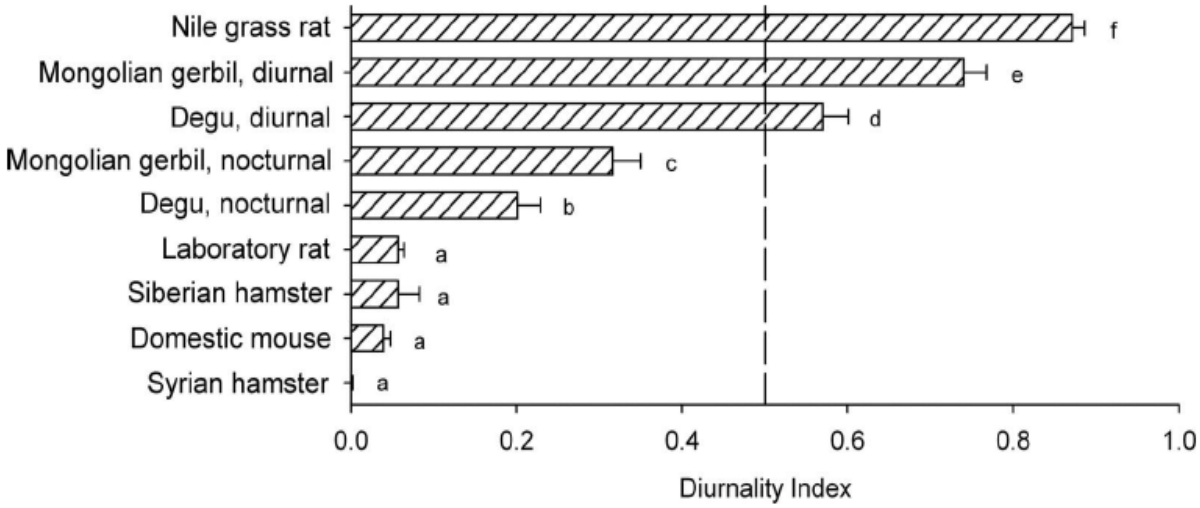


Figure 20. Diurnality gradient from seven rodent species subjected to wheel running activity under LD 12:12 conditions. Means of species bearing different letters (a, b, c, d, e and f) are significantly different from each other ($p<0.05$ family wise). The dashed line indicates the theoretical separation between nocturnal and diurnal animals (i.e., equal amounts of activity during the light and dark phases of the light-dark cycle) (Refinetti, 2006).

In order to understand the anatomical and functional differences of the CTS several diurnal rodent models are being used such as the Asian Chipmunk (*Eutamias asiaticus*) (Honma and Honma, 1999), unstriped Nile Grass Rat (*Arvicanthis niloticus*) (Katona and Smale, 1997; Mahoney *et al*., 2000) as well as *Arvicanthis ansorgei* (Challet *et al*., 2002), European Ground Squirrel (*Spermophilus citellus*) (Hut *et al*., 1999), Golden-mantled Ground Squirrel (*Spermophilus lateralis*) (Mrosovsky *et al*., 2001), Degus (*Octodon degus*) (García-Allegue *et al*., 1999; Lee, 2004), four striped field Mouse (*Rhabdomys pumilio*) (Schumann *et al*., 2005), Mongolian Gerbil (*Meriones unguiculatus*) (Weinert *et al*., 2007), 5 striped North Indian Palm Squirrel (*Funambulus pennanti*) (Rajaratnam and Redman, 1999), 3 striped South Indian palm squirrel (*Funambulus palmarum*) (Navaneethakannan and Kumarasamy, 1986), etc. Indian Palm squirrels, *F. pennanti* and *F. palmarum*, are diurnal and remain active throughout the year. These Palm squirrels associate in large groups and apparently recognize group members (Rajaratnam and Redman, 1999). They are seasonal breeder with pairing and maintenance of territory usually occurring during the

reproductive season (Saxena and Sinha, 2000). The gross locomotor activity of *F. pennanti* showed stable entrainment to LD cycle under laboratory conditions and was diurnally active in both bright and dim LD cycles. The activity onset and offset precisely was defined to onset and off-set of light under LD cycle and that the circadian activity rhythms of *F. pennanti* also respond to other non-photic entraining agents such as exogenous melatonin and ambient temperature cycles (Rajaratnam and Redman, 1997; 1998; 1999).

Based on this literature background the following objectives were planned.

Objectives

Objectives:

I. Characterization of *F. palmarum* through

a) Behavioral studies: Locomotion (Gross locomotor and running wheel activity)

b) Neuroanatomical studies: Neuroanatomy of the SCN

c) Neurotransmitter studies: AVP, VIP and serotonin metabolome rhythms in SCN

d) Clock gene expression studies: *per1*, *per 2*, *cry1*, *cry 2* and *bmal1* rhythms in SCN

e) Effect of Photic (light pulse) and Non-photic (restricted feeding) cues on Locomotor activity rhythms

II. To study the effect of aging on

a) Behavior: Gross locomotor and running wheel activity

b) Neurotransmitters: AVP, VIP and serotonin metabolome rhythms in SCN

c) Clock gene expression: *per 1*, *per 2*, *cry1*, *cry 2* and *bmal1* rhythms in SCN

Materials and Methods

Experimental Animals:

1. Adult male three striped South Indian Palm Squirrels, (*F. palmarum*) (Figure 21), weighing 110 ± 20 g, obtained from a local supplier were maintained in light-dark conditions (LD 12:12) at a room temperature of 24 ± 2 °C, with 55 ± 6% relative humidity for two weeks prior to the experiment. Animals were kept individually in wire cages (60×40×40 cm) contained within well ventilated light proof environmental cabinets isolated in animal facility. Lighting conditions were maintained using an automated timer (Legrad, Germany). Food and water were supplied *ad libitum*. Dim red light (Kodak 1A filter) was used for handling the animals in the dark. Cage changing was done at random intervals (Mammen and Jagota, 2011).

Adult male *F. palmarum* (n=24) were maintained individually under similar conditions for 2.5 years for studies related to age induced changes.

2. Adult male Wistar Rats maintained individually in polypropylene cages under similar conditions (Jagota and Reddy, 2007) were used for comparative neuroanatomical studies.

All experiments were performed as per Institutional Animal Ethics guidelines.

Figure 21. The three striped South Indian Palm Squirrel (*Funambulus palmarum*). This mammal belongs to the Order: *Rodentia*, Suborder: *Sciurognathi*, Family: *Sciuridae*, Genus: *Funambulus* and Species: *palmarum* (Mammen and Jagota, 2011).

Behavioral studies:

Gross locomotor activity

Adult male palm squirrels in three groups (n=6 in each group) were housed individually in cages equipped with infra-red motion detector sensors (KE kits, India) and the data was collected, recorded and analyzed using Chronobiology Kit (Stanford Software Systems, USA) (Figure 22). The animals in Group 1 were subjected to LD 12:12 for 100 days for entire duration of experiment. In Group 2, after 20 days in LD, the animals were kept in constant dark condition (DD) for 57 days. The lighting condition was returned to LD and the animals were maintained in LD for a minimum of 23 days. Group 3 animals were maintained in LD for 20 days followed by 40 days in constant light (LL) and the lighting conditions were reset to LD and the animals were maintained for another 40 days (Rajaratnam and Redman, 1999; Mammen and Jagota, 2011).

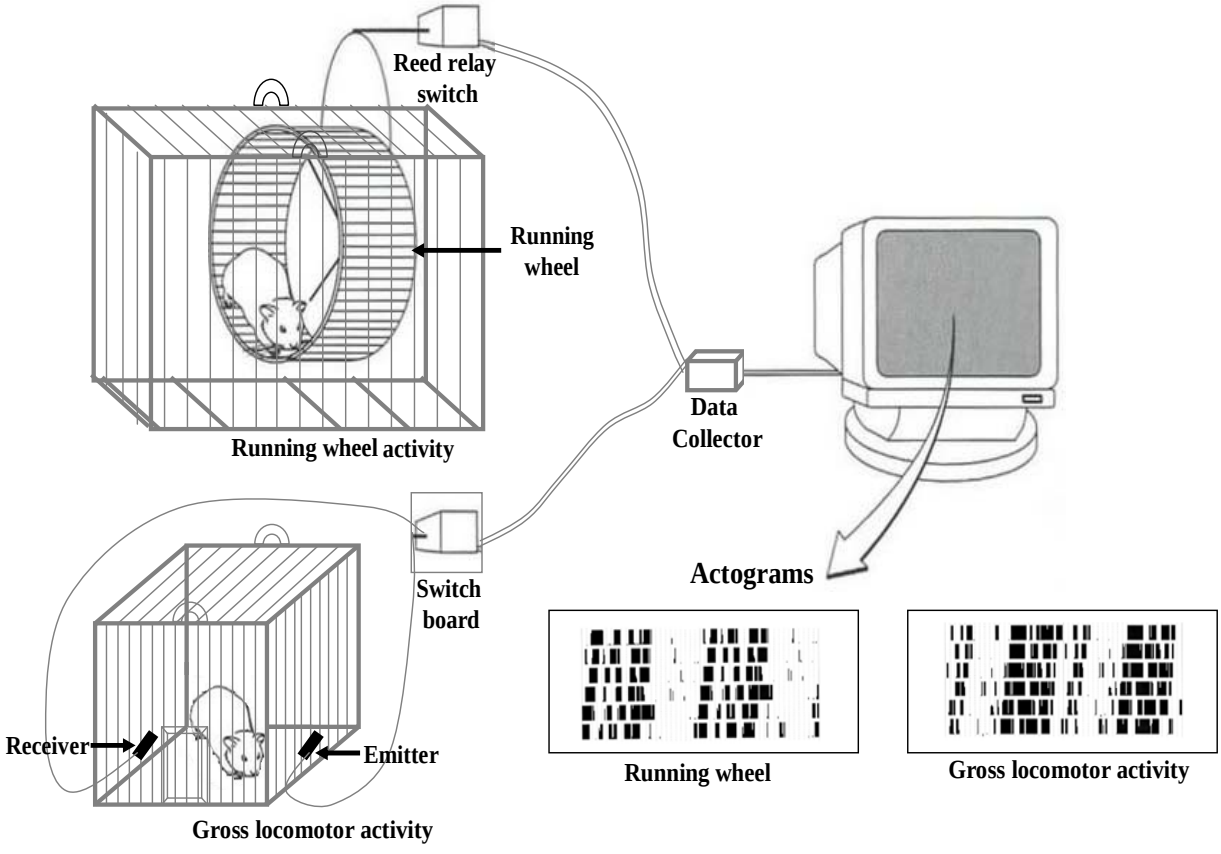


Figure 22. Running wheel and gross locomotor activity set-up for recording locomotor activity.

Wheel running activity

Adult male palm squirrels in four groups (n=6 in each group) were housed in individual cages equipped with running wheels of diameter 17 cm attached with reed relay switch for detecting motion of the wheels. The data was collected, recorded and analyzed using Chronobiology Kit (Stanford Software Systems, USA) (Figure 22). The animals under their respective groups were maintained in a sequence of varying photoperiodic conditions. Group 1 animals were subjected to LD 12:12 for 100 days for entire duration of the experiment. Group 2 animals were subjected to LD for 40 days, DD for 30 days and LD for 30 days. Group 3 animals were subjected to LD for 25 days, LL for 35 days and LD for 40 days. Group 4 animals were in LD for 20 days, LL for 120 days followed by resetting lighting conditions to LD for 20 days (Katona and Smale, 1997).

Effect of light pulse on wheel running activity:

Animals were divided into eight groups (with 3 animals in each group) and maintained under constant darkness (DD) for 2 weeks and then subjected to a light pulse of 500 Lux for 3 hours for Group I at ZT-0/24, Group II at ZT-3, Group III at ZT-6, Group IV at ZT-9, Group V at ZT-12, Group VI at ZT-15, Group VII at ZT-18 and Group VIII at ZT-21 respectively (Figure 23). The phase shifts in running wheel activity were determined and phase response curve (PRC) plotted (Bult *et al*., 1993).

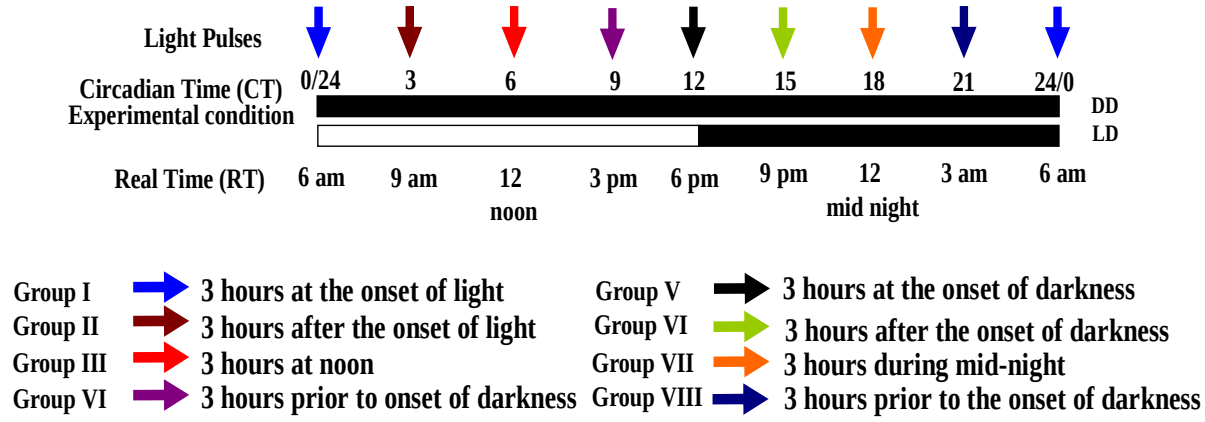


Figure 23. Light pulse schedule for determining the PRC for light. Animals maintained under constant darkness (DD) were subjected to light pulse of 500 Lux for 3 hours for group I at ZT-0/24, group II at ZT-3, group III at ZT-6, group IV at ZT-9, group V at ZT-12, group VI at ZT-15, group VII at ZT-18 and group VIII at ZT-21 respectively.

Effect of restricted feeding (RF) on wheel running activity:

Animal were divided into four groups (6 in each group). These were kept in 12:12 LD cycle for 2 weeks with food and water provided *ad libitum*. Group 1 received 4 hours RF during the light phase (10:00 am to 2:00 pm) and Group 2 received 4 hours RF during the dark phase (6:00 pm to 10:00 pm) in LD 12:12 conditions. Group 3 received 4 hours RF during mid-subjective night under constant dark (DD) conditions (10:00 pm to 2:00 am) and Group 4 received 12 hours RF during subjective night, from 6:00 pm to 6:00 am under DD conditions (Figure 24). The phase shifts in running wheel activity were determined (Hara *et al*., 2001).

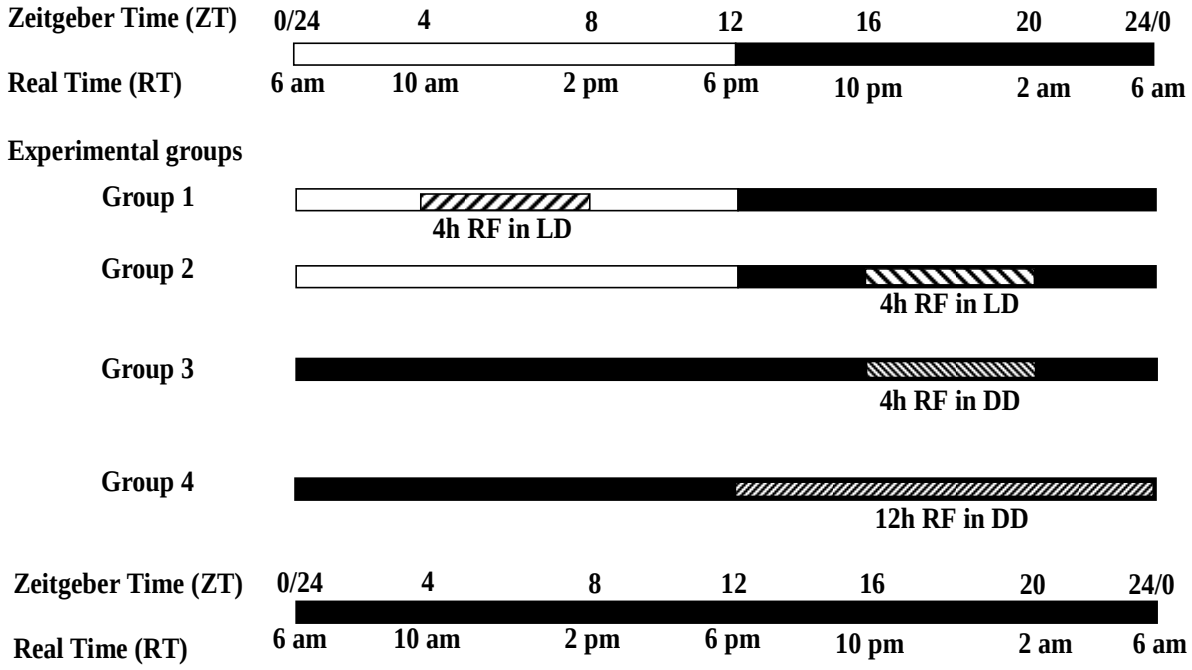


Figure 24. Restricted feeding schedule employed for studying the effect of 4h and 12h RF under LD and DD conditions. Group 1 received 4 hours RF during the light phase (10:00 am to 2:00 pm) and Group 2 received 4 hours RF during the dark phase (6:00 pm to 10:00 pm) in LD 12:12 conditions. Group 3 received 4 hours RF during mid-subjective night under constant dark (DD) conditions (10:00 pm to 2:00 am) and Group 4 received 12 hours RF during subjective night, from 6:00 pm to 6:00 am under DD conditions.

Neuroanatomical studies:

Comparison of gross neuroanatomy of diurnal squirrel and nocturnal rat

Adult Palm Squirrels and Wistar rats were sacrificed and the brains along with optic nerves and eye balls were dissected out. The length and thickness of the optic nerves were compared.

Histology:

Animals were sacrificed and the brains were fixed in 10 % neutral buffered saline for 4 hours. The brain tissues were dehydrated using series of alcohol gradients, after clearing in Xylene the tissues were impregnated with paraffin wax (congealing temperature 65-70° C) (Qualigens, India) and embedded using L-blocks. 8µ thick paraffin sections were taken using Leica RM 2125 microtome. The sections were stained using cresyl violet, mounted and photographed using DP-12 digital camera attached to an Olympus microscope (BX-41). The neuronal counts (Jagota and Habibulla, 1992; Vidal and Lugo, 2006) were determined using Image ProAMS software (Media Cybernetics, USA) (Petersen *et al*., 2006; Mammen and Jagota, 2011).

Immunohistochemistry:

Animals were anesthetized and transcardial perfusion was performed with heparinized 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4). The brains were rapidly removed and post-fixed in 4% paraformaldehyde in 0.1 M PB (pH 7.4) for 2–4 hours and then stored in 20% sucrose in 0.1 M PB (pH 7.4) at 4 °C for 24 hours. 25 µ thick coronal sections of the brain were cut using Leica cryostat (CM 1850) and processed for cresyl violet staining and immunohistochemistry (IHC) (Goel *et al*., 1999). Neu N (neuronal marker) immunohistochemical studies were performed with mouse primary antibody (dilution 1:250) (MAB 377; Chemicon, MA, USA) which was detected using goat anti-mouse IgG-HRP conjugate (HP06, Bangalore Genei, India). Individual sections were incubated with secondary antibody (dilution 1:250) for 1–2 hours at 37°C. 0.1% 3, 3-diaminobenzidine containing 0.3% H_2O_2 were used as substrate in which the sections were incubated for about 15 minutes till colour developed. The reaction was stopped using 2M HCl for 30 seconds and washed immediately (Figure 25) (Caston-Balderrama *et al*., 1998). All sections were arranged in a rostro-caudal axis, photographed and the neurons were

counted (Jagota and Habibulla, 1992; Vidal and Lugo, 2006 and verified using Image ProAMS software (Media Cybernetics, USA) (Petersen *et al*., 2006; Mammen and Jagota, 2011).

Daily rhythms of VIP-ir and AVP-ir in SCN:

For studying daily rhythms of VIP and AVP in SCN, animals were sacrificed at various time points (ZT-0, 6, 12 and 18) (n=4). VIP-ir and AVP-ir were studied using specific primary antibodies raised in mouse (sc-25347; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and rabbit (AHP 372; AbD Serotech, Oxford, UK) respectively. Goat anti-Rabbit IgG-HRP (HP03, Bangalore Genei, India) and Goat anti-Mouse IgG-HRP conjugates (HP06, Bangalore Genei, India) were the corresponding secondary antibodies used. VIP-ir and AVP-ir levels were compared using densitometric analysis with Image Pro AMS software (Figure 25) (Media Cybernetics, USA) (Petersen *et al*., 2006; Mammen and Jagota, 2011).

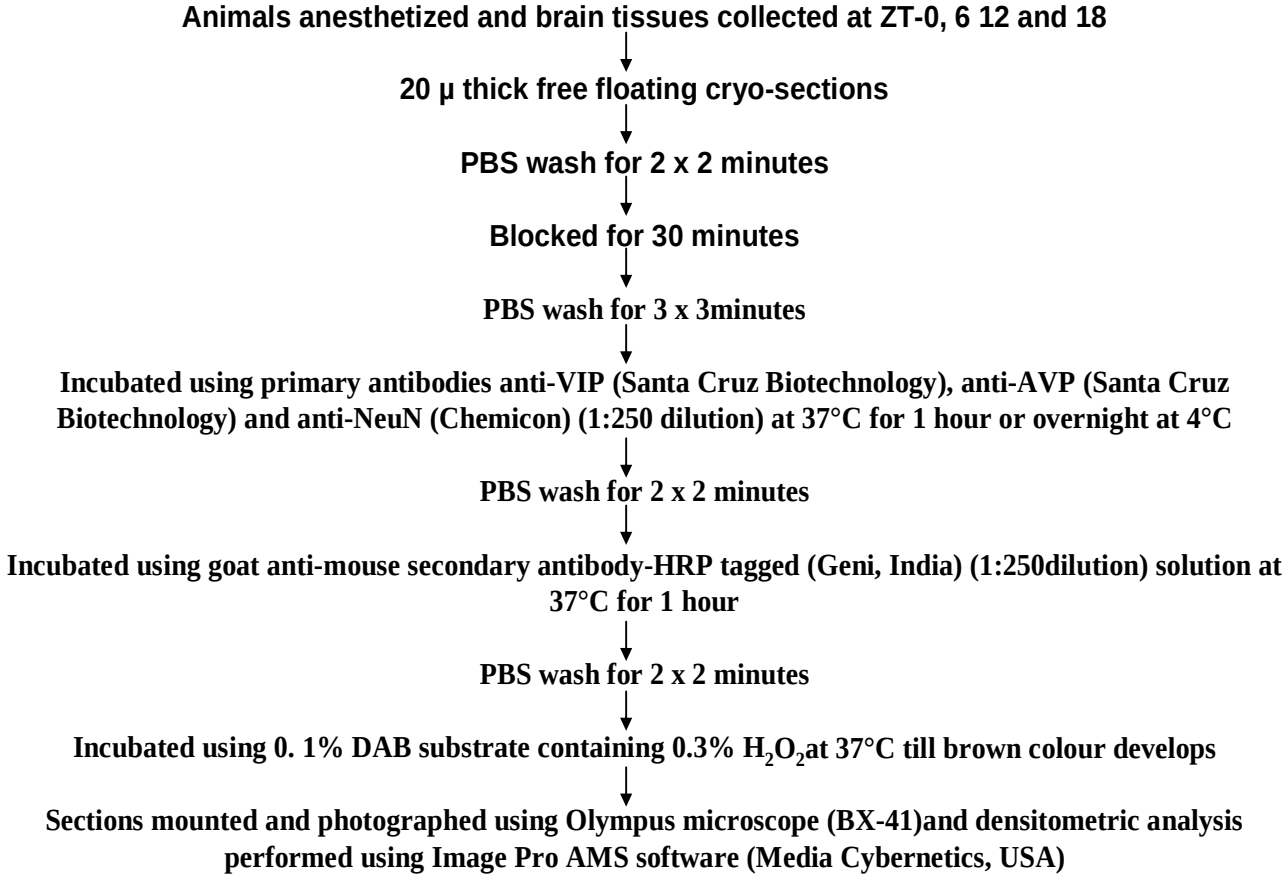


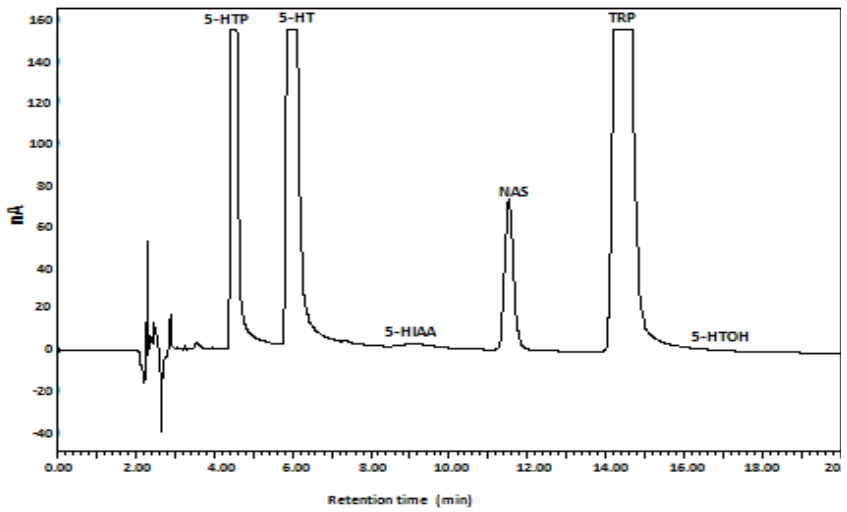
Figure 25. Flow chart for immunohistochemical staining method for detecting AVP, VIP and NeuN in the SCN.

Reverse Phase-High Pressure Liquid Chromatography using Electrochemical Detector (RP-HPLC-ECD):

Animals were acclimatized under laboratory conditions of LD 12:12 for 2 weeks and then sacrificed. The brains were collected at various time points (ZT-0, 6, 12 and 18), pineal glands were separated and the SCN were punched out with the help of a scalpel from 500µ brain slices which were made using a tissue chopper (Jagota and Reddy, 2007). 10% homogenate was prepared using 0.1M perchloric acid containing 1mM sodium bisulphate. The homogenates were then sonicated for 10 seconds at 40 Amp and centrifuged at 12,800xg for 10 minutes at 4ºC. The supernatant was collected and filtered using 0.45µ syringe filter and stored at -80°C until use. The pellets obtained were used for protein estimation by Bradford's method.

Serotonin its precursors and metabolites that include 5-HIAA, NAS, MEL, TRP, 5-HTP, 5-HTOH, 5-MTOH, 5-MIAA and NAT were assayed by RP-HPLC-ECD (Aliance, Waters, USA) method using C_{18} silica column and methanol containing sodium acetate citrate buffer as the mobile phase. Standard curves were prepared by using pure compounds of 5-HT, 5-HIAA, NAS, MEL, TRP, 5-HTP, 5-HTOH, 5-MTOH, 5-MIAA and NAT (Sigma and MP Biomed, USA) (Figure 26). 250µl of clear supernatant were transferred into the carousals and placed in appropriate location on the sample holding tray. The Empower software (Waters, USA) was programmed to aspirate and inject 50 µl of the supernatant. Based on the flow rate and retention time the appropriate metabolites were eluted out from the column and detected by the electrochemical detector (ECD). The concentration of each compounds in the analyte were estimated by comparing the peak area with their respective standards (Figure 27) (Mefford *et al*., 1980; Jagota and Reddy, 2007, Reddy, 2010). Protein concentrations in the samples were measured by Bradford's method by using crystalline bovine serum albumin (Sigma) as standard (Bradford, 1976).

A



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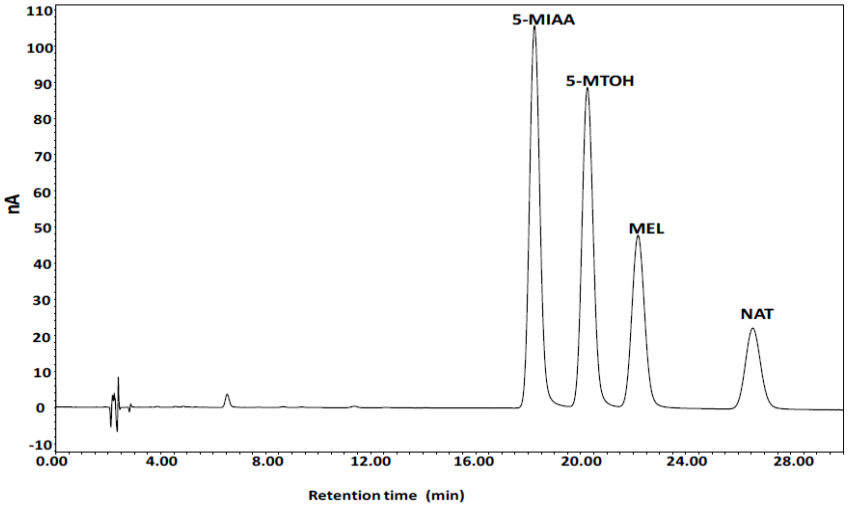


Figure 26. Standard representative peaks of Serotonin and its related compounds. (A) Peaks of 5-HTP, 5-HT, 5-HIAA, NAS, TRP and 5-HTOH obtained using 10% methanol as the mobile phase and (B) peaks of 5-MIAA, 5-MTOH, MEL and NAT obtained using 25% methanol as the mobile phase. The mobile phase consists of methanol prepared in buffer consisting of 0.1N sodium acetate; 0.1N citric acid and 50mg/litre EDTA at pH 4.1. nA refers to nanoampere (Unit of electric current).

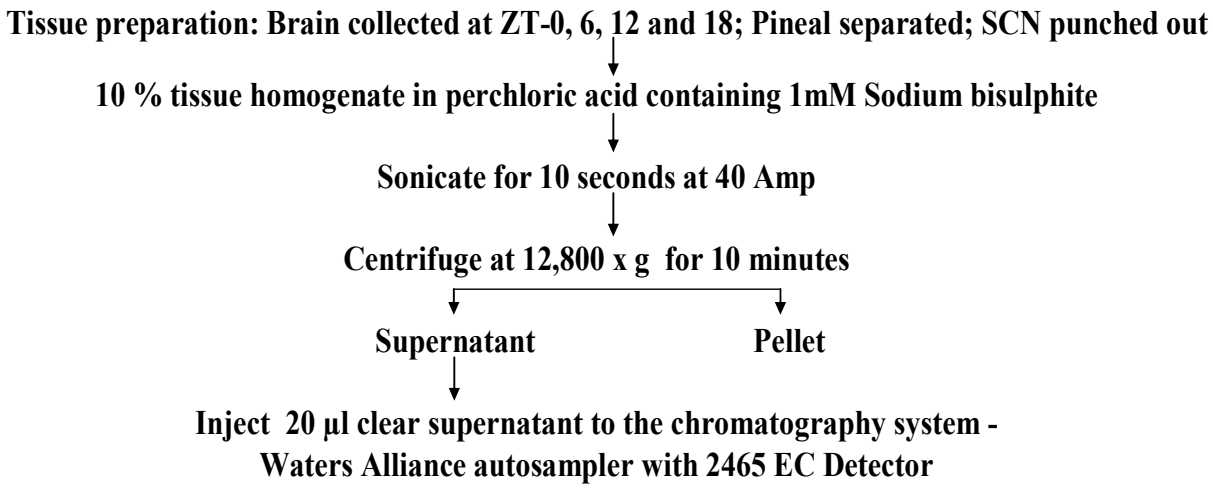


Figure 27. Flow chart for the RP-HPLC-ECD method for estimating the levels of serotonin chronometabolome in the SCN, Pineal and Brain (Reddy, 2010).

Reverse Transcriptase–PCR (RT-PCR) studies:

Animals were acclimatized to laboratory conditions of LD 12:12 for 2 weeks and sacrificed at various time points (ZT-0, 6, 12 and 18). Total RNA was isolated from 100 mg of the tissue by homogenizating in 1 ml of TRI reagent (Sigma). After incubation at room temperature for 5 minutes, 0.2 ml of chloroform was added and mixed vigorously for 16 seconds. The homogenate was allowed to stand at room temperature for 15 minutes and then centrifuged at 12, 000 x g for 15 minutes at 2-8°C. The homogenate separates into three layers and the upper phase containing the RNA was transferred into fresh DEPC treated tubes and 0.5 ml of isopropanol per ml of TRI reagent was added, mixed and incubated at room temperature for 5-10 minutes. The tubes were then centrifuged at 12, 000 x g for 10 minutes at 2-8°C. The supernatant is discarded and the pellet is reconstituted in 75% ethanol and centrifuged at 7,500 x g for 5 minutes at 2-8°C. The supernatant was decanted and the pellet was briefly dried and reconstituted in 100 µl of DEPC treated water and quantitated using nano-drop spectrophotometer (Thermo Fischer 1000) (Figure 28) (Okamura *et al*., 1999; Asai *et al*.,2001).

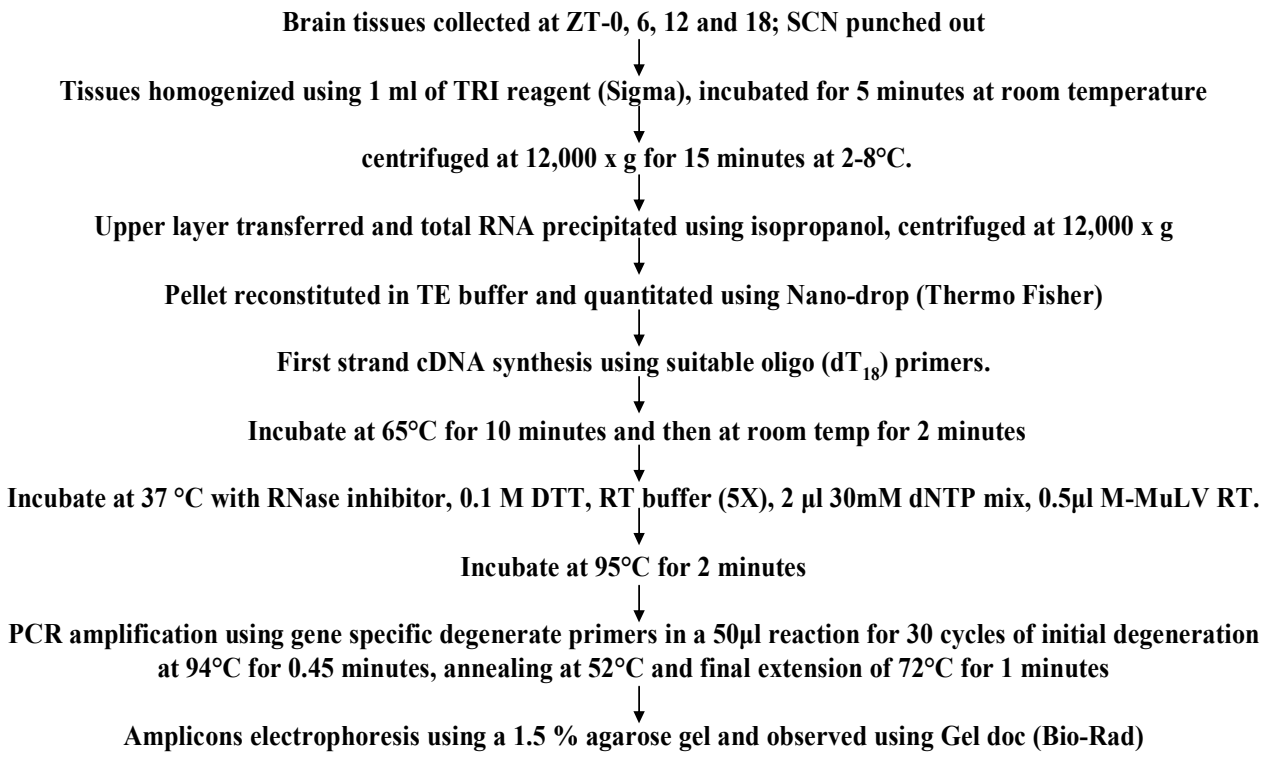


Figure 28. Flow chat for RT-PCR method for clock gene expression studies.

First strand cDNA synthesis was performed using 100 ng of total RNA, to which 1 µl of oligo $(dT)_{18}$ primer was added and incubated at 65°C for 10 minutes. To the solution a 9.5 µl mix consisting of RNase inhibitor, 0.01 M DTT, 5xRT buffer, 30mM dNTP mix, M-MuLV Reverse Transcriptase, wad added and incubated at 37°C for 1 hour followed by a 2 minute incubation at 95°C. The cDNA generated was then used for PCR amplification in a 20 µl master mix containing 10 x assay buffer, 30mM dNTP mix, 1µM of each specific primer and 1.5 units of Taq DNA polymerase. The gene specific degenerate primers used were *m Per 1* [5'-cag caa tgg ctc aag tgg caa-3'(f) (21 mer) and 5'-act gtt cac tgc tgc agc cac -3' (r) (21 mer)], *m per 2* [5'-acg aga act gct cca cgg-3' (f) (18 mer) and 5'-gaa cag cca cag caa aca tat cc-3' (r) (23 mer)], *m Cry 1* [5'-gag gct ggc gtg gaa gtc at -3' (f) (20 mer) and 5'-tat ggg caa ctc ctg tgg-3' (r) (18 mer)], *m Cry 2* [5'-gag aga cct cgg atg aat gc-3' (f) (20 mer) and 5'-tgg atg tgg ctg tcc tgc agt -3' (r) (21 mer)], and *m g3pdh* [5'-gac ctc aac tac atg gtc tac-3' (f) (21 mer) and 5'-tgg ccg tga tgg cgt gga ct-3' (r) (20 mer)] (Okamura *et al*., 1999; Asai *et al.*, 2001). The amplicons were

electrophoresed; documented using UV-Transilluminator (Bio Rad) and densitometric analysis performed using quantity one software (Bio Rad) (Figure 28).

Protein estimation:

Protein estimation was done by using Bradford's method. The SCN, pineal and brain tissues collected at various time points (ZT-0, 6, 12 and 18) were homogenized (5%) and the volume of each tissue sample was adjusted to 100µl with double distilled water. 1ml of Bradford's reagent was added to each sample and mixed thoroughly. The absorbance was measured at 595nm after 2 minutes and within 1hour against a reagent blank of 100µl double distilled water and 1ml of Bradford's reagent. The standard graph was prepared using bovine serum albumin (BSA) of concentrations ranging from 1µg to 10µg and the amount of total protein in the tissue samples were determined (Bradford, 1976).

Statistical analysis:

Data were analyzed using Jandel Scientific Sigma stat software by Student's t-test and one way ANOVA followed by Post hoc Duncan's test (Sigma Stat) for multiple comparisons.

Results

OBJECTIVE - I

Characterization of *F. palmarum* through

a) Behavioral studies: Locomotion (Gross locomotor and running wheel activity)

b) Neuroanatomical studies: Neuroanatomy of the SCN

c) Neurotransmitter studies: AVP, VIP and serotonin metabolome rhythms in SCN

d) Clock gene expression studies: *per1*, *per 2*, *cry1, cry 2* and *bmal1* rhythms in SCN

e) Effect of Photic (light pulse) and Non-photic (restricted feeding) cues on Locomotor activity rhythms

1. Characterization of *F. palmarum* through

(a). Behavioral studies: Gross locomotor activity and Running wheel activity

(i). Gross locomotor activity:

Actograms prepared in LD condition (LD; 12:12) showed a diurnal pattern of activity with robust activity seen in the light phase from ZT-0 (6:00 A.M. lights on) to ZT-12 (6:00 P.M. lights off) with minimum activity in the dark phase (ZT-12 to ZT-0/24) (n=12) (Figure 29, 32 A and D). The circadian period (τ) under these conditions were 24.19 ± 0.12 h (n=12) and animals showed a diurnality of 70.39 ± 3.88% (Table 1).

In constant conditions, *F. palmarum* showed free running pattern with τ of 24.11 ± 0.03 hours (n=6) (Figure 30, 32B) and 24.92 ± 0.03 hours (n=6) (Figure 31, 32C) under DD and LL respectively. The τ in DD was not significantly different from LD; however τ in LL was significantly different from LD as well as DD ($p \leq 0.05$). Activity was anchored to the subjective day with minimum activity during subjective night. On returning to LD, from constant conditions (DD and LL) the animals showed entrainment to LD conditions (Figure 30, 31, 32 B and C). The duration of active period (α) in LD, DD and LL was found to be 12.57 ± 0.49, 12.82 ± 0.76 and 16.03 ± 1.03 hours respectively (Table 1) and the % diurnality was 70.39 ± 3.87, 73.12 ± 7.59 and 55.05 ± 3.81 under similar conditions (Table1). Both α and % diurnality in LL were significantly different from LD and DD ($p \leq 0.05$), however no significant differences were found in LD and DD conditions (Table 1).

(ii). Running wheel activity:

Wheel running activity under LD 12:12 condition showed a diurnal pattern of activity with robust activity during the light phase, from ZT-0 (6:00 A.M. lights on) to ZT-12 (6:00 P.M. lights off) (n=6) (Figure 33, 36A and D), with a τ of 24.01 ± 0.008h and diurnality of 94.94 ± 2.58 % (Table1).

In constant darkness (DD) (n=6) (Figure 34, 36C), *F. palmarum* showed free running pattern of activity with τ of 23.88 ± 0.19 h. Under constant light (LL) a splitting patterns of running wheel activity (n=6) (Figure 35, 36C) were observed with τ of 24.92 ± 0.35 hours. Activity was anchored to the subjective day with minimum activity in subjective night. Under LL, the splitting of activity into two bouts of 2.67 ± 0.71 hours of activity during the subjective

light period and 5.25 ± 1.89 hours of activity during the subjective dark phase. On returning the animals from LL to DD activity were observed through the light period oh 12 hours (Figure 35). The duration of α in LD, DD and LL was found to be 11.62 ± 0.49 h, 11.04 ± 0.72 h and 15.72 ± 0.31 h respectively (Table 1) and % diurnality of 94.94 ± 2.58%, 60.84 ± 5.40% and 62.21 ± 3.82% respectively (Table 1) under similar conditions. On returning to LD 12: 12 from DD and LL, the animals showed entrainment to the LD cycle (Figure 34, 35, 36 B and C). % diurnality showed a significant difference in LD as compared to DD and LL, however no difference was observed between DD and LL ($p \leq 0.05$). Both τ and α under DD and LL was not significantly different from LD (Table 1).

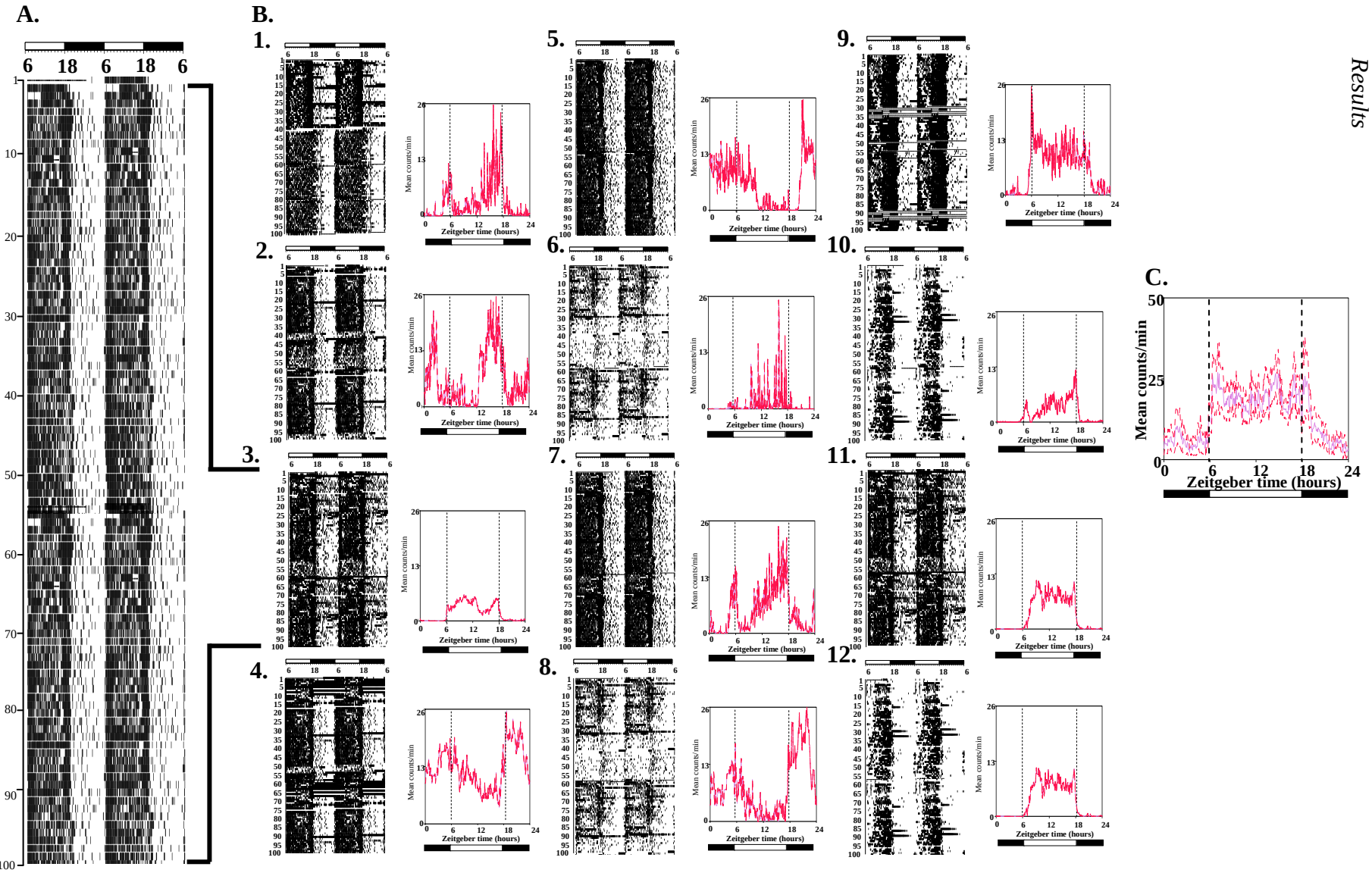


Figure 29. A. Representative double plotted actograms of gross locomotor activity for 100 days in LD 12:12 condition. B (1-12). Actograms and their respective activity profiles of 12 individual animals . C. Mean activity profile (n=12). Each value is mean ± S.E. (n=6).The dashed lines on the activity profile indicates lights on and off for animals in LD (12:12).

