ABSTRACT

Sarath Vijayakumar. THE ROLE OF MEDIATORS OF NEURONAL PLASTICITY IN THE CIRCADIAN REGULATION OF SUPRACHIASMATIC NUCLEUS BY LIGHT. (Under the direction of Dr. Jian Ding) Department of Physiology, August 27, 2009.

Circadian rhythms are highly conserved physiological functions that are present in almost all living organisms. In mammals, circadian rhythms are synchronized to the environmental light:dark cycle by daily adjustments in the hypothalamic suprachiasmatic nucleus (SCN), the location of the master circadian pacemaker. We hypothesize that light entrainment of the circadian clock involves neural plastic adaptations in the SCN. The mechanism of neural plasticity has been intensively studied in the hippocampus and the dentate gyrus. However, the cell and molecular mechanism underlying circadian clock resetting in the SCN remains poorly understood. Thus, we sought to investigate whether modulators that are known to regulate neural plasticity in the hippocampus play a role in the signal transduction of circadian clock resetting. Light induced expression of tissue-type plasminogen activator (tPA) in the SCN, maximal induction was seen one hour following a light pulse at circadian time (CT) 16. A corresponding increase in the tPA proteolytic activity was also observed. tPA-STOP™ (an inhibitor of tPA)-infused animals exhibited attenuated light-induced phase delay of circadian wheel running activity. The levels of cyclin-dependent kinase 5 activators, p35 and p25 were decreased at *Zeitgeber Time* (ZT) 16, at ZT22 the levels were increased whereas, no change was observed at ZT6. The bi-transgenic animal, CK-p25 demonstrated increased phase delay at CT16 and attenuated phase advance at CT22 following transient overexpression of p25. SCN neurons expressing p25 co-localized with phosphorylated-extracellular signal-regulated kinase and Gastrin Releasing Peptide. This is one of the first studies to report the involvement of these neuromodulators in circadian light entrainment. Mounting evidence shows that circadian rhythm

disturbances may be associated with increased health risks, such as jet-lag, cancer development, cardiovascular and metabolic disorders. The findings of this study have improved our understanding of the complex and intricate pathways involved in light entrainment and may lead to development of novel therapeutic avenues in treating circadian rhythm disturbances.

THE ROLE OF MEDIATORS OF NEURONAL PLASTICITY IN THE CIRCADIAN REGULATION OF SUPRACHIASMATIC NUCLEUS BY LIGHT

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DEDICATION

I dedicate this dissertation to my wonderful family:

To my beloved mother, Lekha Vijayakumar, for the unwavering love and moral support that she has given me all these years;

To my respected father, Krishnaru Vijayakumar, who instilled the importance of hard work and perseverance;

To my loving sisters, Drs. Sapna Vijayakumar and Sangeetha Vijayakumar, who persuaded me to come to the United States for advancing my education;

To the memory of my grandmother, HH Uzhithiraru Sreedevi, who believed in the pursuit of academic excellence.

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LIST OF ABBREVIATIONS

3V	third ventricle
AC	adenylyl cyclase
AMPA	alpha-amino-3-hydroxy-r-methyl-4-isoxazoleprrionic acid
AVP	arginine vasopressin
BAD	brain activity dependent
BDNF	brain-derived neurotrophic factor
Bmal1	brain and muscle aryl-hydrocarbon receptor nuclear translocator-like 1
CaMKII	calcium calmodulin-dependent protein kinase II
Cdk5	cyclin-dependent kinase 5
cGMP	cyclic guanosine monophosphate
Clock	circadian locomotor output cycles kaput
CREB	cyclic-AMP response element binding
***Cry* 1, 2**	cryptochrome 1, 2
CT	circadian Time
DAB	3,3'-diaminobenzidine
DMH	dorso medial hypothalamus
ECM	extracellular matrix
EDTA	ethylene diamine tetra acetate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC	guanylyl cyclase
GHT	geniculo-hypothalamic tract

