Age induced changes and the role of Melatonin in the Neurophysiology of Suprachiasmatic Nucleus of Rat

Thesis submitted for the degree of
Doctor of Philosophy

By

Kalyani Dasari

Department of Animal Sciences
School of Life Sciences
University of Hyderabad
Hyderabad 500 046
INDIA

Enrollment No: 01LAPH06
February 2008

UNIVERSITY OF HYDERABAD
(A Central University Established in 1974 By An Act Of Parliament)
HYDERABAD – 500 046, INDIA.

DECLARATION

I hereby declare that the work embodied in this thesis entitled ***"Age induced changes and the role of Melatonin in the Neurophysiology of Suprachiasmatic Nucleus of Rat"*** has been carried out by me under the supervision of **Dr. Anita Jagota,** Department of Animal Sciences. To the best of my knowledge this work has not been submitted for award of any degree or diploma in any other University or Institution.

Dr. Anita Jagota
(Research Supervisor)

D. Kalyani
(Research Scholar)

UNIVERSITY OF HYDERABAD
(A Central University established in 1974 By The Act Of Parliament)
HYDERABAD – 500 046, INDIA

CERTIFICATE

This is to certify that Ms. ***Kalyani. D*** has carried out the research work embodied in the present thesis entitled ***"Age induced changes and the role of Melatonin in the Neurophysiology of Rat Suprachiasmatic Nucleus of Rat"*** under my supervision and guidance towards the degree of Doctor of Philosophy in Animal Sciences. To the best of my knowledge, this work has not been submitted for award of any degree in any other University or Institution.

Dr. Anita Jagota
Supervisor

Prof. S. Dayananda
Head, Dept. of Animal Sciences

Prof. A. S. Raghavendra
Dean, School of Life Sciences

ACKNOWLEDGEMENTS

I express my deep sense of gratitude to my supervisor, ***Dr. Anita Jagota*** *for giving me an opportunity to work under her guidance and for the constant support and scientific discussions throughout my work.*

I am grateful to doctoral committee members, Late ***Prof. Ch. Radha Krishna Murthy****,* ***Prof. Aparna Dutta Gupta*** *and* ***Dr. B. Senthilkumaran*** *for their valuable suggestions and guidance throughout my research work.*

I am thankful to ***Prof. S. Dayananda****, Head, Department of Animal Sciences and the former Heads for the facilities provided in the Department.*

I thank ***Prof. A. S. Raghavendra****, Dean, School of Life Sciences and former Deans for providing necessary facilities to carry out the research work.*

My heartfelt thanks to ***Prof. P. Reddanna*** *for allowing me to work in his lab whenever required. I thank him for letting me use his lab facilities like centrifuge, degassing and filtration unit to process my HPLC samples and radiation safety chamber for my CaMKII samples.*

I am grateful to ***Dr. Seshagiri Rao*** *and* ***Prof. T. Suryanarayana*** *for allowing me to use their spectrofluorimeters during my initial stages of research work.*

I am very much grateful to ***Prof. Aparna Dutta Gupta*** *for readily accepting to use her license to obtain the radioactive material for my research work.*

I am very much thankful to ***Prof. P. Uma Maheswara Reddy****, Osmania University for his valuable suggestions and encouragement given during the assessment of my research work.*

I am thankful to the faculty, School of Life Sciences, especially Dept. of Animal Sciences who taught me various subjects during my post graduation.

I take this opportunity to thank all faculty of School of Life Sciences, for allowing me to work in their labs whenever required.

I thank my lab mates Mr. Kapil M. Shah, Mr. M. Yallamandareddy, Mr. Anumodh P. Mammen, Mr. V. Dileep Kumar Reddy, Miss. M. Ushodaya and Mr. Sudhansu S Choudhury for their friendly nature, cooperation and help throughout my work.

I thank my seniors, School of Life Sciences, Dr. A. Shiva Sreenath, Dr. Valli Maya, Dr. J. Subhashini, Dr. R. Aparna, Dr. Sudar Olli, Dr. N. Kranthi Kumari, Dr. S. Vijay Kumar Mahipal, Dr. M. Mallikarjun Reddy, Dr. Sathya Sai Kumar, Dr. P. Vijay Bhaskar Reddy and Dr. Arif for their timely help during my research work.

Its my pleasure to thank all my M. Sc. classmates Satya, Mukherjee, Madhavi, Abira, Sanghamitra, Gautam, Soumya and batch mates Vinitra, Bhavani, Naresh and Subbu and juniors Katya and Aruna for their friendly nature and help during my M.Sc. days and also throughout the research work.

I would like to thank all the research scholars, School of Life Sciences for their help throughout my research work. I thank Dr. Pavan, Mr. Hussain and Mrs. Anjali for teaching me statistical analysis and densitometric analysis needed for research work.

I thank Mr. Mallesh and Mr. Venkat for their timely and constant help. I especially appreciate Mr. Mallesh for maintaining animals, handling them in dark and helping during dissections.

I thank all the non-teaching staff, Dept. of Animal Sciences, Mr. Ankineedu, Mrs. Bhargavi, Mr. Jagan, Mr. Gopi, Mr. Babu Rao, Mr. Shiva Kumar and Mr. Pandu for timely help.

I express my thanks to Central Instruments Laboratory (C. I. L.) staff, Mr. Murthy and Mr. Suresh for their kind cooperation and help in carrying out spectrofluorimetric assay of my samples.

I take this opportunity to thank Mr. Krishna, Animal House In-charge for providing me animals and Animal House Staff for proper maintenance of animals.

I extend my thanks to Dr. Kalyana Sundaram, Animal House In-charge and Mr. Janaki Ram of National Institute of Nutrition (N. I. N.), Hyderabad, Mr. Jagan Mohan and Mr. Sreenivas for providing me aged animals required for my research work.

I thank all my friends Meena, Anjali, Sharada, Radhika and Salomi for their help and cordial atmosphere during my days on campus.

I thank CSIR, Delhi for fellowship during my research work.

I acknowledge funding to the lab from CSIR, ILS, UPE, DST, ICMR and UGC and funding to the Department from UG-SAP and DST-FIST.

I am greatly indebted to my parents and brothers, Kiran and Sundeep for their love, constant moral support and being my sources of encouragement throughout my career.

I wish to thank each and every person who has helped me directly or indirectly in his/her own possible way and I wish them a great success in all their endeavours.

Finally I express my gratitude to The Almighty for everything He has given me all throughout my life and I owe my success to Him.

Kalyani. D

Dedicated to

The Almighty
&
My Family

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and

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INTRODUCTION

Everyday living organisms perform a wide variety of functions which are controlled by a multitude of periodic processes. Many of these functions are evolutionarily adapted to the continuous changes in environmental conditions for which organisms have acquired an endogenous mechanism. This mechanism exhibits the characteristics of self-sustaining oscillations called biological rhythms.

A biological rhythm is a biological event or function that is repeated through time in the same order with the same interval. These rhythms are generated in two ways, (i) exogenous which are directly driven by external or environmental cues and (ii) endogenous which are driven by a self sustaining oscillator or biological clock, e.g. body temperature, sleep-wake cycle. There are various types of biological rhythms based on the length of the period. Circannual rhythms are the ones which have a period of 365 days. Hence, these are also called yearly rhythms (e.g. gonadal development in some species). Circalunar rhythms follow the lunar cycle which have a period of about 29 days (e.g. menstrual cycle, reproduction in marine organisms). Circadian rhythms have an approximate period of about 24 hour (h) (e.g. sleep-wake cycle). Circatidal rhythms occur due to tidal waves and have a period of 12.4 h (e.g. activity of crab on shore line). Of all these rhythms, circadian rhythms have a major significant effect on organism's physiology (Jagota and Gupta, 2006).

Circadian rhythms regulate the function of living systems at virtually every level of organization from molecular to organismal (Takahashi, 1995). In words of a well known scientist, Aschoff, "circadian rhythms establish a mirror of the changing external world in the internal milieu and thereby prepare the organism for programmed or predictable environmental changes," (1960). Study of biological rhythms and the biological clock is known as 'Chronobiology'. Study of circadian rhythms has many important implications in human life.

Importance of Circadian Rhythms: Human Relevance:

The effect of trans-meridian flight causes jet-lag and continuous changes in light-dark cycles that occur in cases of shift workers lead to altered circadian rhythms. This alteration results in desynchronization of the pacemaker rhythm to the external environment and also affects phase alignments between different peripheral clocks (Yamazaki *et al.,* 2000). Recent findings on shift-working as well as frequent time zone travelling have suggested the disturbances on the circadian system and its effect on health. Several reports demonstrated increased risk of breast cancer (Schernhammer *et al.,* 2006), colorectal cancer (Schernhammer *et al.,* 2003) and prostrate cancer (Kubo *et al.,* 2006). Diseases like heart disease (Fujino *et al.,* 2006) and diabetes (Morikawa *et al.,* 2005) are also reported in shift-workers. All these effects on shift-workers are explained through the disruption of the circadian clock due to phase-shifts in the sleep-wake cycle. The phase-shifts result in desynchronization of multiple physiological functions and alter hormonal status especially melatonin levels (Anon, 2002).

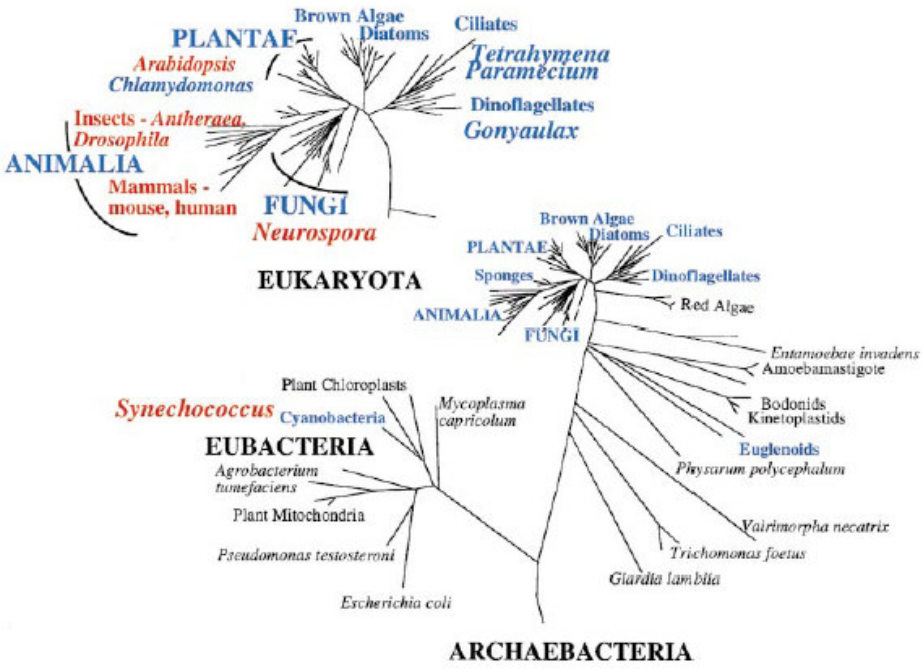
Many physiological and behavioral parameters change within a 24 h cycle. Understanding the natural rhythm and sampling at different times of the day would help in better diagnosis and status of the disease. The efficacy of certain drugs is dependent on time of delivery. Optimizing schedules for drug administration minimize toxic side effects and increase the therapeutic potential (Levi, 1999). There is a circadian variation in the rates of absorption, metabolism, target susceptibility and excretion in the beneficial and toxic effects of drugs (Edery, 2000).

Several disorders such as chronic sleep disturbances, advanced sleep phase syndrome (ASPS), delayed sleep phase syndrome (DSPS), manic depression, seasonal affective disorders (SAD or Winter depression) are associated with altered functions of the circadian timing system (Copinschi *et al.,* 2000). These problems can be alleviated by alterations in the light-dark schedules (Terman *et al.,* 1995).

The rhythms that are generated in the organisms have several intrinsic properties.

Characteristic features of Circadian rhythms:

Circadian rhythms are ubiquitous in nature. They are found in all plant and animal kingdoms including unicellular organisms (Wong and Liao, 2006) (Fig. 1). They can be entrained and adjusted to an exact period by zeitgebers (a German word which means time givers) so that they are suitable for its surroundings. They are affected by light, a major zeitgeber. Rhythms persist even in the absence of zeitgebers under constant conditions such as complete darkness or complete light and they are said to free-run. The rhythms continue to run, but slightly deviates from 24 h as they are not influenced by external factors. The natural free-running period is called 'tau'. The rhythms are genetically determined. Their endogenous and free-running nature suggests that they generate within an organism which involves a complex molecular network. Generation of these rhythms to external cues is pre-adapted driven by a circadian timing system.



(Dunlap, 1999)

Fig. 1: Circadian rhythms in the universal tree of life

The Circadian Timing System:

The circadian system is comprised of three components, (i) input pathways that relay information to the oscillator (ii) the circadian pacemaker or clock that is responsible for the generation of rhythms and (iii) output pathways that provide temporal information to a wide range of physiological and behavioral processes of an organism. The circadian pacemaker or biological clock of the circadian timing system governs rhythm generation and regulates the phases of biological events within the organism in relation to the 24 h environmental cycle (Foster, 2002). The suprachiasmatic nucleus (SCN) is the circadian pacemaker in mammals. For these events to occur in harmony, the circadian system must remain synchronized/ entrained with zeitgebers.

Zeitgebers and Entrainment:

Zeitgebers are the external environmental cues which have the ability to reset the clock or central oscillator. Light is one of the most important environmental cues (Münch *et al.,* 2005). Other potential zeitgebers are magnetic fields, barometric pressure, sound, humidity and social interactions (Mrosovsky, 1996). Hence these cues are mainly categorized into photic and non-photic stimuli. The time of the zeitgeber is known as zeitgeber time (ZT), e.g: in LD: 12:12; ZT-0 is the onset of zeitgeber time (lights on) and ZT-12 is the offset of zeitgeber time (lights off). The time determined by a circadian oscillator under constant conditions is known as circadian time (CT) i.e in the absence of a synchronizing zeitgeber, persistence of rhythmicity. CT-0 is the onset of rhythms and CT-12 is the offset of rhythms (Schibler, 2000). These external stimuli phase shift and entrain circadian rhythms through distinct but interacting mechanisms in the SCN. Phase shift is resetting of a rhythm either by advance or delay in the phase of a biological event to the 24 h cycle. Phase-shifting is an important characteristic feature of circadian clock and a fundamental process of all circadian systems from prokaryotes to *Homo sapiens* (Czeisler *et al.,* 1989). The magnitude and direction of phase shifting in response to a stimulus depends on the circadian phase of stimulation. The

24 h profile for a specific phase resetting stimulus and its characteristic features is known as a phase response curve (PRC). These PRCs help in understanding the responsiveness and sensitivity of the circadian pacemaker to different stimuli (Rosenwasser and Dwyer, 2001). Phase resetting of the clock is important in case of jet-lag, shift workers, people suffering from advanced sleep phase syndrome (ASPS) and delayed sleep phase syndrome (DSPS).

The PRC of light is well established. The intensity and duration of exposure to light affects the rhythms. Photic stimulation during late subjective day or early subjective night (i.e around subjective dusk) causes phase delays. Photic stimulation during late subjective night or early subjective day (i.e around subjective dawn) results in phase advances. Photic stimulation in the mid-subjective day is ineffective (Rosenwasser and Dwyer, 2001). The PRCs of non-photic stimuli are characterized by phase advances during mid-subjective day and phase delays during mid-subjective night. In addition to photic and non-photic cues the pacemaker is also responsive to several neurochemicals and neuropharmacological agents. The PRCs of these agents resemble PRCs of either photic or non-photic cues. Neurotransmitters and neuromodulators like glutamate (Mintz *et al.,* 1999), agonists for acetylcholine, histamine, α-adrenaline, substance P (SP) and pituitary adenylate cyclase activating polypeptide (PACAP) have been reported to have photic like phase shifting effects on the pacemaker. Non-photic like phase-shifting effects are associated with the neurotransmitters and neuropeptides such as serotonin, gamma amino butyric acid (GABA) and neuropeptide Y (NPY) (Mistlberger and Holmes, 2000).

The Circadian Visual System:

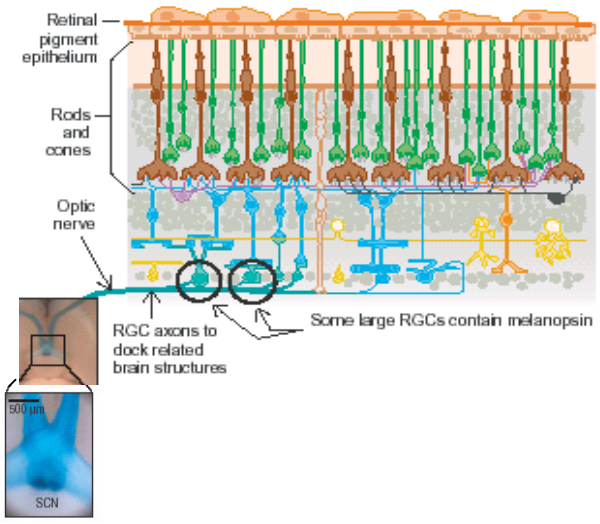
As described in Jagota *et al.,* (1999), the circadian visual system is anatomically and physiologically distinct from the visual system that results in image formation. It consists of a specialized photoreceptive system, subset of ganglion cells formed of type III or type W cells. The recipient neurons respond to changes in light but do not distinguish the temporal and spatial stimuli required for normal vision. Lens of the eye receives light, focuses it on

the retina which then conveys the information to the SCN through several input pathways. These inputs originate from a specific subset of retinal ganglion cells (RGCs) (Moore *et al.,* 1995). The SCN then regulates the preoptic, paraventricular and ventromedial nuclei as well as other nuclei. This visual system is responsible for synchronization of biological clock with the light-dark cycle (Klein *et al.,* 1991), control of pupil size (Lucas *et al.,* 2001), acute suppression of locomotor behavior (Mrosovsky, 1999) and melatonin release (Cajochen *et al.,* 2000).

Photoreceptors:

Photoreceptors are mainly localized in the retina of the eye (Menaker, 2003) (Fig. 2). In lower vertebrates, skin also acts as the photoreceptive system. Three classes of pigments are considered as photoreceptors for the circadian visual system: tetra-pyrrole based heme pigments (Oren, 1996; Campbell and Murphy, 1998), cryptochrome (Bouly *et al.,* 2007) and opsin/retinal based photopigments (Foster, 1998). Tetra-pyrrole based heme pigments are mainly found in humans. Cryptochromes, CRY1 and CRY2 are a kind of blue-light photoreceptors present in mammalian retina and SCN (van Gelder and Sancar, 2003). They contain a compound called pterin/flavin. These photoreceptors absorb light by means of a conjugated derivative of flavin.

Fig. 2: Photoreceptors of the circadian timing system.



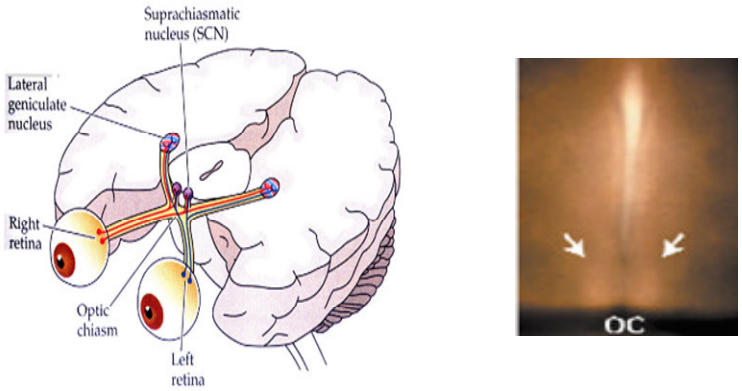
(Menaker, 2003)

In mammals the photoreceptors for entrainment and phase shifting are located in the retina of eye which conveys photic information to the SCN (Kennaway, 2002). The receptor is an opsin, vitamin A based pigment called melanopsin in rodents (Lucas and Foster, 1999). Melanopsin is exclusively expressed in the RGCs. The RGCs with melanopsin form a network of dendritic plexes that allows these cells to capture photic stimuli (Provencio *et al.,* 2002). Melanopsin containing RGCs are intrinsically photosensitive (Berson *et al.,* 2002) and they connect the two lobes of SCN and other areas of brain involved in light responses. They play a major role in photic entrainment. Apart from conveying photic information to the circadian oscillator, melanopsin photoreceptors also contribute to pupillary light reflex and acute alterations in motor activity as well as in a broad range of physiological and behavioral responses to light (Foster *et al.,* 2003).

The Biological Clock: Suprachiasmatic Nucleus:

The biological clock is an internal time keeping mechanism capable of driving or coordinating a rhythm and synchronizes organism's internal functions to the external cues. In vertebrates including the most primitive ones, there are three principal clock structures that interconnect with each other and form the central "circadian axis". They are (i) the retina, (ii) the pineal complex (pineal and parietal eye/organ) and (iii) the suprachiasmatic nucleus (SCN) of the hypothalamus. These structures control the circadian rhythmicity and are capable of sustaining rhythmicity *in vitro*. The retina is found to act as one of the circadian clocks in all vertebrates from pisces to mammals (Sakamoto *et al.,* 2000). In pisces and amphibians retina and pineal act as main clock structures (Cahill, 2002) whereas in reptiles retina, pineal and the parietal eye contain the circadian clocks (Bertolucci *et al.,* 2002). Besharse and Iuvone (1983) demonstrated the retina of a vertebrate (*Xenopus laevis*) as an autonomous circadian clock. The retina, the pineal gland and the hypothalamic oscillator regulate the circadian rhythms in case of birds (Brandstätter, 2002).

The principal clock component of the mammalian circadian system and the master circadian/biological clock in mammals is the SCN (ref: recent review; Jagota, 2006; Meijer *et al.,* 2007). It is a bilateral nucleus present just above the optic chiasm at the base of hypothalamus on either side of the third ventricle (Morin *et al.,* 2006) (Fig. 3). During development hypothalamic primordium gives rise to all types of hypothalamic cells. The SCN is derived from the periventricular zone of the anterior hypothalamic region. Altman and Bayer (1986) extensively studied neuronal generation of the SCN. In rat, SCN is formed from a specialized zone of ventral diencephalic germinal matrix, the suprachiasmatic primordia. The neurons of SCN are generated from embryonic (E) days E14 to E17 with a peak on E15. The cells generated earlier form the ventrolateral division and the later cells form the dorsolateral division except those cells which generate on E17 form the most ventral portion of the SCN adjacent to the third ventricle called the basal suprachiasmatic subnucleus.



(Jagota *et al.,* 2000; Jagota, 2006; Reghunandanan and Reghunandanan, 2006)

Fig. 3: The Suprachiasmatic Nucleus

Functional development of the SCN occurs in two stages (Buijs *et al.,* 2006). First is the development of intrinsic rhythmicity like glucose utilization (Kalsbeek *et al.,* 2006) and firing rate of SCN neurons (Aguilar-Roblero *et al.,* 1992). The second stage is the development of SCN as a circadian pacemaker. This occurs when SCN develops sufficient afferent, intrinsic and efferent connections to function as a neural network. Thus the total development of SCN occurs in four stages (i) development of SCN neurons and establishment of rhythmic function within the nucleus (ii) development of entraining pathways and external regulation of pacemaker function (iii) development of SCN projections and coupling of these to effector systems and (iv) maturation of effector systems for the expression of circadian function (Moore, 1992).

Cellular Architecture:

The neurons of SCN are the smallest in the hypothalamus as well as in the brain. Each nucleus contains about 10,000 small, densely packed neurons, approximately 300 µm in diameter in rat (Moore *et al.,* 2002). The volume of a single neuron of male adult rat ranges from 0.13 to 0.16 mm^3 (van den Pol, 1991). The size of the SCN varies with age and gender (Shirakawa *et al.,* 2001). The SCN is remarkable for the density of dendrodendritic synapses that links the cells together and thus synchronize their activity. These neurons are heterogenous in nature (Kuhlman *et al.,* 2003; Lee *et al.,* 2003) and are classified according to their neuropeptide content (Abrahamson and Moore, 2001a).

Sub divisions:

The SCN is subdivided into two main regions in most of the species. (i) Dorsomedial (shell) region and (ii) Ventrolateral (core) region based on the presence of neuroactive substances and on type of retinal innervation patterns (Kriegsfeld *et al.,* 2004). Dorsomedial region cells are smaller than those cells in ventrolateral region (van den Pol, 1991) and elongated in shape. These are

located along the walls of blood capillaries that course through the SCN. Dorsomedial region is characterized by the presence of arginine-vasopressin (AVP) containing neurons, but do not receive any visual input. Cells in ventrolateral region are spherical in shape. They receive input from retino-hypothalamic tract (RHT), geniculo-hypothalamic tract (GHT) and retino-raphe pathway. Large number of neurons in this region contains vasoactive intestinal peptide (VIP) as the neuroactive substance (Piggins and Cutler, 2003). Neurons of SCN exhibit circadian oscillations even after isolation, with periods ranging from 20-28 h (Honma *et al.,* 2004). Studies using horizontal slices in *in vitro* conditions revealed morning and evening oscillations in SCN (Jagota *et al.,* 2000).

It consists of large number of neurotransmitters which play an important role in its function in addition to AVP and VIP which characterize two regions. They include glutamate, NPY, serotonin (5-hydroxytryptamine/ 5-HT) (Jagota and Reddy, in press), peptide histidine isoleucine (PHI), PACAP, oxytocin (OT), gastrin-releasing peptide (GRP) and SP (Jagota, 2006; Reghunandanan and Reghunandanan, 2006). In addition to these, SCN also contains GABA, angiotensin II, neurotensin (NT), bombesin (BBS), calcitonin gene-related peptide (CGRP), cholecystokinin (CCK), enkephalin (ENK), somatostatin (SS), thyrotropin releasing hormone (TRH) and VGF (a protein induced by nerve growth factor) (Madeira *et al.,* 2004).

Inspite of its heterogenous nature of neuronal cell types, neurochemical organization and function of SCN is able to regulate and synchronize overt rhythms suggesting the strong coordination among the neurons (Jagota, 2006). The central pacemaker itself shows circadian rhythms of metabolic (Perreau-Lenz *et al.,* 2004) and electrical activity (Rohling *et al.,* 2006; Brown *et al.,* 2007). Individual SCN neuron functions as independent oscillator, but at tissue level SCN neurons synchronize by a robust intercellular coupling mechanism (Herzog and Schwartz, 2002). There are evidences for neurons of core projecting to the shell on the ipsilateral side but not for shell to core projections for either ipsilateral (Moore *et al.,* 2002) within a single nucleus or contralateral. Neurotransmitters most importantly GABA and others like VIP,

GRP, prokineticin 2 etc. are required for the synchronization of circadian rhythms (Hastings and Herzog, 2004) and development of action potentials (Yamaguchi *et al.,* 2003) in SCN. Neurons of SCN exhibit circadian rhythmicity of firing rate (Klisch *et al.,* 2006) based on day-night modulations of calcium (Ca^{2+}) currents (Pennartz *et al.,* 2002). Other neurotransmitters like nitric oxide, in the ventrolateral region also acts as a link between ventrolateral and dorsomedial subdivisions of SCN (Reuss *et al.,* 1995).

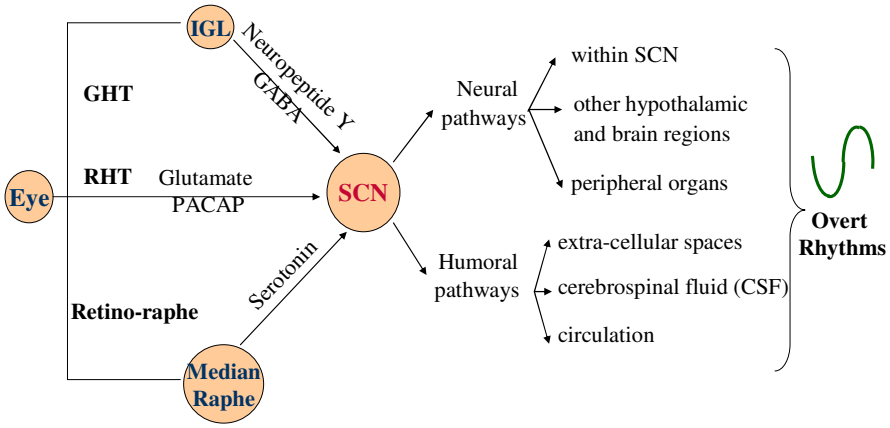
In addition to intra-SCN communication, SCN shows contralateral shell to shell and core to core connection between the nuclei (Moore *et al.,* 2002). The two nuclei of SCN neurons communicate with each other in many ways. Several studies showed that axons of the neurons containing AVP, GRP, VIP, GABA and SS cross between the paired SCN. Serotonergic and tyrosine hydroxylase containing neuronal axons are also found to couple the two lobes of SCN. In addition, axons originating outside the SCN seem to cross the midline of the two SCN (Card *et al.,* 1981). The neurons receive input signal, generate rhythms which have slightly different periods and phases. The average of all these pacemakers constitutes the output signal of SCN (Liu *et al.,* 1997). The input signals to the SCN neurons come from the specialized visual system called circadian visual system.

Afferent Pathways of the SCN:

Of all the stimuli, light is one of the most important stimuli, which entrains the clock. The SCN receives information about the presence, intensity and timing of light via the retina and the optic nerve. In mammals it receives neural innervations from three sources, the retina, the intergeniculate leaflet (IGL) of the lateral geniculate nuclei and the raphe nuclei (Rosenwasser and Dwyer, 2001; Jagota, 2006) (Fig. 4).

Retino-hypothalamic tract: Retino-hypothalamic fibers make monosynaptic contact with SCN neurons and deliver photic information to the SCN directly (Colwell and Menaker, 1996; Ebling, 1996). In rat RHT innervates SCN the day after birth. Glutamate and PACAP are the principal neurotransmitters of

this pathway (Reghunandanan and Reghunandanan, 2006). Light reaches RGCs through lens. Axons of RGCs target neurons containing glutamate in the ventrolateral region of the SCN resulting in secretion of glutamate. This glutamate acts on cells expressing amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) or kainate receptors and smaller population of cells expressing N-methyl D-aspartate (NMDA) receptors. Glutamate plays a critical role in photic regulation of circadian rhythms. Co-localization of PACAP in glutamate containing RGCs is involved in relaying photic information by potentiating the action of glutamate on the SCN (Minami *et al.,* 2002).



(Jagota, 2006)

Fig. 4: Afferent and Efferent Pathways of the SCN

Geniculo-hypothalamic tract: This is a major indirect photic input pathway from IGL to SCN (Card *et al.,* 1991). Retina conveys input signals to IGL via a separate branch of RHT that overlaps with the RHT terminals in the SCN. This pathway is involved in mediating photic as well as non-photic responses such as motor activity necessary for entrainment of circadian rhythms (Menet

et al., 2001). NPY and GABA are the neurotransmitters involved in transmitting the information from IGL to the SCN (Reghunandanan and Reghunandanan, 2006). Neuronal activity of SCN and suppression of firing rate of SCN neurons are under the control of NPY (Cutler *et al.,* 1998).

Retino-raphe-SCN pathway: This is one of the major input pathways that use serotonin as the neurotransmitter in neurons leading to the SCN. The most important afferent inputs terminating in the SCN are the serotonergic neurons (Morin and Allen, 2006). The SCN receives one of the densest serotonergic terminal plexus of the brain. The axons of RGCs receive light information and some of these neurons project into raphe nucleus of brain stem where serotonergic neurons originate. These serotonergic neurons project and terminate in the ventrolateral region of the SCN which contain VIP neurons (Moore and Speh, 2004). Serotonin acts on 5-HT_{2C} receptors of the excitatory interneurons of the SCN. Interneurons synapse with clock cells and reprogram the stimulus. There are evidences for the projections from the SCN to raphe nuclei (Bons *et al.,* 1983).

Efferent pathways from the SCN:

There are two efferent pathways by which SCN regulates the individual circadian rhythm, neural and humoral signals that either drive output rhythm directly or synchronize peripheral oscillators with the day-night cycle (Yamazaki *et al.,* 2000).

Neural pathway: The SCN is composed of different neuronal elements, each having its own specific function. The functional output of the SCN is mainly dependent on intensive interconnection and interaction among the heterogeneous neuronal elements within the SCN. Neural pathway is the communication across synapses. Neural outputs of the SCN primarily reach nearby sites such as hypothalamic and thalamic nuclei from the SCN, particularly to the medial preoptic nucleus, the medial part of the paraventricular nucleus (PVN) of the hypothalamus, the anterior part of the

PVN of thalamus, the medial part of the dorsomedial nucleus of hypothalamus, and the sub-paraventricular zone (Saper *et al.,* 2005). This pathway regulates body temperature, locomotor activity and hormonal levels which occur through the nervous projections to other nuclei of the hypothalamus and other brain regions. The SCN also sends signals to the periphery through autonomic nervous system, via PVN (Buijs and Kalsbeek, 2001) e.g: Sleep-wake cycles are regulated by the projections of the SCN to the dorsomedial hypothalamus and the posterior hypothalamic area (Abrahamson *et al.,* 2001b; Aston-Jones *et al.,* 2001). Secretion of melatonin from the pineal gland is regulated by SCN through adrenergic signalling (Gillette and Mitchell, 2002). In addition to controlling the rhythms of nearby target sites SCN also controls the output rhythms of different organs by means of humoral pathway.

Humoral pathway: This is a non-neuronal pathway which communicates via diffusible signals that can travel in extracellular spaces and/or cerebrospinal fluid (CSF) and through circulation. Cells of SCN release several peptides like AVP, VIP, GRP, Prokineticin 2 and SS into extracellular spaces and CSF (Reghunandanan and Reghunandanan, 2006). A diffusible molecule transforming growth factor (TGFα) synthesized rhythmically in the SCN controls the activity rhythms by the SCN (Silver *et al.,* 1996).

Thus regulation of physiological and behavioral rhythms of an organism involves either neural or humoral outputs or both the outputs from the SCN. The circadian rhythms of the peripheral clocks located in different organs are mainly controlled by the humoral output of the SCN.

Peripheral clocks:

The central pacemaker, SCN regulates the functions of other peripheral organs of the body through its efferent pathways. Peripheral clocks are located in heart, intestine, kidney, liver, lungs and gonads. These peripheral organs also contain their individual circadian clock that is similar to the one present in SCN neurons, but only the SCN shows self-sustainity. Though peripheral

clocks generate circadian rhythms by similar mechanisms as that of the SCN which exhibit same phase relationship (Balsalobre *et al.,* 1998), the events that take place in peripheral clocks are not identical to those occurring in SCN neurons (Oishi *et al.,* 2000). Peripheral clocks exhibit 4 h delay in their circadian gene expression as compared to SCN, suggesting that there is a master-slave relationship between the SCN (master/central clock) and peripheral clocks (Balsalobre *et al.,* 1998). Oscillations of peripheral clocks (2-7 days) dampen very rapidly as compared to SCN (approximately one month) *in vitro* (Yamazaki *et al.,* 2000). The major difference between the central and peripheral clocks is that SCN generates, regulates and entrains rhythms to external cues independently whereas peripheral clocks require SCN output signals to entrain their circadian rhythms (Balsalobre, 2002) which are under the control of SCN.

Generation and entrainment of rhythms is a very complex process that involves a large number of neurotransmitters (serotonin, glutamate, GABA, acetylcholine) a variety of gene expression (clock related genes) and many biochemical processes like phosphorylation. Among many neurotransmitters, serotonergic neurotransmission is important in mammalian circadian clock function and it is implicated in both photic and non-photic regulation of circadian rhythms (Jiang *et al.,* 2000).

***The Pineal Gland*:**

The circadian clock passes on the information to the target organs by efferent pathways through effector follower system. Pineal gland is an important effector follower system in vertebrates and a neuroendocrine gland which secretes the hormone, melatonin (Ganguly *et al.,* 2002). It originates from neural tube and is located at the border between mesencephalon and diencephalon of brain. Lower vertebrates have a single pineal originated intracranially (Fejér *et al.,* 2001). The pineal gland acts as a central clock in a wide range of non-mammalian vertebrates (Wang and Tong, 2004). In reptiles the circadian organization is multi-oscillatory in nature. The retinae, the pineal, the parietal eye and possibly, SCN of the hypothalamus contain

circadian clocks. In these animals, retinae of lateral eyes, pineal and parietal eye all contain photoreceptors (Tosini *et al.,* 2001). Birds have a single pineal located intracranially and developed from epithalamus region (Fejér, *et al.,* 2001). Many of the clock genes are found in avian pineal gland. The temporal profiles of clock gene expression of avian pineal gland are more similar to those observed in the mammalian SCN (Wang and Tong, 2004). The chick pineal gland contains intrapineal photoreceptors and hence it is directly light sensitive (Korf *et al.,* 1998). Mammalian pineal gland is composed of five cell types: (a) pinealocytes (b) interstitial cells, (c) perivascular phagocytes, (d) neurons, and (e) peptidergic neuron-like cells (Møller and Baeres, 2002). Pinealocyte contains the enzymes required for the synthesis of melatonin. Mammalian pinealocytes are derived evolutionarily from the pineal photoreceptors of lower vertebrates. It mainly consists of large cone shaped pinealocytes. Interstitial cells are smaller than pinealocytes, star shaped cells with long and slender processes. Phagocytic cells are mostly confined to perivascular spaces. Neurons in the pineal gland are parasympathetic neurons. Peptidergic-neuron like cells are found to be immunoreactive to vasopressin (Badiu *et al.,* 1999) and oxytocin (Badiu *et al.,* 2001). In mice, the clock proteins that are required for normal rhythm generation by the SCN are also found to be present in pineal gland (Karolczak *et al.,* 2004).

The activity of the pineal gland is regulated by environmental light acting via the nervous system (Zawilska *et al*., 2006). The most important function of mammalian pineal gland is to transmit light information into chemical message to the rest of the organs of the organism. Hence it is called as a neuroendocrine transducer which converts a neural signal to a hormonal signal (Pandi-Perumal *et al.,* 2006). The neural input to the gland is NE and the output is melatonin (Brzezinski, 1997). Axons of SCN neurons innervate into the hypothalamic PVN. Fibers from PVN synapse with the neurons of intermediolateral (IML) column of the spinal cord. The neurons of PVN also innervate the superior cervical ganglia (SCG). The peripheral sympathetic tract arising from the SCG innervates the pinealocytes. Thus endogenous circadian rhythm of melatonin is generated in the SCN and entrained

principally by the light-dark cycle acting via RHT (Arendt, 1998). Melatonin produced in the pineal gland plays an important role in transducing the signal of darkness throughout the body. It also forms a feedback loop with the SCN (Masana *et al.,* 2000).

Molecular events in a SCN neuron:

The molecular basis of circadian timing forms an important model for understanding the cellular and molecular events connecting genes to behaviour. The molecular clockwork in SCN is cell autonomous in nature. Light-dark cycle influences the induction and expression of clock genes and thus the generation of rhythms. The light information received by the retina of the eye is conveyed to the SCN via RHT through glutamate. When glutamate binds to its receptors in the SCN, there is an increase in intracellular Ca^{2+} levels which results in the activation of CaMKII as well as mitogen activated protein kinase (MAPK). The enzyme in turn activates nitric oxide synthase (NOS). Then NOS increases nitric oxide levels and guanynyl cyclase activity which later induces cyclic GMP (cGMP) dependent kinases (cGKs). The MAPKs and cGKs phosphorylate cyclic AMP response element binding (CREB) protein. Brief exposure to light during subjective night dramatically and rapidly increases CREB phosphorylation in the SCN (within 10 minutes (min) after light onset). CREB then binds to cAMP response elements (CRE) containing immediate early genes (IEGs) such as *c-fos*. The expression of late response genes such as clock genes is later induced by *c-fos* (Golombek *et al.,* 2004). Thus when a cell is stimulated, the first wave of gene transcription at the molecular level involves IEG activation.

The IEGs are the genes whose transcription is activated rapidly and transiently within minutes of stimulation (Greenberg *et al.,* 1992). These include *c-fos*, *jun*, *ngfi-A* etc. Transcriptional induction is independent of new protein synthesis but shut off of transcription requires new protein synthesis. The mRNAs transcribed from these genes often have a very short half-life (Sheng and Greenberg, 1990). The proteins encoded by the IEGs are deoxy ribonucleic acid (DNA) binding proteins. Different effects of various

extracellular stimuli on cell physiology are mediated by activation of distinct subsets of IEGs (Bartel *et al.,* 1989). Recent reports suggest that IEGs are involved in the phase-shifting response to light. There is a relationship between the IEG expression and phase-responsiveness of the circadian pacemaker in the SCN as changes in mRNA levels of these genes are necessary for phase-shifting response. This shows that IEG expression is part of the molecular pathway responsible for the behavioral changes (Sutin and Kilduff, 1992). Once translated the protein products of these IEGs re-enter the nucleus and form various complexes, collectively termed as activator protein-1 (AP-1) which bind in a sequence specific manner to recognition sites on many different genes, thereby regulating the transcription of 'late response' target genes.

The c-fos is one of the IEGs that convey light-responsive signals to the SCN. It is a member of *fos* proto-oncogene family which also includes *fos*-like genes *fra-1*, *fra-2* and *fos B* (Milde-Langosch, 2005). The expression *c-fos* is involved in entrainment to the environmental light-dark cycle (Schwartz *et al.,* 2000). The *c-fos* is transiently induced by growth factors, hormones, neurotransmitters and other extracellular signals in a wide variety of systems (Müller, 1986). It has been associated with a variety of physiological functions including proliferation, differentiation and neural excitation (Morgan and Curran, 1991). In different cell types the expression of *c-fos* gene is regulated by three intracellular messenger systems: the Ca^{2+}/phospholipid-dependent protein kinase C (PKC), cyclic adenosine-3′, 5′-monophosphate (cyclic AMP/cAMP) and Ca^{2+}/calmodulin dependent protein kinase II (CaMKII) (Morgan and Curran, 1986).

In the rodent brain, *c-fos* is anatomically restricted to neural elements involved in the photic entrainment of circadian rhythms (Schwartz *et al.,* 1995). Apart from the SCN, the only structure in which light pulses induce *c-fos* is the IGL. It is used as an *in vivo* marker of the SCN intrinsic rhythmicity and photic sensitivity.

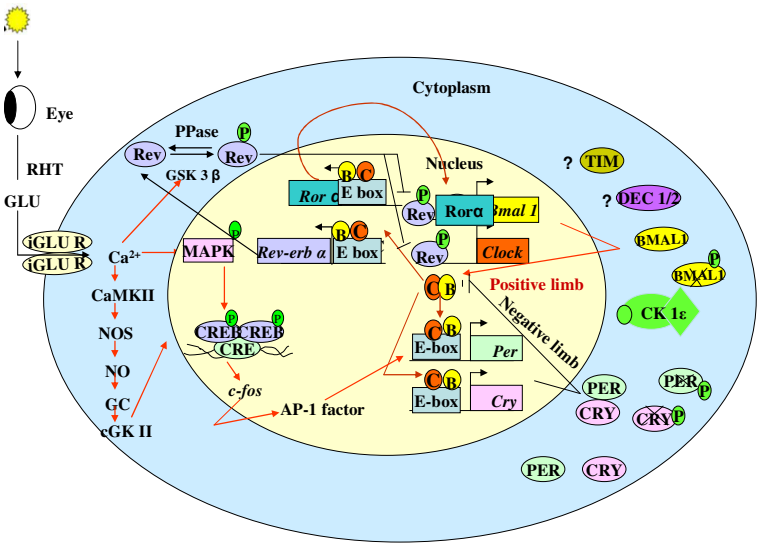
The genes *c-jun, jun-B* and *jun-D* are structurally related to *c-fos* and encode a family of proteins that belong to a large class of DNA-binding

proteins (Bohmann *et al.,* 1987). They are defined by a common structural motif (bZIP) which is composed of a leucine repeat domain (α helix with leucine residues spaced 7 aminoacids apart) (Landschulz *et al.,* 1988) and a domain consisting of highly basic aminoacids of approximately 30 residues lying immediately N-terminal to the leucine repeat. Proteins of this class form dimers when their leucine repeat domains associate as a coiled coil (the leucine zipper) allowing the basic domains to contact DNA for sequence-specific binding (Kouzarides and Ziff, 1988). The bZIP motifs of the Jun proteins are located at their C-terminal regions and appear to be conserved, whereas their N-terminal parts are believed to be responsible for transcriptional activation. Two regulatory elements within the promoter region of *c-fos* gene mediate second messenger effects on *c-fos* transcription. Calcium and cAMP converge to form calcium/cAMP response element (Ca/CRE) (van Haasteren *et al.*, 1999). This is located 60 bp upstream of the transcription start site of *c-fos* gene. In addition to the above, serum and growth factors act through serum response element (SRE) 300 bp upstream of *c-fos* start site (Gilman, 1988).

Light induced expression of *c-fos* is restricted to retinorecipient (Edelstein *et al.,* 2000) ventrolateral part of SCN. Transcription of *c-fos* reaches its peak levels within 30 min after stimulation. It encodes a nuclear phosphoprotein c-Fos which is a part of a sequence-specific DNA binding protein complex AP-1, that regulates transcription of a gene containing AP-1 binding site. It doesn't bind to the AP-1 DNA site on its own (Halozonetis *et al.,* 1988). It forms dimer with the members of another family of IEG, like Jun (Chiu *et al.,* 1988) interacts with DNA at AP-1 binding sequences and modulates the transcription of specific target genes. It preferentially binds to the DNA consensus sequence TGA (C/G) TCA when it is complexed with protein Jun (Rauscher *et al.,* 1988). Both c-Fos and Jun of the heterodimer are responsible for transcriptional activation (Angel *et al.,* 1989). Dimerization of c-Fos with c-Jun enhances the transcription of downstream genes, whereas dimerization of c-Fos with Jun-B inhibits the transcription (Diamond *et al.,* 1990). This c-Fos-Jun complex is also found to act at the cAMP response

element (CRE) present on the DNA of many genes (Ryseck and Bravo, 1991). Understanding the cellular events occurring in the entrainment of circadian rhythms by *c-fos* is important as it is a component of a DNA-binding complex that regulates transcription of many target genes and plays a role in coupling external stimuli to long term cellular responses in other signal transduction programs (Morgan and Curran, 1988).

Clock genes are responsible for the generation and regulation of rhythms which include *Circadian locomotor output cycles kaput (Clock), Brain-muscle-Arnt (Aryl hydrocarbon receptor nuclear translocator)-like-protein 1 (Bmal1* also called *Mop3), Period (Per) 1, 2* and *3, Cryptochromes (Cry 1* and *2), Timeless (Tim), Differentiated embryo chondrocyte* expressed genes *(Dec 1* and *2), Rev-erbα* and *Rorα.* Most of these clock genes are expressed in a well coordinated manner within a circadian cycle. Mutations in clock genes affect the persistence and period length of circadian rhythmicity. In mammals, molecular clock work of SCN consists of interacting positive and negative transcriptional/translational autoregulatory feed back loops (Albrecht, 2004; Ko and Takahashi, 2006) (Fig. 5).