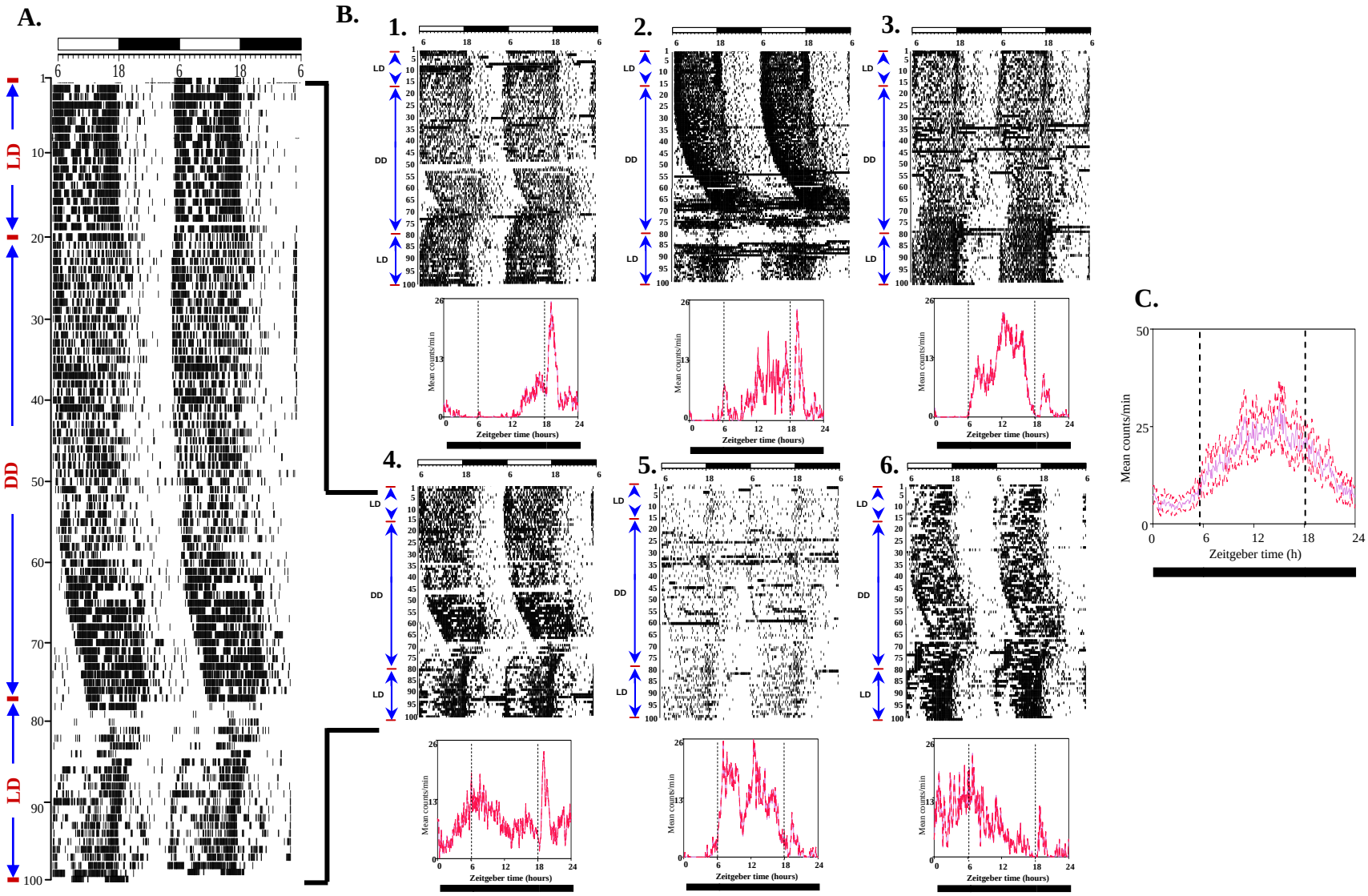


Figure 30. A. Representative double plotted actograms of gross locomotor activity for 100 days in DD condition. B (1-6). Actograms and their respective activity profiles of 6 individual animals under DD condition. C. Mean activity profile of 6 animals showing maximum activity during subjective light phase of DD. Each value is mean ± S.E. (n=6).The dashed lines on the activity profile indicates subjective lights on and off respectively.

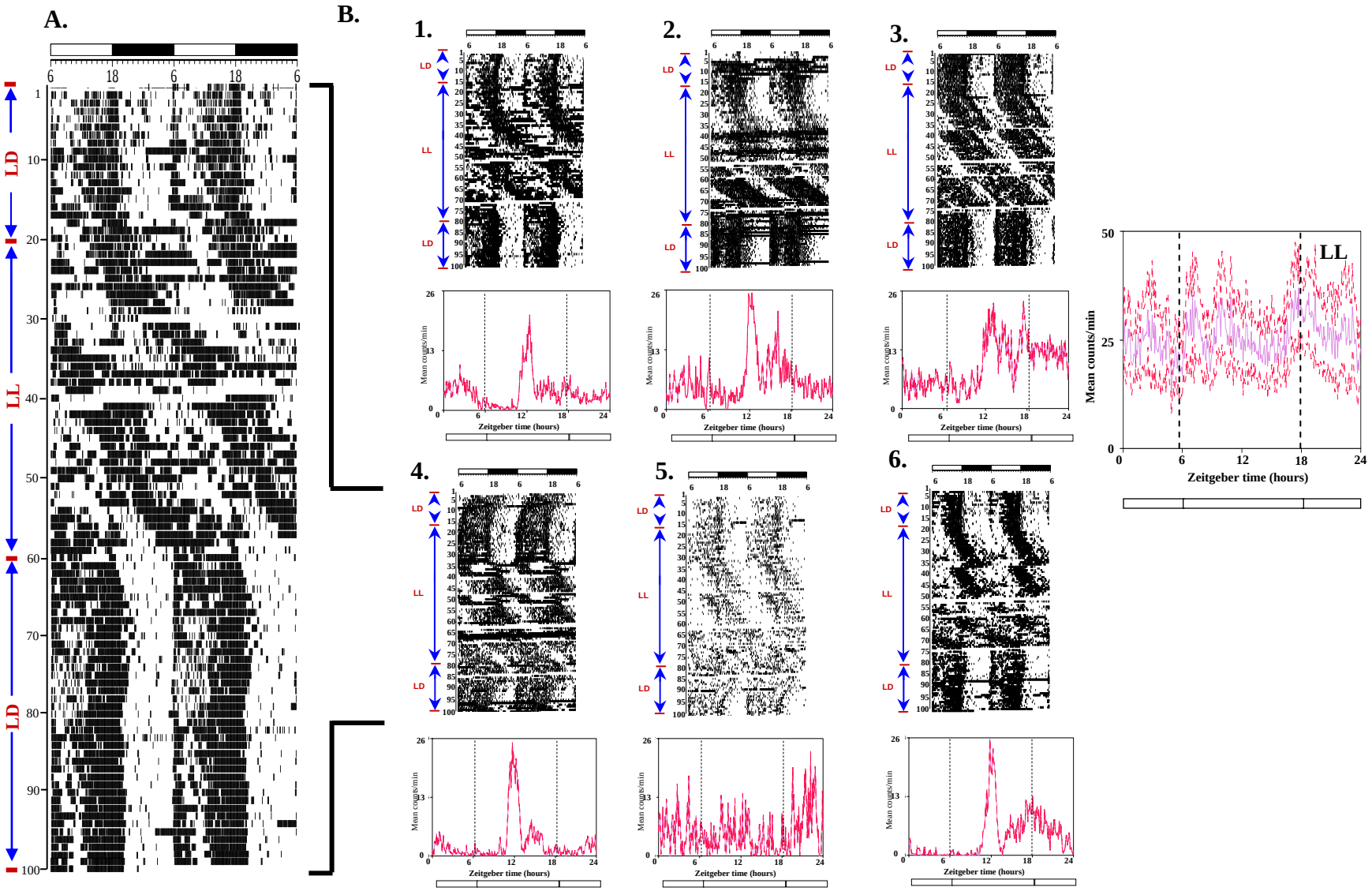


Figure 31. A. Representative double plotted actograms of gross locomotor activity for 100 days in LL condition. B (1-6). Actograms and their respective activity profiles of 6 individual animals under LL condition. C. Mean activity profile of 6 animals. Each value is mean ± S.E. (n=6). The dashed lines on the activity profile indicates subjective lights on and off respectively.

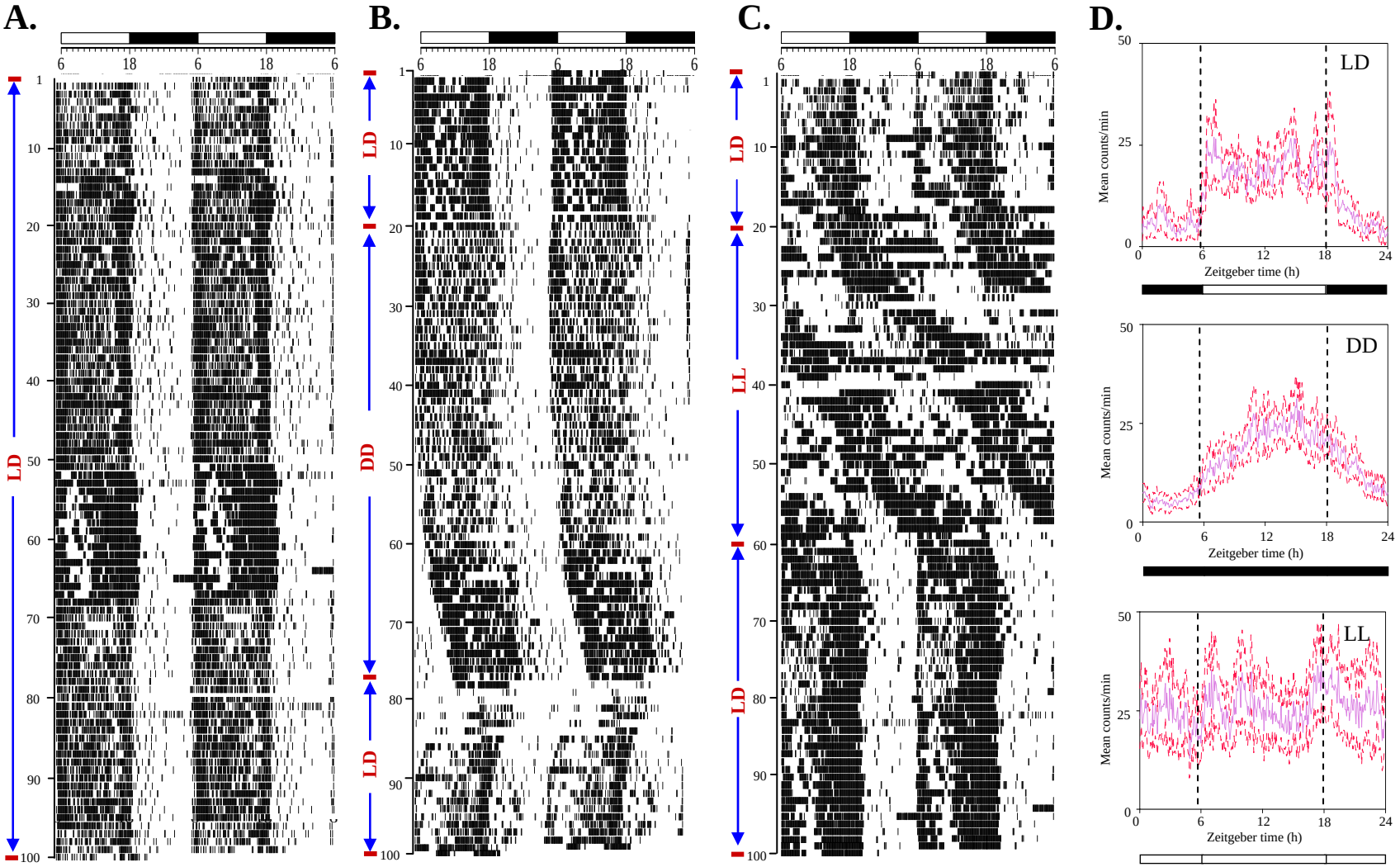


Figure 32. Representative double plotted actograms of gross locomotor activity for 100 days showing activity in various photoperiods LD, DD and LL conditions. A. In LD, a robust activity during the light phase and less activity during the dark phase; B. In DD, a free running activity with τ of 24.11 ± 0.03 hours; C. Under LL, a free running pattern with τ of 24.92 ± 0.35 hours were observed; D. Mean activity profiles under LD (n=12), DD (n=6) and LL (n=6) conditions. Each value is mean ± S.E. (n=6).The dashed lines on the activity profile indicates lights on and off for animals under LD 12:12 condition respectively (Mammen and Jagota, 2011).

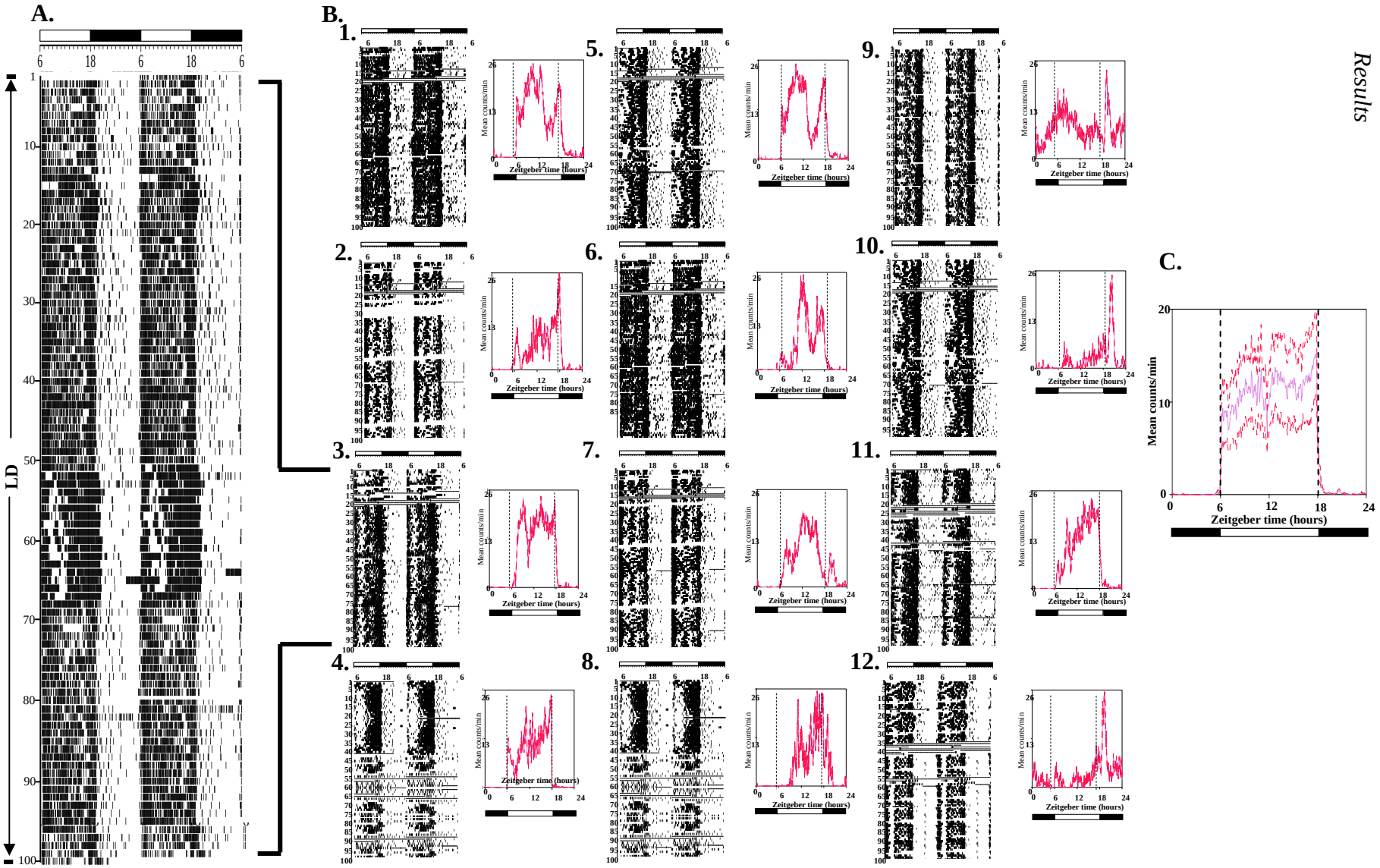


Figure 33. A. Representative double plotted actograms of running wheel activity for 100 days in LD 12:12 condition. B (1-12). Actograms and their respective activity profiles of 12 individual animals. C. Mean activity profile of 12 animals showing maximum activity during light phase of LD 12:12 cycle. Each value is mean ± S.E. (n=6). The dashed lines on the activity profile indicates lights on and off respectively.

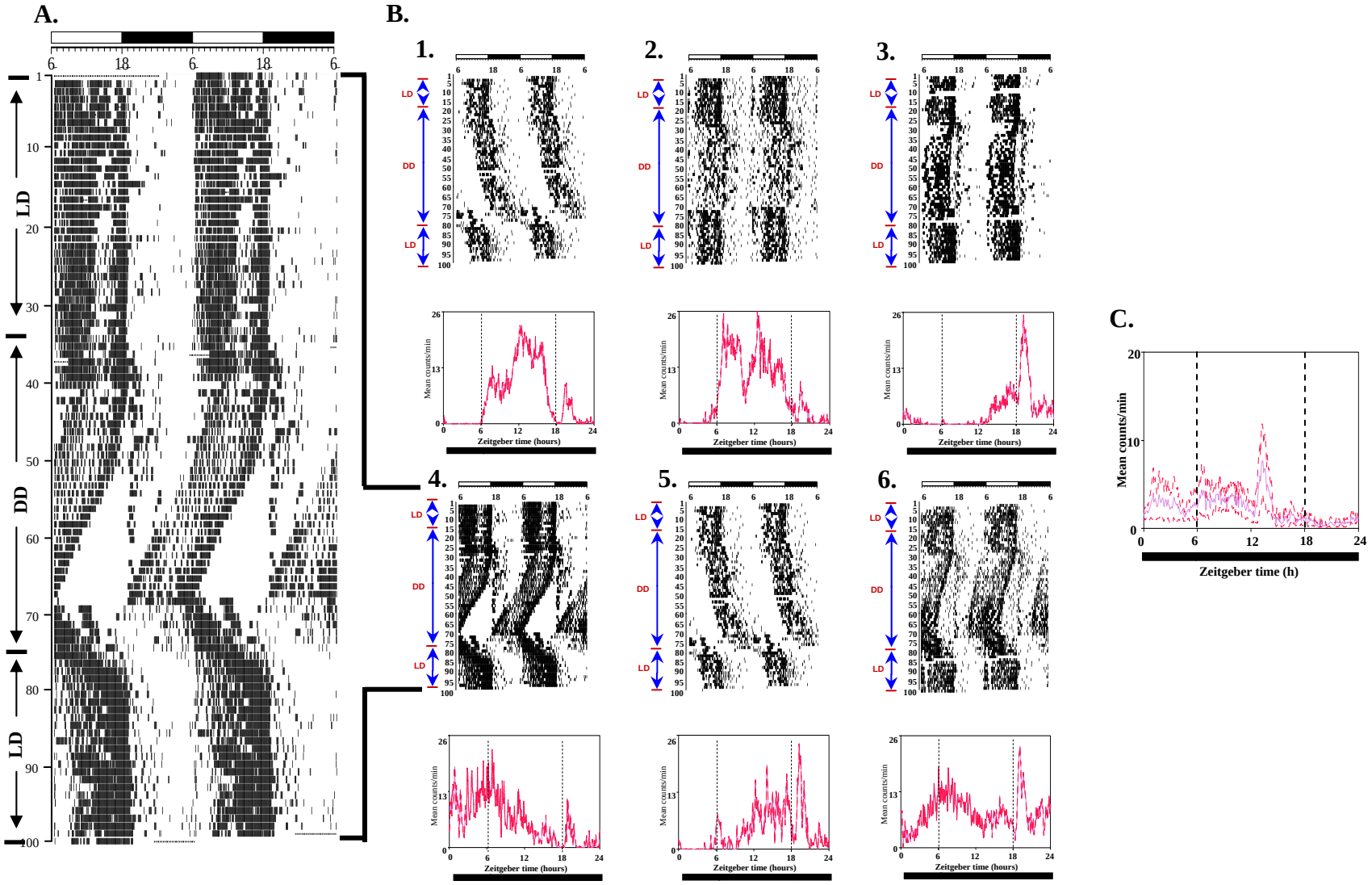


Figure 34. A. Representative double plotted actograms of running wheel activity for 100 days in DD condition. B (1-6). Actograms and their respective activity profiles of 6 individual animals under DD condition. C. Mean activity profile of 6 animals showing maximum activity during subjective light phase of DD. Each value is mean ± S.E. (n=6). The dashed lines on the activity profile indicates subjective lights on and off respectively.

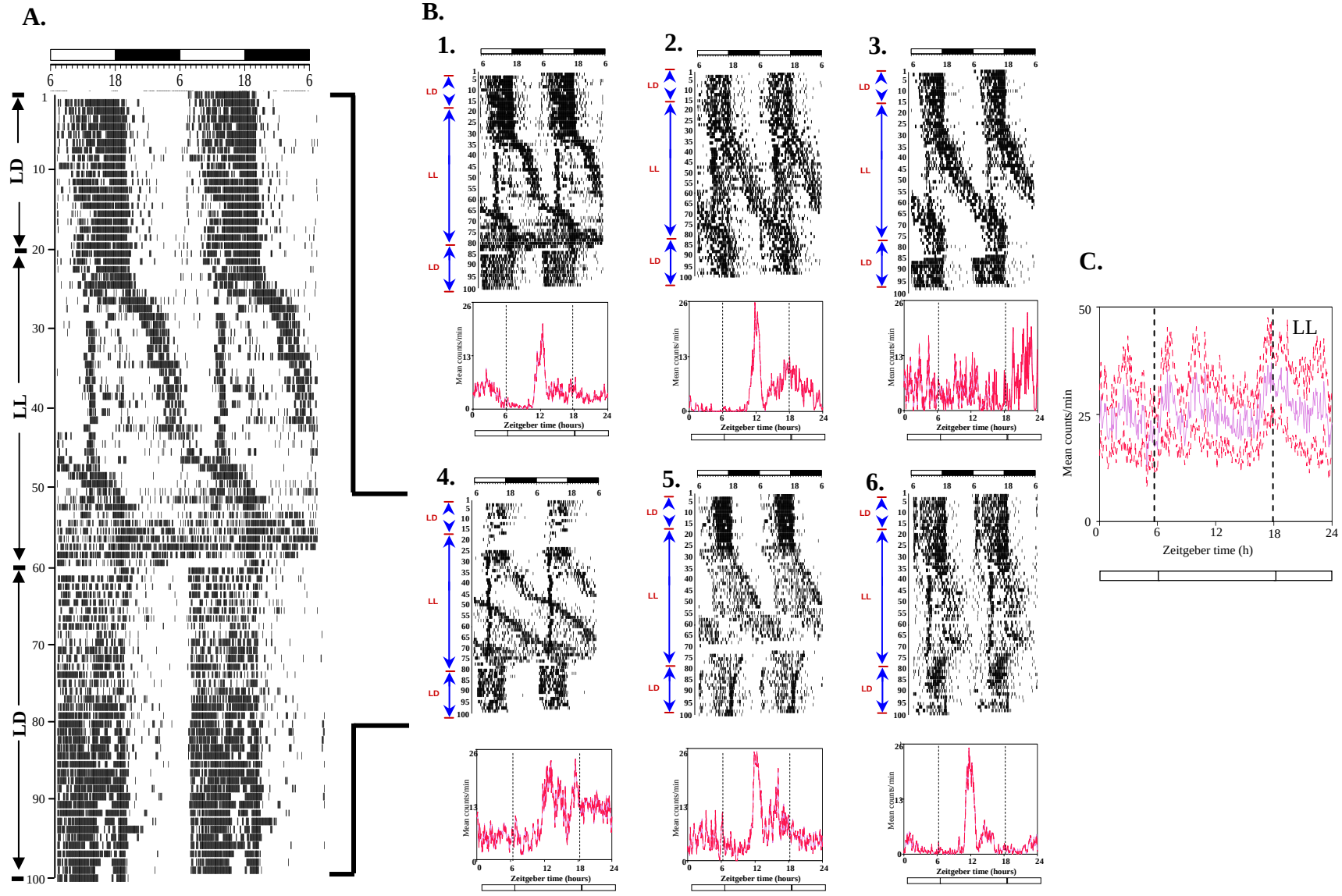


Figure 35. A. Representative double plotted actograms of running wheel activity for 100 days in LL condition showing splitting pattern of activity. B (1-6). Actograms and their respective activity profiles of 6 individual animals under LL condition. All 6 animals showed splitting. C. Mean activity profile of 6 animals. Each value is mean ± S.E. (n=6). The dashed lines on the activity profile indicates subjective lights on and off respectively.

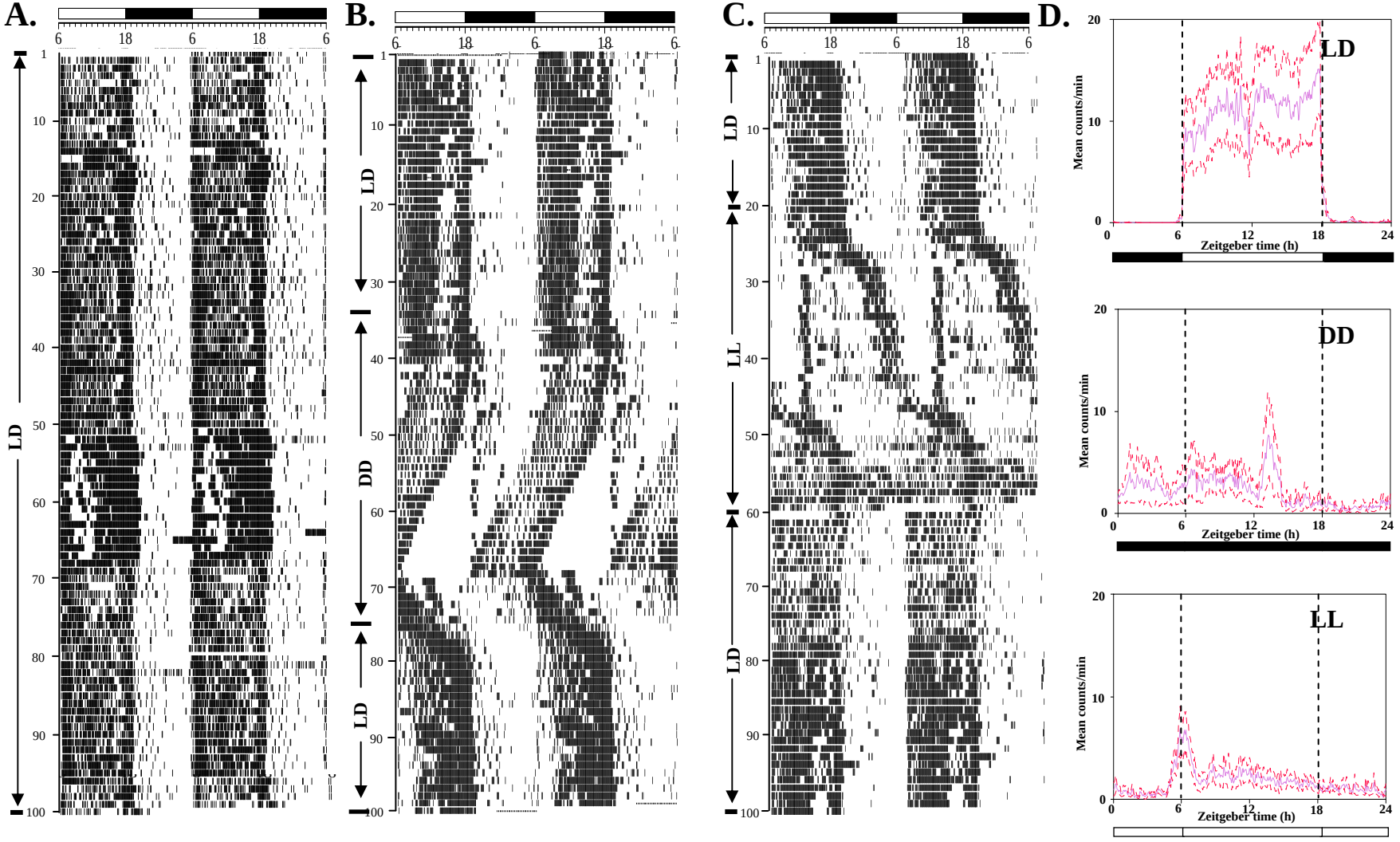


Figure 36. Representative double plotted actograms of running wheel activity for 100 days in various photoperiods LD, DD and LL conditions. A. In LD, a robust activity during the light phase and less activity during the dark phase; B. In DD, a free running activity with τ of 24.11 ± 0.03 hours; C. Under LL, a free running pattern with τ of 24.92 ± 0.35 hours were observed; D. Mean activity profiles under LD (n=12), DD (n=6) and LL (n=6) conditions. Each value is mean ± S.E. (n=6). The dashed lines on the activity profile indicates lights on and off for animals under LD 12:12 condition respectively.

Table-1. Comparison of circadian period (τ), duration of active period (α) and % diurnality between gross locomotor activity and running wheel activity.

Activity parameters	Locomotor activity under constant dark condition (LD)	
	Gross locomotor activity (n=6)	With running wheel (n=6)
τ (hours)	24.196 ± 0.108	24.01 ± 0.008
α (hours)	12.57 ± 0.49	11.62 ± 0.499
% diurnality	70.39 ± 3.88	94.94 ± 2.58*

Activity parameters	Locomotor activity under constant light condition (DD)	
	Gross locomotor activity (n=6)	With running wheel (n=6)
τ (hours)	24.11 ± 0.03	23.88 ± 0.19
α (hours)	12.82 ± 0.76	11.04 ± 0.72
% diurnality	73.13 ± 7.59	60.84 ± 5.40*

Activity parameters	Locomotor activity under constant light condition (LL)	
	Gross locomotor activity (n=6)	With running wheel (n=6)
τ (hours)	24.92 ± 0.35	24.28 ± 0.18
α (hours)	16.03 ± 1.03	15.72 ± 0.31
% diurnality	55.06 ± 3.82	62.21 ± 3.82

* ($p<0.05$) comparison between gross locomotor activity and running wheel activity. Each value is mean ± S.E. (n=6).

% diurnality showed significant increase in wheel running activity as compared to gross locomotor activity under LD condition ($p<0.05$) (Figure 37A), however no significant differences were observed under DD and LL conditions (Figure 37 A). τ and α showed no significant difference between gross locomotor activity and running wheel activity under LD, DD and LL conditions (Figure 37 B and C).

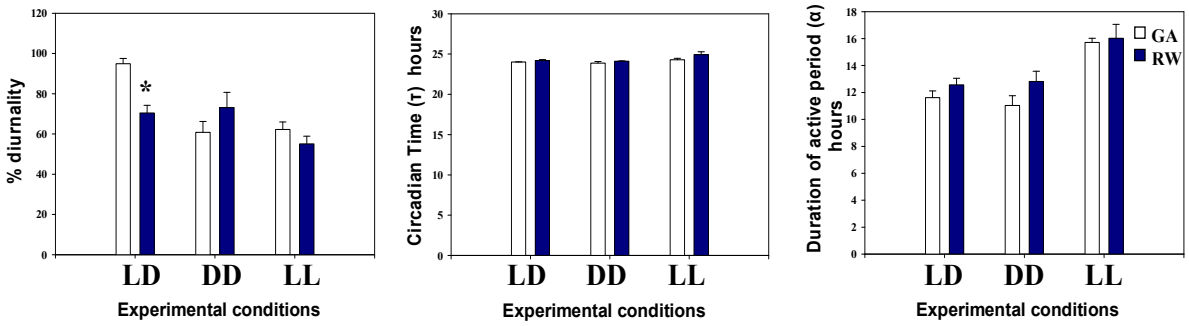


Figure 37. Comparison of activity parameters between running wheel and gross locomotor activity under LD, DD and LL conditions. A. % diurnality under LD of running wheel activity (RW) showed significant difference (*) as compared to gross locomotor activity (GA) (n=12) ($p < 0.05$). No significant difference in % diurnality under constant conditions (DD and LL) (n=6). Each value is mean ± S.E. (n=6). No significant difference was observed in τ (B) and α (C) in both RW and GA under LD (n=12), DD (n=6) and LL (n=6) conditions.

(iv). Locomotor pattern of individual *F. palmarum* under LD, DD and LL conditions

In LD cycle, individual animals (n=12; 6 for gross and 6 for running wheel) exhibited a diurnal pattern of locomotor activity (gross and wheel running), with 50% of activity exhibited during the subjective day (Figure 38A). Under constant darkness (DD), four out of six animals in gross locomotor and all six animals subjected to running wheels showed diurnal pattern of activity (Figure 38B). Under constant Light (LL) five out of six in both gross and wheel running showed diurnal pattern of activity (Figure 38C). The % diurnality was higher in LD as compared with DD and LL, thought with variability (Figure 38D).

(iii). Effect of prolonged LL on Running wheel activity:

F. palmarum (n=6) subjected to prolonged LL for 160 days (5.3 months) showed splitting pattern of activity which repeated for several cycles throughout the period of study (Figure 39 A, 40). On returning to LD condition all animals (n=6) showed entrainment to LD cycle (Figure 39 and 40). No change in τ and α in animals (n=6) subjected to 160 day LL as compared to those subjected to 40 day LL conditions.

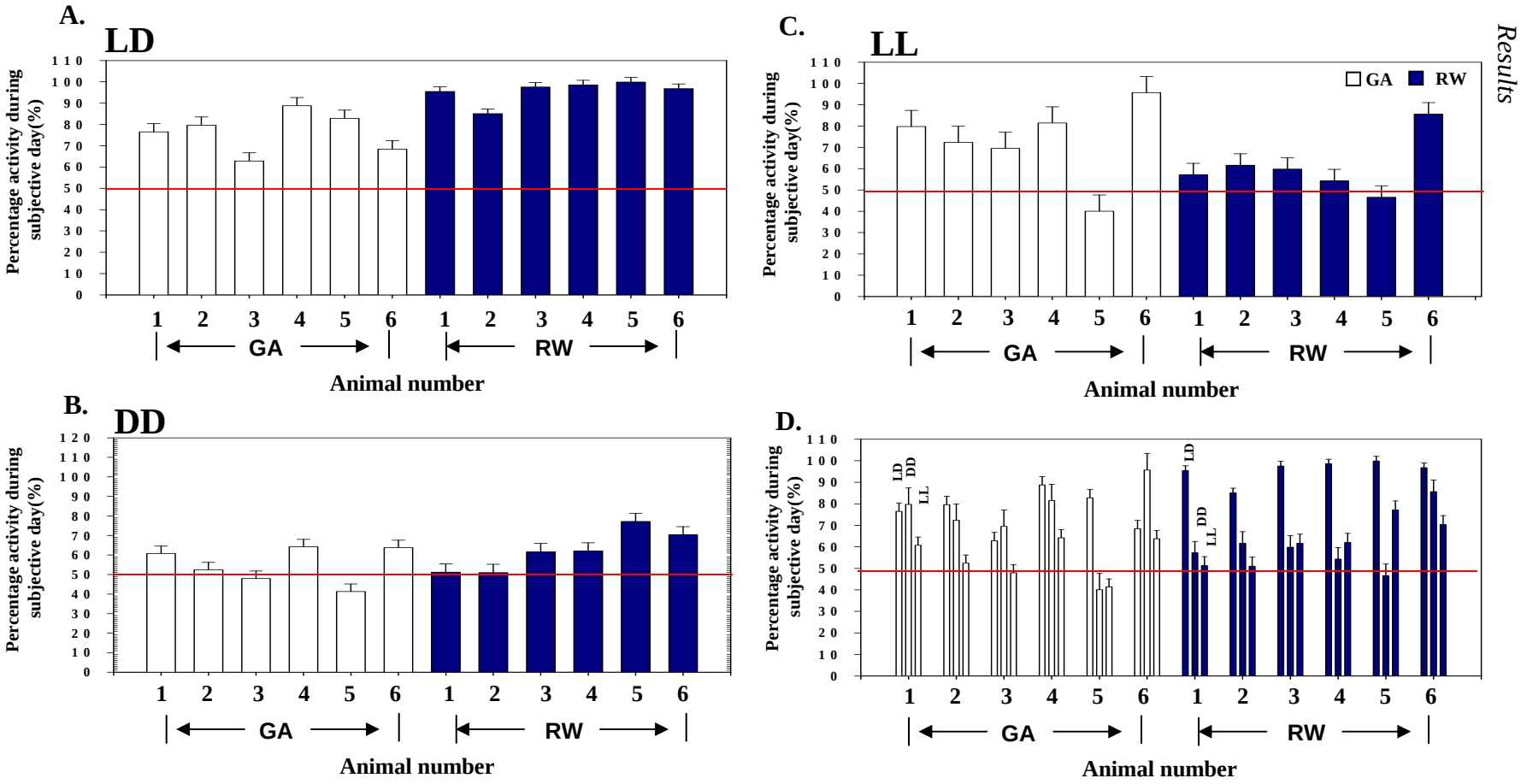


Figure 38. Variation in percentage locomotor activity of individual *F. palmarum* under LD (n=6) (A), DD (n=6) (B) and LL (n=6) (C) conditions. Activity of and above 50 % during the light phase is considered as diurnal. D. Comparison of % diurnality under LD, DD and LL, showing was higher % diurnality in LD as compared to DD and LL in both gross and wheel running activity ($p<0.05$). Each value is mean ± S.E. (n=6).

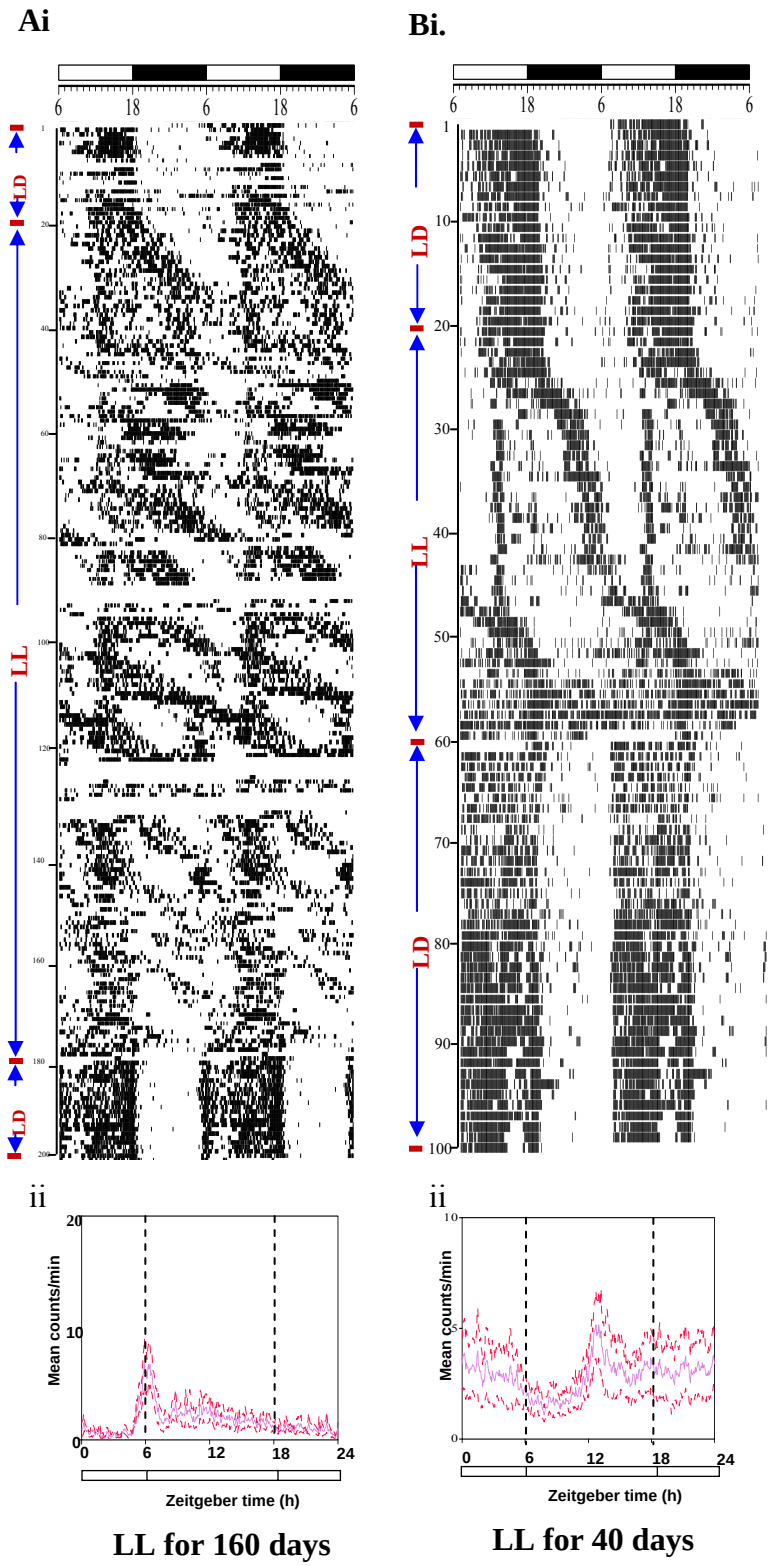


Figure 39. Representative double plotted actograms in constant light (LL) (160 days) on the running wheel activity (A) in comparison to 40 day (B). Splitting pattern sustained for the entire duration of study. The animals showed entrainment on returning to LD cycle (Ai). No change in the circadian period (τ) and duration of active period (α) under prolonged conditions. Mean activity profiles (n=6) showing persistent activity during the subjective light phase of LD cycle (Aii, Bii). Each value is mean ± S.E. (n=6). The dashed lines on the activity profile indicates lights on and off for animals under LD 12:12 condition respectively.

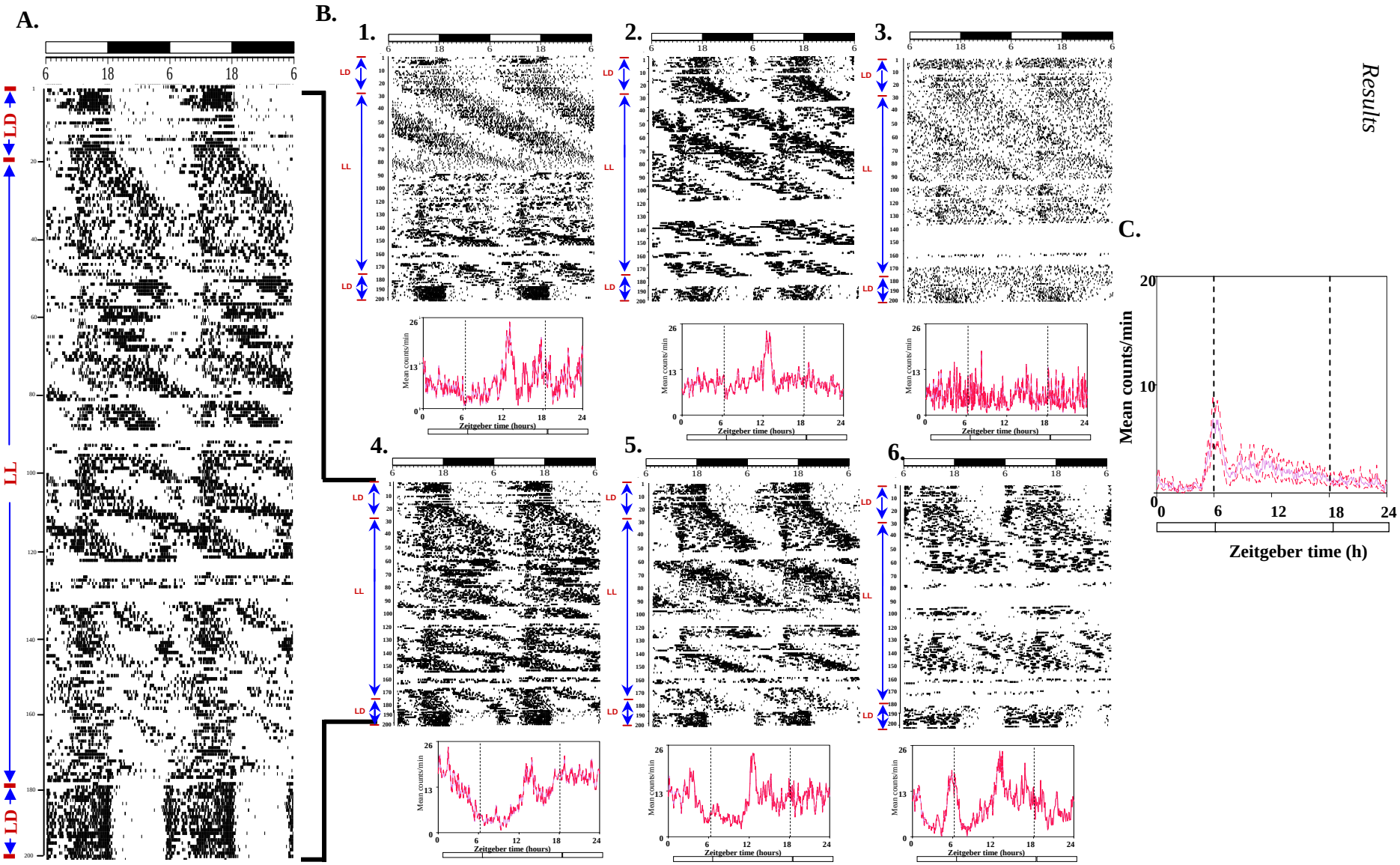


Figure 40. A. Representative double plotted actograms for 160 days-under LL condition showing splitting pattern of activity. B (1-6). Actograms and their respective activity profiles of 6 individual animals under LL condition. All 6 animals showed splitting and on returning to LD cycle showed entrainment. C. Mean activity profile of 6 animals. Each value is mean ± S.E. (n=6). The dashed lines on the activity profile indicates subjective lights on and off for animals under LD 12:12 condition respectively.