Glu	glutamate
GRIP	glutamate receptor-interacting protein
GRP	gastrin releasing peptide
IGL	inter-geniculate leaflet
L:D	Light:Dark
LRP	low density lipoprotein receptor-related protein
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
mGLUR1	metabotropic glutamate receptor group 1
Na_3VO_4	sodium orthovanadate
NGF	nerve growth factor
NHS	normal horse serum
NMDA	N-methyl-D-aspartate
nNOS	neuronal nitric oxide synthase
Npas2	neuronal PAS domain protein 2
OC	optic chiasm
PACAP	pituitary adenylate cyclase activating peptide
PBS	phosphate buffered saline
Per 1, 2	period 1, 2
p-ERK	phosphorylated extracellular signal-regulated kinase
PKA	protein kinase A
PKG	protein kinase G
PMSF	phenylmethanesulphonyl fluoride

PVDF	polyvinylidene fluoride
PVN	para ventricular nucleus
rev-erbα	reverse erythroblastosis virus α
RGC	retinal ganglion cell
RHT	retino-hypothalamic tract
ROR	retinoic-acid receptor related orphan receptor
SCN	suprachiasmatic nucleus
sPVZ	sub paraventricular zone
TBST	tris buffered saline-triton x
tPA	tissue-type plasminogen activator
TrkB	tyrosine receptor kinase B
VIP	vasoactive intestinal polypeptide
ZT	*Zeitgeber Time*

CHAPTER 1 – INTRODUCTION

"Mechanism is not the biologist's only business. He is, or should be, concerned also with questions of both function and history" – **Colin Pittendrigh (1966)**

Circadian Rhythms

Most organisms live in an environment that is changing continuously in a rhythmic manner based on Earth's rotation around its axis. Organisms exhibit daily changes in physiology and behavior based on this astronomical phenomenon (Hastings et al., 2003; Pittendrigh, 1993). These daily changes that occur with a period of around 24 hr are termed circadian from Latin "*circa diem*" meaning "about a day". Circadian rhythms have evolved in most species, ranging from single cell organisms to plants and animals. The cyanobacterium *Synechococcus,* shows circadian rhythmicity in its photosynthetic machinery (Dunlap et al., 2004; Harmer et al., 2001), while the dinoflagellate *Lingulodinium* shows bioluminescent rhythmicity (Dunlap et al., 2004; Lowrey and Takahashi, 2004). In mammals, circadian rhythmicity is seen in the sleep-wake cycle, cardiovascular activity, hormonal secretions, body temperature, hepatic metabolism, gastro-intestinal physiology and many other metabolic and behavioral processes. Circadian rhythms persist with intrinsic periods close to 24 hr even in the absence of any external time cues, indicating the presence of an internal time keeping system or "circadian clock" in every organism. Circadian rhythms expressed in the absence of external cues are defined as "free-running". The "free-running" rhythms often

deviate from 24 hr and need to be adjusted to the environment by external signals, called *Zeitgebers.* This adjustment process is called entrainment. The most potent *Zeitgeber* for animals is the environmental light-dark cycle.

The "circadian clock" has undergone evolutionary changes becoming more complex in higher species (Herzog, 2007). In the marine snail, *Bulla gouldiana*, approximately 100 basal retinal neurons in the eye act as the circadian pacemaker coordinating its daily locomotor activity (Block and Wallace, 1982). In the fruit fly, *Drosophila melanogaster*, a group of neurons located in the lateral and dorsal brain drives rhythms in locomotion, photophobicity and eclosion (Helfrich-Forster, 2005; Stoleru et al., 2004). In birds, for instance, the sparrow, extra-retinal photoreceptors within the brain acts as the "circadian clock" (Menaker, 1968).

The Suprachiasmatic Nucleus

In mammals, the master circadian pacemaker is located in the hypothalamic suprachiasmatic nucleus (SCN). Surgical ablation of the SCN results in a loss of circadian rhythms of locomotor activity, drinking behavior and corticosterone release (Moore and Eichler, 1972; Stephan and Zucker, 1972a, b). Allograft transplant of fetal SCN tissue to SCN-lesioned hosts was able to restore their locomotor activity (Lehman et al., 1987). When fetal SCN taken from mutant hamsters with a 20 hour period was transplanted to SCN-lesioned wild type host with 24 hour period, the restored rhythm of the wild type host became 20 hour circadian period. These studies suggest that the SCN is the master circadian pacemaker that determines the overall circadian rhythm of the

animal (Ralph et al., 1990). All these experiments clearly demonstrate the importance of the SCN in orchestrating circadian rhythms.