(Albrecht, 2004; Ko and Takahashi, 2006)

Fig. 5: Molecular events in a SCN neuron

Positive feed back loop: The *Clock* in mammals is expressed at constant levels throughout the day (Balsalobre, 2002). It was the first clock gene cloned in mammals (King *et al.,* 1997). The two genes, *Clock* and *Bmal1* belong to the members of the basic-helix-loop-helix (bHLH)-PER-ARNT-Single minded (SIM) (PAS) family of proteins and constitute the positive loop. *Bmal1* transcription starts in the dark phase and its mRNA peaks from CT-15 to CT-18 and there occurs a 4 to 6 h delay in its protein rhythm. Increased availability of BMAL1 promotes CLOCK-BMAL1 heterodimerization which occurs at the start of the circadian cycle (CT-0). In the heterodimer, BMAL1 binds to E-box enhancer elements (Hogenesch *et al.,* 1998; Takahata *et al.,* 1998) with a specific nucleotide sequence CACGTG (Darlington *et al.,* 1998) present in the promoter region and CLOCK is essential for the transcriptional activation (Gekakis *et al.,* 1998) of several clock genes such as three *Per1, 2* and *3*, (Takumi *et al.,* 1998) two *Cry1* and *2*, (Okamura *et al.,* 1999) two *Dec1* and *2*, (Honma *et al.,* 2002), *Rev-erbα* (Preitner *et al.,* 2002) and probably *Rorα* (Sato *et al.,* 2004) genes. The protein product of *Rorα* induces *Bmal1* transcription (Sato *et al.,* 2004), whereas *Rev-erbα* represses *Bmal1* transcription. Each of the *Per* gene mRNAs exhibit distinct temporal profiles, *Per1* mRNA rhythm peaks from CT- 4 to 6, *Per3* mRNA from CT- 4 to 8, *Per2* at CT- 8 and *Cry1* at CT- 10.

Negative feed back loop: Translation of PER and CRY proteins form multimeric complexes which then translocate into nucleus. In the nucleus, PER and CRY proteins act as negative regulators by directly interacting with CLOCK: BMAL1 heterodimer at mid circadian day (CT-12) to inhibit their own transcription. At the same time PER2 contributes to rhythmic transcription of *Bmal1*, which expresses a phase opposite to *Per/Cry*. Availability of BMAL1 appears to be rate-limiting and critical step in the clock work to start a new circadian day (Reppert and Weaver, 2001). The stability of PER2 is under the control of CRY proteins (Yagita *et al.,* 2002). Transcription of *Rev-erbα* is negatively regulated by PER and CRY proteins (Preitner *et al.,* 2002).

The genes *Cry1* and *Cry2* in mammals are homologous to plant and *Drosophila cryptochromes* which act as blue-light photoreceptors. The CRYs are pterin/flavin-containing proteins that are structural homologs of the DNA repair enzyme DNA photolyase, but they lack DNA repair activity (Cashmore *et al.,* 1999). Dimerization of CRY1 and CRY2 with PER proteins help in the nuclear translocation of PER proteins (Kume *et al.,* 1999) and the resulting complex regulate their own expression (Shearman *et al.,* 2000). Homologs of *Per* genes are *Drosophila period* genes. There is a PAS domain on PER proteins that allow them to form a heterodimer with CRYs (Shearman *et al.,* 2000). They do not bind to DNA on their own as they lack DNA-binding motifs (Shearman *et al.,* 1997). Mammalian homolog of *Drosophila clock* gene, *Tim* is also believed to play a role in the clock mechanism by interacting with PER (Barnes *et al.,* 2003). However, its function in mammalian clock is not yet clear but knock out of this gene was found to be embryonic lethal (Gotter *et al.,* 2000). The function of CLOCK/BMAL-1 is inhibited by *Dec* genes (Kawamoto *et al.,* 2004).

Once synthesized, protein products of these clock genes undergo post-translational modifications which determine their stability and thus concentration in the cytoplasm, interaction with other proteins and their cellular location. Translational mechanisms like phosphorylation (Lee *et al.,* 2001), degradation (Vielhaber *et al.,* 2000) and nuclear translocation (Yagita *et al.,* 2002) controls the period of oscillations of clock proteins. Clock proteins such as CLOCK, BMAL1, PER 1 and 2, CRY 1 and 2 undergo phosphorylation by casein kinase Iε (CkIε) and also by casein kinase Iδ (CkIδ) probably (Kondratov *et al.,* 2003). The enzyme, CkIε is an ortholog of *Drosophila* DOUBLETIME (DBT). In addition to CRYs, phosphorylation state of PER 1 by CkIε alters its cellular location (Takano *et al.,* 2000). Other kinases such as MAPKs and glycogen synthase kinase-3 (GSK3) also phosphorylate clock proteins (Sanada *et al.,* 2004). CaMKII and MAPKs are involved in *Per* expression. Expression of many of these clock genes are regulated by external cues which are conveyed by several neurotransmitters via different afferent pathways to the SCN.

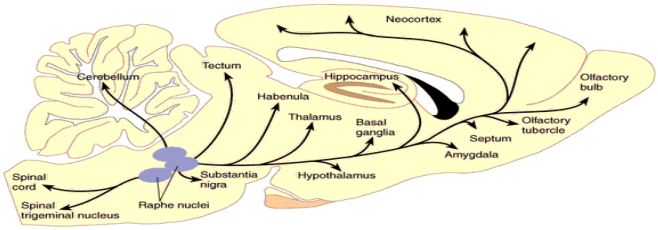
Serotonin:

Serotonin (5-Hydroxytryptamine (5-HT)), a biogenic amine is a neurotransmitter found in a wide variety of sites in the central and peripheral nervous systems (CNS and PNS) (Jacobs and Azmitia, 1992). It was first isolated from serum (sero) as a vascular constricting factor (tonin) (Azmitia, 2002). Hence it is called 'Serotonin'. It is mainly synthesized by the reticular neurons that arise from ancient groups of cell bodies in the brain stem known as raphe nuclei. Some of the raphe cells contain 5-HT and SP, a neuroactive peptide whereas other raphe nuclei contain 5-HT and leu-enkephalin or met-enkephalin/thyrotropin releasing hormone (TRH).

Serotonergic neurons are one of the first brain stem neurons to emerge early in the development of brain and spinal cord, two weeks after gestation and first neurons to differentiate in the brain stem raphe of rats. Raphe neurons synthesize 5-HT one day after their generation. A glial functional protein, S-100β stimulates growth of serotonergic neuron system. It acts as serotonergic neurotrophic factor. Levels of S-100β exhibits clear circadian variation. It even influences most aspects of neural development including neuronal cell division, migration, morphogenesis and synapse formation (Lipton and Kater, 1989).

These neurons diffuse throughout the brain and thus affect various brain functions (Morin, 1999) (Fig. 6). Serotonergic fibers interact in complex

► Schematic Midsagittal Section of a Rat Brain, Showing the Locations of the Most Important Groups of Serotonergic Neurons and the Distribution of Their Axons and Terminal Buttons



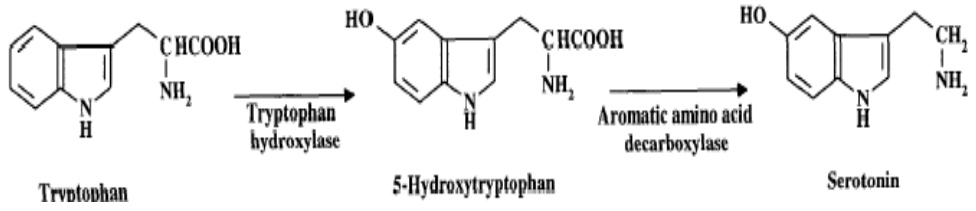
(Morin, 1999)

Fig. 6: Distribution of Serotonergic neurons in rodent brain

ways with various cell types like neurons, glial cells, endothelial cells, ependymal cells and others through their receptors. It also interacts with many other neurotransmitters, either directly through neurons that use both serotonin and other neurotransmitter or by serotonin neurons influencing neurons that primarily use these other neurotransmitters (Azmitia, 2002).

Synthesis of Serotonin:

Serotonin is synthesized from an indole based essential aminoacid, L-tryptophan (Fig. 7). This aminoacid is obtained from dietary sources, contains an indole ring which is unique in light absorbing properties. It is the least common aminoacid in natural proteins. This is one of the essential aminoacids required for *de novo* protein synthesis. It helps in creating a highly lipophilic environment in the protein folds. It is also necessary for the synthesis of kynurenic acid (a neuronal antioxidant) and the reducing cofactors nicotinamide adenine dinucleotide reduced (NADH) and nicotinamide adenine dinucleotide phosphate reduced (NADPH) transfer of tryptophan to the brain competes with several other neutral aminoacids such as phenylalanine, tyrosine, methionine, threonine, leucine, isoleucine and valine. Most effective competitor of tryptophan is phenylalanine (Azmitia, 2002). Tryptophan passes through the blood brain barrier by a carrier protein called neutral amino acid carrier (NAAC).



(Azmitia, 2002)

Fig. 7: Biosynthesis of Serotonin

Tryptophan hydroxylase (TPH), rate limiting enzyme involved in the synthesis of serotonin (Garau *et al.,* 2006). The enzyme, TPH converts tryptophan to 5-hydroxytryptophan (5-HTP) in the presence of oxygen and a pteridine cofactor, tetrahydro-biopterin (BH_4). It is found only in cells that synthesize 5-HT (Boadle-Biber, 1993), the raphe neurons, the pineal gland and enterochromaffin (EC) cells of the gastro-intestinal (GI) tract and thus controls serotonin levels. It exists in two isoforms, TPH1 and TPH 2. The isoform TPH1 is found in pineal and gut whereas TPH2 is found in brain (Sakowski *et al.,* 2006). Enzyme contains 444 aminoacids with a molecular weight of 51 kDa and is 50% homologous with tyrosine hydroxylase, the rate limiting enzyme in catecholamine biosynthesis (Azmitia, 2002). The enzyme aromatic L-aminoacid decarboxylase (AADC) converts 5-HTP into serotonin. This enzyme is present both in serotonergic and catecholaminergic neurons (Frazer and Hensler, 1993).

The activity of tryptophan hydroxylase is regulated by the post translational modification, phosphorylation of the enzyme. This phosphorylation is carried out by CaMKII and cyclic adenosine monophoshate (cAMP)-dependent protein kinase (Banik *et al.,* 1997). These enzymes get activated whenever serotonergic neurons are firing. The phosphate ion is obtained from adenosine triphosphate (ATP) (Azmitia, 2002).

Serotonin storage, release and uptake:

Once synthesized, serotonin is stored in synaptic vesicles, located near the axonal release sites. Before it is stored in the vesicles, 5-HT is protected from its degradative enzyme in the cytosol by a 5-HT binding protein (Tamir and Gershon, 1979). A transporter protein called vesicular monoamine transporter 2 (Vmat 2) packages 5-HT into synaptic vesicles. These vesicles contain a specific protein called serotonin binding protein (SBP) that binds 5-HT with high affinity. The binding of 5-HT to SBP depends on the phosphorylation status of SBP (Aldersberg *et al.,* 1987). When action potential reaches the terminals and calcium enters the cell, a kinase called SBP-kinase, whose activation is Ca^{2+} dependent phosphorylates SBP. Phosphorylated SBP

inhibits the binding of 5-HT to it. Under this condition, vesicles fuse with the plasma membrane and 5-HT along with SBP is released into extracellular matrix by exocytosis (Sanders-Bush and Martin, 1982) and interacts with 14 distinct receptors. The release of 5-HT in the SCN is photically regulated. The activity of 5-HT is terminated by binding of 5-HT molecules to specific transporter proteins, 5-HTR located on serotonergic neurons. It is a plasma membrane glycoprotein that controls the synaptic concentration of 5-HT by selectively removing 5-HT from the synaptic cleft. Glial cells are also capable of taking up serotonin by a high-affinity transport system. High affinity transporter of serotonin (SERT) and Vmat 2, transporters of 5-HT are present in non-serotonergic neurons allowing them to capture 5-HT that is released or leaked out from the 5-HT producing neurons. The uptake of serotonin is an active process i.e. temperature dependent and requires external Na^+ and Cl^-.

Catabolism of Serotonin:

Serotonin that is synthesized is either converted to melatonin, a neurohormone or it is first degraded to 5-hydroxyindole acetaldehyde (5-HIA) by the enzyme monoamine oxidase-B (MAO-B) in brain (Fagervall and Ross, 1986). This is again either reduced to 5-hydroxytryptophol by the NADH-dependent aldehyde reductase or oxidized to 5-hydroxyindole acetic acid (5-HIAA) by the enzyme NAD^+ dependent aldehyde dehydrogenase. Oxidation or reduction of 5-HIA depends on the NAD^+/ NADH ratio present in the tissue. The primary metabolite of serotonin in brain is 5-HIAA (Azmitia, 2002).

Serotonergic Receptors:

Once released into extracellular matrix, serotonin acts on distinct receptors to exert its diverse physiological functions. Fifteen genes have been known to encode 5-HT receptors in mammalian brain (van Hooft and Yakel, 2003). Two are 5-HT gated ion channel receptors ($5\text{-}HT_{3A}$ and $5\text{-}HT_{3B}$) and the rest are G-protein coupled receptors (Raymond *et al.,* 2001). Many of these receptors are broadly distributed throughout the central nervous system

(Uphouse, 1997). They are found on all types of neurons, glial cells and astrocytes. Of the multiple receptor subtypes described, binding sites have demonstrated the presence of 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2C}, 5-HT_{5A} and $5HT_7$ receptors in the SCN.

The 5-HT_{1A} receptors are expressed early in the embryonic life mainly in the raphe neurons, hippocampus and transiently expressed in motor neurons and cerebellum after birth (Talley *et al.,* 1998). Activation of these receptors stimulates neurogenesis in dentate gyrus and in subventricular zone (Brezun and Daszuta, 2000). 5-HT_{1B} receptors are expressed early in development. They are expressed in raphe nucleus, striatum, cerebellum and the RGCs (Boschert *et al.,* 1994). They are localized presynaptically on axon terminals and modulate the release of glutamate in relation to incoming neural activity. They affect axon growth (Lotto *et al.,* 1999). In mammals these receptors inhibit cAMP production and calcium entry in axon terminals (Chen and Regehr, 2003).

Serotonin's effect on circadian rhythm generation and regulation has been extensively studied. Serotonin's effect on photic responses in SCN and IGL are mediated by 5-$HT_{1A/\ 7}$ and 5-HT_{1B} receptors. Also 5-HT_{5A} receptors are present in the four important components of the circadian timing system, the SCN, IGL, DRN and MRN of syrian hamster. Immunoreactivity of 5-HT_{5A} receptor is co-localized with serotonin immunoreactivity. This receptor plays an important role in the serotonergic regulation of circadian time keeping and it also acts as a presynaptic autoreceptor regulating serotonergic neuronal activity (Duncan *et al.,* 2000). In the SCN, 5-HT_7 receptors mediate serotonergic induction of phase shifts (Lovenberg *et al.,* 1993).

Physiological functions of serotonin:

Serotonin exhibits wide range of biological and behavioral functions, including aggression, appetite, sex, locomotor activity, learning and memory, sleep, thermoregulation, cerebral blood flow, hormonal secretion (Azmitia and Whitaker-Azmitia, 1991) than any other neurotransmitter in brain which is mediated through its receptors. It is involved in peristaltic movement and

initiating secretory reflexes in the gastrointestinal tract. Serotonin is implicated in a variety of illnesses such as depression (Graeff *et al.,* 1996), attention deficit disorders (Saudou *et al.,* 1994), Alzheimer's disease, anorexia nervosa, bulimia, autism, schizophrenia. Serotonin is also the precursor of melatonin, the internal zeitgeber.

Serotonin and SCN:

Serotonergic neurons innervate the SCN from the midbrain raphe nuclei that terminate predominantly in the retinorecipient ventrolateral region of the SCN. They form one of the important afferent pathways to the SCN implicated in the modulation of circadian rhythms (Varcoe *et al.,* 2003). Plexus formation of serotonergic fibers in the SCN occurs between 5 to 14 days after birth. Serotonin is known to exert multiple actions on SCN neurons. It regulates SCN neurons by both pre- and post-synaptic inhibitory mechanisms (Jiang *et al.,* 2000). (i) It inhibits the release of glutamate mainly from RHT in the presynaptic terminals. (ii) 5-HT reduces spontaneous and evoked release of GABA from presynaptic terminals. (iii) 5-HT acts directly on the post-synaptic membranes inducing inhibitory action in a subpopulation of SCN neurons. (iv) It induces an excitatory inward current in a subset of SCN neurons. All these actions of serotonin in the SCN are mediated by several receptors which include 5-HT_{1A}, 5-HT_{1B} and few 5-HT_{1C} and 5-HT_{2C} (Prosser *et al.,* 1993). Thus serotonin is involved in phase resetting of the clock.

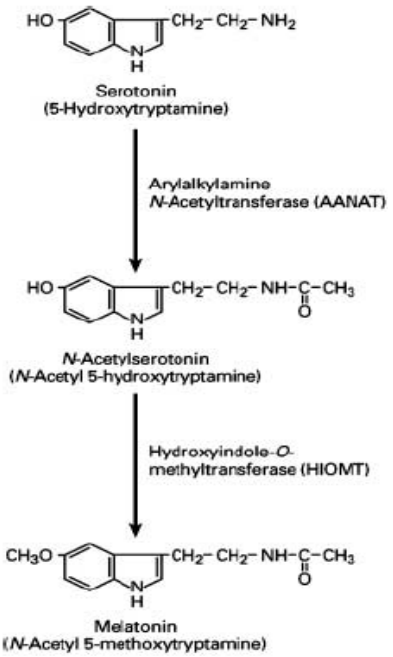
Melatonin:

Melatonin is an ancient hormone, found even in some single-cell organisms and in some plants. Melatonin has been associated with aging as its levels are known to decline upon aging (Rúzsás and Mess, 2000). It is widely used to both characterize and to treat the circadian rhythm disorders (Arendt and Skene, 2005) such as jet lag syndrome. Melatonin, a derivative of serotonin is a neurohormone produced by the pineal gland. This was

discovered by Lerner *et al.,* in 1959 from bovine pineal glands in search of the amphibian skin-lighting factor. Melatonin ('mel' from melanin and 'tonin' means 'to contract'). Melatonin is a low molecular weight (232.3 Da) lipophilic indoleamine hormone. It is diffusible, rapidly carried by blood and cerebrospinal fluid to all tissues of the organism (Moore, 1996).

Synthesis of Melatonin:

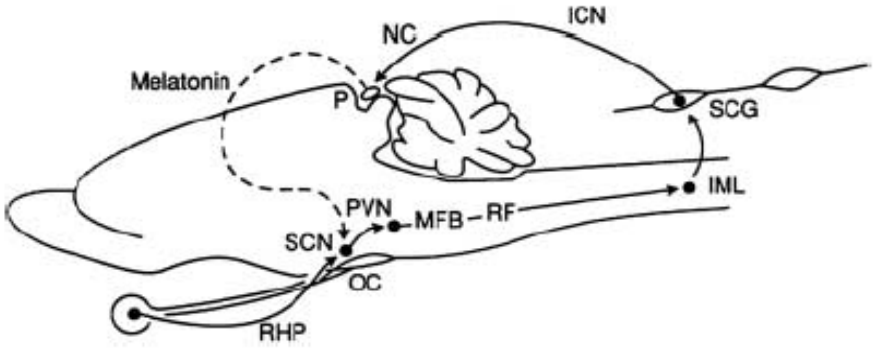
Melatonin is synthesized from serotonin (Fig. 8). Serotonin is converted to *N*-acetyl serotonin (NAS) by the enzyme *N*-acetyl transferase (NAT) in the presence of acetyl coenzyme A (acetyl CoA). The enzyme hydroxy indole-*O*-methyl transferase (HIOMT) converts NAS to melatonin (*N*-acetyl 5-methoxytryptamine) in the presence of *S*-adenosyl methionine (Ganguly *et al.,* 2002). The rhythmic nature of the synthesis and secretion of pineal melatonin are controlled by the light-dark environment, acting through the hypothalamic SCN. Apart from pineal gland, melatonin is also synthesized in retina, harderian gland and gastrointestinal tract (Huether, 1994).



(Ganguly *et al.,* 2002)

Fig. 8: Synthesis of melatonin

A multisynaptic neural pathway from the SCN to the pineal gland controls production of melatonin (Chen and Baler, 2000) (Fig. 9). Its synthesis is driven by the circadian rhythm in NAT also called arylalkylamine N-acetyltransferase (AANAT) (Illnerová *et al.,* 1983). The NAT rhythm is controlled by the SCN which in turn is regulated by light-dark cycle (Klein and Moore, 1979). Projections from SCN innervate PVN of the hypothalamus. Cells from PVN innervate the SCG of the spinal cord. Noradrenergic cells from SCG innervate the pinealocytes of the pineal gland. This sympathetic innervation is known to mediate all biochemical and physiological functions of pineal gland which releases NE. NE release is low during the day and high at nights (Chen and Baler, 2000). NE when released interacts with α_1-adrenergic receptors on the pinealocytes, activates phosphoinositide pathway and enhances intracellular calcium concentration (Vacas *et al.,* 1985). This results in potentiation of β_1-adrenergic receptors on the pinealocytes which increases intracellular cAMP levels, NAT activity resulting in melatonin synthesis (Schomerus and Korf, 2005).



RHP: Retino-hypothalamic projection; OC: Optic chiasm; SCN: Suprachiasmatic nucleus; PVN: Paraventricular nucleus; MFB: Median forebrain bundle; RF: Reticular formation IML: Intermediolateral cell column; SCG: Superior cervical ganglia; ICN: Inferior carotid nerve; NC: Nervi conari; P: Pineal gland.

(Ganguly *et al.,* 2002)

Fig. 9: Regulation of melatonin synthesis

Melatonin secretion is rhythmic, with peak levels occurring in the night irrespective of animal's diurnal or nocturnal activity (von Gall *et al.,* 2002). Melatonin synthesized during night in the pinealocytes does not have any storage site and directly enters into the blood stream through passive diffusion. Thus the circulating melatonin parallel's the activity of pineal gland. This is the major route of transport of endogenous melatonin to its target sites.

Melatonin Receptors:

Melatonin has three types of receptors which belong to two distinct classes of receptors, the seven transmembrane G-protein coupled receptor superfamily (MT_1, MT_2) (Dubocovich and Markowska, 2005) and the quinone reductase enzyme family (MT_3). This makes their function unique at the molecular level. In mammals majority of melatonin receptors reside in the SCN (Reppert *et al.,* 1996). The sensitivity of receptors to specific cues fluctuates throughout a 24 h cycle and their sensitivity can be modulated in a homologous fashion, i.e. by melatonin and in a heterologous fashion, i.e. by other cues such as photoperiod and estrogen. Melatonin receptors also exhibit variation in their density throughout the 24 h cycle which is out of phase with circulating melatonin levels. MT_1 and MT_2 receptors couple to multiple and distinct signal transduction cascades and their activation lead to unique cellular responses (Witt-Enderby *et al.,* 2003). Melatonin has highly sensitive and specific binding sites in mammals (Vaněček *et al.,* 1987). There is a great variability in the distribution of melatonin receptors in mammalian brain (Carlson *et al.,* 1991). The SCN contains high affinity melatonin binding sites and pars tuberalis is the most intensely labeled site for melatonin receptors (Weaver *et al.,* 1989) in most of the mammalian species including humans. In addition to SCN and pars tuberalis, melatonin receptors are also found in dorsomedial and ventromedial hypothalamic nuclei, anterior hypothalamus, medial preoptic area, paraventricular thalamic nuclei, hippocampus, cerebral cortex, area prostrema, amygdala and retina of brain (Morgan *et al.,* 1994).

Melatonin released from the pineal activates high affinity melatonin receptors which are located in the SCN and pituitary pars tuberalis. These two

receptor subtypes show 60% homology at the aminoacid level (Reppert *et al.,* 1995). Melatonin has high affinity for MT_1 receptors. It is present in picomolar concentrations. Activation of MT_1 receptors inhibits adenylase cyclase activity in target cells thus inhibiting cyclic AMP production and activates phospholipase Cβ. This is involved in the retinal function, circadian rhythms and reproduction. There are two MT_1 isoforms, MT_{1a} and MT_{1b}. The MT_{1a} is expressed in hypophysial pars tuberalis and SCN, the sites of reproductive and circadian actions of melatonin respectively. The MT_{1b} is expressed mainly in retina and to a lesser extent in brain. Melatonin has low affinity for MT_2 receptors. MT_2 receptor mRNA is also present in rodent SCN (Wan *et al.,* 1999) and hippocampus as well as in human retina and brain (Hunt *et al.,* 2001). MT_2 receptor is present in nanomolar concentrations. Activation of these receptors is coupled to the stimulation of phophoinositide hydrolysis. Melatonin receptors present in the SCN regulate circadian rhythms. High affinity melatonin receptors are also present in pars tuberalis of pituitary, a relay 'station' between the central and peripheral nervous systems. Melatonin affects the endocrine system through pars tuberalis (Morgan, 2000). These receptors regulate reproductive function (Lincoln *et al.,* 2003). Receptors in peripheral tissues regulate cardiovascular function, body temperature etc. (Brugger and Herold, 1995). In CNS, melatonin may modify neurotransmitter function.

Melatonin also acts at intracellular sites. Intracellularly, melatonin binds to cytosolic calmodulin with high affinity (Benítez-King *et al.,* 1993) and may directly affect calcium signaling by interacting with target enzymes like adenylate cyclase and phosphodiesterases and also with structural proteins (Valenti and Giusti, 2002).

Physiological functions of Melatonin:

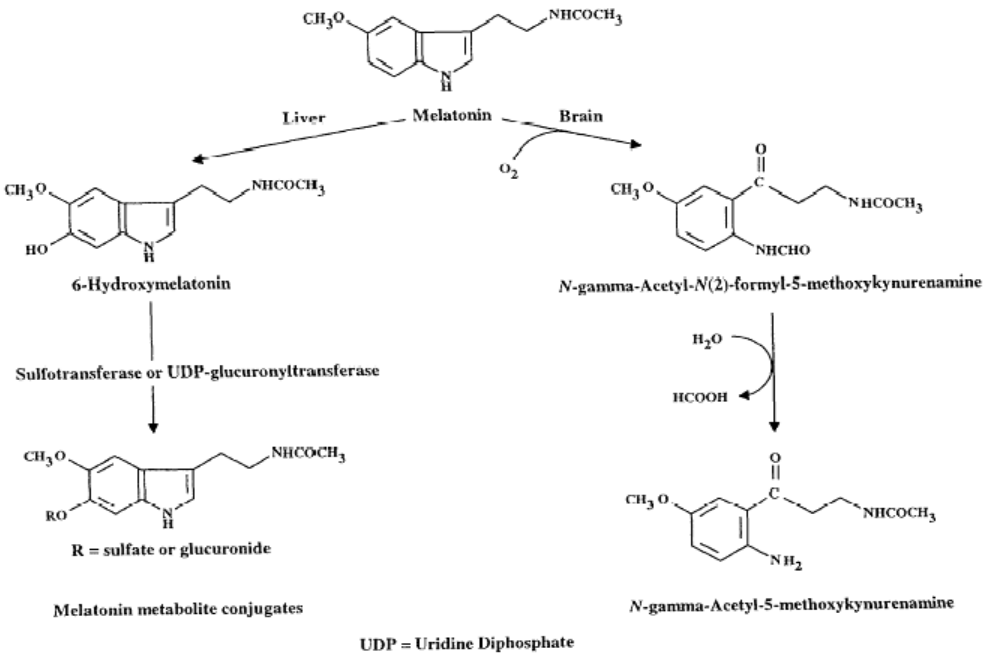
Melatonin activates membrane receptors and putative cytoplasmic and nuclear sites to mediate a variety of physiological responses. Its physiological effects are pleiotropic and it is regarded as "regulator of regulators" (Reiter, 1991). Melatonin has a wide range of biological effects ranging from

physiological to behavioral responses of an organism. The primary physiological function of melatonin is to convey information concerning the daily cycle of light and darkness to body physiology (Claustrat *et al.,* 2005). The most important role of melatonin is the modulation of sleep-wake cycle. Melatonin is a potent free radical scavenger of highly toxic radicals and other oxygen centered radicals (Karasek and Reiter, 2002). Thus it has protective effects against oxidative stress and provides protection from diseases that cause degenerative, proliferative changes by shielding macromolecules especially DNA. Thus it plays an important role in cellular aging, especially in the brain. Melatonin stimulates production of interleukin 4 (IL-4) in bone marrow T-helper cells and granulocyte-macrophage colony stimulating factor (GMCSF) in stromal cells. It protects bone marrow cells from apoptosis induced cytotoxic compounds. It is an anti-cancer agent. It has a wide spectrum of metabolic and other physiological effects including hypothermic, sedative, hypnotic, analgetic, myorelaxing, cardio- and neuroprotective effects (Vijayalaxmi *et al.,* 2002). Physiological functions like metabolism, behavior and reproduction of many vertebrate species depend upon the changes in day length. Melatonin is also known to regulate reproduction, the most important physiological role of melatonin (Underwood and Goldman, 1987).

Metabolism of Melatonin:

Melatonin is metabolized in liver. Circulating plasma melatonin has a very short half-life and 90% of this gets cleared in liver (Huether, 1994) (Fig. 10). Melatonin hydroxylase converts melatonin to 6-hydroxymelatonin that is then converted to a sulfate (60-70%) or glucoronide (20-30%) for urinary excretion (Webb and Puig-Domingo, 1995). In tissues, especially in the central nervous system melatonin undergoes pyrrole ring cleavage. The primary cleavage product is *N*1-acetyl-*N*2-formyl-5-methoxykynuramine (AFMK), which is deformylated, either by arylamine formamidase or hemoperoxidases to *N*1-acetyl-5-methoxykynuramine (AMK). Other oxidative catabolites are cyclic 3-hydroxymelatonin (c3OHM), which can also be metabolized to AFMK. Additional hydroxylated or nitrosated metabolites also appear and represent

minor quantities. AFMK and AMK also form metabolites by interactions with reactive oxygen and nitrogen species (Hardeland *et al.,* 2006).



(Huether, 1994)

Fig. 10: Metabolism of Melatonin

Melatonin and SCN:

In the rodent brain, SCN is a major site of melatonin binding (Dubocovich *et al.,* 1996; Gillette and McArthur, 1996). Melatonin inhibits neuronal firing in SCN, most effectively observed at times of high SCN neuronal activity (subjective day time) as well as in the subjective night, when melatonin levels are normally high (van den Top *et al.,* 2001). This suppression of neuronal activity by melatonin is important for SCN's sensitivity to entraining agents (von Gall *et al.,* 2002). In the SCN, melatonin inhibits phosphorylation of the transcription factor CREB induced by PACAP (Kopp *et al.,* 1997) by MT_1 receptor (von Gall *et al.,* 2000), but does not affect glutamate induced CREB phosphorylation (von Gall *et al.,* 1998). Melatonin can entrain circadian rhythms whose effect is time dependent restricted to dusk (Weaver, 1999). PRC studies by some workers showed that melatonin is

most effective if administered at CT-11 (Cardinali *et al.,* 2002). Generally melatonin levels peak at mid-night. This suggests that endogenous melatonin may contribute to circadian organization but exogenous administration of melatonin can be used as a pharmacological tool for resetting the clock related disorders.

Mode of action of Melatonin:

Melatonin's action is mediated by several mechanisms. It acts by binding to neural and non-neural membrane receptors (Dubocovich, 1995), by binding to calmodulin (Benítez-King and Antón-Tay, 1993) and to nuclear proteins (Steinhilber *et al.,* 1995). Melatonin when it binds to its receptors, there is an influx of Ca^{2+} which then activates calmodulin by binding to it. This Ca^{2+}-calmodulin complex binds to CaMKII and activates it. CaMKII also gets autophosphorylated and both forms phosphorylate intracellular targets such as tryptophan hydroxylase, synapsin I and *c-fos*.

Ca^{2+}/Calmodulin- dependent protein Kinase II:

The enzyme CaMKII is a member of a family of Ca^{2+}/calmodulin-regulated protein kinases which also include Ca^{2+}/calmodulin-dependent protein kinase I, III and myosin light chain kinase and phosphorylase kinase (Nairn *et al.,* 1985). It is also known as synapsin kinase (Kennedy *et al.,* 1983) and glycogen synthase kinase (Payne *et al.,* 1983). It is a multifunctional serine / threonine protein kinase and is one of the most abundant protein kinases in the mammalian brain (McGuinness *et al.,* 1985). In addition to brain, CaMKII is also found in liver (Payne *et al.,* 1983), Woodgett *et al.,* 1983), heart (Iwasa *et al.,* 1986), pancreas (Wang *et al.,* 2005), lungs (Schulman *et al.,* 1985), parathyroid (Kinder *et al.,* 1987), mammary gland (Brooks and Landt, 1985) and intestinal brush border tissue (Reiker *et al.,* 1987) of mammals. Many of its substrates are involved in neuronal signaling. CaMKII modulates both neurotransmitter synthesis and release (Erondu and Kennedy, 1985).

Subcellular Distribution:

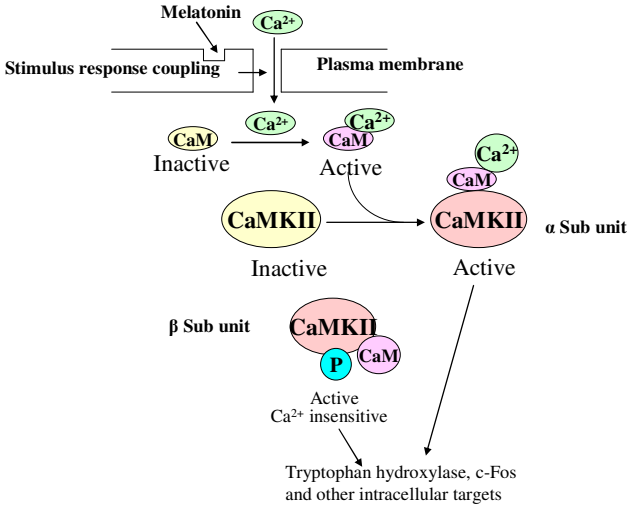
Subcellular distribution of CaMKII varies from tissue to tissue. There are two pools of CaMKII, cytosolic and a particulate pool where the enzyme is associated with certain membranes and cytoskeletal structures like post synaptic density (PSD). This PSD is found to be rich in CaMKII as compared to other subcellular regions (Rostas and Dunkley, 1992). Rostas and Margrie (1997) suggest that both cytosolic and particulate CaMKII exist in dynamic equilibrium *in vivo*, actively regulated by unknown intracellular control mechanisms which in response to many developmental, physiological and pathological stimuli alter the proportions in these fractions. In the neuron, CaMKII is distributed in the spines, somata, axons, dendrites and nerve terminals, with little in the nuclei (Ouimet *et al.,* 1984). There are five isoforms of CaMKII in the rat α, β, β', γ and δ. The aminoacid sequence of these isoforms is highly conserved. The α and β isoforms are primarily expressed in brain (Saha *et al.,* 2006) whereas γ and δ isoforms are expressed in various tissues (Tobimatsu and Fujisawa, 1989).

Structure of CaMKII:

The CaMKII is a heteropolymer with different subunits ranging from 50-62 kDa depending on the type of tissue and species. The subunits have regulatory as well as catalytic functions. In the rat brain, all subunits contain ATP binding, catalytic activity and calmodulin binding domains (Colbran *et al.,* 1989). All of them exhibit 91% homology at N-terminal end, 76% homology at C-terminal region and comparatively less homology in the central region (Bulliet *et al.,* 1988). ATP binding domain and catalytic activity of the enzyme reside in the N-terminal region whereas calmodulin binding domain is located between aminoacid residues 290 and 314 in α subunit. The C-terminal region may be involved in its subcellular localization (Colbran *et al.,* 1989). The determinants for substrate specificity of CaMKII lie in the three arginine residues at N-terminal region (Payne *et al.,* 1983).

CaMKII activation and regulation:

Protein kinases are known to be regulated by a number of mechanisms such as activators like cyclic nucleotides and Ca^{2+}, proteins and peptides (Beale *et al.,* 1977), substrates (Miyamoto *et al.,* 1973) and phosphorylation (Geahlen *et al.,* 1981) etc. CaMKII requires Ca^{2+}/calmodulin for its activity (Fig. 11). In the presence of Ca^{2+}/calmodulin, CaMKII undergoes intramolecular autophosphorylation (Kuret and Schulman, 1985) before phosphorylating any exogenous substrate (Kwiatkowski *et al.,* 1988). The inactive CaMKII attains partially Ca^{2+}-independent form which is completely reversible by treatments with phosphoprotein phosphatases (Lai *et al.,* 1986). In the presence of phosphatases and ATP, enzyme phosphorylates a suitable substrate, thus regulating different physiological processes.



(Ikeda *et al.,* 1991)

Fig. 11: Mode of action of CaMKII

Activation and inactivation of the enzyme is regulated by the regulatory domain. The regulatory domain contains calmodulin-binding domain and inhibitory domain. Calmodulin-binding domain spans from 295-315 amino acid residues in the α subunit (Hanley *et al.,* 1987). Inhibitory domain is located within 281-309 residues, close to the calmodulin-binding domain which supresses the kinase activity in the absence of Ca^{2+}/calmodulin (Kelly *et al.,* 1988).

Ca^{2+}/calmodulin when binds to calmodulin-binding domain of the enzyme induces conformational changes which disrupts interaction of inhibitory domain at ATP-binding site making the enzyme active. Once ATP binds to its respective site kinase will either undergo autophosphorylation or phosphorylate exogenous substrates (Colbran *et al.,* 1989). The phosphorylated enzyme remains active until it is dephosphorylated even after a decrease in Ca^{2+} levels suggesting its active role for a longer duration to transient increase in intracellular Ca^{2+} levels (Ochiishi *et al.,* 1993). Inhibitory domain blocks ATP binding site that is competitive and also autophosphorylation site (Thr^{286}) thus making the enzyme inactive (Kelly *et al.,* 1988).

Physiological functions of CaMKII:

The CaMKII plays an important role in the regulation of the synthesis and secretion of neurotransmitters, receptor function, structural modification of cytoskeletal proteins, microtubule assembly/disassembly, axonal transport and in long term potentiation in the brain (Soderling, 1990). This kinase phosphorylates many proteins *in vitro* which include synapsin (Kennedy *et al.,* 1983), tyrosine hydroxylase (Vulliet *et al.,* 1984), tryptophan hydroxylase (Yamauchi and Fujisawa, 1984) and glycogen synthase (Payne *et al.,* 1983). It also regulates expression of IEGs like *c-fos* and phosphorylation of many proteins and enzymes required for their activation. Thus it acts as one of the important enzymes essential for the generation of rhythms.

SCN is rich in CaMKII and it is known to be involved in transmission of photic information (Weber *et al.,* 1995) and phase resetting of the circadian

clock upon light exposure (Agostino *et al.,* 2004). Recent studies have shown high frequency oscillations in Ca^{2+} in SCN neurons in brain slices. These oscillations alter membrane potential of the SCN neurons that result in membrane depolarization and spontaneous firing of SCN neurons. Phosphorylation of CaMKII is rhythmic both under free-running and entrained conditions with peak levels during the subjective day (Agostino *et al.,* 2004).

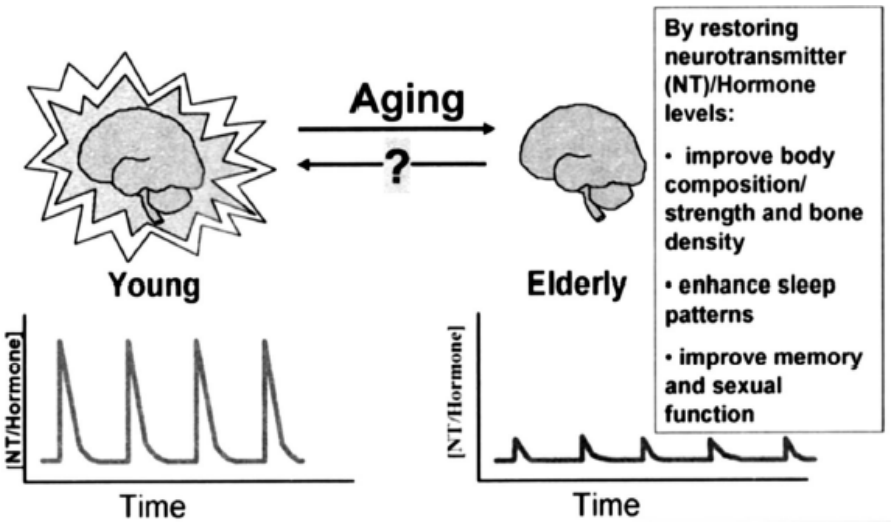
Aging:

Aging is the most important factor that influences or alters the functioning of the circadian timing system. Aging is the progressive deterioration in the functions of an organism (Karasek and Reiter, 2002; Jagota, 2005). These functions are governed by a number of complex interactions among the biochemical, morphological and anatomical aspects of an organism and thus the process of aging is multifactorial. There seems to be a reduction in the complexity of physiological and behavioral control systems with increase in age and in disease conditions (Lipsitz and Goldberger, 1992) due to loss or defect in the control systems (Vaillancourt and Newell 2002).

Aging causes many structural, biochemical, functional and neurochemical changes (Hussain and Mitra, 2000). Biochemical changes like accumulation of pigment called lipofuscin occurs with age. Lipofuscin is a byproduct of autophagia and lipid peroxidation which might interfere with intracellular function. Masses of fibrous substances, neurofibrillary tangles and neuritic plaques are found extracellularly and intracellularly with normal aging. Some workers have reported an increase in transcription of glial fibric acidic protein (GFAP) mRNA in the brain of aging humans and rats (Nichols *et al.,* 1993) that results in the increased amount of GFAP protein. In addition to these various changes, blood-brain barrier also shows increased permeability leading to increased drug sensitivity and susceptibility to pathological conditions.

Neurological changes upon aging have been attributed to the loss of neurotransmitters, their receptors and responsiveness to neurotransmitters (Arivazhagan and Panneerselvam, 2002) (Fig. 12). Degeneration of

monoaminergic neurons (Watabe *et al.,* 2005) and alterations in the metabolism of brain monoaminergic neurotransmitters (Slotkin *et al.,* 2000) were also demonstrated in the aging brain. Neuroendocrine changes occur with aging (Ferrari *et al.,* 2000) and are characterized by changes in pulse, amplitude and irregularity in the periodicity of hormone and neurotransmitter releases that respond to various physiological and behavioral functions (Matsumoto *et al*., 2000). Several reports suggest that alteration of neurotransmitter metabolism might control the process of aging (Goldberg *et al.,* 2004) by the agents that stimulate hypothalamic neuroendocrine transducer cells (Samorajski, 1977). The selective cell death in the brain is also implicated in progressive loss of function, behavioral changes and the onset of age-related diseases. In addition, enzymatic (protein kinases) and metabolic alterations are also present with aging (Jin and Saitoh 1995).



(Smith *et al.,* 2005)

Fig. 12: Decline in the activity of brain during aging

The most widely accepted theory of aging is oxidative stress due to increased free radical generation and several reports suggest a close connection between aging, age-related pathologies and oxidative stress

(Balaban *et al.,* 2005). Recent studies suggest that clock proteins such as BMAL1 and PER are directly involved in regulation of free radical levels in cells and thus control aging (Kondratov, 2007). Many metabolic processes are associated with aging and changes in the metabolic processes induced by metabolic diseases like diabetes and obesity also contribute to the aging process. Recently it was demonstrated that circadian system is actively involved in synchronization of metabolic processes and the control of mammalian energy balance (Kondratov, 2007).

Aging and Circadian rhythms:

Aging affects the circadian timing systems of wide range of animals from invertebrates to vertebrates. *Aplysia*, a mollusc exhibits reduced rhythm amplitude in optic nerve impulse frequency with aging (Sloan *et al*., 1999). In mammals, old mice show delayed activity onsets, take longer time to for phase resetting. They exhibited increased fragmentation in their wheel running activity (Weinert and Waterhouse, 1999). There were disruptions in the phase shifting ability of mice and hamsters to photic (Benloucif *et al*., 1997a) and non-photic stimuli (Van Reeth *et al*., 1993) as a consequence of aging. In older rats, there was reduced amplitude in circadian drinking behavior (Burwell *et al*., 1992), locomotor activity rhythms (Dawson *et al*., 1987) and body temperature rhythms (Li and Satinoff, 1995). In aged individuals, rhythms are less precise, shorter in period, smaller in amplitude, slow in resynchronization to external stimuli (Sharma, 2001).

The circadian clock properties are altered with aging. There is desynchronization of rhythms and the efficacy of input and output pathways to and from the circadian pacemaker and the functioning of the central pacemaker. Aging results in neuronal deterioration (Mirmiran *et al.,* 1992), decrease in protein levels (Laitinen *et al.,* 1992), changes in the rhythms of glucose (Van Cauter *et al.,* 1997), and a reduction of dendritic surface (Swaab *et al.,* 1985). All these changes lead to the aperiodic pattern of firing of circadian rhythms in the SCN neurons (Satinoff *et al.,* 1993). It has been demonstrated that *Bmal1* null mutant mice show early signs of aging. It is an

important protein required for normal tissue homeostasis in mice (Kondratov *et al.,* 2006). Mice mutant for *Per1* and *Per2* showed early onset of aging with faster decline of fertility and loss of soft tissue (Lee, 2005). Witting *et al.,* (1993) correlated age-related changes in circadian rhythmicity with decreased sensitivity of the circadian system to light. According to Aujard *et al.,* (2001), there are several hypotheses to explain the observed decrease in sensitivity to light in the SCN with aging: (1) a modification in the kinetics of the activation of signaling pathways in the SCN; (2) a reduction in the amplitude of photic information transmitted by the retina to the clock; (3) age-related changes within the clock mechanism of the SCN itself. There are several evidences of aging affecting SCN function. These were demonstrated by previous studies which showed disruption of circadian and seasonal rhythms in vasopressin and a progressive loss of vasopressinergic cells with aging (Hofman and Swaab, 1995). The functional activity of the SCN is also altered with a loss of day/night differences in vasoactive polypeptide mRNA levels of aged rats (Kawakami *et al.,* 1997), alteration in glucose utilization (Wise *et al.,* 1988), and in cAMP-response element-binding protein phosphorylation (Zhang *et al.,* 1996).

Objectives

Objectives of our study:

i. Age induced changes in serotonin rhythms in brain and SCN of rat

ii. Effect of melatonin administration on age related changes in serotonin rhythms in the SCN of rat.

iii. Age induced changes and the effect of melatonin administration on *N*-acetyl transferase (NAT) activity rhythms in the SCN of rat.

iv. Age induced changes and the effect of melatonin administration on Ca^{2+}/Calmodulin-dependent protein kinase II (CaMKII) activity rhythms in SCN and Pineal gland of rat.

v. Age induced changes and the effect of melatonin administration on c-Fos levels in the SCN and Pineal gland of rat.

CHAPTER 1

Age induced changes in serotonin rhythms in brain and SCN of rat

CONTENTS

INTRODUCTION:

Neurotransmitters behave as growth regulators (Lauder, 1993) during specific developmental periods (Brezun and Daszuta, 2000) and modulate the construction and plasticity of brain circuits during development (Gaspar *et al*., 2003). Serotonin is the neurotransmitter found to be present in most organisms. Serotonin is synthesized in neuronal as well as non-neuronal tissues like pineal gland, enterochromaffin cells of the gut, neuroepithelial bodies of the lung (Azmitia, 2002). The rate limiting enzyme, tryptophan hydroxylase (TPH) is involved in the synthesis of 5-HT in the neuronal and non-neuronal tissues (Walther *et al.,* 2003). Serotonin affects morphogenesis of gastrointestinal tract, cardiovascular system and craniofacial organization through its $5\text{-}HT_{2B}$ receptor in rat, mouse and chicken (Gaspar *et al.,* 2003).