(b). Neuroanatomical studies: Neuroanatomy of the SCN

(i). Gross anatomy of the brain

The brain of adult *F. palmarum* was almost similar in size and weight as compared to adult rat. The major differences were seen in the structures associated with the circadian timing system, the optic nerves and pineal gland (Figure 41). The optic nerves of *F. palmarum* were short, thick and stout in comparison to rat where it is paired and slender. The optic chiasm was also found to be prominent in *F. palmarum* (Figure 41 Aii) as compared to rat (Figure 41 Bii). The pineal gland was found submerged embedded deep at the junction of the cerebral hemispheres in *F. palmarum* (Figure 41 Ai) as compared to the rat where it is superficial (Figure 41Bi).

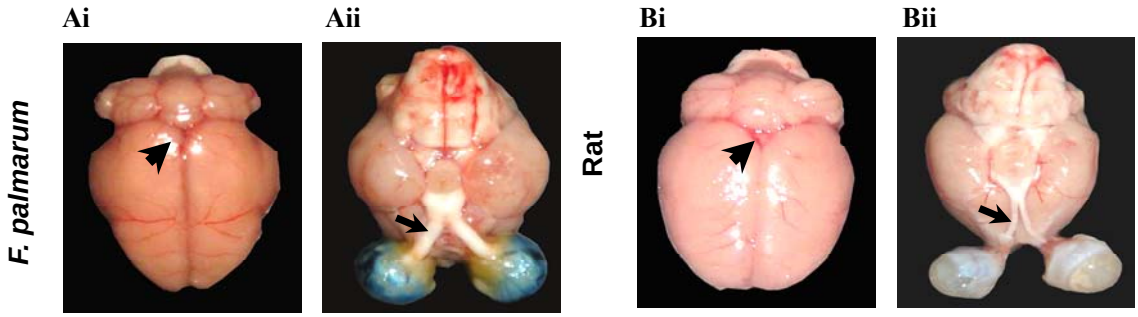


Figure 41. Comparison of the brain of *F. palmarum* (diurnal) (A) and Wistar Rat (nocturnal) (B). Prominent differences were seen in the pineal gland (Ai) and optic nerves (Aii) of *F. palmarum* as compared to rat (Bi and ii). The pineal gland was found submerged and embedded deep at the junction of cerebral hemispheres (Ai) as compared to the superficial location in the rat (Bi). The optic nerves were thick, short and stout (Aii) as compared to rat where it is thin, long and slender (Bii) (Mammen and Jagota, 2011).

(ii). Microscopic observation:

(i) Suprachiasmatic nuclei

The SCN were discrete, bilateral and elliptical in shape nuclei above the optic chiasm (n=12) (Figure 42 A and C) as compared to the spherical shape in the rat (n=4) (Figure 42 B). The nuclei were found completely separated by the third ventricle throughout the rostrocaudal length (Figure 42 A). The cells in the SCN were evenly distributed with a heterogeneous population of densely packed neurons of size varying from 3-10µ. The SCN was approximately 300µm in the rostrocaudal dimension with a neuronal count of 30851 ±

195 (n=12) ($p<0.05$). The third ventricle extended completely between the two SCN upto the optic chiasm (n=12) (Figure 42 Aiii)) (Mammen and Jagota, 2011).

(ii) AVP-ir and VIP-ir distribution in SCN

AVP-ir cell bodies were concentrated in the dorsal SCN in proximity to optic chiasm and third ventricle. AVP-ir projections were located dorsomedially and in proximity to third ventricle (n=4) (Figure 43 A). VIP-ir cell bodies were focused to the ventrolateral region of the SCN with very close proximity to the optic chasm. Dense concentrations of VIP-ir fibers were seen and the largest concentration of VIP-ir efferents appeared to exit the SCN dorsally (n=4) (Figure 43 B) (Mammen and Jagota, 2011).

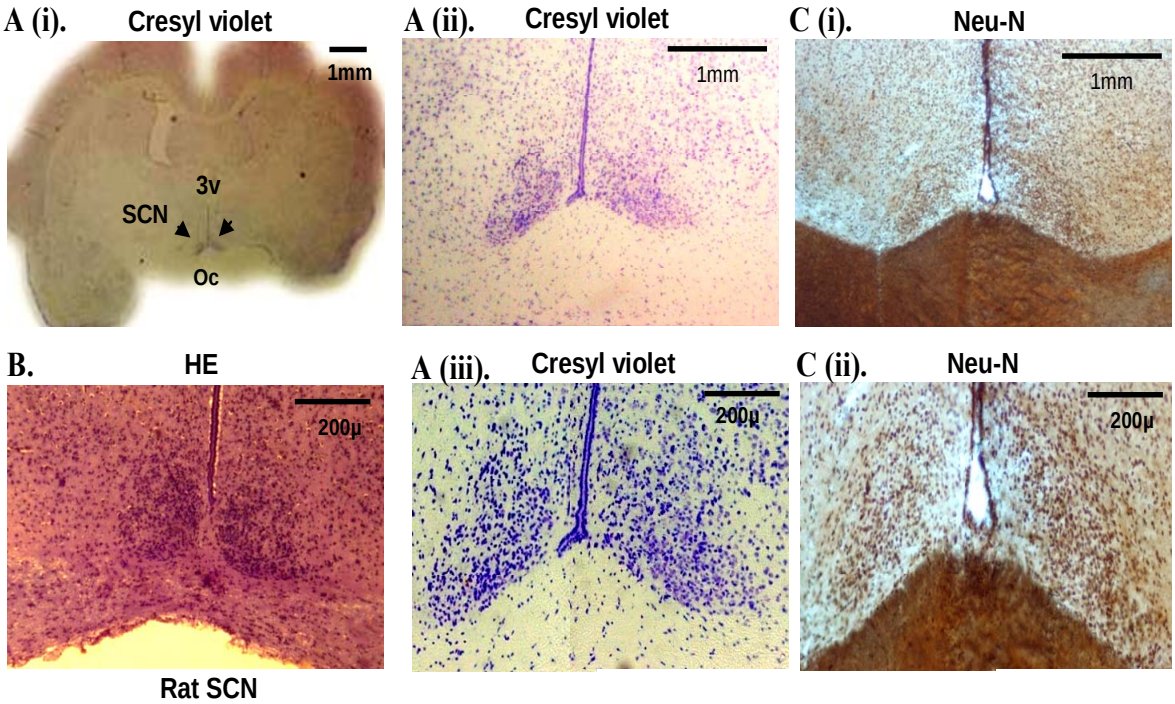


Figure 42. The suprachiasmatic nucleus of *F. palmarum*. (A) Cresyl violet staining of the coronal section showing the SCN in *F. palmarum*. A (i-iii) show the SCN at variable magnification. The SCN appeared elliptical in shape as compared to rat SCN (n=12) (B) which is spherical in shape (n=4). C. Neuronal marker Neu N-IHC of the SCN confirming the shape of the SCN (n=12) Scale bar = 1 mm for Ai,ii and Ci; 200µm for Aiii, B, Cii. (Top panel - Mammen and Jagota, 2011).

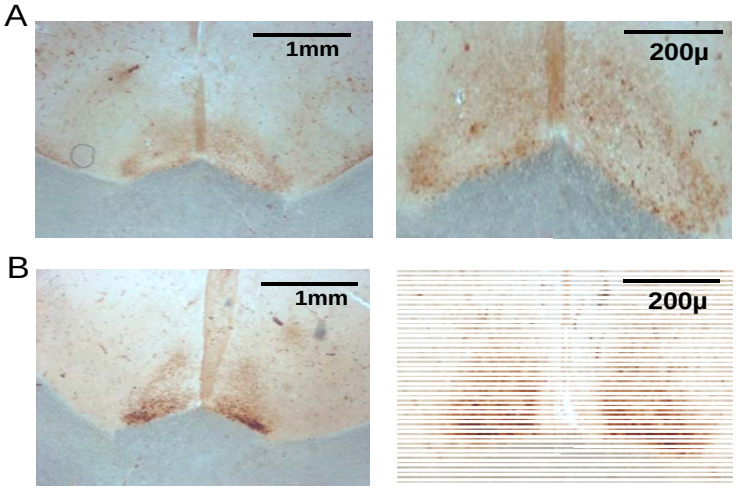


Figure 43. Core and shell differentiation of the SCN of *F. palmarum*. A. AVP-ir showed a dorsomedial (shell) pattern of distribution (n=4) and B. VIP-ir showed ventrolateral (core) pattern of distribution in the SCN (n=4). This pattern was similar to that reported in nocturnal rodents. Scale bar = 1 mm for left panel; 200 µm for right panel (Mammen and Jagota, 2011).

(c). Neurotransmitter studies:

(i). Daily profile of AVP-ir and VIP-ir under LD 12:12 condition

AVP-ir showed daily rhythmicity with maximum levels at ZT-12 (on-set of darkness) and minimum at ZT-0 (on-set of light) (n=4) (Figure 44 Ai and 44Bi, ia). AVP-ir levels at all ZT's studied were significantly different from each other (n=4) ($p \leq 0.05$). The daily mean of AVP-ir in LD 12:12 condition was significantly higher by 39.4 folds as compared to DD and by 34.25 folds as compared to LL condition (n=4) ($p < 0.05$) (Table 2). The maximum : minimum ratios of AVP-ir under LD 12: 12 was significantly higher by 1.93 folds as compared to DD and by 1.64 folds as compared to LL condition (n=4) ($p < 0.05$) (Table 2). No AVP-ir was also seen at the median pre-optic region (MOP) and sub-paraventricular zone (sPVHz) region (Figure 44 Ai).

Daily rhythmicity were observed in VIP-ir with maximum levels at ZT-6 (mid subjective day) and minimum levels at ZT-0 (On-set of light) (n=4) (Figure 44 Aii and Bii,iib). VIP-ir level at ZT-12 was neither significantly lower from ZT-6 nor significantly higher from ZT-18 though ZT-6 and ZT-18 were significantly different. However, VIP-ir level at ZT-0 was significantly different from ZT-6, 12 and 18 ($p_a \leq 0.05$) and ZT-18 was significantly higher from ZT-6 ($p_b \leq 0.05$). The daily mean and maximum : minimum ratios of

VIP-ir under LD 12:12 were not significantly different as compared to DD and LL condition (n=4) ($p<0.05$) (Table 2). VIP-ir was also observed at the median pre-optic region (MOP) and sub-paraventricular zone (sPVHz) at ZT-12 and ZT-18 respectively (Figure 44Aii).

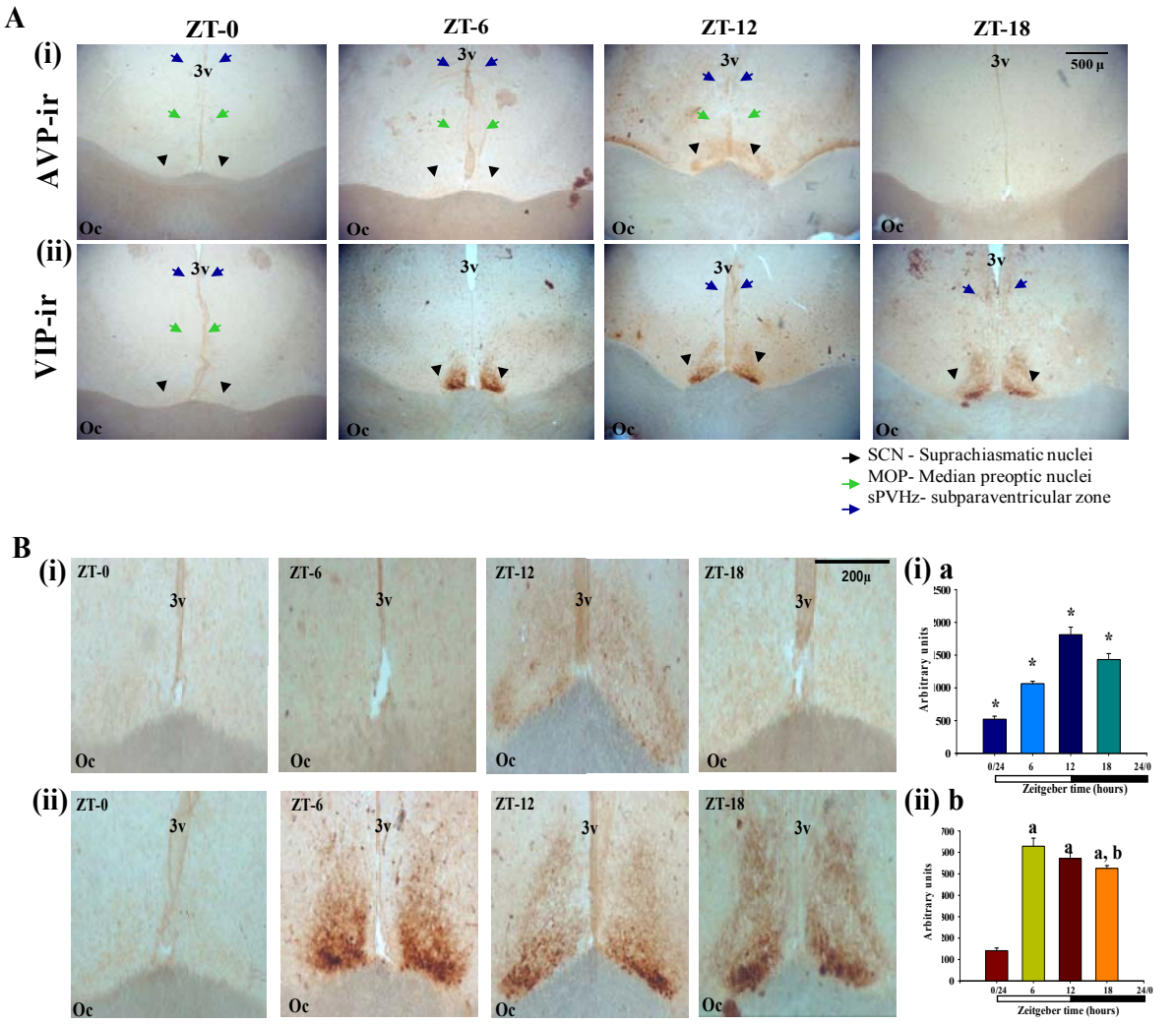


Figure 44 A. Daily profile of AVP-ir and VIP-ir in the SCN (black arrow), MOP (green arrow) and sPVHz (blue arrow) under LD, 12:12 condition (n=4). (i). VIP-ir was observed the MOP and sPVHz region at ZT-12 and ZT-18 respectively (n=4). (ii). No AVP-ir was observed at MOP and sPVHz regions (n=4). Scale bar= 500 µm. **B.** Daily profile of AVP-ir and VIP-ir in the SCN under LD, 12:12 condition. Both AVP (i) and VIP (ii) showed daily profile in its expression. Densitometric analysis showed AVP-ir maximum at ZT-12 and minimum at ZT-0 (ii.a) while VIP-ir maximum at ZT-6 and minimum at ZT-0 (ii.b). Each value is mean ± S.E (n=4). * AVP-ir at various ZT's was significantly different from each other ($p\leq0.05$). VIP-ir showed significant difference between ZT's ($p_a\leq0.05$ and $p_b\leq0.05$) (where a and b refers to comparison with ZT-0 and ZT-6 respectively). No significant difference between ZT-12 and ZT-18 were observed for VIP-ir. Scale bar= 200 µm (Mammen and Jagota, 2011).

(ii). Circadian profile of AVP-ir and VIP-ir under constant darkness (DD)

VIP-ir showed significant difference between ZT-12 and ZT-0, 6 and 18 (n=4) ($p<0.05$). VIP-ir levels at ZT-0 was not significantly lower as compared to ZT-6 and ZT-18 respectively (n=4) ($p<0.05$) (Figure 45Bii). VIP-ir under DD did not show significant difference in daily mean and maximum : minimum ratios as compared to LD and LL conditions (n=4) ($p<0.05$) (Table 2). VIP-ir maximum levels showed a phase delay of 6 hours as compared to LD 12:12 conditions (Figure 45 Aii and Bii) and a phase delay of 12 hours as compared to LL condition (Figure 45 Aii and Cii).

AVP-ir showed an abolition in circadian rhythm with no significant difference between different time points (n=4) ($p<0.05$) (Figure 46 Bi). Circadian rhythmicity was observed in VIP-ir with maximum levels at ZT-0 and minimum levels at ZT-12 (Figure 45 Bi). The daily mean levels and maximum : minimum ratios of AVP-ir under DD was significantly decreased as compared to LD but not significantly different as compared to LL (n=4) ($p<0.05$) (Table-2).

(ii). Circadian profile of AVP-ir and VIP-ir under constant light (LL)

VIP-ir showed circadian rhythmicity in its levels with maximum levels at ZT-12 and minimum levels at ZT-6 (Figure 45 C). VIP-ir levels at ZT-12 were significantly different to that at ZT-0, ZT-6 and ZT-18 (n=4) ($p<0.05$) (Figure 45 Cii). However VIP-ir levels at ZT-0, ZT-6 and ZT-18 were not significantly different between each other (n=4) (Figure 45 Cii). Under LL VIP-ir levels maximum level at ZT-12 showed a phase advance of 12 hours as compared to LD 12:12 conditions (Figure 45 A and C) and DD conditions (Figure 45 B and C). The daily mean levels and maximum : minimum ratios of VIP-ir under LL did not show significant difference as compared to LD and DD conditions (n=4) (Table 2).

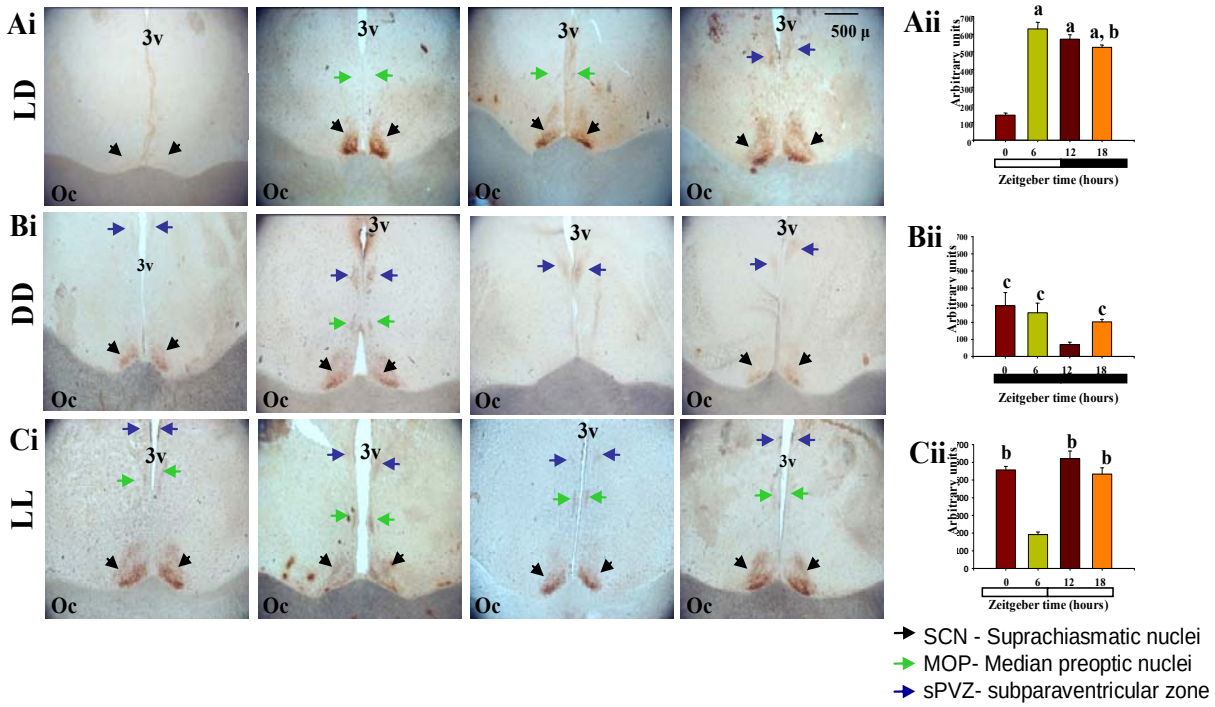


Figure 45. Effect of variable photoperiods on daily VIP-ir rhythms in the SCN, MOP and sPVHz of *F. palmarum* under LD, DD and LL conditions. VIP-ir in the SCN (black arrow heads) showed rhythmicity under LD (A), DD (B) and LL (C) condition. VIP-ir were also observed at the MOP (green arrow heads) and sPVHz (blue arrow heads) regions also. Densitometric analysis showing VIP-ir in the SCN with maximum levels in LD at ZT-6 (n=4) ($p<0.05$) (Aii) is phase advanced 6h to ZT-0, under constant dark (DD) (n=4) ($p<0.05$) (Bii). VIP-ir then showed phase delay of 6 hours, to ZT-12, under constant light (LL) (n=4) (C ii) condition as compared to LD 12:12 condition. Each value is mean ± S.E (n=4). VIP-ir showed significant difference between ZT's ($p_a \leq 0.05$, $p_b \leq 0.05$ and $p_c \leq 0.05$) (where a, b and c refers to comparison of ZT-0, ZT-6 and ZI-12 with other ZT's respectively). Scale bar= 500µm.

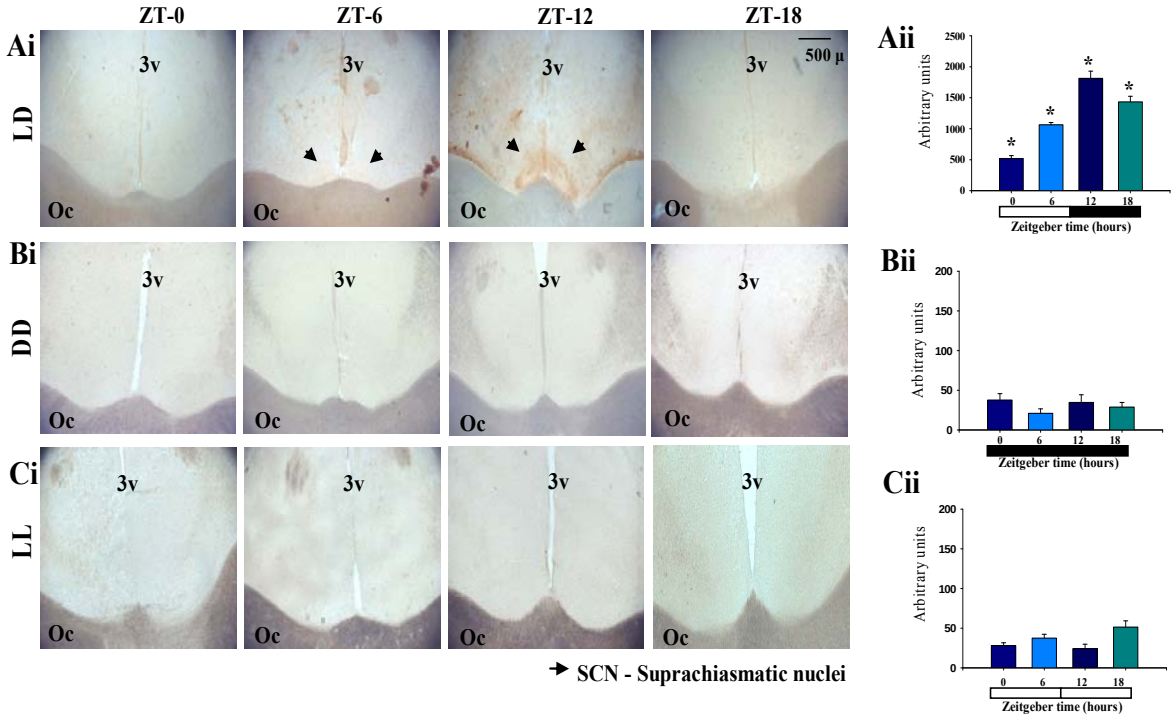


Figure 46. Effect of variable photoperiods on AVP-ir rhythms in the SCN of *F. palmarum*. *A*VP-ir in the SCN showed daily rhythmicity under LD (n=4) (A) but the rhythmicity was abolished under constant conditions DD (n=4) (B) and LL (n=4) (C) respectively. No AVP-ir were observed at the MOP and sPVHz regions. Each value is mean ± S.E (n=4). * The levels of AVP-ir in the SCN at ZT-0 were significantly different to that at ZT-6, ZT-12 and ZT-18 (n=4) ($p \leq 0.05$). No significant difference between ZT's were observer under DD and LL conditions (n=4) ($p \leq 0.05$). Scale bar=500µm.

Table 2. Comparison of Mean (A) and maximum : minimum ratios (B) of AVP and VIP under LD, DD and LL condition.

A

Experimental condition	AVP	VIP
LD	1207.42 ± 275.28	466.79 ± 110.76
DD	30.63 ± 3.69 [a]	206.33 ± 49.55
LL	35.25 ± 6.05 [bc]	475.96 ± 96.48

B

Experimental condition	AVP	VIP
LD	3.48 ± 0.36	4.47 ± 0.29
DD	1.80 ± 0.15[a]	4.26 ± 0.79
LL	2.12 ± 0.41[bc]	3.23 ± 0.63

Each value is mean ± S.E (n=4). The mean and maximum: minimum ratios levels of AVP-ir in LD were significantly different from that in DD and LL. AVP-ir was also significantly different between DD and LL ($p_a \leq 0.05$; $p_b \leq 0.05$ and $p_c \leq 0.05$; where a refers to comparison between LD and DD, b refers to comparison between LD and LL & c refers to comparison between DD and LL). VIP mean and maximum : minimum ratios showed no significant difference between conditions ($p<0.05$).

Under LL conditions, AVP-ir showed an abolished pattern of circadian rhythm (n=4) ($p<0.05$) (Figure 46Ci) similar to that under DD (Figure 46 Bi). The AVP-ir levels at ZT-18 were significantly different to that at ZT-0 and ZT-12 (n=4) ($p<0.05$) (Figure 46 Cii). No significant difference were observed in the levels between ZT-6 and ZT-12 (n=4) ($p<0.05$) (Figure 46 Cii). The daily mean of AVP-ir in LL was significantly different as compared to LD but was not significantly different as compared to DD (n=4) ($p<0.05$) (Table 2). The maximum : minimum levels of AVP-ir in LL were not significantly different as compared to LD and DD condition (n=4) ($p<0.05$) (Table 2).

(iv). Daily rhythm of serotonin and its related compounds in the SCN, pineal and brain

Serotonin chronometabolome studies in the SCN showed daily rhythmicity in the levels of all compounds. 5-HTP, 5-HIAA, 5-HT, NAS and 5-MIAA showed maximum levels during the light phase and minimum during the dark phase under LD 12:12 conditions ($p \leq 0.05$) (Figure 47). TRP, 5-HTOH, 5-MTOH, MEL and NAT showed maximum levels during the dark phase of LD 12:12 (Figure 50). The mean for 24 hours was maximum for 5-HIAA and minimum for 5-HTOH (n=6) ($p<0.05$) where as the max: min ratios were highest for 5-HTP and minimum for melatonin (n=6) ($p<0.05$) (Table 3).

In the pineal, serotonin chronometabolome showed daily rhythmicity in the levels of all metabolites. 5-HTP, 5-HT, TRP, 5-HTOH, NAS, 5-MIAA and NAT showed maximum levels during the light phase where as 5-HIAA, 5-MTOH and MEL showed maximum levels during the dark phase (Figure 48). The mean 24 hours was maximum for 5-HTP and minimum for TRP (n=6) ($p<0.05$) where as the maximum: minimum ratios were maximum for TRP and minimum for 5-HTOH (n=6) ($p<0.05$) (Table 4).

In the brain 5-HTP, 5-HT, 5-HIAA, and NAT showed daily rhythmicity with maximum levels during the light phase (Figure 49). The mean 24 hours was maximum for NAS and minimum for 5-HTP (n=6) ($p<0.05$) where as the maximum: minimum ratios were maximum for TRP and minimum for NAS (n=6) ($p<0.05$) (Table 5).

The mean levels of 5-HTP, TRP, NAS, 5-MIAA, 5-MTOH, MEL and NAT were higher in the brain as compared to SCN (n=6) ($p_b<0.05$) and pineal (n=6) ($p_c<0.05$) (Figure 50 A). Mean levels of 5-HIAA and 5-HT were higher in the SCN as compared to brain (n=6) ($p_b<0.05$) and pineal (n=6) ($p_c<0.05$) (Figure 50 A). The maximum : minimum ratio of 5-HTP, 5-HT, TRP showed significantly higher levels in the SCN as compared to Pineal (n=6) ($p_a<0.05$) and brain (n=6) ($p_b<0.05$). Only TRP levels in the pineal was higher as compared to brain (n=6) ($p_c<0.05$) (Figure 50 B).

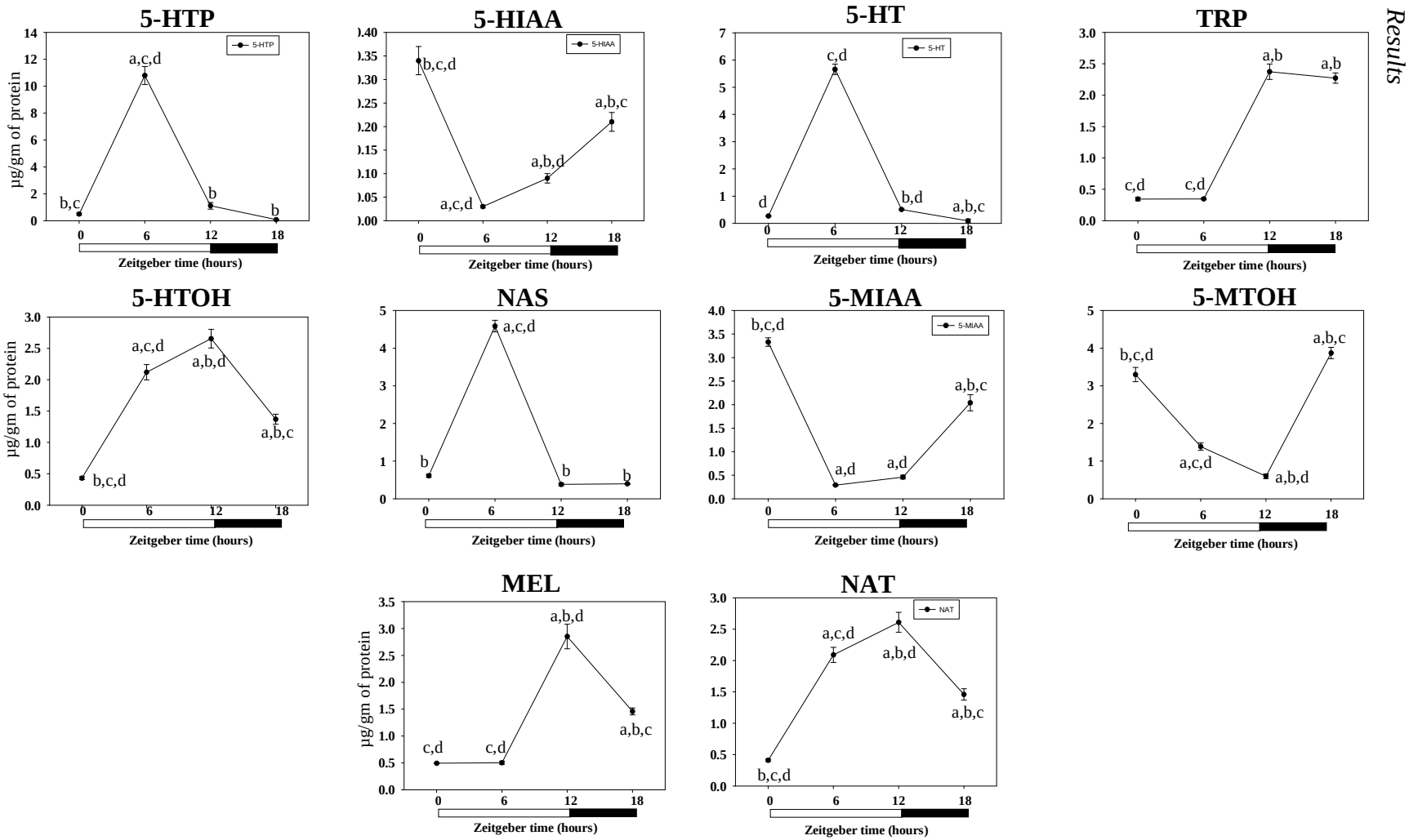


Figure 47. Daily profile of serotonin and its related compounds in the SCN of *F. palmarum.* All compounds showed daily rhythmicity. *pa < 0.05*; *pb < 0.05*; *pc < 0.05* and *pd < 0.05* (where a, b, c and d refers to comparison between ZT-0, 6, 12 and 18 respectively).

Table 3. Comparison of serotonin and its related compounds in the SCN of 3 months old *F. palmarum*.

Metabolites	Max Levels (µ mol/gm)	Max Time point	Min Levels (µ mol/gm)	Min Time point	Ratio Max/Min	Daily Mean levels (µ mol/gm)
5-HTP	10.80 ± 0.67	ZT- 6	0.08 ± 0.01*	ZT-18	140.35 ± 59 b,c,d,e,f,g,h,i,j	0.55 ± 0.03 b,e,h,i
5-HIAA	0.34 ± 0.03	ZT-0	0.03 ± 0.003*	ZT-6	11.45 ± 10.99	6.23 ± 0.59 a,c,d.e,f,g,h,i,j
5-HT	5.66 ± 0.19	ZT-6	0.09 ± 0.07*	ZT-18	65.65 ± 0.04	0.58 ± 0.09 a,b,d,e,f,i
TRP	2.37 ± 0.12	ZT-12	0.34 ± 0.03 *	ZT-0	6.89 ± 0.22	0.33 ± 0.03 b,e,h,i
5-HTOH	2.66 ± 0.15	ZT-12	0.43 ± 0.02*	ZT-0	6.16 ± 5.11	2.22 ± 0.25 a,b,c,d,f,g,j
NAS	4.59 ± 0.15	ZT-6	0.38 ± 0.04*	ZT-12	12.01 ± 4.27	1.01 ± 0.19 a,b,e,i
5-MIAA	3.33 ± 0.99	ZT-0	0.29 ± 0.01*	ZT-6	11.21 ± 6.18	0.35 ± 0.03 b,e,h,i
5-MTOH	3.87 ± 0.15	ZT-18	0.59 ± 0.06*	ZT-12	6.47 ± 2.42	1.62 ± 0.18 a,b,c,d,g
MEL	2.85 ± 0.23	ZT-12	0.49 ± 0.01*	ZT-0	5.78 ± 5.45	2.21 ± 0.25 a,b,c,d,f,g,j
NAT	2.61 ± 0.16	ZT-12	0.41 ± 0.02*	ZT-0	6.30 ± 5.61	1.07 ± 0.12 a,b,e,i

Daily mean: 5-HIAA > 5-HTOH > MEL > 5-MTOH > NAT > NAS > TRP > 5-HTP > 5-MIAA > 5-HTOH
Max/min ratios: 5-HTP > 5-HT > NAS > 5-HIAA > 5-MIAA > TRP > 5-MTOH > NAT > 5-HTOH > MEL

The mean levels in descending order of levels in the SCN are as follows 5-HIAA, 5-HTOH, MEL, 5-MTOH, NAT, NAS, TRP, 5-HTP, 5-MIAA and 5-HTOH. Maximum: minimum ratio in the descending order of their levels are 5-HIAA, 5-HTOH, MEL, 5-MTOH, NAT, NAS, TRP, 5-HTP, 5-MIAA and 5-HTOH. The maximum levels of metabolites were significantly higher as compared to their minimum levels (*)($p<0.05$). Each value is Mean ± S.E (n=6). p_a, p_b, p_c, p_d, p_e, p_f, p_g, p_h, p_i, $p_j < 0.05$ (where a, b, c, d, e, f, g, h, i and j refers to comparison with 5-HTP, 5-HIAA, 5-HT, TRP, 5-HTOH, NAS, 5-MIAA, 5-MTOH, MEL and NAT respectively. * refers to the comparison between maximum and minimum levels (n=6) ($p<0.05$).

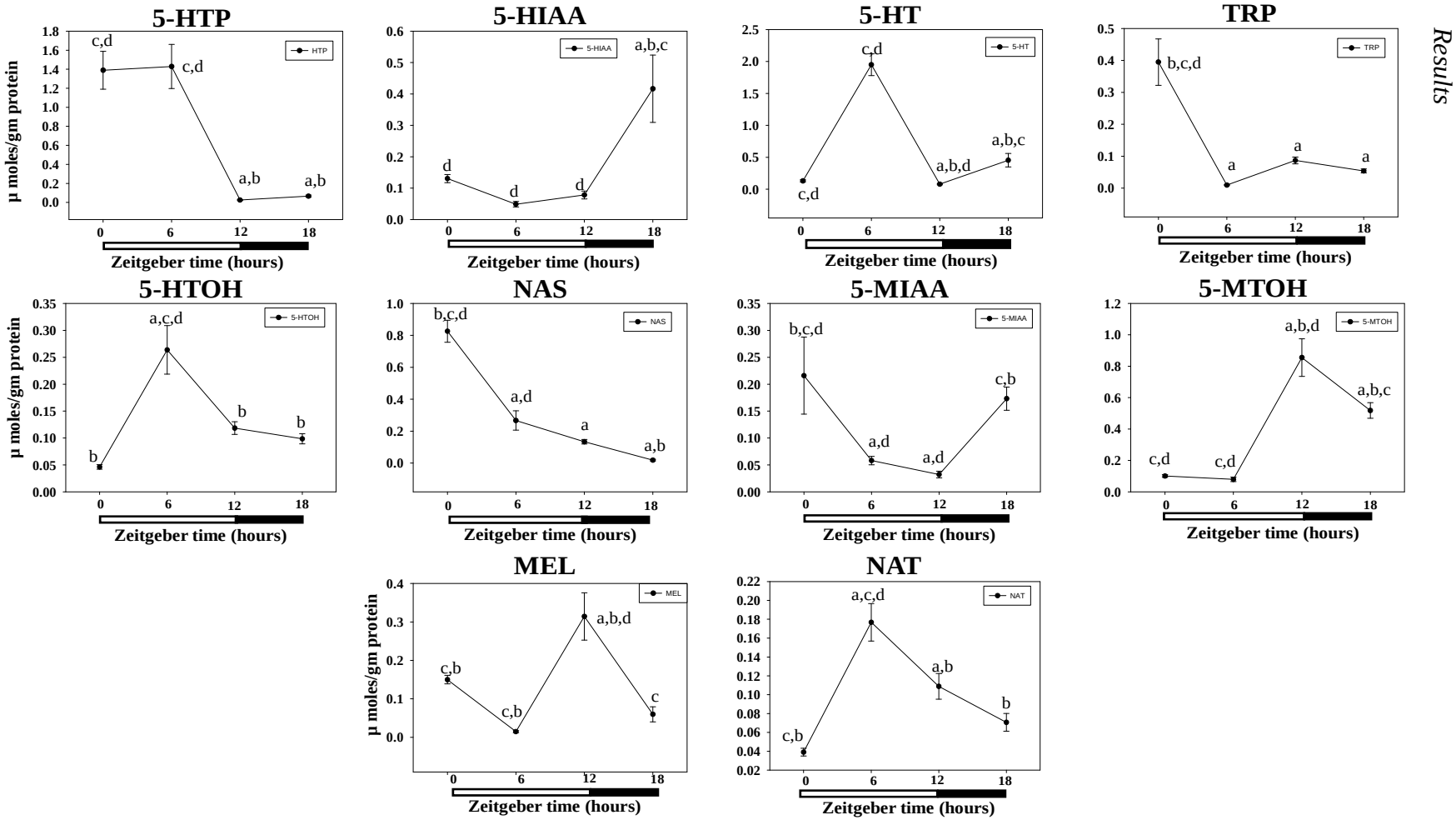


Figure 48. Daily profile of serotonin and its related compounds in the pineal of *F. palmarum.* All compounds showed daily rhythmicity. 5-HTP, 5-HT, TRP, 5-HTOH, NAS, 5-MIAA and NAT showed maximum levels at light phase and minimum at dark phase. 5-HIAA, 5-MTOH and MEL showed maximum levels during the dark phase and minimum during the light phase. Each value is Mean ± S.E (n=6). *pa < 0.05*; *pb < 0.05*; *pc < 0.05* and *pd<0.05* (where a, b, c and d refers to comparison between ZT-0, 6, 12 and 18 respectively).

Table 4. Comparison of serotonin and its related compounds in the Pineal of 3 months old *F. palmarum.*

Metabolites	Max Levels (µ mol/gm)	Max Time point	Min Levels (µ mol/gm)	Min Time point	Ratio Max/Min	Daily Mean levels (µ mol/gm)
5-HTP	1.43 ± 0.23	ZT-6	0.02 ± 0.004*	ZT-12	2.41 ± 0.24 ^{d}	1.25 ± 0.12^{b,c,d,e,f,g,h,i,j}
5-HIAA	0.42 ± 0.11	ZT-18	0.05 ± 0.008*	ZT-6	2.64 ± 1.64 ^{d}	0.67 ± 0.11^{a,c,d,e,f,g,h,i,j}
5-HT	1.95 ± 0.17	ZT-6	0.08 ± 0.01*	ZT-12	2.85 ± 0.54 ^{d}	0.18 ± 0.03^{a,b}
TRP	0.39 ± 0.07	ZT-0	0.009 ± 1.73	ZT-6	7.55 ± 1.56 ^{a,b,c,d,e,f,g,h,i,j}	0.03 ± 0.005^{a,b,h}
5-HTOH	0.26 ± 0.04	ZT-6	0.05 ± 0.004*	ZT-0	0.73 ± 0.17 ^{d}	0.19 ± 0.03^{a,b}
NAS	0.83 ± 0.07	ZT-0	0.019 ± 0.005*	ZT-18	1.31 ± 0.42 ^{d}	0.06 ± 0.005^{a,b,h}
5-MIAA	0.22 ± 0.07	ZT-0	0.03 ± 0.006*	ZT-12	3.18 ± 0.86 ^{d}	0.05 ± 0.005^{a,b,h}
5-MTOH	0.86 ± 0.12	ZT-12	0.08 ± 0.01*	ZT-6	2.68 ± 0.72 ^{d}	0.26 ± 0.03^{a,b,d}
MEL	0.31 ± 0.06	ZT-12	0.02 ± 0.002*	ZT-6	1.23 ± 0.177	0.12 ± 0.006^{a,b,j}
NAT	0.18 ± 0.02	ZT-6	0.04 ± 0.004*	ZT-0	1.59 ± 0.43 ^{d}	0.08 ± 0.006^{a,b,h}

Daily mean: 5-HTP > 5-HIAA > 5-MTOH > 5-HT > 5-HTOH > MEL > NAT > NAS > 5-MIAA > TRP
Max/min ratios: TRP > 5-MIAA > 5-HT > 5-MTOH > 5-HT > 5-HTP > NAT > NAS > MEL > 5-HTOH

The mean levels in descending order of levels in the SCN are as follows 5-HTP, 5-HIAA, 5-MTOH, 5-HT, 5-HTOH, MEL, NAT, NAS, 5-MIAA and TRP. Maximum: minimum ratio in the descending order of their levels are TRP, 5-MIAA, 5-HT, 5-MTOH, 5-HT, 5-HTP, NAT, NAS, MEL and 5-HTOH. The maximum levels of metabolites were significantly higher as compared to their minimum levels (*)($p<0.05$). TRP did not show significant difference between maximum and minimum levels. Each value is Mean ± S.E (n=6). p_a, p_b, p_c, p_d, p_e, p_f, p_g, p_h, p_i, p_j < *0.05* (where a, b, c, d, e, f, g, h, i and j refers to comparison with 5-HTP, 5-HIAA, 5-HT, TRP, 5-HTOH, NAS, 5-MIAA, 5-MTOH, MEL and NAT respectively. * refers to the comparison between maximum and minimum levels (n=6) ($p<0.05$).

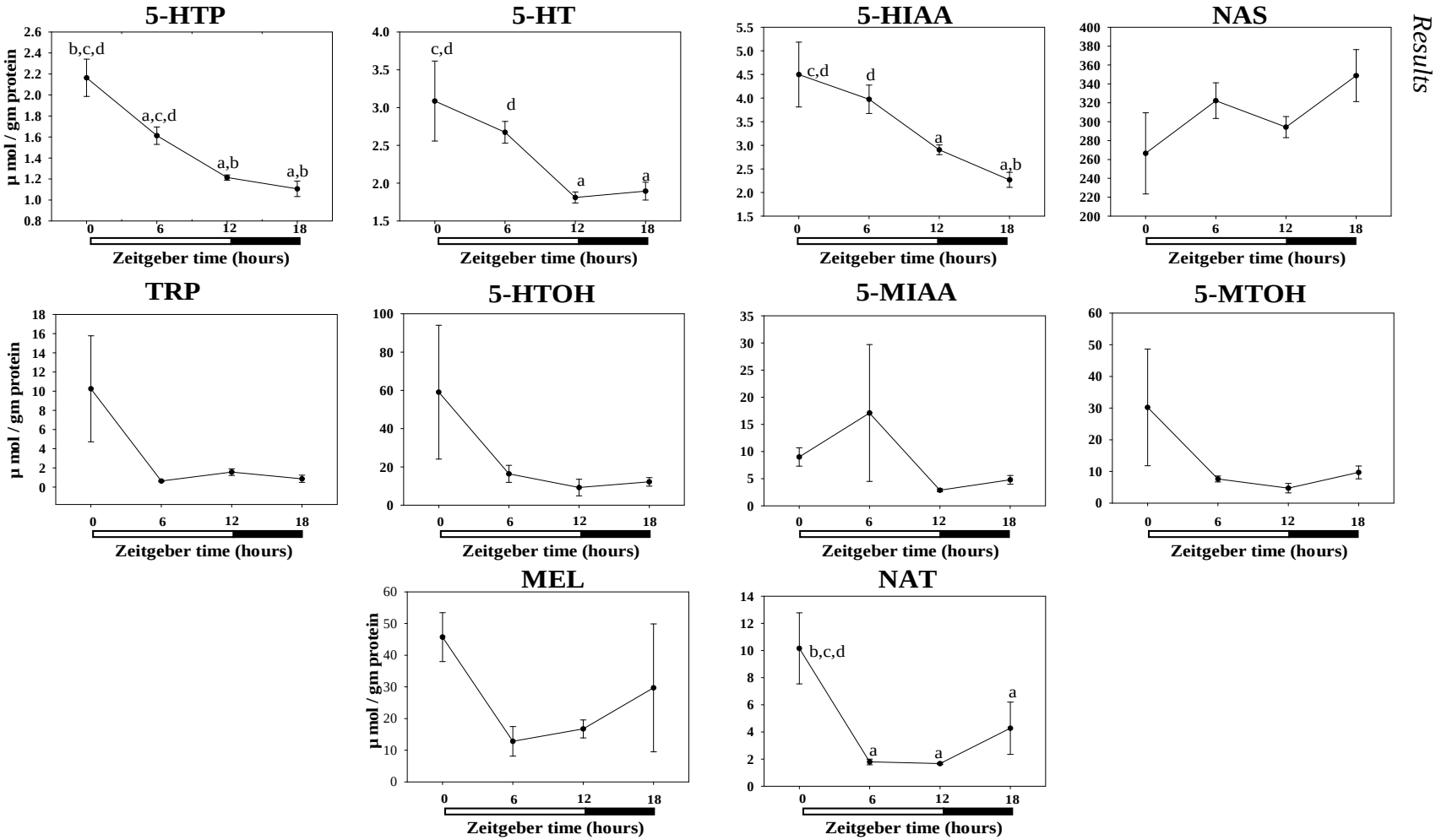


Figure 49. Daily profile of serotonin and its related compounds in the brain of *F. palmarum.* 5-HTP, 5-HT, 5-HIAA, and NAT showed daily rhythmicity with maximum levels during the light phase. Each value is Mean ± S.E (n=6). *pa < 0.05*; *pb < 0.05*; *pc < 0.05* and *pd<0.05* (where a, b, c and d refers to comparison between ZT-0,6, 12 and 18 respectively).

Table 5. Comparison of serotonin and its related compounds in the Brain of 3 months old *F. palmarum.*

Metabolites	Max Levels (µ mol/gm)	Max Time point	Min Levels (µ mol/gm)	Min Time point	Ratio Max/Min	Daily Mean levels (µ mol/gm)
5-HTP	2.16 ± 0.35	ZT- 0	1.11 ± 0.15[a]	ZT- 12	1.96 ± 0.1317	1.52 ± 0.24*
5-HT	3.09 ± 1.06	ZT- 0	1.81 ± 0.15	ZT- 12	1.75 ± 0.37	2.37 ± 0.31*
5-HIAA	4.49 ± 1.37	ZT- 0	2.27 ± 0.21	ZT- 18	2.02 ± 0.33	3.41 ± 0.51*
NAS	348.79 ± 55.07	ZT- 18	266.41 ± 85.91	ZT- 0	1.44 ± 0.22	307.91 ± 17.76
TRP	10.25 ± 0.11	ZT- 0	0.64 ± 0.13[a]	ZT- 6	17.79 ± 10.37	3.33 ± 2.32*
5-HTOH	59.06 ± 69.98	ZT- 0	9.22 ± 0.867	ZT- 12	11.30 ± 7.30	24.19 ± 11.71*
5-MIAA	17.09 ± 0.25	ZT- 6	2.86 ± 0.53	ZT- 12	5.85 ± 4.27	8.43 ± 3.16*
5-MTOH	30.22 ± 3.69	ZT- 0	4.70 ± 2.98	ZT- 12	11.84 ± 6.43	13.05 ± 5.81*
MEL	45.71 ± 7.70	ZT- 0	12.79 ± 0.93[a]	ZT- 6	15.55 ± 12.57	26.23 ± 7.43*
NAT	10.15 ± 5.23	ZT- 0	1.66 ± 0.13	ZT- 12	6.281 ± 1.687	4.46 ± 1.98*

Daily mean: NAS > MEL > 5-HTOH > 5-MTOH > 5-MIAA > NAT > 5-HIAA > TRP > 5-HT > 5-HTP

Max/min ratios: TRP > MEL > 5-MTOH > 5-HTOH > NAT > 5-MIAA > 5-HIAA > 5-HTP > 5-HT > NAS

The mean levels in descending order of levels in the SCN are as follows NAS, MEL, 5-HTOH, 5-MTOH, 5-MIAA, NAT, 5-HIAA, TRP, 5-HT and 5-HTP. Maximum: minimum ratio in the descending order of their levels are TRP, MEL, 5-MTOH, 5-HTOH, NAT, 5-MIAA, 5-HIAA, 5-HTP, 5-HT and NAS. Each value is Mean ± S.E (n=6). The maximum levels of 5-HTP, TRP and MEL were significantly higher from their minimum levels ($p_a<0.05$). No significant difference between maximum and minimum levels were observed in other metabolites. The mean levels of NAS was significantly different (*) from that of 5-HTP, 5-HIAA, 5-HT, TRP, 5-HTOH, 5-MIAA, 5-MTOH, MEL and NAT ($p<0.05$). No significant difference in the mean levels were seen in the comparison between other metabolites. The maximum: minimum levels did not show any significant difference in the comparison between various metabolites. * refers to the comparison between maximum and minimum levels (n=6) ($p<0.05$).

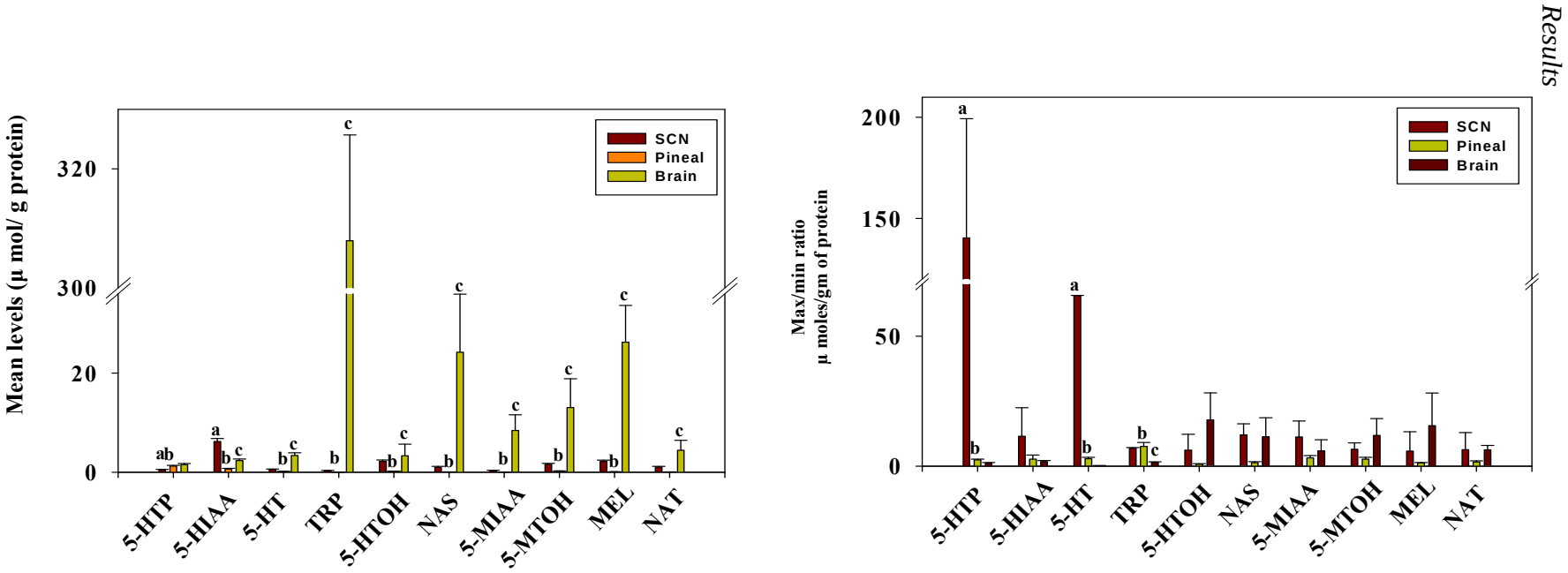


Figure 50. Mean (A) and daily pulse (B) of serotonin and its metabolites in the SCN, Pineal and Brain of *F. palmarum.* Each value is Mean ± S.E (n=6). * One way anova for the comparison of levels of metabolites where a, b, c represent comparison of SCN with pineal, SCN with brain and pineal with brain (*pa*, *pb*, *pc<0.05*).

(d). Clock gene expression studies: *per1, per2, cry1, cry2* and *bmal1* expression

The *per1, per2, cry1* and *cry2* genes expression showed a diurnal pattern of expression in the SCN with maximum and minimum levels at ZT-0 and ZT-18 respectively. However, *bmal1* gene showed rhythmicity with maximum levels at ZT-18 and minimum at ZT-0 respectively (Figure 51A). Densitometric analysis showed *per1* levels with maximum levels of 451.28 ± 1.03 arbitrary units at ZT-0 and minimum levels of 263.54 ± 2.58 arbitrary units at ZT-18. The *per2* levels showed maximum levels of 452.47 ± 0.59 arbitrary units at ZT-0 and minimum levels of 210.24 ± 2.14 arbitrary units at ZT-18. The *cry1* levels showed maximum levels of 447.73 ± 1.02 arbitrary units at ZT-0 and minimum levels of 210.84 ± 6.98 arbitrary units at ZT-18. The cry*2* levels showed maximum levels of 448.91 ± 2.96 arbitrary units at ZT-0 and minimum levels of 258.81 ± 3.29 arbitrary units at ZT-18. However, the *bmal1* levels were maximum at ZT18 with levels of 121.41 ± 3.60 units and minimum levels of 39.89 ± 1.03 units at ZT-0 respectively (Figure 51B). The daily pulses of *per1*, *per2*, *cry1*, *cry2* and *bmal1* genes were 1.7 ± 0.005, 2.15 ± 0.005, 2.11 ± 0.005, 2.03 ± 0.011 and 3.106 ± 0.0017 (n=6) ($p<0.05$) respectively.

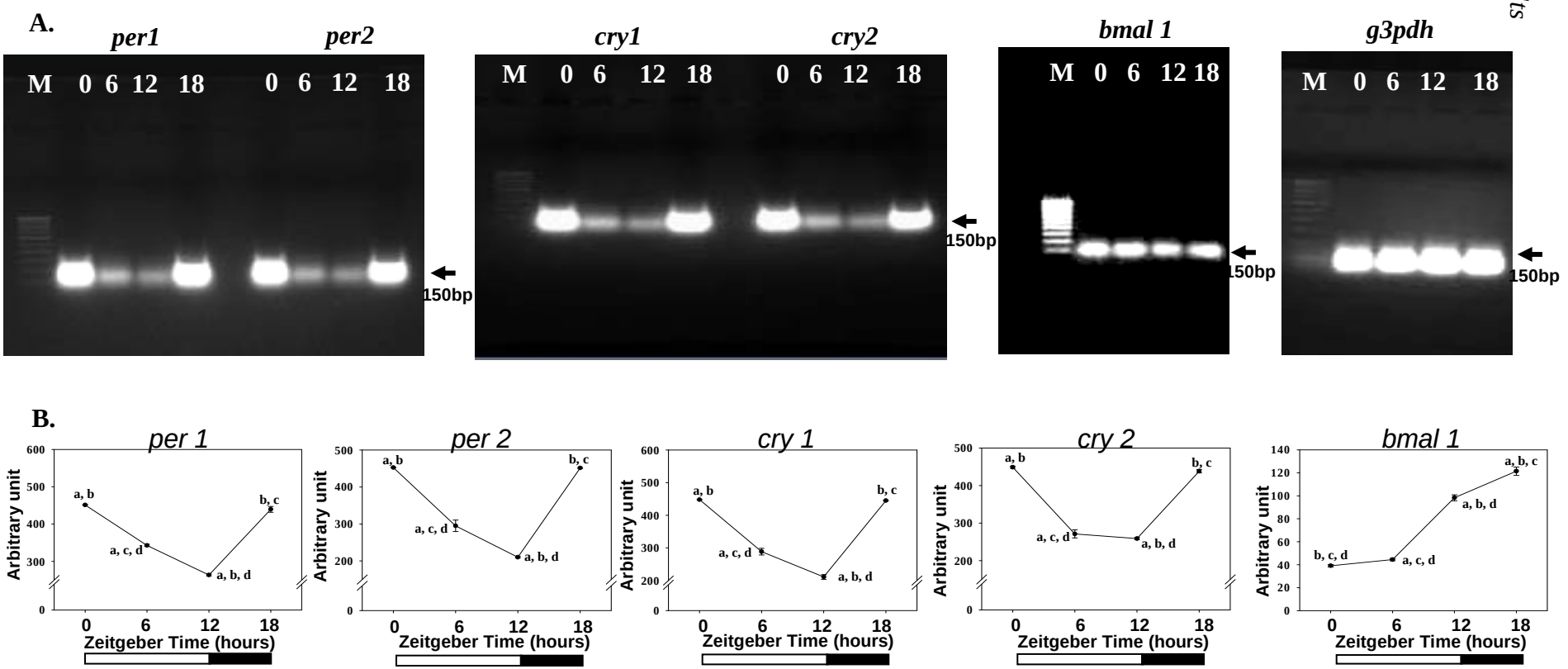


Figure 51. Daily rhythms of *per1*, *per2*, *cry1*, *cry2* and *bmal1* expression in the SCN of 3 months old *F. palmarum*. A. Amplicons of *per1*, *per2*, *cry1*, *cry2* and *bmal1* in the SCN of *F. palmarum.* B. Densitometry analysis showing daily rhythmicity of *per1*, *per2*, *cry1* and *cry2* with maximum levels at ZT-0 and minimum at ZT-12 (n=6) ($p<0.05$). *bmal1* gene showed max at ZT-18 and min at ZT- 0 ($p<0.05$). Each value is Mean ± S.E (n=6). $pa < 0.05$; $pb < 0.05$; $pc < 0.05$ and $pd<0.05$ (where a, b, c and d refers to comparison between ZT-0, 6,12 and 18 respectively).

(e). Photic and Non-photic (restricted feeding) cues on locomotor activity rhythms

(i). Photic cues (light pulse):

Light pulse of 20 minutes and 60 minutes did not show any effect, however a light pulse of 3 hours induced a phase shift in the onset of wheel running activity. Light pulse at ZT-12 (onset of darkness) induced a 2.3 hour phase delay in the onset of wheel running activity where as at ZT-21 the onset of activity was delayed for 2.5 hours (Figure 52). There was no phase shift at ZT- 3, 6, 9 or 24. The PRC for light showed phase delays corresponding to ZT-12 and ZT-21 respectively (Figure 53).

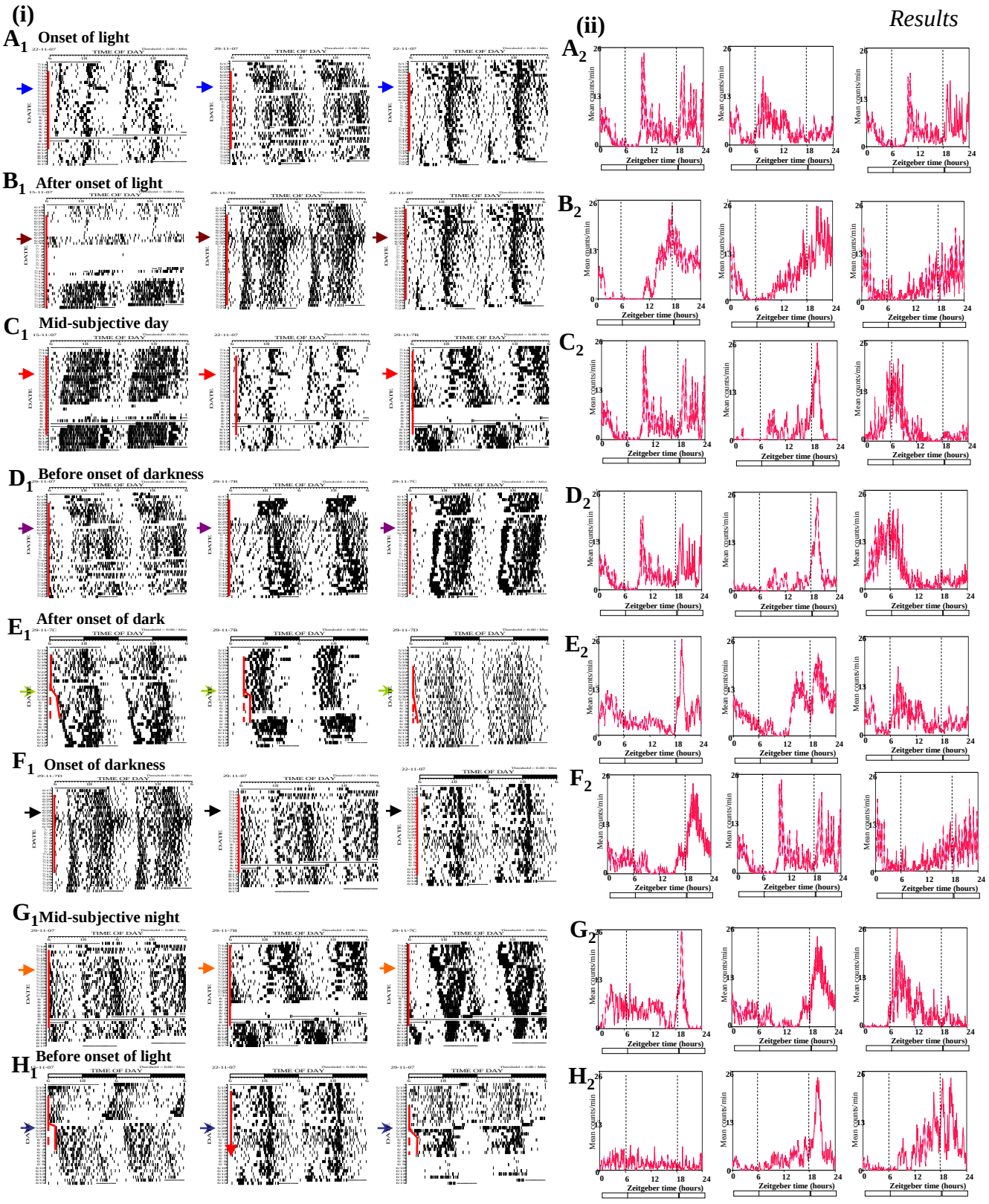


Figure 52. Double plotted actograms showing the effect of light pulse on wheel running activity (i) and their respective activity profiles (ii). Light pulse induced phase delay in the onset of activity were observed at ZT-12 and ZT-21 respectively (n=3). Each value is mean ± S.E. (n=3). The dashed lines on the activity profile indicates lights on and off for animals under LD 12:12 condition respectively.

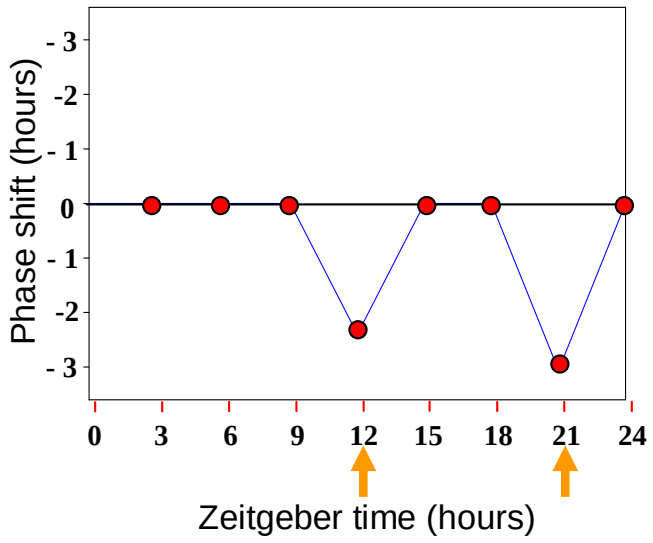
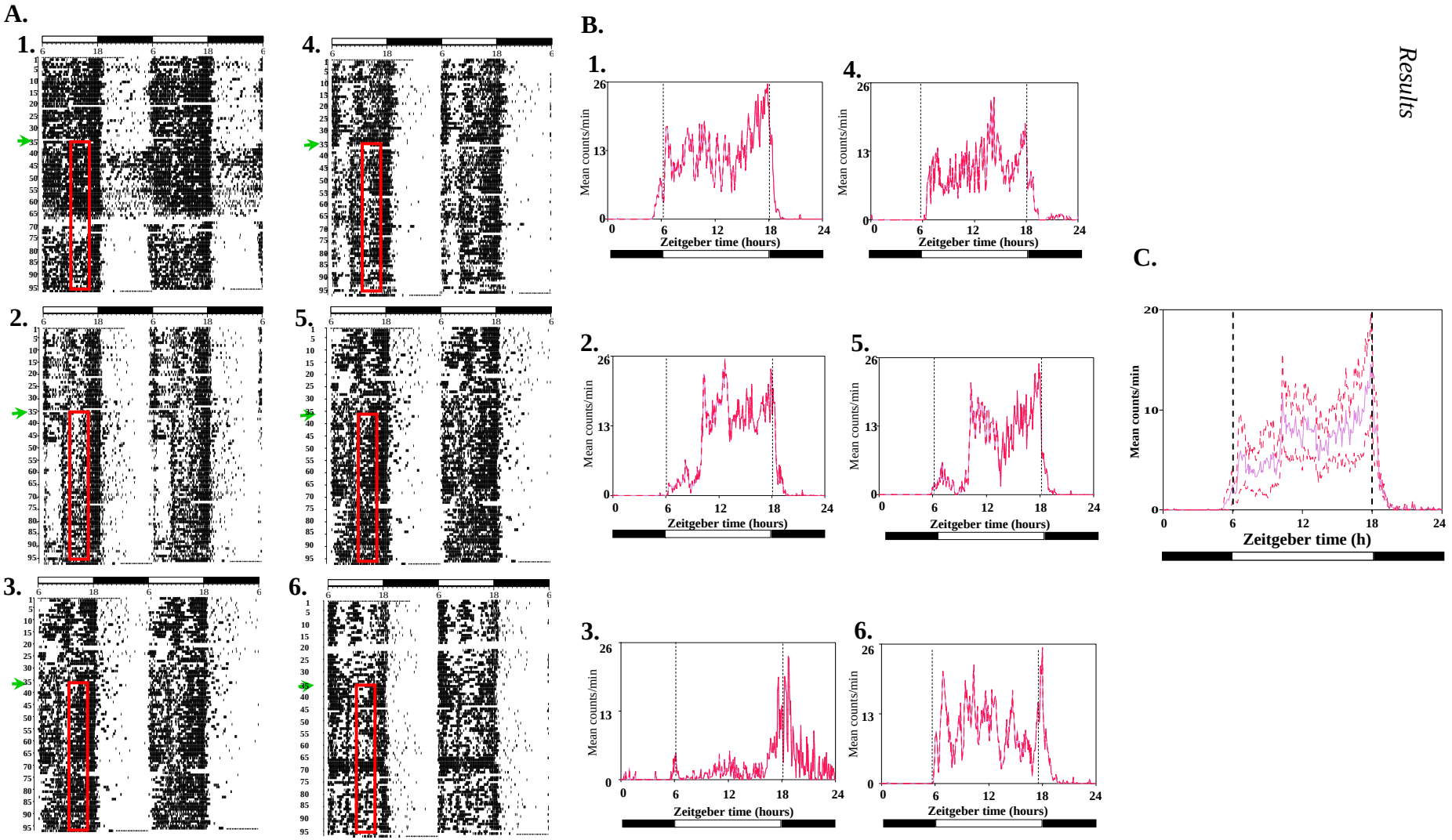


Figure 53. Phase Response Curve (PRC) of 3 hours light pulse on running wheel activity. PRC for light showed phase delay at ZT-12 and ZT-21 (arrow). Red dots represent the amount of phase shift in hours. Each value is mean ± S.E. (n=3).

(b) Non-photic stimuli (restricted feeding):

Food restriction (RF) for 4 hours between ZT-4 to ZT-8 during mid-subjective day of LD 12:12 condition did not induce any phase shift in the onset of wheel running activity (Figure 54). When animals were subjected to 4 hours RF between ZT-18 to ZT-22 during the dark phase of LD 12:12 cycle, a short bout of activity, almost 3 hours after onset of dark phase (ZT-21) were observed (Figure 55). However, there was no major change in the usual activity pattern. In constant dark (DD) condition the animals subjected to 4 hours RF during mid-subjective night during ZT-4 to ZT-8 (Figure 56) and 12 hours RF during subjective night, between ZT-18 to ZT-6, did not show any effect on the running wheel pattern of activity (Figure 57).



Duration of RF (4 hours) during light phase from ZT-4 to 8 (10:00 am to 2:00 pm) and the period of study; n=3

Indicate initiation of RF

Figure 54. Effect of RF (ZT-4 to 8) during light phase of LD on wheel running activity. A (1-6) Double plotted actograms of animals subjected to 4 hours RF (red outlined box) and their corresponding activity profiles (B.1-6). C. Mean activity profile of all animals (n=6). No effect of RF seen in animals (n=6). Green arrow represents the onset of RF in animals maintained under LD 12:12 with food and water provided *ad libitu*m. Each value is mean ± S.E. (n=6). The dashed lines on the activity profile indicates lights on and off for animals under LD 12:12 condition respectively.

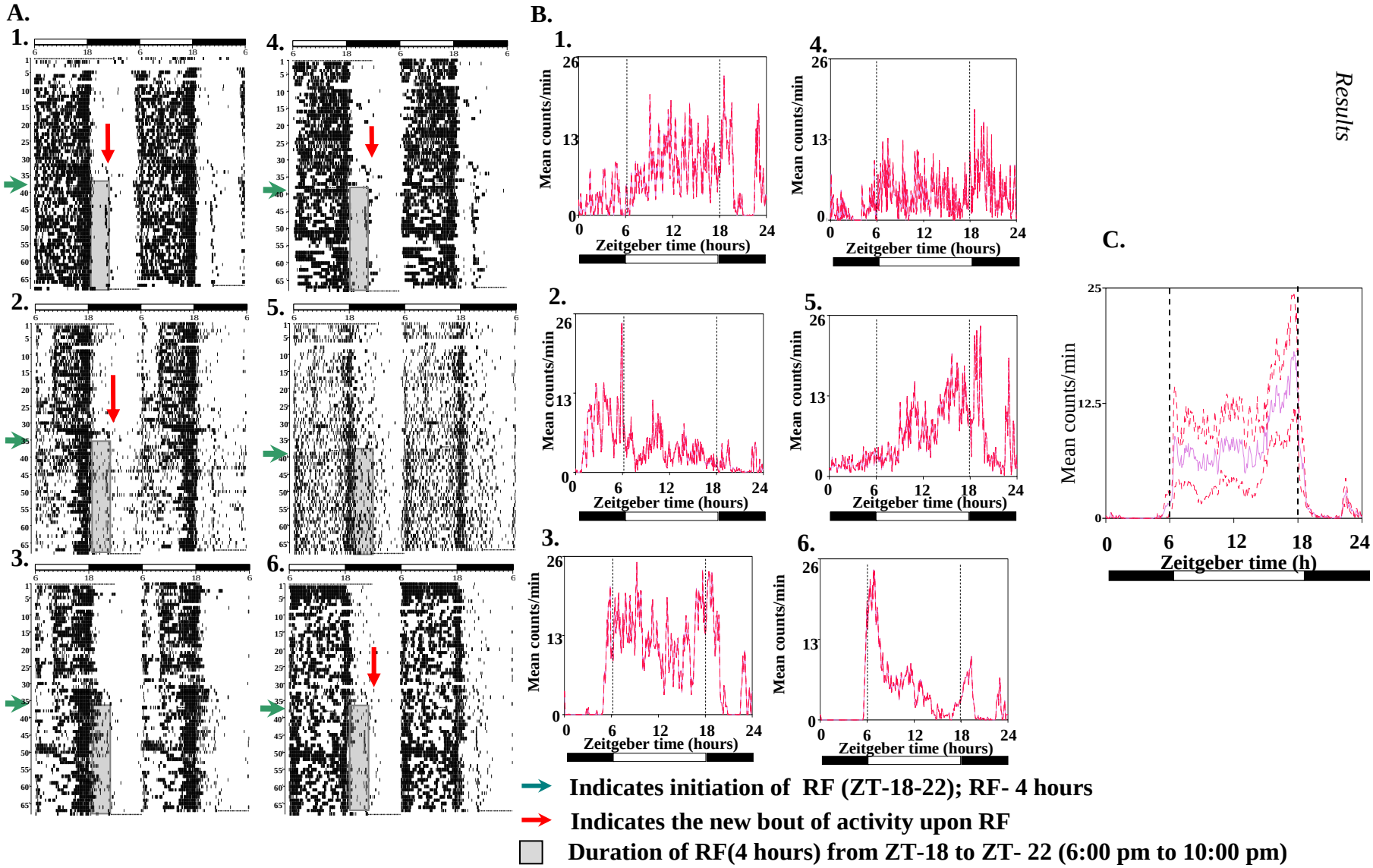


Figure 55. Effect of 4 hours RF during dark phase of LD on wheel running activity. A(1-6) representative double plotted actograms of animals subjected to 4 hours RF (red outlined box) and their corresponding activity profiles (B.1-6). C. Mean activity profile of all animals (n=6). Short bout of activity observed 3 hours after providing food in 4 out of 6 animals. Green arrow represents the onset of RF in animals maintained under LD 12:12 with food and water provided *ad libitu*m. Each value is mean ± S.E. (n=6). The dashed lines on the activity profile indicates lights on and off for animals under LD 12:12 condition respectively.

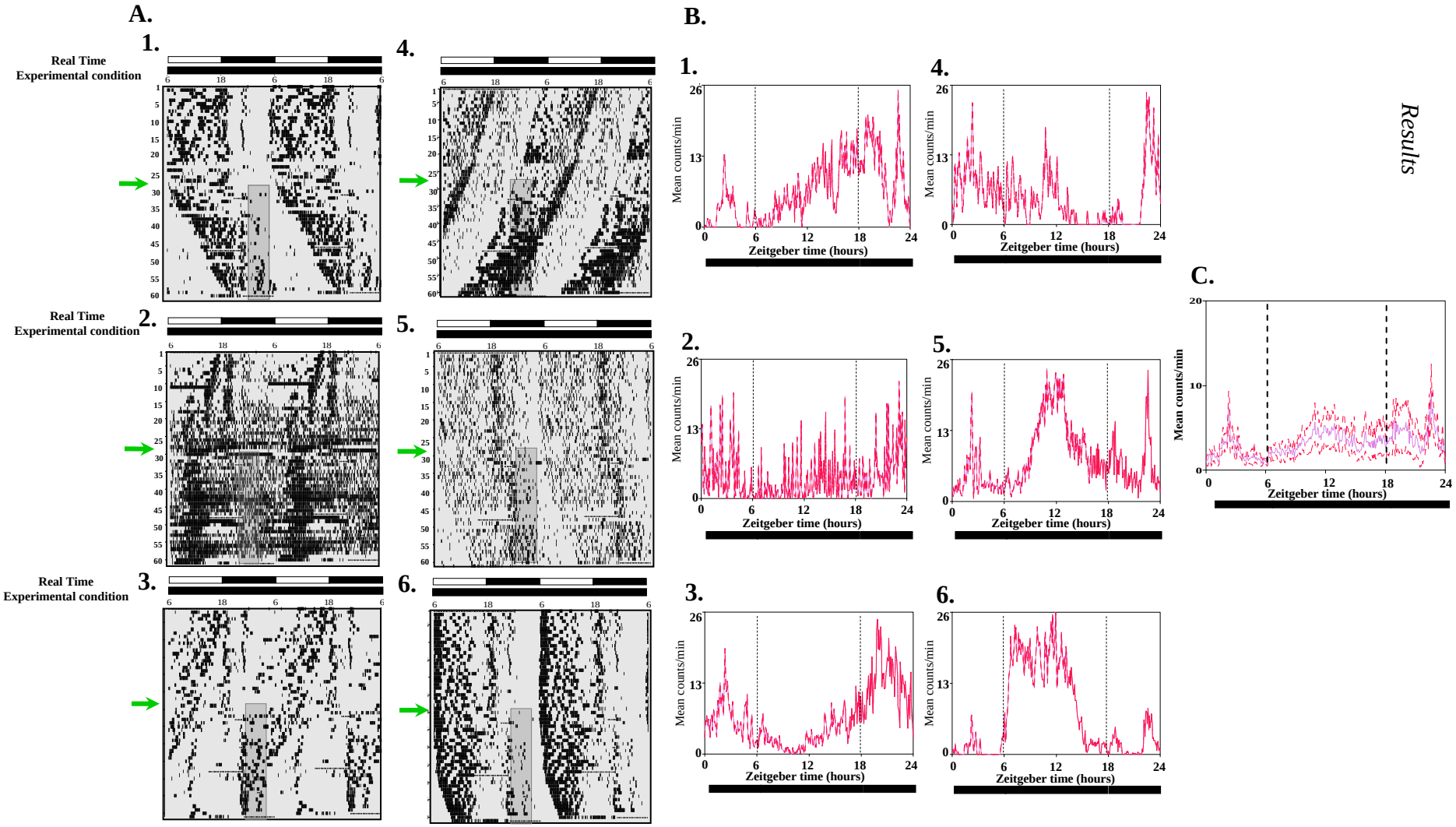


Figure 56. Effect of 4 hours RF during mid-subjective night of DD photoperiod condition on running wheel activity. A(1-6) representative double plotted actograms of animals subjected to 4 hours RF (red outlined box) and their corresponding activity profiles (B.1-6). C. Mean activity profile of all animals (n=6). No effect of RF were observed in any animals (n=6). Green arrow represents the onset of RF in animals maintained under DD with food and water provided *ad libitu*m. Each value is mean ± S.E. (n=6). The dashed lines on the activity profile indicates lights on and off for animals under LD 12:12 condition respectively.

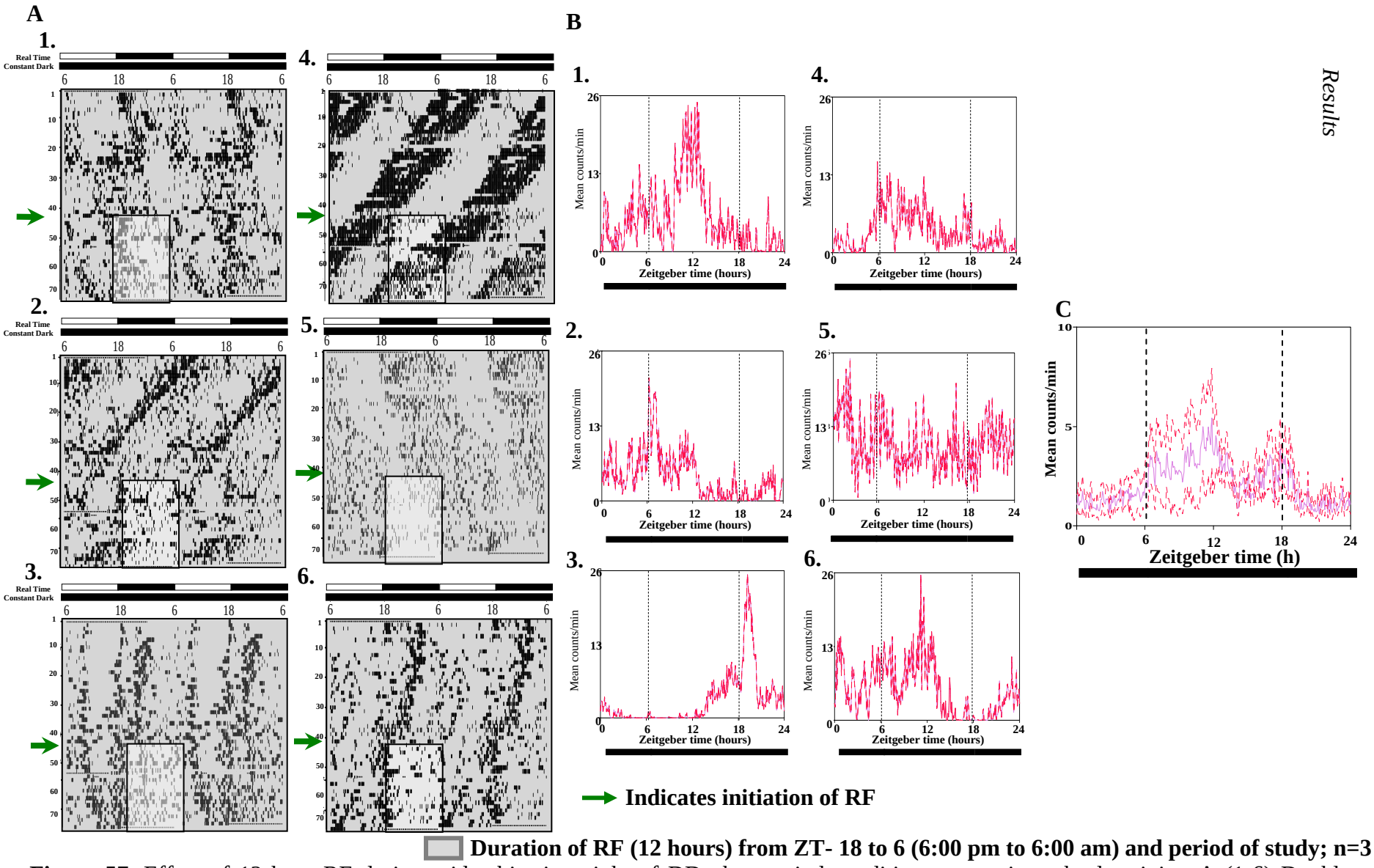


Figure 57. Effect of 12 hour RF during mid-subjective night of DD photoperiod condition on running wheel activity. A (1-6) Double plotted actograms of animals subjected to 4 hours RF (red outlined box) and their corresponding activity profiles (B.1-6). C. Mean activity profile of all animals (n=6). 12 hour RF did not show any overt effect in any animals (n=6). Green arrow represents the onset of RF in animals maintained under DD with food and water provided *ad libitu*m. Each value is mean ± S.E. (n=6). The dashed lines on the activity profile indicates lights on and off for animals under LD 12:12 condition respectively.

Objective-II.

To study the effect of aging on

a) Behavior: Gross locomotor and running wheel activity

b) Neurotransmitters: AVP, VIP and serotonin metabolome rhythms in SCN

c) Clock gene expression: *per 1, per 2*, *cry1, cry 2* and *bmal1* rhythms in SCN

II. To study the effect of aging:

(a). Behavioural pattern under various photoperiodic conditions:

The actograms of gross locomotor activity of 30 months old *F. palmarum* showed a robust diurnal pattern under LD condition (n=6) (Figure 58, 61 A) (LD; 12:12) as compared to 3 months old (Figure 29, 32A). The circadian period (τ) and (α) showed no significant difference in aged animals as compared to 3 months old animals (n=6) (Figure 68, Table 6). Significant decreases in % diurnality in aged animals were observed as compared to adults (n=6) ($p \leq 0.05$) (Figure 68, Table 6). In constant conditions of DD the animals showed a free running pattern of gross locomotor activity (Figure 59, 61 B and D, 66 Dii and Eii). The mean activity profile in DD appeared similar to that in LD (n=6) (Figure 66 Eii and Ei). However, in LL the mean activity profile were almost twice as that compared to that in LD and DD (n=6) (Figure 61, 66 Eiii). No significant changes in τ and α were observed in both DD and LL. However, the % diurnality of the 30 months old animals showed a significant decrease in both LD and DD as compared to 3 months old (n=6) ($p \leq 0.05$) (Figure 68, Table 6). However, the % diurnality showed no age associated changes in LL condition (Table 6).

The running wheel activity of aged animals showed a robust diurnal pattern of activity under LD 12:12 condition (n=6) (Figure 62, 65 A) with no significant differences in τ and α as compared to 90 day old (n=6) (Figure 68, Table 6). The % diurnality showed a significant decrease in aged animals as compared to adults under LD and DD conditions (n=6) ($p \leq 0.05$) (Table 6). However, there was no effect of aging on % diurnality in LL condition (Table 6). In DD, the 30 months old animals showed free running pattern in DD and LL conditions (n=6) (Figure 62, 65 B, 67). The mean activity counts were higher during the light phase in LD and DD condition with mean levels appearing similar in both LD and DD (Figure 65 A, B, D). Under LL, also aged animals showed free running pattern with no splitting (n=6) (Figure 64, 65 C, 67). Under DD and LL no significant change in τ and α were observed in aged animals. However, % diurnality showed a significant decrease in DD and LL as compared to LD condition in aged animals as compared to 90 day old animals (n=6) ($p \leq 0.05$) (Figure 68, Table 6).

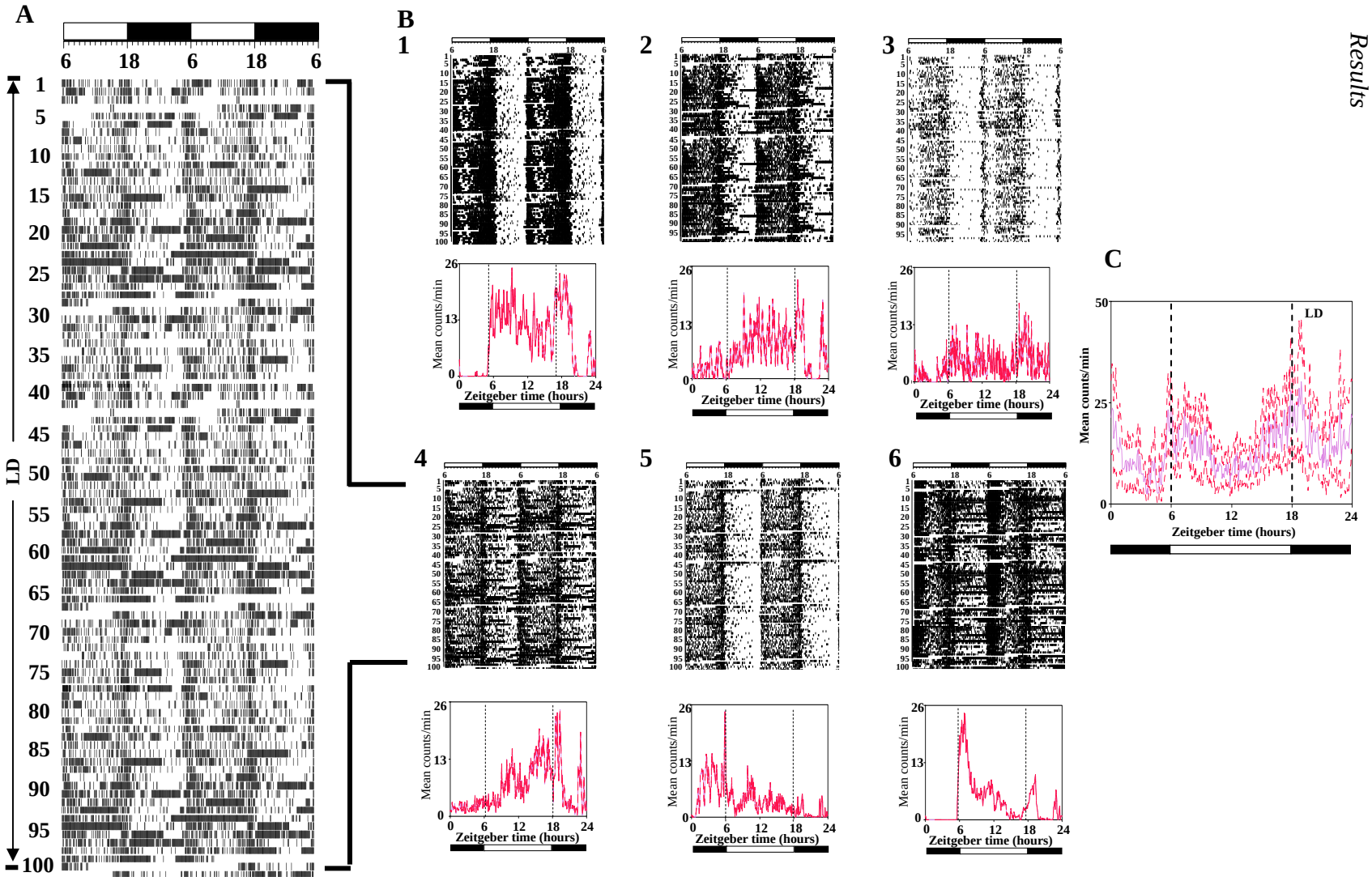


Figure 58. A. Representative double plotted actograms of gross locomotor activity for 100 days of 30 months old *F. palmarum* under LD 12:12 condition. Actograms and their respective activity profiles of 6 individual animals (B1-6). C. Mean activity profile of 6 animals showing maximum activity during light phase of LD 12:12 cycle. Each value is mean ± S.E. (n=6). The dashed lines on the activity profile indicates lights on and off for animals under LD 12:12 condition respectively.