Serotonergic neurons are the first neurons to be generated, on embryonic (E) days 10-12 in mouse and in primates during the first month of gestation (Levitt and Rakic, 1982). Neurons containing 5-HT are known as B1-B9 cell groups (Dahlstrom and Fuxe, 1964). There are around 20,000 serotonergic neurons as compared to the total 10^{10} neurons in the central nervous system of rat (Jacobs and Azmitia, 1992). These neurons are located in the raphe nuclei, on the midline of rhombencephalon (Dahlstrom and Fuxe, 1964). Neurons of raphe nuclei synthesize serotonin, one day after their generation and profusely extend through their axons to the rostral and caudal ends which project into forebrain and spinal cord respectively (Lidov and Molliver, 1982). Several reports suggest that maternal serotonin determines normal development of fetus (Côté *et al.,* 2007).

Serotonin in Nervous system development:

Development of nervous system arises from the ectoderm of the three germinal layers (Jessell and Sanes, 1991). It is a series of events that involves neuron formation, migration, differentiation, death, synapse formation, process elimination and establishment of function (Moore, 1992). Serotonin (5-

hydroxytryptamine, 5-HT) was the first neurotransmitter known to act as a developmental regulator (Levin *et al.,* 2006) especially nervous system development (Richerson, 2004; Sodhi and Sanders-Bush 2004). Serotonergic neurons are one of the first neurons to emerge and differentiate in the brain of many species (Djalali *et al.,* 2005). 5-HT regulates development of its own neurons (autoregulation) as well as development of target tissues (Whitaker-Azmitia, 2001). It also acts as a trophic factor and influences functional state of neurons in the central nervous system (Djavadian, 2004). Development of several neural networks depends on the action of serotonin on various multiple, hetero- and autoreceptor subtypes (Lesch, 2001). 5-HT receptors are expressed early in embryonic life and are regulated dynamically during pre- or postnatal development. 5-HT acts on different target receptors at different times and in different tissues during development (Gaspar *et al*., 2003). Each and every type of neuron (motor neurons, neurosecretory neurons, ganglion neurons and different types of inter-neurons) receives serotonergic signals and has 5-HT receptors. Its receptors are located on glial cells including astrocytes, oligodendroglial cells and microglial cells. Serotonin is involved in a number of developmental events like cell division (Eddahibi *et al*., 1999), neuronal migration (Lipton and Kater, 1989), neural differentiation (Azmitia, 2001), axon outgrowth (Lesch, 2001), synaptogenesis, synaptic modeling, maturation of synapses (Zhang, 2006), enhancement of synapse refinement in brain (Bethea and Sikich, 2007) and regulation of spontaneous activity (Zhang, 2006). Because of its diverse cellular targets and its receptors, serotonin is involved in an enormous number of functions like appetite, hormonal secretion, locomotor activity, learning and memory (Buhot *et al*., 2000), mood (aggression and anxiety) and sleep (Azmitia and Whitaker-Azmitia, 1991). Alteration in serotonin homeostasis cause permanent changes to adult behavior and modify the fine wiring of brain connections that lead to the pathophysiology of the brain (Gaspar *et al*., 2003). Thus, serotonergic system has been implicated in a variety of illnesses such as depression (Graeff *et al.,* 1996), attention deficit disorders (Saudou *et al*., 1994), anorexia nervosa, bulimia, autism (Whitaker-Azmitia, 2001) and pathological

conditions like Alzheimer's disease, Schizophrenia and hepatic encephalopathy (Azmitia, 2002).

Serotonin in Brain:

Serotonin is extensively distributed in the central nervous system (Jacobs and Azmitia, 1992). In the brain it is present in the raphe nuclei of brain stem. These neurons branch out profusely to every area of the brain and spinal cord by extensive and diffuse collateralization of their axons and have multiple cellular targets. It plays an important role in regulating the development and maturation of mammalian brain through the release of an astroglial protein, S100β. This protein plays a role in neurite extension, microtubule and dendritic stabilization which are key elements in the production of synapses (Mazer *et al.,* 1997). Serotonergic fibres innervate different types of cells such as ependymal cells that line the ventricles, choroid plexus which make cerebrospinal fluid and endothelial cells that form blood vessels (Azmitia, 2002). In the brain the endocrine centres, the pituitary and the pineal gland (Boadle-Biber, 1993) also receive serotonin. Serotonin has multiple physiological functions as a neurotransmitter to a growth factor (Buznikov *et al.,* 2001). It acts as a neuroprotective agent in cortical neurons (Stankovski *et al.,* 2007). Serotonin plays a critical role in the initiation of neurogenesis in hippocampus which is associated with learning, memory and responsible for emotional responses (Chen *et al.,* 2007). Serotonin plays an important role in many physiological functions. All these functions are determined by the identity of cells and tissues which is defined by the genes they express, the time and order of their expression that are under circadian clock, SCN (Hastings *et al*., 2003).

Serotonin in SCN:

Serotonin is an important regulator of the mammalian circadian clock (Garau *et al.,* 2006). Malek *et al.,* (2005) suggested that 5-HT synthesis and release in the median raphe nuclei within the circadian system is under the control of the SCN directly or indirectly. Circadian 5-HT synthesis in

serotonergic neurons projecting to the circadian system is due to the rhythmic transcription of the *tph2* gene in the raphe nuclei (Malek *et al.,* 2005). Serotonin and its agonists have various phase resetting affects on the SCN (Graff *et al.,* 2007). Serotonergic neurons modulate the phase of the circadian clock and this is affected by the amount of prior serotonin signaling present in the SCN. This signaling alters the density of surface 5-HT receptors on SCN neurons (Prosser *et al.,* 2006). Non-photic phase-shifting of mammalian circadian rhythms is partly mediated by serotonin acting in the SCN (Duncan *et al.,* 2005). Serotonin modulates the effects of light on circadian behavior by acting on 5-HT1B receptors on retinohypothalamic (RHT) terminals in the SCN (Sollars *et al.,* 2006). In the SCN, serotonin has a long lasting effect on differentiation of VIP and vasopressin (VP) and 5-HT is involved in the release of these peptides in the SCN (Mirochnik *et al.,* 2005). It is known to stimulate glutamate release which is involved in arginine-vasopressin release, one of the important input pathways from the SCN (Isobe and Nishihara, 2002). Rhythms in serotonin synthesis and release in the SCN of rat has been studied earlier (Barassin *et al.,* 2002). Its afferents are known to modulate VIP and gastrin releasing peptide (GRP) expression in the ventrolateral neurons of the SCN by activating the 5-HT_{1B} receptor in the RHT (Hayashi *et al.,* 2001).

Role of serotonin in Circadian rhythms:

Serotonin is one of the important neurotransmitters with a wide variety of physiological functions in an organism. It is involved in the input pathway of circadian system and rhythm modulation. Regional distribution of extracellular 5-HT and 5-HIAA concentrations had been studied earlier by Adell *et al.,* (1991). The presence of serotonin in discrete areas of rat brain had been demonstrated earlier (Saavedra, 1977).

SCN is one of the important target areas of serotonergic projection. Serotonin is one of the principal neurotransmitters that convey information about external cues through retino-raphe pathway to the SCN. 5-HT influences many aspects of circadian rhythms, including phase shifts, onset of locomotor activity,

period length, integrity of rhythms during exposure to constant light (Duncan *et al.,* 2000) and also in modulation of circadian rhythms in response to photic (Pickard and Rea, 1997) and non-photic stimuli (Cutrera *et al.,* 1994). SCN receives serotonergic projection from median raphe nucleus (Challet *et al.,* 1998). In the SCN, increase in 5-HT release results in behavioral arousal during the subjective day (Grossman *et al.,* 2000). Disruption in serotonergic projections to the SCN has been shown to affect circadian behavioral and neuroendocrine rhythms in rodents (Morin and Blanchard, 1991).

Serotonergic neurotransmission is an important element in the neurochemical basis of circadian rhythm generation. Serotonin also plays an important role in the development of nervous system. In this chapter, serotonin daily rhythms in brain and SCN in various age groups were studied.

MATERIALS and METHODS:

Male Wistar rats of different age groups (15, 30, 60, 90, 120, 180, 270, 365, 545 and 730 day old) were taken and maintained under laboratory conditions, 06.30h (ZT-0)-18.30h (ZT-12) light phase; 18.30h (ZT-12)-06.30h (ZT-24) dark phase, two weeks prior to the experiments. All rats were kept individually in polypropylene cages at room temperature (20+2^{o}C) with relative humidity (55+6%). Food and water were supplied *ad libitum*. Dim red light was used for handling the animals in the dark. Cage changing was done at random intervals. Serotonin levels were measured at various time points (ZT-0, 6, 12, 18 and 24) in the rat brain and SCN by spectrofluorimeter (Hitachi, F-4010).

1) Brain tissue preparation:

Rats were decapitated and brains were removed carefully.

2) SCN tissue preparation:

Rats were decapitated and brains were removed carefully. 500µ brain slices were made using tissue chopper and SCN tissue was carefully punched out

with the help of a sharp scalpel (Gillette, 1986; Prosser and Gillette, 1989).

All chemicals and reagents used in this study were of analytical grade. Standard serotonin was obtained from Sigma chemicals.

3) Fluorimetric determination of Serotonin:

5% tissue homogenate (cold acetone/1N formic acid 95:5 v/v) was made from each sample by using tissue homogenizer (Remi, RQ 127A). 40µl of homogenate was taken and 80µl of 0.01N HCl containing 0.01% of ascorbic acid was added and kept for 30 minutes (min) at 0^{o}C for extraction. This was centrifuged at 1600 rpm for 10 min at -10^{o}C in a refrigerated centrifuge (Remi, C-24). Supernatant was taken and to it 160µl of freshly made heptane/chloroform (8:1 v/v) was added and centrifuged at 1000 rpm for 5 min. Aqueous layer was taken and evaporated to dryness by passing nitrogen gas, obtained locally. To the residue obtained, 40µl of 0.1N HCl containing 0.5% ascorbic acid, 84µl of 2% EDTA, 0.04g of NaCl and 160µl of butyl acetate were added. Contents were shaken for 5 min and then centrifuged at 2500 rpm for 10 min.

Aqueous layer was taken and to 40µl of aqueous layer, 3µl of 2M Na_2CO_3 was added to set the pH at 9.8. Then 100µl of NaCl saturated butanol was added, mixed well and then centrifuged at 2000 rpm for 5 min. Butanol layer was transfered to tubes containing 1ml of borate buffer and centrifuged for 5 min at 2000 rpm. 80µl of butanol layer was taken from the above and 140µl of heptane and 40µl of 0.1 N HCl were added and centrifuged at 2000 rpm for 10 min. Aqueous layer containing serotonin was made 3N by adding 10N HCl and its fluorescence was measured by spectrofluorimetry with an excitation at 300 nm and emission at 545 nm (Fischer and Aprison, 1972; Jagota and Habibulla, 1992) (Fig. 13).

4) Protein Estimation:

Protein estimation for brain samples was done by Lowry's method (Lowry *et al.,* 1951). 20µl of 5% homogenate was used for the protein estimation of each brain sample studied (15, 30, 60, 90, 120, 180, 270, 365, 540 and 730 day old). Each

sample was made to 500µl by adding double distilled water. To this 0.1ml of 2N NaOH was added and incubated for 10 min at 100°C in water bath. Then 1ml of freshly made complex reagent (2% Na_2CO_3, 1% $CuSO_4.5H_2O$ and 2% Sodium potassium tartarate) was added to the above mixture and incubated at room temperature for 10 min. After incubation, 0.1 ml of 1N Folin's reagent was added. This mixture was then incubated at room temperature for 30-60 min and

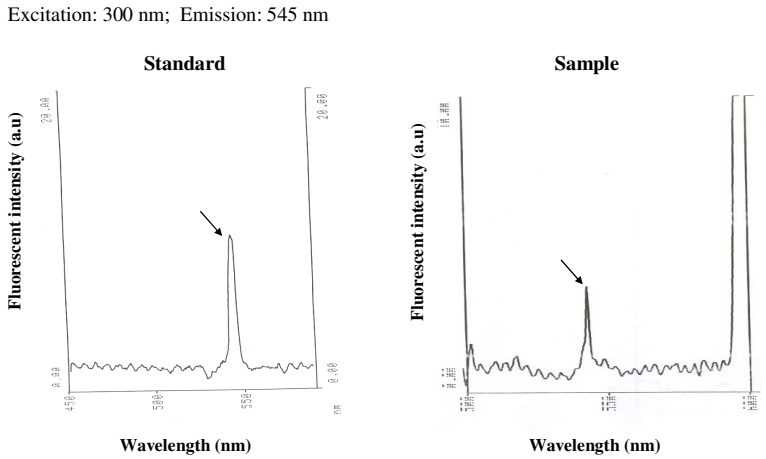


Fig. 13: Spectrofluorimetric assay of Serotonin

absorbance was measured at 550 nm. The standard was prepared using bovine serum albumin (BSA) of concentrations ranging from 10µg to 100µg. 1ml of reagent was used for each standard sample. Standard graph was plotted by taking concentration of protein sample on x-axis against the corresponding absorbance obtained on y-axis.

Protein estimation for SCN samples was done by using Bradford's method (Bradford, 1976) because the sample size of SCN was too less to be estimated by Lowry's method. 10µl of 5% homogenate was used for the protein estimation of each SCN sample in all the age groups of rats studied (15, 30, 60, 90, 120, 180, 270, 365, 540 and 730 day old). Volume of each tissue sample was adjusted to

100µl with double distilled water. 1ml of Bradford's reagent was added to each sample and contents were mixed properly. The absorbance was measured at 595nm after 2 min and before 1hr against a reagent blank of 100µl double distilled water and 1ml of Bradford's reagent. The standard was prepared using bovine serum albumin (BSA) of concentrations ranging from 1µg to 10µg. 1ml of reagent was used for each standard sample. Standard graph was plotted by taking concentration of protein sample on X-axis against the corresponding absorbance obtained on Y-axis.

STATISTICAL ANALYSIS:

One Way ANOVA with Tukey test was done for all the age groups (15D, 30D, 60D, 120D, 180D, 270D, 365D, 545D and 730D at all zeitgeber times) studied by taking 90 day values as control. *t-test* was done to compare maximum and minimum amount of 5-HT/g protein within the same age group and their ratio values were compared in all the age groups studied with 90 day as control.

RESULTS:

Serotonin daily rhythms in the brain:

In this study all the age groups showed a distinct pattern of rhythmicity in brain serotonin levels (Table 1; Fig. 14). 5-HT levels increased from 15 day to 90 day except in 30 day, there was decrease in serotonin levels at all zeitgeber times. Serotonin levels then decreased from 90 day to 730 day. Rhythmicity in serotonin levels was observed in the age groups, 15, 30, 60, 90, 120 and 180 day with highest levels at mid-day (ZT-6) and lowest levels observed at mid-night (ZT-18). Rhythms were abolished in 270 and older age groups up to 730 day. We observed a phase advance in the maximum levels of serotonin at 270 day (ZT-0/24), phase delay at 365 and 545 day (ZT-18) and at ZT-6 in 730 day.

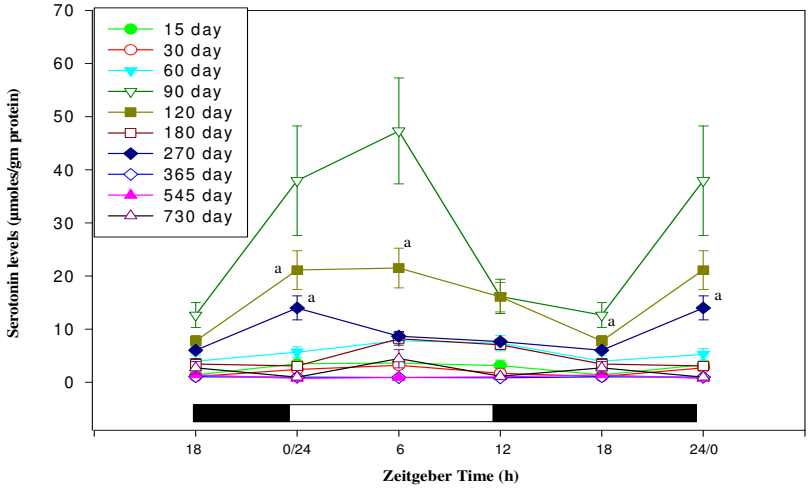
The maximum levels of 5-HT observed were 3.61 ± 0.42, 3.21 ± 0.28, 7.82 ± 0.49, 47.3 ± 9.98, 21.51 ± 3.72, 8.22 ± 1.21, 14.01 ± 2.23, 1.054 ± 0.32, 1.42 ± 0.26 and 4.44 ± 1.73 µmol/g protein in 15, 30, 60, 90, 120, 180, 270, 365,

545 and 730 day respectively (Table 2; Fig. 15). There was a significant difference ($p_a \leq 0.05$) in serotonin levels at ZT-6 in 15, 30, 60, 120, 180, 270, 365, 545 and 730 day as compared with 90 day, adult.

Table 1: Age related changes in daily serotonin rhythms in the rat brain (LD; 12:12)

S.No	Age (days)	Serotonin levels (µmol/g protein) at different zeitgeber times				
		0/24	6	12	18	24/0
1	15	3.49 ± 0.03 [a]	3.61 ± 0.42 [a]	3.14 ± 1.02 [a]	1.37 ± 0.39 [a]	3.31 ± 0.23 [a]
2	30	2.44 ± 0.28	3.2 ± 0.28 [a]	1.68 ± 0.32 [a]	1.07 ± 0.17 [a]	2.74 ± 0.31 [a]
3	60	5.69 ± 1.03 [a]	7.82 ± 0.49 [a]	7.37 ± 1.43 [a]	4.0 ± 1.0 [a]	5.27 ± 1.10 [a]
4	90	37.95 ± 10.29	47.3 ± 9.98	16.17 ± 3.25	12.65 ± 2.35	37.95 ± 10.29
5	120	21.13 ± 3.65	21.51 ± 3.72 [a]	16.06 ± 2.77	7.76 ± 1.03	21.13 ± 3.65
6	180	3.09 ± 0.36 [a]	8.22 ± 1.21 [a]	7.07 ± 0.9 [a]	3.43 ± 1.02 [a]	3.09 ± 0.36 [a]
7	270	14.01 ± 2.23 [a]	8.69 ± 0.87 [a]	7.67 ± 0.48	6.03 ± 0.5 [a]	14.01 ± 2.23 [a]
8	365	0.973 ± 0.13 [a]	0.915 ± 0.23 [a]	0.846 ± 0.07 [a]	1.054 ± 0.32 [a]	0.973 ± 0.13 [a]
9	545	0.773 ± 0.11 [a]	0.91 ± 0.13 [a]	1.028 ± 0.12 [a]	1.42 ± 0.26 [a]	0.773 ± 0.11 [a]
10	730	0.977 ± 0.027 [a]	4.442 ± 1.734 [a]	1.154 ± 0.057 [a]	2.739 ± 1.162 [a]	0.977 ± 0.027 [a]

Each value is mean ± S.E, (n=6); Zeitgeber Time (ZT): ZT-0 = 6.30 h (Lights on); ZT-12 = 18.30 h (Lights off).
One Way Anova: $p_a \leq 0.05$ (a refers to comparison with 90D)



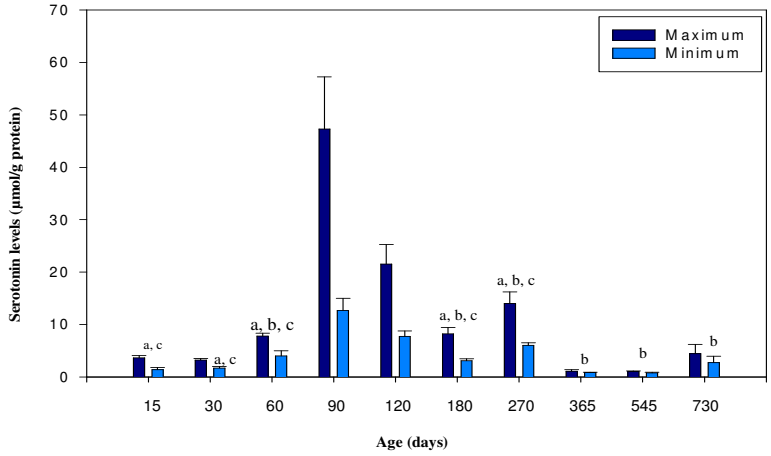
Each value is mean ± S.E, (n=6); Zeitgeber Time (ZT): ZT-0 = 6.30 h (Lights on); ZT-12 = 18.30 h (Lights off).

Fig. 14: Age related changes in daily serotonin rhythms in rat brain (LD; 12:12)

Table 2: Daily pulses of Serotonin levels in the rat brain (LD; 12:12)

Age (days)	Serotonin levels (µmol/g protein)		Ratio Maximum: Minimum
	Maximum	Minimum	
15	3.616 ± 0.427 [a]	1.375 ± 0.393 [a]	4.096 ± 0.476 [c]
30	3.206 ± 0.284 [a]	1.68 ± 0.32 [a]	3.323 ± 0.204 [c]
60	7.82 ± 0.495 [a]	4.0 ± 1.0 [a]	2.818 ± 0.319 [b, c]
90	47.3 ± 9.98	12.65 ± 2.35	4.431 ± 0.505 [c]
120	21.51 ± 3.72 [a]	7.76 ± 1.03	3.49 ± 0.31 [c]
180	8.22 ± 1.21 [a]	3.09 ± 0.36 [a]	2.84 ± 0.18 [b, c]
270	14.01 ± 2.23 [a]	6.03 ± 0.5 [a]	2.45 ± 0.26 [b, c]
365	1.054 ± 0.32 [a]	0.846 ± 0.07 [a]	1.38 ± 0.48 [b]
545	1.028 ± 0.12 [a]	0.773 ± 0.11 [a]	1.33 ± 1.09 [b]
730	4.442 ± 1.734 [a]	2.739 ± 1.162 [a]	1.7 ± 0.448 [b]

Each value is mean ± S.E, (n=6); Zeitgeber Time (ZT): ZT-0 = 6.30 h (Lights on); ZT-12 = 18.30 h (Lights off)
One Way Anova: $p_a \leq 0.05$ (a refers to comparison with 90D)
$p_b \leq 0.05$ (b refers to comparison of ratio values between a given age group and 90D)
t-test: $p_c \leq 0.05$ (c refers to comparison between maximum and minimum values in the same age group)



Each value is mean ± S.E, (n=6); Zeitgeber Time (ZT): ZT-0 = 6.30 h (Lights on); ZT-12 = 18.30 h (Lights off).
One Way Anova: $p_a < 0.05$ (a refers to comparison with 90D)
$p_b < 0.05$ (b refers to comparison of ratio values between a given age group and 90D)
t-test: $p_c < 0.05$ (c refers to comparison between maximum and minimum values in the same age group)

Fig. 15: Daily pulses of Serotonin levels in Rat Brain (LD; 12:12)

Minimum levels of serotonin observed were 1.37 ± 0.39, 1.07 ± 0.17, 4.0 ± 1.0, 12.65 ± 2.35, 7.76 ± 1.03, 3.09 ± 0.36, 6.03 ± 0.5, 0.84 ± 0.07, 0.77 ± 0.11 and 0.97 ± 0.02 µmol/g protein in 15, 30, 60, 90, 120, 180, 270, 365, 545 and 730 day respectively (Table 2; Fig. 15). 5-HT levels at ZT-18 in 15, 30, 60, 180, 270, 365, 545 and 730 day brain were significantly different ($p_a \leq 0.05$) from the adult, 90 day.

Serotonin daily rhythms in the SCN:

We observed age-related changes in serotonin levels and circadian rhythmicity in the SCN of all the age groups studied (15, 30, 60, 90, 120, 180, 270, 365, 545 and 730 day) (Table 3; Fig. 16). There was increase in serotonin levels from 15 day old to 120 day, except in 30 day serotonin levels decreased. With increase in age, 120 day to 730 day serotonin levels decreased.

Serotonin daily rhythms in SCN were observed from 15 day to 180 day with maximum serotonin levels at ZT-6 and minimum serotonin levels at ZT-18 and at ZT-12 in 30 day. However, rhythmicity in serotonin levels was not observed in 30, 270 day and other older age groups up to 730 day. Robust increase in the amplitude of serotonin rhythms in 90 day was observed as compared to serotonin rhythms in other age groups. Rhythmicity was abolished in older age groups with maximum levels at ZT-0/24, ZT-12, ZT-0/24 and ZT-6 in 270, 365, 545 and 730 day respectively.

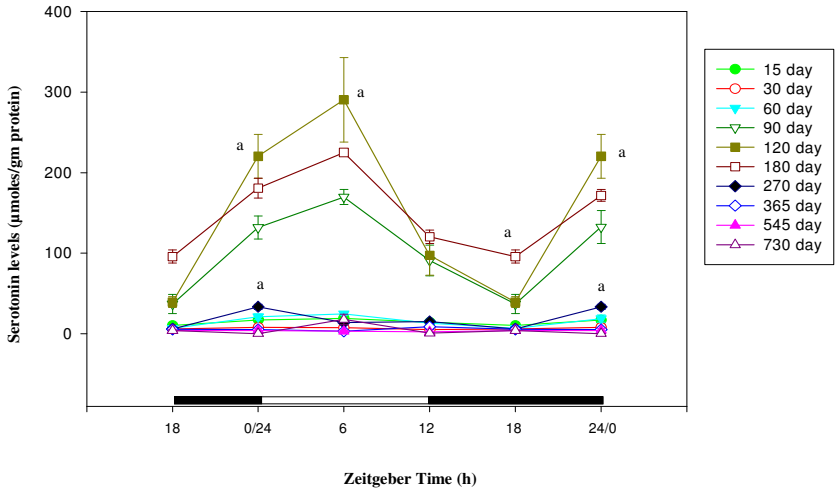
Maximum serotonin levels observed were 19.26 ± 2.2, 7.66 ± 0.74, 24.69 ± 1.71, 169.75 ± 9.21, 290.53 ± 52.49, 225.10 ± 3.66, 33.53 ± 3.3, 8.97 ± 3.45, 4.14 ± 2.4 and 17.66 ± 6.63 µmol/g protein in the age groups studied from 15 day to 730 day respectively (Table 4; Fig. 17). Minimum levels of serotonin observed were 10.4 ± 3.44, 5.21 ± 1.09, 6.9 ± 1.58, 36.96 ± 12.0, 39.33 ± 6.92, 95.84 ± 8.07, 5.96 ± 0.32, 3.02 ± 0.32, 2.54 ± 0.83 and 0.21 ± 0.13 µmol/g protein in 15, 30, 60, 90, 120, 180, 270, 365, 545 and 730 day respectively (Table 4; Fig. 17). There was a significant difference ($p_a \leq 0.05$) in serotonin levels in 15, 30, 60, 270, 365, 545 and 730 day at all zeitgeber times as compared to the adult (90

day). Serotonin levels in 120 day were significant at ZT-0/24 and ZT-6 ($p_a \leq 0.05$) and in 180 day, levels were significant at ZT-18 ($p_a \leq 0.05$).

Table 3: Age related changes in daily serotonin rhythms in the SCN of Rat (LD; 12:12)

S.No	Age (days)	Serotonin levels (µmol/g protein) at different zeitgeber times				
		0/24	6	12	18	24/0
1	15	17.33 ± 2.71[a]	19.26 ± 2.2 [a]	14.28 ± 2.3 [a]	10.4 ± 3.44 [a]	16.98 ± 2.85 [a]
2	30	7.88 ± 1.52 [a]	7.66 ± 0.74 [a]	5.21 ± 1.09 [a]	6.06 ± 1.72 [a]	7.88 ± 1.52 [a]
3	60	21.05 ± 3.66 [a]	24.69 ± 1.71[a]	13.11 ± 1.97 [a]	6.9 ± 1.58 [a]	18.87 ± 4.52 [a]
4	90	131.91 ± 14.28	169.75 ± 9.21	91.07 ± 19.03	36.96 ± 12.0	132.47 ± 20.45
5	120	220.26 ± 27.03 [a]	290.53 ± 52.49 [a]	97.56 ± 24.68	39.33 ± 6.92	220.26 ± 27.03 [a]
6	180	180.84 ± 12.46	225.10 ± 3.66	120.41 ± 8.33	95.84 ± 8.07 [a]	171.73 ± 7.40
7	270	33.53 ± 3.30 [a]	13.94 ± 0.67 [a]	15.2 ± 0.96 [a]	5.96 ± 0.32 [a]	33.53 ± 3.30 [a]
8	365	5.26 ± 1.82 [a]	3.018 ± 0.32 [a]	8.97 ± 3.45 [a]	5.54 ± 1.77 [a]	5.26 ± 1.82 [a]
9	545	4.14 ± 2.4 [a]	3.193 ± 0.865 [a]	2.54 ± 0.83 [a]	3.88 ± 1.45 [a]	4.14 ± 2.4 [a]
10	730	0.212 ± 0.129 [a]	17.661 ± 6.633 [a]	0.659 ± 0.369 [a]	4.067 ± 0.779 [a]	0.212 ± 0.129 [a]

Each value is mean ± S.E, (n=6); Zeitgeber Time (ZT): ZT-0 = 6.30 h (Lights on); ZT-12 = 18.30 h (Lights off)
One Way Anova: $p_a \leq 0.05$ (a refers to comparison with 90D)



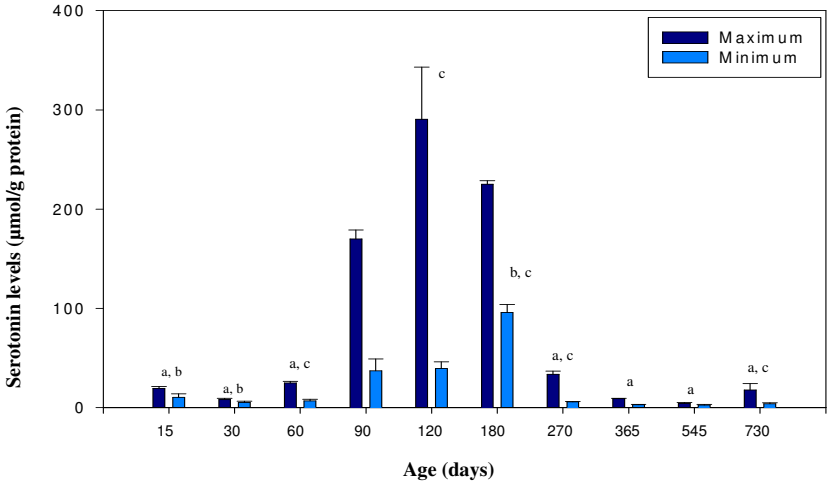
Each value is mean ± S.E, (n=6); Zeitgeber Time (ZT): ZT-0 = 6.30 h (Lights on); ZT-12 = 18.30 h (Lights off).

Fig. 16: Age related changes in daily serotonin rhythms in the SCN of rat (LD; 12:12)

Table 4: Daily pulses of Serotonin levels in the SCN of Rat (LD; 12:12)

Age (days)	Serotonin levels (µmol/g protein)		Ratio Maximum: Minimum
	Maximum	Minimum	
15	**19.267 ± 2.206 [a]**	**10.4 ± 3.44**	**1.852 ± 0.641 [b]**
30	**7.887 ± 1.525 [a]**	**5.21 ± 1.09 [a]**	**1.883 ± 0.279 [b]**
60	**24.69 ± 1.713 [a]**	**6.91 ± 1.586 [a]**	**4.652 ± 0.46 [c]**
90	**169.75 ± 9.21**	**36.96 ± 12.0**	**6.912 ± 0.979 [c]**
120	**290.53 ± 52.49 [a]**	**39.33 ± 6.92**	**13.70 ± 3.28 [c]**
180	**225.10 ± 3.66**	**95.84 ± 8.07 [a]**	**3.01 ± 0.28 [b, c]**
270	**33.53 ± 3.30 [a]**	**5.96 ± 0.32 [a]**	**5.56 ± 0.37 [c]**
365	**8.97 ± 0.54 [a]**	**3.018 ± 0.32 [a]**	**2.97 ± 1.68**
545	**4.14 ± 1.2 [a]**	**2.54 ± 0.83 [a]**	**1.63 ± 1.4 [b]**
730	**17.661 ± 6.63 [a]**	**4.06 ± 0.779 [a]**	**3.94 ± 1.04 [c]**

Each value is mean ± S.E, (n=6); Zeitgeber Time (ZT): ZT-0 = 6.30 h (Lights on); ZT-12 = 18.30 h (Lights off).
One Way Anova: $p_a \leq 0.05$ (a refers to comparison with 90D)
$p_b \leq 0.05$ (b refers to comparison of ratio values between a given age group and 90D)
t-test: $p_c \leq 0.05$ (c refers to comparison between maximum and minimum values in the same age group)



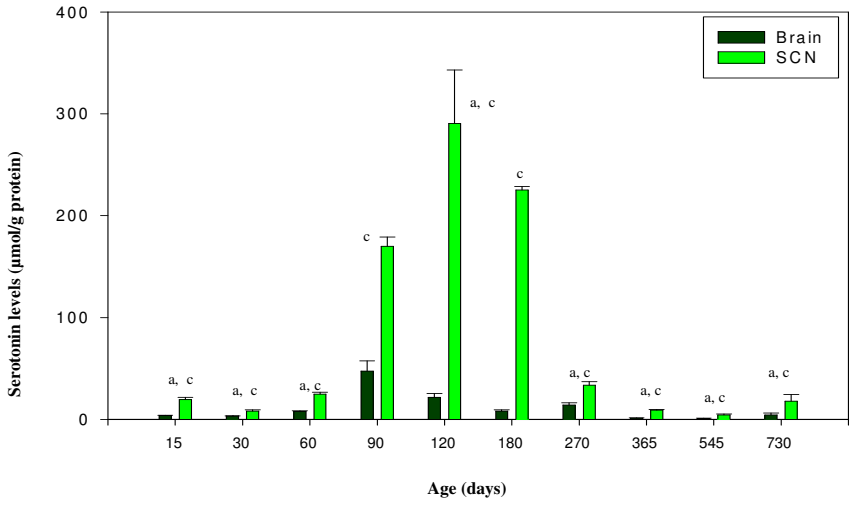
Each value is mean ± S.E, (n=6); Zeitgeber Time (ZT): ZT-0 = 6.30 h (Lights on); ZT-12 = 18.30 h (Lights off).
One Way Anova: $p_a \leq 0.05$ (a refers to comparison with 90D)
$p_b \leq 0.05$ (b refers to comparison of ratio values between a given age group and 90D)
t-test: $p_c \leq 0.05$ (c refers to comparison between maximum and minimum values in the same age group)

Fig. 17: Daily pulses of Serotonin levels in the SCN of Rat (LD; 12:12)

Table 5: Age related changes in serotonin levels in Brain and SCN of Rat (LD; 12:12)

Age (days)	Maximum serotonin levels (µmol/g protein)		Ratio SCN: Brain
	SCN	Brain	
15	19.26 ± 2.2 [a]	3.62 ± 0.43 [a]	5.9 ± 1.22 [c]
30	7.66 ± 0.74 [a]	3.21 ± 0.28 [a]	2.46 ± 0.25 [c]
60	24.69 ± 1.71[a]	7.82 ± 0.49 [a]	3.26 ± 0.4 [c]
90	169.75 ± 9.21	47.3 ± 9.98	4.41 ± 0.82 [c]
120	290.53 ± 52.49 [a]	21.51 ± 3.72 [a]	12.21 ± 0.84 [b, c]
180	225.10 ± 3.66	8.22 ± 1.21[a]	27.15 ± 1.69 [b, c]
270	33.53 ± 3.3 [a]	14.01 ± 2.23 [a]	3.97 ± 0.51[c]
365	8.97 ± 0.54 [a]	1.054 ± 0.32 [a]	8.51± 1.68 [c]
545	4.14 ± 1.2 [a]	1.028 ± 0.12 [a]	4.02 ± 0.82 [c]
730	17.661 ± 6.63 [a]	4.442 ± 1.734 [a]	3.975 ± 3.823 [c]

Each value is mean ± S.E, (n=6); Zeitgeber Time (ZT): ZT-0 = 6.30h (Lights on); ZT-12 = 18.30h (Lights off).
One Way Anova: $p_a \leq 0.05$ (a refers to comparison with 90D)
$p_b \leq 0.05$ (b refers to comparison of ratio values between a given age group and 90D)
t-test: $p_c \leq 0.05$ (c refers to comparison between SCN and Brain values in the same age group)



Each value is mean ± S.E, (n=6); Zeitgeber Time (ZT): ZT-0 = 6.30h (Lights on); ZT-12 = 18.30h (Lights off).
One Way Anova: $p_a \leq 0.05$ (a refers to comparison with 90D)
t-test: $p_c \leq 0.05$ (c refers to comparison between SCN and Brain values in the same age group)

Fig. 18: Age related changes in serotonin levels in Brain and SCN of Rat (LD; 12:12)

Serotonin levels in SCN were high as compared to brain 5-HT levels. There was about 4 fold and 27 fold difference in 5-HT levels of SCN and brain in 90 day (adult) and 180 day respectively.

DISCUSSION:

In the present study, we report that aging results in decreased serotonin levels and arrhythmicity in the brain (Fig. 14) as well as in the SCN (Fig. 16) of rat.

The SCN showed significant changes in serotonin levels as well as in the rhythmicity with increase in age. Serotonin levels increased from 15 day to 120 day, but not in 30 day (Fig. 16). Robust increase in rhythmicity was observed in 60, 90 and 120 day, but no rhythmicity was observed in 30 day (Fig. 17). According to earlier reports, SCN in rodents is rhythmic in nature at birth and responds to light (Ferguson *et al*., 2000) and hence rhythmicity in serotonin levels was observed in 15 day. The phase of establishment of SCN as a circadian clock was observed by the changes in daily serotonin pulses. Serotonin daily pulses decreased in SCN by 1.85 and 1.88 folds ($p_b \leq 0.05$) in 15 day and 30 day respectively which is significantly very low as compared to 90 day. Serotonin daily pulses in SCN increased significantly in 60, 90 and 120 day by 4.6, 7 and 14 folds respectively (Table 4; Fig. 17). The arrhythmicity in serotonin levels at 30 day but not in 15 day or 60 day shows that, 30 day could be the stage at which SCN gets established as a circadian clock on its own in the individual. Daily pulses in serotonin levels further decreased from 120 day and 1.6 folds were observed in 545 day and 3.9 fold in 730 day. The arhythmicity with either phase advances or delays in 5-HT levels as well as decrease in serotonin levels with increase in age from 120 day to 730 day attributes the role of serotonin in age related circadian disorders such as advanced sleep phase syndrome (ASPS) or delayed sleep phase syndrome (DSPS) as 5-HT plays an important role in the sleep-wake cycle of an organism. Our results are in agreement with such workers who have reported age related changes in 5-HT afferents to the SCN (Turek,

1994; Penev *et al.,* 1995). Age-related decline in postsynaptic receptors of serotonin has been reported (Meltzer *et al.,* 1998). This could be resulting in alterations of SCN functions as serotonin forms one of the input pathways to the SCN.

In brain, serotonin levels increased significantly from 15 day to 90 day, except in 30 day levels decreased as compared to 15 day 5-HT levels (Table 1). Serotonin levels in 15 day could be more than in 30 day as 15 day old were still in the weaning stage. Rhythmicity in serotonin levels was seen in 15, 30 and 90 day but not in 60 day old brain (Fig. 14). Robust increase in the amplitude of serotonin levels in 90 day old was observed. Significant change in the daily pulses of serotonin levels was observed which decreased from 4 folds in 15 day to 3 folds in 30 day and 2.8 folds ($p_b \leq 0.05$) in 60 day whereas in 90 day daily pulses increased robustly by about 4 folds. Thus 60 day could be the stage at which serotonin rhythms get established in the brain. However, 4 fold serotonin daily pulses in 15 and 90 day old suggest that maternal influence on serotonin rhythms is almost similar to that observed in the adult. These daily pulses in 5-HT levels decreased further with the advancement of age from 4 fold (90 day) to 1.7 fold (730 day). This is in agreement with earlier workers who reported marked decline in the brain serotonin levels in age rats (Petkov *et al.,* 1987).

The occurrence of rhythmicity in serotonin levels during weaning stage (15 day old) in both SCN (Table 3; Fig.16) and brain (Table 1; Fig. 14) suggests that 5-HT is under maternal regulation (Reppert *et al*., 1988) and it has a role in early development as reported by Levitt and Rakic (1982) in other species. The early establishment of individual serotonin daily rhythms in SCN (at 30 day) (Table 3; Fig. 16) as compared to brain (at 60 day) (Table 1; Fig. 14) conveys the stage specific and tissue specific organization of circadian rhythm generation. This once again proves that SCN is the master circadian clock and once it gets established as an individual clock it regulates the rhythms in other regions of brain as well as in peripheral tissues. This is also supported by earlier workers, which includes the establishment of intrinsic rhythmicity first and the maturation of effector follower systems for the expression of circadian function in other areas

(Moore, 1992). In all the age groups studied serotonin levels were high in SCN as compared to brain (Table 5; Fig. 18). Earlier studies report that brain serotonin originates from a relatively few serotonergic neurons, which profusely branch out to all areas of central nervous system (Jacobs and Azmitia, 1992). Also SCN receives one of the densest serotonergic terminal plexes in the brain (Morin, 1999). Maximum levels of serotonin in SCN were 4 ($p_c \leq 0.05$) times higher as compared to the brain serotonin levels in the adult (90 day) whereas there was 27 fold difference in the 5-HT levels of SCN and brain in 180 day (Table 5). This suggests the importance of serotonergic innervation in SCN in the circadian rhythm generation and entrainment. The decrease and arrhythmicity in serotonin levels in both brain and SCN with increase in age implies the importance of serotonin in the circadian timing system and alteration in serotonin homeostasis could lead to age related circadian disorders such as ASPS, DSPS, later life depression (Meltzer *et al.,* 1998). The changes could be due to serotonergic neuronal degeneration or changes in the metabolism of serotonin.

Thus present study helps us in understanding age induced changes in serotonin rhythms in SCN and brain of rat. These observations may be helpful in understanding the aging process and age related neurological disorders.

CHAPTER 2

Effect of melatonin administration on age related changes in serotonin rhythms in the SCN of rat

CONTENTS

INTRODUCTION:

Aging has been related with changes in structure and functions of neurotransmitter systems. Maintenance of physiological concentrations of serotonin in the organism is important because it has a great therapeutic significance as depletion of serotonin levels causes depression and other age related changes in elderly (Lozeva-Thomas, 2004). There are limited and conflicting data in the literature regarding changes in the 5-HT system in normal aging. Earlier workers have reported effects of aging on 5-hydroxyltryptamine-immunoreactive (5-HT-IR) neurons in raphe and extrarapheal nuclei of rats (Lolova, 1996). Age-related changes in behaviors such as sleep are linked to serotonergic function which suggests decline in 5-HT function (Klöppel *et al.,* 2001; Meltzer *et al.,* 2001). There are also reports that development of major depression is implicated with age-related deficit in serotonergic neurotransmission. It has been postulated that 5-HT may play an important role in age-related memory impairment (Buhot *et al.,* 2000). Disruptions in serotonergic system have been implicated in age related disorders such as Alzheimer's disease where a combination of disturbances in cholinergic and serotonergic function may play a role in cognitive impairment in Alzheimer's disease (AD) (Lorke *et al.,* 2006), Schizophrenia (Stone and Pilkowsky, 2007).

The SCN and pineal gland alterations have been suggested to be the basis of circadian rhythm disturbances during aging (Wu and Swaab, 2007). Age related changes in circadian function could also be due to decreased exposure or response of the pacemaker to entraining effects of photic and non-photic stimuli (Van Cauter *et al.,* 1998). Aging alters the synchronization of rhythms by the SCN in humans (Touitou *et al.*, 1997). Many of the circadian functions such as neuroendocrine rhythms (Smith *et al.,* 2005), locomotor activity rhythms, feeding and drinking rhythms decline with the progression of age (Turek, 1995). In the SCN neurons, firing rate and the amplitude of the rhythms are primarily controlled by the genes at the level of transcription. Increase in age results in irregular firing rate and reduced amplitude in the

rhythms of these neurons (Edery, 2000). Decrease in amplitude shows the decrease in neuronal activity in SCN (Rúzsás and Mess, 2000; Van Someren, 2000). The quality of sleep and sleep-wake patterns that are regulated by the SCN are known to be altered with aging (Dijk and Lockley, 2002). Age associated changes in circadian rhythms are known to influence metabolism like glucose regulation (Van Cauter *et al.,* 1997), alterations in carbohydrate and lipid metabolism that causes excess deposition of fat at the expense of muscle (Bjorntorp, 1999).

In the circadian timing system, SCN and IGL, receive serotonergic projections from median raphe nucleus (MRN) and dorsal raphe nucleus (DRN) respectively. Serotonergic projections to these structures have different functions in the circadian responses such as rhythm modulation by the SCN to photic and non-photic stimuli (Duncan *et al.,* 2005). Several workers studied serotonergic activity in relation to characteristics of circadian rhythms generated by SCN. Endogenous serotonin and serotonergic drugs influence many aspects of circadian rhythms, including phase shifts, onset of locomotor activity, period length and integrity of rhythms in the SCN (Duncan *et al.,* 2000). Serotonin agonists inhibit RHT mediated responses in the SCN to photic signaling (Ying and Rusak, 1994) and IEG activation (Rea and Pickard, 2000). Decrease in serotonin levels inhibit phase resetting evoked by locomotor activity (Sumova *et al.,* 1996; Marchant *et al.,* 1997) and there are evidences for the release of serotonin in the SCN to non-photic phase resetting stimuli such as wheel running (Dudley *et al.,* 1998). It is known to phase reset the circadian clock both *in vitro* and *in-vivo* (Ehlen *et al.,* 2001). The circadian activity of SCN serotonergic neurons affects circadian rhythms in the secretions of several anterior pituitary hormones in old animals (Simpkins and Millard, 1987). Serotonergic afferents to the SCN (Turek, 1994; Penev *et al.,* 1995) and serotonin's action on light entrainment of SCN-driven rhythms (Penev *et al*., 1997) have been implicated as a site of neural aging in mammals. In mouse, serotonergic afferents to the SCN are necessary for activity dependent entrainment (Edgar *et al.,* 1997). Ehlen *et al.,* (2001) suggested that serotonin may directly act on SCN to induce *in vivo* non-photic

phase-resetting. Therefore, changes in endogenous serotonin levels or serotonin receptors could play a role in age-related changes and functioning of SCN.

In addition to these physiological functions which alter with aging, serotonin connects the nervous system with the endocrine system as it is the precursor of a neurohormone, melatonin (Aparicio *et al.,* 2006). Melatonin has been associated with aging and its levels decline with aging. Melatonin metabolite, 6-hydroxymelatonin levels were significantly higher than free melatonin levels in tissues like cerebral cortex, serum, heart, liver and kidney of mice (Lahiri *et al*., 2004). Age related changes in melatonin production could be due to (i) a marked decline in the neuronal mass including SCN, which regulates its production (ii) an overall disturbance of all SCN driven circadian rhythms, (iii) general disturbances related to circadian clock with aging, (iv) dysfunction or insensitivity of neural processes involved in entrainment of circadian clock, (v) dysfunction of pineal gland and (vi) insufficient exposure to zeitgebers.