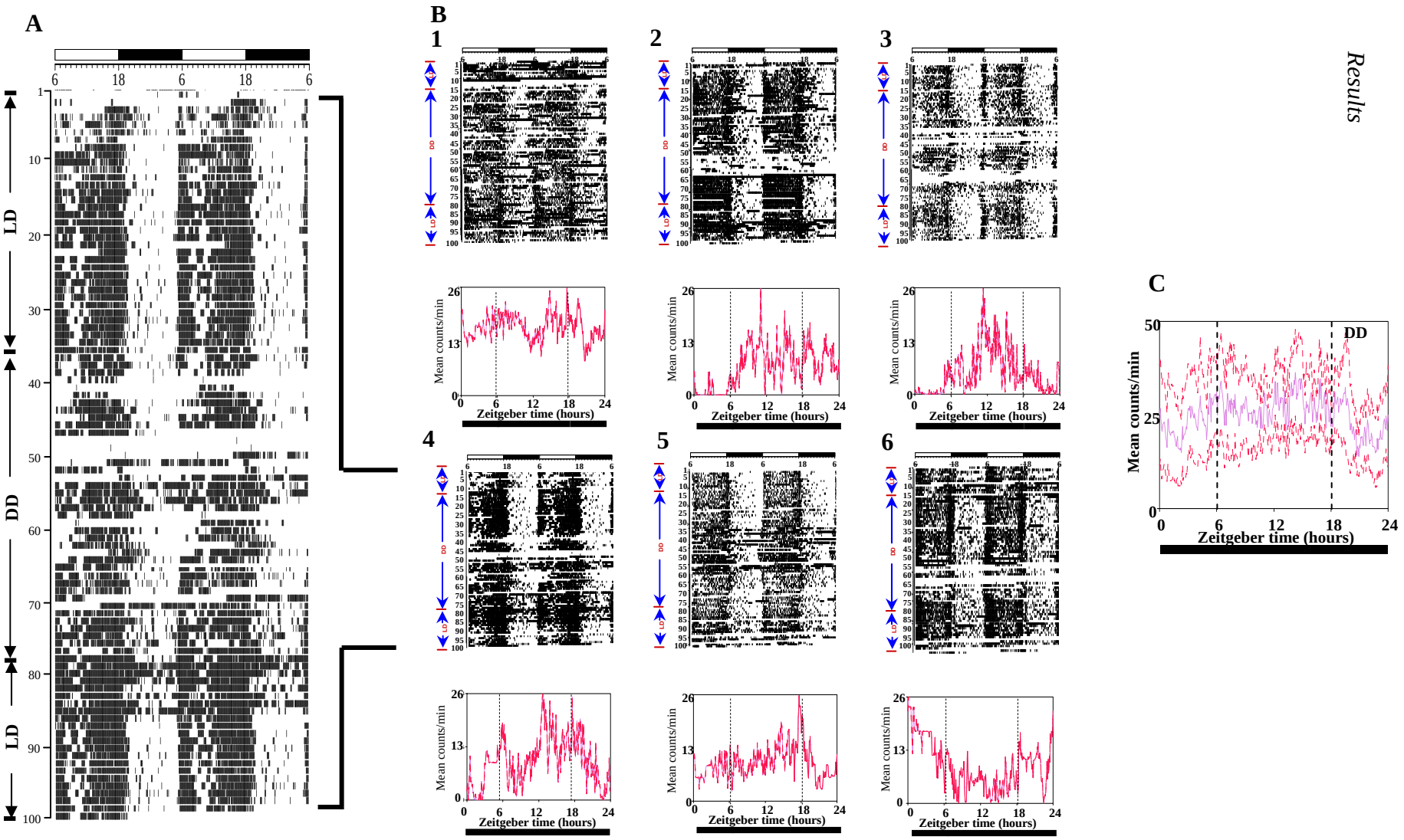


Figure 59. A. Representative double plotted actograms of gross locomotor activity for 100 days of 30 months old *F. palmarum* under DD condition. B (1-6). Actograms and their respective activity profiles of 6 individual animals under DD condition. C. Mean activity profile of 6 animals showing maximum activity during subjective light phase of DD. Each value is mean ± S.E. (n=6). The dashed lines on the activity profile indicates subjective lights on and off for animals under LD 12:12 condition respectively.

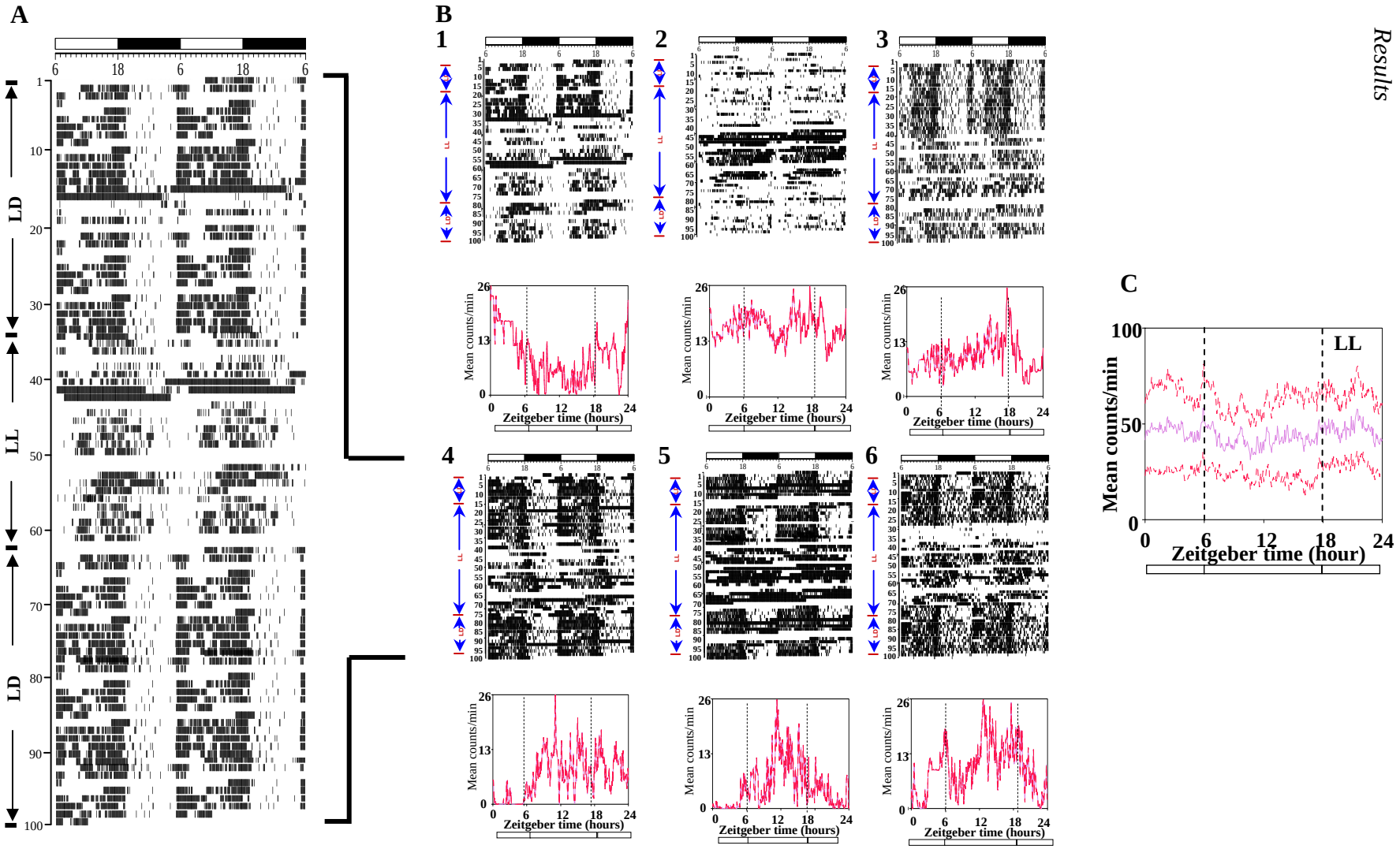


Figure 60. A. Representative double plotted actograms of gross locomotor activity for 100 days of 30 months old *F. palmarum* under LL condition. B (1-6). Actograms and their respective activity profiles of 6 individual animals under LL condition. C. Mean activity profile of 6 animals. Each value is mean ± S.E. (n=6). The dashed lines on the activity profile indicates subjective lights on and off for animals under LD 12:12 condition respectively.

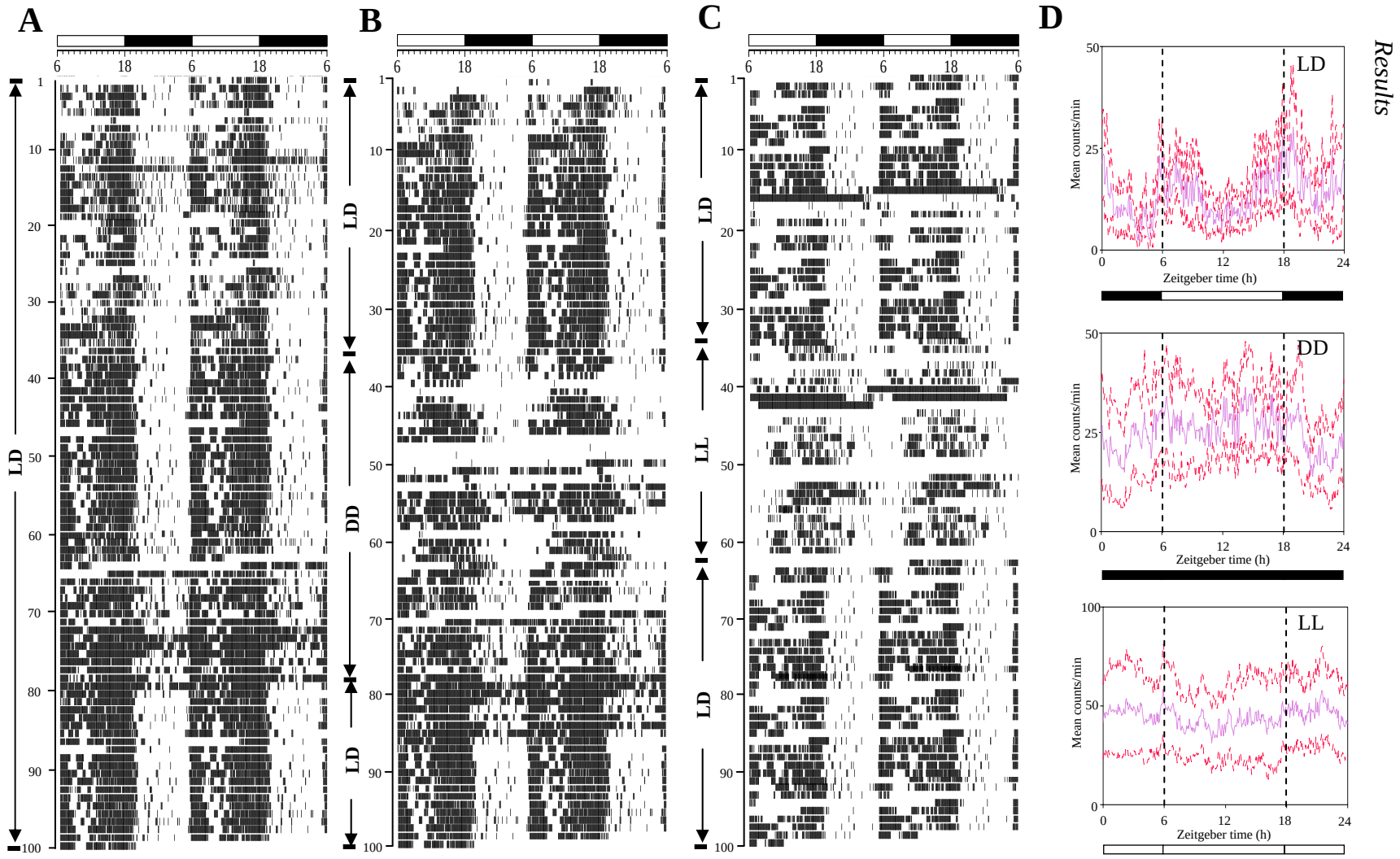


Figure 61. Representative double plotted actograms of gross locomotor activity for 100 days showing activity in under various photoperiods LD, DD and LL conditions. A. In LD, a robust activity during the light phase and less activity during the dark phase; B. In DD, a free running activity with τ of 23.84 ± 0.24 hours; C. Under LL, a free running pattern with τ of 24.32 ± 0.24 hours were observed; D. Mean activity profiles in LD, DD and LL conditions. Each value is mean ± S.E. (n=6). The dashed lines on the activity profile indicates lights on and off for animals under LD 12:12 condition respectively.

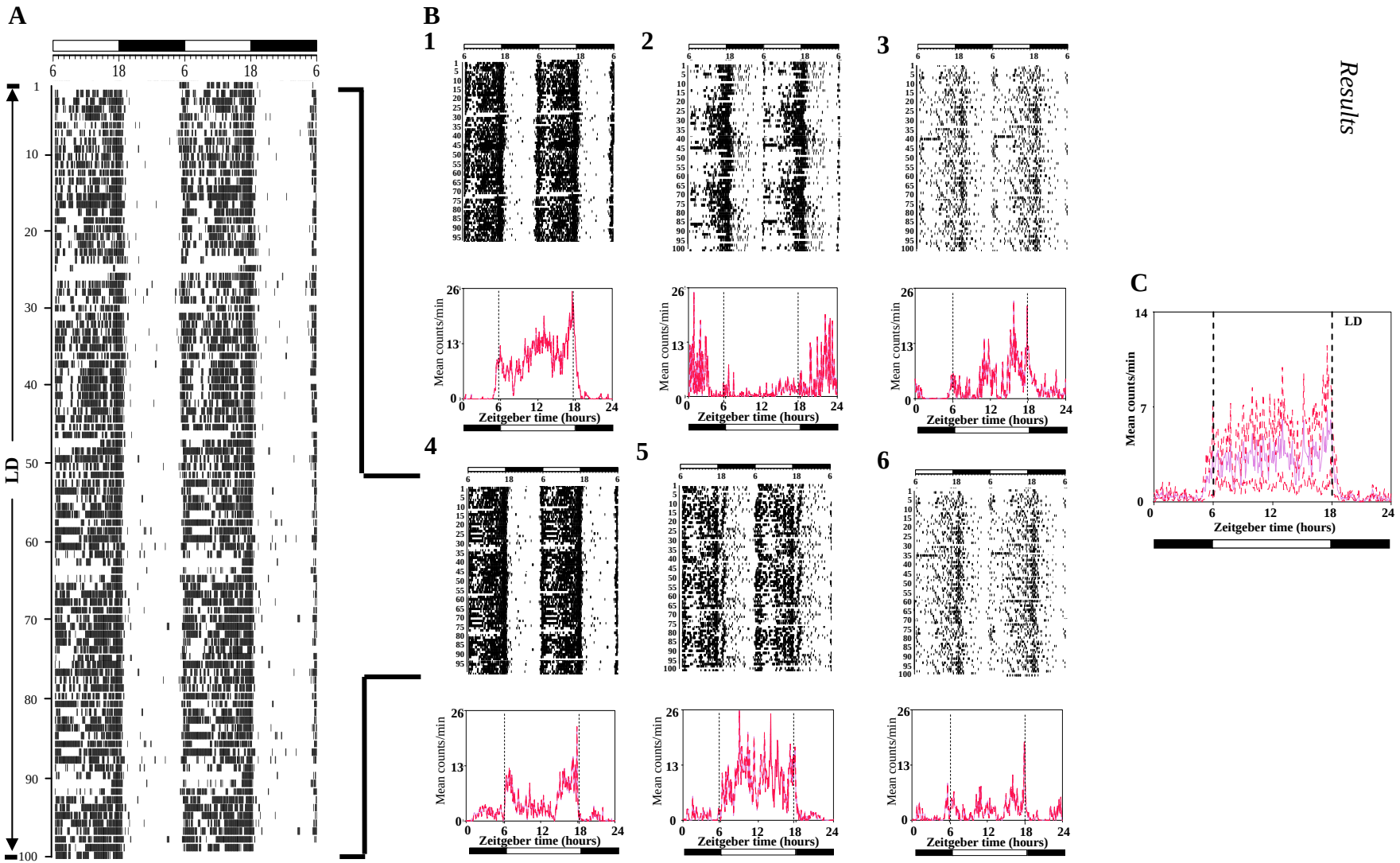


Figure 62. A. Representative double plotted actograms of running wheel activity for 100 days of 30 months old *F. palmarum* under LD 12:12 condition. B (1-6). Actograms and their respective activity profiles of 6 individual animals. C. Mean activity profile of 6 animals showing maximum activity during light phase of LD 12:12 cycle. Each value is mean ± S.E. (n=6). The dashed lines on the activity profile indicates subjective lights on and off for animals under LD 12:12 condition respectively.

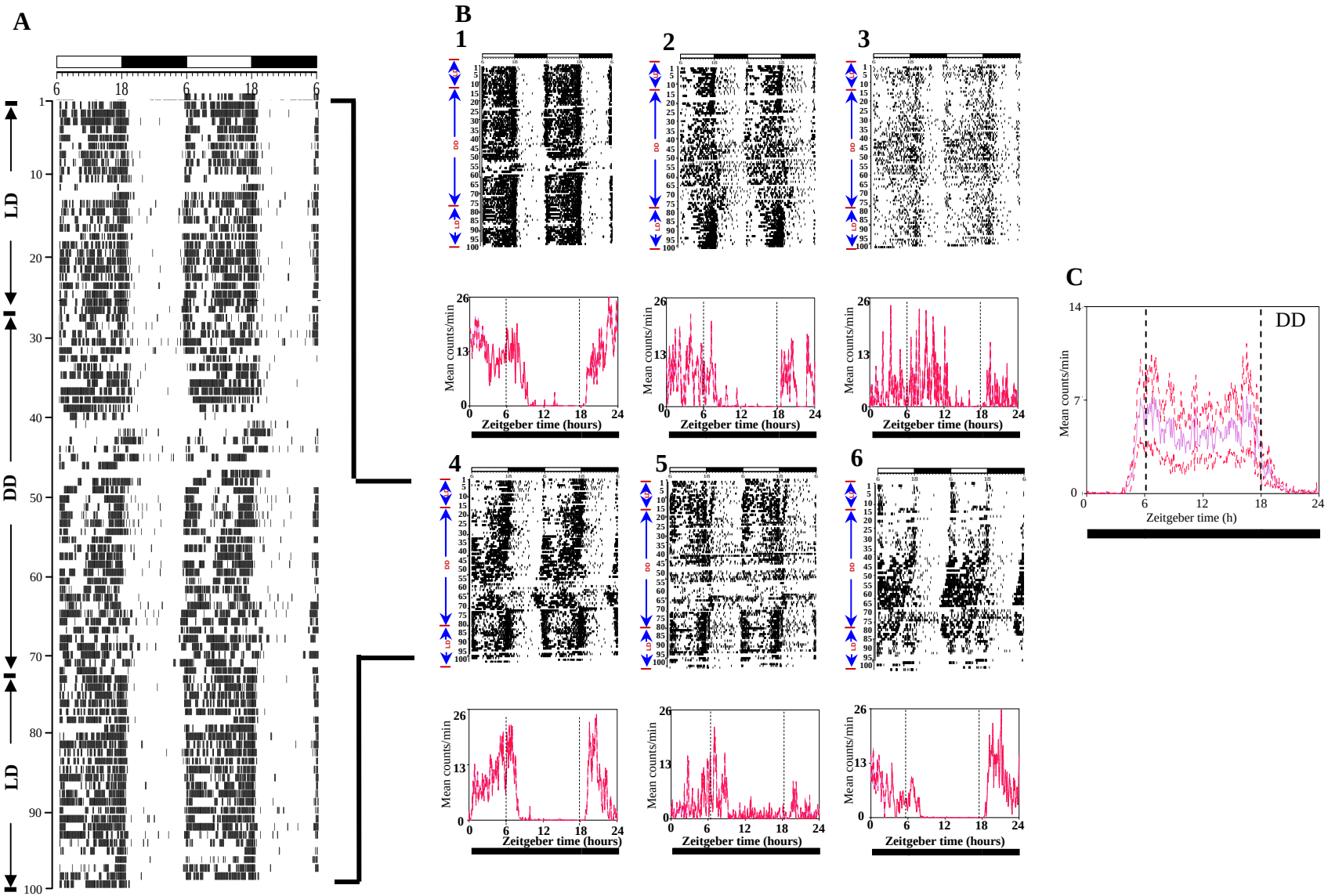


Figure 63. A. Representative double plotted actograms of running wheel activity for 100 days of 30 months old *F. palmarum* under DD condition. B (1-6). Actograms and their respective activity profiles of 6 individual animals under DD condition. C. Mean activity profile of 6 animals showing maximum activity during subjective light phase of DD. Each value is mean ± S.E. (n=6). The dashed lines on the activity profile indicates subjective lights on and off for animals under LD 12:12 condition respectively.

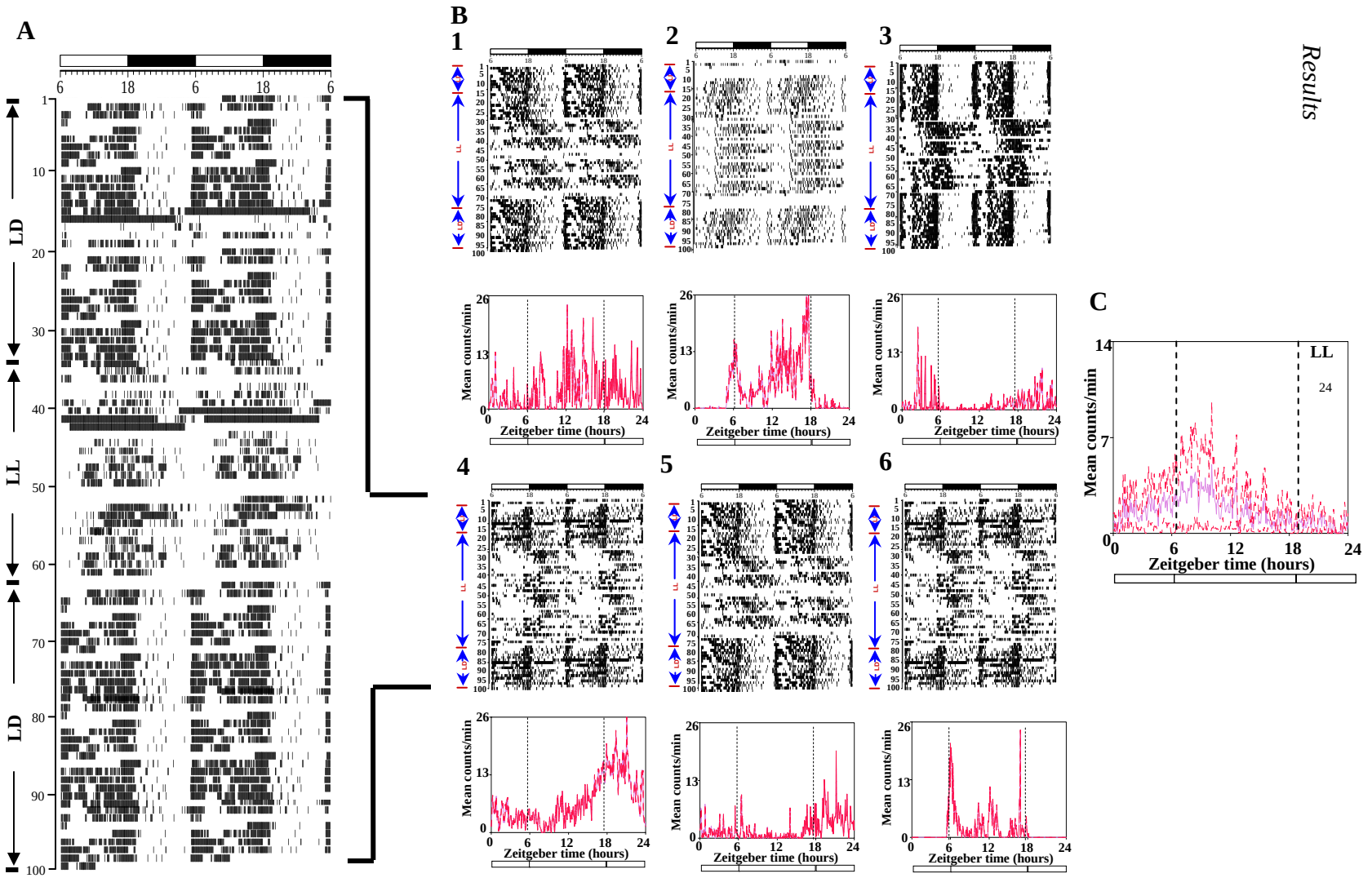


Figure 64. A. Representative double plotted actograms of running wheel activity for 100 days of 30 months old *F. palmarum* under LL condition showing splitting pattern of activity. B (1-6). Actograms and their respective activity profiles of 6 individual animals under LL condition. All 6 animals showed splitting. C. Mean activity profile of 6 animals. Each value is mean ± S.E. (n=6). The dashed lines on the activity profile indicates subjective lights on and off for animals under LD 12:12 condition respectively.

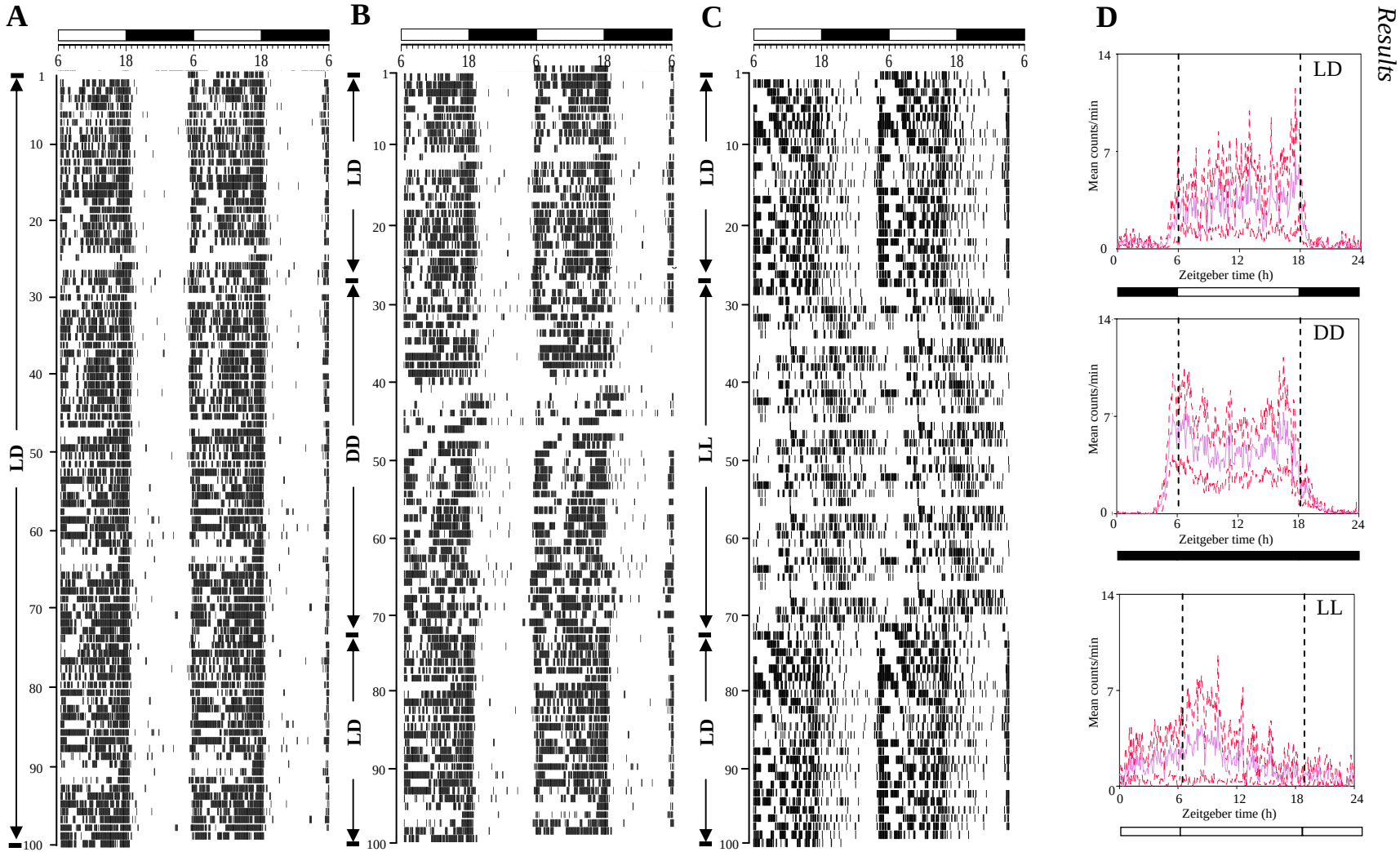


Figure 65. Representative double plotted actograms of running wheel activity for 100 days of 30 months old *F. palmarum* under various photoperiods LD, DD and LL conditions. A. In LD, a robust activity during the light phase and less activity during the dark phase; B. In DD, a free running activity with τ of 24.14 ± 0.12 hours; C. Under LL, a free running pattern with τ of 24.16 ± 0.15 hours were observed; D. Mean activity profiles under LD, DD and LL conditions. Each value is mean ± S.E. (n=6). The dashed lines on the activity profile indicates lights on and off for animals under LD 12:12 condition respectively.

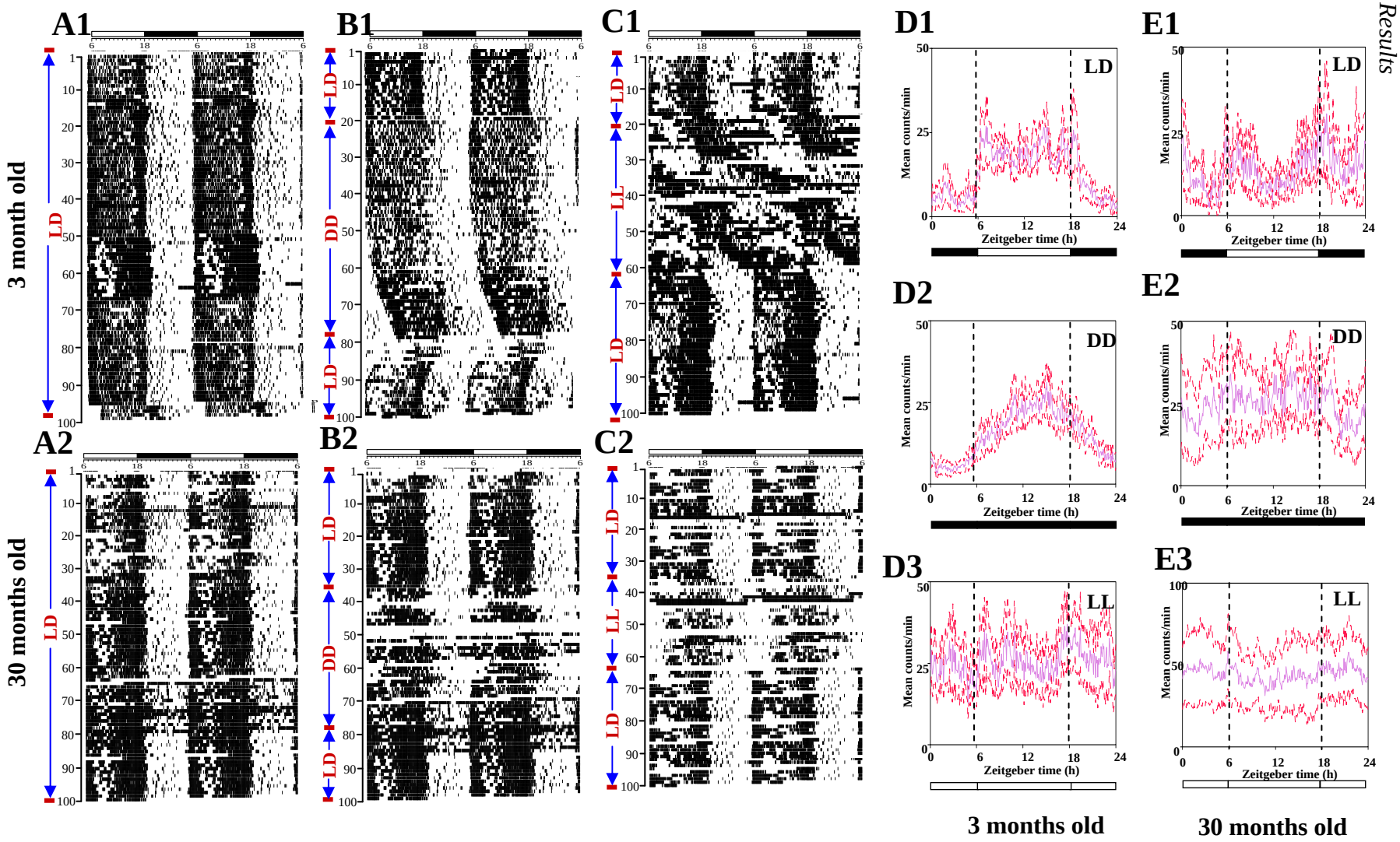


Figure 66. Comparison of representative double plotted actograms of gross locomotor activity between 3 months old (A1, B1 and C1) and 30 months old (A2. B2 and C2) *F. palmarum* under LD (A), DD (B) and LL (C) conditions. Mean activity profiles of 3 months old (D) and 30 months old (E) under LD (D1, E1), DD (D2, E2) and LL (D3, E3) represented as mean (dark pink) along with their standard error (red). Each value is mean ± S.E. (n=6). The dashed lines on the activity profile indicates subjective lights on and off for animals under LD 12:12 condition respectively.

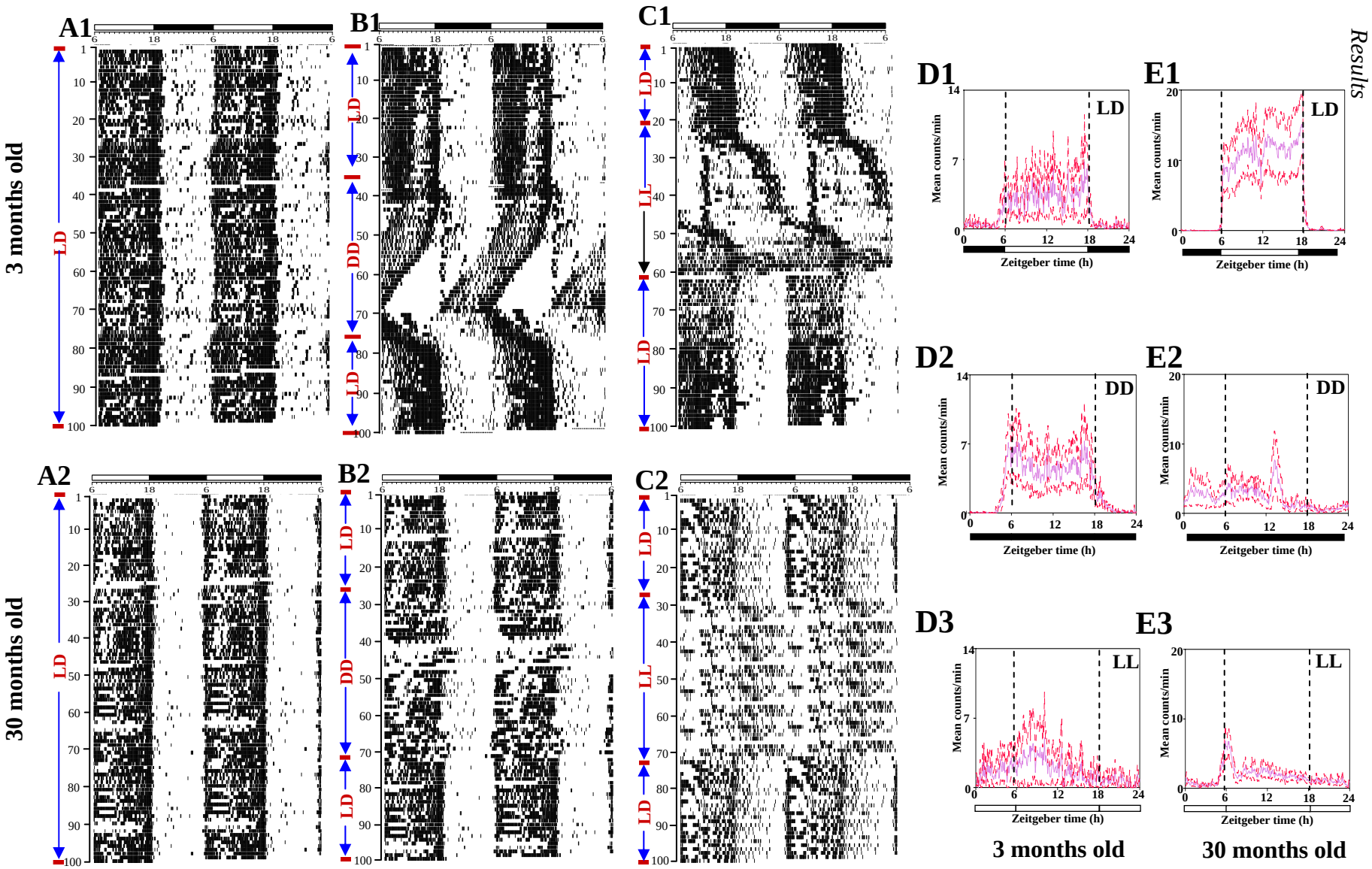


Figure 67. Comparison of representative double plotted actograms of running wheel activity between 3 months old (A1, B1 and C1) and 30 months old (A2. B2 and C2) *F. palmarum* under LD (A), DD (B) and LL (C) conditions. Mean activity profiles of 90 day old (D) and 2.5 year old (E) under LD (D1, E1), DD (D2, E2) and LL (D3, E3) represented as mean (dark pink) along with their standard error (red). Each value is mean ± S.E. (n=6). The dashed lines on the activity profile indicates subjective lights on and off for animals under LD 12:12 condition respectively.

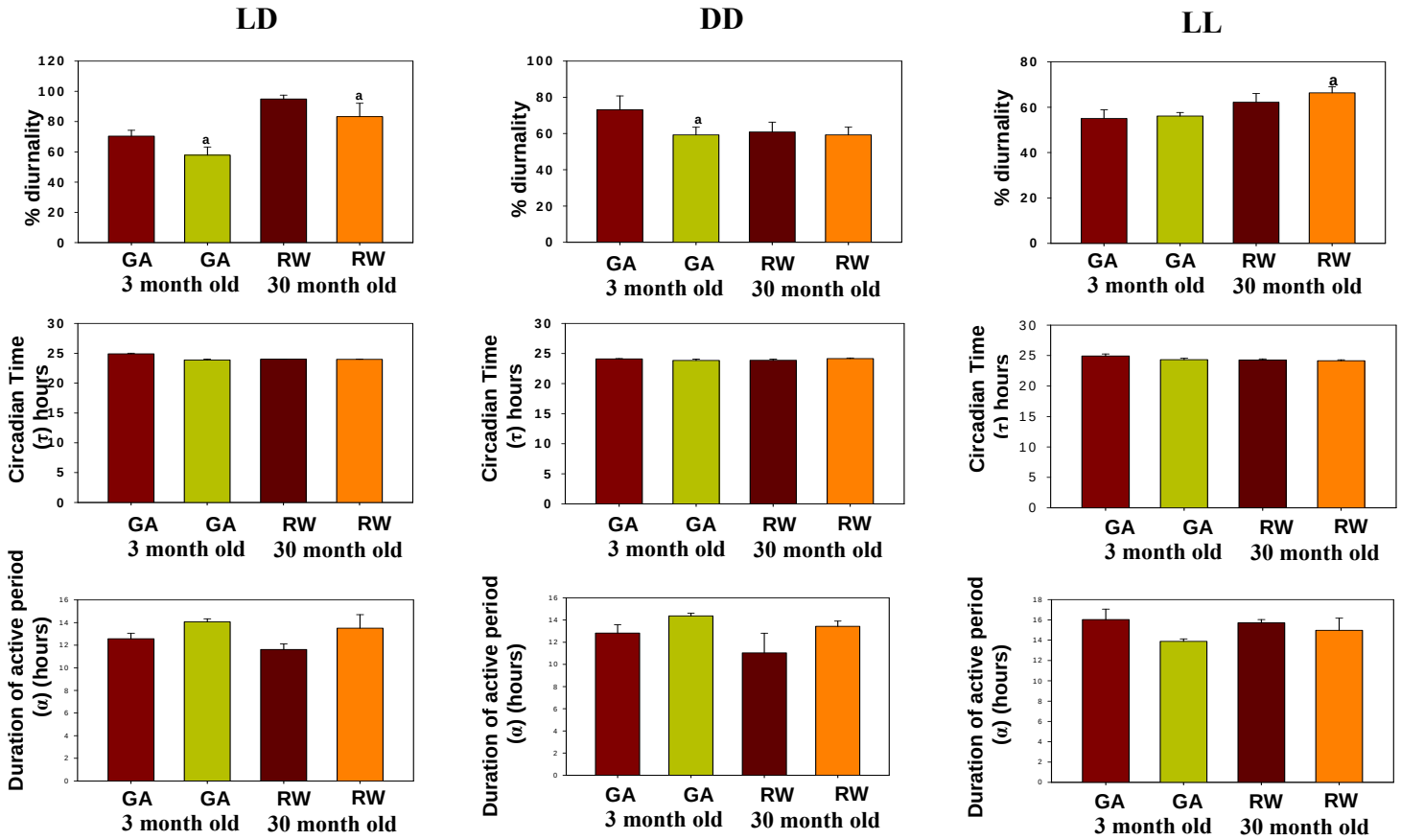


Figure 68. Effect of aging on % diurnality (A), τ (B) and α (C) under LD, DD and LL conditions. Gross locomotor activity showed significant decrease in % diurnality in LD and DD as compared to 3 months adult ($p_a < 0.05$). Running wheel activity showed significant decrease % diurnality in LD ($p_a < 0.05$) and an increase in LL ($p_a < 0.05$). τ and α showed no age induced changes in both gross locomotor activity and running wheel activity ($p_a < 0.05$). $p_a < 0.05$; where *a* refers to the comparison between 3 months old and 30 months old age groups. Each value is mean ± S.E. (n=6).

Table 6. Effect of aging on various circadian parameters under LD, DD and LL conditions.

Experimental conditions		Gross locomotor activity		Running wheel	
		3 months old	30 months old	3 months old	30 months old
LD	Circadian Time (τ) (hours)	24.91 ± 0.1	23.88 ± 0.13	24.01 ± 0.008	24.001 ± 0.009
	Duration of active period (α) (hours)	12.57 ± 0.49	14.06 ± 0.27	11.62 ± 0.49	13.51 ± 1.19
	% diurnality	70.39 ± 3.88	57.81 ± 5.43 [a]	94.94 ± 2.58[a]	83.34 ± 8.76 [a, b]
DD	Circadian Time (τ) (hours)	24.11 ± 0.03	23.84 ± 0.24	23.88 ± 0.19	24.14 ± 0.12
	Duration of active period (α) (hours)	12.82 ± 0.76	14.38 ± 0.25	11.04 ± 1.77	13.44 ± 0.47
	% diurnality	73.13 ± 7.59	59.23 ± 4.27 [a]	60.84 ± 5.40[b]	59.24 ± 4.27 [a, b]
LL	Circadian Time (τ) (hours)	24.92 ± 0.35	24.32 ± 0.24	24.28 ± 0.18	24.16 ± 0.15
	Duration of active period (α) (hours)	16.03 ± 1.03	13.89 ± 0.24	15.72 ± 0.31	14.98 ± 1.18
	% diurnality	55.06 ± 3.82[a,c]	56.1 ± 1.54	62.21 ± 3.82[c]	66.3 ± 2.72 [c, d]

Each value is mean ± S.E. (n=6). $p_a<0.05$, $p_b<0.05$ and $p_c<0.05$ (where a represents comparison between 3 months old and 30 months old, b represents comparison between LD and DD, c represents comparison between LD and LL and d represents comparison between gross locomotor activity and running wheel activity respectively.

(b). Neurotransmitter level:

(i) Daily profile of AVP-ir and VIP-ir in the SCN

The daily profile of VIP-ir in the SCN of aged *F. palmarum* showed rhythmicity with significant difference in levels between zeitgeber time points (n=4) ($p<0.05$) (Figure 69 Ai). The levels of VIP-ir at ZT-0 and 18 showed an increase of 30.12 and 67.33% respectively as compared to 3 months old adult (n=4) ($p<0.05$) (Figure 69 Aii). However, the VIP-ir levels at ZT-6 and 12 were 55.89 and 39.20% respectively decreased in 30 months old squirrels as comparison to 3 months old adults. A 12 hour phase reversal in the maximum levels was seen in aged animals as compared to 90 day old adult (Figure 69 Aii). The maximum : minimum ratios were not significantly different as compared to 90 day old (n=4) (Figure 69 Aiii).

AVP-ir rhythmicity was abolished upon aging in the SCN (Figure 69Bi). The levels of AVP-ir were significantly decreased at ZT-0, 6, 12 and 18 by 44.70, 8.68, 10.77 and 8.67% respectively as compared to 90 day old (n=4) ($p<0.05$) (Figure 69 Bii). The maximum :

minimum ratios in aged animals were not significantly different as compared to adults (n=4) (Figure 69 Biii).

(ii) Daily profile of serotonin metabolome in the SCN and Pineal

In the SCN of 30 months old *F. palmarum*, all compounds studied showed daily rhythmicity except for 5-HTP, however the mean 24 hour levels of all compounds were decreased as compared to adults (Figure 70). 5-HIAA, 5-HT, 5-HTOH, 5-MIAA, 5-MTOH and NAT showed 6 hour phase advance in their levels as compared to adults (Figure 70). The mean 24 hour levels were maximum for TRP and minimum for 5-HTP in aged animals where as in the adult it was maximum for 5-HIAA and minimum for 5-HTOH. The maximum: minimum levels were maximum for 5-MIAA and minimum for 5-HTOH in aged animals as compared to adults where it was maximum for 5-HIAA and minimum for 5-HTOH (Table 7).