Melatonin has various effects on SCN function (von Gall *et al.,* 2002). Pineal melatonin modulates clock function through a direct action on G-protein coupled melatonin receptors in the SCN (Dubocovich *et al.,* 2003). It inhibits SCN neuronal firing (Hunt *et al.,* 2001) through MT-1 receptor, which plays a role in the sensitivity of SCN to phase-shifting stimuli (Gerdin *et al.,* 2004) and it entrains mammalian circadian rhythms (Lewy *et al.,* 2006). Therefore, a multiple and complicated reciprocal feed-forward and feed-back regulatory mechanism appears to act between the SCN and pineal gland (Mess and Rúzsás, 1986).

Several studies indicated that there is a great interaction between melatonin and central serotonin (Miguez *et al.,* 1997). Serotonin along with its methylated derivative is known to regulate arousal and sleep-wake cycles (Roskoski, 1996). Effect of melatonin administration on the changes in serotonin levels and its turnover in different hypothalamic nuclei of pinealectomized rats had been studied earlier by Miguez *et al*., (1996). There are evidences for the mediatory role of serotonergic neurons to the melatonin

signals in the brain (Cardinali *et al.,* 1985; Ruzsás *et al*., 1986). There have been reports suggesting that melatonin administration changes the hypothalamic serotonin uptake and release (Miguez *et al.,* 1995).

Age-related events have been related to an alteration in amplitude and pulsatile pattern of hormone and neurotransmitter release (Wise *et al.,* 1999). The frequency of release of a hormone is as important or more important in some cases, than the amount of hormone released. Target cells respond most effectively to exogenous hormonal stimulation when the frequency of stimulation approaches the endogenous frequency (Goldbeter, 1996). The age-related decline of pineal melatonin production is due to the degenerative changes of the neural structures (serotonergic and noradrenergic neuron systems) innervating the pineal gland and the suprachiasmatic nuclei rather than to the degeneration of the pineal tissue itself (Rúzsás and Mess, 2000). The endocrine system affects neuronal signaling and neuronal integrity. Therefore, age-dependent endocrine changes influence structure and function of the CNS (Smith *et al.,* 2005). Decline in melatonin production during aging might be a consequence of the age-related alterations of the brain neuronal systems regulating pineal activity (Rúzsás and Mess, 2000). Dietary supplementation with melatonin resulted in a significant increase in serum and other tissue melatonin levels tested in mice. Thus age-related decline of tissue melatonin gets reversed by supplementation with dietary melatonin in such studies (Lahiri *et al.,* 2004).

The effect of exogenous melatonin administration is well established both *in vivo* and *in vitro*. Melatonin is known to exert both long term effects such as synchronizing the free running locomotor activity by daily melatonin injections (Pitrosky *et al.,* 1999; Slotten *et al.,* 1999) and also immediate effects (Poirel *et al.,* 2003). Melatonin, when administered in the late subjective day inhibits metabolic activity of SCN (Cassone *et al.,* 1988) and immediately phase shifts the clock *in vivo* (Warren *et al.,* 1993) as well as mice locomotor activity (Sharma *et al.,* 1999). *In vitro* application of melatonin on SCN slices inhibits neuronal electrical activity immediately

(Shibata *et al.,* 1989), phase advances SCN circadian neuronal activity (Hunt *et al.,* 2001) and inhibits vasopressin (AVP) synthesis (Watanabe *et al.,* 1998).

SCN and circadian systems of several mammalian species have been indicated to be highly sensitive to exogenous melatonin (Cassone, 1992) and it has a chronobiotic effect (Pévet *et al.,* 2002). In our 1st chapter, we found decrease as well as abolition of rhythmicity in serotonin levels in SCN with aging (Table 3 and 4; Chapter 1). The rhythmicity in serotonin levels was altered from 270 day to 730 day (Fig. 16 and 17; Chapter 1). Melatonin treatments have been shown to alter hypothalamic serotonin metabolism (Miguez *et al.,* 1991). We studied effect of melatonin administration on serotonin levels and daily rhythms in aging SCN.

MATERIALS and METHODS:

Male Wistar rats of different age groups (90, 180, 270 and 730 day old) were maintained under laboratory conditions, 06.30h (ZT-0)-18.30h (ZT-12) light phase; 18.30h (ZT-12)-06.30h (ZT-24) dark phase, two weeks prior to the experiments. All rats were kept individually in polypropylene cages at room temperature (20+2^{o}C) with relative humidity (55+6%). Food and water were supplied *ad libitum*. Dim red light was used for handling the animals in the dark. Cage changing was done at random intervals. Serotonin levels were measured at various zeitgeber times in the rat brain and SCN by spectrofluorimetry (Jagota and Habibulla, 1992).

1) SCN tissue preparation:

SCN was dissected out as described in Chapter 1.

2) Melatonin administration:

30µg/Kg body weight of melatonin was administered subcutaneously via 10% ethanol in physiological saline, 1 h (ZT-11) before the onset of darkness (Cardinali *et al*., 2002). This treatment was given for 11 days. On the 12th day animals were sacrificed and serotonin levels were measured spectrofluorimetrically.

3) Fluorimetric assay of Serotonin:

This assay was done as described in Chapter 1.

4) Protein Estimation:

Protein estimation was done as given in Chapter 1

STATISTICAL ANALYSIS: Statistical analysis was done by one way ANOVA and student's t-test.

RESULTS:

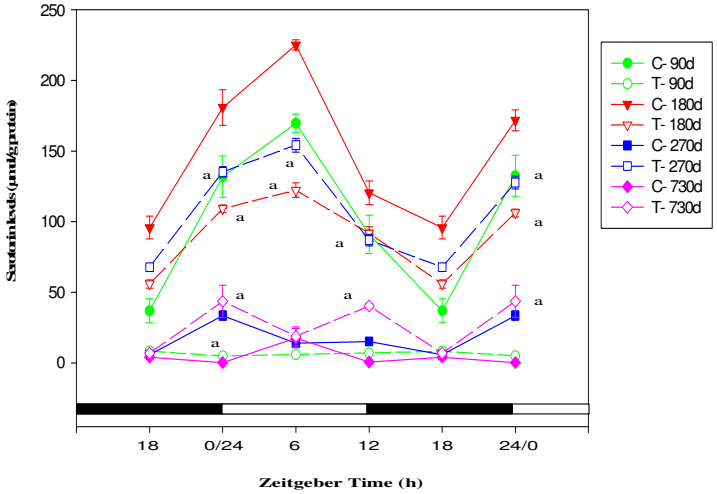
Effect of melatonin administration on serotonin levels and rhythmicity in the SCN of aging rat:

Melatonin administration had a significant effect on serotonin levels in the SCN with increase in age (Table 6 and 7; Fig. 19a and 19b). This was studied in 90, 180, 270 and 730 day. Upon melatonin administration, maximum serotonin levels decreased significantly in 90 and 180 day by 21.48 ± 2.63 ($p_c \leq 0.05$) and 1.84 ± 0.047 ($p_c \leq 0.05$) folds respectively but levels increased by 4.64 ± 0.27 folds ($p_c \leq 0.05$) in 270 day SCN as compared to their respective controls. Melatonin administration decreased serotonin levels by 2.69 ± 0.79 folds ($p_c \leq 0.05$) in 730 day SCN as compared to its control. The maximum serotonin levels observed in treated SCN were 8.267 ± 1.727, 122.3 ± 5.1, 154.17 ± 4.85 and 43.63 ± 11.39 µmol/g protein in 90, 180, 270 and 730 day respectively as compared to their controls 169.75 ± 6.51, 225.10 ± 3.66, 33.53 ± 3.3 and 17.66 ± 6.63 µmol/g protein. Upon melatonin administration, the maximum 5-HT levels were observed at ZT-18, ZT-6, ZT-6 and at ZT-0 in 90, 180, 270 and 730 day respectively. Minimum serotonin levels observed in melatonin treated SCN were 4.91 ± 0.98, 56.02 ± 2.95, 67.79 ± 2.2 and 6.89 ± 1.47 µmol/g protein in 90, 180, 270 and 730 day respectively as compared to their controls 36.96 ± 8.49, 95.84 ± 8.07, 5.96 ± 0.32 and 0.212 ± 0.129 µmol/g protein. Upon melatonin administration, serotonin levels were

Table 6: Effect of melatonin administration on serotonin rhythms in the SCN of rat (LD; 12:12)

Age (days)		Serotonin levels (µmol/g protein) at different zeitgeber times				
		0/24	6	12	18	24/0
90	C	131.91 ± 14.68	169.75 ± 6.51	91.07 ± 13.58	36.96 ± 8.49	132.47 ± 14.46
	T	4.91 ± 0.98 [a]	5.947 ± 0.911 [a]	7.08 ± 1.38 [a]	8.267 ± 1.727 [a]	4.91 ± 0.98 [a]
180	C	180.84 ± 12.46	225.10 ± 3.66	120.41 ± 8.33	95.84 ± 8.07	171.73 ± 7.40
	T	109.22 ± 2.72 [a]	122.3 ± 5.1 [a]	91.77 ± 4.66 [a]	56.02 ± 2.95 [a]	106.22 ± 2.81 [a]
270	C	33.53 ± 3.30	13.94 ± 0.67	15.2 ± 0.96	5.96 ± 0.32	33.53 ± 3.30
	T	135.28 ± 3.69 [a]	154.17 ± 4.85 [a]	86.92 ± 4.08 [a]	67.79 ± 2.20 [a]	127.75 ± 4.5 [a]
730	C	0.212 ± 0.129	17.661 ± 6.63	0.659 ± 0.369	4.06 ± 0.779	0.212 ± 0.129
	T	43.63 ± 11.39 [a]	18.86 ± 6.63	40.34 ± 2.21 [a]	6.89 ± 1.47	43.63 ± 11.39 [a]

Each value is mean ± S.E, (n=6); Zeitgeber Time (ZT): ZT-0 = 6.30h (Lights on); ZT-12 = 18.30h (Lights off).
t-test: $p_a \leq 0.05$ (a refers to comparison of control and treated values within the age groups)



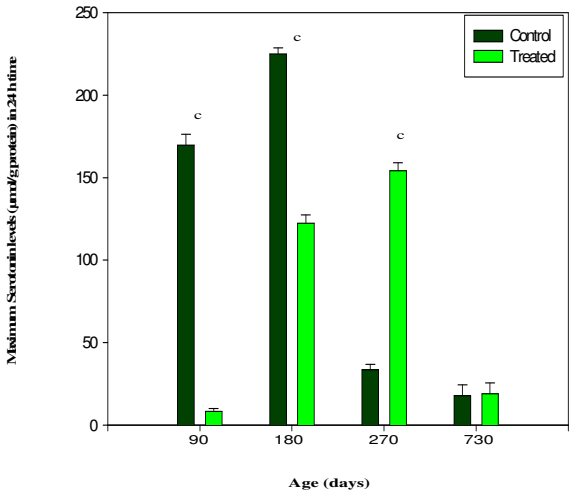
Each value is mean ± S.E, (n=6); Zeitgeber Time (ZT): ZT-0 = 6.30h (Lights on); ZT-12 = 18.30h (Lights off).
t-test: *p*a ≤ 0.05 (a refers to comparison of control and treated values within the age groups)

Fig. 19a: Effect of melatonin administration on serotonin rhythms in the SCN of rat (LD; 12:12)

Table 7: Daily pulses of melatonin administration on serotonin rhythms in the SCN of rat (LD; 12:12)

Age (days)	Maximum Serotonin levels (µmol/g protein)		Ratio Control: Treated
	Control	Treated	
90	169.75 ± 6.51	8.26 ± 1.72	21.48 ± 2.63 [c]
180	225.10 ± 3.66	122.3 ± 5.1	1.84 ± 0.047 [c]
270	33.53 ± 3.3	154.17 ± 4.85	4.64 ± 0.27 [c]
730	43.63 ± 11.39	17.66 ± 6.63	2.69 ± 0.79

Each value is mean ± S.E, (n=6); Zeitgeber Time (ZT): ZT-0 = 6.30h (Lights on); ZT-12 = 18.30h (Lights off)
t-test: $p_c \leq 0.05$ (c refers to comparison between maximum control and treated values in the same age group)



Each value is mean ± S.E, (n=6); Zeitgeber Time (ZT): ZT-0 = 6.30h (Lights on); ZT-12 = 18.30h (Lights off)
t-test: $pc \leq 0.05$ (c refers to comparison between maximum control and treated values in the same age group)

Fig. 19b: Effect of melatonin administration on serotonin rhythms in the SCN of rat (LD; 12:12)

minimum at ZT-0 in 90 day and at ZT-18 in 180, 270 and 730 day. Rhythmicity in serotonin levels was not persistent in 90 day upon melatonin administration. There was restoration in the rhythmicity of serotonin levels in both 180 and 270 day upon melatonin administration whereas in 730 day, serotonin levels increased but rhythmicity was not restored.

DISCUSSION:

Aging causes disturbances in the functioning of the rhythm generating system, due to increase in monoamine oxidase activity, decrease in serotonin and melatonin concentrations (Kabuto *et al.,* 1995), alterations in concentrations of receptors for hormones and neuropeptides in the central nervous system (Smith *et al.,* 2005). This leads to altered overt rhythms. Thus changes in the serotonergic system could be an important neurophysiological aspect during aging.

Melatonin administration resulted in significant decrease in 5-HT levels in the SCN of rat (Fig. 19a). Melatonin administration decreased maximum serotonin levels by about 21 and 2 folds in 90 and 180 day respectively. This is in agreement with earlier workers who have reported drastic changes in serotonin metabolism in hypothalamus upon melatonin administration (Miguez *et al*., 1994). At 270 day, physiological disturbances such as biochemical alterations would have initiated because of age. We found decrease in serotonin levels as well as abolition of serotonin rhythms with aging. Upon melatonin administration, maximum 5-HT levels in 270 day increased by about 4.6 folds and hence serotonin levels and rhythmicity were restored in 270 day. There was about 2.7 fold decrease in maximum serotonin levels in 730 day (2 years). Some workers reported that younger rats were more sensitive to hormonal control and treatment as compared to older rats (Maines *et al.,* 1999). This could be due to the loss of inherent capacity of the tissue with aging causing changes in molecular, biochemical, anatomical and morphological aspects resulting in functional deterioration. Therefore, exogenous melatonin could not restore serotonin levels and its rhythmicity.

Alterations in catecholaminergic levels have pharmacological effects also. The effects of drugs on cellular neurochemistry have been found to change with age. Dopamine and serotonin release in response to drugs is variable with age in rats and therefore, the effects of various drugs may differ between younger and older rats due to changes in neurochemistry with age (Yurek *et al.,* 1998; Gerhardt and Maloney, 1999). The time of administration of melatonin at one hour before the onset of darkness (ZT-11) doesn't coincide with the physiological peak levels of melatonin (ZT-18). Thus the changes observed in serotonin levels as well as in its rhythmicity could be due to exogenous melatonin administration.

This work suggests that melatonin could be playing an important regulatory role in the modulation of rhythms upon aging. This would also suggest that melatonin is essential for maintaining serotonin levels during aging. The decline of pineal melatonin with age could be a consequence of a deficit in the pathway of serotonin utilization as conversion of serotonin to melatonin could be getting affected. This may be linked to impaired pineal catecholaminergic neurotransmission (Miguez *et al.,* 1998).

CHAPTER 3

Age induced changes and the effect of melatonin administration on N-acetyl transferase (NAT) activity rhythms in the SCN of rat

CONTENTS

INTRODUCTION:

The endocrine system plays a key role in conveying environmental information to changes in physiology (Foulkes *et al.,* 1997). The daily synthesis of melatonin is one of the important output signals of SCN to the organism. Melatonin synthesis is catalyzed by two enzymatic reactions. Serotonin is first acetylated to NAS by the enzyme NAT. The NAS is then methylated by HIOMT to form melatonin (Sun *et al.,* 2002; Simonneaux and Ribelayga, 2003). Melatonin is secreted only during the dark phase of the light-dark cycle (Hamada *et al.,* 1999).

The daily rhythm of melatonin synthesis in the rat pineal gland is controlled by the SCN, via a multi-synaptic pathway that include neurons of the PVN of the hypothalamus, sympathetic preganglionic neurons of the IML cell column of the spinal cord and NE containing sympathetic neurons of the SCG (Perreau-Lenz *et al.,* 2005). Induction of melatonin production occurs during the first phase of darkness. The sympathetic nerve fibres from the SCG release NE which acts on both α_1- and β-adrenergic receptors present on the pinealocytes of pineal gland. The β-adrenergic receptors stimulate adenylate cyclase and α_1 adrenergic receptors potentiate the β- induced cAMP production (Foulkes *et al.,* 1997). This later increases the concentration of cAMP. Increased levels of cAMP lead to the activation of cAMP-dependent protein kinase A (PKA) (Maronde *et al.,* 1999). The PKA phosphorylates a group of transcription factors such as CREB (Spessert *et al.,* 2000). Phosphorylation of CREB is an important step in the signal transduction cascade of melatonin biosynthesis (Maronde *et al.,* 1997) and is phosphorylated constitutively with a transient fall occurring at the beginning of night (Foulkes *et al.,* 1997). Phosphorylation of CREB is regulated by multiple entraining agents in the SCN, thus plays a role in the clock entrainment (Hastings *et al.,* 1997). Phosphorylated-CREB (P-CREB) binds to the CREs present on the cAMP response genes such as *N-acetyl transferase (Nat)* and stimulates its transcription (Chen and Baler, 2000) leading to 100-150 fold increase in *Nat* mRNA levels (Klein *et al.,* 2003) and translation with 70 fold nocturnal increase in protein levels (Obsil *et al.,* 2001; Ganguly *et al.,* 2002) and

also maintains the enzyme in its active form (Takahashi, 1994). Activation of NAT results in a 10 fold increase in melatonin synthesis and secretion, approximately 5-6 h after the onset of night (Drijfhout *et al.,* 1996). The cAMP also triggers the expression of a negative transcription factor, an inducible cAMP early repressor (ICER). This ICER competes with phosphorylated CREB for the CREs in the *Nat* promoter (Stehle *et al.,* 1993). *Nat* gene expression is suppressed when there is a decrease in P-CREB together with an increase in ICER. Increase ICER levels inhibit transcription of CRE-induced genes late in the night (Maronde *et al.,* 1999). Melatonin synthesis is inhibited during the second phase of darkness which includes events like inhibition of NE secretion by SCN, withdrawal of adrenergic inputs and reversal of events that take place in the first phase of darkness (Gupta *et al.,* 2005). Decline in NAT protein levels occur due to proteasomal proteolysis (Fukuhara *et al.,* 2001; Iuvone *et al.,* 2002). The mechanism involved in photoperiodic control of pineal metabolism involves two important links; photoperiodic regulation of *Nat* gene expression and photoperiodic regulation of HIOMT activity which occurs at the transcriptional level (Ribelayga *et al.,* 1999). The mRNA levels of HIOMT exhibit circadian variation with a peak at mid-light phase in *in vivo* as well as *in vitro* conditions (Grève *et al.,* 1996). The SCN controls melatonin rhythm in the pineal by using inhibitory signal, GABA during day time and stimulatory signal, glutamate at night time (Perreau-Lenz *et al.,* 2005). The decrease in melatonin synthesis at the end of the night depends on post-translational mechanisms triggered by termination of NE release from ganglionic terminals.

N-acetyl transferase, is the key regulatory enzyme in melatonin biosynthesis (Touitou, 2005). It is a member of the GCN-5-related N-acetyl transferase (GNAT) superfamily of enzymes (Dyda *et al.,* 2005). These enzymes catalyze a wide range of biologically important acetyl transfer reactions from antibiotic resistance to chromatin remodeling (Scheibner *et al.,* 2002). The members of the super family are characterized by a common substrate, acetyl CoA and a structural fold where acetyl CoA binds to them (Neuwald and Landsman, 1997). Several species exhibit remarkable differences in the molecular mechanisms involved in regulation of NAT activity. In rat, *Nat* gene

expression is transcriptionally regulated by cAMP and circadian regulation of NAT mRNA occurs transcriptionally and post-transcriptionally (Klein *et al.,* 1997). Maintaining the mRNA stability is an important mechanism in controlling gene expression (Tae-Don *et al.,* 2005). Regulation of NAT also occurs at protein level (Fukuhara *et al.,* 2001). Sheep *Nat* mRNA levels exhibit relatively little change within a circadian cycle and enzyme activity is primarily regulated at protein level. In chicken, *Nat* mRNA rhythmicity is driven by a non-cAMP dependent mechanism linked to the clock within the pineal gland (Klein *et al.,* 1997).

In pineal gland of mammals, NAT activity is dependent on two mechanisms, cAMP/P-CREB stimulation of *Nat* expression in rats and post transcriptional regulation of NAT protein in ungulates (Garidou *et al.,* 2002). Several studies suggested that age related decline in melatonin synthesis in pineal is due to degenerative changes of neural structures (serotonergic and noradrenergic neuron systems) innervating the pineal gland and the SCN rather than to the degeneration of pineal tissue itself (Rúzsás and Mess, 2000) resulting in the advancement of age (Pazo *et al.,* 2002).

The activity of NAT has been reported in SCN by Hamada *et al.,* (1999) and melatonin production in the SCN by Gachon *et al.,* (2004). The primary function of melatonin is to co-ordinate circadian responses to the external cues. The secondary function is to co-ordinate a variety of seasonal photoperiodic responses (Poirel *et al.,* 2003). Middle-aged rats show decreased levels of α-adrenergic receptors in the SCN. The diurnal rhythm of α-adrenergic receptor expression, characteristic of young rats, disappears by middle age (Weiland and Wise, 1990). Earlier studies showed that alterations in neurotransmitter release result in age-associated changes in hormone secretion (Simpkins and Millard, 1987).

Serotonin levels decreased with increase in age in brain and SCN (Chapter 1). The decrease in serotonin levels could be due to either decreased synthesis of serotonin or altered NAT activity i.e. increase in NAS levels but not in melatonin levels, because melatonin levels were shown to decline with age (Rúzsás and Mess, 2000). Melatonin is known to have a feedback effect on

serotonin and that reduced melatonin levels may give signal for 5-HT to enter into melatonin synthesis pathway. It has been shown that aging results in circadian system disorders and treatment for these disorders include light therapy and melatonin (Rivkees, 1997). Exogenous melatonin affects all levels of circadian network by acting on the circadian clock. It has been investigated that in rat, REV-ERBα is the initial molecular target for the chronobiotic effect of melatonin (Pévet *et al.,* 2006). Exogenous melatonin administration either subcutaneously or directly into the SCN was shown to exhibit a direct action on the amplitude of clock oscillations in addition to its phase-shifting effect (Bothorel *et al.,* 2002). Thus in order to understand the effect of aging on NAS levels and to know if melatonin administration can reset age induced changes in NAS levels altered with age, we studied age related changes and the effect of melatonin on NAT activity measured in terms of NAS levels in the SCN of rat.

MATERIALS and METHODS:

Based on our results in Chapter 1 and 2, the onset of age related changes occurred by middle age. Therefore we concentrated on middle age and also age related changes appeared reversible by melatonin administration in 270 day as compared to 2 years. Male Wistar rats of 90, 180 and 270 day were taken for present study but not 2 year old because of the non-availability of aged rats. Animals were maintained as described in Chapter 1. Melatonin administration was given by the method of Cardinali *et al.,* (2002) as described in Chapter 2. NAT activity was assayed by reverse phase high pressure liquid chromatography (RP-HPLC) (Waters, 2465) using fluorescence detector measured in terms of NAS formed from serotonin (Slominski *et al*., 2002) in different age groups of rat SCN.

All chemicals and reagents used in this study were of HPLC grade. Standard NAS was obtained from Sigma chemicals. HPLC grade or Milli Q water was used for preparation of solutions. Solutions were degassed and filtered through 0.22 μm thick solvent filters (Millipore) and samples were also filtered through 0.22 μm thick syringe filters (Millipore) before injecting into HPLC system.

1) SCN tissue preparation:

SCN was dissected out as described in Chapter 1.

***N*-Acetyltransferase Activity Assay:**

Animals were decapitated and the SCN were removed carefully and rapidly. Tissue was homogenized in an ice-cold 0.25 M potassium phosphate buffer (pH-6.8) containing 1mM DTT, 1mM EGTA, protease inhibitor cocktail (2 µl/ml homogenization mixture) and 0.625mM acetyl CoA. Homogenates were centrifuged at 15000g for 10 min at 4ºC. Enzymatic activity was measured by taking 80 µl of supernatant and mixed with 20 µl of 5mM serotonin in 0.25 M potassium phosphate buffer (pH-6.8). The final concentrations of acetyl CoA and substrate were 0.5mM and 1mM respectively. The reaction mixture was incubated for 1 hr at 37ºC and then reaction was stopped by the addition of 20 µl of 6 M $HClO_4$. The above mixture was centrifuged at 15000g at 4ºC. 20 µl of supernatant was subjected to HPLC system equipped with C_{18} reverse-phase column (150 X 5 mm, I.D.) and fluorimetric detector. The excitation and emission wavelengths were set at 285 and 360 nm respectively for detection. Elution was carried out isocratically at ambient temperature with a flow rate of 1.5 ml/min. The mobile phase contained 4mM sodium 1-octanesulfonate as ion-pairing agent, 50mM ammonium formate (pH-4.0) versus methanol (80:20 v/v). Elution peaks of NAS were identified by retention time. The peaks of samples were verified by running standards. For background controls, the reaction mixture was incubated without substrate.

1 mg of standard NAS was taken and dissolved in 1 ml of mobile phase containing 4mM sodium 1-octanesulfonate in 50mM ammonium formate (pH-4.0) and methanol in 80: 20 v/v. From this 1 µg/ml stock, different concentrations such as 5nM, 10nM, 15nM and 20nM were taken and run for HPLC. The unknown was compared with the standard (Fig. 20).

Protein estimation was done by Bradford's method as described in Chapter 1.

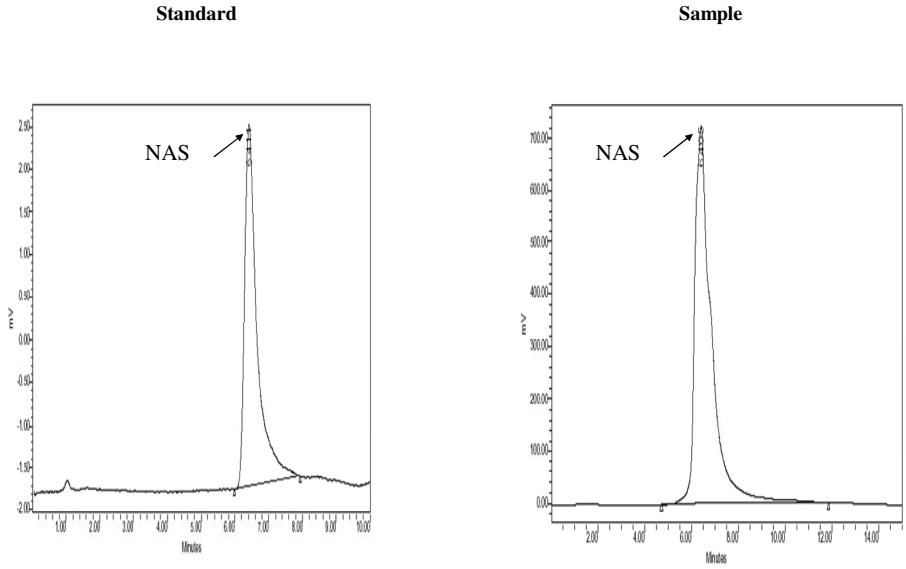


Fig. 20: *N*-Acetyl transferase (NAT) activity assay by RP-HPLC

STATISTICAL ANALYSIS: Statistical analysis was done by one way ANOVA and student's t-test.

RESULTS:

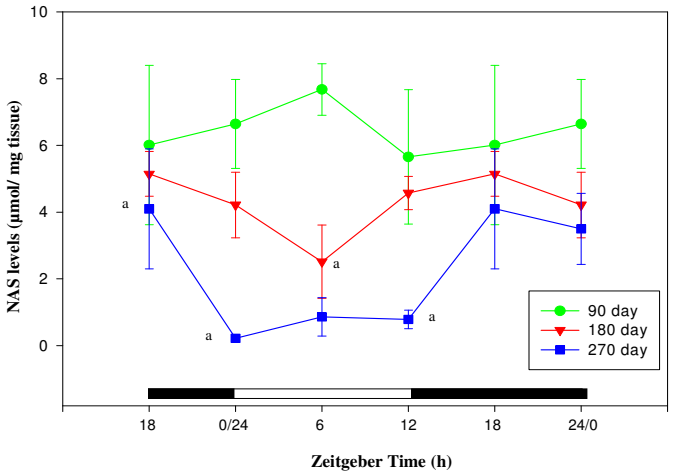
Effect of age related changes on the NAT enzyme activity rhythms in the SCN:

NAT enzyme activity rhythms were studied in the aging SCN (90, 180 and 270 day old). Our results showed highest enzyme activity at 90 day but no significant rhythmicity. We observed rhythmicity but decreased NAT activity with increase in age from 180 to 270 day (Table 8; Fig. 21a). NAT activity was highest at ZT-6 in 90 day whereas in 180 and 270 day highest activity was observed at ZT-18. NAS levels observed were 7.67 ± 0.77, 5.14 ± 0.67 and 4.09 ± 1.79 µmol/ mg tissue in 90, 180 and 270 day respectively. There is a significant difference in NAT activity in 180 day ($p_a \leq 0.05$) at ZT-6 and 270 day ($p_a \leq 0.05$) at ZT-0, 6 and 12 to that of the activity in 90 day old. Lowest activity of NAT was observed at ZT-12, 6 and 0 in 90, 180 and 270 day respectively. Lowest

Table 8: Age related changes in NAT activity rhythms in the SCNof rat (LD; 12:12)

S. N0	Age (days)	NAS levels (µmol/ mg tissue) at different zeitgeber times					
		0/24	6	12	18	24/0	Ratio Max : Min
1	90	6.645 ± 1.33	7.675 ± 0.77	5.657 ± 2.016	6.013 ± 2.38	6.645 ± 1.33	1.35 ± 0.38
2	180	4.217 ± 0.98	2.514 ± 1.09 [a]	4.57 ± 0.49	5.144 ± 0.67	4.217 ± 0.98	2.04 ± 0.61
3	270	0.22 ± 0.11 [a]	0.862 ± 0.57[a]	0.785 ± 0.27[a]	4.097 ± 1.79	3.497 ± 1.06	18.62 ± 6.46[b]

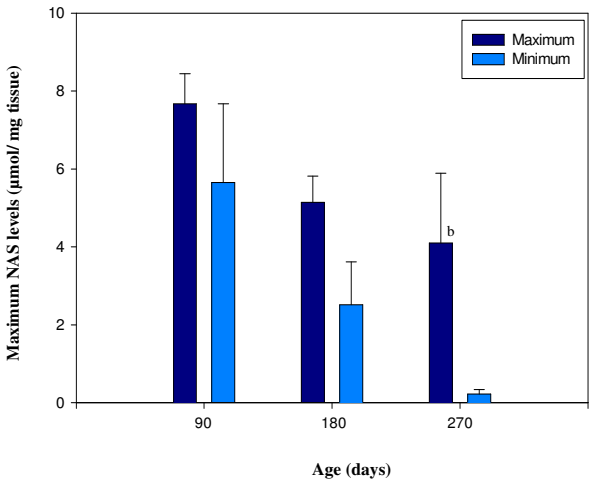
Each value represents HPLC-FC measurement of enzymatically formed N-Acetyl Serotonin (NAS) (µmol/ mg tissue)
Each value is mean ± S.E, (n=6); Zeitgeber Time (ZT): ZT-0 = 6.30 h (Lights on); ZT-12 = 18.30 h (Lights off)
One Way ANOVA: $p_a \leq 0.05$ (a refers to comparison with 90D)
t-test: $p_b \leq 0.05$ (b refers to comparison between maximum and minimum values within an age group)



Each value represents HPLC -FC measurement of enzymatically formed N -Acetyl Serotonin (NAS) (µmol/ g tissue)
Each value is mean ± S.E, (n=6); Zei tgeber Time (ZT): ZT -0 = 6.30 h (Lights on); ZT -12 = 18.30 h (Lights off).
One Way ANOVA: $p_a \leq 0.05$ (a refers to comparison with 90D)

Fig. 21a: Age related changes in the NAT activity rhythms in the SCN of rat (LD; 12:12)

NAS levels observed were 5.65 ± 2.01, 2.51 ± 1.098 and 0.22 ± 0.115 µmol/ mg tissue in 90, 180 and 270 day respectively. We observed 1.35 ± 0.38, 2.04 ± 0.61 and 18.62 ± 6.46 (Table 8; Fig. 21b) fold difference between the maximum and minimum NAT activity in 90, 180 and 270 day respectively.



Each value represents HPLC-FC measurement of enzymatically formed *N*-acetyl serotonin (NAS) (µmol/ g tissue)
Each value is mean ± S. E, (n=6); Zeitgeber Time (ZT): ZT-0=6.30 H (Lights on); ZT-12= 18.30 h (Lights off)
t-test: $p_b \leq 0.05$ (b refers to comparison of ratio values between a given age group)

Fig. 21b: Age related changes in the NAT activity rhythms in the SCN of rat (LD; 12:12)

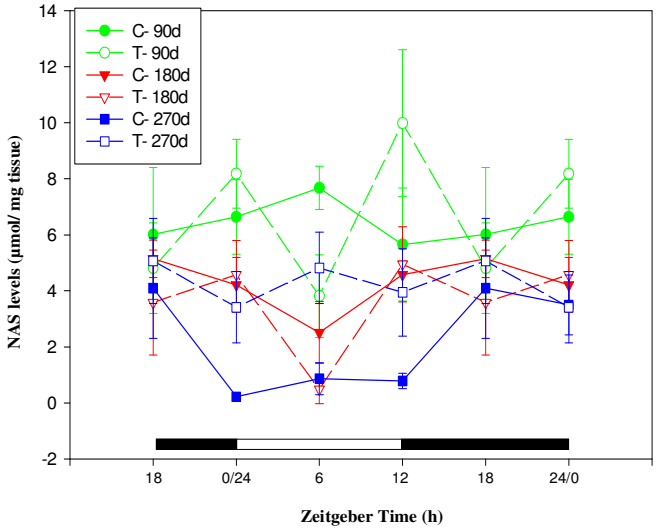
Effect of melatonin administration on NAT enzyme activity rhythms in the SCN of aging rat:

NAT activity did not show any significant change in 90 and 180 day, but a little increase in the activity was observed in 270 day upon melatonin administration (Table 9; Fig. 22a). We observed a phase delay in the maximum NAT activity from ZT-6 to ZT-12 at 90 day. NAT at 180 day, showed a phase advance in the maximum activity from ZT-18 to ZT-12. However we observed a significant decrease in NAT activity at ZT-6 in 180 day as compared to its control. Maximum and minimum activities were observed at ZT-18 and ZT-0

Table 9: Effect of melatonin administration on age related changes in the NAT activity rhythms of rat SCN (LD; 12:12)

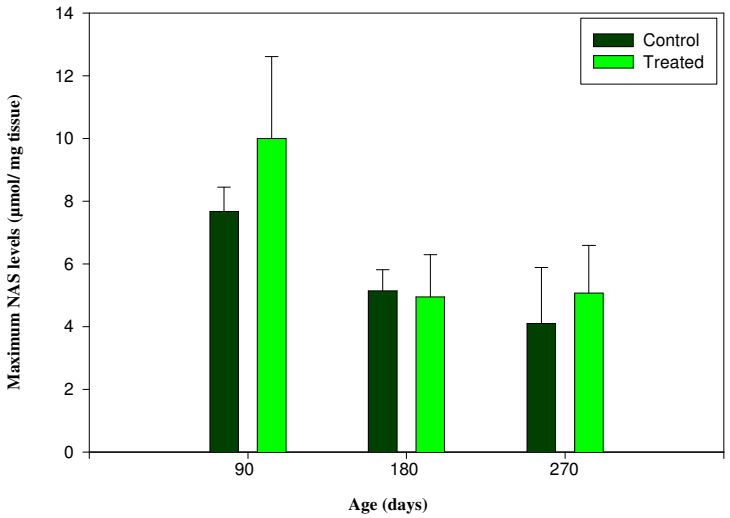
S. No	Age (days)		NAS levels (µmol/ mg tissue) at different zeitgeber times					Ratio
			0/24	6	12	18	24/0	Max : Min
1	90	C	6.64 ± 1.33	7.675 ± 0.77	5.657 ± 2.01	6.013 ± 2.38	6.645 ± 1.334	1.35 ± 0.3
		T	8.17 ± 1.23	3.815 ± 1.47[a]	9.993 ± 2.61	4.813 ± 1.61	8.179 ± 1.23	2.61 ± 1.77
2	180	C	4.21 ± 0.98	2.514 ± 1.09	4.57 ± 0.49	5.144 ± 0.67	4.217 ± 0.98	2.04 ± 0.61
		T	4.57 ± 1.22	0.513 ± 0.49	4.949 ± 1.34	3.583 ± 1.87	4.576 ± 1.22	9.64 ± 2.73
3	270	C	0.22 ± 0.11	0.862 ± 0.57	0.785 ± 0.27	4.097 ± 1.79	3.497 ± 1.06	18.62 ± 6.46[b]
		T	3.41 ± 1.27	4.817 ± 1.27 [a]	3.946 ± 1.55	5.071 ± 1.52	3.417 ± 1.27	1.48 ± 1.19

Each value represents HPLC-FC measurement of enzymatically formed N-Acetyl Serotonin (NAS) ((µmol/ mg tissue)
Each value is mean ± S.E, (n=6); Zeitgeber Time (ZT): ZT-0 = 6.30h (Lights on); ZT-12 = 18.30h (Lights off)
t-test: $p_a \leq 0.05$ (a refers to comparison of control and treated values within age groups)
t-test: $p_b < 0.05$ (b refers to comparison of ratio values between a given age group and 90D control)



Each value represents HPLC-FC measurement of enzymatically formed N-Acetyl Serotonin (NAS) ((µmol/ mg tissue)
Each value is mean ± S.E, (n=6); Zeitgeber Time (ZT): ZT-0 = 6.30 h (Lights on); ZT-12 = 18.30 h (Lights off)

Fig. 22a: Effect of melatonin administration on age related changes in the NAT activity rhythms of rat SCN (LD; 12:12)



Each value represents HPLC-FC measurement of enzymatically formed N-Acetyl Serotonin (NAS) ((µmol/ mg tissue)
Each value is mean ± S.E, (n=6); Zeitgeber Time (ZT): ZT-0 = 6.30 h (Lights on); ZT-12 = 18.30 h (Lights off)

Fig. 22b: Effect of melatonin administration on age related changes in the NAT activity rhythms of rat SCN (LD; 12:12)

respectively as observed in controls. There was no significant change in rhythmicity of NAT activity at 270 day upon melatonin administration. Melatonin treatment increased NAT activity significantly in 270 day old at all time points except at ZT-0 and 18. Maximum activity observed upon melatonin administration was 9.99 ± 2.61, 4.94 ± 1.34 and 5.07 ± 1.52 µmol/ mg tissue in 90, 180 and 270 day respectively. Minimum activity observed was 3.81 ± 1.47, 0.51 ± 0.49 and 3.41 ± 1.27 µmol/ mg tissue in 90, 180 and 270 day respectively.

DISCUSSION:

Several workers studied neural regulation of melatonin synthesis in various organs, species, both in nocturnal and diurnals and at different experimental conditions. It is well established that NAT activity which is under the control of SCN plays an important role in melatonin synthesis. Melatonin levels decline with age. The exact mechanism for the decrease in melatonin levels is yet to be determined. Decline in melatonin levels with age could be due to less

availability of its precursor, serotonin and alteration (decreased or shift) in the activity of enzymes involved in the synthesis or due to changes in the signal transduction pathway. The results in the previous chapters showed that serotonin levels decrease with increase in age. This could be a reason for the decrease in melatonin levels with age.

In the present work, we studied daily NAT activity rhythms in rat SCN of different age groups. Our results indicated that aging reduces the amplitude of daily NAT activity in the SCN (Table 8; Fig. 21). Earlier *in vivo* studies showed that different subpopulations of SCN neurons have different peak time of their activity (Saeb-Parsy and Dyball, 2003). Similarly the nocturnal neuronal activity in specific SCN neurons could play an important role in stimulation of NAT activity in the SCN. VIP is the neuropeptide through which SCN regulates output rhythms. Ibata *et al.,* (1999) suggested that the amount of VIP mRNA reduces in the SCN of aged rats.

Another group of workers showed that lesions in the PVN lead to reduced activity of melatonin synthesizing enzymes and thus results in low melatonin levels. Glutamatergic signaling within the PVN plays an important role in melatonin synthesis. Thus, for the stimulation of melatonin synthesis in the pineal gland, nocturnal neuronal activity in specific SCN cell populations as well as activity in PVN would be crucial (Perraeu-Lenz *et al.,* 2004).

It was reported that decline in circadian activity of suprachiasmatic nucleus serotoninergic neurons may account for the blunting of circadian rhythms in the secretions of several anterior pituitary hormones in old animals. Decrease in hypothalamic NE turnover has been known with aging (Simpkins and Millard, 1987). Though NE induces nocturnal increase in pineal *Nat* gene expression, Garidou *et al*., (2001) suggested that neurotransmitters other than NE are involved in the day time inhibition and night time stimulation of pineal metabolism. Serotonin has been shown to enhance the release of NE from the adrenals (Lefebvre *et al.,* 1998). Previous reports showed that there was a decrease in the density of β-adrenergic receptors on the pinealocytes of rat with increasing age (Henden *et al.,* 1992). Earlier studies suggested that 5-HT release may play a role in the full expression of β-adrenergically induced NAT activity

and thus contribute to optimal melatonin synthesis at night (Miguez *et al.,* 1997). Our present results suggested that the decrease in serotonin levels (Chapter 1) were not because of increased NAS levels as we found decrease in NAS levels with aging. The decrease in NAT activity which was measured in terms of NAS formed could be related to decrease in serotonin levels with aging. Thus in addition to transcriptional and post-transcriptional regulation of NAT, serotonin levels as well as its release seem to play a crucial role in inducing NAT activity in the pineal. This could be the same in SCN also for the induction of NAT activity.

The same mechanism appears to be involved in a gland called submaxillary gland (Ellison *et al.,* 1972) as observed in pineal gland but lacks circadian rhythmicity in NAT. Thus, this suggests that regulation of NAT activity differs from tissue to tissue and pineal gland adapts specific mechanism to regulate rhythmicity of NAT in the pineal gland (Ellison *et al.,* 1972). Thus regulation of NAT activity in SCN could be different from that in pineal gland as it does not involve the multisynaptic pathway and the exact mechanism is yet to be elucidated. It was reported that the specificity, stability and inhibition by melatonin are the factors that regulate the activity of NAT which differs from tissue to tissue (Voisin *et al.,* 1984). Hamada *et al.,* (1999) suggested that in the SCN of rat, post-transcriptional mechanisms such as phosphorylation of NAT by PKA might play a dominant role in regulating NAT activity. Melatonin secretion was found to diminish with the advancement of age due to insufficient environmental illumination (Mishima *et al.,* 2001). This is supported by a clear change in habitual light exposure patterns associated with aging (Kawinska *et al.,* 2005). This suggests that the response of SCN to photic cues alters with aging and hence results in changes in circadian rhythms of many physiological functions.

Several studies have reported that timed melatonin administration can help with re-adjusting the circadian system after jet-lag and shift-work. Melatonin administration helped in improving the quality of sleep and/or timing of sleep in some patients of insomnia (Rajaratnam and Arendt, 2001). Melatonin is known to accelerate the re-entrainment of circadian rhythms (locomotor activity as well as

NAT activity) in rats subjected to a shift in the LD cycle (Redman and Armstrong 1988). Thus the probable chronobiotic effect of melatonin may result from a direct action on the SCN. The rhythmic synthesis of melatonin by the pineal is a direct output of the clock. Exogenous melatonin exerts its effects on SCN through its receptors and it could also affect the endogenous melatonin rhythm (Pévet *et al.,* 2002). Thus we studied the effect of exogenous melatonin on age related changes in NAT activity.

Our results showed that there was no significant increase in the NAT activity upon melatonin administration in the SCN of aging rats (Table 9; Fig. 22a and 22b). Previous report suggests that age related decline in melatonin production is a consequence of increased oxidation of its precursors (Lerchl, 1994). Recent studies by Liu and Borjigin (2005) showed that NAT is not the rate limiting enzyme in melatonin biosynthesis in the pineal gland. They demonstrated that (i) night time NAS was in excess as compared to melatonin in pineal (ii) increase in NAT protein levels didn't induce melatonin production and (iii) increase in NAS didn't increase in melatonin output. They suggest that HIOMT and NAT determine the level of melatonin synthesis in the pineal at night. HIOMT activity of pineal gland was found to reduce by 17 to 55% in old rats (18 months) (Dax and Sugden, 1988). Additional factors may be playing an important role in regulation of NAT activity and hence exogenous melatonin would not have significant effect on NAT activity in the SCN.

This could also be explained by the number of melatonin receptors present on the tissue. The affinity of binding sites for melatonin is similar in all brain regions and doesn't change with circadian timing. Receptor autoradiography studies showed that the density of melatonin receptors in the hypothalamus decreased significantly with the advancement of age (Pevet *et al.,* 2002). In rat SCN melatonin receptors were shown to exhibit circadian variation with low levels during the night (Gauer *et al.,* 1993, Tenn and Niles, 1993). Earlier reports showed that aging reduces MT_1 receptor mRNA expression in the SCN during day but not at night (Benloucif *et al.,* 1997b). This supports that there is a great correlation between the density of melatonin receptors within the SCN and the ability of exogenous melatonin administration in the entrainment of

clock. It was suggested that along with high affinity melatonin receptors, there could be other mechanisms involved in entraining effect of melatonin like high dosage (Pevet *et al.,* 2002).

It was reported that intraperitoneal administration of melatonin could restore the amount of VIP mRNA in aged rats to that of the levels in young ones (Ibata *et al.,* 1999). This suggests that different routes of administration of melatonin might have different targets in the same tissue. Melatonin mechanism of signal transduction shows both species and tissue specificity (McArthur *et al.,* 1997). Studies on hamsters revealed that melatonin can entrain rhythms only under particular experimental conditions such as long term infusions (Schuhler *et al.,* 2002). This could be the reason for no significant increase in NAT activity in the SCN of aging rats upon melatonin administration. The present work suggests that melatonin synthesized in the SCN itself might play a role in entraining the clock along with exogenous melatonin. Thus our present study suggests to separately elucidate the role of endogenous melatonin that is synthesized in pineal gland as well as in SCN itself. Also the exact mechanism of exogenous melatonin action on the SCN is to be determined.

CHAPTER 4

Age induced changes and the effect of melatonin administration on Ca^{2+}/Calmodulin-dependent protein kinase II (CaMKII) activity rhythms in SCN and Pineal gland of rat

CONTENTS

INTRODUCTION:

The circadian oscillator of organisms is composed of autoregulatory transcriptional/translational feedback loops (Kondratov *et al.,* 2003). Post-transcriptional regulation of clock proteins plays an important role in rhythm generation and entrainment (Lowrey *et al.,* 2000). Entrainment of mammalian circadian rhythms involves several signal transduction pathways such as activation of transcription factors (Gau *et al.,* 2002) and related kinases (Golombek *et al.,* 2003). Mutations in key protein kinases have been shown to affect the chronobiological properties of different animal models (Lowrey *et al.,* 2000). Cellular processes as diverse as the transcription and translation of genes, fertilization and cell division, metabolism, membrane transport and permeability, secretion, contractility, neurotransmission and even memory are all regulated by post-translational modifications (Ceseña *et al.,* 2007; Whitmarsh, 2007). Various studies demonstrated that post-translational modification is critical to all circadian mechanisms sometimes more important than regulated transcription.

The common feature of all the post translational modifications in circadian systems is phosphorylation of one or several clock proteins (Merrow *et al.,* 2006; Vanselow *et al.,* 2006). In eukaryotes, phosphorylation mediates the circadian timing through regulation of proteins of the transcription-translation feedback loop (Young and Kay, 2001; Brunner and Schafmeier, 2006). Transcriptional factors which play an important role in clock function are the largest group of proteins to be phosphorylated (Ptacek *et al.,* 2005). Phosphorylation determines the cellular localization and stability of clock proteins, a critical process for building time delays into the 24 h rhythms of molecular mechanism (Young 2000; Denault *et al.,* 2001).

Clock proteins in all molecular circadian systems exhibit a temporally distinct phosphorylation patterns. In *Drosophila*, DBT, which is most closely related to mammalian CKIε phosphorylates PER, thereby influencing PER turnover (Price *et al.,* 1998). In mammals, phosphorylation appears to control critical aspects of mCRY: mPER interactions necessary for normal clock

function (Lee *et al.,* 2001). Phosphorylation status of the transcription factors may be a determining character for the transcriptional competency of the heterodimer. Phosphorylation regulates the transcriptional activity of other bHLH transcription factors (Neufeld *et al.,* 2000; Park *et al.,* 2000). It may also be important for the formation of protein complexes that inhibit CLOCK: BMAL1-mediated transcription, but it does not appear to alter CLOCK: BMAL1 heterodimerization or binding to DNA. Studies of the *tau* mutation in Syrian hamsters (a spontaneous, semi-dominant mutation leading to marked shortening of the circadian period) revealed that it encodes a missense mutation within CKIε. This results in the mutant enzyme deficient in its ability to phosphorylate the mPER proteins (Lowrey *et al.,* 2000). A human genetic disorder characterized by shortened circadian period and advanced sleep phase is associated with a missense mutation in human PER2 and the mutant protein is less effectively phosphorylated by CKIε *in vitro* (Toh *et al.,* 2001). Several studies suggested that pharmacological modulation of cellular protein phosphorylation has yielded useful information on the molecular events involved. Phosphorylation state of many proteins is fine-tuned by a balance between kinases and phosphatases.

Protein kinases are important regulators of many cellular processes. These kinases modify the functions of enzymes, receptors, channels, transporters and others by phosphorylation. Second messengers like Ca^{2+}, cAMP, cGMP and phospholipase C activate the protein kinases. Several protein kinases like CKIδ (Lee *et al.,* 2001) are known to play an important role in mammalian clock function.

Calcium as intracellular messenger in the circadian system:

Cellular calcium concentrations act as important components of signal transduction pathways (D'Souza and Johri, 2003). It plays a key role in the light resetting of the circadian clock. It regulates diverse cellular processes like membrane potential, neurotransmitter release, gene expression. Calcium is compartmentalized into cytosolic and nuclear Ca^{2+} and this has been described in several cell types. The gradients of cytosolic and nuclear Ca^{2+} depend on

the type of cell and stimulants (Ikeda *et al.,* 2003). Studies by Ikeda *et al.,* (2003) showed that Ca^{2+} levels in the cytosol but not the nucleus of SCN neurons exhibit circadian rhythmicity and nuclear Ca^{2+} response in SCN neurons might play an important role in circadian regulation. Neurotransmitter release is generally dependent on cytosolic Ca^{2+} $[Ca^{2+}]_c$ (Ikeda *et al.,* 2003). Cytosolic free Ca^{2+} mediates circadian signal from the core loop to membrane potential. Calcium transmits both the input and out put signals to and from the core molecular clock in the SCN neurons (Honma and Honma, 2003). $[Ca^{2+}]_c$ is important for output pathways via neuronal circuits (Aston-Jones *et al.,* 2001) as well as humoral pathways from SCN neurons.

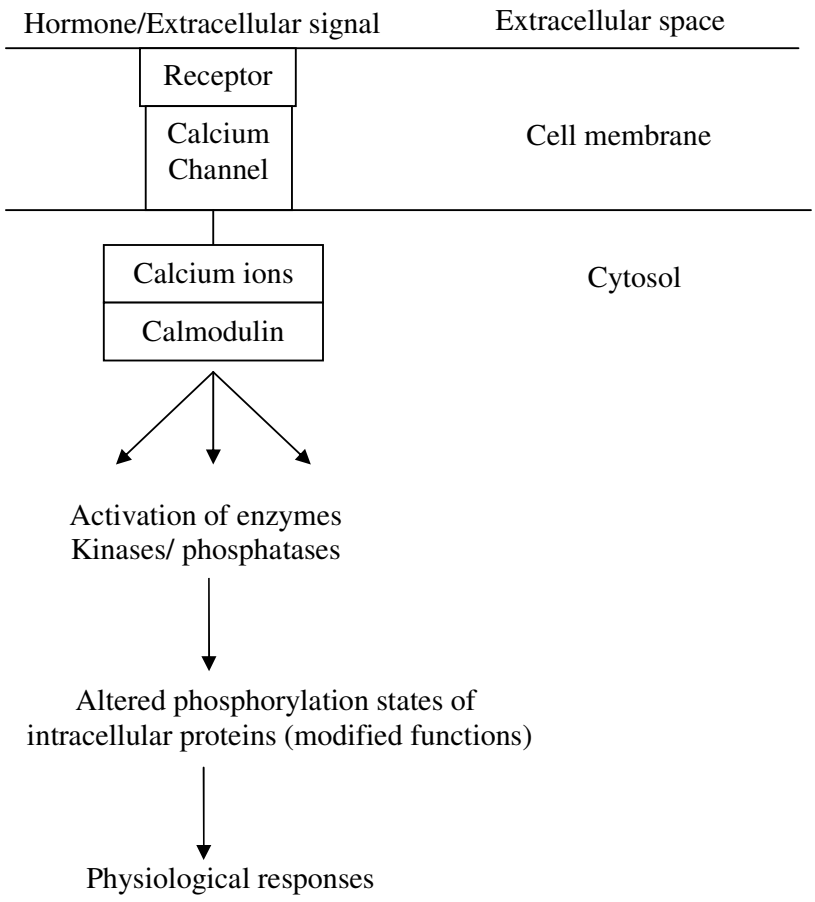
Calcium plays an important role in neuronal aging and based on 'calcium hypothesis of aging' (Khachaturian, 1994), dysfunction of intracellular calcium $[Ca^{2+}]_i$ homeostasis and neuronal loss are important alterations that are age dependent (Raza *et al.,* 2007). $[Ca^{2+}]_c$ increases (0.1µM resting state to 1-10µM in the stimulated state) either by release from intracellular stores or by influx from the extracellular space (Machaca, 2003). It mediates the effects of many hormones and neurotransmitters on the target tissues (Colbran *et al.,* 1989). The first link in the chain of events is generally a hormone or transmitter reacting with a specific receptor (Fig. 23). The primary intracellular receptor of increased calcium is calmodulin (CaM) (Cheung, 1980).

Calmodulin:

Calmodulin is a highly conserved and most widely distributed Ca^{2+}-binding protein (Turjanski *et al.,* 2004). It has a dumbbell shape with two Ca^{2+}-binding domains (Barbato *et al.,* 1992). It is an important sensor of intracellular Ca^{2+} and upon activation it undergoes conformational change. It is known to interact with a large number of Ca^{2+}-dependent intracellular signaling. It helps in the control of various cellular processes such as muscle contraction, fertilization, cell proliferation, vesicular fusion and apoptosis (Berridge *et al.,* 1998). It functions as a regulatory element for its target proteins. The principal action of Ca^{2+}/calmodulin complex is alteration of

phosphorylation states of intracellular proteins and enzymes (Manalan and Klee, 1984) which modulates important cellular functions. Small hydrophobic molecules bind to CaM and modify its function by inhibiting the interaction with other proteins (Harmat *et al.,* 2000). There are two major classes of Ca^{2+}-dependent protein kinases, phosphatidylserine-dependent kinases (Ca^{2+}/PS Kinases) (Nishizuka, 1984) and Ca^{2+}/calmodulin-dependent protein kinases (CaM Kinases) (Schulman and Greengard, 1978).

Fig. 23: Generalised Mechanism of Calcium mediated actions of Hormones and extracellular signals



(Cohen, 1988)

Ca^{2+}/calmodulin-dependent protein kinases:

Elevated calcium levels trigger CaM kinases (Schulman, 1993) which coordinate cellular responses to external stimuli. These responses include phosphorylation of proteins involved in neurotransmitter synthesis, neurotransmitter release, carbohydrate metabolism, ion flux and neuronal plasticity. The kinase is relatively inactive in its basal state by the presence of an autoinhibitory domain. Binding of Ca^{2+}/calmodulin allows the kinase to phosphorylate its substrates, as well as itself. This autophosphorylation significantly slows dissociation of CaM, thereby trapping CaM even when Ca^{2+} levels are sub-threshold. Once CaM dissociates, CaM kinase remains partially active until it is dephosphorylated (Schulman and Hanson, 1993).

The CAM kinases are mostly located within the cytosol or loosely associated with the plasma membrane (Nairn *et al.,* 1985). They include phosphorylase kinase, myosin light chain kinase and Ca^{2+}/calmodulin dependent protein kinases I, II III and IV. Ca^{2+}-dependent intracellular signaling is an important regulatory mechanism in neural tissues which contain high concentrations of Ca^{2+}/calmodulin regulated proteins. Some of these CaM-binding proteins are involved in regulating the synthesis or degradation of signaling systems and also protein phosphatases (Hashimoto *et al.,* 1988).

The enzyme CaMKII is abundantly expressed in the rat SCN (Agostino *et al.,* 2004). It was reported that light exposure results in phosphorylation of CaMKII in the SCN (Yokota *et al.,* 2001). It is implicated in the resetting of the circadian clock by light exposure (Fukushima *et al.,* 1997). Golombek and Ralph (1994) suggested that activation of CaMKII mediates the circadian responses to light via CREB phopsphorylation. It is known to induce *Per1* and *Per2* mRNA in the hamster SCN as well as phase shifting upon light-exposure (Yokota *et al.,* 2001). Earlier workers reported that CaMKII inhibitor, KN-62 suppressed light-induced phase shift of activity rhythm (Golombek and Ralph, 1995) c-Fos expression (Fukushima *et al.,* 1997), CREB phosphorylation in the SCN (Golombek and Ralph, 1995). The enzyme CaMKII is rhythmically

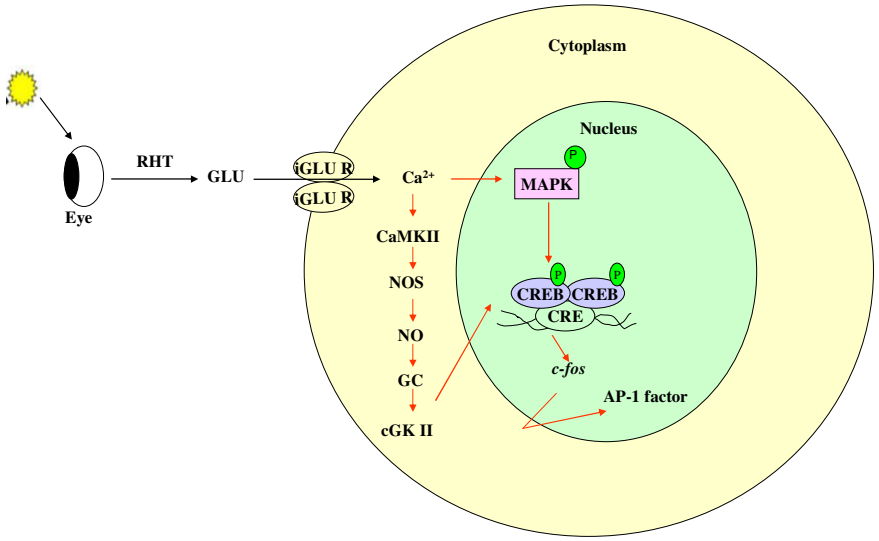
phosphorylated in the SCN both under entrained and free-running (constant dark) conditions (Golombek *et al.,* 2004). Its activity was found to be reduced in ischemia. Loss of activity of CaMKII was suggested to play an important role in initiating the changes involved in ischemia induced- cell death (Shackelford *et al.,* 1995).

Hormones and Extracellular signals:

Hormones, neurotransmitters and other extracellular signals transmit information to the interior of the cell by activating transmembrane signaling systems that control the production of chemical mediators called 'second messengers' such as cAMP and Ca^{2+} (Hashimoto and Soderling, 1987). These second messengers regulate protein kinase and phosphatase activities and alter the phosphorylation states and hence the activities of many intracellular proteins resulting in the physiological response. Intracellular free Ca^{2+} $[Ca^{2+}]_i$ levels have been estimated in the SCN. Various cellular processes are regulated in a well-coordinated manner due to the cross talk between the membrane-associated cell signaling processes (Shenolikar, 1988). In the SCN, $[Ca^{2+}]_i$ levels are higher during the light phase than in the dark (Colwell, 2000). It was also suggested that $[Ca^{2+}]_i$ rhythm is a result of circadian firing rhythms of the SCN neurons (Honma and Honma, 2003).

Light exposure in the night results in the release of glutamate at the terminals of the retinohypothalamic tract which reach SCN neurons. Glutamate interacts with ionotropic glutamate receptors on the SCN neuron leading to calcium influx. Influx of Ca^{2+} activates a series of events (Obrietan *et al.,* 1998). Calcium activates CaMKII and triggers NOS activation. This results in increased levels of nitric oxide which stimulates soluble guanylyl cyclase. Guanylyl cyclase increases cGMP concentrations and thus activates cGMP-dependent protein kinase II (cGKII) (Liu *et al.,* 1997). This is later involved in regulating light induced *Per* expression (Oster *et al.,* 2003). In addition to CaMKII, Ca^{2+} also activates mitogen activated protein kinases (Yokota *et al.,* 2001). The kinase phosphorylates cAMP responsive element

binding protein (CREB) and ultimately leads to the expression of clock controlled genes (Fig. 24).



(Gurudutt and Albrecht, 2005)

Fig. 24: Role of CaMKII in a SCN neuron

Calcium dependent protein kinases have been shown to play a major role in the regulation of serotonin synthesis and release (Ramakrishnan *et al.,* 2005). The rate-limiting step in the synthesis of 5-HT is the activity of TPH (Malek *et al.,* 2005) whose activity in the brain is mainly dependent on two factors: the concentration of L-tryptophan in the brain and the impulse activity in the serotonergic neurons. The activity of TPH is again dependent on its phosphorylation status by the CaMKII.

Melatonin effects are known to be mediated by several mechanisms. It can act by binding to neural and non-neural membrane receptors (Dubocovich, 1995), by binding to CaM (Turjanski *et al.,* 2004), to nuclear proteins (Acuña-Castroveijo *et al.,* 1994; Steinhilber *et al.,* 1995) and also acts as a free radical scavenger (Reiter *et al.,* 1995). Recent evidence suggests that a melatonin

mechanism of action may be through modulation of Ca^{2+}-activated CaM (Benitez-King *et al.,* 1996).

We observed decrease in serotonin levels and its rhythmicity with age (Chapter 1). Melatonin levels are known to decline with age and one reason for the decrease in melatonin levels could be decreased serotonin levels with age. Our results, (Chapter 3) showed that NAT activity rhythms also alter with age. The CaMKII is known to regulate the TPH activity. So, CaMKII might play a role in regulating serotonin levels and hence its rhythmicity indirectly which was shown to alter with age and these serotonin levels could help in maintainance of normal melatonin levels involved in maintaining various physiological circadian functions. Thus we studied the age induced changes in CaMKII activity rhythms and the effect of exogenous melatonin administration on CaMKII activity rhythms in SCN and pineal gland.

MATERIALS and METHODS:

[γ^{32}P] ATP (2000-4000 cpm/pmol) was obtained from Board of Radiation and Isotope Technology (BRIT JONAKI, CCMB, Hyderabad, India). Okadaic acid, Syntide-2 and CaM were purchased from Sigma Chemicals. Phosphocellulose filter (P-81) was purchased from Whatman (Canlab Corp., Mississauga, ON, Canada). All other chemicals were of analytical grade and obtained from standard commercial suppliers.

Male Wistar rats of different age groups (90, 180 and 270 day old) were taken and maintained under laboratory conditions, 06.30h (ZT-0)-18.30h (ZT-12) light phase; 18.30h (ZT-12)-06.30h (ZT-24) dark phase, two weeks prior to the experiments. All rats were kept individually in polypropylene cages at room temperature (20+2^{o}C) with relative humidity (55+6%). Food and water were supplied *ad libitum*. Dim red light was used for handling the animals in the dark. Cage changing was done at random intervals.

SCN preparation:

SCN was dissected out as described in Chapter 1.

Pineal preparation:

Pineal gland is located just rostro-dorsal to the superior colliculus and behind and beneath the stria medullaris, between the laterally positioned thalamic bodies. It is part of the epithalamus. Rats were decapitated and brains were removed carefully. Pineal glands were carefully removed with the help of curved forceps as described by Jagota *et al.,* (1999).

Melatonin administration:

Melatonin administration was given as described in Chapter 2 and 3.

CaMKII activity assay:

1 mg of tissue was homogenized in 50mM Tris-Cl (pH-7.4), 1mM EDTA, 1mM EGTA, 50mM NaF, a protease inhibitor cocktail, 10µM okadaic acid and 0.32M sucrose. Activity of CaMKII was assayed by the method of Fukunaga *et al.,* (1989). 50µl reaction mixture contained 50mM HEPES (pH-7.5), 10 mM $MgCl_2$, 0.1 mM [γ^{32}P] ATP (2000-4000 cpm/pmol), 30µM syntide-2, 2µM CaM, 1 mM $CaCl_2$ and 20 µg of homogenate protein. The reaction mixture was incubated at 30ºC for 1 min and the reaction was stopped by adding 10µl of 0.4 M EDTA. The radioactivity was measured by the method of Roskoski, (1983). 50 µl of sample was spotted on to 2 x 2 cm phosphocellulose strips. The strips were immersed in 75 mM phosphoric acid (10 ml per strip) and swirled gently for 2 min. The phosphoric acid was decanted and the phosphocellulose strips were washed twice (1 min each) gently in 75 mM phosphoric acid. After drying the strips, radioactivity was measured by liquid scintillation counter (Tricarb, 2100R Liquid Scintillation Analyzer) using toluene scintillation fluid.

RESULTS:

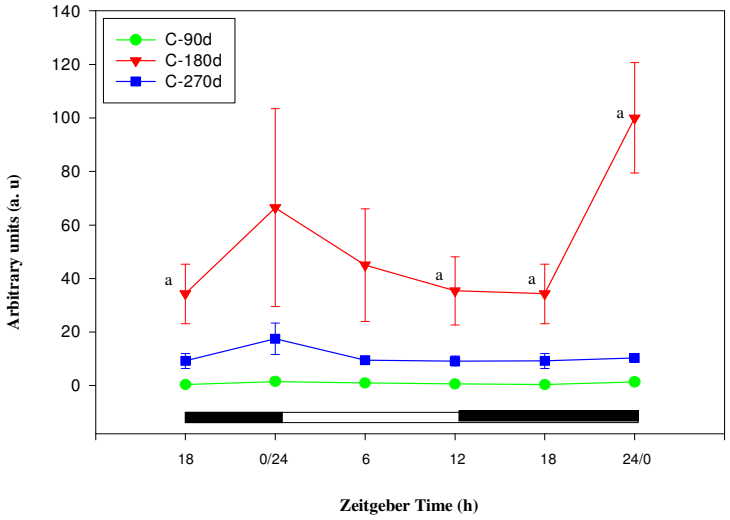
Age induced changes in the CaMKII activity rhythms in the SCN:

CaMKII activity and its rhythmicity varied significantly with increasing age in SCN (Table 10; Fig. 25a). CaMKII activity was observed to be rhythmic with maximum amplitude at ZT-0/24 i.e at the onset of light and minimum at ZT-18, mid-night in all the age groups studied. Aging had a significant effect on CaMKII activity. Maximum CaMKII activity was observed at 180 day. CaMKII activity increased from 90 day to 180 day and then decreased from 180 day to 270 day. The maximum CaMKII activity observed was 1.52 ± 0.602, 66.46 ± 36.94 and 17.48 ± 5.86 arbitrary units (a.u) at ZT-0 in 90, 180 and 270 day respectively. In 180 day maximum CaMKII activity was 100 ± 20.61 at ZT-24/0. Minimum CaMKII activity observed was 0.33 ± 0.06, 34.28 ± 11.13 and 9.15 ± 1.84 a.u. at ZT-18 in 90, 180 and at ZT-12 in 270 day. Activity of CaMKII was significantly different in 180 day at ZT-12, 18 and 24/0 ($p_a < 0.05$) as compared to 90 day. The maximum CaMKII activity increased by 78 folds from 90 to 180 day and decreased by about 6.5 folds from 180 day to 270 day.

Table 10: Effect of melatonin on age related changes in CaMKII activity rhythms in the rat SCN (LD; 12:12)

S.No	AGE (days)		CaMKII activity (a.u) at different zeitgeber times				
			0/24	6	12	18	24/0
1	90	C	1.52 ± 0.602	1.0 ± 0.045	0.61 ± 0.087	0.33 ± 0.06	1.4 ± 0.12
		T	46.67 ± 2.12^{b}	18.71 ± 5.72$^{a, b}$	7.52 ± 2.27^{b}	12.67 ± 4.11$^{a, b}$	24.75 ± 4.5$^{a, b}$
2	180	C	66.46 ± 36.94	44.99 ± 20.98	35.39 ± 12.73^{a,}	34.28 ± 11.13^{a}	100 ± 20.61^{a}
		T	23.28 ± 2.08^{b}	15.33 ± 2.76$^{a, b}$	14.44 ± 3.05$^{a, b}$	10.86 ± 1.32$^{a, b}$	29.12 ± 4.03$^{a, b}$
3	270	C	17.48 ± 5.86	9.52 ± 1.37	9.15 ± 1.84	9.2 ± 2.78	10.34 ± 1.54
		T	65.33 ± 45.13	9.09 ± 1.02	10.52 ± 1.17$^{a, b}$	10.53 ± 2.65$^{a, b}$	13.7 ± 0.64

Each value is mean ± S.E, (n=6); Zeitgeber Time (ZT): ZT-0 = 6.30 h (Lights on); ZT-12 = 18.30 h (Lights off)
One Way ANOVA: $p_a \leq 0.05$ (a refers to comparison with 90 D control (C))
$p_b \leq 0.05$ (b refers to comparison of control and treated values within age groups)



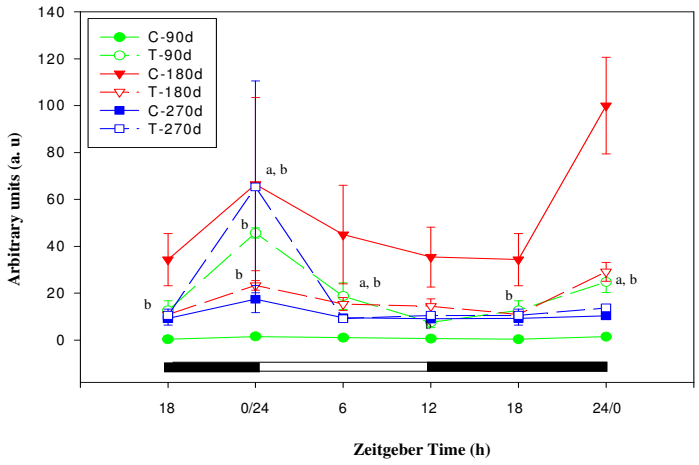
Each value is mean ± S.E, (n=6); Zeitgeber Time (ZT): ZT-0 = 6.30 h (Lights on); ZT-12 = 18.30 h (Lights off)
One Way ANOVA: $p_a \leq 0.05$ (a refers to comparison with 90 D control (C))

Fig. 25a: Age related changes in CaMKII activity rhythms in the SCN of rat (LD; 12:12)

Effect of melatonin administration on CaMKII activity rhythms in the SCN of aging rat:

Melatonin treatment had a significant effect on CaMKII activity in the SCN (Table 10; Fig. 25b). Melatonin treatment resulted in increased CaMKII activity in 90 and 270 day but not in 180 day. In 90 and 180 day, upon melatonin administration, CaMKII activity increased significantly at all times as compared to controls of 90 day CaMKII activity ($p_a < 0.05$). There was a significant difference in the CaMKII activity of 270 day upon melatonin treatment as compared to the control CaMKII activity in 90 day at all zeitgeber times except at ZT-0 ($p_a < 0.05$). The maximum CaMKII activity observed after melatonin administration was 46.67 ± 2.12 ($p_b < 0.05$), 29.12 ± 4.03 ($p_b < 0.05$) and 65.33 ± 45.13 a.u at ZT-0/24 in 90, 180 and 270 day respectively as compared to their respective controls. Upon melatonin administration, the maximum CaMKII activity increased in 90 and 270 day by

about 36 and 4 folds respectively as compared to their controls. We observed a decrease in the activity at 180 day by 3.5 fold but, melatonin played a role in controlling the fluctuations in the activity at 180 day. Though there was an increase in the CaMKII activity of melatonin treated 270 day rats at all zeitgeber times except at ZT-6, the increase was not significant as compared to its controls. The CaMKII activity observed at ZT-0 and ZT-24/0 in 270 day was 65.33 ± 45.13 and 13.7 ± 0.64 a.u. respectively. The minimum CaMKII activity observed after melatonin treatment was 7.52 ± 2.27 (ZT-12), 10.86 ± 1.32 (ZT-18) and 9.09 ± 1.02 a. u. (ZT-6) respectively.



Each value is mean ± S.E, (n=6); Zeitgeber Time (ZT): ZT-0 = 6.30 h (Lights on); ZT-12 = 18.30 h (Lights off)
One Way ANOVA: $p_a \leq 0.05$ (a refers to comparison with 90 D control (C))
$p_b \leq 0.05$ (b refers to comparison of control and treated values within age groups)

Fig. 25b: Effect of melatonin administration on age related changes in CaMKII activity rhythms in the SCN of rat (LD; 12:12)

Age induced changes in the CaMKII activity in the pineal gland:

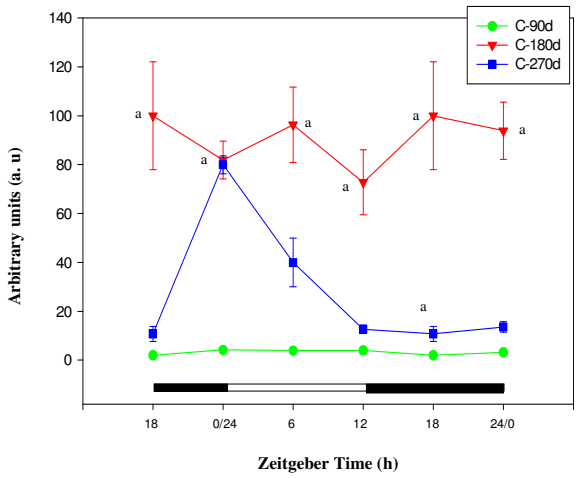
We found that there was a similar change in the activity of CaMKII in pineal gland (Table 11; Fig. 26a) as was observed in SCN with increase in age. Activity increased from 90 day to 180 day and then decreased from 180 day to 270 day. The maximum CaMKII activity was observed at ZT-0, i.e at the onset of light in 90 and 270 day whereas in 180 day maximum activity was observed at ZT-18. In 180 day, CaMKII activity was higher at all zeitgeber

times as compared to the activity with respect to 90 and 270 day, but rhythmicity was not observed. In 270 day rhythmicity was observed but with decreased activity as compared to that of the activity in 180 day. The activity of CaMKII increased significantly at all zeitgeber times in 180 day ($p_a < 0.05$) as compared to 90 day. In 270 day CaMKII activity was significantly different from 90 day activity at ZT-0 and 18 ($p_a < 0.05$). The maximum CaMKII activity observed was 4.19 ± 0.5, 100 ± 2.21 and 79.97 ± 3.67 a.u in 90, 180 and 270 day respectively. The increase in maximum activity from 90 day to 180 day was about 24 folds and decrease in maximum activity from 180 day to 270 day was about 1.25 folds. The minimum activity observed was 1.95 ± 0.17, 72.77 ± 13.34 and 10.76 ± 3.11 a.u in 90, 180 and 270 day respectively. The minimum activity was observed at ZT-18 in 90 and 270 day i.e at mid-night and at ZT-12 at 180 day. The increase in minimum activity from 90 day to 180 day was about 37.32 folds and decrease in minimum activity from 180 day to 270 day was about 6.76 folds respectively. The activity of the enzyme was significantly high in pineal as compared to the SCN in all the age groups.

Table 11: Effect of melatonin on age related changes in CaMKII activity rhythms in the rat pineal gland (LD; 12:12)

S.No	AGE (days)		CaMKII activity (a.u) at different zeitgeber times				
			0/24	6	12	18	24/0
1	90	C	4.19 ± 0.5	3.92 ± 0.27	3.96 ± 0.21	1.95 ± 0.17	3.14 ± 0.26
		T	48.58 ± 8.75$^{a, b}$	41.09 ± 10.5$^{a, b}$	38.72 ± 4.77$^{a, b}$	27.32 ± 9.28^{b}	45.7 ± 8.82$^{a, b}$
2	180	C	81.91 ± 7.74^{a}	96.28 ± 15.41^{a}	72.77 ± 13.34^{a}	100 ± 2.21^{a}	93.87 ± 11.77^{a}
		T	4.44 ± 1.43^{b}	5.41 ± 2.14^{b}	6.1 ± 1.21^{b}	7.68 ± 1.91^{b}	12.05 ± 3.68^{b}
3	270	C	79.97 ± 3.67^{a}	39.95 ± 9.96	12.63 ± 1.68	10.76 ± 3.11^{a}	13.59 ± 2.15
		T	34.45 ± 7.99$^{a, b}$	3.36 ± 0.39^{b}	3.25 ± 0.55^{b}	3.96 ± 0.18	34.39 ± 7.72$^{a, b}$

Each value is mean ± S.E, (n=6); Zeitgeber Time (ZT): ZT-0 = 6.30 h (Lights on); ZT-12 = 18.30 h (Lights off)
One Way ANOVA: $p_a \leq 0.05$ (a refers to comparison with 90 D control (C))
$p_b \leq 0.05$ (b refers to comparison of control and treated values within age groups)



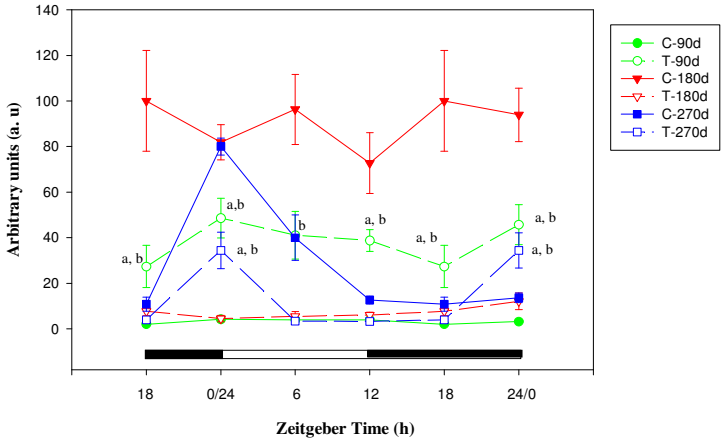
Each value is mean ± S.E, (n=6); Zeitgeber Time (ZT): ZT-0 = 6.30 h (Lights on); ZT-12 = 18.30 h (Lights off)
One Way ANOVA: $p_a \leq 0.05$ (a refers to comparison with 90 D control (C))

Fig. 26a: Age related changes in CaMKII activity in the pineal gland of rat (LD; 12:12)

Effect of melatonin administration on CaMKII activity rhythms in the pineal gland of aging rat:

The effect of melatonin treatment on CaMKII activity rhythms in the pineal gland (Table 11: Fig. 26b) was significant and different from SCN. Upon melatonin administration, there was an increase in CaMKII activity in 90 day but significant decrease was observed in 180 and 270 day. Rhythmicity was maintained after melatonin administration in 90 day but not in 180 and 270 day. In 90 day treated, CaMKII activity was significantly increased as compared to the respective control values at all zeitgeber times ($p_a < 0.05$). In treated 180 and 270 day, CaMKII activity at ZT-18 and 24/0 were significantly different from that of control values of 90 day ($p_a < 0.05$). The maximum CaMKII activity observed after melatonin administration was 48.58

± 8.75, 12.05 ± 3.68 and 34.45 ± 7.99 a.u ($p_b < 0.05$) as compared to their controls in 90, 180 and 270 day respectively. Maximum CaMKII activity was observed at ZT-0/24 (i.e. onset of light) in 90, 180 and 270 day melatonin treated rats. There was about 11.62 fold increase in maximum CaMKII activity in 90 day, 9.15 fold and 1.68 fold decrease in maximum CaMKII activity in 180 and 270 day after melatonin administration with respect to their controls. The minimum activity observed after melatonin administration was 27.32 ± 9.28, 4.44 ± 1.43 and 3.25 ± 0.55 a.u in 90, 180 and 270 day respectively. Minimum CaMKII activity in melatonin treated 90, 180 and 270 day was observed at ZT-18, 0 and 12 respectively. There was about 14 fold increase in minimum CaMKII activity in 90 day, 18.3 and 3.4 folds decrease in minimum CaMKII activity in 180 and 270 day after melatonin administration.



Each value is mean ± S.E, (n=6); Zeitgeber Time (ZT): ZT-0 = 6.30 h (Lights on); ZT-12 = 18.30 h (Lights off)
One Way ANOVA: $p_a \leq 0.05$ (a refers to comparison with 90 D control (C))
$p_b \leq 0.05$ (b refers to comparison of control and treated values within age groups)

Fig. 26b: Effect of Melatonin administration on age related changes in CaMKII activity in the pineal gland of rat (LD; 12:12)

DISCUSSION:

We report here that aging had a significant effect on CaMKII activity rhythms in both SCN and pineal. In the hamster SCN, phosphorylated CaMKII had been shown to exhibit varying levels under both diurnal and circadian conditions. This suggests that CaMKII is under both photic and clock regulated control in hamster SCN (Agostino *et al.,* 2004). We report for the first time age related changes in CaMKII activity rhythms in SCN and pineal of rat. Ca^{2+} and CaM act as activators of the enzyme CaMKII. The decrease in CaMKII activity could be due to decreased influx of Ca^{2+} ions and also decreased number of CaM molecules that are synthesized and the alteration in the binding affinity of the Ca^{2+}-CaM complex to the enzyme. It has been shown that Ca^{2+} levels regulate CaMKII in the hamster SCN and the free $[Ca^{2+}{}_i]$ in the cytoplasm results from highly regulated balance between the rates of Ca^{2+} influx and removal/buffering (Agostino *et al.,* 2004). Our results on age related changes in the CaMKII activity rhythms suggest that 180 day SCN exhibits maximum CaMKII activity among the three age groups studied. Also, serotonin levels were highest at 180 day as compared with 90, 180 and 270 day age groups. This suggests that CaMKII activity plays an important role in phosphorylating tryptophan hydroxylase which is essential for serotonin synthesis. Thus, CaMKII activity is related with age related changes in serotonin synthesis.

As CaMKII activity has an important role in many physiological functions and its activity decreased with increase in age in both SCN and pineal gland we studied effect of melatonin treatment on age related changes in CaMKII activity rhythms in these two tissues. Melatonin treatment in the SCN resulted in increased CaMKII activity at 90 day at all zeitgeber times. The amplitude in the activity at ZT-0 in melatonin treated 90 day was almost similar to the amplitude of CaMKII activity of 180 day controls (Table 10; Fig. 26a). In case of 180 day, melatonin administration decreased CaMKII activity but not significantly. This suggests that SCN maintains a maximum threshold activity of CaMKII and melatonin administration could not exhibit

its effect on the CaMKII activity beyond that threshold maximum, but rather decreased its activity. CaMKII in 180 day SCN showed decreased activity upon melatonin administration but tight regulation of its amplitude was observed after melatonin administration. In 270 day, there was no significant change in CaMKII activity upon melatonin administration except at ZT-0. At ZT-0, CaMKII activity increased to that of the activity observed at ZT-0 of 270 day control. Significant increase in CaMKII activity at ZT-0 and not at other zeitgeber times suggests that the effect of melatonin was immediate and not consistent. This suggests that melatonin administration had differential effect in different age groups.

Aging had a significant effect on CaMKII activity rhythms in the pineal gland. The activity decreased and rhythmicity was abolished with increase in age. This suggests that the decrease in pineal melatonin levels with age could be due to decreased CaMKII activity which phosphorylates TPH enzyme. This would lead to decreased serotonin synthesis, the precursor of melatonin. Melatonin treatment in pineal gland resulted in increased CaMKII activity in 90 day only but not in 180 and 270 day. The increase in activity in 90 day SCN and pineal could be due to binding of hormone to its receptors enhances influx of Ca^{2+} levels and thus resulting in the Ca^{2+}/calmodulin complex. Melatonin receptors were found to decrease with age and their densities could be altered in the pineal with aging and hence no significant change was observed in 180 and 270 day CaMKII activity upon melatonin administration. This suggests that aging not only causes biochemical changes in SCN but also leads to functional alteration. The whole circadian machinery seems to be altered with increase in age. Earlier workers suggested that the circadian machinery could be responsible for circadian rhythms in phosphorylated CaMKII in the SCN (Agostino *et al.,* 2004).

According to Welsh *et al.,* (1995), the basic mechanism responsible for rhythm generation is intrinsic to individual SCN neurons with individual circadian frequencies. The circadian oscillations result from synchronization of neurons in the SCN which are mediated by intercellular communication between them. This intercellular communication in tissue and in between cells

is mediated by protein phosphorylation that later control various physiological functions (Sáez *et al.,* 1998). Thus maintainence of CaMKII activity rhythms is important for the normal circadian and physiological functions.

Several reports suggested that CaMKII has a pharmacological role in circadian regulation. It is known that psychotropic drugs selectively affect presynaptic CaMKII and thus change the local synaptic mechanisms for pharmacological regulation of kinase (Celano *et al.,* 2003). It is also involved in long term antidepressant drug action on post receptor signaling mechanisms and modulation of transmitter release is the primary action of psychotropic drugs (Consogno *et al.,* 2001). Antidepressants mostly are monoamine reuptake inhibitors and they induce an increase in autophosphorylation and activity of kinase in nerve terminals of hippocampus (Consogno *et al.,* 2000). Thus studies on the post-transcriptional and post-translational modifications especially phosphorylation status of various proteins by kinases and phosphatases would help in understanding the clock function. Thus our study suggests that, more work has to be done on the effect of melatonin treatment on age induced changes in CaMKII activity rhythms.

CHAPTER 5

Age induced changes and the effect of melatonin administration on c-Fos levels in the SCN and Pineal gland of rat

CONTENTS

INTRODUCTION:

The molecular genetic approach to the circadian timing system is associated with circadian synchronization and its rhythmic output (Ikonomov *et al.,* 1994). Neurotransmitter driven signal transduction about extracellular stimuli activates immediate early genes that control cellular activity by initiating or repressing transcription of their target genes in neural and neuroendocrine cells (Koch *et al.,* 2003). The protein products of these genes are transcription factors, which can bind on to DNA and regulate the expression of other genes. Previous studies suggested that IEGs such as *c-fos* and *jun-B* may act as molecular signals involved in the time keeping mechanisms within the mammalian SCN (Wôllnik *et al.,* 1995).

The circadian profile of the *c-fos* expression is opposite to other SCN-intrinsic circadian rhythms. The inducibility *c-fos* in rat SCN was reported as early as on embryonic day 18 (E18). The induction of *c-fos* in the fetus is mediated through D1-dopamine receptors and does not demonstrate circadian variations (Weaver *et al*., 1992). In adults, c-*fos* expression is gated by a circadian clock (Jáć *et al.*, 2000). Expression is primarily restricted to the retinorecipient i.e ventral region of the SCN (Edelstein *et al.,* 2000). Photic induction of *c-fos* expression is phase dependent and is the target of circadian pacemaker. *c-fos* serves as a measure of the duration of the SCN's photosensitivity at night (Schwartz *et al.,* 2001). Light induces *c-fos* expression at night but not during the day (Hastings *et al.,* 1995). There are strong correlations between photic induction of *c-fos* and phase shifts of circadian rhythmic locomotor activity (Schwartz *et al.,* 2001). The *c-fos and jun-B* induction occurs in hamsters after light pulses as short as 5 minutes at CT-19 (subjective night) and reaches maximal mRNA level only 30 minutes after light exposure (Kornhauser *et al.,* 1992).

Light phase-shifts the clock through glutamatergic stimulation of NMDA and non-NMDA receptors (Beaulé and Amir, 1999; Guido *et al.,* 1999) and by IEGs, like *c-fos* (Sutin and Kilduff, 1992). The best characterized photoinducible protein that is expressed in circadian visual

system is c-Fos (Kornhauser *et al.,* 1996). Expression of *c-fos* is observed in the rods/cones of retina. In the absence of these photoreceptors, light will induce *c-fos* expression in RGCs with melanopsin (Semo *et al.*, 2007).

The IEGs in the IGL may not be directly related to photic resetting of the circadian clock. Light seldom induces c-Fos protein in IGL neurons projecting directly to the SCN (Peters *et al.,* 1996). Recent data suggests, however, that the IGL is critical for entrainment to a skeleton photoperiod (Edelstein and Amir, 1999). In the IGL, Jun-B may not be the only protein that dimerizes with c-Fos to mediate the effects of light on the circadian system (Beaulé and Amir, 1999). Non-photic cues like serotonergic agonists were also shown to phase-shift the clock through cAMP, activating protein kinase A and by opening K^+ channels (Prosser, 2003; Duncan *et al.,* 2005). Quipazine, a 5-$HT_{1/2}$ agonist has been reported to induce *c-fos* expression at night in rat SCN *in vivo* mimicking effects of light (Neumaier *et al.*, 2001).

Transcription of *c-fos* induction is calcium dependent (Curran and Morgan, 1987). Calcium enters via low voltage sensitive Ca^{2+} channels (L-VSCC) and *c-fos* mRNA gets elevated within minutes and returns to baseline by 30 minutes. Exposure to NMDA also leads to *c-fos* transcription. Transcription of *c-fos* is regulated by calcium response element (CaRE), CRE, CREB protein and CREB binding protein (CBP). The regulatory region of *c-fos* gene contains a sequence called CRE. Increase in intracellular cAMP content or Ca^{2+} activity triggers CREB phosphorylation. The P-CREB binds the CRE and turns on *c-fos* transcription. CREB phosphorylation takes place only during night when light pulses induce *c-fos* transcription. CREB is not phosphorylated during the subjective day. Thus the circadian control of the *c-fos* stimulus-transcription cascade lies upstream to CREB phosphorylation (Ikonomov *et al.,* 1994). The c-Fos containing heterodimer AP-1 was among the first inducible transcription factors identified. It has been widely used for mapping brain areas activated by various stimuli including drugs (Semba *et al.*, 1999).

In the rat pineal gland, c-Fos is induced upon the onset of darkness and induction abolishes after the removal of superior cervical ganglion (Carter, 1990). This suggests that there is a relation between melatonin and c-Fos induction as melatonin synthesis occurs at dark phase and requires signaling from superior cervical ganglion. Studies by Trávnícková *et al*., (1996) suggested that *c-fos* gene expression could be involved in photic resetting of pineal NAT rhythm. The differential photic and circadian regulation in separate cell populations implies that the function of the gene in circadian time keeping is likely to be cell specific (Schwartz *et al*., 2001).

Hormones have been known to modulate gene transcription (Smith *et al.,* 2005). We have observed significant effect of melatonin administration on age induced changes in CaMKII. Some workers have reported regulation of *c-fos* expression by CaMKII (Golombek *et al.,* 2004; Zayzafoon *et al.,* 2005). Melatonin has been reported to bind to nuclear proteins (Kilduff *et al.,* 1992) and c-Fos is one such nuclear phosphoprotein. Melatonin levels (Rúzsás and Mess, 2000) as well as CaMKII activity (Chapter 4) decline with age. Thus, we studied the age induced changes on c-Fos expression and the effect of melatonin treatment on age induced changes on c-Fos expression in SCN and pineal of rat.

MATERIALS and METHODS:

Male Wistar rats of different age groups (90, 180 and 270 day old) were taken and maintained under laboratory conditions, 06.30h (ZT-0)-18.30h (ZT-12) light phase; 18.30h (ZT-12)-06.30h (ZT-24) dark phase, two weeks prior to the experiments. All rats were kept individually in polypropylene cages at room temperature (20+2^{o}C) with relative humidity (55+6%). Food and water were supplied *ad libitum*. Dim red light was used for handling the animals in the dark. Cage changing was done at random intervals.

All chemicals and reagents used in this study were of analytical grade from standard companies.

Tissue preparation:

SCN tissue and pineal gland were dissected out as described in Chapter 1 and Chapter 4.

Melatonin administration:

Melatonin treatment was given by the method of Cardinali *et al*., (2002) as described in Chapter 2.

Western blotting for c-Fos:

Animals adapted to LD; 12:12 light-dark cycles for two weeks were transferred to continuous dark conditions for 48 h. A light pulse of ~200 lux was delivered for 30 min at mid-subjective day (ZT-6) and mid-subjective night (ZT-18) before sacrifice for controls. Brains were rapidly removed, SCN and pineal were dissected as described earlier and immediately frozen on dry ice. Extraction of nuclear proteins was conducted at 4°C (Best *et al.,* 1999). Tissues were homogenized in 400 µl of homogenization buffer containing: 10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mg/ml aprotinin, and 2 mg/ml pepstatin. The nuclear fraction was precipitated by centrifugation for 2 min at 14,000 rpm and the pellet was resuspended in 36 µl of ice-cold extraction buffer (10 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 5 mM DTT, 1 mM PMSF, 10 mg/ml aprotinin, and 2 mg/ml pepstatin) and incubated on ice for 20 min. The mixture was then centrifuged at 14,000 rpm for 2 min and the supernatant was collected and used for western blotting. The protein content was determined by Bradford's method (Bradford, 1976).