In the pineal also all compounds showed daily rhythmicity except for 5-MIAA. TRP and 5-HTOH showed a phase shift of 6 hours in their peak levels as compared to adults (Figure 71). The mean 24 hour levels were maximum for TRP and minimum for 5-HTP in aged animals where as in the adult it was maximum for 5-HTP and minimum for TRP (Figure 71). The maximum : minimum levels were maximum for 5-MIAA and minimum for 5-HTOH in aged animals as compared to adults where it was maximum for TRP and minimum for 5-HTOH (Table 8).

The mean levels of 5-HIAA, 5-HTOH, NAS, 5-MTOH, MEL and NAT showed significant decrease upon aging in the SCN compared to 90 day old (Figure 72 Ai). The daily pulse in the SCN showed significant decrease in the 5-HTP and 5-HT levels, with the level of all other metabolites decreased (Figure 72 Aii). In the pineal, mean levels of 5-HTP and 5-HIAA showed significant increase upon aging as compared to adults (Figure 72 Bi). The daily pulse of 5-HT, TRP and 5-MIAA showed significant decrease while that of 5-HTOH increase upon aging (Figure 72 Bii).

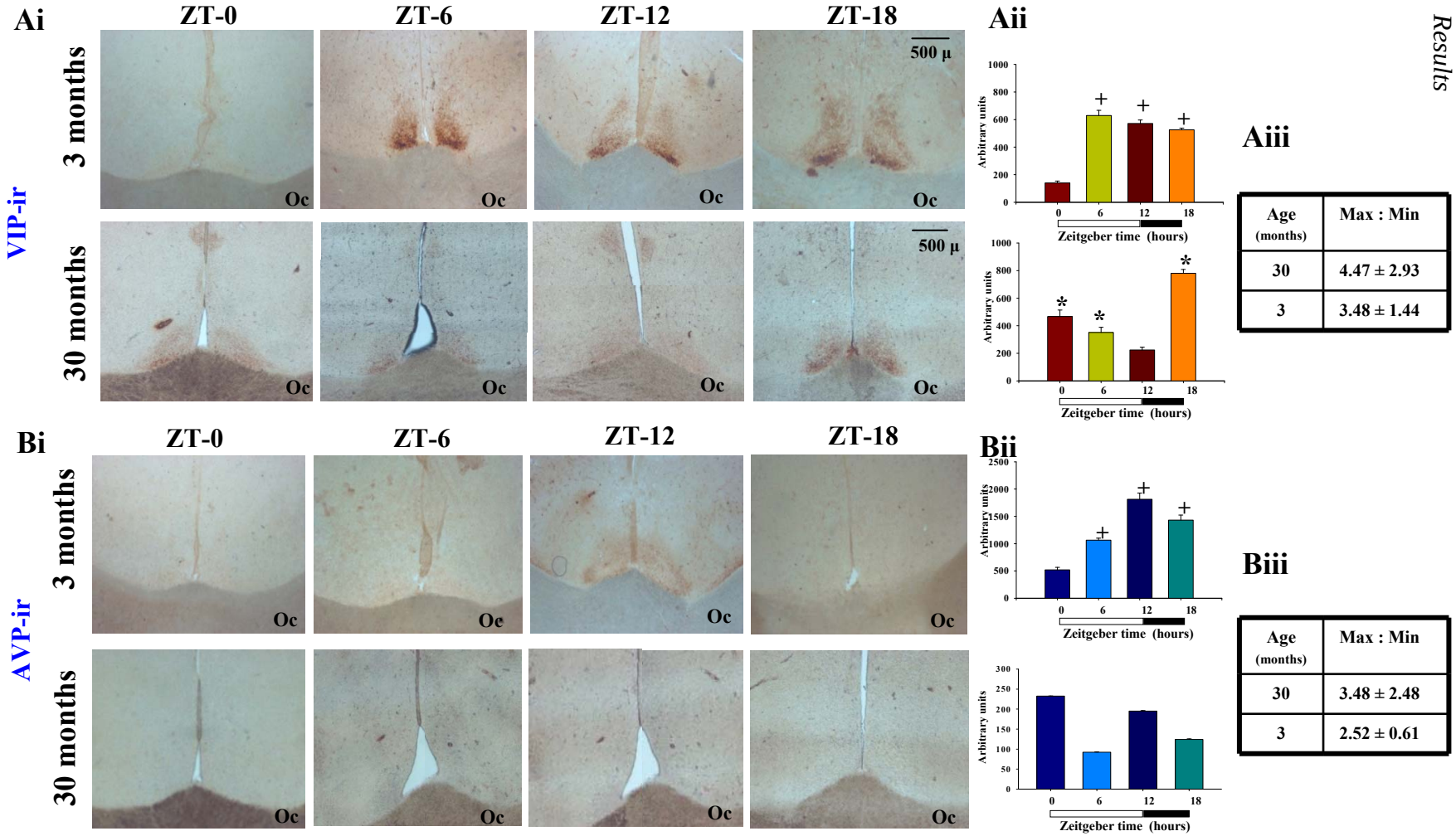


Figure 69. Effect of aging on daily rhythm on VIP (A) and AVP (B) in the SCN of *F. palmarum*. Densitometry analysis showing VIP-ir daily rhythmicity in its levels in aged *F. palmarum* (n=4) ($p<0.05$) with maximum levels at ZT-18 and minimum levels at ZT-12, and phase shift of 12 hours in the maximum levels (Aii). AVP-ir levels were significantly decreased with abolition of rhythmicity in the 30 months old SCN as compared to 3 months old ($p<0.05$) (Bii). Table showing the comparison of max: min ratio of VIP-ir (Aiii) and VIP-ir (Biii). Each value is mean ± S.E. (n=4). $^{+,*}$ represents comparison of lowest level of ZT with other time points.

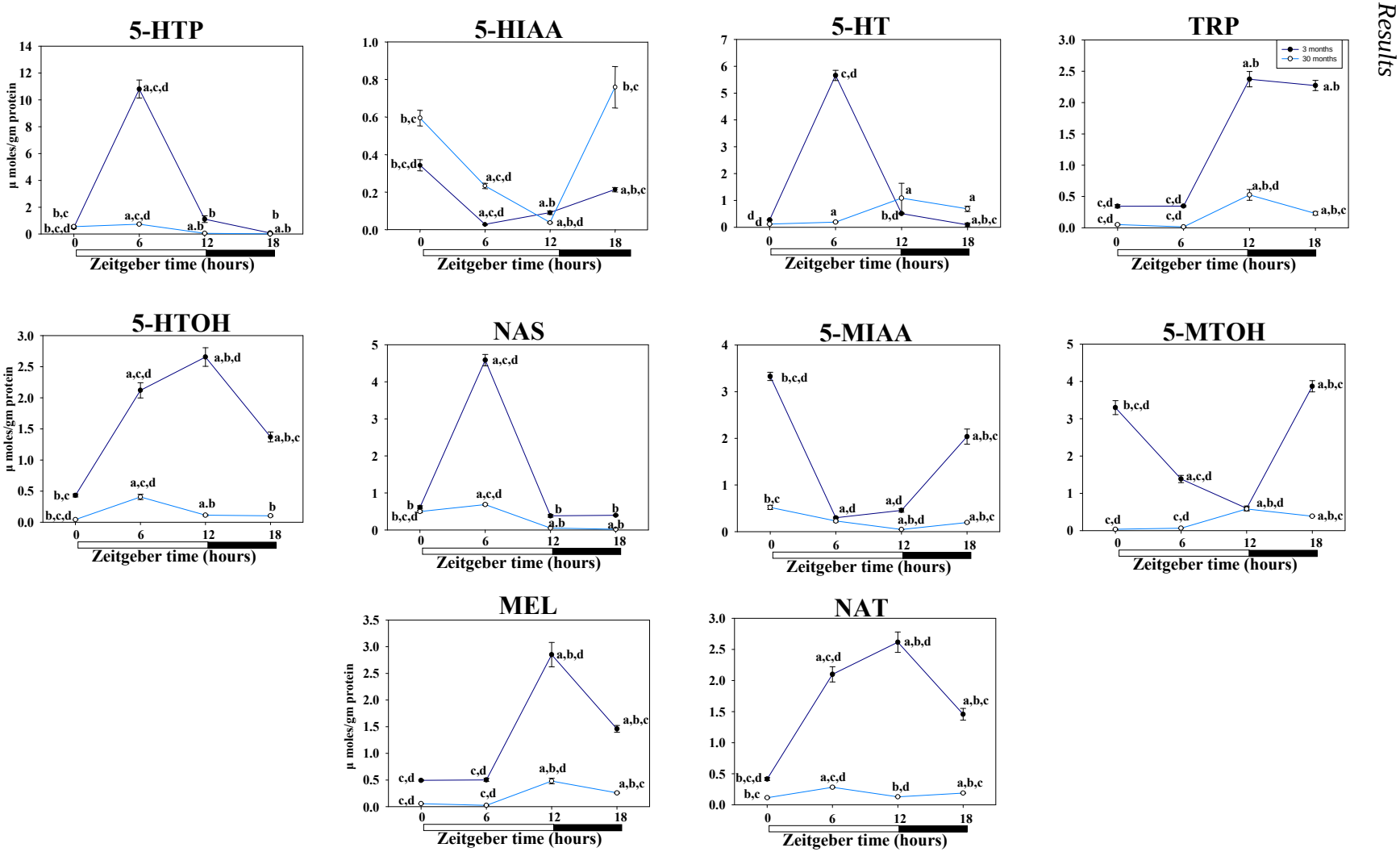


Figure 70. Comparison of daily rhythms of serotonin and its related compounds in the SCN of 3 months old and 30 months old *F. palmarum.* Each value is mean ± S.E. (n=6). $p_a < 0.05$; $p_b < 0.05$; $p_c < 0.05$ and $p_d < 0.05$ (where a, b, c and d refers to comparison between ZT-0, 6,12 and 18 respectively).

Table 7. Age induced changes in serotonin and its related compounds in the SCN of 3 months and 30 months old *F. palmarum.*

Metabolites	Age groups	Max Levels (µ mol/gm)	Max Time point	Min Levels (µ mol/gm)	Min Time point	Ratio Max/Min	Daily Mean levels (µ mol/gm)
5-HTP	3 months old	0.74 ± 0.04	ZT-6	0.009 ± 0.0008*	ZT-18	2.13 ±1.21	0.68 ± 0.14
	30 months old	10.80 ± 0.67	ZT- 6	0.08 ± 0.01*	ZT-18	140.35 ± 59 b,c,d,e,f,g,h,i,j	0.55 ± 0.03 b,e,h,i
5-HIAA	3 months old	0.76 ± 0.11	ZT-18	0.038 ± 0.004*	ZT-12	0.57 ± 0.02	0.63 ± 0.04
	30 months old	0.34 ± 0.03	ZT-0	0.03 ± 0.003*	ZT-6	11.45 ± 10.99	6.23 ± 0.59 a,c,d.e,f,g,h,i,j
5-HT	3 months old	1.08 ± 0.56	ZT-6	0.12 ± 0.007*	ZT-0	0.96 ± 0.18	0.05 ± 0.003a,b,e,f,h
	30 months old	5.66 ± 0.19	ZT-6	0.09 ± 0.07	ZT-18	65.65 ± 0.04	0.58 ± 0.09 a,b,d,e,f,i
TRP	3 months old	0.52 ± 0.087	ZT-12	0.01 ± 0.005*	ZT-6	2.12 ± 1.92	0.02 ± 0.003a,b,e,h,f
	30 months old	2.37 ± 0.12	ZT-12	0.34 ± 0.03 *	ZT-0	6.89 ± 0.22	0.33 ± 0.03 b,e,h,i
5-HTOH	3 months old	0.41 ± 0.04	ZT-6	0.04 ± 0.004*	ZT-0	0.86 ± 0.099	0.53 ± 0.03a
	30 months old	2.66 ± 0.15	ZT-12	0.43 ± 0.02*	ZT-0	6.16 ± 5.11	2.22 ± 0.25 a,b,c,d,f,g,j
NAS	3 months old	0.69 ± 0.024	ZT-6	0.02 ± 0.009*	ZT-18	0.89 ± 0.11	0.24 ± 0.009a,b,e,h
	30 months old	4.59 ± 0.15	ZT-6	0.38 ± 0.04*	ZT-12	12.01 ± 4.27	1.01 ± 0.19 a,b,e,i
5-MIAA	3 months old	0.52 ± 0.05	ZT-0	0.05 ± 0.002*	ZT-12	0.84 ± 0.05	0.056 ± 0.007a,b,e,h,f
	30 months old	3.33 ± 0.99	ZT-0	0.29 ± 0.01*	ZT-6	11.21 ± 6.18	0.35 ± 0.03 b,e,h,i
5-MTOH	3 months old	0.58 ± 0.05	ZT-12	0.04 ± 0.002*	ZT-0	0.31 ± 0.07	0.41 ± 0.05a,b,e
	30 months old	3.87 ± 0.15	ZT-18	0.59 ± 0.06*	ZT-12	6.47 ± 2.42	1.62 ± 0.18 a,b,c,d,g
MEL	3 months old	0.48 ± 0.05	ZT-12	0.02 ± 0.006*	ZT-6	0.35 ± 0.03	0.099 ± 0.008a,b,e,h,f
	30 months old	2.85 ± 0.23	ZT-12	0.49 ± 0.01*	ZT-0	5.78 ± 5.45	2.21 ± 0.25 a,b,c,d,f,g,j
NAT	3 months old	0.28 ± 0.007	ZT-6	0.12 ± 0.007*	ZT-0	0.36 ± 0.03	0.14 ± 0.013a,b,e,h,f
	30 months old	2.61 ± 0.16	ZT-12	0.41 ± 0.02*	ZT-0	6.30 ± 5.61	1.07 ± 0.12 a,b,e,i

The mean levels in descending order of levels in the SCN are as follows TRP, 5-HT, 5-MIAA, MEL, NAT, NAS, 5-MTOH, 5-HTOH, 5-HIAA and 5-HTP. Maximum: minimum ratio in the descending order of their levels are 5-HIAA, TRP, 5-HTP, 5-MTOH, MEL, NAT, 5-MIAA, 5-HTOH, NAS and 5-HT. Each value is Mean ± S.E (n=6). *pa<0.05, pb<0.05, pc<0.05, pd<0.05, pe<0.05, pf<0.05, pg<0.05, ph<0.05, pi<0.05, pj < 0.05* (where a, b, c, d, e, f, g, h, i and j refers to comparison with 5-HTP, 5-HIAA, 5-HT, TRP, 5-HTOH, NAS, 5-MIAA, 5-MTOH, MEL and NAT respectively. * refers to the comparison between maximum and minimum levels (n=6) (*p<0.05*).

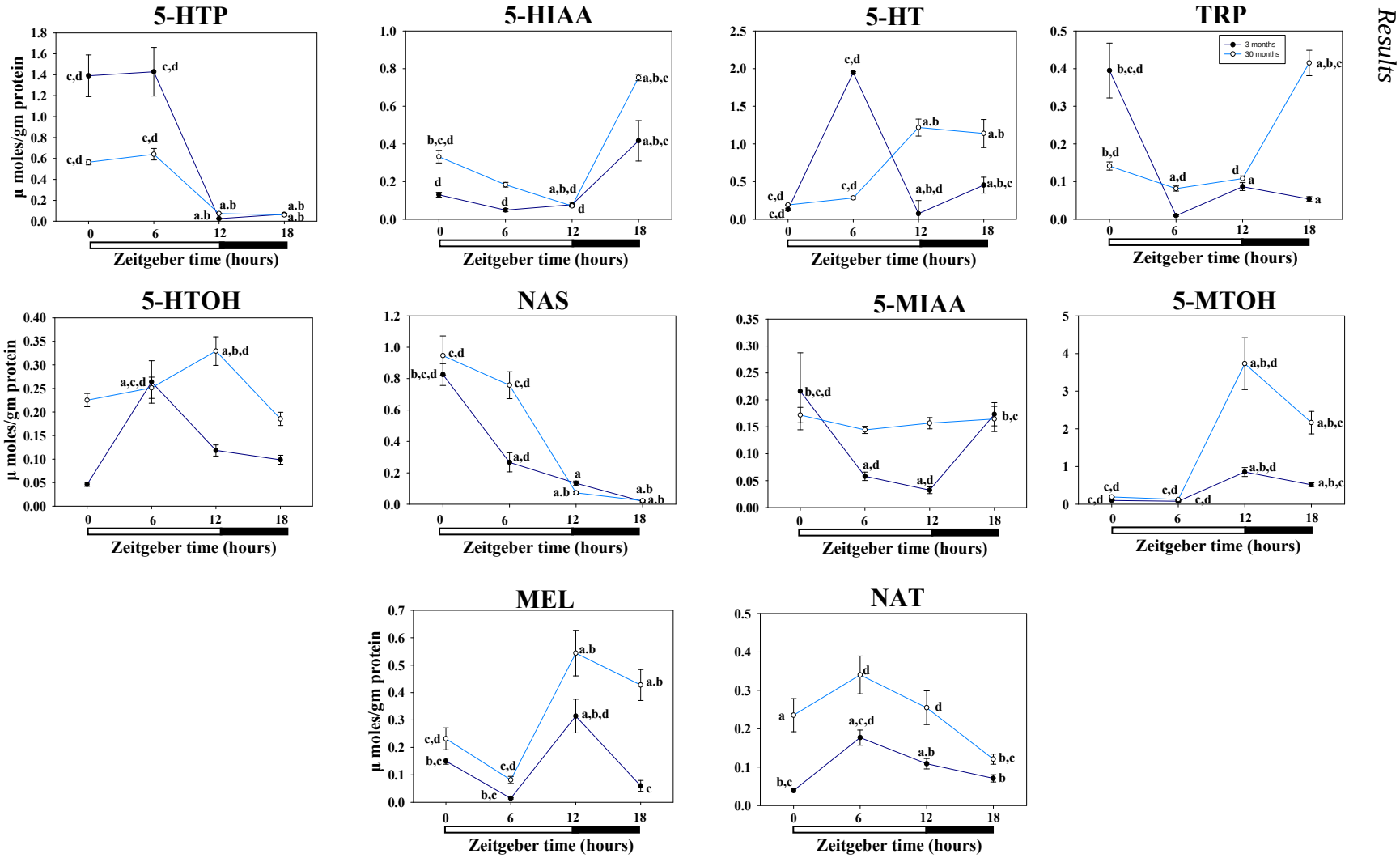


Figure 71. Comparison of daily rhythms of serotonin and its related compounds in the Pineal of 3 months old and 30 months *F. palmarum. pa < 0.05; pb < 0.05; pc < 0.05* and *pd<0.05* (where a, b, c and d refers to comparison between ZT-0, 6, 12 and 12 respectively).

Table 8. Age induced changes in serotonin and its related compounds in the Pineal of 3 months old and 30 months old *F. palmarum.*

Metabolites	Age (months)	Max Levels (µ mol/gm)	Max Time point	Min Levels (µ mol/gm)	Min Time point	Ratio Max/Min	Daily Mean levels (µ mol/gm)
5-HTP	3 months old	1.43 ± 0.23	ZT-6	0.02 ± 0.004*	ZT-12	2.41 ± 0.24 ^{d}	1.25 ± 0.12^{b,c,d,e,f,g,h,i,j}
	30 months old	0.64 ± 0.006	ZT-6	0.06 ± 0.01*	ZT-18	0.37 ± 0.05^{e,b,j}	1.61 ± 0.31^{b,c,d,e,f,g,h,i,j}
5-HIAA	3 months old	0.42 ± 0.11	ZT-18	0.05 ± 0.008*	ZT-6	2.64 ± 1.64 ^{d}	0.67 ± 0.11^{a,c,d,e,f,g,h,i,j}
	30 months old	0.75 ± 0.02	ZT-18	0.07 ± 0.004*	ZT-12	3.01 ± 0.47^{a,c,d,f,g,g,h,i,j}	1.18 ± 0.152^{a,c,d,e,f,g,h,i,j}
5-HT	3 months old	1.95 ± 0.17	ZT-6	0.08 ± 0.01*	ZT-12	2.85 ± 0.54 ^{d}	0.18 ± 0.03^{a,b}
	30 months old	0.21 ± 0.11	ZT-12	0.28 ± 0.002	ZT-18	0.34 ± 0.05^{e,b,j}	0.13 ± 0.02^{a,b}
TRP	3 months old	0.39 ± 0.07	ZT-0	0.009 ± 1.73	ZT-6	7.55 ± 1.56 ^{a,b,c,d,e,f,g,h,i,j}	0.03 ± 0.005^{a,b,h}
	30 months old	0.42 ± 0.03	ZT-18	0.08 ± 0.006*	ZT-6	1.04 ± 0.21^{e,b,j}	0.06 ± 0.006^{a,b}
5-HTOH	3 months old	0.26 ± 0.04	ZT-6	0.05 ± 0.004*	ZT-0	0.73 ± 0.17 ^{d}	0.19 ± 0.03^{a,b}
	30 months old	0.33 ± 0.03	ZT-12	0.19 ± 0.01*	ZT-18	3.24 ± 0.49^{a,c,d,f,g,g,h,i,j}	0.29 ± 0.04^{a,b}
NAS	3 months old	0.83 ± 0.07	ZT-0	0.019 ± 0.005*	ZT-18	1.31 ± 0.42 ^{d}	0.06 ± 0.005^{a,b,h}
	30 months old	0.95 ± 0.13	ZT-0	0.02 ± 0.002*	ZT-18	0.47 ± 0.08^{e,b,j}	0.29 ± 0.03^{a,b}
5-MIAA	3 months old	0.22 ± 0.07	ZT-0	0.03 ± 0.006*	ZT-12	3.18 ± 0.86 ^{d}	0.05 ± 0.005^{a,b,h}
	30 months old	0.17 ± 0.01	ZT-0	0.14 ± 0.006*	ZT-6	0.32 ± 0.03^{e,b,j}	0.17 ± 0.02^{a,b}
5-MTOH	3 months old	0.86 ± 0.12	ZT-12	0.08 ± 0.01*	ZT-6	2.68 ± 0.72 ^{d}	0.26 ± 0.03^{a,b,d}
	30 months old	3.73 ± 0.69	ZT-12	0.13 ± 0.009*	ZT-6	0.71 ± 0.14^{e,b,j}	0.38 ± 0.05^{a}
MEL	3 months old	0.31 ± 0.06	ZT-12	0.02 ± 0.002*	ZT-6	1.23 ± 0.177	0.12 ± 0.006^{a,b,j}
	30 months old	0.55 ± 0.08	ZT-12	0.082 ± 0.013*	ZT-6	0.62 ± 0.07^{e,b,j}	0.24 ± 0.02^{a,b}
NAT	3 months old	0.18 ± 0.02	ZT-6	0.04 ± 0.004*	ZT-0	1.59 ± 0.43 ^{d}	0.08 ± 0.006^{a,b,h}
	30 months old	0.34 ± 0.05	ZT-6	0.12 ± 0.013*	ZT-18	1.62 ± 0.18^{acefghi}	0.18 ± 0.02^{a,b}

The mean levels in descending order of levels in the SCN are as follows TRP, 5-HT, 5-MIAA, NAT, MEL, NAS, 5-HTOH, 5-MTOH, 5-HIAA and 5-HTP. Maximum: minimum ratio in the descending order of their levels are 5-MIAA, 5-HT, 5-HTP, NAS, MEL, 5-MTOH, TRP, NAT, 5-HIAA and 5-HTOH. Each value is Mean ± S.E (n=6). *pa<0.05*, *pb<0.05*, *pc<0.05*, *pd<0.05*, *pe<0.05*, *pf<0.05*, *pg<0.05*, *ph<0.05*, *pi<0.05*, *pj < 0.05* (where a, b, c, d, e, f, g, h, i and j refers to comparison of each metabolite, i.e. 5-HTP, 5-HIAA, 5-HT, TRP, 5-HTOH, NAS, 5-MIAA, 5-MTOH, MEL and NAT. * refers to the comparison between maximum and minimum levels (n=6) (*p<0.05*).

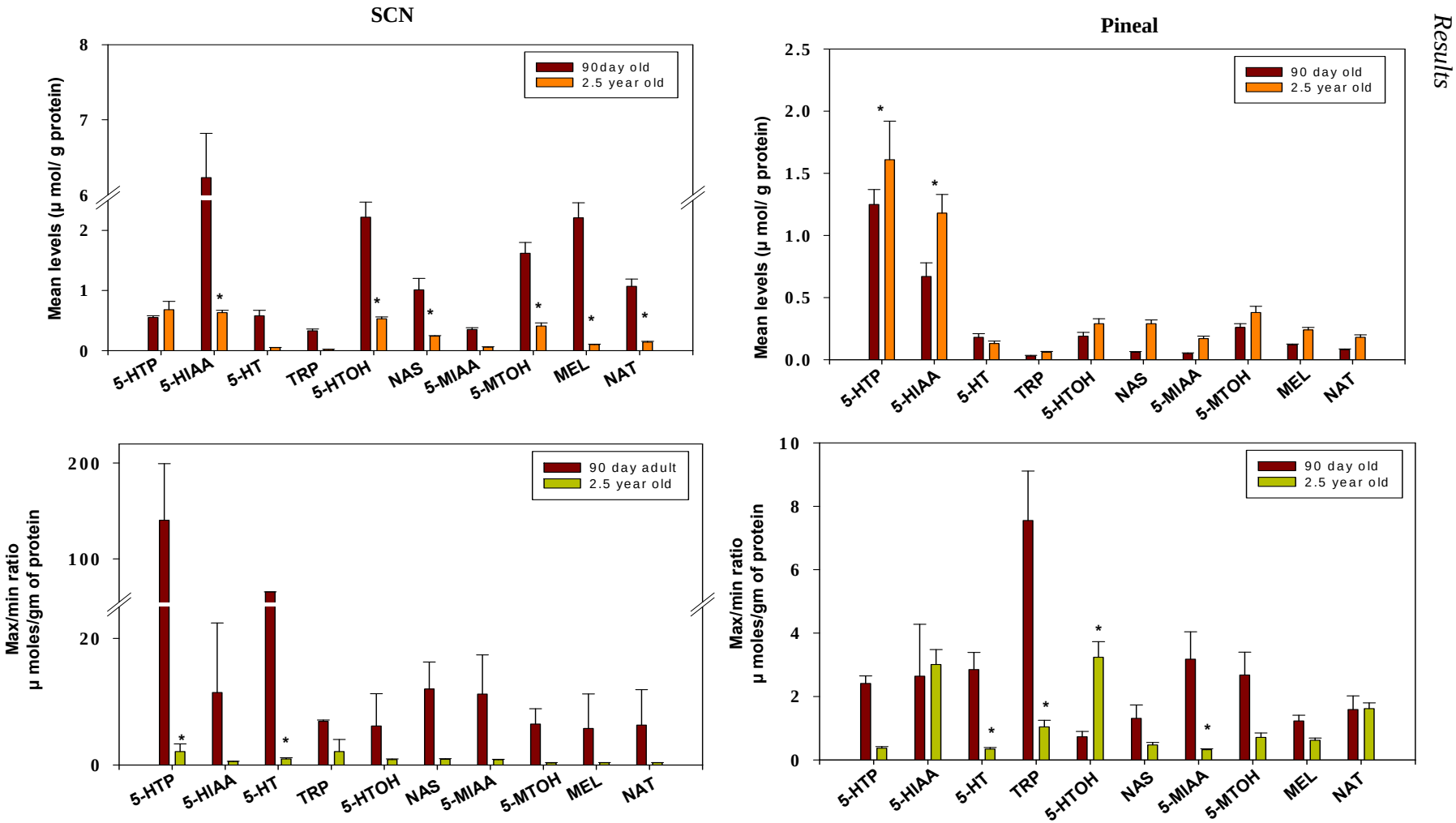


Figure 72. Comparison of mean and daily pulse in the SCN (A) and Pineal (B) of 3 months old and 30 months old *F. palmarum.* Each value is Mean ± S.E (n=6).* comparison between 3 month old and 30 month old showing significant difference ($p<0.05$).

(c). Molecular level: *per1, per2, cry1, cry2* and *bmal1* expression

Upon aging *per1*, *cry1* and *bmal 1* showed daily rhythmicity where as *per2* and *cry2* showing abolished rhythmicity (n=4) ($p<0.05$) in the SCN (Figure 73 A and B). *per1*and *per2* showed maximum level at ZT-0 and minimum at ZT-6 in the SCN of 30 months old. In comparison to adult the maximum levels of *per 1*and *per 2* were similar to that in adults. However, the minimum levels of *per1*and *per 2* were at ZT-12, which was 6 hours advanced in aged *F. palmarum* as compared to adults (Figure 73 B). The *cry1* levels were maximum at ZT-12 and minimum at ZT-18 in aged SCN which was different as compared to adults where it was maximum at ZT-0 and minimum at ZT-12. The *cry 2* levels were maximum at ZT-18 and minimum at ZT-12, where as in the adults the maximum levels were at ZT-0 and minimum at ZT-12. The *bma l1* levels were maximum at ZT-0 and minimum at ZT-6 upon aging which was different as compared to adults where the levels are maximum at ZT-0 and minimum at ZT-6. The expression levels of *per1, per2, cry1, cry2 and bmal1* genes were decreased by 13.36, 15.71, 10.44, 16.77 and 50.24 % (n=6; $p<0.05$) respectively upon aging in the SCN as compared to adults (n=4) ($p< 0.05$) (Figure 73 B).

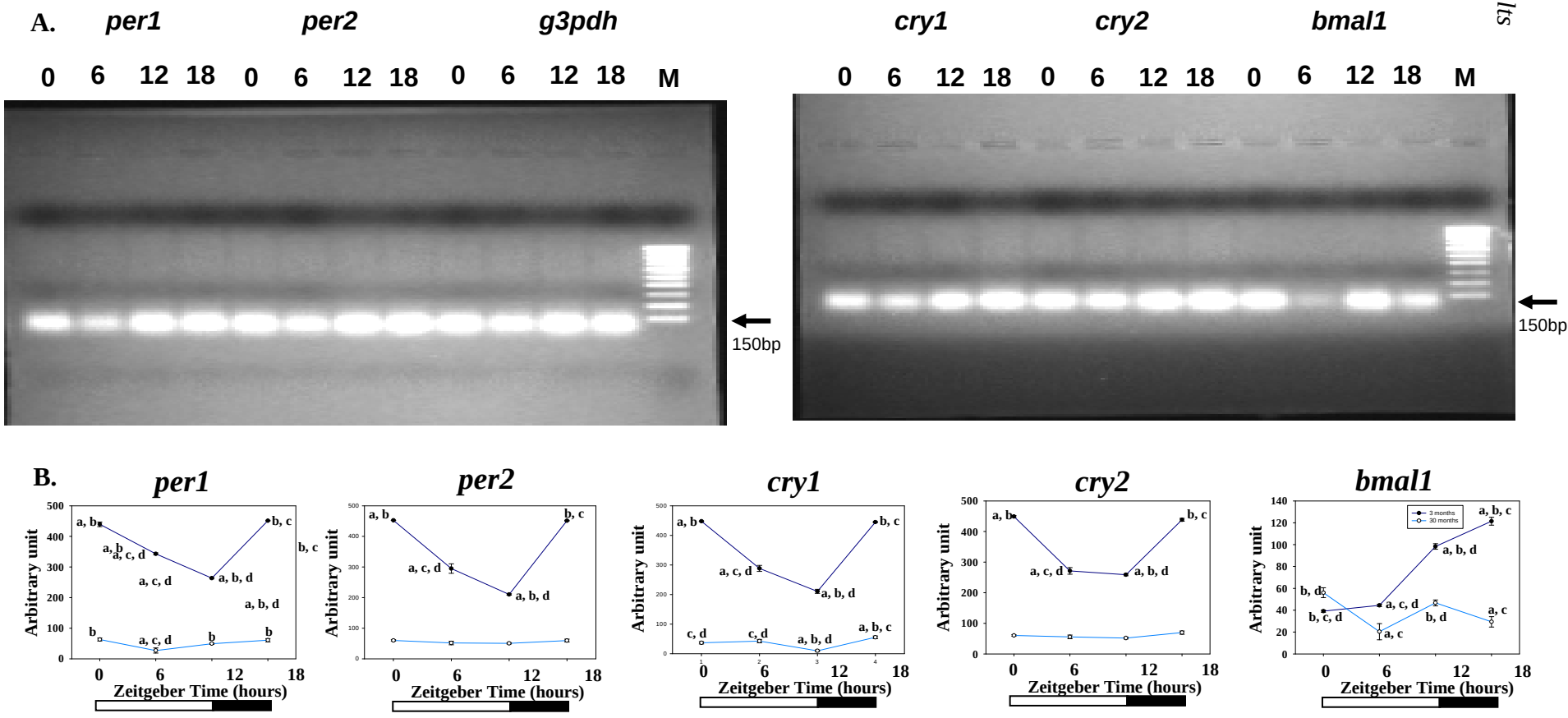


Figure 73. Effect of aging on daily rhythms of *per1*, *per2*, *cry1*, *cry2* and *bmal1* expression in the SCN. A. Representative gel showing the amplicons of *per1*, *per2*, *cry1*, *cry2*, *bmal1* and *g3phd* in the SCN of 30 months old *F. palmarum* (n=4). B. Densitometry analysis showing the comparison of *per1*, *per2*, *cry1*, *cry2* and *bmal1* in the SCN of 3 months old and 30 months old *F. palmarum*. *pa < 0.05; pb < 0.05; pc < 0.05* and *pd<0.05* (where a, b, c and d refers to comparison between ZT-0, 6,12 and 18 respectively).

Discussion

1. Characterization of *F. palmarum* through

(a). Behavioral studies: Locomotion (Gross locomotor activity and Running wheel activity)

The locomotor activity of three striped South Indian Palm Squirrel, *F. palmarum* exhibited a diurnal pattern of activity under LD, 12:12 condition for both gross and running wheel activity. All animals showed stable entrainment to LD cycles (Figure 29) (Mammen and Jagota, 2011). This suggests that, as in most other mammals; the LD cycle is a potent zeitgeber (Zucker, 1976). Activity on-set and off-set were precisely defined with the animals starting and ending their activity at approximately the same time each day. Similar on-set and off-set of activity and entrainment to LD cycle were reported in *F. pennanti* by Rajaratnam and Redman, 1999. These findings in Palm Squirrels indicate that the animals possess robust diurnal CTS. Gross locomotor activity showed a % diurnality of 70.39 ± 3.88% and a circadian period (τ) of 24.19 ± 0.12 h (Mammen and Jagota, 2011). A similar pattern of entrainment was also reported earlier in *F. pennanti* by Rajaratnam and Redman, 1999 with 88% diurnality. Other diurnal rodents such as *A. niloticus* and *R. pumilio* have been reported to show diurnality of 60% (Katona and Smale, 1997) and 68% (Schumann *et al*., 2005) respectively. The amount of activity during the light phase (% diurnality) in diurnal rodents appear to be comparatively less as compared to the amount of activity during the dark phase (% nocturnality) in nocturnal rodents such as 93 % for Sprague-Dawley rats and 99% for Syrian hamsters respectively (Zucker and Stephan, 1973). In *F. palmarum*, a free running circadian rhythm in locomotor activity was exhibited under constant darkness (DD) (Figure 30) as well as constant light (LL) (Figure 31) condition with a τ of 24.11 ± 0.03 and 24.92 ± 0.35 hours respectively suggesting that the functional endogenous pacemaker is able to express rhythms of locomotor activity (Mammen and Jagota, 2011). Similar free running pattern *A. niloticus* and *O. degus* also showed a free running pattern of gross locomotor activity under DD with a τ of 23.9 (Refinetti, 2004) and 23.33 ± 0.23 hours (Garcia-Allegue *et al.,* 1999) respectively. In *F. palmarum* the mean activity under DD showed a diurnal preference, however in LL the activity an even preference throughout the light and dark period (Mammen and Jagota, 2011). A free-running rhythm reflects the presence of an endogenous pacemaker and also signifies a functional circadian timing system that is able to sustain rhythmicity even in the absence of external cues. A preference for diurnal or

nocturnal pattern of activity could be an inherent property of the pacemaker of an individual species based on the temporal niche it occupies. In our study *F. palmarum* showed a diurnal preference in DD. This is in contrast to that reported in nocturnal rodents where they show nocturnal preference in DD and LL (Schöttner *et al*., 2006).

Gross locomotor activity depicts the general behavioral pattern of animals and can also be determined by subjecting animals to running wheels. However, studies have shown that when diurnal animals such as *O. degus*, *A. niloticus*, etc. were subjected to running wheel, a switch in the behavior of locomotor activity, from diurnal types to nocturnal types, were reported. Hence, *F. palmarum* were subjected to running wheel under various photoperiods such as LD, DD and LL. The running wheel activity of *F. palmarum* exhibited a diurnal pattern of activity under LD, 12:12 conditions with a τ of 24.01 ± 0.008 hours (Figure 33). *F. palmarum* showed a percentage diurnality of 94.94 ± 2.58 % in running wheel which was significantly higher as compared to gross locomotor activity. Similar but lower percentage diurnality of running wheel activity of 77% has been reported in the diurnal zebra mouse (*Lemniscomys barbarus*) (Lahmam *et al*., 2008). However, in the diurnal *O. degus* the percentage diurnality of running wheel activity was reported to be only 31% but with nocturnal preference. This is due to switching pattern reported in *O.degus* in the presence of running wheel (Ocampo-Garcés *et al*., 2005). Domestic mice, laboratory rats, Syrian hamsters, and Siberian hamsters are traditionally considered nocturnal species showing 52%, 21%, 70 % and 43% of running wheel activity restricted to the dark phase (Refinetti, 2008). In *F. palmarum* the amount of activity on running wheel (percentage diurnality) was higher as compared to the running wheel activity (percentage nocturnality) in nocturnal rodents. Under constant dark (DD) conditions *F. palmarum* showed a free running pattern of running wheel activity with a τ of 23.88 ± 0.19 hours (Figure 34). Similar free running endogenous period of running wheel activity with a τ of 23.8 hours has been reported in the diurnal zebra mouse (*Lemniscomys barbarus*) (Lahmam *et al*., 2008). In our study under DD the τ was not affected in the presence of running wheel as compared to the τ for gross locomotor activity. This shows that the endogenous pacemaker in *F. palmarum* is diurnal and that it is able to sustain a diurnal activity rhythm even in the presence of external factors such as the running wheel. *F. palmarum* subjected running wheel under LL conditions showed a splitting pattern in the locomotor activity with τ of 24.28 ± 0.18h (Figure 35). Similar splitting of activity

rhythms under LL reported in the several diurnal rodents such as arctic ground squirrels (*Spermophilus parryii*) (Pittendrigh, 1960), uinta chipmunk (*Neotamias umbrinus*) (Swade and Pittendrigh, 1967), diurnal tree shrew (*Tupata belangeri*) (Hoffmann, 1971), diurnal ground squirrels (*Eutamias sibiricus* and *Ammospermophilus leucurus*) (Pickard *et al*., 1993), starlings (*Sturnus vulgaris*) (Gwinner, 1974) and lizards (*Anolis carolinensis*) (Underwood, 1977). Splitting patterns of activity rhythms, body temperature rhythm, feeding and drinking rhythms and SCN electrophysiological activity has earlier been reported in nocturnal animals, such as hamsters (*Mesocricetus auratus*) and rats (*Rattus norvegicus*) upon exposure to constant light (Lees *et al*., 1983). Splitting has been attributed to the presence of dual oscillator system within the SCN neurons (Pittendrigh and Daan, 1976; Daan and Berde, 1978). Though the mechanism involved in splitting has not been elucidated yet, it could be due to changes in τ causing changes in the time-interval between split components resulting in transient behavior. During splitting the running wheel activity dissociates into two components that free run with different periods until they become stably coupled at 180º (i.e. 12 hours) apart. Each of the dual-oscillators thus acts as a precise and stable circadian pacemaker with one synchronized to the sunrise at dawn and the other to the sunset at dusk (de la Iglesia *et al*., 2000; Jagota *et al*., 2000). These events progress slowly until the split components regains their pre-pulse phase relationship (Daan and Berde, 1978). In our study on *F. palmarum* the splitting was sustained for upto 160 days after which the animals showed entrainment to LD cycle (Figure 39 and 40). Splitting can also be caused by factors such as constant darkness, changes in hormonal milieu of the subject, etc. (Morin and Cummings, 1982).

Both the gross locomotor activity as well as the wheel running activity of *F. palmarum* exhibited a diurnal pattern in the present study. This pattern of diurnal locomotor activity under laboratory conditions is in agreement with the daily activity pattern of squirrels exhibited in their natural habitat (Prater, 1980). In the present study, all animals subjected to locomotor activity, i.e. both gross as well as running wheel activity, showed only a diurnal pattern of activity. However, inter-individual variations among individuals in the presence of running wheel were reported under laboratory conditions. For *A. niloticus* housed with running wheel exhibited inter- individual variations with some individuals exhibiting nocturnal activity while some retained diurnal pattern of locomotor activity (Katona and

Smale, 1997; Blanchong *et al*., 1999; Redlin and Mrosovsky, 2004). Similar studies in *O. degus* also shows inter- individual variation with diurnal, noctournal and crepuscular pattern of running wheel activity (Kas and Edgar, 1999; 2000; Ocampo-Garcés *et al*., 2005; Refinetti, 2006). *O. degus* are considered to be diurnal in the wild (Kenagy *et al*., 2002) and in the laboratory if monitored by Automated Radio Telemetry System (ARTS), i.e. using automated battery powered microcomputer to record locomotor activity with little human attention, showed a diurnal pattern of gross locomotor activity (Refinetti, 1996). However, large inter-individual variability in the phase angle of entrainment has been reported in *O. degus* monitored by ARTS (Labyak and Lee, 1997). Similarly, Mongolian gerbils (*M. unguiculatus*) also showed great intra-species variability, with 24% of the animals being predominantly nocturnal and 76% being predominantly diurnal (Refinetti, 2008). ARTS recordings of the Central American agouti (*Dasyprocta punctata*) also displayed a typical diurnal pattern of activity, with peak activity occurring shortly after dawn and just before dusk. However, these rodents exhibited bouts of nocturnal activity when they were exposed to risk of predation by ocelots (*Leopardus pardalis*), changes in environmental conditions and resource abundance (Lambert *et al*., 2009). Intra-species variability in behavior, with some individuals showing predominantly diurnal activity patterns and others showing predominantly nocturnal activity patterns, has been occasionally described in other species. For instance, in goldfish (*Carassius auratus*), about 80% of individuals tested in the laboratory were found to be diurnal; whereas 10% were nocturnal, and 10% displayed very weak rhythmicity (Iigo and Tabata, 1996). In carpenter ants (*Camponotus compressus*), approximately 70% of individually tested animals were found to be nocturnal, whereas 30% were diurnal (Sharma *et al*., 2004). Likewise, in the subterranean mole rats of various species, some members of the species were found to be diurnal and some were found to be nocturnal (Oster *et al*., 2002). Wolves (*Canis lupus*) are normally nocturnal; however, when traveling over long distances, they travel during the day (Merrill and Mech, 2003). Conversely migratory birds are normally diurnal, but they do most of their migratory flight at night (Cochran, 1987; Rattenborg *et al*., 2004). Inter-individual variation in the locomotor activity can be attributed to masking. Masking occurs when external cues that entrain the CTS also directly affect activity rhythms of an organism (Aschoff and von Goetz, 1988). Masking being an evolutionary advantage for an organism, enables species not to be rigidly

confined to being active by day or by night and can adjust their rhythms according to the environmental circumstances. Understanding these cases could give insight into mechanisms employed when switching of temporal niche occurs as part of the life cycle, or in response to circumstances such as availability of food. In the laboratory condition switching can be induced in the presence of running wheel or using stimuli such as food availability, temperature, social pressures, seasonal cues, developmental changes or as a result of mutations, genetic manipulations or brain lesions (Mrosovsky and Hattar, 2005).

The distinction between diurnality and nocturnality could depend on two relatively independent processes: the establishment of an adequate phase relationship between the circadian clock and the entrainment of activity, and the establishment of an adequate pattern of photic masking i.e. negative masking in nocturnal organisms and positive masking in diurnal organisms (Redlin and Mrosovsky, 2004). Studies on the phase relationship between the circadian clock and the entrainment of activity using mutant mice and hamsters with abnormally long or short endogenous periods show that animals with a typically nocturnal circadian system will entrain to the LD cycle with a diurnal phase angle (Ralph and Menaker 1988; Low-Zeddies and Takahashi, 2001). When the circadian period is lengthened or shortened without a concomitant change in the photic phase-response curve, entrainment to a standard LD cycle can only be attained by longer exposure to light, which requires a shift of the active phase into daylight. During masking, as reported in several studies, have shown that light exposure during the night often inhibits activity in nocturnal animals and elicits activity in diurnal animals (Redlin *et al*., 1999; Redlin and Mrosovsky, 2004). Genetic manipulations of photic receptor systems (rod and cone opsins, cryptochrome, and melanopsin) in mice have been shown to mimic temporal niche switching from nocturnal to diurnal (Mrosovsky and Hattar, 2005). Mice deficient in photic receptors exhibited much more activity during the light phase than did normal mice and also exhibited positive masking in response to short pulses of light. This appearance of positive masking in a species that usually exhibits negative masking suggests that the niche inversion was achieved partially by an inversion in masking mechanisms. The interplay of mechanisms of entrainment and masking thus creates a distinction between diurnality and nocturnality and generates different chronotypes that are purely diurnal, nocturnal or bimodal as observed in Mongolian gerbils, degus and Nile grass rat (Refinetti, 2008).

Nocturnal and diurnal rodents respond differently to light and have opposite effect on their pacemaker (Carpenter and Grossberg, 1984). Aschoff (1960) proposed that the locomotor activity of diurnal species exhibit a longer τ in DD than in LL with a τ greater than 24 h in DD (Moore-Ede *et al*., 1982). In our study, the locomotor activity of *F. palmarum* did not confirm to Aschoff's rules. The gross locomotor activity of *F. palmarum* showed a τ greater in LL than DD. Running wheel activity also showed a greater τ in LL than at DD but the τ was less than 24 h in DD. Similar findings were reported in the *A. niloticus*, where the τ was greater in LL than DD. However, species differences in the time period were reported with a τ shorter than 24 h in DD in *A. niloticus* (Katona and Smale, 1997) and a τ greater than 24 h in LL than dim red light for *A. ansorgei* (Challet *et al*., 2002) and *O. degus* (Lee and Labyak, 1997). These findings in diurnally active species could be a result of their being normally exposed to light over a substantial portion of the day. The pacemaker in mammals regulates the activity-rest cycles based on the intensity of light and feedback mechanisms within the pacemaker. Light enhances the locomotor activity of diurnal species and suppresses activity of the nocturnal species. In both diurnal and nocturnal species the feedback mechanisms inhibit the pace of locomotor activity. This asymmetrical action of light and the feedback actions on the pacemaker enable mammals to generate chronotypes such as diurnal and nocturnal based on the environment that it occupies (Carpenter and Grossberg, 1984). Diurnal mammals are thus subjected to both continuous modulation of circadian period (parametric entrainment) as well as to discrete phase-shifting effects (non-parametric entrainment) by light in their natural environment (Daan 1977; Boulos *et al*., 1996).

(b). Neuroanatomical studies: Neuroanatomy of the SCN

(i). Gross anatomy of the brain

The optic structures and brain regions that receive visual and photic input have been reported by some workers to differ between diurnal and nocturnal rodents (Goel *et al*., 1999; Smale and Boverhof, 1999). Differences in eye size, corneal size, retinal morphology, opsin distribution and spectral sensitivities of photopigments, reflected the lifestyle of the animal and show considerable evolutionary flexibility in the visual system (Ankel-Simons and Rasmussen, 2008). Nocturnal mammals tend to have larger eyes than diurnal mammals

and that nocturnal species have eyes with larger anterior segment, pupil with relatively thick and strongly curved lenses (Schmitz and Motani, 2010). Differences in the anatomical organization of the circadian timing system have been reported to differ between nocturnal and diurnal animals (Smale *et al*., 1991). The darker eyes found in *F. palmarum*, could be due to presence of pigmentation in lens (Mammen and Jagota, 2011). Lens pigmentation has been reported as an apparent feature of diurnal animals and is absent in nocturnal species (Jacobs *et al*., 2003). The presence of short, thick and stout optic nerves in *F. palmarum* as compared to nocturnal rats (Figure 41) (Mammen and Jagota, 2011) is in agreement with earlier studies on nocturnal (Kita and Oomura, 1982; Pickard, 1982) and other diurnal rodents (Goel *et al*., 1999; Smale and Boverhof, 1999). Ecosystems are complex and comprise ecological communities, in which each and every species has it own specific niche, characterized by its relationships with the abiotic environment and with other organisms. These apparent neuroanatomical features of diurnal animals as distinct from nocturnal animals could be related to their better adaptation in diurnal environment with more light input pathways for locating food, communication and protection from predators in diurnal animals (reviewed in Kronfeld-Schor and Dayan, 2008). Being active in a diurnal environment exposes animals to very different environmental challenges, both biotic and abiotic that include chances in ambient day time temperatures, humidity, food availability, predation risk, prey availability, competitors, mates, etc. Diurnal animals usually use vision for locating food, while nocturnal animals use tactile probing, smell, and hearing. Nocturnal animals usually communicate using vocal stimuli as well as aroma and use camouflage for concealment from their diurnal predators during the day (e.g. moths and owls). Diurnal animals on the other hand use visual signals such as aposematic coloration, i.e. warning coloration that makes it appear dangerous, poisonous, or foul-tasting (Daan, 1981) or camouflage coloration to blend to the environment or attractive coloration during breeding seasons, and alarm calling to escape from predators (Shelley and Blumstein, 2005). Retinas of nocturnal and diurnal mammals differ in their photoreceptors and adaptations to vision at a given light level. This not only improves the circadian fitness at that light level but also tend to reduce the efficiency of activity at other times (Jacobs, 1993; van Schalk and Griffiths, 1996) as reported in the wild-caught antelope ground squirrels (*A. leucurus*) when deprived of their SCN behaved normally, but became arrhythmic and active during the night. This

disadvantage of having nocturnal activity enable nocturnal desert predators such as rattlesnakes, kit foxes, bobcats, coyotes, barn owls, and feral cats to take advantage of the squirrels' limited visual activity at night and prey upon them (DeCoursey *et al*., 1997; DeCoursey, 2004). The environment of an organism is divided into times when it is optimal to perform a certain activity, and at times when performing the same activity may even be disadvantageous. Thus most animals have a distinct activity pattern confined to a certain part of the diel cycle, which is typical to the species, and to which they are adapted behaviorally, anatomically and physiologically (reviewed in Kronfeld-Schor and Dayan, 2008).

The pineal gland, an effector organ of SCN, in *F. palmarum* appeared submerged in the cerebral junction unlike that in nocturnal rodents where it appeared superficial (Figure 41) (Mammen and Jagota, 2011). This could be due to the redundant photoreceptive function of pineal in diurnal rodents as compared to nocturnal species. Mammals are unique among vertebrates, in that they appear to have lost extraocular photoreceptors. Extraocular photoreceptors, the deep brain and pineal photoreceptors play a critical role in the regulation of circadian clocks of non-mammalian vertebrate species (Foster, 2002). The mammalian pinealocytes also contain ultrastructural features associated with photoreceptors that include a range of photoreceptor proteins including opsin. However, the mammalian pineal contains no chromophores, neither 11 cis retinal nor all-trans-retinaldehyde suggesting that the opsins present within the mammalian pineal is not a part of the functional photopigment and that the pineal plays no role as a photoreceptor for entrainment of circadian rhythms (reviewed in Roenneberg and Foster, 1997). Though pineal have no photoreceptive functions they play a major role in conveying the message of darkness by the production of melatonin in all species irrespective of their behavioral type (Vivanco *et al*., 2007).

(ii). Suprachiasmatic nucleus

The neuroarchitecture of the SCN of *F. palmarum* showed an elliptical shape as compared to other rodents such as rat, mice and hamsters where it is spherical in shape (Figure 42) (Mammen and Jagota, 2011). The proximity of the *F. palmarum* SCN was more to the optic chiasm than to the third ventricle as seen in rats. Similar elliptical shaped SCN have been reported in the *O. degus* and *A. niloticus* (Refinetti, 2004; Smale *et al*., 1991). The neuronal counts of the *F. palmarum* SCN were significantly higher as compared to nocturnal

species where it is about 20,000. The higher neuronal numbers could play a role in higher challenges these animals have to encounter in their diurnal niche where the risk of predation is much higher (Kronfeld-Schor and Dayan, 2008). The SCN of *F. palmarum*, showed a neuronal density that was evenly distributed through out the SCN. This could be due to presence of more number of neurons associated to the input pathway as supported by the prominent optic chiasm of these animals (Mammen and Jagota, 2011). A similar cell density pattern was reported in the *O. degus* (Goel *et al*., 1999), however, in the *S. lateralis* the cell density appears to be greatest in the ventral half of the SCN (Smale *et al*., 1991). The neuronal counts in the paired SCN of 3-striped South Indian palm Squirrel were significantly higher as compared to nocturnal species (van den Pol, 1991; van Esseveldt *et al*., 2000). Though similar data is not available from other diurnal species, the higher neuronal count obtained in SCN of *F. palmarum* could be a distinct feature related to diurnality (Kronfeld-Schor and Dayan, 2008). Though SCN occupy a significant role in the entrainment of physiological functions, its size is comparatively smaller than other nuclei in the hypothalamus and could be sufficient to generate and/or coordinate the neural time-keeping process in mammals, irrespective of the size of the organism (Hofman *et al*., 1988).

The SCN produces entrained rhythms based on the input signals received through afferent pathway and are mediated by various neuropeptides. In the SCN neuropeptides such as AVP, VIP, GRP, etc. have been reported to show daily rhythmicity with region specific expression (Dardente *et al*., 2004; Caldwell *et al*., 2008). In the SCN of *F. palmarum* AVP-ir and VIP-ir studies showed a dorso-medial and ventrolateral distribution respectively, indicating a core-shell differentiation. This pattern of core-shell distribution of AVP and VIP was similar to that found in other diurnal as well as nocturnal species (Goel *et al*., 1999). Presence of dense AVP-ir and VIP-ir fibre that efferent from the SCN, towards the sPVHz is similar to that in both diurnal (Gomez *et al*., 1997; Smale *et al*., 2008) and nocturnal rodents (Lu *et al*., 2001). The sPVHz has been reported to play important role in regulation of circadian physiology (Watts, 1991) and is related to influence diurnal physiology (Smale *et al*., 2008) by its efferents to several regions of the brain. sPVHz mediates the production of melatonin by the pineal gland through multi-synaptic pathway via the intermediolateral cell column of the upper thoracic spinal cord (Moore, 1996; Pyner, 2008; Mammen and Jagota, 2011).

(c). Neurotransmitter studies: AVP, VIP and serotonin metabolome rhythms in SCN

(i). AVP-ir and VIP-ir daily rhythmicity in the SCN

VIP-ir and AVP-ir showed daily rhythmicity in the SCN of *F. palmarum* (Figure 43) (Mammen and Jagota, 2011). Similar diurnal pattern of AVP and VIP rhythmicity have been reported in the diurnal *A. ansorgei* (Dardente *et al*., 2004). However, in diurnal humans VIP does not exhibit diurnal fluctuations though vasopressin (VP) has been reported to exhibit daily rhythmicity (Hofman and Swaab, 1994; Shinohara *et al*., 1994; Hofman *et al*., 1996). In our study, in the SCN *F. palmarum* VIP-ir levels were maximum and minimum at ZT-6 and ZT-0 respectively (Figure 44) (Mammen and Jagota, 2011). This is in contrast to that reported in the nocturnal rat SCN where the VIP maximum and minimum levels were reported to be at ZT-0 and ZT-12 respectively (Shinohara *et al*., 1993; Isobe and Nishino, 1998). The VIP maximum levels were thus 6 hours phase advanced in diurnal rodent while the minimum levels were 12 hours phase delayed in the diurnal rodents as compared to nocturnal rodents (Mammen and Jagota, 2011). However, in the diurnal *A. ansorgei*, VIP has been reported maximum at ZT-18 in light dark condition (Dardente *et al*., 2004). This could be attributed to the inter-individual variations exhibited by these animals. AVP-ir showed maximum and minimum levels at ZT-12 and ZT-0 respectively where as in the rat the maximum and minimum levels were at ZT-4 and ZT-12 respectively (Isobe and Nishino, 1998). In *A. ansorgei* also the maximum AVP levels have been reported at ZT-12 (Dardente *et al*., 2004). High VIP-ir and AVP-ir levels during the light phase in our study on *F. palmarum* were similar to that reported in rats. However, the maximum and minimum VIP-ir levels are only 6 h apart in *F. palmarum* though 12 h apart in rat and the maximum and minimum AVP-ir levels are 12 h apart in *F. palmarum* though only 8 h apart in rats. In rats, VIP rhythm was ahead of AVP but VIP levels were six times higher as that of AVP (Shinohara *et al*., 1994). Additionally, the SCN activity such as metabolism as well as the multiple-unit activity is maximum during the day though melatonin levels are maximum during midnight in nocturnal animals (Schwartz *et al*., 2004). VIP in the rat SCN has a strong inhibitory effect where as in the diurnal rodent it has a stimulatory effect on corticosterone rhythms (Kalsbeek *et al*., 2008). Studies using VIP and $VPAC_2$ receptor knockout mice have shown to induce arrhythmicity in entrained running wheel behavior indicating that VIP

signaling via the VPAC2 receptor is controlled by the prevailing lighting conditions (Hannibal and Fahrenkrug, 2003).

F. palmarum subjected to constant conditions of DD and LL showed circadian rhythmicity in VIP levels where as that of AVP is abolished in the SCN. This pattern of rhythmicity was reversed as compared to nocturnal rodents where AVP showed endogenous rhythmicity and VIP rhythmicity was abolished under constant conditions (Shinohara *et al*., 1993). In *F. palmarum* under DD conditions, VIP maximum levels were at ZT-0. However in the diurnal *A. ansorgei* in DD the maximum levels were reported to be at at CT-12. This indicates that VIP levels in diurnal species maximize during the light phase and hence indicating a pacemaker that sustains diurnal pattern of activity. This is supported by the fact that genes that are part of the basic molecular feedback loop that drives circadian oscillations in single cells are regulated by light (King and Takahashi, 2000, Reppert and Weaver, 2001). Studies on VIP and VPAC2 knock out mice show absence of *per* gene expression which is normally expressed during exposure to light (Harmar *et al*., 2002). These observations suggest that VIP plays a significant role in coupling of the circadian oscillator to the environment and thus could be associated with diurnality (Colwell *et al*., 2003). In *F. palmarum* VIP neurons in the ventrolateral SCN are responsive to photic cycle (LD cycle) and also under constant conditions enabling SCN to maintain endogenous rhythmicity. Unlike that in the nocturnal rodent's where AVP in the dorsomedial SCN mediates internal generation of circadian rhythms (Shinohara *et al*., 1993). *F. palmarum* being diurnal, under constant conditions, non-photic cues such as social contacts, sound, etc. may be acting as an input signal to entrain the SCN (Novak *et al*., 2008).

(ii). Serotonin chronometabolome daily rhythmicity in the SCN, Pineal and Brain

5-HT is a major neurotransmitter in the input and output pathways of the SCN (Jagota, 2006). Serotonin chronometabolome in the SCN of adult *F. palmarum* showed daily rhythmicity in all the compounds. In the SCN of *F. palmarum* 5-HT showed a diurnal pattern in its peak timings similar to that compared to nocturnal rats. However, distinct difference in mean levels and maximum : minimum ratios were seen in comparison with nocturnal rodents. In the SCN of *F. palmarum*, the maximum levels were 12 % decreased while the minimum levels showed a decrease of 0.71 % as compared to that of adult rat SCN (Jagota

and Kalyani, 2008). The mean 24 hour levels of 5-HT were decreased by 2 % while the maximum : minimum ratios increased by 2.3 folds in the SCN *of F. palmarum* as compared to the SCN of rat (Jagota and Kalyani, 2008). The 5-HT/5-HIAA ratios were maximum at ZT-6 and minimum at ZT-18 in squirrels in the present study, which is 6 hours advanced as compared to adult rat 5-HT/ 5-HIAA ratios in the SCN (Jagota and Reddy, 2007). These results indicate serotonin to play a major role in determining diurnal rhythm patterns by the SCN as in the nocturnal rodents 5-HT has been implicated in a variety of functions such as the modulation of photic signaling in the SCN and the resetting of circadian phase, light-induced phase shifts in free-running locomotor activity, light-induced Fos protein immunoreactivity in SCN cells, electrical activity of light-responsive SCN cells and SCN field potentials evoked by electrical stimulation of the optic nerve, phase shifting effects on the circadian locomotor activity rhythm and on the circadian rhythm of neuronal activity in SCN slices (Dudley *et al*., 1998). In Syrian hamsters entrained to LD cycle, serotonin showed daily rhythm with increased daytime levels that was temporally associated with active waking behaviors. 5-HIAA daily rhythmicity also show day time peaks in the SCN of rat, Syrian and Siberian hamster (Dudley *et al*., 1998). In the pineal of *F. palmarum* also serotonin chrono-metabolome showed daily rhythmicity in all compounds. The daily rhythms of 5-HT showed diurnal pattern similar to that in nocturnal rodents, however maximum levels were 6 hours delayed and minimum levels were 6 hours advanced in *F. palmarum* as compared to adult rats (Jagota and Reddy, 2007). The mean 24 hour levels of 5-HT were drastically reduced in *F. palmarum* as compared to adult rat (Jagota and Reddy, 2007). The 5-HT/5-HIAA ratios were maximum at ZT-6 and minimum at ZT-0 in the pineal of *F. palmarum*. The maximum levels were 6 hours advanced and minimum levels were 12 hours delayed as compared to adult rat 5-HT/ 5-HIAA ratios in the pineal (Jagota and Reddy, 2007). The levels of TRP, 5-HTP and 5-HT were lower by 1.2, 2.5 and 0.6 % in the SCN and by 2.7, 0.23 and 0.18 % in the pineal respectively of *F. palmarum* as compared to serum levels in normal adult humans. Though indole metabolites freely cross the blood brain barrier (Cornford *et al*., 1979), this decrease could be attributed to the physiological state of the animals or the differential presence of the compounds in circulation at the time of study.

The levels of serotonin and melatonin showed a similar pattern with serotonin showing day time peak and melatonin peaking during the dark phase. In all vertebrate

species, melatonin peaks during the night, regardless of whether the animal shows nocturnal or diurnal behavior (Reiter, 1991). In diurnal species, the nocturnal rise in melatonin coincides with the physiological rest period resulting in increased sleepiness, decreased body temperature and locomotor activity, and increase in the immune responses (van den Heuvel *et al*., 2005). By contrast, in nocturnal species, the stable endogenous signal of nocturnal melatonin is associated with wakefulness causing increased locomotor activity, body temperature and decreased sleepiness. Endogenous melatonin acts differentially to promote behaviors associated with night, in other words, resting in diurnal species and activity in nocturnal species (Mendelson *et al*., 1980; Vivanco *et al*., 2007). Studies on the rate limiting enzyme arylalkylamine N-acetyltransferase (AANAT) have shown that the post transcriptional / translational regulation play a role in modulating diurnal behavioral pattern in rodents (Lee *et al*., 2009). Studies show that phylogenetic position of an organism, not their diurnality or nocturnality of behavior, determines regulatory mechanisms of melatonin synthesis (Garidou *et al*., 2002). In *F. palmarum* 5-MTOH levels were higher during on-set of dark and minimum during mid-subjective day in the pineal, however in humans the 5-MTOH have been shown to have maximum levels during day and minimum during the night in the pineal (Hofman *et al*., 1995). In humans, the serotoninergic system plays an important role in neuroendocrine control of reproductive hormone secretion such as the daily pulsatility of GnRH a function essential for reproductive cycle processes (Comai *et al*., 2010). Though the role of all indole metabolites have not yet been elucidated, they could be contributing to the extensive network of serotonergic system throughout the nervous system that mediate a wide variety of physiological functions and may play an important role in behavior, depression, mood regulation, cognitive function, anxiety, sleep, appetite, sexual function, blood flow to the brain, and many other complex functions in mammals that are under the regulation of the biological clock (Sa´nchez *et al*., 2008).

(d). Clock gene expression studies: *per1, per2, cry1, cry2* and *bmal1* gene expression

The expression of circadian clock genes *per1*, *per 2*, *cry1* and *cry2* in the SCN of *F. palmarum* showed a daily pattern with maximum levels at ZT-0 and minimum levels at ZT-18 respectively. The *bmal 1* gene expression also showed daily rhythmicity with maximum levels at ZT-18 and minimum levels at ZT-0 respectively. Similar daily rhythms of gene

expression have been reported in other diurnal rodents. In *A. niloticus*, the *per1* expression was maximum at ZT-6 and minimum at ZT-18 while the *per2* expression was maximum at ZT-10 and minimum at ZT-24/0 (Lambert *et al*., 2005; Ramanathan *et al*., 2006). Studies in *A. ansorgei* also show a similar per1 and *per2* expression pattern with maximum levels at CT-4 and 8 respectively during the light phase and minimum levels at CT-16 and 18 respectively during the dark phase. The *bmal1* gene expression in *A. ansorgei* was maximum between CT-12 to CT-18 and minimum between CT-20 to CT-8. The *cry 2* expression also exhibited daily rhythmicity with peak expression levels during the light phase in *A. ansorgei* (Caldelas *et al*., 2003). In the diurnal *Spermophilus tridecemlineatus* also both *per1* and *per2* showed maximum levels at ZT-5 and minimum level at ZT-13 for *per1* and ZT-17 for *per2* respectively (Mrosovsky *et al*., 2001). Studies in the diurnal sheep also showed a similar pattern of expression with *per l* and *per 2* showing maximum at ZT-3 and ZT-7; *cry l* and *cry 2* expressions peaking at ZT-11 and *bmal 1* expression peaking at ZT-19 respectively (Lincoln *et al*., 2002). This daily rhythmicity of clock gene expression is similar to that reported in several nocturnal mammals such as mouse, rat and hamsters. In mouse the *per1* mRNA expression in the SCN peaks between CT-4 and 6 hours (Shearman *et al*., 1997; Oster *et al*., 2003), the *per 2* mRNA peaks at CT-10 (Shearman *et al*., 1997; Oster *et al*., 2003), the *cry*1 mRNA peaks at CT-10 (Shearman *et al*., 2000; Oster *et al*., 2003) and the *cry* 2 mRNA is expressed without a clear defined rhythm (Kume *et al*., 1999). The temporal patterns of circadian clock genes expression in the SCN appear to be similar in both nocturnal and diurnal species. This similarity at the molecular level could attribute to SCN cellular functioning such as reported in daily rhythms of multiple-unit activity (MUA) (Schwartz *et al*., 2004), the rates of glucose utilization (Schwartz, 1991), expression of immediate early gene product Fos which in turn regulates the production of melatonin during the night in both diurnal and nocturnal species (Tournier *et al*., 2003; Lambert *et al*., 2005). However, output pathways from the SCN to other brain regions, that are responsible for complex functions, could be playing a role in manifesting diurnal behavioral patterns (Guilding and Piggins, 2007; Ramanathan *et al*, 2008).

(e). Effect of Photic and Non-photic (restricted feeding) cues on locomotor activity rhythms.

(i). Photic cues

The phase-response curve (PRC) of light in *F. palmarum* was insensitive during most of subjective day and a phase delay was observed during the on-set and off-set of darkness. No phase advance was observed at any other time points. Though the sample size was limited to only 3 animals (n=3) at each time points, and needs to be confirmed with more animals, the PRC for light was in contrast to that reported in other diurnal and nocturnal rodents (Roenneberg and Foster, 1997). The PRC for light of *A. niloticus* showed an inactive zone during most of subjective day, a phase delay region during early subjective night and a phase advance was seen during late subjective night (Refinetti, 2004; Mahoney *et al*., 2001). A similar PRC for light have been reported for *O. degus* with both phase advance and delay (Kas and Edgar, 2000). In the diurnal zebra mouse (*L. barbarus*), a 30 min light pulse of 200 lux revealed both phase delays and advances at CT-11 and 21 respectively with no dead zone (Lahmam *et al*., 2008). The largest phase shifts occurring during the subjective night, with phase delays around dusk and advances around dawn (Mahoney *et al*., 2001). These features indicate that the twilight zones are the main driving force shaping the phase response characteristics of all circadian systems, irrespective of the temporal niche (e.g. day or night active) and independent of the rhythmic function within an organism (e.g. eclosion and activity in the fruitfly) as during twilight zones all light qualities change including their radiance, irradiance and spectral properties (reviewed in Roenneberg and Foster, 1997).

The intensity of the light pulse also plays a role in the form of PRC. In *F. palmarum* a light pulse of 20 minutes could not induce an onset in the locomotor activity; however a 3 hours light pulse induced a phase shift. In *A. niloticus* and *O. degus*, a 20 minutes light pulse was able to induce a phase shift in the onset of locomotor activity (Mahoney *et al*., 2001; Kas and Edgar, 2000). The high intensity of light that these diurnal *F. palmarum* are exposed to in the environment could make them less sensitive to the effects of artificial light. Several studies on diurnal mammals show that the circadian pacemaker being less sensitive to the phase-resetting or non-parametric effects of light than those of nocturnal mammals (DeCoursey, 1972; Pohl, 1982; 1983; Meijer *et al*., 1989). The PRC of the diurnal antelope

ground squirrel, *A. leucurus,* shows phase shifts in response to 6-h light pulses but were not affected by 1-h light pulses, even at an intensity of 500 lux (Pohl, 1983). Similarly, in the diurnal primate, *Saimiri sciureus* (squirrel monkey), exposure to 1-h light pulses at 600 lux could induce clear phase shifts (Hoban and Sulzman, 1985). However, in hamster's 1-h light pulses at 1 lux induced phase shifts (Boulos *et al*., 1982). The phase shifts obtained in diurnal rodents are comparable to that in nocturnal rodents where clear phase shifts in response to 5-15 minutes of light pulses at much lower intensity levels have been reported (Nelson and Takahashi, 1991).

(ii). Restricted feeding

Restricted feeding (RF) is a major non-photic cue that entrains the SCN in mammals. In our study, on diurnal *F. palmarum*, the effect of 4 hours restricted food availability induced a single bout of activity in the dark phase of LD, 12:12 conditions. Similar findings in nocturnal mice, when food was provided at times when the animal is normally inactive, induces a bout of locomotor activity anticipating the time of feeding (Mistlberger, 1994; Stephan, 2002). This food-anticipatory activity is driven by a food-entrainable clock synchronized with the SCN (Weinert, 2005). Though the exact mechanism of this anticipation for food in diurnal rodents is not known it could be associated with competition for food in their natural diurnal environment or could not be a stronger zeitgeber as compared to photic cues (Stephan, 2002; Refinetti, 2008). However, in the diurnal *O. degus* subjected to 2 hours restricted access of food have been shown to shift the pattern of running wheel activity and body temperature rhythms. *O. degus* subjected to RF have been shown to induce various chronotypes in the running wheel activity similar to that when these animals are subjected to running wheel with *ad libitum* food (*Vivanco et al*., 2010). Thus RF could be acting as a stress, similar to that in the presence of running wheel, inducing switching in behavioral types. However, in nocturnal rodents RF induces an increase in locomotor activity and adrenal corticosterone secretion 2–3 hours prior to the feeding time (food-anticipatory activity). RF had no entraining (synchronizing) effect on oscillation components of activity rhythm driven by the SCN. The food-anticipatory activity was not affected by SCN bilateral lesions, which disrupted the free-running component of circadian behavioral rhythms (Stephan *et al*., 1979). Moreover, the circadian expression rhythms of clock genes in mouse,

such as *per1* and *per2* in the SCN were unchanged by RF (Damiola *et al*., 2000; Hara *et al*., 2001). These findings suggested that RF cannot reset the SCN, and that the food-anticipatory activity is regulated by another oscillator located either within the brain or in the peripheral tissues that entrains to the RF (food-entrainable oscillator) (Abe *et al*., 2007).

F. palmarum could not induce a phase shift under DD when food was restricted for 4 and 12 hours respectively. However, entrainment of the free-running circadian rhythm to RF has been reported in hamsters under particular feeding schedules and in rats under prolonged LL conditions. In Syrian hamsters, RF entrains free-running behavioral rhythm under DD (Abe and Rusak, 1992). RF induces both behavioral rhythmicity and PER 2 protein rhythm in the SCN of rats that are arrhythmic in prolonged housing under LL (Lamont *et al*., 2005). RF studies in CS mice and C57BL/6J mice under DD showed a free running rhythm without significant changes as compared to *ad libitum* conditions (Abe *et al*., 2007). This suggest the possibility that the SCN pacemaker can be reset by an unknown input signal associated with feeding schedule under specific conditions (Abe *et al*., 2007). The site of the food-entrainable oscillator has not been successfully identified. For instance, the ablations of feeding associated brain areas, such as the lateral hypothalamus, ventromedial hypothalamus, and paraventricular nucleus did not eliminate the food-anticipatory activity under RF (reviewed in Stephan, 2002). Cell-specific lesions in the dorsomedial hypothalamic nucleus (DMH) abolished the food-anticipatory activity in rats, suggesting that the neurons of the DMH have a critical role in the expression of food-anticipatory activity (Gooley *et al*., 2006). Lesions of the DMH in rats did not abolish food-anticipatory activity when the behavior was measured more specifically by motion detectors situated overhead and at a food-access window, suggesting that the DMH is most likely situated on the output side of the food-entrainable oscillator critical for anticipatory activity (Landry *et al*., 2006). The food-entrainable oscillator could be a population of oscillating cells in the brain areas outside the SCN or in the peripheral tissues. Genes, which code metabolic factors in peripheral cells, such as neuronal PAS domain protein 2 and retinoid-related orphan receptor have been found to act as clock-related genes involved in the Per and BMAL1 transcription/translation autoregulatory loop, suggesting that these genes play a significant role in food-entrainment in peripheral oscillating cells (Nakajima *et al*., 2004; Rutter *et al*., 2001).

II. To study the effect of aging

(a). Behavior: gross locomotor and running wheel activity

Aging affects the functioning of the biological clock that is characterized by shortening of the circadian period, alteration in the phase angle of entrainment to the light-dark (LD) cycle, fragmentation of the activity rhythm, decreased precision in onset of daily activity, altered rates of re-entrainment following a shift in the LD cycle, and alterations in the response to the phase-shifting effects of light and non-photic stimuli in both diurnal human and nocturnal rodents (Penev *et al*., 1998; Kolker *et al*., 2003). Age-related changes in circadian rhythms have been well documented in several nocturnal species such as mice, rat and hamsters. However, limited reports are available on the effect of aging on diurnal rodents. In our study on the diurnal *F. palmarum*, age associated decrease in robustness of both gross locomotor activity and running wheel activity were observed under LD cycle (Figure 61). The mean activity appeared decreased upon aging with a shift towards the onset of darkness. However the mean activity of the running wheel activity was increased as compared to adults but retaining a diurnal pattern. The gross and the running wheel activity showed a decrease in percentage diurnality under LD and DD conditions. There was no effect on the onset of activity however an increase in fragmentation of activity across the 24-h day were observed. The fragmentation of circadian locomotor behavior in hamsters has been linked with impaired performance on memory task (Antoniadis *et al*., 2000). Delay in activity and variability in the onset of activity have been reported in aged mice (Valentinuzzi *et al*., 1997; Kolker *et al*., 2003). Free-access to a running wheel shortens the period of free-running rhythm in blinded nocturnal rats (Tamada *et al*., 1988). Studies in aged nocturnal hamsters show less sensitivity to photic inputs and delay to adjust to the phase of daily rhythms generated by the pacemaker (Zhang *et al*., 1996). In LL conditions the mean activity of gross locomotor activity of diurnal *F. palmarum* were doubled as compared to adults with even distribution during the light and dark phase (Figure 63). However the mean levels of running wheel activity showed a diurnal preference with higher mean levels as compared to adults. Splitting was not exhibited by aged *F. palmarum* under constant light condition in both gross locomotor activity and running wheel activity as compared to adults where a robust splitting was observed in the running wheel activity. This shows that aging might not be a strong factor to induce splitting in these diurnal *F. palmarum*. However, in nocturnal

hamsters aging reduced the incidence of spontaneous and dark-pulse induced splitting of the circadian rhythm (Duncan and Deveraux, 2000; McAuley *et al*., 2002). Age associated increase in splitting was however also reported in SAMP8 mice, a mutant nocturnal rodent under constant dark conditions as compared to adults (McAuley *et al*., 2002). Age associated physiological changes that animals undergo in the wild are harmful to their survival especially in the day active animals. In the natural environment diurnal tree shrew (*Crocidura russula*) decrease their body temperature and slow down their heart and respiratory rates along with other fundamental physiological functions, to allow healthy adults to reduce their energy expenditure during daylight hours (Fons *et al*., 1997). But as the circadian rhythms begin to dephase upon aging, animals lose their ability to increase their daily energy expenditure and thus exposing them to a higher probability of predation. However, when these diurnal tree shrews (*Crocidura russula*) were maintained in captivity they exhibit a longer senescent phase by switching from their expected lifespan, determined by extrinsic factors in the wild, to an intrinsic longer lifespan. The mechanisms as to how this is achieved are not yet known. Similar mechanisms might be operating in these diurnal Palm Squirrels (*F. palmarum*), that might be responsible for stable locomotor activity patterns in the 30 months old animals as compared to adults. However the time period as to how long *F. palmarum* survive in the wild and laboratory conditions is not yet known. Studies on the effect of aging on diurnal animal could provide a portal through which the appearance of senescence and the loss of daily activity rhythm can be elucidated.

(b). Neurotransmitters:

(i). AVP-ir and VIP-ir daily rhythmicity

Our study on aged diurnal *F. palmarum* showed daily rhythmicity in VIP where as an abolition of AVP rhythmicity (Figure 72). The levels of VIP and AVP were decreased with 12 hours phase reversal in the peak levels as compared to adults. No reports of the effect of aging on VIP and AVP-ir are available on diurnal rodents; however reports of decrease in levels of immunoreactivity and cell number have been reported in nocturnal rat SCN (Krajnak *et al*., 1998). In the diurnal humans, marked day-night oscillation in the number of AVP-ir neurons was reported in the SCN of young individuals, whereas such oscillation seemed to be reduced and shifted by aging (Hofman and Swaab, 1994). In both rats and

humans, AVP rhythms in the SCN have been shown to deteriorate with advancing age (van der Zee *et al*., 1999). Studies in AVP-deficient nocturnal Brattleboro rat show that the circadian organization of running wheel activity was not affected but the generation of locomotor activity was reduced. In *F. palmarum* also the locomotor activity was reduced with a decrease in the levels of AVP as well as a loss of rhythmicity. The reduction in locomotor activity precision could be due to reduced SCN functioning and not to failure of expression of locomotor activity itself upon aging (van der Zee *et al*., 1999). In humans, age associated decrease in the VIP levels and cell number have been reported in the SCN as compared to young individuals (Zhou *et al*., 1995; Hofman *et al*., 1996). Similar findings of decreased VIP levels as well as rhythmicity were obtained in our study on *F. palmarum*. Aging also decreased SCN expression of GRP mRNA which along with the decreases VIP mRNA during aging may contribute to age-related loss of sensitivity to photic phase shifting signals (Kolker *et al*., 2003).

Decrease in the levels of neuropeptides content in the SCN due to aging could be attributed to loss of robustness of locomotor activity in our study on *F. palmarum* (Figure 72). Decreased expression of VIP in the SCN of nocturnal rats upon aging has been shown to contribute to age related deficits in circadian rhythm amplitude and coupling. Aging decreases SCN expression of VIP mRNA in the nocturnal hamster and rat SCN (Krajnak *et al*., 1998; Duncan *et al*., 2001) and reduces the number of VIP-immunoreactive cells in both rat and human SCN (Pereira *et al*., 2005). In nocturnal mice, genetic deletion of either VIP or its receptor VPAC2 disrupts circadian activity rhythms during exposure to constant darkness, as well as the circadian rhythms of firing rate of SCN neurons *in-vitro* (Aton *et al*., 2005). The circadian rhythms in the SCN of mutant mice show that rhythms of VIP mRNA are characterized by lower amplitude and expression of more than one period length, suggesting desynchrony among multiple circadian oscillators (Harmar *et al*., 2003; Aton *et al*., 2005). These studies suggest that VIP plays an important role in oscillator coupling and a reduction in its levels upon aging could enhance the uncoupling process in the SCN. Microinjections of VIP into the hamster SCN induce photic-like phase shifts (Piggins *et al*., 1995). Thus, VIP acting through VPAC2 receptors participates in light-induced phase shifts and is necessary for consolidated high-amplitude circadian rhythms. VIP is also an important SCN output signal regulating circadian endocrine rhythms, such as the glucocorticoid rhythm and the

preovulatory luteinizing hormone surge, which exhibit attenuation with aging (Gerhold *et al*., 2005; Loh *et al*., 2008). Therefore, age-related decreases in VIP mRNA expression probably contribute to age-related deficits in photic phase shifts as well as age-related attenuation of rhythm amplitude and perhaps fragmentation or loss of synchrony of rhythms (Duncan *et al*., 2010). Reports on the effect of aging have shown that it does not alter the size of the SCN or the number of cells (Madeira *et al*., 1995); and thus, the changes in circadian rhythmicity associated with aging cannot be explained by a process such as cell death or atrophy. Age related changes observed in the behavior and physiology of the whole animal could be due to changes in SCN neural function as that seen in the amplitude of the daily rhythm of glucose utilization which is decreased in the SCN of old rats (Wise *et al*., 1988). Furthermore, the daily rhythm of the neural firing rate of the SCN maintained *in-vitro* is also dampened in old rats (Satinoff *et al*., 1993). This suggests that age-related changes in the interactions among cells within the SCN and other brain regions could be affected (Krajnak *et al*., 1998) in both diurnal and nocturnal rodents.

(ii). Serotonin chronometabolome daily rhythmicity in the SCN

Serotonin metabolome showed an age associated decrease in the levels of all compounds in the SCN and Pineal of *F. palmarum*, in the present study (Figure 73 and 74). All compounds showed daily rhythmicity in the SCN and pineal except for 5-HTP in the former and 5-MIAA in the latter upon aging. Though the exact role of indole metabolites in mediating diurnality has not yet been elucidated but they have been shown to be affected upon aging. Age associated decrease in levels of 5-HT have been reported form our laboratory, both in the SCN and Pineal of nocturnal rat (Jagota and Kalyani, 2007; Jagota and Kalyani, 2010). Age associated decrease in melatonin levels have been reported in several studies in both diurnal humans and nocturnal laboratory animals (Jagota, 2005). In humans a decrease in levels as well as a loss of sensitivity to melatonin upon aging has been shown to be responsible for disruption of circadian rhythms and sleep (Cagnacci *et al*., 1995). Age associated decrease in levels of indole metabolites could be due to either a reduced availability or a reduction in the uptake of tryptophan. Age associated decrease in serotonin and melatonin levels have been shown to be due to reduced N-acetyl transferase activity, the key enzyme for pineal-melatonin synthesis in rats (Touitou, 2001). The rate of pineal-

melatonin synthesis is dependent on the serotonin (5-HT) levels in the pinealocytes and studies using drugs which increase it also stimulate melatonin synthesis (Huether, 1993). Inhibition of 5-HT synthesis by parachlorophenylalanine (PCPA) also inhibited melatonin production similar to that in aging conditions in rats (Miquez *et al*., 1997). Studies in rat show that increased tryptophan intake raised the availability of brain tryptophan levels and consequently the 5-HT levels (Huether, 1993; Esteban *et al*., 2004), which during the night, when the synthesis of melatonin is activated; the increased tryptophan levels can stimulate melatonin production (Hajak *et al*., 1991; Esteban *et al*., 2004). Thus upon aging the melatonin fall could be attributed due to 5-HT deficiencies. Also upon aging the blood- brain barrier shows a reduced permeability to tryptophan in rats (Tang and Melethil, 1995). Aging also caused an increase in the downstream enzymatic activity such as the kynurenine pathway which activates the metabolism of tryptophan thus causing a reduction in the plasma levels of tryptophan in humans (Frick *et al*., 2004) and aged rats (Comai *et al*., 2005). However, studies of external administration of melatonin in diurnal trees shrews and humans have shown to combat the effects of senescence and thus prolong the aging process (Magnanou *et al*., 2009).

(c). Clock gene expression: *per1, per2, cry1, cry2* and *bmal1* gene expression

In the SCN of *F. palmarum*, *per 1, cry1* and *bmal 1* gene showed daily rhythmicity where as *per2* and *cry2* showing abolished rhythmicity upon aging. The levels of *per1*, *per2*, *cry1*, *cry2* and *bmal1* were decreased upon aging (Figure 75). Upon aging cells undergo alterations in various activities including DNA damage signaling and cell stress response as well as in their energy and glucose metabolism (Oh *et al*., 2007). In addition, a number of recent studies on tissues from aged animals show that the expression of several genes involved in cellular activities are either upregulated or downregulated during aging (Kayo *et al*., 2001). In our study, the expression levels of circadian genes were reduced and could be associated with decreased AVP, VIP and serotonin levels. Studies in the nocturnal hamster have shown age related decline in *bmal1* levels without corresponding declines in either *per 1* or *per 2* levels (Kolker *et al*., 2003). Similarly in the nocturnal rat SCN no difference in either *per1* or *per2* expression were reported upon aging as compared to young rats but there was a slight decrease in *cry1* expression (Asai *et al*., 2001). In mice, aging has been found to

diminish the amplitude of *per2* but not *per1* expression (Weinert *et al*., 2001). Thus, aging showed differential expression of clock genes in several species and appears to be independent of whether the animal is diurnal or nocturnal. As the molecular mechanism of circadian rhythm generation involves various stages, alterations in the mRNA's and protein levels, changes in transcriptional or translational regulation (Roy *et al*., 2002), mRNA stability (Brewer, 2002) and proteasome-mediated protein degradation (Goto *et al*., 2001) could be affected by aging process. Studies in the *per1* and *cry2* knock out mice show specific alterations in the regulation of the circadian core oscillator in an age-related manner. The levels of *mper2* and *bmal1* mRNA levels are strongly reduced in the SCN and in the kidney of old *per1-/-* and *cry2 -/-* mice. Additionally, CRY1 protein levels are elevated, indicating an impaired degradation of mCRY1 protein upon aging. However, *cry1* mRNA cycling is not affected in contrast to *per2* and *bmal1* transcripts, indicating that the regulation of *cry1* differs from that of *per2* and *bmal1*. *per1-/-* and *cry2-/-* mice display not only altered gene expression of core clock components but also altered the expression of clock output gene that regulate AVP levels. This indicates that physiological pathways influenced by AVP are affected in these aging mice (Oster *et al*., 2003). This is similar to our study where an abolition of *per2* rhythmicity could be associated with a loss of AVP rhythmicity upon aging in *F. palmarum*. However there was no effect on the behavioral pattern of locomotor activity in these animals.

There are several possible explanations for the differential effects of aging on the robustness of neuronal and molecular rhythms of the SCN. Age-related changes in electrical activity due to alterations in the amplitude and regulation of molecular components of the clock other than *per1* or *per2* could be a reason. Other molecular components of the biological clock of SCN may be more profoundly affected by aging, and their behavior may contribute to the changes in the electrical and chemical rhythm (Asai *et al*., 2001). Another possibility is that aging acts ''downstream'' of the molecular feedback loop, either on clock-regulated gene expression or directly on the biochemical and biophysical events involved in the production and expression of the electrical impulse rhythm. However, it is not clear how either the molecular or the electrical events within the SCN are coupled to behavioral or physiological outputs and gets affected upon aging (Silver *et al*., 1996; Kramer *et al*., 2001. The effects of aging on behavioral and physiological rhythmicity are likely to be complex

and could result due to changes at several levels of circadian organization in both diurnal and nocturnal species (Yamazaki *et al*., 2002).

Summary and Conclusion

The circadian timing system is the primary neural site for the generation and synchronization of circadian rhythms in mammals. The CTS ensures maximum survivability of mammals in their respective temporal niches and thus differentiates their behavior into diurnal, nocturnal and crepuscular types. Diurnal and nocturnal species are profoundly different in terms of the temporal organization of daily rhythms in physiology and behavior; however the neural basis for these divergent patterns are at present unknown. In this study we have used the three striped South Indian Palm Squirrel, *F. palmarum*, to understand the functioning of diurnal CTS. *F. palmarum* exhibited a robust diurnal pattern in both gross and running wheel activity rhythms under LD and a free running pattern in DD and LL photoperiodic conditions. All individuals showed a similar diurnal pattern of locomotor activity with no inter-individual variations. However, the running wheel activity exhibited a splitting in the locomotor activity under constant light conditions. The neuro-anatomy of the optic nerves, pineal and the SCN showed distinct features specific to diurnal species. The neuropeptides VIP and AVP showed a core and shell pattern of distribution in the SCN. VIP showed rhythmicity under LD, DD and LL photoperiodic conditions. AVP showed rhythmicity only in LD and the rhythmicity was abolished in DD and LL. Interestingly the rhythmic pattern of both VIP and AVP show distinct patterns which could be related to diurnal mechanism in the functioning of SCN. Further study of the serotonin chronometabolome however showed rhythmicity patterns that were similar to nocturnal rodents in both the SCN and Pineal. At the molecular level the clock genes *per1*, *per2*, *cry1*, *cry2* and *bmal1* showed a temporal pattern of expression that is similar to nocturnal species. Thus, indicating that diurnality may not be just a reversal of nocturnality but an extended functionality.

Studies on the effect of aging on *F. palmarum* showed a decreased robustness, increased fragmentation and decreased percentage diurnality in both gross locomotor as well as running wheel activity. All animals retained the diurnal pattern of locomotor activity without inter-individual variations. Age associated changes in the functioning of the SCN were evident at the neuropeptide, neurotransmitter and clock gene expression levels. VIP levels showed daily rhythmicity however AVP daily rhythmicity was abolished upon aging in the SCN with decreased levels in both AVP and VIP. Aging caused changes in the maximum: minimum levels as well as decreased daily mean levels of serotonin chronometabolome in the SCN and Pineal. In the SCN the maximum : minimum levels of 5-HIAA were highest as compared to 3 month old adult where it was highest for 5-HTP. The daily mean levels of TRP were higher in the SCN of

aged animals as compared 3 month old adults where the daily mean levels of 5-HIAA were highest. In the pineal, the maximum : minimum levels of 5-MIAA were highest as compared 3 months old adult where it was highest for TRP while the daily mean levels of TRP were higher in aged animals as compared to 5-HTP in the 3 months old pineal. The *per 1*, *cry1* and *bmal1* showed daily rhythmicity while *per2* and *cry2* rhythmicity were abolished in the SCN of aged animals. The levels of all the genes studied showed a significant decrease in their level as compared to adults. Thus upon aging a reduction in the neuronal functioning of the CTS at the cellular level contributes to the multiple effect on the physiology, however maintaining the diurnal behavioral trait of these rodents (Figure 74).