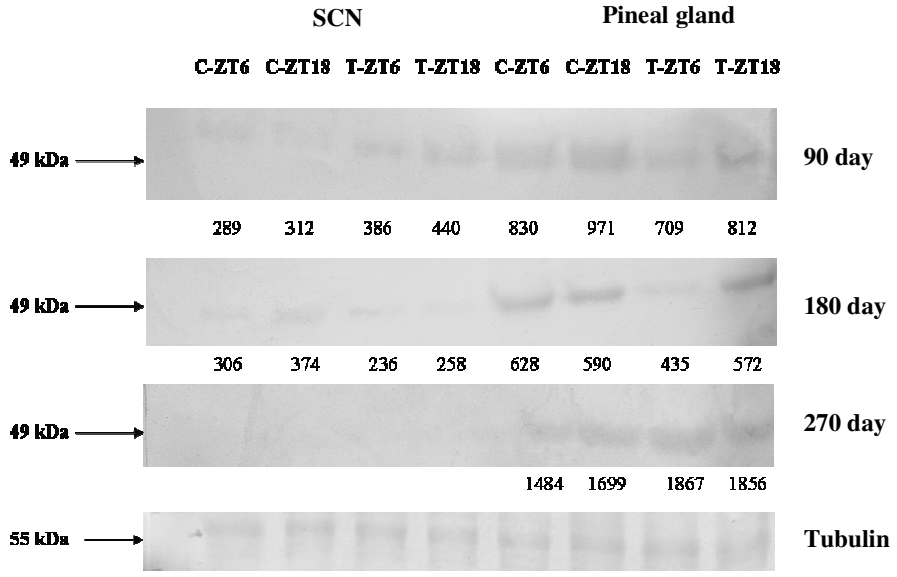
Nuclear extracts containing 15 µg of protein were separated on a 3% stacking 12% SDS-polyacrylamide gel and electro-transferred along with protein molecular weight standards at 70 V for 2 h to nitrocellulose membranes. Membranes were stained with 0.5% Ponceau *S* in 1% acetic acid to check the transfer. The membranes were blocked for 60 min at room temperature in 4% non

fat milk (NFM) (Nestle, Everyday) in TBS and then incubated overnight in TBS buffer (1X Tris-buffered saline, 0.05% Tween 20, 2.5% NFM) containing c-Fos antisera (1:1500) (Genetix) with gentle shaking at 4˚C. The blot was washed for 30 min in 2.5% NFM and 1:200 Tween 20 in TBS and then incubated for 60 min in 1:2000 alkaline phosphatase anti-mouse antibody (Bangalore Genei). The blots were then washed in 2.5% NFM in TBS for 15 min, developed with 2 ml of the substrate for alkaline phosphatase, i.e nitro-blue tetrazolium chloride/ 5-bromo-4-chloro-3-indolylphosphate toluidine (NBT/ BCIP) (Bangalore Genei). The blue coloured bands were visualized with the help of a gel documentation system (Biorad, Quantity One Software). The blots were probed with tubulin to confirm equal loading (Best *et al.,* 1999).

Densitometric Analysis: Densitometric analysis was done by using Scion image software.

RESULTS:

Fig. 27: Effect of Melatonin administration on age related changes in c-Fos levels



Effect of aging on c-Fos levels in the SCN:

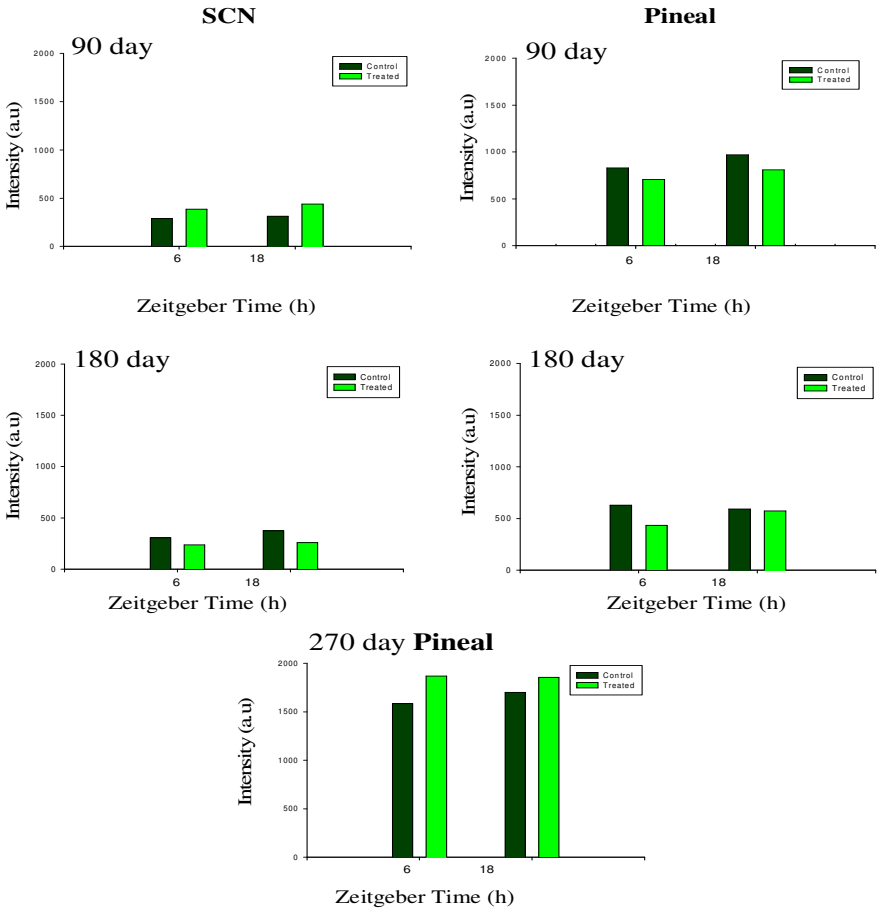
The c-Fos levels were studied at two zeitgeber times, mid-subjective day (CT-6) and mid-subjective night (CT-18) in 90, 180 and 270 day SCN (Fig. 27). The levels increased from 90 day to 180 day but c-Fos levels could not be detected in 270 day. Densitometric analysis was done for c-Fos levels (Fig. 28). At 90 day the observed c-Fos levels were 289 and 312 a. u. at CT-6 and CT-18 respectively. The c-Fos levels observed at 180 day were 306 and 374 a. u. at CT-6 and CT-18 respectively. The levels were observed to be high at CT-18 as compared to CT-6 in both the age groups, 90 and 180 day old.

Effect of melatonin administration on c-Fos levels in the SCN of aging rat:

Melatonin administration had a significant effect on c-Fos levels in the SCN (Fig. 27). The levels increased upon melatonin administration in 90 day old. c-Fos levels observed were 386 and 440 a. u. at CT-6 and CT-18 respectively in 90 day SCN (Fig. 28). There was about 1.3 and 1.4 fold increase in c-Fos levels at CT-6 and CT-18 upon melatonin administration in 90 day SCN. There was a decrease in c-Fos expression at 180 day upon melatonin administration. The levels observed were 236 and 258 a. u. at CT-6 and CT-18 respectively. Levels decreased by about 1.3 and 1.4 fold at CT-6 and CT-18 respectively at 180 day. The levels could not be detected in 270 day even upon melatonin treatment.

Age related changes in c-Fos levels in the pineal gland:

There was a decrease in c-Fos levels in the pineal gland from 90 day to 180 day but then increased significantly at 270 day at both zeitgeber times, ZT-6 as well as ZT-18 (Fig. 27). The c-Fos levels by densitometric analysis were observed to be 830 and 971 a. u. in 90 day and 628 and 590 a. u. in 180 day at ZT-6 and ZT-18 respectively (Fig. 28). We observed a 1.32 and 1.64 fold decrease in c-Fos levels at ZT-6 and ZT-18 respectively from 90 day to 270 day. However in 270 day, c-Fos levels increased drastically and the levels



In 270 day SCN, c-Fos levels could not be detected however, in 270 day pineal c-Fos levels were more than 2 times to that 90 day pineal levels.

Fig. 28: Densitometric analysis of c-Fos levels

observed were 1484 and 1699 a. u. at ZT-6 and ZT-18 respectively. There was about approximately 2.4 and 3 fold increase in c-Fos levels from 180 day to 270 day at ZT-6 and ZT-18 respectively whereas there was about 1.8 and 1.75 folds increase in c-Fos levels from 90 day to 270 day at both zeitgeber times, ZT-6 and ZT-18.

Effect of melatonin administration on c-Fos levels in the pineal gland of aging rat:

Melatonin administration decreased c-Fos levels in 90 and 180 day but increased levels were observed in 270 day at both zeitgeber times, ZT-6 and ZT-18 respectively as compared to their controls (Fig. 27). The levels observed were 709 and 812 a. u. in 90 day and 435 and 572 a. u. in 180 day at ZT-6 and ZT-18 respectively (Fig. 28). There was about 1.2 folds decrease in c-Fos levels at both zeitgeber times at 90 day. At 180 day, upon melatonin administration c-Fos levels decreased by about 1.4 and 1.03 folds at ZT-6 and ZT-18. In 270 day, melatonin administration increased c-Fos levels. The levels observed were 1867 and 1856 a. u. at ZT-6 and ZT-18 respectively. There was about 0.8 and 0.9 folds increase in c-Fos levels at ZT-6 and ZT-18 in 270 day.

DISCUSSION:

We found that c-Fos levels increased from 90 day to 180 day at ZT-6 as well as at ZT-18 in SCN. There was a drastic decrease in c-Fos levels by 270 day in SCN and was not detected by immunoblotting. At 90 and 180 days, c-Fos levels were higher at ZT-18 than at ZT-6. These results were in agreement with previous reports which suggested that elevation of c-Fos levels in the mammalian SCN occurs only during the night (Hastings *et al.,* 1995). According to Kilduff *et al.*, (1992), phase shifting of the circadian clock also occurs during the subjective night. This once again suggests that c-Fos could be playing an important role in phase shifting of the locomotor activity rhythms of the circadian clock.

Expression of c-Fos in the SCN indicates presence of light-activated retinorecipient neuronal involvement in photic entrainment (Amir *et al.,* 1998). We observed that c-Fos levels were high at mid-night (ZT-18) when melatonin levels are highest. The high levels of c-Fos as well as melatonin at mid-night suggest the role of melatonin in circadian rhythm generation and modulation. Thus our results suggested that aging reduced c-Fos levels in the SCN. This is in agreement with earlier reports which showed age related

changes in circadian rhythms with decreased sensitivity of the circadian system to light demonstrated by reduced c-Fos expression in the SCN of rodents (Zhang *et al.*, 1996; Benloucif *et al.,* 1997).

Aging had a significant effect on pineal gland c-Fos levels. There was a decrease in c-Fos levels from 90 day to 180 day and then increased dramatically by 270 day. Levels were found to be higher at ZT-18 in 90 and 270 day but not in 180 day. In 90 day pineal gland c-Fos levels were found to be similar as was observed in 90 day SCN. Previous studies in rodents showed a severe alteration of pineal physiology with aging (Miguez *et al.*, 1998). Thus this could be the reason for changing c-Fos levels with aging.

In SCN, c-Fos levels increased from 90 day to 180 day but in pineal gland levels decreased by 180 day. This could be due to age related loss of regulation of pineal function by SCN or disruption of the downstream pathway from SCN to pineal gland. This also suggests that aging affects the intrinsic rhythmicity of SCN and also acts on its ability to control the function of peripheral organs. Disturbances in c-Fos levels with aging in pineal could also be due to uncoupling of central (SCN) and peripheral pacemakers (pineal) with aging. Aging had a differential effect on SCN and pineal and at various times on c-Fos.

Upon melatonin administration in SCN, c-Fos levels increased in 90 day at both mid-day (ZT-6) and mid-night (ZT-18) whereas in 180 day levels decreased upon melatonin administration at both zeitgeber times. In 270 day c-Fos levels could not be detected even after melatonin administration. In the pineal gland, upon melatonin administration c-Fos levels decreased at ZT-6 as well as at ZT-18 in 90 day and 180 day. However c-Fos levels increased upon melatonin administration but not significantly at 270 day. This suggests that melatonin administration seems to have dose-dependent effect on c-Fos expression with increasing in age and response of the circadian clock to both photic and non-photic stimuli is altered in advanced age (Turek *et al.*, 1995). Earlier reports suggest that SCN output signals alter with age that lead to changes in rhythms of those cells that receive the signals (Smale *et al.,* 2003). Earlier workers reported that circadian and seasonal rhythmicity characterizes

the action of many hormones and elicit long-term physiological adaptations that are ultimately mediated by changes in gene expression (Krieger, 1979). Hence we found melatonin had differential effect on age related changes in the c-Fos levels in the SCN as well as in pineal gland.

Molecular mechanisms underlying the effect of exogenous melatonin on the SCN endogenous rhythmicity is not yet clear (Poirel *et al*., 2003). Recent *in vitro* studies suggested that the transcriptional activity of the CLOCK: BMAL1 heterodimer can be modulated directly by nuclear hormone receptors and redox potential (McNamara *et al.*, 2001; Rutter *et al.*, 2001). That means melatonin may bind to its nuclear receptors and activate *c-fos* transcription whose protein products bind to the target genes *Clock* and *Bmal1*. CLOCK and BMAL1 later heterodimerize and act on other genes thus regulate the circadian cycle and rhythm generation. Studies on the IEGs would help in unraveling the cellular transduction cascade involved in rhythm generation because light activation of immediate early genes, including *c-fos,* ultimately, appears to result in the up-regulation of two of the core clock genes *Per1* and *Per 2* (Reppert and Weaver, 2002). Our present study suggests more work to be done on age related changes in c-Fos expression as it induces the target genes of molecular clock.

Conclusion

CONCLUSION:

The endogenous timekeeper, SCN regulates an enormous array of physiological systems, altering their activity rhythmically on both the daily and seasonal time scales (Loudon *et al.,* 2000). Generation of circadian rhythm and its regulation is a complex process which involves many molecular and biochemical processes. In order to understand circadian rhythm generation and regulation, different biochemical parameters were studied. Initially serotonin levels and daily serotonin rhythms in brain and SCN in various age groups such as 15, 30, 60, 90, 120, 180, 270, 365, 545 and 730 day were studied. Serotonin rhythms appeared to be maternally regulated at 15 day in both brain and SCN. Individual rhythmicity in serotonin levels was established by 90 day in brain and 60 day in SCN which was observed to be a little early in SCN as compared to whole brain. This suggests that SCN is the master circadian clock and it regulates other peripheral clocks. With advancement of age the robustness and amplitude of serotonin rhythms decreased in both brain and SCN finally leading to abolition of rhythmicity by 270 day. These changes in serotonin rhythms would have an impact on SCN function. We hypothesized that the age induced changes in serotonin levels and rhythmicity could be due to either decreased anabolism of serotonin or increased catabolism of serotonin or alteration in the conversion of serotonin to melatonin.

Upon subcutaneous administration of melatonin, we found restoration of serotonin levels as well as its daily rhythmicity in 90, 180 and 270 day but not in 730 day SCN. So, in order to understand how melatonin levels are decreasing with age, we have studied NAT activity rhythms in the SCN. Our results showed decreased NAT activity with aging. This suggests that both serotonin levels as well as NAT activity could be responsible for low melatonin levels with the advancement of age. The decrease in melatonin levels with aging would be certainly affecting the proper functioning of the circadian clock. Upon melatonin administration, there was no significant increase in the NAT activity in 90 and 180 day but there was significant phase advancement in the activity in these age groups. In 270 day, though there was

increase in NAT activity but no phase shift was observed. This suggests that exogenous melatonin may result in phase shifting and can alter the amplitude of NAT activity rhythms in the aging SCN.

Several reports showed that post-translational modifications such as phosphorylation play an important role in circadian rhythm regulation and phase shifts. As serotonin levels are decreasing with age and CaMKII phosphorylates TPH (Yamauchi and Fujisawa, 1983), we wanted to know age induced changes in CaMKII activity rhythms in SCN and pineal of rat and also the effect of melatonin on CaMKII as melatonin is synthesized from serotonin primarily in pineal gland and plays a crucial role in SCN functions. Our studies revealed that aging reduces CaMKII activity in both SCN and pineal. Melatonin administration resulted in increased amplitude of CaMKII activity in 90 and 270 day but not in 180 day which was found to be highest in control 180 day in both SCN and pineal. This suggests that melatonin influences the amplitude of CaMKII activity.

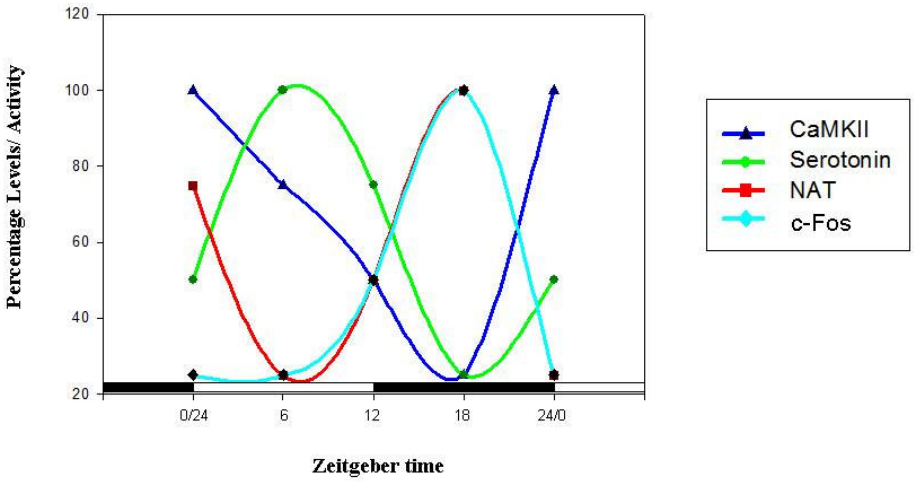
It is well known that c-Fos expression is an important functional marker for SCN neuronal activity. Aging had a significant but differential effect on c-Fos expression in SCN and pineal. There was a reduced c-Fos expression with aging and by 270 day c-Fos expression could not be detected in SCN whereas we observed a decrease in c-Fos expression but then drastic increase in c-Fos expression was observed by 270 day in pineal. Melatonin had no significant effect on c-Fos levels.

Our studies revealed that in the SCN of young rat (90 day old) within a 24h daily rhythm, (Fig. 29) CaMKII activity was maximum at ZT-0 and serotonin levels were highest at mid-day (ZT-6). Thus the peak activity of CaMKII at ZT-0 could be activating TPH by phosphorylating it. Phosphorylated TPH catalyzes the conversion of tryptophan to 5-hydroxytryptophan resulting in serotonin synthesis which peaks by ZT-6. The NAT activity in SCN peaks at ZT-18 as was observed in pineal gland. This showed that SCN neurons are also capable of synthesizing melatonin within them. The c-Fos levels were observed to be high at ZT-18 at the time when melatonin levels are highest. This suggests that c-Fos and melatonin could

have some direct relationship as c-Fos is involved in circadian phase shifts in the SCN and melatonin has a feedback effect on the SCN.

Our studies on age induced changes in daily rhythms of serotonin levels, NAT activity, CaMKII activity and c-Fos levels indicate that aging is a complex, multi-factorial and interconnected process. This study gives us an insight on how daily rhythms play a role in age related diseases. Exogenous melatonin had differential effect on serotonin rhythms, NAT and CaMKII activity rhythms and c-Fos levels such as age specificity and tissue specificity. Therefore our study suggests that the effect of various dosages, durations and frequencies of melatonin on age induced changes should be tried to get the optimum restoration of various biochemical parameters.

Fig. 29: Probable model of neural regulation of circadian clock



In addition, the age related changes in the SCN function could be probably restored by targeting multiple therapies such as light therapy and melatonin treatment. As reported earlier, light and exogenous melatonin represent two different kinds of zeitgebers but their functional properties of entrainment resemble each other closely. This suggests that entrainment to melatonin or light involves at one level or another, a common mechanism even if their input pathway to the pacemaker differ (Pevet *et al.,* 2002).

Much work has to be done to know whether melatonin acts in a synergistic manner along with serotonin or independently through its various mechanisms of action. The interaction of melatonin with serotonin has to be elucidated at both biochemical and molecular levels for the better understanding of the SCN functions. Thus our studies provide new insights into the effect of aging on the underlying mechanisms and signal transduction pathways in circadian rhythm regulation as well as the role of melatonin in the effective treatment of age related circadian disorders and age-associated pathologies.

REFERENCES:

Abrahamson F. F. and Moore R. Y. (2001a). Suprachiasmatic nucleus in the mouse: retinal innervation, intrinsic organization and efferent projections. Brain Research. 916: 172-191.

Abrahamson F. F., Leak R. K. and Moore R. Y. (2001b). The suprachiasmatic nucleus projects to posterior hypothalamic arousal system. Neuroreport 12: 435-440.

Acuña-Castroviejo D., Reiter R. J., Menendez-Palaez A., Pablos M. I. and Bargos A. (1994). Characterization of high affinity melatonin binding sites in purified cell nuclei of rat liver. Journal of Pineal Research. 16: 100-112.

Adell A., Carceller A. and Artigas F. (1991). Regional distribution of extracellular 5-hydroxytryptamine and 5-hydroxyindole acetic acid in the brain of freely moving rats. Journal of Neurochemistry. 56: 709-712.

Adlersberg M., Liu K. P., Hsiung S. C., Ehrlich Y. and Tamir H. (1987). A Ca^{2+}-dependent protein kinase activity associated with serotonin binding protein. Journal of Neurochemistry. 49: 1105-1115.

Agostino P. V., Ferreyra G. A., Murad A. D., Watanabe Y. and Golombek D. A. (2004). Diurnal, circadian and photic regulation of calcium/calmodulin-dependent kinase II and neuronal nitric oxide synthase in the hamster suprachiasmatic nuclei. Neurochemistry International. 44: 617-625.

Aguilar-Roblero R., Shibata S., Speh J. C., Drucker-Colín R. and Moore R. Y. (1992). Morphological and functional development of the suprachiasmatic nucleus in transplanted fetal hypothalamus. Brain Research. 580: 288-296.

Albrecht U. (2004). The mammalian circadian clock: a network of gene expression. Frontiers in Biosciences. 9: 48-55.

Altman J. and Bayer S. (1986). The development of the rat hypothalamus. Advances in Anatomy, Embryology and Cell Biology. 100: 1-178.

Amir S., Robinson B., Ratovitski T., Rea M. A., Stewart J. and Simantov R. (1998). A role for serotonin in the circadian system revealed by the distribution of serotonin transporter and light-induced Fos immunoreactivity in the suprachiasmtic nucleus and intergeniculate leaflet. Neuroscience. 84: 1059-1073.

Angel P., Smeal T., Meek J. and Karin M. (1989). Jun and v-Jun contain multiple regions that participate in transcription activation in an interdependent manner. New Biology. 1: 35-43.

Anon N. (2002). Hormonal resynchronization – an occupational hazard. Lancet Oncology. 3: 323.

Aparicio S., Garau C., Nicolau M. C., Rial R. V. and Esteban S. (2006). Opposite effects of tryptophan intake on motor activity in ring doves (diurnal) and rats (nocturnal). Comparitive Biochemistry and Physiology A Molecular Integrative Physiology. 144: 173-179.

Arendt J. and Skene D. J. (2005). Melatonin as a chronobiotic. Sleep Medicine Reviews. 9: 25-39.

Arivazhagan P. and Panneerselvam C. (2002). Neurochemical changes related to ageing in the rat brain and the effect of D, L-alpha-lipoic acid. Experimental Gerontology. 37: 1489-1494.

Aschoff J. (1960). Exogenous and endogenous components in circadian rhythms. Cold Spring Harbor Symposium. Quantum Biology. 25: 11-26.

Aston-Jones G., Chen S., Zhu Y. and Oshinsky M. L. (2001). A neural circuit for circadian regulation of arousal. Nature Neuroscience. 4: 732-738.

Aujard F., Dkhissi-benyahya A. O., Fournier B. I., Claustrat C. B., Schilling C. A., Cooperb A. H. M. and Perreta M. (2001). Artificially accelerated aging by shortened photoperiod alters early gene expression (*fos*) in the suprachiasmatic nucleus and sulfatoxymelatonin excretion in a small primate, *Microcebus murinus*. Neuroscience. 105: 403-412.

Azmitia E. C and Whitaker-Azmitia P. M. (1991). Awakening the sleeping giant: anatomy and plasticity of the brain serotonergic system. Journal of Clinical Psychiatry. 52: 4-16.

Azmitia E. C. (2001). Modern view on an ancient chemical: serotonin effects on proliferation, maturation and apoptosis. Brain Research Bulletin. 56: 414-424.

Azmitia E. C. G. (2002). Serotonin. Encyclopedia of Life Sciences. 123-133.

Badiu C., Badiu L., Coculescu M., Vilhardt H. and Møller M. (2001). Presence of oxytocinergic neuronal-like cells in the bovine pineal gland: an immunocytochemical and in situ hybridization study. Journal of Pineal Research. 31: 273-280.

Badiu C., Coculescu M. and Moller M. (1999). Arginine vasotocin mRNA revealed by in situ hybridization in bovine pineal gland cells. Cell and Tissue Research. 295: 225-229.

Balsalobre A., Damiola F. and Schibler U. (1998). A serum shock induces circadian gene expression in mammalian tissue culture cells. Cell. 93: 929–937.

Balsalobre A. (2002). Clock genes in mammalian peripheral tissues. Cell and Tissue Research. 309: 193-199.

Balaban R. S., Nemoto S. and Finkel T. (2005). Mitochondria, oxidants and aging. Cell. 120: 483-495.

Banik U, Wang GA, Wagner PD, Kaufman S. (1997). Interaction of phosphorylated tryptophan hydroxylase with 14-3-3 proteins. Journal of Biological Chemistry. 272: 26219-26225.

Barassin S., Raison S., Saboureau M., Bienvenu C., Maître M., Malan A. and Pévet P. (2002). Circadian tryptophan hydroxylase levels and serotonin release in the suprachiasmatic nucleus of the rat. European Journal of Neuroscience. 15: 833-840.

Barbato G., Ikura M., Kay L.E., Pastor R.W. and Bax A. (1992). Backbone dynamics of calmodulin studied by 15N relaxation using inverse detected two-dimensional NMR spectroscopy: The central helix is flexible. Biochemistry. 31: 5269-5278.

Barnes J. W., Tischkau S. A., Barnes J. A., Mitchell J. W., Burgoon P. W., Hickok J. R. and Gillette M. U. (2003). Requirement of mammalian timeless for circadian rhythmicity. Science. 302: 439-442.

Bartel D., Sheng M., Lau L. F. and Greenberg M. E. (1989). Growth factors and membrane depolarization activate distinct programs of early response gene expression: dissociation of *fos* and *jun* induction. Genes and Development. 3: 304-313.

Beale E. G., Dedman J. R. and Means A. R. (1977). Isolation and characterization of a protein from rat testis which inhibits cyclic AMP-dependent protein kinase and phosdiesterase. Journal of Biological Chemistry. 252: 6322-6327.

Beaulé C. and Amir S. (1999). Photic entrainment and induction of immediate-early genes within the rat circadian system. Brain Research. 821: 95-100.

Benítez-King G. and Antón-Tay F. (1993). Calmodulin mediates melatonin cytoskeletal effects. Experientia. 49: 635-641.

Benítez-King G., Huerto-Delgadillo L. and Antón-Tay F. (1993). Binding of 3H-melatonin to calmodulin. Life Sciences. 53: 201-207.

Benitez-King G., Rios A., Martinez A. and Anton-Tay F. (1996). *In vitro* inhibition of Ca2+/calmodulin-dependent kinase II activity by melatonin. Biochimica et Biophysica Acta. 1290:191-196.

Benloucif S., Masana M. I. and Dubocovich M. L. (1997a). Light induced phase shifts of circadian activity rhythms and immediate early gene expression in the suprachiasmatic nucleus are attenuated in old C3H/HeN mice. Brain Research. 747: 34-42.

Benloucif S., Masana I. M. and Dubocovich M. L. (1997b). Responsiveness to melatonin and its receptor expression in the aging circadian clock of mice. American Journal of Physiology Regulatory Integrative Comparitive Physiology. 273:1855-1860.

Berridge M. J., Bootman M. D. and Lipp P. (1998). Calcium-A life and death signal. Nature. 395: 645-648.

Berson D. M., Dunn F. A. and Takao M. (2002). Phototransduction by retinal ganglion cells that set the circadian clock. Science. 295: 1070-1073.

Bertolucci C., Foà A. and Van't Hof T. J. (2002). Seasonal variations in circadian rhythms of plasma melatonin in ruin lizards. Hormones and Behavior. 41: 414-419.

Besharse J. C. and Iuvone P. M. (1983). Circadian clock in Xenopus eye controlling retinal serotonin N-acetyltransferase. Nature. 305:133-135.

Best J. D., Maywood E. S., Smith K. L. and Hastings M. H. (1999). Rapid Resetting of the Mammalian Circadian Clock. The Journal of Neuroscience. 19: 828-835.

Bethea T. C. and Sikich L. (2007). Early pharmacological treatment of autism: a rationale for developmental treatment. Biological Psychiatry. 61: 521-537.

Bjorntorp P. (1999). Neuroendocrine perturbations as a cause of insulin resistance. Diabetes Metabolism Research and Reviews. 15: 427-441.

Boadle-Biber M. C. (1993). Regulation of serotonin synthesis. Progress in Biophysical Molecular Biology. 60:1-15.

Bohmann D., Bos T. J., Admon A., Nishimura T., Vogt P. K. and Tijan R. (1987). Human proto-oncogene *c-jun* encodes a DNA binding protein with structural and functional properties of transcription factor AP-1. Science. 238: 1386-1392.

Bons N., Combes A., Szafarczyk A. and Assenmacher I. (1983). Efferences dextrahypothalamiques de noyau suprachiasmatique chez le rat. Comptes rendus de l'Académie des sciences. 297:347-350.

Boschert U., Amara D. A., Segu L. and Hen R. (1994). The mouse 5-hydroxytryptamine1B receptor is localized predominantly on axon terminals. Neuroscience. 58:167-182.

Bothorel B., Barassin S., Saboureau M., Perreau S., Vivien-Roels B., Malan A. and Pevet P. (2002). In the rat, exogenous melatonin increases the amplitude of pineal melatonin secretion by a direct action on the circadian clock. European Journal of Neuroscience. 16: 1090-1098.

Bouly J. P., Schleicher E., Dionisio-Sese M., Vandenbussche F., Van Der Straeten D., Bakrim N., Meier S., Batschauer A., Gall P., Bittl R. and Ahmad M. (2007). Cryptochrome blue light photoreceptors are activated through interconversion of flavin redox states. Journal of Biological Chemistry. 282: 9383-9391.

Bradford M. M. (1976). A rapid and sensitive method for the quantitationof microgram quantities of protein utilizing the principle of protein-dye binding. Analaytical Biochemistry. 72: 248–254.

Brandstätter J. H. (2002). Glutamate receptors in the retina: the molecular substrate for visual signal processing. Current Eye Research. 25: 327-331.

Brezun J. M. and Daszuta A. (2000). Serotonin may stimulate granule cell proliferation in the adult hippocampus, as observed in rats grafted with fetal raphe neurons. European Journal of Neuroscience. 12: 391–396.

Brooks C. L. and Landt M. (1985). Calmodulin-dependent protein kinase in acini from lactating rat mammary tissue: subcellular locale, characterization, and solubilization. Archives of Biochemistry Biophysics. 240: 663-673.

Brown T. M., Colwell C. S., Waschek J. A. and Piggins H. D. (2007). Disrupted neuronal activity rhythms in the suprachiasmatic nuclei of vasoactive intestinal polypeptide-deficient mice. Journal of Neurophysiology. 97: 2553-2558.

Brugger P. and Herold M. (1995). Impaired nocturnal secretion of melatonin in coronary heart disease. The Lancet. 345:1408.

Brunner M. and Schafmeier T. (2006). Transcriptional and post-transcriptional regulation of the circadian clock of cyanobacteria and *Neurospora*. Genes and Development. 20: 1061-1074.

Brzezinski A. (1997). Melatonin in humans. The New England Journal of Medicine. 336:186-195.

Buhot H. C., Martin S. and Segu L. (2000). Role of serotonin in memory impairment. Annals in Medicine. 32: 210-221.

Buijs R. M. and Kalsbeek A. (2001). Hypothalamic integration of central and peripheral clocks. Nature Reviews Neuroscience. 2: 521-526.

Buijs R. M., Scheer F. A., Kreier F., Yi C., Bos N., Goncharuk V. D. and Kalsbeek A. (2006). Organization of circadian functions: interaction with the body. Progress in Brain Research. 153: 341-360.

Bulliet R. F., Bennett M. K., Molloy S. S., Hurley J. B. and Kennedy M. B. (1988). Conserved and variable regions in the subunits of brain type II Ca^{2+}/calmodulin-dependent protein kinase. Neuron. 1: 63-72.

Burwell R. D., Whealin J. and Gallagher M. (1992). Effects of aging on the diurnal pattern of water intake in rats. Behavioral and Neural Biology. 58:196–203.

Buznikov G. A., Lambert H. W. and Lauder J. M. (2001). Serotonin and serotonin-like substances as regulators of early embryogenesis and morphogenesis. Cell and Tissue Research. 305: 177-186.

Cahill G. M. (2002). Clock mechanisms in zebrafish. Cell and Tissue Research. 309: 27-34.

Cajochen C., Zeitzer J. M., Czeisler C. A. and Dijk D. J. (2000). Dose-response relationship for light intensity and ocular and electroencephalographic correlates of human alertness. Behavioral Brain Research. 115: 75- 83.

Campbell S. S. and Murphy P. J. (1998). Extraocular circadian phototransduction in humans. Science. 279: 396-399.

Card J. P., Brecha N., Karten H. J. and Moore R. Y. (1981). Immunocytochemical localization of vasoactive intestinal polypeptide-containing cells and processes in the suprachiasmatic nucleus of the rat: light and electron microscopic analysis. Journal of Neuroscience. 1:1289-1303.

Card J. P., Whealy M. E., Robbins A. K., Moore R. Y. and Enquist L. W. (1991). Two alpha-herpesvirus strains are transported differentially in the rodent visual system. Neuron. 6: 957-969.

Cardinali D. P., Vacas M I., Keller Sarmiento M. I. and Morguenstern E. (1985). Melatonin action: sites and possible mechanisms in brain. In, The Pineal Gland and its Endocrine role. Eds. Axelrod J., Fraschini F. and Velo G. Plenum Press, New York. pp. 277-302.

Cardinali D. P., Pazo D., Cano P., Reyes Toso C. A. and Esquifino A. I. (2002). Age-related changes in 24-hour rhythms of norepinephrine content and serotonin turnover in rat pineal gland: Effect of melatonin treatment. Neurosignals. 11: 81-87.

Carlson L. L., Weaver D. R. and Reppert S. M. (1991). Melatonin receptors and signal transduction during development in Siberian hamsters (*Phodopus sungorus*). Brain Research Developmental Brain Research. 59: 83-88.

Carter D. A. (1990). Temporally defined induction of *c-fos* in the rat pineal. Biochimical Biophysical Research Communications. 166: 589-594.

Cashmore A. R., Jarillo J. A., Wu Y. J. and Liu D. (1999). Cryptochromes: blue light receptors for plants and animals. Science. 284: 760-765.

Cassone V. M. (1992). The pineal gland influences rat circadian activity rhythms in constant light. Journal of Biological Rhythms. 7: 27-40.

Cassone V. M., Roberts M. H. and Moore R.Y. (1988). Effects of melatonin on 2-deoxy-[1-^{14}C] glucose uptake within rat suprachiasmatic nucleus. American Journal of Physiology. 255: R332-R337.

Celano E., Tiraboschi E., Consogno E., D'Urso G., Mbakop M. P., Gennarelli M., de Bartolomeis A., Racagni G. and Popoli M. (2003). Selective regulation of presynaptic calcium/calmodulin-dependent protein kinase II by psychotropic drugs. Biological Psychiatry. 53: 442-449.

Ceseña T. I., Cui T. X., Piwien-Pilipuk G., Kaplani J., Calinescu A. A., Huo J. S., Iñiguez-Lluhí J. A., Kwok R. and Schwartz J. (2007). Multiple mechanisms of growth hormone-regulated gene transcription. Molecular Genetics and Metabolism. 90: 126-133.

Challet E., Scarbrough K., Penev P. D. and Turek F. W. (1998). Roles of suprachiasmatic nuclei and intergeniculate leaflets in mediating the phase-shifting effects of a serotonergic agonist and their photic modulation during subjective day. Journal of Biological Rhythms. 13: 410-421.

Chen C. and Regehr W. G. (2003). Presynaptic modulation of retinogeniculate synapse. Journal of Neuroscience. 23: 3130-3135.

Chen S. J., Kao C. L., Chang Y. L., Yen C. J., Shui J. W., Chien C. S., Chen I. L., Tsai T. H., Ku H. H. and Chiou S. H. (2007). Antidepressant administration modulates neural stem cell survival and serotoninergic differentiation through *bcl-2*. Currents in Neurovascular Research. 4:19-29.

Chen W. and Baler R. (2000). The rat arylalkylamine *N*-acetyltransferase E-box: differential use in a master vs. a slave oscillator. Molecular Brain Research. 81: 43–50.

Cheung W. Y. (1980). Calmodulin plays a pivotal role in cellular regulation. Science. 207: 19-27.

Chiu R., Boyle W. J., Meek J., Smeal T., Hunter T. and Karin M. (1988). The c-Fos protein interacts with c-Jun/AP-1 to stimulate transcription of AP-1 responsive genes. Cell. 54: 541-552.

Claustrat B., Brun J. and Chazot G. (2005). The basic physiology and pathophysiology of melatonin. Sleep Medicine Reviews. 9: 11-24.

Cohen. (1988). Protein phosphorylation and hormone action. Proceedings of Royal Society of London B. 234: 115-144.

Colbran R. J., Schworer C. M., Hashimoto Y., Fong Y. L., Rich D. P., Smith M. K. And Soderling T. R. (1989). Calcium/calmodulin-dependent protein kinase II. Biochemistry Journal. 258:313-325.

Colwell C. S. (2000). Circadian modulation of calcium levels in cells in the SCN. European Journal of Neuroscience. 12: 571-576.

Colwell C. S. and Menaker M. (1996). Regulation of circadian rhythms by excitatory amino acids. In Excitatory amino acids: Their role in neuroendocrine function. Eds: Brann D. W., Mahesh V.B. New York: CRC Press. pp: 223-252.

Consogno E., Dorigo C. and Popoli G. R. M. (2000). Modification of presynaptic CaM kinase II affinity for ATP in hippocampus after long term blockade of serotonin reuptake. Life Sciences. 67: 1959-1967.

Consogno E., Racagni G. and Popoli M. (2001). Modifications in brain CaM kinase II after long-term treatment with desmethylimipramine. Neuropsychopharmacology. 24: 21-30.

Copinschi G., Spiegel K., Leproult R. and Van Cauter E. (2000). Pathophysiology of human circadian rhythms. Novartis Found Symposium. 227:143-62.

Côté F., Fligny C., Bayard E., Launay J. M., Gershon M. D., Mallet J. and Vodjdani G. (2007). Maternal serotonin is crucial for murine embryonic development. Proceedings in National Academy of Sciences. 104: 329–334.

Curran T. and Morgan J. I. (1987). Memories of *fos*. Bio Essays. 7: 255-258.

Cutler D. J., Piggins H. D., Selbie L. A. and Mason R. (1998). Responses to neuropeptide Y in adult hamster suprachiasmatic nucleus neurones *in vitro.* European Journal of Pharmacology. 345: 155-162.

Cutrera R. A., Kalsbeek A. and Pevet P. (1994). Specific destruction of the serotonergic afferents to the suprachiasmatic nuclei prevents triazolam-induced phase advances of hamster

activity rhythms. Behavioral Brain Research. 62: 21-28.

Czeisler C. A., Kronauer R. E., Allan J. S., Duffy J. F., Jewett M. E., Brown E. N. and Ronda J. M. (1989). Bright light induction of strong (type 0) resetting of the human circadian pacemaker. Science. 244: 1328-1333.

Dahlstrom A. and Fuxe K. (1964). Localization of monoamines in the lower brain stem. Experientia. 20: 398-399.

Darlington T. K., Wager-Smith K., Ceriani M. F., Staknis D., Gekakis N., Steeves T. D., Weitz C. J., Takahashi J. S. and Kay S. A. (1998). Closing the circadian loop: CLOCK-induced transcription of its own inhibitors *per* and *tim*. Science. 280:1599-603.

Dawson K. A., Crowne D. P., Richardson C. M. and Anderson E. (1987). Effects of age on nocturnal activity rhythms in rats. Progress in Clinical and Biological Research. 227: 107-110.

Dax E. M. and Sugden D. (1988). Age-associated changes in pineal adrenergic receptors and melatonin synthesizing enzymes in the Wistar rat. Journal of Neurochemistry. 50:468-472.

Denault D., L. Loros J. J. and Dunlap J. C. (2001). WC-2 mediates WC-1-FRQ interaction within the PAS protein-linked circadian feedback loop of *Neurospora*. European Molecular Biology Organization Journal. 20:109-117.

Diamond M. I., Miner J. N., Yoshinaga S. K. and Yamamoto K. R. (1990). Transcription factor interactions: selectors of positive and negative regulation from a single DNA element. Science. 249: 1266-1272.

Dijk D. J. and Lockley S. W. (2002). Integration of human sleep-wake regulation and circadian rhythmicity. Journal of Applied Physiology. 92: 852-862.

Djalali S., Holtje M., Grosse G., Rothe T., Stroh T., Grosse J., Deng D. R., Hellweg R., Grantyn R., Hortnagl H. and Ahnert-Hilger G. (2005). Effects of brain-derived neurotrophic factor (BDNF) on glial cells and serotonergic neurons during development. Journal of Neurochemistry. 92: 616-627.

Djavadian R. L. (2004). Serotonin and neurogenesis in the hippocampal dentate gyrus of adult mammals. Acta Neurobiologiae Experimentalis (Wars). 64: 189-200.

Drijfhout W. J., van der Linde A. G., De Vries J. B., Grol C. J. and Westerink B. H. C. (1996). Microdialysis reveals dynamics of coupling between noradrenaline release and melatonin secretion in conscious rats. Neuroscience Letters. 202:185-188.

D'Souza J. S. and Johri M. M. (2003). Purification and characterization of a Ca^{2+}-dependent/calmodulin-stimulated protein kinase from moss chloronema cells. Journal of Biosciences. 28:223-233.

Dubocovich M. L. (1995). Melatonin receptors: are there multiple subtypes? Trends in Protein Sciences. 16: 50-56.

Dubocovich M. L., Benloucif S. and Masana M. I. (1996). Melatonin receptors in the mammalian suprachiasmatic nucleus. Behavioral Brain Research. 73: 141-147.

Dubocovich M. L., Rivera-Bermudez M. A., Gerdin M. J. and Masana M. I. (2003). Molecular pharmacology, regulation and function of mammalian melatonin receptors. Frontiers in Biosciences. 8: d1093-d1108.

Dubocovich M. L. and Markowska M. (2005). Functional MT1 and MT2 melatonin receptors in mammals. Endocrine. 27: 101-110.

Dudley T. E., Di Nardo L. A. and Glass J. D. (1998). Endogenous regulation of serotonin release in the hamster suprachiasmatic nucleus. Journal of Neuroscience. 18: 5045-5052.

Duncan M. J., Franklin K. M., Davis V. A., Grossman G. H., Knoch M. E. and Glass J. D. (2005). Short-term constant light potentiation of large-magnitude circadian phase shifts induced by 8-OH-DPAT: Effects on serotonin receptors and gene expression in the hamster suprachiasmatic nucleus. European Journal of Neuroscience. 22: 2306-2314.

Duncan M. J., Jennes L., Jefferson J. B. and Brownfield M. S. (2000). Localization of serotonin$_{5A}$ receptors in discrete regions of the circadian timing system in the Syrian hamster. Brain Research. 869: 178-185.

Dunlap J. C. (1999). Molecular bases for circadian clocks. Cell. 96: 271-290.

Dyda F., Klein D. C. and Hickman A. B. (2005). GCN5-related N-acetyltransferases: a structural overview. Annual Reviews in Biophysical Biomolecules Structure. 29: 81-103.

Ebling F. J. P. (1996). The role of glutamate in the photic regulation of the suprachiasmatic nucleus. Progress in Neurobiology. 50:109-132.

Eddahibi S., Fabre V., Boni C., Martres M. P., Raffestin B., Hamon M. and Adnot S. (1999). Induction of serotonin transporter by hypoxia in pulmonary vascular smooth muscle cells. Relationship with the mitogenic action of serotonin. Circulation Research. 84: 329-36.

Edelstein K. and Amir S. (1999). The role of intergeniculate leaflet in entrainment of circadian rhythms to a skeleton photoperiod. Journal of Neuroscience. 19: 372–380.

Edelstein K., Beaulé C., D'Abramo R. and Amir S. (2000). Expression profiles of Jun B and c-Fos proteins in the rat circadian system. Brain Research. 870: 54-65.

Edery I. (2000). Circadian rhythms in a nutshell. Physiological Genomics 3: 59-74.

Edgar D. M., Reid M. S. and Dement W. C. (1997). Serotonergic afferents mediate activity-dependent entrainment of the mouse circadian clock. American Journal of Physiology. 273: R265-R269.

Ehlen J. C., Grossman G. H. and Glass J. D. (2001). *In vivo* resetting of the hamster circadian clock by 5-HT7 receptors in the suprachiasmatic nucleus. Journal of Neuroscience. 21: 5351-5357.

Ellison N., Weller J. L. and Klein D. C. (1972). Development of a circadian rhythm in the activity of pineal serotonin N-acetyltransferase. Journal of Neurochemistry. 19:1335-1341.

Erondu N. E. and Kennedy M. B. (1985). Regional distribution of type II Ca^{2+}/calmodulin-dependent protein kinase in rat brain. Journal of Neuroscience. 5: 3270-3277.

Fagervall I. and Ross S. B. (1986). A and B forms of monoamine oxidase within the monoaminergic neurons of the rat brain. Journal of Neurochemistry. 47: 569-576.

Fejér Z., Röhlich P., Szél A., Dávid C., Zádori A., Manzano M. J. and Vígh B. (2001). Comparative ultrastructure and cytochemistry of the avian pineal organ. Microscopy Research and Techniques. 53:12-24.

Ferguson S. A., Rowe S. A., Krupa M. and Kennaway D. J. (2000). Prenatal exposure to the

dopamine agonist SKF-38393 disrupts the timing of the initial response of the suprachiasmatic nucleus to light. Brain Research. 858: 284-289.

Ferrari E., Arcaini A., Gornati R., Pelanconi L., Cravello L., Fioravanti M., Solerte S. B. and Magri F. (2000). Pineal and pituitary-adrenocortical function in physiological aging and in senile dementia. Experimental Gerontology. 35: 1239-1250.

Fischer C. A. and Aprison M. H. (1972). Determination of nanomole levels of 5-hydroxytryptophan, 5-hydroxytryptamine and 5-hsydroxyindoleacetic acid in the same sample. Analytical Biochemistry. 46: 67-81.

Foster R. G. (1998). Shedding light on the biological clock. Neuron. 20: 829-832.

Foster R. G. (2002). Keeping an eye on the time: the Cogan Lecture. Investigative Ophthalmology & Visual Science. 43:1286-1298.

Foster R. G., Hankins M., Lucas R. J., Jenkins A., Mufiaz M., Thompson S., Appleford J. M. and Bellingham J. (2003). Non-rod, non-cone photoreception in rodents and teleost fish. Molecular clocks and light signal. Novartis Foundaton Symposium. 253: 3-30.

Foulkes N. S., Whitmore D. and Sassone-Corsi P. (1997). Rhythmic transcription: the molecular basis of circadian melatonin synthesis. Biology of Cell. 89: 487-494.