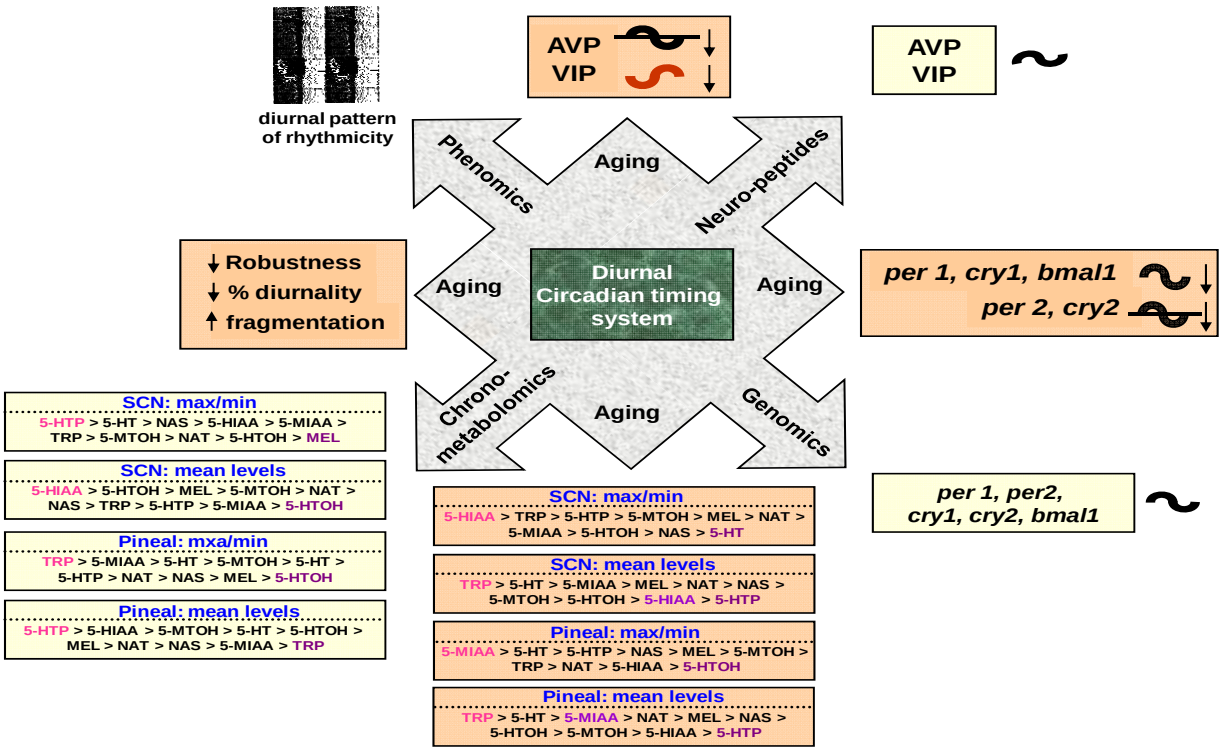


Figure 74. Diagrammatic representation of the diurnal circadian timing system and age induced changes in the South Indian Palm Squirrel (*F. palmarum*) on the phenomics, neuropeptide, neurotransmitters, chronometabolome and genomics.

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Figure 32. Representative double plotted actograms of running wheel activity for 100 days showing activity in under various photoperiods LD, DD and LL conditions. A. In LD, a robust activity during the light phase and less activity during the dark phase; B. In DD, a free running activity with τ of 24.11 ± 0.03 hours; C. Under LL, a free running pattern with τ of 24.92 ± 0.35 hours were observed; D. Mean activity profiles under LD (n=12), DD (n=6) and LL (n=6) conditions.

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Figure 34. A. Representative double plotted actograms of running wheel activity for 100 days-under DD condition. B (1-6). Actograms and their respective activity profiles of 6 individual animals under DD condition. C. Mean activity profile of 6 animals showing maximum activity during subjective light phase of DD. Each value is mean ± S.E. (n=6). The dashed lines on the activity profile indicates subjective lights on and off respectively.

Figure 35. A. Representative double plotted actograms of running wheel activity for 100 days under LL condition showing splitting pattern of activity. B (1-6). Actograms and their respective activity profiles of 6 individual animals under LL condition. All 6 animals showed splitting. C. Mean activity profile of 6 animals.

Figure 36. Representative double plotted actograms of running wheel activity for 100 days under various photoperiods LD, DD and LL conditions. A. In LD, a robust activity during the light phase and less activity during the dark phase; B. In DD, a free running activity with τ of 24.11 ± 0.03 hours; C. Under LL, a free running pattern with τ of 24.92 ± 0.35 hours were observed; D. Mean activity profiles under LD (n=12), DD (n=6) and LL (n=6) conditions.

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Figure 64. A. Representative double plotted actograms of running wheel activity for 100 days of 2.5 year old *F. palmarum* under LL condition showing splitting pattern of activity. B (1-6). Actograms and their respective activity profiles of 6 individual animals under LL condition. All 6 animals showed splitting. C. Mean activity profile of 6 animals. Each value is mean ± S.E. (n=6). The dashed lines on the activity profile indicates subjective lights on and off for animals under LD 12:12 condition respectively.

Figure 65. Representative double plotted actograms of running wheel activity for 100 days of 2.5 year old *F. palmarum* under various photoperiods LD, DD and LL conditions. A. In LD, a robust activity during the light phase and less activity during the dark phase; B. In DD, a free running activity with τ of 24.14 ± 0.12 hours; C. Under LL, a free running pattern with τ of 24.16 ± 0.15 hours were observed; D. Mean activity profiles under LD (n=12), DD (n=6) and LL (n=6) conditions. Each value is mean ± S.E. (n=6). The dashed lines on the activity profile indicates lights on and off for animals under LD 12:12 condition respectively.

Figure 66. Comparison of representative double plotted actograms of gross locomotor activity between 90 day old (Ai, Bi and Ci) and 2.5 year old (Aii. Bii and Cii) *F.palmarum* under LD (A), DD (B) and LL (C) conditions. Mean activity profiles of 90 day old (D) and 2.5 year old (E) under LD (Di, Ei), DD (Dii, Eii) and LL (Diii, Eiii) represented as mean (dark pink) along with their standard error (red). Each value is mean ± S.E. (n=6). The dashed lines on the activity profile indicates subjective lights on and off for animals under LD 12:12 condition respectively.

Figure 67. Comparison of representative double plotted actograms of running wheel activity between 90 day old (Ai, Bi and Ci) and 2.5 year old (Aii. Bii and Cii) *F.palmarum* under LD (A), DD (B) and LL (C) conditions. Mean activity profiles of 90 day old (D) and 2.5 year old (E) under LD (Di, Ei), DD (Dii, Eii) and LL (Diii, Eiii) represented as mean (dark pink) along with their standard error (red). Each value is mean ± S.E. (n=6). The dashed lines on the activity profile indicates subjective lights on and off for animals under LD 12:12 condition respectively.

Figure 68. Effect of aging on % diurnality (A), τ (B) and α (C) under LD, DD and LL conditions. Gross locomotor activity showed significant decrease in % diurnality in LD and DD as compared to 90 day adult ($pa < 0.05$). Running wheel activity showed significant decrease % diurnality in LD ($pa < 0.05$) and an increase in LL ($pa < 0.05$). τ and α showed no age induced changes in both gross locomotor activity and running wheel activity ($pa < 0.05$). $pa < 0.05$; where *a* refers to the comparison between 90 day old and 2.5 years old age groups. Each value is mean ± S.E. (n=6).

Figure 69. Effect of aging on daily rhythm on VIP (A) and AVP (B) in the SCN of F. palmarum. Densitometry analysis showing VIP-ir daily rhythmicity in its levels (n=4) ($p<0.05$) with maximum levels at ZT-18 and minimum levels at ZT-12, and phase shift of 12 hours in the maximum levels (Aii). AVP-ir levels were significantly decreased with abolition of rhythmicity in the aged SCN as compared to adults ($p<0.05$) (Bii). Table showing the comparison of max: min ratio of VIP-ir (Aiii) and VIP-ir (Biii). Each value is mean ± S.E. (n=4).

Figure 70. Comparison of daily rhythms of 90 day old and 2.5 year old serotonin and its related compounds in the SCN of *F. palmarum.* Each value is mean ± S.E. (n=6). $p_a < 0.05$; $p_b < 0.05$; $p_c < 0.05$ and $p_d < 0.05$ (where a refers to comparison of ZT-0 with ZT-6,12 and 18; b refers to comparison of ZT6 with ZT-0,12 and 18; c refers to comparison of ZT-12

with ZT-0,6 and 18 and d refers to comparison of ZT-18 with ZT-0, ZT-6 and ZT-12 respectively).

Figure 71. Comparison of daily rhythms of 90 day old and 2.5 year old serotonin and its related compounds in the Pineal of *F. palmarum.* $p_a < 0.05$; $p_b < 0.05$; $p_c < 0.05$ and $p_d < 0.05$ (where a refers to comparison of ZT-0 with ZT-6,12 and 18; b refers to comparison of ZT6 with ZT-0,12 and 18; c refers to comparison of ZT-12 with ZT-0,6 and 18 and d refers to comparison of ZT-18 with ZT-0, ZT-6 and ZT-12 respectively).

Figure 72. Comparison of mean and daily pulse in the SCN (A) and Pineal (B) of 90 day and 2.5 year old *F. palmarum.* Each value is Mean ± S.E (n=6).* comparison between 90 day old and 2.5 year old showing significant difference ($p<0.05$).

Figure 73. Effect of aging on daily rhythms of *per1*, *per2*, *cry1, cry2* and *bmal1* expression in the SCN. A. Representative gel showing the amplicons of *per1*, *per2*, *cry1*, *cry2*, *bmal1* and *g3phd* in the SCN of 2.5 year old *F. palmarum* (n=4). B. Densitometric analysis showing the comparison of *per1*, *per2*, *cry1, cry2* and *bmal1* in the SCN of 90 day old and 2.5 year old *F. palmarum*. * represents one way anova showing significant difference between lowest level of ZT with other ZT's within an age group ($p<0.05$).

Figure 74. Diagrammatic representation of the diurnal circadian timing system and age induced changes in the South Indian Palm Squirrel (*F. palmarum*) on the phenomics, neuropeptide, neurotransmitters, chronometabolome and genomics.

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
Amp	:	Ampere
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
GPCRs	:	G protein-coupled receptors
GRP	:	Gastrin releasing peptide
IP3	:	Inositol Phosphate 3
ISAAC	:	Insitu Ag/AgCl electrode
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
NGF	:	Nerve Growth Factor
NPY	:	Neuropeptide Y
PCR	:	Polymerase chain reaction

Per	:	*Period*
PHI	:	Peptide histidine isoleucine
PRC	:	Phase response curve
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
SAD	:	Seasonal Affective Disorder
SCG	:	Superior cervical ganglion
SCN	:	Suprachiasmatic nucleus
SS	:	Somatostatin
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

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BRAIN RESEARCH

Research Report

Immunocytochemical evidence for different patterns in daily rhythms of VIP and AVP peptides in the suprachiasmatic nucleus of diurnal *Funambulus palmarum*

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ABSTRACT

The suprachiasmatic nucleus (SCN) is the principal pacemaker that coordinates circadian rhythmicity in mammals. The studies on understanding the circadian system in diurnal rodents are limited. In this study, we have used the 3 striped South Indian Palm Squirrel (*Funambulus palmarum*). The locomotor activity showed a diurnal pattern of activity in LD 12:12, constant darkness (DD) and light (LL) conditions with circadian periods (τ) of 24.19±0.1, 24.11±0.03 and 24.92±0.35 h respectively. Anatomical study of the brain revealed that this animal had short, thick and stout optic nerves with SCN elliptical in shape with a higher neuronal population as distinct from nocturnal rodents. Since the neuropeptides, vasoactive intestinal polypeptide (VIP) and arginine vasopressin (AVP) play important roles in photic entrainment and relay of information respectively in nocturnal rodents, we studied the distribution and daily rhythms of VIP-ir and AVP-ir in squirrel SCN. The VIP-ir and AVP-ir cells in the SCN showed a ventrolateral and dorsomedial distribution with daily rhythmicity in their levels. The peak time of VIP-ir rhythm was found ahead of AVP-ir. The VIP-ir levels were higher for longer duration than AVP-ir levels. The maximum and minimum VIP-ir levels were at ZT-6 and ZT-0 respectively and AVP-ir levels at ZT-12 and ZT-0 respectively. Thus, VIP and AVP maximum and minimum levels appeared 6 and 12 h apart respectively in squirrel, though 12 and 8 h apart in rat. These findings in the present report could be a step towards underpinning the mechanisms regulating diurnality.

1. Introduction

Mammals show circadian rhythms in physiology and behaviour that are regulated by the hypothalamic suprachiasmatic nucleus (SCN) (Klein et al., 1991; Jagota et al., 2000; van Esseveldt et al., 2000; Morin and Allen, 2005). Animals are adapted to entrain their behaviour to environmental zeitgeber, mainly the external light dark (LD) cycle. Based on the proportion of their activity during the day, night or twilight, the behavioural pattern of animals are manifested as diurnal, nocturnal or crepuscular respectively (Schumann et al., 2005). The SCN is a bilaterally symmetrical collection of tightly packed cells, located immediately above the optic chiasm on either side of the third ventricle (Klein et al., 1991). The SCN of both diurnal and nocturnal

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rodents show similarity in various aspects such as daily rhythms in multiple-unit activity (MUA) (Schwartz et al., 2004), the rates of glucose utilization (Schwartz, 1991), and expression of immediate early gene product Fos (Smale and Boverhof, 1999; Novak et al., 2000). However, nocturnal and diurnal species typically differ with respect to the temporal organization of a wide range of physiological and behavioural functions including locomotor activity, copulatory behaviour, parturition, body temperature and sleep (Challet, 2007). A recent approach is to understand the functioning of SCN in diurnal species (Refinetti, 2008; Smale et al., 2008; Cuesta et al., 2009).

The cytoarchitecture of SCN has been characterized in several nocturnal species (Cohen et al., 2010; Dibner et al., 2010; Welsh et al., 2010). Various diurnal rodent models such as the Asian Chipmunk (*Eutamias asiaticus*) (Honma and Honma, 1999), unstriped Nile Grass Rat (*Arvicanthis niloticus*) (Katona and Smale, 1997; Mahoney et al., 2000) as well as *Arvicanthis ansorgei* (Challet et al., 2002), European Ground Squirrel (*Spermophilus citellus*) (Hut et al., 1999), 5 striped North Indian Palm Squirrel (*Funambulus pennanti*) (Rajaratnam and Redman, 1999), Golden-mantled Ground Squirrel (*Spermophilus lateralis*) (Mrosovsky et al., 2001), Degus (*Octodon degus*) (García-Allegue et al., 1999; Lee, 2004), four striped Field Mouse (Schumann et al., 2005), Mongolian Gerbil (Weinert et al., 2007), etc., have been reported. However, detailed anatomical description of the SCN of most of these diurnal rodents has not been reported.

The mechanism responsible for the differences in nocturnality and diurnality could be present either within the SCN or downstream of it or both (Smale et al., 2008). The SCN mediates the overt rhythms by various neurotransmitters and neuropeptides (Moore and Silver, 1998; Jagota, 2006; Reghunandanan and Reghunandanan, 2006). Anatomically, the SCN is composed of two subdivisions, which are distinguished by the differential concentrations of chemically distinct neurons as well as the terminal arborization of visual afferents (Card and Moore, 1991; van den Pol, 1991; Goel et al., 1999; Smale and Boverhof, 1999). The ventrolateral SCN contains neurons with VIP, gastrin-releasing peptide (GRP) and NPY immunoreactivity (ir) (Shinohara et al., 1993) and receives visual efferents (Ibata et al., 1999). The dorsomedial SCN lacking visual input contains AVP and somatostatin (SS)-ir neurons and efferent pathways thus regulating internal generation of circadian rhythms. Both VIP and AVP peptide and mRNA show daily rhythmicity in their expression in the SCN of several nocturnal rodents under LD 12:12 conditions (Duncan et al., 1995; Isobe and Muramatsu, 1995; Isobe and Nishino, 1998; Dardente et al., 2004) which is influenced by circulating glucocorticoids (Larsen et al., 1994). The role of gap junction communication in synchronization of sub population of the rat SCN has been reported (Shinohara et al., 2000). The studies on rhythmic profiles of VIP and AVP levels in diurnal rodents are limited. In our laboratory using a diurnal rodent, three striped South Indian Palm Squirrel (*Funambulus palmarum*) (Fig. 1) belonging to the suborder *Scuiromorpha* and family *Scuiridae*, we have studied daily locomotor activity rhythms in LD, DD and LL to confirm diurnality. We then studied neuronal architecture of SCN. Since VIP and AVP are important neuropeptides in the SCN of nocturnal rodents in the input and output pathways respectively, we have studied daily VIP and AVP rhythms in SCN of Indian Palm Squirrel.

Fig. 1 – Three striped South Indian Palm Squirel, *Funambulus palmarum*, a diurnal rodent belonging to suborder: Scuiromorpha and family Scuiridae.

2. Results

2.1. *Locomotor activity*

Actograms prepared in LD condition (LD; 12:12) showed diurnal pattern of activity with robust activity seen in the light phase (ZT-0 6:00 A.M. lights on) to ZT-12 (6:00 P.M. lights off) with no activity in the dark phase (ZT-12 to ZT-0/24). The circadian period (τ) appeared 24.19±0.12 h (n=6) and animals showed 70.39±3.88% diurnality (Fig. 2A).

In constant conditions, *F. palmarum* showed free running pattern with τ as 24.11±0.03 (n=6) and 24.92±0.03 (n=6) in DD and LL respectively. The τ in DD was not significantly different from LD; however τ in LL was significantly different from LD as well as DD ($p \leq 0.05$). Activity was anchored to the subjective day with minimum activity in subjective night. On returning to LD, the animals showed entrainment to LD conditions (Figs. 2B and C). The duration of active period (α) in LD, DD and LL was found to be 12.57±0.49, 12.82±0.76 and 16.03±1.03 h respectively. The % diurnality was also found to be different in LD and constant conditions. These were 70.39±3.87, 73.12±7.59 and 55.05±3.81 in LD, DD and LL respectively (Fig. 2D, Table 1). Both α and % diurnality in LL were significantly different from LD and DD ($p \leq 0.05$), however α and % diurnality were not significantly different in LD and DD.

2.2. *Neuroanatomical observations*

The brain of adult *F. palmarum* was almost similar in size and weight as compared to adult rat. The major difference was seen in the optic nerves and location of the pineal gland (Figs. 3 A, B). The optic nerves were short, thick and stout in comparison to those of rats which were long and slender. The optic chiasm was found to be prominent in the squirrel as compared to rat. The eye balls were darker as compared to rat.

2.3. *Immunohistochemical characterization of SCN of Indian Palm Squirrel*

The SCN were discrete, bilateral and elliptical in shape with elongation in the ventro-lateral direction as revealed in both cresyl violet as well as Neu N-ir (Figs. 4A, B). The

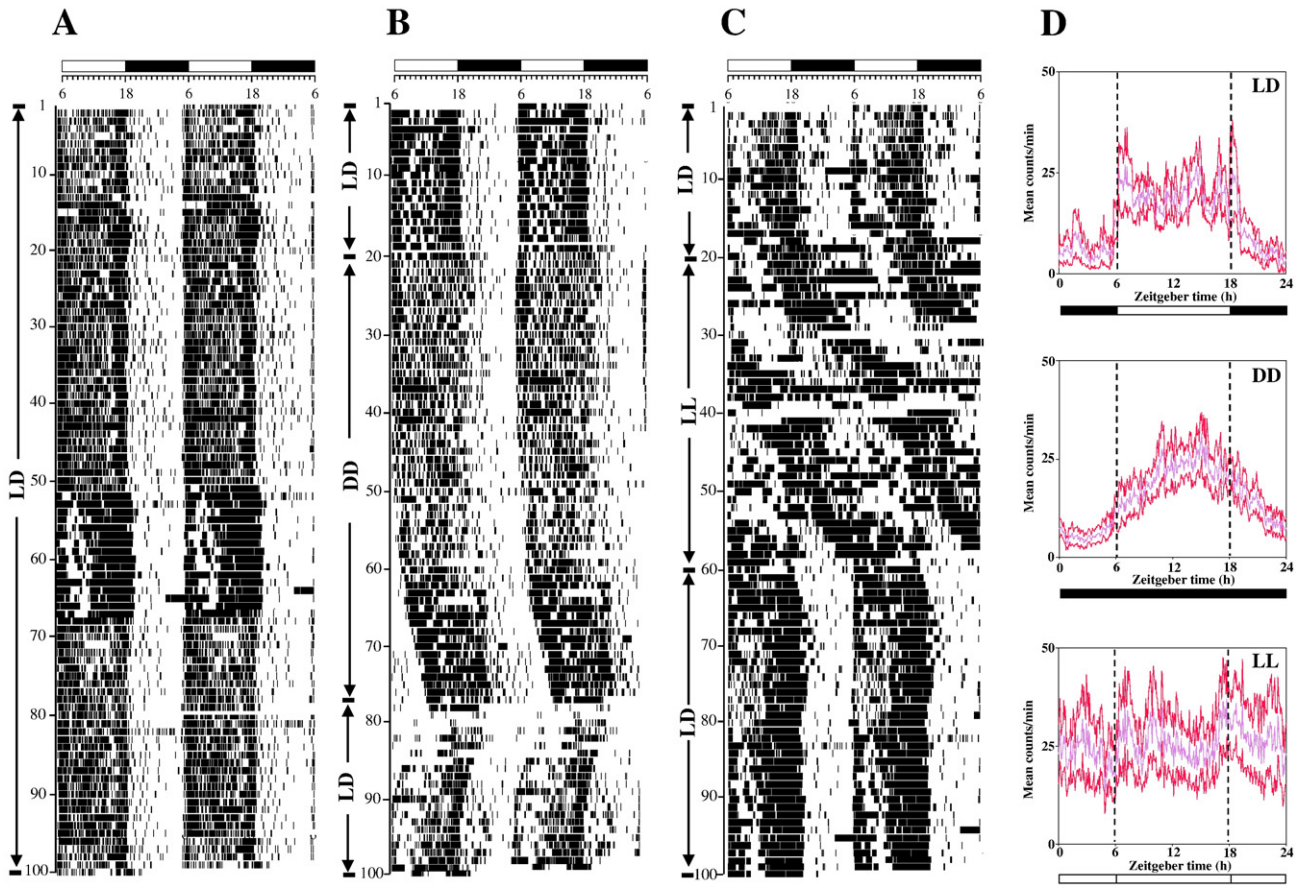

Fig. 2 – Representative Actograms for *F. palmarum* showing double-plotted locomotor activity during A. LD (12:12), B. DD and C. LL. Return from constant conditions to LD (12:12) shows entrainment to LD (B, C). D. Activity profiles of animals in 3 groups LD, DD and LL (n = 6 animals in each group). Each value is mean ± S.E. (n = 6). The dashed lines on the activity profile indicates lights on and off for animals in LD (12:12).

nuclei were found completely separated by the third ventricle throughout the rostrocaudal length. The cells in the SCN were evenly distributed with a heterogeneous population of densely packed neurons of size varying from 3 to 10 μ. The SCN was approximately 300 μm in the rostrocaudal axis, 450 μm in the dorso-ventral axis and 200 μm in the mediolateral axis. The neuronal count in the paired SCN of the Indian Palm Squirrel was 30851 ± 195 (n = 12) with right and left SCN showing 15338 ± 172 and 15513 ± 217 respectively.

Table 1 – Effect of different photoperiods on various parameters in the activity profiles.

Experimental condition	Circadian time (τ) (h)	Duration of activity period (α) (h)	% diurnality
LD	24.19 ± 0.12	12.57 ± 0.49	70.39 ± 3.88
DD	24.11 ± 0.03	12.82 ± 0.76	73.13 ± 7.59
LL	24.92 ± 0.35*	16.03 ± 1.03*	55.06 ± 3.82*

Each value is mean ± S.E. (n = 6).
*Significantly different from both LD and DD ($p \leq 0.05$). No significant difference was found in τ, α as well as % diurnality between LD and DD.

2.4. *Daily rhythms of VIP-ir and AVP-ir*

The cell bodies with VIP-ir were abundant and appeared more in the ventrolateral region of the SCN in close proximity to the optic chiasm. However, VIP-ir fibres appeared more in the dorsal region. VIP-ir showed daily rhythm with maximum and minimum at ZT-6 and ZT-0 respectively (Figs. 5A, B). Interestingly

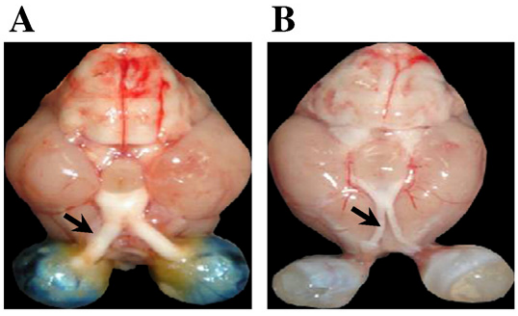

Fig. 3 – Comparison of ventral view of brain of A. 3 striped South Indian Palm Squirrel showing short, stout and thick optic nerves B. compared to rat showing long and slender optic nerves.

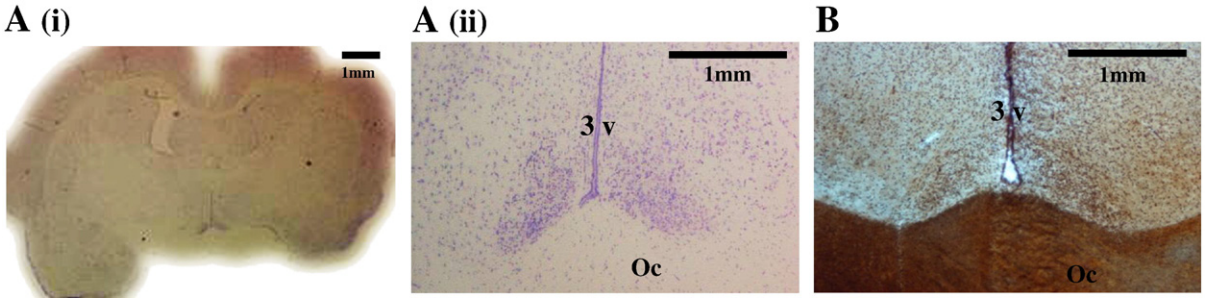

Fig. 4 – Suprachiasmatic nucleus (SCN) of *F. palmarum* showing cresyl violet stain (A i and ii) and neuron specific NeuN immunoreactivity (B i and ii) in coronal brain sections. The SCN is elliptical in shape with two nuclei separated by the third ventricle. Oc — optic chiasm; 3v — third ventricle. Scale bar = 1 mm.

VIP-ir level at ZT-12 was neither significantly lower from ZT-6 nor significantly higher from ZT-18 though ZT-6 and ZT-18 were significantly different. However VIP-ir level at ZT-0 was significantly different from ZT-6, 12 and 18 ($p_a \leq 0.05$) and ZT-18 was significantly higher from ZT-6 ($p_b \leq 0.05$).

The cell bodies with AVP-ir were concentrated in the dorsal SCN in proximity to optic chiasm and third ventricle. AVP-ir projections were located dorso-medially and in proximity to third ventricle. AVP-ir also showed a daily rhythm with maximum and minimum immunoreactivity at ZT-12 and ZT-0 respectively (Figs. 6A, B). AVP-ir levels at all ZTs studied were significantly different from each other ($p \leq 0.05$) (n = 4).

Thus, the VIP-ir level profile here was distinct from AVP-ir level profile over 24 h period as the peak time of VIP-ir level rhythm was found ahead of AVP rhythm with maximum and minimum VIP-ir levels at ZT-6 and ZT-0 respectively and AVP-ir levels at ZT-12 and ZT-0 respectively. In addition, the VIP-ir level remained higher for longer duration than AVP-ir level.

3. Discussion

In our study, the gross locomotor activity of *F. palmarum* exhibited a diurnal pattern of activity under LD condition with 70.39 ± 3.88% diurnality and circadian period (τ) of 24.19 ± 0.12 h. A similar pattern of entrainment was also reported earlier from related species *F. pennanti* (Rajaratnam and Redman, 1999) with ≈88% diurnality and other rodents such as *A. niloticus* with 60% (Katona and Smale, 1997), *R. pumilio* with 68% (Schumann et al., 2005) diurnality. In *F. Palmarum*, a free running circadian rhythm in locomotor activity was exhibited under constant darkness as well as constant light condition with a τ of 24.11 ± 0.03 h and 24.92 ± 0.35 h respectively.

Aschoff (1960) proposed that activity rhythms in nocturnal and diurnal rodents respond differently to light in that diurnal rodent activity rhythms exhibit: a longer tau in DD than LL, a tau greater than 24 h in DD. Gross locomotor activity in *F. palmarum*

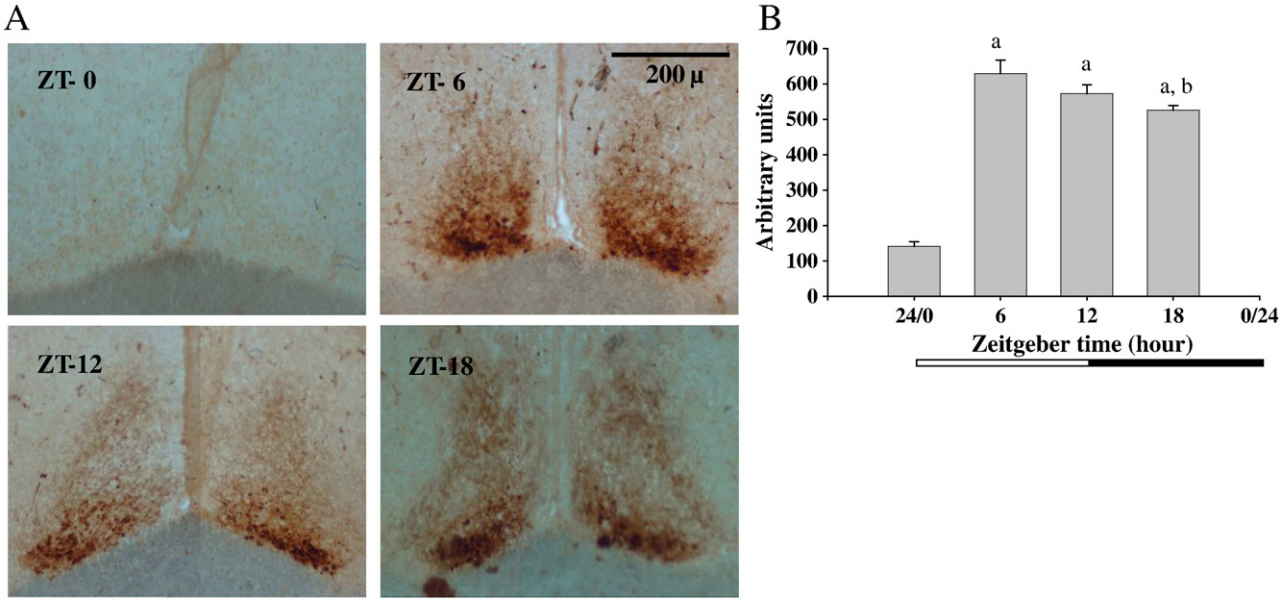

Fig. 5 – A. VIP-ir in the coronal brain sections (25 µm) of SCN of *F. palmarum* sacrificed at various zeitgeber times (ZT) 0, 6, 12 and 18. Scale bar = 200 µm. B. Densitometric analysis using Image Pro AMS software. The VIP-ir appears maximum at ZT-6 and minimum at ZT-0. Oc — optic chiasm; 3v — third ventricle. Each value is mean ± S.E (n = 4). ($p_a \leq 0.05$ and $p_b \leq 0.05$) (where a and b refers to comparison with ZT-0 and ZT-6 respectively. No significant difference in ZT-12 and ZT-18 were observed).

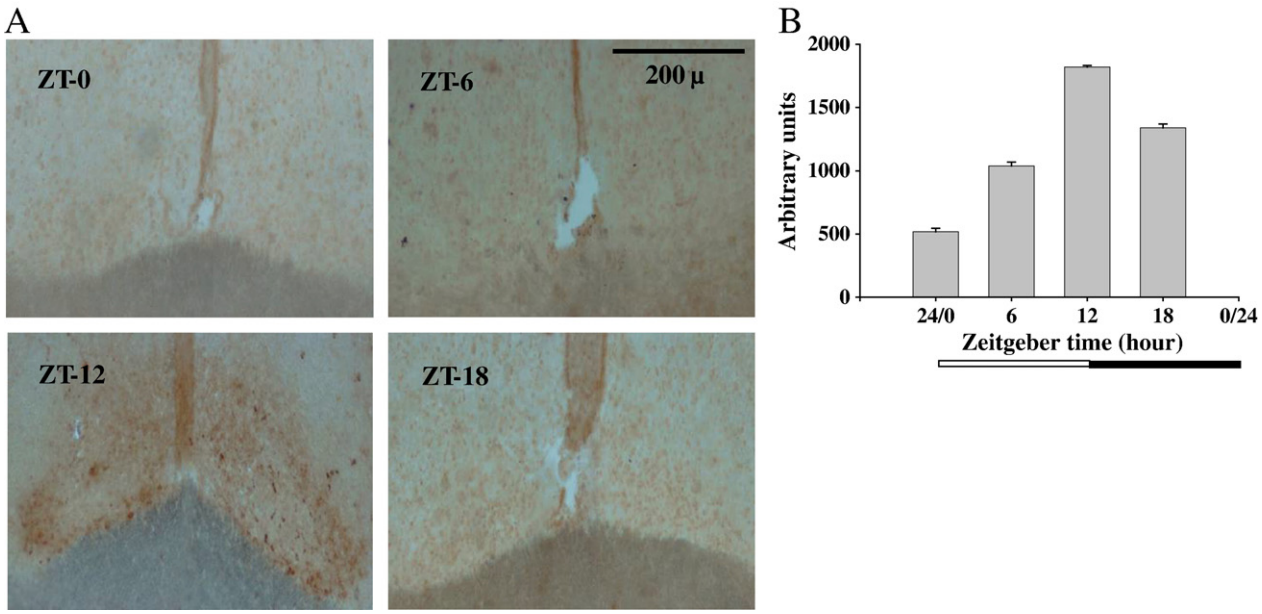

Fig. 6 – A. AVP-ir in the coronal brain sections (25 µm) of SCN of *F. palmarum* sacrificed at various zeitgeber times (ZT) 0, 6, 12 and 18. Scale bar = 200 µm. B. Densitometric analysis using Image Pro AMS software. The AVP-ir appears maximum at ZT-12 and minimum at ZT-0. AVP-ir levels at all ZTs studied were significantly different from each other ($p \leq 0.05$). Oc — optic chiasm; 3v — third ventricle. Each value is mean ± S.E. (n = 4).

showed however a τ greater in LL than DD but the τ was greater than 24 h in DD. Numerous studies have shown that not all diurnal species confirm to these expectations (Moore-Ede et al., 1982). Similar findings are reported in *A. niloticus*, tau was greater in LL than DD and was generally shorter than 24 h in DD (Katona and Smale, 1997), *A.ansorgei*, with a larger τ in constant light than dim red light (Challet et al., 2002), and *O. degus* (Lee and Labyak, 1997). Similarly α was found to be significantly high in LL though there was no significant difference in DD.

The optic structures and brain regions that receive visual and photic input have been reported by some workers to differ between diurnal and nocturnal rodents (Goel et al., 1999; Smale and Boverhof, 1999). The darker eyes found in *F. palmarum*, could be due to presence of pigmentation in lens. Lens pigmentation has been reported as an apparent feature of diurnal animals and is absent in nocturnal species (Jacobs et al., 2003). The presence of short, thick and stout optic nerves in *F. palmarum* as compared to nocturnal rats is in agreement with earlier studies on nocturnal (Kita and Oomura, 1982; Pickard, 1982) and other diurnal rodents (Goel et al., 1999; Smale and Boverhof, 1999). These apparent neuroanatomical features of diurnal animals as distinct from nocturnal animals could be related to their better adaptation in diurnal environment with more light input pathways.

Interestingly the shape and size of SCN of diurnal rodents reported so far appear to be different as compared to that of nocturnal rodents such as rat, mice and hamsters where it is oval in shape. The SCN in South Indian Palm Squirrel appeared similar to that of diurnal *O. degus* (Goel et al., 1999) and *S. lateralis* (Smale et al., 1991) elliptical in shape with elongation in the ventrolateral direction but oval in *A. niloticus* (Smale and Boverhof, 1999). The size of SCN in *F. palmarum* also appeared similar to other diurnal rodents such as *A. niloticus* with 200 µm in the mediolateral axis, 200 µm in the dorsoventral axis and 600 µm in the rostrocaudal axis. In the *O. degus* the SCN was 1.3 mm in the rostrocaudal axis (Goel et al., 1999). The SCN of *S. lateralis* appear as compact cell groups adjacent to the third ventricles with a length of 700 µm and height of 400 µm (Smale et al., 1991). In the nocturnal rats a dimension of 425, 400 and 950 µm in the mediolateral, dorsoventral and rostrocaudal axis respectively with a volume of 0.13–0.16 mm^3 had been reported (van den Pol, 1991). However, some other workers reported the rat SCN has a dimension in the mediolateral, dorsoventral and rostrocaudal axis as 300, 300 and 700 µm respectively with the SCN volume as 0.036 ± 0.006 mm^3 (Ibata et al., 1999; Moore et al., 2002).

The cell density in the present study in SCN appeared evenly distributed; however the proximity of the SCN was more to the optic chiasm. This could be due to presence of more number of neurons associated to the input pathway as supported by the prominent optic chiasm of these animals (Figs. 3A, 4A(i)). A similar cell density pattern was reported in the *O. degus* (Goel et al., 1999), however, in the *S. lateralis* the cell density appears to be greatest in the ventral half of the SCN (Smale et al., 1991). The neuronal counts in the paired SCN of 3-striped South Indian palm Squirrel were significantly higher as compared to nocturnal species (van den Pol, 1991; van Esseveldt et al., 2000). Though data is not available from other diurnal species, the higher neuronal count obtained in SCN could be a distinct feature related to diurnality (Kronfeld-Schor and Dayan, 2008).

In the SCN of *F. palmarum* VIP-ir cells were found in the ventro-medial and ventro-lateral SCN with proximity towards the optic chiasm where as AVP-ir cell bodies were found in the dorsomedial and ventral regions with a central region of low immunoreactivity. This pattern is similar to that found in other diurnal as well as nocturnal species (Goel et al., 1999). Presence of dense VIP-ir and AVP-ir fibres in the SCN towards the subparaventricular zone (sPVNz) is similar to that in the diurnal

(Gomez et al., 1997; Smale et al., 2008) and nocturnal rodents (Lu et al., 2001). The sPVNz has been reported to play important role in regulation of circadian physiology (Watts, 1991) and is related to influence diurnal physiology (Smale et al., 2008) by its efferents to several regions of the brain. sPVNz mediates the production of melatonin by the pineal gland through multi--synaptic pathway via the intermediolateral cell column of the upper thoracic spinal cord (Moore, 1996).

The SCN produces entrained rhythms based on the input signals received through afferent pathway. Neuropeptides such as AVP, VIP, GRP, etc. have been reported to show rhythmicity (Mikkelsen and Fahrenkrug, 1994; Isobe and Nishino, 1996; Dai et al., 1997; Krajnak et al., 1998; Lucas et al., 1998; Jacomy et al., 1999; Mirochnik et al., 2003; Dardente et al., 2004; Caldwell et al., 2008). In the present study the levels of both VIP and AVP showed daily rhythmicity in the SCN of *F. palmarum*. VIP-ir levels in the palm squirrel SCN were maximum and minimum at ZT-6 and ZT-0 respectively where as in the rat the maximum and minimum levels were reported at ZT-0 and ZT-12 respectively (Shinohara et al., 1993; Isobe and Nishino, 1998). On the other hand some workers have reported rat SCN with no VIP rhythmicity (Albers et al., 1987). However, in diurnal humans VIP does not exhibit diurnal fluctuations though vasopressin (VP) has been reported to exhibit daily rhythmicity (Hofman and Swaab, 1994; Shinohara et al., 1994; Hofman et al., 1996). In diurnal *A. ansorgei* VIP has been reported maximum at ZT-18 in light dark condition and in continuous dark condition at CT-12 (Dardente et al., 2004). AVP-ir showed maximum and minimum levels at ZT-12 and ZT-0 respectively where as in the rat the maximum and minimum levels were at ZT-4 and ZT-12 respectively (Isobe and Nishino, 1998). In *A. ansorgei* also the maximum AVP levels have been reported at ZT-12 (Dardente et al., 2004). High VIP-ir and AVP-ir levels during the light phase in squirrel found in the present study are similar to that in rats. However, the maximum and minimum VIP-ir levels are only 6 h apart in squirrel though 12 h apart in rat and the maximum and minimum AVP-ir levels are 12 h apart in squirrel though only 8 h apart in rats. The peak time of VIP rhythm was found ahead of AVP rhythm in *F. palmarum*. In addition, the VIP-ir levels were higher for longer duration than AVP-ir level. Furthermore, the total AVP-ir levels over a 24 h period were about 2.5 times as that of VIP-ir levels. It has been also reported in nocturnal rats that VIP rhythm was ahead of AVP but VIP levels were, over a 24 h period, six times higher as that of AVP (Shinohara et al., 1994). Additionally the SCN activity such as metabolism as well as the multiple-unit activity is maximum during the day though melatonin levels are maximum during midnight in nocturnal animals (Schwartz et al., 2004). Interestingly VIP from rat SCN has a strong inhibitory where as from diurnal rodent has a stimulatory effect on corticosterone rhythms (Kalsbeek et al., 2008).

Thus, three striped South Indian Palm Squirrel (*Funambulus palmarum*) shows differences in anatomy as well as neuroarchitecture as compared to nocturnal rodents. Daily rhythms of VIP-ir and AVP-ir levels showed distinct patterns as compared to nocturnal rodents. Our studies on VIP-ir and AVP-ir rhythms in diurnal rodents *F. palmarum* showed distinct pattern which were not simply reversal of the pattern in nocturnal rodent. Thus indicating the understanding of diurnality could be related to many obscure or unknown parameters. Hence, our results indicate that diurnality may not be just a reversal of nocturnality but an extended functionality. Further studies are in progress in understanding the neural mechanisms involved in regulation of SCN functions in this diurnal rodent.

4. Experimental procedures

4.1. *Animals*

Adult male 3 striped South Indian Palm Squirrels weighing 110±20 g, supplied by a local supplier, were maintained in light–dark conditions (LD 12:12), at room temperature (24±2 °C) with relative humidity (55±6%), two weeks prior to experiment. Animals were kept individually in wire cages (60×40×40 cm) contained within well ventilated light proof environmental cabinets isolated in animal facility. Food and water were supplied *ad libitum*. Dim red light (Kodak 1A filter) was used for handling the animals in the dark. Cage changing was done at random intervals.

Adult male Wistar Rats maintained in polypropylene cages individually under similar conditions (Jagota and Reddy, 2007) such as LD 12:12, temperature 24±2 ° C and relative humidity 55±6% were used for comparative neuroanatomy.

All experiments were performed as per Institutional Animal Ethics.

4.2. *Gross locomotor activity*

Adult male palm squirrels in three groups (n=6 in each group) were housed individually in cages equipped with infra-red motion detector sensors and the data was recorded using Chronobiology Kit (Stanford Software Systems, USA). The animals in Group 1 were subjected to LD 12:12 for 100 days for entire duration of experiment (Fig. 2A). In Group 2 after 20 days in LD the timer were set to constant dark (DD) where the animals were maintained for 57 days. The lighting conditions were then reset to LD and the animals were maintained for a minimum of 23 days (Fig. 2B). In group 3, the animals were kept in LD for 20 days and then lighting condition were set as constant light condition (LL) for 40 days. The lighting was then returned to LD where the animals were maintained for another 40 days (Fig. 2C).

4.3. *Data analysis*

Actograms and mean activity profiles were prepared using the Chronobiology Kit and analyzed using the Kit Analyze software (Stanford Software systems, USA). The duration of the active period (α), circadian period (τ) and percentage diurnality were calculated as described by Schumann et al., 2005. α started with activity onset and ended with activity offset. Results were represented as mean±standard error (Table 1).

4.4. *Comparison of gross neuroanatomy of diurnal squirrel and nocturnal rat*

Adult Palm Squirrels and Wistar rats were sacrificed and the brains along with optic nerves and eye balls were dissected out. The length and thickness of the optic nerves were compared (Figs. 3A, B).

4.5. *Immunohistological characterization of SCN*

Animals were sacrificed after anesthetizing and perfusing transcardially with heparinized 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4). The brains were rapidly removed and post-fixed in 4% paraformaldehyde 0.1 M PB (pH 7.4) for 2–4 h and then stored in 20% sucrose in 0.1 M PB (pH 7.4) at 4 °C for 24 h. 25 μ thick coronal sections of the brain were cut using Leica cryostat (CM 1850) and processed for cresyl violet and immunohistochemistry (IHC) (Goel et al., 1999).

Neu N (neuronal marker) immunohistochemical studies were performed with Mouse primary antisera generated against Neu N (MAB 377; Chemicon, MA, USA) and detected using respective secondary goat anti-Mouse IgG-HRP conjugate (HP06, Bangalore Genei, India). The sections were then placed in the respective secondary antibody (dilution 1:250) for 1–2 h at 37 °C and were subjected to 0.1% 3, 3-diaminobenzidine containing 0.3% H_2O_2 and incubated for about 15 min till colour developed (Caston-Balderrama et al., 1998). All sections were then arranged in a rostro-caudal axis, photographed using DP-12 digital camera attached to Olympus microscope (BX-41) and neurons were counted (Jagota and Habibulla, 1992; Vidal and Lugo, 2006). The counts were later verified by using Image Pro AMS software (Media Cybernetics, USA) (Petersen et al., 2006).

4.6. *Daily rhythms of VIP-ir and AVP-ir in SCN*

For studying daily rhythms of VIP and AVP in SCN, animals were sacrificed at various zeitgeber times (ZT-0, 6, 12 and 18) (n=4). VIP-ir and AVP-ir were studied using Mouse primary antisera generated against VIP (sc-25347; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and Rabbit primary antisera generated against AVP (AHP 372; AbD Serotech, Oxford, UK) and secondary antibodies, Goat anti-Rabbit IgG-HRP (HP03, Bangalore Genei, India) and Goat anti-Mouse IgG-HRP conjugate (HP06, Bangalore Genei, India). The IHC was performed as described above. VIP-ir and AVP-ir levels were compared using densitometric analysis using Image Pro AMS software (Media Cybernetics, USA).

4.7. *Statistical analysis*

Data were analyzed using Jandel Scientific Sigma stat software by Student's t-test and one way ANOVA followed by Post hoc Duncan's test for multiple comparisons.

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