Frazer A. and Hensler J. G. (1993). In Basic Neurochemistry. Eds: Siegel G. J., Agranoff B. W., Albers R. W. and Molinoff P. B. Raven press. 285-286.

Fujino Y., Iso H., Tamakoshi A., Inaba Y., Koizumi A., Kubo T. and Yoshimura T. (2006). A prospective cohort study of shift work and risk of ischemic heart disease in Japanese male workers. American Journal of Epidemiology.164: 128-135.

Fukuhara C., Dirden J. C. and Tosini G. (2001). Photic regulation of melatonin in rat retina and the role of proteasomal proteolysis. Neuroreport. 12:3833-3837.

Fukunaga K., Rich D. P., and Soderling T. R. (1989). Generation of the Ca^{2+}-independent form of Ca^{2+}/calmodulin-dependent protein kinase II in cerebellar granule cells. Journal of Biological Chemistry. 264: 21830-21836.

Fukushima T., Shimazoe T., Shibata S., Watanabe A., Ono M., Hamada T. and Watanabe S., (1997). The involvement of calmodulin and Ca^{2+}/calmodulin-dependent protein kinase II in the circadian rhythms controlled by the suprachiasmatic nucleus. Neuroscience Letters. 227: 45-48.

Gachon F., Nagoshi E., Brown S. A., Ripperger J. and Schibler U. (2004). The mammalian circadian timing system: from gene expression to physiology. Chromosoma. 113:103-112.

Ganguly S., Coon S. L. and Klein D. C. (2002). Control of melatonin synthesis in the mammalian pineal gland. Cell and Tissue Research. 309: 127-137.

Garau C., Aparicio S., Rial R. V., Nicolau M. C. and Esteban S. (2006). Age-related changes in circadian rhythm of serotonin synthesis in ring doves: Effects of increased tryptophan ingestion. Experimental Gerontology. 41: 40-48.

Garidou M. L., Ribelayga C., Pevet P. and Simonneaux V. (2001). Syrian hamster and rat display developmental differences in the regulation of pineal arylalkylamine *N*-acetyltransferase. Journal of Neuroendocrinology. 14: 861-868.

Garidou M. L., Ribelayga C., Pevet P. and Simonneaux V. (2002). Syrian hamster and rat display developmental differences in the regulation of pineal arylalkylamine N-

acetyltransferase.
Journal of Neuroendocrinology. 14:861-868.

Gaspar P., Cases O. and Maroteaux L. (2003). The developmental role of serotonin: news from mouse molecular genetics. Nature Reviews Neuroscience. 4: 1002-1012.

Gau D., Lemberger T., Von Gall C., Kretz O., Le Minh N., Gass P., Schmid W., Schibler U., Korf H. W. and Schütz G. (2002). Phosphorylation of Ser^{142} regulates light-induced phase-shifts of the circadian clock. Neuron. 34: 245-253.

Gauer F., Masson-Pévet M., Skene D. J., Vivien-Roels B. and Pévet P. (1993). Daily rhythms of melatonin binding sites in the rat pars tuberalis and suprachiasmatic nuclei: evidence for a regulation of melatonin receptors by melatonin itself. Neuroendocrinology. 57:120-126.

Geahlen R. L., Allen S. M. and Krebs E. G. (1981). Effect of phosphorylation on the regulatory subunit of the type I cAMP-dependent protein kinase. Journal of Biological Chemistry. 256: 4536-4540.

Gekakis N., Staknis D., Nguyen H. B., Davis F. C, Wilsbacher L. D., King D. P., Takahashi J. S. and Weitz C. J. (1998). Role of the CLOCK protein in the mammalian circadian mechanism. Science. 280: 1564-1569.

Gerdin M. J., Masana M. I., Rivera-Bermúdez M. A., Hudson R. L., Earnest D. J., Gillette M. U. and Dubocovich M. L. (2004). Melatonin desensitizes endogenous MT2 melatonin receptors in the rat suprachiasmatic nucleus: relevance for defining the periods of sensitivity of the mammalian circadian clock to melatonin. The Federation of American Societies for Experimental Biology. 8: 1646-1656.

Gerhardt G. A. and Maloney R. E. Jr. (1999). Microdialysis studies of basal levels and stimulus-evoked overflow of dopamine and metabolites in the striatum of young and aged Fischer 344 rats. Brain Research. 816: 68-77.

Gillette M. U. (1986). The suprachiasmatic nuclei: Circadian shifts induced at the time of hypothalamic slice preparation are preserved *in vitro*. Brain Research. 379: 176-181.

Gillette M. U. and McArthur A. J. (1996). Circadian actions of melatonin at the suprachiasmatic nucleus. Behavioral Brain Research. 73: 135-139.

Gillette M. U. and Mitchell J. W. (2002). Signaling in the suprachiasmatic nucleus: selectively responsive and integrative. Cell and Tissue Research. 309: 99-107.

Gilman M. Z. (1988). The *c-fos* serum response element responds to protein kinase C-dependent and -independent signals but not to cyclic AMP. Genes and Development. 2: 394-402.

Goldberg S., Smith G. S., Barnes A., Ma Y., Kramer E., Robeson K., Kirshner M., Pollock B. G. and Eidelberg D. (2004). Serotonin modulation of cerebral glucose metabolism in normal aging. Neurobiol of Aging. 25: 167-174.

Goldbeter A. (1996). Biochemical oscillations and cellular rhythms. Cambridge, UK: Cambridge University Press.

Golombek D. A. and Ralph M. R. (1994). KN-62, an inhibitor of Ca^{2+}/calmodulin kinase II attenuates circadian responses to light. Neuroreport. 5: 1638-1640.

Golombek D. A. and Ralph M. R. (1995). Circadian responses to light: the calmodulin connection. Neuroscience Letters. 192: 101-104.

Golombek D. A., Ferreyra G. A., Agostino P. V., Murad A. D., Rubio M. F., Pizzio G. A., Katz M. E., Marpegan L. and Bekinschtein T. A. (2003). From light to genes: moving the hands of the circadian clock. Frontiers in Biosciences. 8: 56-70.

Golombek D. A. Agostino P. V., Plano S. A. and Ferreyra G. A. (2004). Signaling in the mammalian circadian clock: the NO/cGMP pathway. Neurochemistry International. 45: 929-936.

Gotter A. L., Manganaro T., Weaver D. R., Kolakowski L. F. Jr., Possidente B., Sriram S., MacLaughlin D. T. and Reppert S. M. (2000). A time-less function for mouse timeless. Nature Neuroscience. 3: 755-756.

Graeff F. G., Guimaraes F. S., De Andrade T. G. C. S. and Deakin J. F. W. (1996). Role of 5-HT in stress, anxiety, and depression. Pharmacology and Biochemistry and Behavior. 54: 129-141.

Graff C., Challet E., Pévet P. and Wollnik F. (2007). 5-HT3 receptor-mediated photic-like responses of the circadian clock in the rat. Neuropharmacology. 52: 662-671.

Greenberg M. E., Thompson M. A. and Sheng M. (1992). Calcium regulation of immediate early gene transcription. Journal of Physiology, Paris. 86: 99-108.

Greve P., Voisin P., Grechez-Cassiau A., Bernard M., Collin J. P. and Guerlotte J. (1996). Circadian regulation of hydroxyindole-O-methyltransferase mRNA in the chicken pineal gland *in vivo* and *in vitro*. Biochemistry Journal. 319: 761-766.

Grossman G. H., Mistlberger R. E., Antle M. C., Ehlen J. C. and Glass J. D. (2000). Sleep deprivation stimulates serotonin release in the suprachiasmatic nucleus. Neuroreport. 11: 1929-1932.

Guido M. E., Goguen D., de Guido L., Robertson H. A. and Rusak B. (1999). Circadian and photic regulation of immediate-early gene expression in the hamster suprachiasmatic nucleus. Neuroscience 90: 555-571.

Gupta B. B., Spessert R. and Vollrath L. (2005). Molecular components and mechanism of adrenergic signal transduction in mammalian pineal gland: regulation of melatonin synthesis. Indian Journal of Experimental Biology. 43: 115-149.

Gurudutt P. and Albrecht U. (2005). Circadian rhythms, glutamate and behavior. Proceedings of the Indian National Science Academy, Part B. 71: 207-218.

Halozonetis T. D., Georgopoulos K., Greenberg M. E. and Leder P. (1988). c-Jun dimerizes with itself and with c-Fos, forming complexes of different DNA binding affinities. Cell. 55: 917-924.

Hamada T., Ootomi M., Horikawa K., Niki T., Wakamatu H. and Ishida N. (1999). The expression of the melatonin synthesis enzyme: Arylalkylamine N-acetyltransferase in the suprachiasmatic nucleus of rat brain. Biochemical and Biophysical Research Communications. 258: 772-777.

Hanley R. M., Means A. R., Ono T., Kemp B. E., Burgin K. E., Waxham N. and Kelly P. T. (1987). Functional analysis of a complementary DNA for the 50-kilodalton subunit of calmodulin kinase II. Science. 237: 293-297.

Hardeland R., Pandi-Perumal S. R. and Cardinali D. P. (2006). Melatonin. International Journal of Biochemistry and Cell Biology. 38: 313-316.

Harmat V., Bocskei Z., Naray-Szabo G., Bata I., Csutor A.S., Hermecz I., Aranyi P., Szabo B., Liliom K. and Vertessy B. G. (2000). A new potent calmodulin antagonist with arylalkylamine structure: Crystallographic, spectroscopic and functional studies. Journal of Molecular Biology. 297: 747-755.

Hashimoto Y. and Soderling T. R. (1987). Ca^{2+}/calmodulin-dependent protein kinase II and calcium phospholipid-dependent protein kinase activities in rat tissues assayed with a synthetic peptide. Archives of Biochemistry and Biophysics. 252: 418-425.

Hashimoto Y., King M. M. and Soderling T. R. (1988). Regulatory interactions of calmodulin-binding proteins: Phosphorylation of calcineurin by autophosphorylated Ca^{2+}/calmodulin-dependent protein kinase II. Proceedings of the National Academy of Sciences. 85: 7001-7005.

Hastings M. H., Ebling F. J. P., Grosse J., Herbert J., Maywood E. S., Mikkelsen J. D. and Sumová A. (1995). Immediate-early genes and the neuronal bases of photic and non-photic entrainment. In Circadian clocks and their adjustment. Ciba Foundation Symposium 183. Eds: Chadwick D. J. and Wiley A. K. Chichester, UK. pp: 175-197.

Hastings M. H., Duffield G. E., Ebling F. J., Kidd A., Maywood E. S. and Schurov I. (1997). Non-photic signaling in the suprachiasmatic nucleus. Biology of Cell. 89: 495-503.

Hastings M. H., Reddy A. B. and Maywood E. S. (2003). A clock work web: circadian timing in brain and periphery, in health and disease. Nature Reviews Neuroscience. 4: 649-661.

Hastings M. H. and Herzog E. D. (2004). Clock genes, oscillators and cellular networks in the suprachiasmatic nuclei. Journal of Biological Rhythms. 19: 400-413.

Hayashi S., Ueda M., Amaya F., Matusda T., Tamada Y., Ibata Y. and Tanaka M. (2001). Serotonin modulates expression of VIP and GRP mRNA via the 5-HT (1B) receptor in the suprachiasmatic nucleus of the rat. Experimental Neurology. 171: 285-292.

Henden T., Stokkan K. A., Reiter R. J., Nonaka K. O., Lerchl A. and Jones D. J. (1992). Age-associated reduction in pineal beta-adrenergic receptor density is prevented by life-long food restriction in rats. Biological Signals. 1: 34-39.

Herzog E. D. and Schwartz W. J. (2002). A neural clockwork for encoding circadian time. Journal of Applied Physiology. 92: 401-408.

Hofman M. A. and Swaab D. F. (1995). Influence of aging on the seasonal rhythm of the vasopressin-expressing neurons in the human suprachiasmatic nucleus. Neurobiology of Aging. 16: 965-971.

Hogenesch J. B., Gu Y. Z., Jain S. and Bradfield C. A. (1998). The basic-helix-loop-helix-PAS orphan MOP3 forms transcriptionally active complexes with circadian and hypoxia factors. Proceedings of the National Academy of Sciences. U S A. 95: 5474-5479.

Honma S., Kawamoto T., Takagi Y., Fujimoto K., Sato F., Noshiro M., Kato Y. and Honma K. (2002). *Dec1* and *Dec2* are regulators of the mammalian molecular clock. Nature. 419: 841-844.

Honma S. and Honma K. (2003). The biological clock: Ca^{2+} links the pendulum to the hands. Trends in Neurosciences. 26: 650-653.

Honma S., Nakamura W., Shirakawa T. and Honma K. (2004). Diversity in the circadian periods of single neurons of the rat suprachiasmatic nucleus depends on nuclear structure and intrinsic period. Neuroscience Letters. 358: 173-176.

Huether G. (1994). Melatonin synthesis in the gastrointestinal tract and the impact of nutritional factors on circulating melatonin. Annals of the New York Academy Sciences. 719: 146-158.

Hunt A. E., Al-Ghoul W. M., Gillette M. U. and Dubocovich M. L. (2001). Activation of MT(2) melatonin receptors in rat suprachiasmatic nucleus phase advances the circadian clock. American Journal of Physiology and Cell Physiology. 280: C110-C118.

Hunt A. E., Al-Ghoul W. M., Gillette M. U. and Dubocovich M. L. (2001). Activation of MT (2) melatonin receptors in rat suprachiasmatic nucleus phase advances the circadian clock. The American Journal of Physiology. 280: C110-C118.

Hussain A. M and Mitra A. K. (2000). Effect of aging on tryptophan hydroxylase in rat brain: implications on serotonin level. Drug Metabolism and Disposition. 28: 1038-1042.

Ibata Y., Okamura H., Tanaka M., Tamada Y., Hayashi S., Iijima N., Matsuda T., Munekawa K., Takamatsu T., Hisa Y., Shigeyoshi Y. and Amaya F. (1999). Functional morphology of the suprachiasmatic nucleus. Frontiers in Neuroendocrinology. 20: 241-268.

Ikeda A., Okuno S. and Fujisawa H. (1991). Journal of Biological Chemistry. 266: 11582.

Ikeda M., Sugiyama T., Wallace C. S., Gompf H. S., Yoshioka T., Miyawaki A. and Allen C. N. (2003). Circadian dynamics of cytosolic and nuclear Ca^{2+} in single suprachiasmatic nucleus neurons. Neuron. 38: 253-263.

Ikonomov O. C., Stoynev A. G. and Shisheva A. C. (1994). Circadian function of Suprachiasmatic nuclei: molecular and cellular biology. Chronobiologia. 21: 71-77.

Illnerová H., Vaněček J. and Hoffmann K. (1983). Regulation of the pineal melatonin concentration in the rat (*Ratus norvegicus*) and in the Djungarian hamster (*Phodopus sungorus*). Comparitive Biochemistry and Physiology. 73: 155-159.

Isobe Y. and Nishihara K. (2002). Serotonin-stimulated glutamate release from an SCN explant culture was higher during light period. Brain Research Bulletien. 58: 401-404.

Iuvone P. M., Brown A. D., Haque R., Weller J., Zawilska J. B., Chaurasia S. S., Ma M. and Klein D. C. (2002). Retinal melatonin production: role of proteasomal proteolysis in circadian and photic control of arylalkylamine N-acetyltransferase. Investigative Ophthalmology and Visual Science. 43: 564-572.

Iwasa T., Inoue N., Fukunaga K., Isobe T., Okuyama T. and Miyamoto E. (1986). Purification and characterization of a multifunctional calmodulin-dependent protein kinase from canine myocardial cytosol. Archives of Biochemistry and Biophysics. 248: 21-29.

Jáć M., Sumová A. and Illnerová H. (2000). c-Fos rhythm in subdivisions of the rat suprachiasmatic nucleus under artificial and natural photoperiods. American Journal of Physiology Regulatory Integrative Comparitive Physiology. 279: R2270-R2276.

Jacobs B. L. and Azmitia E. C. (1992). Structure and function of the brain serotonergic system. Physiological Reviews. 72: 165-229.

Jagota A. and Habibulla M. (1992). The frontal ganglionic system: Cauterization effects on serotonin circadian rhythms of the cockroach corpora allata and corpora cardiaca. Insect Biochemistry and Molecular Biology. 22: 747-755.

Jagota A., Olcese J., Harinarayana Rao S. and Gupta P. D. (1999). Pineal rhythms are synchronized to light-dark cycles in congenitally anopthalmic mutant rats. Brain Research. 825: 95-103.

Jagota A., de la Iglesia H. O. and Schwartz W. J. (2000). Morning and evening circadian oscillations in the suprachiasmatic nucleus *in vitro.* Nature Neuroscience. 3: 372-376.

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

Jagota A. (2006). Suprachiasmatic nucleus: center for circadian timing system in mammals. Proceedings of the Indian National Science Academy. B-71: 275-288.

Jagota A. and Gupta P. D. (2006). Living clocks. V. Brijratan Publications, Bikaner, India.

Jagota A. and Reddy M. Y. (in press). The effect of Curcumin on ethanol induced changes in suprachiasmatic nucleus (SCN) and pineal. Cell and Molecular Neurobiology.

Jessell T. M. and Sanes J. R. (1991). The induction and patterning of the nervous system. In; Principles of Neuralscience. Eds: Kandel E. R., Schwartz J. H. and Jessel T. M. The McGraw-Hill Companies, Inc. pp: 1021.

Jiang Z. G., Teshima K., Yang Y., Yoshioka T. and Allen C. N. (2000). Pre- and post-synaptic actions of serotonin on rat suprachiasmatic nucleus neurons. Brain Research. 866: 247-256.

Jin L. W. and Saitoh T. (1995). Changes in protein kinases in brain aging and Alzheimer's disease. Implications for drug therapy. Drugs and Aging. 6: 136-149.

Kabuto H., Yokoi I., Mori A., Murakami M. and Sawada S. (1995). Neurochemical changes related to ageing in the senescence-accelerated mouse brain and the effect of chronic administration of nimodipine. Mechanism of Ageing and Development. 80: 1–9.

Kalsbeek A., Ruiter M., La Fleur S. E., Cailotto C., Kreier F. and Buijs R. M. (2006). The hypothalamic clock and its control of glucose homeostasis. Progress in Brain Research. 153: 283-307.

Karasek M. and Reiter R. J. (2002). Melatonin and aging. Neuroendocrinology Letters. 1: 14-26.

Karolczak M., Burbach G. J., Sties G., Korf H. W. and Stehle J. H. (2004). Clock gene mRNA and protein rhythms in the pineal gland of mice. European Journal of Neuroscience. 19: 3382-3388.

Kawakami F., Okamura H., Tamada Y., Maebayashi Y., Fukui K. and Ibata Y. (1997). Loss of day-night differences in VIP mRNA levels in the suprachiasmatic nucleus of aged rats. Neuroscience Letters. 222: 99-102.

Kawamoto T., Noshiro M., Sato F., Maemura K., Takeda N., Nagai R., Iwata T., Fujimoto K., Furukawa M., Miyazaki K., Honma S., Honma K. and Kato Y. (2004). A novel autofeedback loop of *Dec1* transcription involved in circadian rhythm regulation. Biochemical Biophysical Research Communications. 313: 117-124.

Kawinska A., Dumont M., Selmaoui B., Paquet J. and Carrier J. (2005). Age modifications of melatonin circadian rhythm in the middle years of life related to habitual patterns of light exposure? Journal of Biological Rhythms. 20: 451-460.

Kelly P. T., Weinberger R. P. and Waxham M. N. (1988). Active site-directed inhibition of Ca^{2+}/calmodulin-dependent protein kinase type II by a bifunctional calmodulin-binding peptide. Proceedings of the National Academy of Sciences. U.S.A. 85: 4991-4995.

Kennaway D. J. (2002). Programming of the fetal suprachiasmatic nucleus and subsequent adult rhythmicity. Trends in Endocrinology and Metabolism. 13: 398-402.

Kennedy M. B., McGuinness T. and Greengard P. (1983). A calcium/calmodulin-dependent protein kinase from mammalian brain that phosphorylates Synapsin I: partial purification and characterization. Journal of Neuroscience. 3: 818-831.

Khachaturian Z. S. (1994). Calcium hypothesis of Alzheimer's disease and brain aging. Annals of the New York Academy of Sciences. 747: 1-11.

Kilduff T. S., Landel H. B., Nagy G. S., Sutin E. L., Dement W. C. and Heller H. C. (1992). Melatonin influences Fos expression in the rat suprachiasmatic. Brain Research Molecular Brain Research. 16: 47-56.

Kinder B. K., Delahunt N. G., Jamieson J. D. and Gorelick F. S. (1987). Calcium-calmodulin-dependent protein kinase in hyperplastic human parathyroid glands. Endocrinology. (Baltimore). 120: 170-177.

King D. P., Zhao Y., Sangoram A. M., Wilsbacher L. D., Tanaka M., Antoch M. P., Steeves T. D., Vitaterna M. H., Kornhauser J. M., Lowrey P. L., Turek F. W. and Takahashi J. S. (1997). Positional cloning of the mouse circadian clock gene. Cell. 89: 641-653.

Klein D. C. and Moore R. J. (1979). Pineal N-acetyltransferase and hydroxyindole-O-methyltransferase: Control by the retinal hypothalamic tract and the suprachiasmatic nucleus. Brain Research. 174: 245-262.

Klein D. C., Moore R. Y. and Reppert S. M. (1991). Suprachiasmatic nucleus: The Mind's clock. Oxford University Press. New York.

Klein D. C., Coon S. L., Roseboom P. H., Weller J. L., Bernard J. A., Gastel J. A., Zatz M., Iuvone P. M., Rodriguez I. R., Begay V., Falcon J., Cahill G. M., Cassone V. M. and Baler R. (1997). The melatonin rhythm-generating enzyme: molecular regulation of serotonin *N*-acetyltransferase in the pineal gland. Recent Progress in Hormone Research. 52: 307-358.

Klein D. C., Ganguly S., Coon S. L., Shi Q., Galidrat P., Morin F., Weller J. L., Obsil T., Hickman A. and Dyda F. (2003). 14-3-3 proteins in pineal photoneuroendocrine transduction: How many roles? Journal of Neuroendocrinology. 15: 370-377.

Klisch C., Mahr S. and Meissl H. (2006). Circadian activity rhythms and phase-shifting of cultured neurons of the rat suprachiasmatic nucleus. Chronobiology International. 23: 181-190.

Klöppel S., Kovacs G. G., Voigtländer T., Wanschitz J., Flicker H., Hainfellner J. A., Gueutcheu M. and Budka H. (2001). Serotonergic nuclei of the raphe are not affected in human aging. Neuroreport. 12: 669-671.

Ko C. H. and Takahashi J. S. (2006). Molecular components of the mammalian circadian clock. Human Molecular Genetics. 15: R271-R277.

Koch M., Mauhin V., Stehle J. H., Schomerus C. and Korf H. W. (2003). Dephosphorylation of pCREB by protein serine/threonine phophatases is involved in inactivation of *Aanat* gene transcription in rat pineal gland. Journal of Neurochemistry. 85: 170-179.

Kondratov R. V., Chernov M. V., Kondratova A. A., Gorbacheva V. Y., Gudkov A. V. and Antoch M. P. (2003). BMAL1-dependent circadian oscillation of nuclear CLOCK: posttranslational events induced by dimerization of transcriptional activators of the mammalian clock system. Genes and Development. 17: 1921-1932.

Kondratov R. V., Kondratova A. A., Gorbachevas V. Y., Vykhovanets O. V. and Antoch M. P. (2006). Early aging and age-related pathologies in mice deficient in BMAL1, the core component of the circadian clock. Genes and Development. 20: 1868–1873.

Kondratov R. V. (2007). A role of the circadian system and circadian proteins in aging. Ageing Research Reviews. 6: 12–27.

Kopp M., Meissl H. and Korf H. W. (1997). The pituitary adenylate cyclase–activating polypeptide-induced phosphorylation of the transcription factor CREB (cAMP response element binding protein) in the rat suprachiasmatic nucleus is inhibited by melatonin. Neuriscience. Letters. 227: 145-148.

Korf H. W., Schomerus C. and Stehle J. H. (1998). The pineal organ, its hormone melatonin and the photoneuroendocrine system. Advances in Anatomy, Embryology and Cell Biology. 146: 1-100.

Kornhauser J. M., Nelson D. E., Mayo K. E. and Takahashi J. S. (1992). Regulation of *jun-B* messenger RNA and AP-1 activity by light and a circadian clock. Science. 255: 1581-1584.

Kornhauser J. M., Mayo K. E. and Takahashi J. S. (1996). Light, immediate-early genes and circadian rhythms. Behavioral Genetics. 26: 221-240.

Kouzarides T. and Ziff E. (1988). The role of the leucine zipper in the *fos-jun* interaction. Nature. 336: 646-651.

Krieger D. T. (1979). Endocrine Rhythms. Raven Press.

Kriegsfeld L. J., Leak R. K., Yackulic C. B., Le Sauter J. and Silver R. (2004). Organization of suprachiasmatic nucleus projections in Syrian hamsters (*Mesocricetus auratus*): an anterograde and retrograde analysis. Journal of Comparitive Neurology. 468: 361-379.

Kubo T., Ozasa K., Mikami K., Wakai K., Fujino Y., Watanabe Y., Miki T., Nakao M., Hayashi K., Suzuki K., Mori M., Washio M., Sakauchi F., Ito Y., Yoshimura T. and Tamakoshi A. (2006). Prospective cohort study of the risk of prostate cancer among rotating-shift workers: findings from the Japan collaborative cohort study. American Journal of Epidemiology. 164: 549-555.

Kuhlman S. J., Silver R., Le Sauter J., Bult-Ito A., McMahon D. G. (2003). Phase resetting light pulses induce Per1 and persistent spike activity in a subpopulation of biological clock neurons. Journal of Neuroscience. 23: 1441-1450.

Kume K., Zylka M. J., Sriram S., Shearman L. P., Weaver D. R., Jin X., Maywood E. S., Hastings M. H. and Reppert S. M. (1999). mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. Cell. 98: 193-205.

Kuret J. and Schulman H. (1985). Mechanism of autophosphorylation of the multifunctional Ca^{2+}/calmodulin-dependent protein kinase. Journal of Biological Chemistry. 260: 6427-6433.

Kwiatkowski A. P., Shell D. J. and King M. M. (1988). The role of autophosphorylation in activation of the type II calmodulin-dependent protein kinase. Journal of Biological Chemistry. 263: 6484-6486.

Lahiri D. K., Ge Y. W., Sharman E. H. and Bondy S. C. (2004). Age-related changes in serum melatonin in mice: higher levels of combined melatonin and 6-hydroxymelatonin sulfate in the cerebral cortex than serum, heart, liver and kidney tissues. Journal of Pineal Research. 36: 217-223.

Lai Y., Nairn A. C. and Greengard P. (1986). Autophosphorylation reversibly regulates the Ca^{2+}/calmodulin-dependence of Ca^{2+}/calmodulin-dependent protein kinase II. Proceedings of the National Academy of Sciences. U.S.A. 83: 4253-4257.

Laitinen J. T., Viswanathan M., Vakkuri O. and Saavedra J. M. (1992). Differential regulation of melatonin receptors: selective age-associated decline and lack of melatonin-induced changes. Endocrinology. 130: 2139-2144.

Landschulz W. H., Johnson P. F. and McKnight S. L. (1988). The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science. 240: 1759-1764.

Lauder J. M. (1993). Neurotransmitters as growth regulatory signals: role of receptors and second messengers. Trends in Neurosciences. 16: 233-240.

Lee C., Etchegaray J. P., Cagampang F. R., Loudon A. S. and Reppert S. M. (2001). Posttranslational mechanisms regulate the mammalian circadian clock. Cell. 107: 855-867.

Lee C. C. (2005). The circadian clock and tumor suppression by mammalian period genes. Methods in Enzymology. 393: 852-861.

Lee H. S., Billings H. J. and Lehman M. N. (2003). The suprachiasmatic nucleus: a clock of multiple components. Journal of Biological Rhythms. 18: 435-449.

Lefebvre H., Contesse V., Delarue C., Vaudry H. and Kuhn J. M. (1998). Serotonergic regulation of adrenocortical function. Hormones Metabolism and Research. 30: 398-403.

Lerchl A. (1994). Increased oxidation of pineal serotonin as a possible explanation for reduced melatonin synthesis in the aging Djungarian hamster (*Phodopus sungorus*). Neuroscience Letters. 176: 25-28.

Lerner A. B., Case J. D. and Heinzelman R. V. (1959). Structure of melatonin. Chemical Society. 81: 6084-6085.

Lesch K. P. (2001). Serotonergic gene expression and depression: implications for developing novel antidepressants. Journal of Affective Disorders. 62: 57-76.

Levi F. (1999). Cancer prevention: epidemiology and perspectives. European Journal of Cancer. 35: 1912-1924.

Levin M., Buznikov G. A. and Lauder J. M. (2006). Of minds and embryos: left-right asymmetry and the serotonergic controls of pre-neural morphogenesis. Developmental Neurosciences. 28: 171-185.

Levitt P. and Rakic P. (1982). The time of genesis, embryonic origin and differentiation of the brain stem monoamine neurons in the rhesus monkey. Brain Research. 256: 35-57.

Lewy A. J., Emens J., Jackman A. and Yuhas K. (2006). Circadian uses of melatonin in humans. Chronobiology International. 23: 403-412.

Li H. and Satinoff E. (1995). Changes in circadian rhythms of body temperature and sleep in old rats. American Journal of Physiology. 269: R208-R214.

Lidov H. G. and Molliver M. E. (1982). An immunohistochemical study of serotonin neuron development in the rat: ascending pathways and terminal fields. Brain Research Bullitein. 8: 389-430.

Lincoln G. A., Andersson H. and Loudon A. S. I. (2003). Clock genes in calendar cells as the basis of annual time keeping in mammals-a unifying hypothesis. Journal of Endocrinology. 179: 1-13.

Lipsitz L. A. and Goldberger A. L. (1992). Loss of 'complexity' and aging. Potential applications of fractals and chaos theory to senescence. The Journal of the American Medical Association. 267: 1806-1809.

Lipton S. A. and Kater S. B. (1989). Neurotransmitter regulation of neuronal out growth, plasticity and survival. Trends in Neurosciences. 12: 265-270.

Liu C., Ding J. M., Faiman L. E. and Gillette M. U. (1997). Coupling of muscarinic cholinergic receptors and cGMP in nocturnal regulation of the suprachiasmatic circadian clock. Journal of Neuroscience. 17: 659-666.

Liu T. and Borjigin J. (2005). *N*-acetyltransferase is not the rate limiting enzyme of melatonin synthesis at night. Journal of Pineal Research. 39: 91-96.

Lorke D. E., Lu G., Cho E. and Yew D. T. (2006). Serotonin 5-HT_{2A} and 5-HT_6 receptors in the prefrontal cortex of Alzheimer and normal aging patients. Bio Med Central Neuroscience. 27: 7-36.

Lotto B., Upton L., Price D. J. and Gaspar P. (1999). Serotonin receptor activation enhances neurite outgrowth of thalamic neurones in rodents. Neuroscience Letters. 269: 87-90.

Loudon A. S., Semikhodskii A. G. and Crosthwaite S. K. (2000). A brief history of circadian time. Trends in Genetics. 16: 477-481.

Lovenberg T. W., Baron B. M., de Lecea L., Miller J. D., Prosser R. A., Rea M. A., Foye P. E., Racke M., Slone A. L., Siegel B. W., Danielson P. E., Sutcliffe J. G. and Erlander M. G. (1993). A novel adenylyl cyclase activating serotonin receptor (5-HT) implicated in the regulation of 7 mammalian circadian rhythms. Neuron. 11: 449-458.

Lowrey P. L., Shimomura Z., Antoch M. P., Yamazaki S., Zemenideds P. D., Ralph M. R., Menaker M. and Takahashi J. S., (2000). Positional syntenic cloning and functional characterization of a mammalian circadian mutation *tau.* Science. 288: 483-491.

Lowry O. H., Roserbrough N. J., Farr A. L. and Randall R. J. (1951). Protein measurement with the folin phenol reagent. Journal of Biological Chemistry. 193: 265-275.

Lozeva-Thomas V. (2004). Serotonin brain circuits with a focus on hepatic encephalopathy. Metabolic Brain Disease. 19: 413-420.

Lucas R. J. and Foster R. G. (1999). Photoentrainment in mammals: a role for cryptochrome? Journal of Biological Rthythms. 14: 4-10.

Lucas R. J., Douglas R. H. and Foster R. G. (2001). Characterization of an ocular photopigment capable of driving pupillary constriction in mice. Nature Neuroscience. 4: 621-626.

Machaca M. K. (2003). Ca^{2+}-Calmodulin dependent protein kinase II potentiates store-operated Ca^{2+} current. Journal of Biological Chemistry. 278: 33730-33737.

Madeira M. D., Pereira P. A., Silva S. M., Cadete-Leite A. and Paula-Barbosa M. M. (2004). Basal forebrain neurons modulate the synthesis and expression of neuropeptides in the rat suprachiasmatic nucleus. Neuroscience. 125: 889-901.

Maines L. W., Keck B. J., Smith J. E. (1999). Lakoski J. M. Corticosterone regulation of serotonin transporter and 5-HT1A receptor expression the aging brain. Synapse. 32: 58-66.

Malek Z. S., Dardente H., Pevet P. and Raison S. (2005). Tissue-specific expression of tryptophan hydroxylase mRNAs in the rat midbrain: anatomical evidence and daily profiles. European Journal of Neuroscience. 22: 895-901.

Manalan A. S. and Klee C. B. (1984). Calmodulin. Advances in cyclic nucleotide and protein phosphorylation research. 18: 227-278.

Marchant E. G., Watson N. V. and Mistlberger R. E. (1997). Both neuropeptide Y and serotonin are necessary for entrainment of circadian rhythms in mice by daily treadmill running schedules. Journal of Neuroscience. 17: 7974-7987.

Maronde E., Schomerus C., Stehle J. H. and Korf H. W. (1997). Control of CREB phosphorylation and its role for induction of melatonin synthesis in rat pinealocytes. Biology of Cell. 89: 505-511.

Maronde E., Pfeffer M., Olcese J., Molina C. A., Schlotter F., Dehghani F., Korf H-W. and Stehle J. H. (1999). Transcription factors in neuroendocrine regulation: Rhythmic changes in pCREB and ICER levels frame melatonin synthesis. Journal of Neuroscience. 19: 3326-3336.

Masana M. I., Benloucif S. and Dubocovich M. L. (2000). Circadian rhythm of MT1 melatonin receptor expression in the suprachiasmatic nucleus of the C3H/HeN mouse. Journal of Pineal Research. 28: 185-192.

Matsumoto A. M., Marck B. T., Gruenewald D. A., Wolden-Hanson T. and Naai M. A. (2000). Aging and the neuroendocrine regulation of reproduction and body weight. Experimental Gerontology. 35: 1251-1265.

Mazer C., Muneyyirci J., Taheny K., Raio N., Borella A. and Whitaker-Azmitia P. (1997). Serotonin depletion during synaptogenesis leads to decreased synaptic density and learning deficits in the adult rat: a possible model of neurodevelopmental disorders with cognitive deficits. Brain Research.760: 68-73.

McArthur A. J., Hunt A. E. and Gillette M. U. (1997). Melatonin action and signal transduction in the rat suprachiasmatic circadian clock: Activation of protein kinase C at Dusk and Dawn. Endocrinology. 138: 627-634.

McGuinness T. L., Lai Y. and Greengard P. (1985). Ca^{2+}/calmodulin-dependent protein kinase II. Isozymic forms from rat forebrain and cerebellum. Journal of Biological Chemistry. 260: 1696-1704.

McNamara P., Seo S. B., Rudic R. D., Sehgal A., Chakravarti D. and Fitz Gerald G.A. (2001). Regulation of CLOCK and MOP4 by nuclear hormone receptors in the vasculature: a humoral mechanism to reset a peripheral clock. Cell. 105: 877-889.

Meijer J. H., Michel S. and Vansteensel M. J. (2007). Processing of daily and seasonal light information in the mammalian circadian clock. General and Comparitive Endocrinology. 152: 159-164.

Meltzer C. C., Smith G., DeKosky S. T., Pollock B. G., Mathis C. A., Moore R. Y., Kupfer D. J. and Reynolds III C. F. (1998). Serotonin in aging, late-life depression, and Alzheimer's disease: The emerging role of functional imaging. Neuropsychopharmacology 18: 407-430.

Meltzer C. C., Drevets W. C., Price J. C., Mthis C. A., Lopresti B., Greer P. J., Villemagne V. L., Holt D., Mason N. S., Houck P. R., Reynolds C. F. 3rd and DeKosky S. T. (2001). Gender specific aging effects on the serotonin 1A receptor. Brain Research. 895: 9-17.

Menaker M. (2003). Circadian rhythms. Circadian photoreception. Science. 299: 213-214

Menet J., Vuillez P., Jacob N., Pevet P. (2001) Intergeniculate leaflets lesion delays but does not prevent the integration of photoperiodic change by the suprachiasmatic nuclei. Brain Research: 906: 176-179.

Merrow M., Mazzotta G., Chen Z. and Roenneberg T. (2006). The right place at the right time: regulation of daily timing by phosphorylation. Genes and Development. 20: 2629-2633.

Mess B. and Rúzsás C. (1986). Relationship between suprachiasmatic nuclei and rhythmic activity of the pineal gland. In: Advances in Pineal Research. Eds: Reiter R. J. and Karasek M. London: John Libbey Ltd. 1: 149-158.

Miguez J. M., Martin F. J., Miguez I. and Aldegunde M. (1991). Long-term pinealectomy alters hypothalamic serotonin metabolism in the rat. Journal of Pineal Research. 11: 75-79.

Miguez J. M., Martin F. J. and Aldegunde M. (1994). Effect of single doses and daily melatonin treatments on serotonin metabolism in rat brain regions. Journal of Pineal Research. 17: 170-176.

Miguez J. M., Simonneaux V. and Pevet P. (1995). Evidence for a regulatory role of melatonin on serotonin release and uptake in the pineal gland. Journal of Neuroendocrinology. 7: 949-956.

Míguez J. M., Simonneaux V. and Pévet P. (1996). Changes in pineal indoleamines in rats after single melatonin injections: evidence for a diurnal sensitivity to melatonin. Journal of Neuroendocrinology. 8: 611-616.

Miguez J. M., Martin F. J. and Aldegunde M. (1997). Melatonin effects on serotonin synthesis and metabolism in the striatum, nucleus accumbens and dorsal and median raphe nuclei of rats. Neurochemical Research. 22: 87-92.

Miguez J. M., Recio J., Sanchez-Barcelo E. and Aldegunde M. (1998). Changes with age in daytime and nighttime contents of melatonin, indoleamines, and catecholamines in the pineal gland: a comparative study in rat and Syrian hamster. Journal of Pineal Research. 25: 106-115.

Milde-Langosch K. (2005). The Fos family of transcription factors and their role in tumourigenesis. European Journal of Cancer. 41: 2449-2461.

Minami Y., Furuno K., Akiyama M., Moriya T. and Shibata S. (2002). Pituitary adenylate cyclase-activating polypeptide produces a phase shift associated with induction of mPer expression in the mouse suprachiasmatic nucleus. Neuroscience. 113: 37-45.

Mintz E. M., Marvel C. L., Gillespie C. F., Price K. M., Albers H. E. (1999). Activation of NMDA receptors in the suprachiasmatic nucleus produces light-like phase shifts of the circadian clock *in vivo*. Journal of Neuroscience. 19: 5124-5130.

Mirmiran M., Swaab D. F., Kok J. H., Hofman M. A., Witting W. and Van Gool W. A. (1992). Circadian rhythms and the suprachiasmatic nucleus in perinatal development, aging and Alzheimer's disease. Progress in Brain Research. 93: 151-162.

Mirochnik V., Bosler O., Tillet Y., Calas A. and Ugrumov M. (2005). Long-lasting effects of serotonin deficiency on differentiating peptidergic neurons in the rat suprachiasmatic nucleus. International Journal in Developmental Neuroscience. 23: 85-91.

Mishima K., Okawa M., Shimizu T. and Hishikawa Y. (2001). Diminished melatonin secretion in the elderly caused by insufficient environmental illumination. Journal of Clinical Endocrinology and Metabolism. 86: 129-134.

Mistlberger R. E. and Holmes M. M. (2000). Behavioral feedback regulation of circadian rhythm phase angle in light-dark entrained mice. American Journal of Physiology Regulative Integrative Comparitive Physiology. 279: R813-R821.

Miyamoto E., Petzold G. L., Kuo J. F. and Greengard P. (1973). Dissociation and activation of adenosine 3', 5'-monophosphate-dependent and guanosine 3', 5'-monophosphate-dependent protein kinases by cyclic nucleotides and by substrate proteins. Journal of Biological Chemistry. 248: 179-189.

Møller M. and Baeres F. M. (2002). The anatomy and innervation of the mammalian pineal gland. Cell and Tissue Research. 309: 139-50.

Moore R. Y. (1992). Development of the suprachiasmatic nucleus. In Suprachiasmatic Nucleus: The mind's clock. Eds: Klein D. C., Moore R. Y. and Reppert S. M. pp: 391-404.

Moore R. Y., Speh J. C. and Card J. P. (1995). The retinohypothalamic tract originates from a distinct subset of retinal ganglion cells. Journal of Comparitive Neurology. 352: 351-366.

Moore R. Y. (1996). Neural control of the pineal gland. Behavior Brain Research. 73: 125-130.

Moore R. Y., Speh J. C. and Rehana K. L. (2002). Suprachiasmatic nucleus organization. Cell and Tissue Research. 309: 89-98.

Moore R. Y. and Speh J. C. (2004). Serotonin innervation of the primate suprachiasmatic nucleus. Brain Research. 1010: 169-173.

Morgan J. I. and Curran T. A. (1986). Role of ion flux in the control of *c-fos* expression. Nature. 322: 552-555.

Morgan J. I. and Curran T. A. (1988). Calcium as a modulator of the immediate-early gene cascade in neurons. Cell Calcium. 9: 303-311.

Morgan J. I. and Curran T. A. (1991). Stimulus-transcription coupling in the nervous system: involvement of the inducible proto-oncogenes *fos* and *jun*. Annual Reviews of Neuroscience. 14: 421-451.

Morgan P. J., Barret H., Howell E. and Helliwell R. (1994). Melatonin receptors: localization, molecular pharmacology and physiological significance. Neurochemistry International. 24: 101-146.

Morgan P. J. (2000). The pars tuberalis: the missing link in the photoperiodic regulation of prolactin secretion. Journal of Neuroendocrinology. 12: 287-295.

Morikawa Y., Nakagawa H., Miura K., Soyama Y., Ishizaki M., Kido T., Naruse Y., Suwazono Y. and Nogawa K. (2005). Shift work and the risk of diabetes mellitus among Japanese male factory workers. Scandinavian Journal on Work Environment and Health. 31: 179-183.

Morin L. P. and Blanchard J. (1991). Depletion of brain serotonin by 5, 7-DHT modifies hamster circadian rhythm response to light. Brain Research. 566: 173-185.

Morin L. P. (1999). Serotonin and the regulation of mammalian circadian rhythmicity. Annals of Medicine. 31: 12-33.

Morin L. P. and Allen C. N. (2006). The circadian visual system. Brain Research Reviews. 51: 1-60.

Morin L. P., Shivers K. Y., Blanchard J. H and Muscat L. (2006). Complex organization of mouse and rat suprachiasmatic nucleus. Neuroscience. 137: 1285-1297.

Mrosovsky N. (1996). Locomotor activity and non-photic influences on circadian clocks. Biological Reviews of the Cambridge Philosophical Society (London). 71: 343-372.

Mrosovsky N. (1999). Masking: history, definitions, and measurement. Chronobiology International. 16: 415-429.

Müller R. (1986). Cellular and viral *fos* genes: structure, regulation of expression and biological properties of their encoded products. Biochimica Biophysica et Acta. 823: 207-225.

Münch M., Cajochen C. and Wirz-Justice A. (2005). Sleep and circadian rhythms in ageing. Journal of Gerontology and Geriatrics. 38: I21-I23.

Nairn A. C., Bhagat B. and Palfrey H. C. (1985). Identification of calmodulin-dependent protein kinase III and its major Mr 100,000 substrate in mammalian tissues. Proceedings of the National Academy of Sciences. U.S.A. 82: 7939-7943.

Neufeld B., Grosse-Wilde A., Hoffmeyer A., Jordan B. W., Chen P., Dinev D., Ludwig S. and Rapp U. R. (2000). Serine/Threonine kinases 3pK and MAPK-activated protein kinase 2 interact with the basic helix-loop-helix transcription factor E47 and repress its transcriptional activity. Journal of Biological Chemistry 275: 20239-20242.

Neumaier J. F., Sexton T. J., Yracheta J., Diaz A. M. and Brownfield M. (2001). Localization of 5-HT(7) receptors in rat brain by immunocytochemistry, *in situ* hybridization and agonist stimulated c-Fos expression. Journal of Chemical Neuroanatomy. 21: 63-73.

Neuwald A. F. and Landsman D. (1997). GCN5-related histone N-acetyltransferases belong to a diverse superfamily that includes the yeast SPT10 protein. Trends in Biochemical Sciences. 22: 154-155.

Nichols N. R., Johanthan R. D., Nicholas J. L., Steven A. J. and Celeb E. F. (1993). GFAP mRNA increases with age in rat and human brain. Neurobiology of Aging. 14: 421-429.

Nishizuka Y. (1984). Turnover of inositol phospholipids and signal transduction. Science. 225: 1365-1370.

Obrietan K., Impey S., and Storm D. R. (1998). Light and circadian rhythmicityregulate MAP kinase activation in the suprachiasmatic nuclei. Nature Neuroscience. 1: 693-700.

Obsil T., Ghirlando R., Klein D. C., Ganguly S. and Dyda F. (2001). Crystal structure of the 14-3-3zeta: serotonin *N*-acetyltransferase complex. a role for scaffolding in enzyme regulation. Cell. 105: 257-267.

Ochiishi T., Suigiura H. and Yamauchi T. (1993). Characterization and autophosphorylation of Ca^{2+}/calmodulin-dependent protein kinase in the postsynaptic density of the rat forebrain. Brain Research. 610: 97-107.

Oishi K., Fukui H. and Ishida N. (2000). Rhythmic expression of BMAL1 mRNA is altered in Clock mutant mice: differential regulation in the suprachiasmatic nucleus and peripheral tissues. Biochemical Biophysical Research Communications. 268: 164-171.

Okamura H., Miyake S., Sumi Y., Yamaguchi S., Yasui A., Muijtjens M., Hoeijmakers J. H., van der Horst G. T. (1999). Photic induction of *mPer1* and *mPer2* in cry-deficient mice lacking a biological clock. Science. 286: 2531-2534.

Oren D. (1996). The impact of light on the secretion of melatonin in humans. Neuroscientist. 2: 207-210.

Oster H., Werner C., Magnone M. C., Mayser H., Feil R., Seeliger M. W., Hofman F. and Albrecht U. (2003). cGMP-dependent protein kinase II modulates *mPer1* and *mPer2* gene induction and influences phase shifts of the circadian clock. Current Biology. 13: 725-733.

Ouimet C. C., McGuinness T. L. and Greengard P. (1984). Immunocytochemical localization of calcium/calmodulin-dependent protein kinase II in rat brain. Proceedings of the National Academy of Sciences. U. S. A. 81: 5604-5608.

Pandi-Perumal S. R., Srinivasan V., Maestroni G. J., Cardinali D. P., Poeggeler B. and Hardeland R. (2006). Melatonin: Nature's most versatile biological signal? Federation of European Biochemical Socities Journal. 273: 2813-2838.

Park S., Henry E. C. and Gasiewicz T. A. (2000). Regulation of DNA binding activity of the ligand-activated aryl hydrocarbon receptor by tyrosine phosphorylation. Archives of Biochemistry and Biophysics. 381: 302-312.

Payne M. E., Schworer C. M. and Soderling T. R. (1983). Purification and characterization of rabbit liver calmodulin-dependent glycogen synthase kinase. Journal of Biological Chemistry. 258: 2376-2382.

Pazo D., Cardinali D. P., Cano P., Reyes Toso C. S. and Esquifino A. I. (2002). Age related changes in 24-hour rhythms of norepinephrine content and serotonin turnover in rat pineal gland: effect of melatonin treatment. Neurosignals. 11: 81-87.

Penev P. D., Zee P. C., Wallen E. P. and Turek F. W. (1995). Aging alters the phase-resetting properties of a serotonin agonist on hamster circadian rhythmicity. American Journal of Physiology. 268: R293-R298.

Penev P. D., Zee P. C., Wallen E. P. and Turek F. W. (1997). Aging alters the serotonergic modulation of light-induced phase advances in golden hamsters. Regulatory Integrative Comparitive Physiology. 41: R509- R513.

Pennartz C. M., de Jeu M. T., Bos N. P., Schaap J. and Geurtsen A. M. (2002). Diurnal modulation of pacemaker potentials and calcium current in the mammalian clock. Nature. 416: 286-290.

Perreau-Lenz S., Kalsbeek A., Pevet P. and Bujis R. M. (2004). Glutamatergic clock output stimulates melatonin synthesis at night. European Journal of Neuroscience. 19: 318-324.

Perreau-Lenz S., Kalsbeek A., Van Der Vliet J., Pevet P. and Buijs R. M. (2005). *In vivo* evidence for a controlled offset of melatonin synthesis at dawn by the suprachiasmatic nucleus in the rat. Neuroscience. 130: 797-803.

Peters R. V., Aronin N., Schwartz W. J. (1996). c-Fos expression in the rat intergeniculate leaflet: photic regulation, co-localization with Fos-B, and cellular identification. Brain Research. 28: 231-241.

Petkov V. D., Stancheva S. L., Petkov V. V. and Alova L. G. (1987). Age-related changes in brain biogenic monoamines and monoamine oxidase. General Pharmacology: The Vascular System.18: 397-401.

Pévet P., Bothorel B., Slotten H. and Saboureau M. (2002). The chronobiotic properties of melatonin. Cell and Tissue Research. 309: 183-191.

Pévet P, Agez L, Bothorel B, Saboureau M, Gauer F, Laurent V, Masson-Pévet M. (2006). Melatonin in the multi-oscillatory mammalian circadian world. Chronobiology International. 23: 39-51.

Pickard G. E. and Rea M. A. (1997). Serotonergic innervation of the hypothalamic suprachiasmatic nucleus and photic regulation of circadian rhythms. Biology of the Cell. 89: 513-523.

Piggins H. D. and Cutler D. J. (2003). The roles of vasoactive intestinal polypeptide in the mammalian circadian clock. Journal of Endocrinology. 177: 7-15.

Pitrosky B., Kirsch R., Malan A., Mocear E. and Pévet P. (1999). Organization of rat circadian rhythms during daily infusion of melatonin or S20098, a melatonin agonist. American Journal of Physiology. 277: R812-R828.

Poirel V. J., Boggio V., Dardente H., Pevet P., Masson-pevet M. and Gauer F. (2003). Contrary to other non-photic cues, acute melatonin injection does not induce immediate changes of clock gene mRNA expression in the rat suprachiasmatic nuclei. Neuroscience. 120: 745-755.

Preitner N., Damiola F., Lopez-Molina L., Zakany J., Duboule D., Albrecht U. and Schibler U. (2002). The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. Cell. 110: 25-260.

Price J. L., Blau J., Rothenfluh. A., Adodeely M., Kloss B. and Young, M. W., (1998). *double-time* is a new *Drosophila* clock gene that regulates PERIOD protein accumulation. Cell. 94: 83-95.

Prosser R. A. and Gillette M. U. (1989). The mammalian circadian clock in the suprachiasmatic nuclei is rest *in vitro* by cAMP. Journal of Neuroscience. 9: 1073-1081.

Prosser R. A., Dean R. R., Edgar D. M., Heller H. C. and Miller J. D. (1993). Serotonin and the mammalian circadian system: I. In vitro phase shifts by serotonergic agonists and antagonists. Journal of Biological Rhythms. 8: 1-16.

Prosser R. A. (2003). Serotonin phase-shifts the mouse suprachiasmatic circadian clock *in vitro*. Brain Research. 966: 110-115.

Prosser R. A., Lee H. M. and Wehner A. (2006). Serotonergic pre-treatments block in vitro serotonergic phase shifts of the mouse suprachiasmatic nucleus circadian clock. Neuroscience. 142:547-555.

Provencio I., Rollag M. D. and Castrucci A. M. (2002). Photoreceptive net in the mammalian retina. This mesh of cells may explain how some blind mice can still tell day from night. Nature. 415: 493.

Ptacek J., Devgan G., Michaud G., Zhu H., Zhu X., Fasolo J., Guo H., Jona G., Breitkreutz A., Sopko R., McCartney R. R., Schmidt M. C., Rachidi N., Lee S. J., Mah A. S., Meng L., Stark M. J., Stern D. F., De Virgilio C., Tyers M., Andrews B., Gerstein M., Schweitzer B., Predki P. F. and Snyder M. (2005). Global analysis of protein phosphorylation in yeast. Nature. 438: 679-684.

Rajaratnam S. M. and Arendt J. (2001). Health in a 24-hr society. The Lancet. 358: 999-1005.

Ramakrishnan R., Prabhakaran K., Jayakumar A. R., Gunasekaran P., Sheeladevi R. and Suthanthirarajan N. (2005). Involvement of Ca2+/calmodulin-dependent protein kinase II in the modulation of indolamines in diabetic and hyperglycemic rats. Journal of Neuroscience Research. 80: 518-528.

Rauscher F. J., Sambucetti L. C., Curran T., Distel R. J. and Spiegelman B. M. (1988). A common DNA binding site for Fos protein complexes and transcription factor AP-1. Cell. 52: 471-480.

Raymond J. R., Mukhin Y. V., Gelasco A., Turner J., Collinsworth G., Gettys T. W., Grewal J. S. and Garnovskaya M. N. (2001). Multiplicity of mechanisms of serotonin receptor signal transduction. Pharmacology Therapy. 92: 179-212.

Raza M., Deshpande L. S., Blair R. E., Carter D. S., Sombati S. and DeLorenzo R. J. (2007). Aging is associated with elevated intracellular calcium levels and altered calcium homeostatic mechanisms in hippocampal neurons. Neuroscience Letters. 418: 77-81.

Rea M. A. and Pickard G. E. (2000). Serotonergic modulation of photic entrainment in the Syrian hamster. Biological Rhythm Research. 31: 284-314.

Redman J. R. and Armstrong S. M. (1988). Reentrainment of rat circadian activity rhythms: effects of melatonin. Journal of Pineal Research. 5: 203-215.

Reghunandanan V. and Reghunandanan R. (2006). Neurotransmitters of the suprachiasmatic nuclei. Journal of Circadian Rhythms. 4: 2-22.

Reiker J. P., Swanljung-Collins. H. and Collins J. H. (1987). Purification and characterization of a calmodulin-dependent myosin heavy chain kinase from intestinal brush border. Journal of Biological Chemistry. 262: 15262-15268.

Reiter R. J. (1991). Neuroendocrine effects of light. International Journal of Biometeorology. 35: 169-175.

Reiter R. J., Melchiorri D., Sewerynek E., Poeggeler B., Barlow-Walden L., Chuang J., Ortiz G. G. and Acuña-Castroviejo D. (1995). A review of the evidence supporting melatonin's role as an antioxidant. Journal of Pineal Research. 18: 1-11.

Reppert S. M., Weaver D. R. and Rivkees S. A. (1988). Maternal communication of circadian phase to the developing mammal. Psychoneuroendocrinology. 13: 63-78.

Reppert S. M., Godson C. G., Mahle C. D., Weaver D. R., Slaugenhaupt S. A. and Gusella J. F. (1995). Molecular characterization of a second melatonin receptor expressed in human retina and brain: the Mel_{1b}-melatonin receptor. Proceedings of the National Academy of Sciences. USA. 92: 8734-8738.

Reppert S. M., Weaver D. R. and Godson C. (1996). Melatonin receptors step into the light: Cloning and classification of subtypes. Trends in Pharmacological Sciences. 17: 100-102.

Reppert S. M. and Weaver D. R. (2001). Molecular analysis of mammalian circadian rhythms. Annual Reviews of Physiology. 63: 647-76.

Reppert S. M. and Weaver D. R. (2002). Coordination of circadian timing in mammals. Nature. 418: 935-941.

Reuss S., Decker K., Rösseler L., Layes E., Schollmayer A. and Spessert R. (1995). Nitric oxide synthase in the hypothalamic suprachiasmatic nucleus of rat: evidence from histochemistry, immunohistochemistry and western blot; and colocalization with VIP. Brain Research. 695: 257-262.

Ribelayga C., Garidou M. L., Malan A., Gauer F., Calgari C., Pevet P. and Simonneaux V. (1999). Photoperiodic control of the rat pineal arylalkylamine-N-acetyltransferase and

hydroxyindole-O-methyltransferase gene expression and its effect on melatonin synthesis. Journal of Biological Rhythms. 14: 105-115.

Richerson G. B. (2004). Serotonergic neurons as carbon dioxide sensors that maintain pH homeostasis. Nature Reviews Neurosciences. 5: 449-461.

Rivkees S. A. (1997). Developing circadian rhythmicity. Basic and clinical aspects. Pediatric Clinics of North America. 44: 467-487.

Rohling J., Meijer J. H., VanderLeest H. T. and Admiraal J. (2006). Phase differences between SCN neurons and their role in photoperiodic encoding; a simulation of ensemble patterns using recorded single unit electrical activity patterns. Journal of Physiology. Paris. 100: 261-270.

Rosenwasser A. M. and Dwyer S. M. (2001). Circadian phase shifting: Relationships between photic and nonphotic phase-response curves. Physiology and Behavior. 73: 175-183.

Roskoski R. Jr. (1983). Assays of protein kinase. Methods in Enzymology. 99: 3-6.

Roskoski R. Jr. (1996). Biochemistry-Neurotransmitters. W. B. Saunders Company, A division of Harcout Brace & Company, Philadelphia, Pennsylvania. pp: 396-417.

Rostas J. A. P. and Dunkley P. R. (1992). Multiple forms and distribution of calcium/calmodulin–stimulated protein kinase in brain. Journal of Neurochemistry 59: 1191-1202.

Rostas J. A. P. and Margrie T. W. (1997). Subcellular translocation of Ca^{2+}/Calmodulin-dependent protein kinase II: Fact or artifact? Journal of Neurochemistry. 69: 435-436.

Rutter J., Reick M., Wu L. C. W. and McKnight S. L. (2001). Regulation of CLOCK and NPAS2 DNA binding by the redox state of NAD cofactors. Science. 293: 510-514.

Ruzsás C., Fraschini F., Peschke E., Esposti D. and Esposti G. (1986). Brain neurotransmitters mediating neuroendocrine activity of melatonin. In Advances in Pineal Research. Eds. Reiter R. J. and Karasek M. John Libbey and Co. Ltd, London. pp: 159-166.

Rúzsás C. and Mess B. (2000). Melatonin and aging. A brief survey. Neuroendocrinology Letters 21: 17–23

Ryseck R. P. and Bravo R. (1991). *c-jun*, *jun B* and *jun D* differ in their binding affinities to AP-1 and CRE consensus sequences: effects of Fos proteins. Oncogene. 6: 533-542.

Saavedra J. M. (1977). Distribution of serotonin and synthesizing enzymes in discrete areas of the brain. Federation Proceedings. 36: 2134-2141.

Saeb-Parsy K. and Dyball R. E. (2003). Responses of cells in the rat suprachiasmatic nucleus in vivo to stimulation of afferent pathways are different at different times of the light/dark cycle. Journal of Neuroendocrinology. 15: 895-903.

Sáez J. C., Martínez A. D., Brañes M. C. and González H. E. (1998). Regulation of gap junctions by protein phosphorylation. Brazilian Journal of Medical and Biological Research. 31: 593-600.

Saha S., Ramanathan A. and Rangarajan P. N. (2006). Regulation of Ca^{2+}/calmodulin kinase II inhibitor alpha (CaMKII Ialpha) in virus-infected mouse brain. Biochemical and Biophysical Research Communications. 350: 444-449.

Sakamoto K., Oishi K, Shiraishi M., Hamano S., Otsuka H., Miyake Y. and Ishida N. (2000). Two circadian oscillatory mechanisms in the mammalian retina. Neuroreport. 11: 3995-3997.

Sakowski S. A., Geddes T. J., Thomas D. M., Levi E., Hatfield J. S. and Kuhn D. M. (2006). Differential tissue distribution of tryptophan hydroxylase isoforms 1 and 2 as revealed with monospecific antibodies. Brain Research. 1085: 11-18.

Samorajski T. (1977). Central neurotransmitter substances and aging: a review. Journal of American Geriatric Society. 25: 337-348.

Sanada K., Harada Y., Sakai M., Todo T. and Fukuda Y. (2004). Serine phosphorylation of mCRY1 and mCRY2 by mitogen-activated protein kinase. Genes Cells. 9: 697-708.

Sanders-Bush E. and Martin L. L. (1982). Storage and release of serotonin. In Biology of Serotonin Transmission. Ed: N. N. Osborne. New York: Wiley. pp: 95-118.

Saper C. B., Lu J., Chou T. C. and Gooley J. (2005). The hypothalamic integrator for circadian rhythms. Trends in Neurosciences. 28: 152-157.

Satinoff E., Li H., Tcheng T. K., Liu C., McArthur A. J., Medanic M. and Gillette M. U. (1993). Do the suprachiasmatic nuclei oscillate in old rats as they do in young ones? The American Journal of Physiology. 265: R1216-R1222.

Sato T. K., Panda S., Miraglia L. J., Reyes T. M., Rudic R. D., McNamara P., Naik K. A., FitzGerald G. A., Kay S. A. and Hogenesch J. B. (2004). A functional genomics strategy reveals Rorα as a component of the mammalian circadian clock. Neuron. 43: 527-537.

Saudou F., Amara D. A., Dierich A., LeMeur M., Ramboz S., Segu L., Buhot M. C. and Hen R. (1994). Enhanced aggressive behavior in mice lacking 5-HT1B receptor. Science. 265: 1875-1878.

Scheibner J., Trendelenburg A. U., Hein L., Starke K. and Blandizzi C. (2002). Alpha 2-adrenoceptors in the enteric nervous system: a study in alpha 2A-adrenoceptor-deficient mice. British Journal of Pharmacology. 135: 697-704.

Schernhammer E. S., Laden F., Speizer F. E., Willett W. C., Hunter D. J., Kawachi I., Fuchs C. S. and Colditz G. A. (2003). Night-shift work and risk of colorectal cancer in the nurses' health study. Journal of the National Cancer Institute. 95: 825-828.

Schernhammer E. S., Holly J. M., Hunter D. J., Pollak M. N. and Hankinson S. E. (2006). Insulin-like growth factor-I, its binding proteins (IGFBP-1 and IGFBP-3), and growth hormone and breast cancer risk in The Nurses Health Study II. Endocrine Related Cancer. 13: 583-592.

Schibler U. (2000). Circadian clocks. Heartfelt enlightenment. Nature. 404: 27-28.

Schomerus C. and Korf H. W. (2005). Mechanisms regulating melatonin synthesis in the mammalian pineal organ. Annals of New York Academy of Sciences. 1057: 372-383.

Schuhler S., Pitrosky B., Kirsch R. and Pévet P. (2002). Entrainment of locomotor activity rhythm in pinealectomized adult Syrian hamsters by daily melatonin infusion. Behavioral Brain Research. 133: 343-350.

Schulman H. and Greengard P. (1978). Ca^{2+}-dependent protein phosphorylation system in membranes from various tissues, and its activation by "calcium-dependent regulator". Proceedings of the National Academy of Sciences U. S. A. 75: 5432-5436.

Schulman H., Kuret L., Jefferson A. B., Nose P. S. and Spitzer K. H. (1985). Ca^{2+}/calmodulin-dependent microtubule-associated protein 2 kinase: broad substrate specificity and multifunctional potential in diverse tissues. Biochemistry. 24: 5320-5327.

Schulman H. (1993). The multifunctional Ca^{2+}/calmodulin-dependent protein kinases. Current Opinions in Cell Biology. 5: 247-253.

Schulman H. and Hanson P. I. (1993). Multifunctional Ca^{2+}/calmodulin-dependent protein kinase. Neurochemical Research. 18: 65-77.

Schwartz W. J., Aronin N., Takeuchi J., Bennett M. R. and Peters R. V. (1995). Towards a molecular biology of the suprachiasmatic nucleus; Photic and temporal regulation of *c-fos* gene expression. Seminars in Neurosciences. 7: 53-60.

Schwartz W. J. (1996). Internal time keeping. Science and medicine. 44-53.

Schwartz W. J., Caprio A. Jr., de la Iglesia H. O., Baler R., Klein D. C., Nakabeppu Y. and Aronin N. (2000). Differential regulation of *fos* family genes in the ventrolateral and dorsomedial subdivisions of the rat suprachiasmatic nucleus. Neuroscience. 98: 535-547.

Schwartz W. J., de la Iglesia H. O., Zlomanczuk P. and Illnerova H. (2001). Encoding Le Quattro Stagioní within the mammalian brain: Photoperiodic orchestration through the suprachiasmatic nucleus. Journal of Biological Rhythms. 16: 302-311.

Semba J., Sakai M. W., Suhara T. and Akanuma N. (1999). Differential effects of acute and chronic treatment with typical and atypical neuroleptics on c-fos mRNA expression in rat forebrain regions using non-radioactive in situ hybridization. Neurochemistry International. 34: 269-277.

Semo M., Lupi D., Peirson S N., Butler J. S. and Foster R. G. (2007). Light-induced *c-fos* in melanopsin retinal ganglion cells of young and aged rodless/coneless (*rd/rd cl*) mice. European Journal of Neuroscience.18: 3007-3011.

Shackelford D. A., Yeh R. Y., Hsu M., Buzsáki G. and Zivin J. A. (1995). Effect of cerebral ischemia on calcium/calmodulin-dependent protein kinase II activity and phosphorylation. Journal of Cerebral Blood Flow and Metabolism. 15: 450-461.

Sharma V. K., Singaravel M., Subbaraj R. and Chandrashekaran M. K. (1999). Timely administration of melatonin accelerates reentrainment to phase-shifted light-dark cycles in the field mouse *Mus booduga*. Chronobiology International. 16: 163-170.

Sharma V. K. (2001). Do biological clocks age like their owners? Proceedings of Indian National Science Academy. 6: 373-388.

Shearman L. P., Zylka M. J., Weaver D. R., Kolakowski L. F. Jr. and Reppert S. M. (1997). Two period homologs: circadian expression and photic regulation in the suprachiasmatic nuclei. Neuron. 19: 1261-1269.

Shearman L .P., Sriram S., Weaver D. R., Maywood E. S., Chaves I., Zheng B., Kume K., Lee C. C., vander Horst G. T., Hastings M. H. and Reppert S. M. (2000). Interacting molecular loops in the mammalian circadian clock. Science. 288: 1013-1019.

Sheng M. and Greenberg M. E. (1990). The regulation and function of c-fos and other immediate early genes in the nervous system. Neuron. 4: 477-485.

Shenolikar S. (1988). Protein phosphorylation: hormones, drugs and bioregulation. The Federation of American Societies for Experimental Biology. The Federation of American Societies for Experimental Biology Journal. 2: 2753-2764.

Shibata S., Cassone V. M. and Moore R. Y. (1989). Effects of melatonin on neuronal activity in the rat suprachiasmatic nucleus in vitro. Neuroscience Letters. 97: 140-144.

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

Silver R., Le Sauter J., Tresco P. A. and Lehman M. N. (1996). A diffusible coupling signal from the transplanted suprachiasmatic nucleus controlling circadian locomotor rhythms. Nature. 382: 810-813.

Simonneaux V. and Ribelayga C. (2003). Generation of melatonin endocrine message in mammals: a review of the complex regulation of melatonin synthesis by norepinephrine, peptides and other pineal transmitters. Pharmacological Reviews. 55: 325-395.

Simpkins J. W. and Millard W. J. (1987). Influence of age on neurotransmitter function. Endocrinology and Metabolism Clinics of North America. 16: 893-917.

Sloan M. A., Levenson J., Tran Q., Kerbeshian M., Block G. D. and Eskin A. (1999). Aging affects the ocular circadian pacemaker of *Aplysia californica*. Journal of Biological Rhythms. 14: 151-159.

Slominski A., Semak I., Pisarchik A., Sweatman T., Szczesniewski A. and Wortsman J. (2002). Conversion of L-tryptophan to serotonin and melatonin in human melanoma cells. Federation European Biochemical Ssocieties Letters. 511: 102-106.

Slotkin T. A., Seidler F. J. and Ali S. F. (2000).Cellular determinants of reduced adaptability of the aging brain: neurotransmitter utilization and cell signaling responses after MDMA lesions. Brain Research. 879: 163-173.

Slotten H. A., Pitrosky B. Pévet P. (1999). Influence of the mode of daily melatonin administration on entrainment of rat circadian rhythms. Journal of Biological Rhythms. 14: 347-353.

Smale L., Lee T. and Nunez A. A. (2003). Mammalian diurnality: Some facts and gaps. Journal of Biological Rhythms. 18: 356-366.

Smith R. G., Betancourt L. and Sun Y. (2005). Molecular endocrinology and physiology of the aging central nervous system. Endocrine Reviews. 26: 203-250.

Soderling T. R. (1990). Protein kinases. Regulation by autoinhibitory domains. Journal of Biological Chemistry. 265: 1823-1826.

Sodhi M. S. and Sanders-Bush E. (2004). Serotonin and brain development.International Review in Neurobiology. 59: 111-174.

Sollars P. J., Ogilvie M. D., Simpson A. M. and Pickard G. E. (2006). Photic entrainment is altered in the 5-HT1B receptor knockout mouse. Journal of Biological Rhythms. 21: 21-32.

Spessert R., Rapp M., Jastrow H., Karabul N., Blum F. and Vollrath L. (2000). A differential role of CREB phosphorylation in cAMP-inducible gene expression in the rat pineal. Brain Research. 864: 270-280.

Stankovski L., Alvarez C., Ouimet T., Vitalis T., El-Hachimi K. H., Price D., Deneris E., Gaspar P. and Cases O. (2007). Developmental cell death is enhanced in the cerebral cortex of

mice lacking the brain vesicular monoamine transporter. Journal of Neuroscience. 27: 1315-1324.

Stehle J. H., Foulkes N. S., Molina C. A., Simonneaux V., Pevet P. and Sassone-Corsi P. (1993). Adrenergic signals direct rhythmic expression of transcriptional repressor CREM in the pineal gland. Nature. 365: 314-320.

Steinhilber D., Brungs M., Werz O., Weisenberg I., Danielsson C., Kahlen J. P., Nayeri S., Schräder M. and Carlberg C. (1995). The nuclear receptor for melatonin represses 5-lipoxygenase gene expression in human B lymphocytes. Journal of Biological Chemistry. 270: 7037-7040.

Stone J. M. and Pilkowsky L. S., (2007). Novel targets for drugs in schizophrenia. CNS and Neurological Disorders-Drug Targets. 6: 265-272.

Sumova A., Maywood E. S., Selvage D., Ebling F. J. P. and Hastings M. H. (1996). Serotonergic antagonists impair arousal-induced phase shifts of the circadian system of the Syrian hamster. Brain Research. 709: 88-96.

Sun X., Deng J., Liu T, and Borjigin J. (2002). Circadian 5-HT production regulated by adrenergic signaling. Proceedings of the National Academy of Sciences. U. S. A. 99: 4686-4691.

Sutin E. L. and Kilduff T. S. (1992). Circadian and light induced expression of immediate early gene mRNAs in the rat suprachiasmatic nucleus. Molecular Brain Research. 15: 281-290.

Swaab D. F., Fliers E. and Partiman T. S. (1985). The suprachiasmatic nucleus of the human brain in relation to sex, age and senile dementia. Brain Research. 342: 37-44.

Tae-Don K., Kyung-Chul W., Sungchan C., Dae-Cheong H., Sungkey J. and Kyong-Tai K. (2005). Rhythmic control of AANAT translation by hnRNP Q in circadian melatonin production. Neuroscience Research. 58: S166.

Takahashi J. S. (1994). ICER is nicer at night (sir!). Current Biology. 4: 165-168.

Takahashi J. S. (1995). Molecular neurobiology and genetics of circadian rhythms in mammals. Annual Reviews of Neuroscience. 18: 531-553.

Takahata S., Sogawa K., Kobayashi A., Ema M., Mimura J., Ozaki N. and Fujii-Kuriyama Y. (1998). Transcriptionally active heterodimer formation of an Arnt-like PAS protein, Arnt3, with HIF-1a, HLF and clock. Biochemica Biophysics Research Communications. 248: 789-794.

Takano A., Shimizu K., Kani S., Buijs R. M., Okada M. and Nagai K. (2000). Cloning and characterization of rat casein kinase 1ε. Federation of European Biochemical Societies Letters. 477: 106-112.

Takumi T., Taguchi K., Miyake S., Sakakida Y., Takashima N., Matsubara C., Maebayashi Y., Okumura K., Takekida S., Yamamoto S., Yagita K., Yan L., Young M. W. and Okamura H. (1998). A light-independent oscillatory gene *mPer3* in mouse SCN and OVLT. The European Molecular Biology Oraganization Journal 17: 4753-4759.

Talley E. M., Sadr N. N. and Bayliss D. A. (1998). Postnatal development of serotonergic innervation, 5-HT1A receptor expression, and 5-HT responses in rat motoneurons. Journal of Neuroscience. 17: 4473-4485.

Tamir H. and Gershon M. D. (1979). Storage of serotonin and serotonin binding protein in synaptic vesicles. Journal of Neurochemistry. 33: 35-44.

Tenn C. and Niles L. P. (1993). Physiological regulation of melatonin receptors in rat suprachiasmatic nuclei: diurnal rhythmicity and effects of stress. Molecular and Cellular Endocrinology. 98: 43-48.

Terman M., Lewy A. J., Dijk D. J., Boulos Z., Eastman C. I. and Campbell S. S. (1995). Light treatment for sleep disorders: consensus report. IV. Sleep phase and duration disturbances. Journal of Biological Rhythms. 10: 135-147.

Tobimatsu T. and Fujisawa H. (1989). Tissue-specific expression of four types of rat calmodulin-dependent protein kinase II mRNAs. Journal of Biological Chemistry. 264: 17907-17912 .

Toh K. L., Jones C. R., He Y., Eide E. J., Hinz W. A., Virshup D. M., Ptacek L. J. and Fu Y. H., (2001). An *hPer2* phosphorylation site mutation in familial advanced sleep phase syndrome. Science. 291. 1040–1043.

Tosini G., Bertolucci C. and Foà A. (2001). The circadian system of reptiles: a multioscillatory and multiphotoreceptive system. Physiology and Behavior. 72: 461-471.

Touitou Y., Bogdan A., Haus E. and Touitou C. (1997). Modifications of circadian and circannual rhythms with aging. Experimental Gerontology. 32: 603-614.

Touitou Y. (2005). Melatonin: what for? Bulletin de l Academie Nationale de Medecine (Paris). 189: 879-889.

Trávnícková Z., Sumová A., Peters R., Schwartz W. J. and Illnerová H. (1996). Photoperiod-dependent correlation between light-induced SCN *c-fos* expression and resetting of circadian phase. American Journal of Physiology. 271: R825-R831.

Turek F. W. (1994). Circadian rhythms. In Recent Progress in Hormone Research. Ed: Bardin W. Academic Press, New York. pp: 43-90.

Turek F. W. (1995). Effects of age on the circadian system. Neuroscience and Behavioral Reviews. 19: 53-58.

Turek F. W., Penev P., Zhang Y., Van Reeth O., Takahashi J. S. and Zee P. (1995). Alterations in the circadian system in advanced age. Ciba Foundation Symposium 183: 212-226.

Turjanski A. G., Estrin D. A., Rosenstein R. E., Mccormick J. E., Martin S. R., Pastore A., Biekofsky R. R. and Martorana V. (2004). NMR and molecular dynamics studies of the interaction of melatonin with calmodulin. Protein Science. 13: 2925-2938.

Underwood H. and Goldman B. D. (1987). Vertebrate circadian and photoperiodic systems: role of the pineal gland and melatonin. Journal of Biological Rhythms. 2: 279-315.

Uphouse L. (1997). Multiple serotonin receptors: Too many, not enough, or just the right number? Neuroscience Biobehavioral Reviews. 21: 679-698.

Vacas M. I., Keller Sarmiento M. I. and Cardinali D. P. (1985). Interaction between alpha- and beta-adrenoceptors in rat pineal adenosine cyclic 3', 5'-monophosphate phosphodiesterase activation. Journal of Neural Transmission. 62: 295-304.

Vaillancourt D. E. and Newell K. M. (2002). Changing complexity in human behavior and physiology through aging and disease. Neurobiology of Aging. 23:1-11.

Valenti S. and Giusti M. (2002). Melatonin participates in the control of testosterone secretion from rat testis: an overview of our experience. Annals of New York Academy of Sciences. 966: 284-289.

Van Cauter E., Plat L., Leproult R. and Copinschi G. (1998). Alterations of circadian rhythmicity and sleep in aging: endocrine consequences. Hormonal Research. 49: 147-152.

Van Cauter E., Polonsky K. S. and Scheen A. J. (1997). Roles of circadian rhythmicity and sleep in human glucose regulation. Endocrine Reviews. 18: 716-738.

van den Pol A. N. (1991). Glutamate and aspartate immunoreactivity in hypothalamic presynaptic axons. Journal of Neuroscience. 11: 2087-2101.

van den Top M., Bujis R. M., Ruijter J. M., Delagrange P., Spanswick D. and Hermes M. L. (2001). Melatonin generates an outward potassium current in rat suprachiasmatic nucleus neurons *in vitro* independent of their circadian rhythm. Neuroscience. 107: 99-108.

van Gelder R. N. and Sancar A. (2003). Cryptochromes and inner retinal non-visual irradiance detection. Novartis Foundation Symposium. 253: 31-42.

van Haasteren G., Li S., Muda M., Susini S. and Schlegel W. (1999). Calcium signaling and gene expression. Journal of Receptor and Signal Transduction Research. 19: 481-492.

van Hooft J. A. and Yakel J. L. (2003). 5-HT3 receptors in the CNS: 3B or not 3B? Trends in Pharmacological Sciences. 24: 157-160.

Van Reeth O., Zhang Y., Reddy A., Zee P. and Turek F. W. (1993). Aging alters the entraining effects of an activity-inducing stimulus on the circadian clock. Brain Research. 607: 286-292.

Van Someren E. J. W. (2000). Circadian and sleep disturbances in the elderly. Experimental Gerontology. 35: 1229-1237.

Vaněček J., Pavlĩk A. and Illnerová H. (1987). Hypothalamic melatonin receptor sites revealed by autoradiography. Brain Research. 435: 359-362.

Vanselow K., Vanselow J. T., Westermark P. O., Reischl S., Maier B., Korte T., Herrmann A., Herzel H., Schlosser A. and Kramer A. (2006). Differential effects of PER2 phosphorylation: molecular basis for the human familial advanced sleep phase syndrome (FASPS). Genes and Development. 20: 2660-2672.

Varcoe T. J., Kennaway D. J., Voultsios A. (2003). Activation of 5-HT2C receptors acutely induces *Per* gene expression in the rat suprachiasmatic nucleus at night. Brain Research Molecular Brain Research. 119: 192-200.

Vielhaber R., Eide E., Rivers A., Gao Z. H. and Virshupp D. M. (2000). Nuclear entry of the circadian regulator mPER1 is controlled by casein kinase I epsilon. Molecular Cell Biology. 20: 4888-4899.

Vijayalaxmi Thomas, C. R., Reiter, R. J. and Herman T. S. (2002). Melatonin: from basic research to cancer treatment clinics. Journal of Clinical Oncology. 20: 2575-2601.

Voisin P., Namboodiri M. A. and Klein D. C. (1984). Arylamine N-acetyltransferase and arylalkylamine N-acetyltransferase in the mammalian pineal gland. Journal of Biological Chemistry. 253: 10913-10918.

von Gall C., Duffield G. E., Hastings M. H., Kopp M. D., Dehghani F., Korf H. W. and Stehle J. H. (1998). CREB in the mouse SCN: a molecular interface coding the phase-adjusting stimuli light, glutamate, PACAP, and melatonin for clockwork access. Journal of Neuroscience. 18: 10389-10397.

von Gall C., Weaver D. R., Kock M., Korf H. W. and Stehle J. H. (2000). Melatonin limits transcriptional impact of phosphor CREB in the mouse SCN via the Mel_{1a} receptor. Neuroreport. 11: 1803-1807.

von Gall C., Stehle J. H. and Weaver D. R. (2002). Mammalian melatonin receptors: molecular biology and signal transduction. Cell and Tissue Research. 309: 151-162.

Vulliet P. R., Woodgett J. R. and Cohen P. (1984). Phosphorylation of tyrosine hydroxylase by calmodulin-dependent multiprotein kinase. Journal of Biological Chemistry. 259: 13680-13683.

Walther D. J., Peter J. U., Bashammakh S., Hortnagl H., Voits M., Fink H. and Bader M. (2003). Synthesis of serotonin by a second tryptophan hydroxylase isoform. Science. 299: 76.

Wan Q., Man H. Y., Liu F., Braunton J., Niznik H. B., Pang S. F., Brown G. M. and Wang Y. T. (1999). Differential modulation of GABA receptor function by Mel1a and Mel1b receptors. Nature Neuroscience. 2: 401-403.

Wang G. Q. and Tong J. (2004). Advances in study on molecular mechanism of circadian clock in pineal gland. Sheng Li Ke Xue Jin Zhan. 35: 210-214.

Wang Z., Ramanadham S., Ma Z. A., Bao S., Mancuso D. J., Gross R. W. and Turk J. (2005). Group VI A phospholipase A2 forms a signaling complex with the calcium/calmodulin-dependent protein kinase IIbeta expressed in pancreatic islet beta-cells. Journal of Biological Chemistry. 280: 6840-6849.

Warren W. S., Hodges D. B. and Cassone V. M. (1993). Pinealectomized rats entrain and phase-shift to melatonin injections in a dose-dependent manner. Journal of Biological Rhythms. 8: 233-245.

Watabe Y., Yoshimoto K., Eguchi M. and Ueda S. (2005). Degeneration of monoaminergic fibers in the aged micrencephalic rat. Neuroscience Letters. 385: 82-86.

Watanabe K., Yamaoka S. and Vanecek J. (1998). Melatonin inhibits spontaneous and VIP-induced vasopressin release from suprachiasmatic neurons. Brain Research. 801: 216-219.

Weaver D. R., Rivkees S. A. and Reppert S. M. (1989). Localization and characterization of melatonin receptors in rodent brain *by in vitro* autoradiography. Journal of Neuroscience. 9: 2581-2590.

Weaver D. R., Rivkees S. A. and Reppert S. M. (1992). D1-dopamine receptors activate *c-fos* expression in the fetal suprachiasmatic nuclei. Proceedings of the National Academy of Sciences. U. S. A. 89: 9201-9204.

Weaver D. R. (1999). Melatonin and circadian rhythms in vertebrates: Physiological roles and pharmacological effects. In: Neurobiology of Sleep and Circadian Rhythms. Eds: Turek F. W. and Zee P. C. Marcel Dekker. pp: 197-262.

Webb S. M. and Puig-Domingo M. (1995). Role of melatonin in health and disease. Clinical Endocrinology (Oxford). 42: 221-234.

Weber E. T., Gannon R. L. and Rea M. A. (1995). cGMP-dependent protein kinase inhibitor blocks light-induced phase advances of circadian rhythms *in vivo*. Neuroscience Letters. 197: 227-230.

Weiland N. G. and Wise P. M. (1990). Aging progressively decreases the densities and alters the diurnal rhythms of alpha 1-adrenergic receptors in selected hypothalamic regions. Endocrinology. 126: 2392-2397.

Weinert D. and Waterhouse J. (1999). Daily activity and body temperature rhythms do not change simultaneously with age in laboratory mice. Physiology and Behavior. 66: 605-612.

Welsh D. K., Logothetis D. E., Meister M. and Reppert S. M. (1995). Individual neurons dissociatred from rat suprachiasmatic nucleus express independently phased circadian firing rhythms. Neuron. 14: 697-706.

Whitaker-Azmitia P. M. (2001). Serotonin and brain development: role in human developmental diseases. Brain Research Bulletin. 56: 479-485.

Whitmarsh A. J. (2007). Regulation of gene transcription by mitogen-activated protein kinase signaling pathways. Biochimica et Biophysica Acta. 1773: 1285-1298.

Wise P. M., Cohen I. R., Weiland N. G. and London E. D. (1988). Aging alters the circadian rhythm of glucose utilization in the Suprachiasmatic nucleus. Proceedings of the National Academy of Sciences. U. S. A. 85: 5305-5309.

Wise P. M., Smith M. J., Dubal D. B., Wilson M. E., Krajnak K. M. and Rosewell K. L. (1999). Neuroendocrine influences and repercussions of the menopause. Endocrine Reviews. 20: 243-248.

Witt-Enderby P. A., Bennett J., Jarzynka M. J., Firestine S. and Melan M. A. (2003). Melatonin receptors and their regulation: biochemical and structural mechanisms. Life Sciences. 72: 2183-2198.

Witting W., Mirmiran M., Bos N. P. and Swaab D. F. (1993). Effect of light intensity on diurnal sleep-wake distribution in young and old rats. Brain Research Bullitein. 30: 157-162.

Wollnik F. W., Brysch E., Uhlmann F., Gillardon R., Bravo M., Zimmermann K., Schlingensiepen H. and Herdegen T. (1995). Block of c-Fos and JunB expression by antisense oligonucleotides inhibits light-induced-phase shifts of the mammalian circadian clock. European Journal of Neuroscience. 7: 388.

Wong W. W., Liao J. C. (2006). The design of intracellular oscillators that interact with metabolism. Cellular and Molecular Life Sciences. 63: 1215-1220.

Woodgett J. R., Davison M. T. and Cohen P. (1983). The calmodulin-dependent glycogen synthase kinase from rabbit skeletal muscle. Purification, subunit structure and substrate specificity. European Journal of Biochemistry. 136: 481-487.

Wu Y. H. and Swaab D. F. (2007). Disturbance and strategies for reactivation of the circadian rhythm system in aging and Alzheimer's disease. Sleep Medicine. 6: 623-636.

Yagita K., Tamanini F., Yasuda M., Hoeijmakers J. H., van Der Horst G. T. and Okamura H. (2002). Nucleocytoplasmic shuttling and mCRY dependent inhibition of ubiquitylation of the mPER2 clock protein. The European Molecular Biology Oraganization Journal. 21: 1301-1314.

Yamaguchi S., Isejima H., Matsuo T., Okura R., Yagita K., Kobayashi M. and Okamura H. (2003). Synchronization of cellular clocks in the suprachiasmatic nucleus. Science. 302: 1408-1412.

Yamauchi T. and Fujisawa H. (1983). Purification and characterization of the brain calmodulin-dependent protein kinase (kinase II), which is involved in the activation of tryptophan 5-monooxygenase. European Journal of Biochemistry. 132: 15-21.

Yamauchi T. and Fujisawa H. (1984). Calmodulin-dependent protein kinase (kinase II) which is involved in the activation of tryptophan 5-monooxygenase catalyzes phosphorylation of tubulin. Archives in Biochemisry and Biophysics. 234: 89-96.

Yamazaki S., Numano R., Abe M., Hida A., Takahashi R., Ueda M., Block G. D., Sakaki Y., Menaker M. and Tei H. (2000). Resetting central and peripheral circadian oscillators in transgenic rats. Science. 288: 682-685.

Ying S-W. and Rusak B. (1994). Effects of serotonergic agonists on firing rates of photically responsive cells in the hamster suprachiasmatic nucleus. Brain Research. 651: 37-46.

Yokota S., Yamamoto M., Moriya T., Akiyama M., Fukunaga K., Miyamoto E. and Shibata S. (2001). Involvement of calcium-calmodulin protein kinase but not mitogen-activated protein kinase in light-induced phase delays and *Per* gene expression in the suprachiasmatic nucleus of the hamster. Journal of Neurochemistry. 77: 618-627.

Young M. W. (2000). Life's 24-hour clock: molecular control of circadian rhythms in animal cells. Trends in Biochemical Sciences. 25: 601-606.

Young M. W. and Kay S. A. (2001). Time zones: a comparative genetics of circadian clocks. Nature Reviews Genetics. 2: 702-715.

Yurek D. M., Hipkens S. B., Hebert M. A., Gash D. M. and Gerhardt G. A. (1998). Age-related decline in striatal dopamine release and motoric function in brown Norway/Fischer 344 hybrid rats. Brain Research. 791: 246-256.

Zawilska J. B., Lorenc A., Berezijska M., Vivien-Roels B., Pévet P. and Skene D. J. (2006). Diurnal and circadian rhythms in melatonin synthesis in the turkey pineal gland and retina. General and Comparative Endocrinology. 145: 162-168.

Zayzafoon M., Fulzele K. and McDonald J. M. (2005). Calmodulin and calmodulin-dedendent kinase II alpha regulate osteoblast differentiation by controlling *c-fos* expression. Journal of Biological Chemistry. 280: 7049-7059.

Zhang Y., Kornhauser J. M., Zee P. C., Mayo K. E., Takahashi J. S. and Turek F. W. (1996). Effects of aging on light-induced phase-shifting of circadian behavioral rhythms, Fos expression and CREB phosphorylation in the hamster suprachiasmatic nucleus. Neuroscience. 70: 951-961.

Zhang Z. W. (2006). Canadian Association of Neurosciences Review: Postnatal development of the mammalian neo-cortex: role of activity revisited. Canadian Journal of Neurological Sciences. 33: 158-169.

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ABBREVIATIONS

5-HIA	:	5-Hydroxyindole acetaldehyde
5-HIAA	:	5-Hydroxy indole acetic acid
5-HT	:	5-Hydroxytryptamine
5-HTP	:	5-Hydroxytryptophan
AADC	:	L-aminoacid decarboxylase
AANAT	:	Arylalkylamine N-acetyltransferase
Acetyl CoA	:	Acetyl coenzyme A
AFMK	:	*N*1-acetyl-*N*2-formyl-5-methoxykynuramine
AMK	:	*N*1-acetyl-5-methoxykynuramine
AMPA	:	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP-1	:	Activator protein-1
APS	:	Ammonium per sulphate
ARNT	:	Aryl hydrocarbon receptor nuclear translocator
ASPS	:	Advanced sleep phase syndrome
ATP	:	Adenosine triphosphate
AVP	:	Arginine vasopressin
bHLH	:	basic helix loop helix
Bmal1	:	Brain-muscle-Arnt-like-protein 1
Ca^{2+}	:	Calcium
Ca/CRE	:	Calcium/cAMP response element
CaM	:	Calmodulin
CaM Kinases	:	Ca^{2+}/calmodulin-dependent kinases
CaMKII	:	Ca^{2+}/Calmodulin-dependent protein kinase II
cAMP	:	cyclic adenosine monophoshate
CGRP	:	Calcitonin gastrin releasing peptide
CkIε	:	Casein kinase Iε
CkIδ	:	Casein kinase Iδ
c3OHM	:	cyclic 3-hydroxymelatonin
Clock	:	Circadian locomotor output cycles kaput
CNS	:	Central nervous system
Ca/CRE	:	Calcium/ cAMP response element
CRE	:	cAMP response element
CREB	:	cAMP-responsive element binding protein
Cry	:	*Cryptochrome*
CSF	:	Cerebrospinal fluid
DNA	:	Deoxy ribonucleic acid
DRN	:	Dorsal raphe nuclei
DSPS	:	Delayed sleep phase syndrome
EC cells	:	Enterochromaffin cells
GABA	:	Gamma amino butyric acid
GFAP	:	Glial fibrillary acidic protein

GHT	:	Geniculohypothalamic tract
GI tract	:	Gastro-intestinal tract
GMCSF	:	Granulocyte-macrophage colony stimulating factor
GNAT	:	GCN-5-related N-acetyl transferase
GRP	:	Gastrin releasing peptide
HIOMT	:	Hydroxyl indole-*O*-methyl transferase
ICER	:	Inducible cAMP early repressor
IEG	:	Immediate early gene
IGL	:	Intergeniculate leaflet
IL-4	:	Interleukin 4
kDa	:	Kilodalton
LD cycle	:	Light-dark cycle
MAO	:	Monoamine oxidase
MRN	:	Median raphe nuclei
mg	:	milligram
ml	:	milliliter
mM	:	millimolar
mRNA	:	messenger ribonucleic acid
NAAC	:	Neutral amino acid carrier
NAD^+/NADH	:	Nicotinaminde adenine dinucleotide oxidised/reduced
NAT	:	*N*-acetyl transeferase
NE	:	Norepinephrine
nm	:	nanometers
NMDA	:	*N*-methyl *D*-aspartate
NPY	:	Neuropeptide Y
PACAP	:	Pituitary adenylate cyclase activating polypeptide
PAGE	:	Polyacrylamide gel electrophoresis
PAS	:	PER-ARNT-Single minded
P-CREB	:	Phosphorylated cAMP-responsive element binding protein
Per	:	*Period*
PHI	:	Peptide histidine isoleucine
PKA	:	Protein kinase A
PKC	:	Protein kinase C
PNS	:	Peripheral nervous system
PSD	:	Post synaptic density
PVN	:	Paraventricular nucleus
PVZ	:	Paraventricular zone
RGCs	:	Retinal ganglion cells
RHT	:	Retino hypothalamic tract
RP-HPLC	:	Reverse phase high pressure liquid chromatography
SBP	:	Serotonin binding protein
SCG	:	Superior cervical ganglia

SCN	:	Suprachiasmatic nucleus
SDS	:	Sodium dodecyl sulphate
SERT	:	Transporter of serotonin
SP	:	Substance P
SS	:	Somatostatin S
TEMED	:	*N, N,N□,N□*-Tetramethylethylenediamine
TGFα	:	Transforming growth factor
TPH	:	Tryptophan hydroxylase
TRH	:	Thyrotropin releasing hormone
Tris	:	Tris-(Hydroxymethyl) aminoethane
VIP	:	Vasoactive intestinal peptide
Vmat 2	:	Vesicular monoamine transporter 2
µl	:	micro litre
µM	:	micro molar
^{0}C	:	degree centigrade/ degree celsius