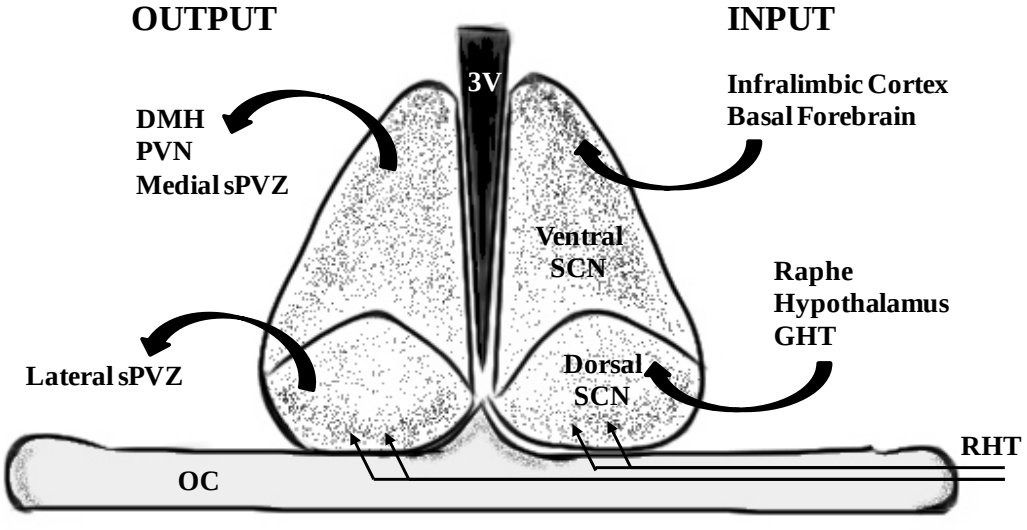
SCN Input and Output Pathways

The SCN is a paired nucleus consisting of approximately 10,000 neurons each and is located on either side of the third ventricle above the optic chiasm in the hypothalamus (Abrahamson and Moore, 2001; Van den Pol, 1980). The SCN receives input from many brain regions (Fig. 1). The main input is via the retino-hypothalamic tract (RHT), a monosynaptic pathway originating from an unique set of non-image forming retinal ganglions cells (RGCs) that express the photopigment melanopsin and are intrinsically photo sensitive (Berson et al., 2002; Johnson et al., 1988; Moore and Lenn, 1972). Glutamate (Glu) and the neuropeptide Pituitary Adenylyl Cyclase Activating Peptide (PACAP) are the principal neurotransmitters released from the RHT terminals to the SCN (Ebling, 1996; Hannibal, 2002; Hannibal et al., 1997). SCN also receives non photic input via the geniculo-hypothalamic tract that projects from the intergeniculate leaflet (IGL) carrying neuropeptide Y, and projections from the mesencephalic raphé nuclei that are serotonergic. These pathways project mainly to the ventral SCN (Moga and Moore, 1997; Moore and Card, 1990; van Esseveldt et al., 2000). The dorsal SCN receives input mainly from the basal forebrain and infralimbic cortex (Moga and Moore, 1997). There are several other minor pathways that project to the SCN from the hypothalamic tuberomammillary, arcuate and supraoptic nuclei (Krout et al., 2002; Morin and Blanchard, 2001; Saeb-Parsy et al., 2000).

The SCN has three major output pathways (Fig. 1). The pathway that contains the largest portion of SCN efferents runs in an arc dorsally and caudally. This pathway projects to regions surrounding the SCN, mainly the paraventricular nucleus, the subparaventricular zone, and the dorsomedial hypothalamus (Leak et al., 1999). The other two pathways are to the medial preoptic area and to the retrochiasmatic area and the capsule of the ventromedial nucleus. Additionally, the SCN has sparse projections to other brain areas, i.e., the tuberomammillary nucleus, the ventrolateral preoptic nucleus, and an indirect projection to the limbic system via the paraventricular nucleus (Abrahamson et al., 2001; Chou et al., 2002; Peng and Bentivoglio, 2004).

Figure 1: SCN input and output pathways.

The diagram depicts the major input and output pathways to and from the SCN. The ventral SCN containing VIP neurons and the dorsal SCN containing AVP neurons are outlined. DMH (dorsomedial hypothalamus), PVN (paraventricular nucleus), sPVZ (sub paraventricular zone), RHT (retino-hypothalamic tract), 3V (third ventricle), OC (optic chiasm), GHT (geniculo-hypothalamic tract). The inputs and outputs are shown on separate sides for clarity.

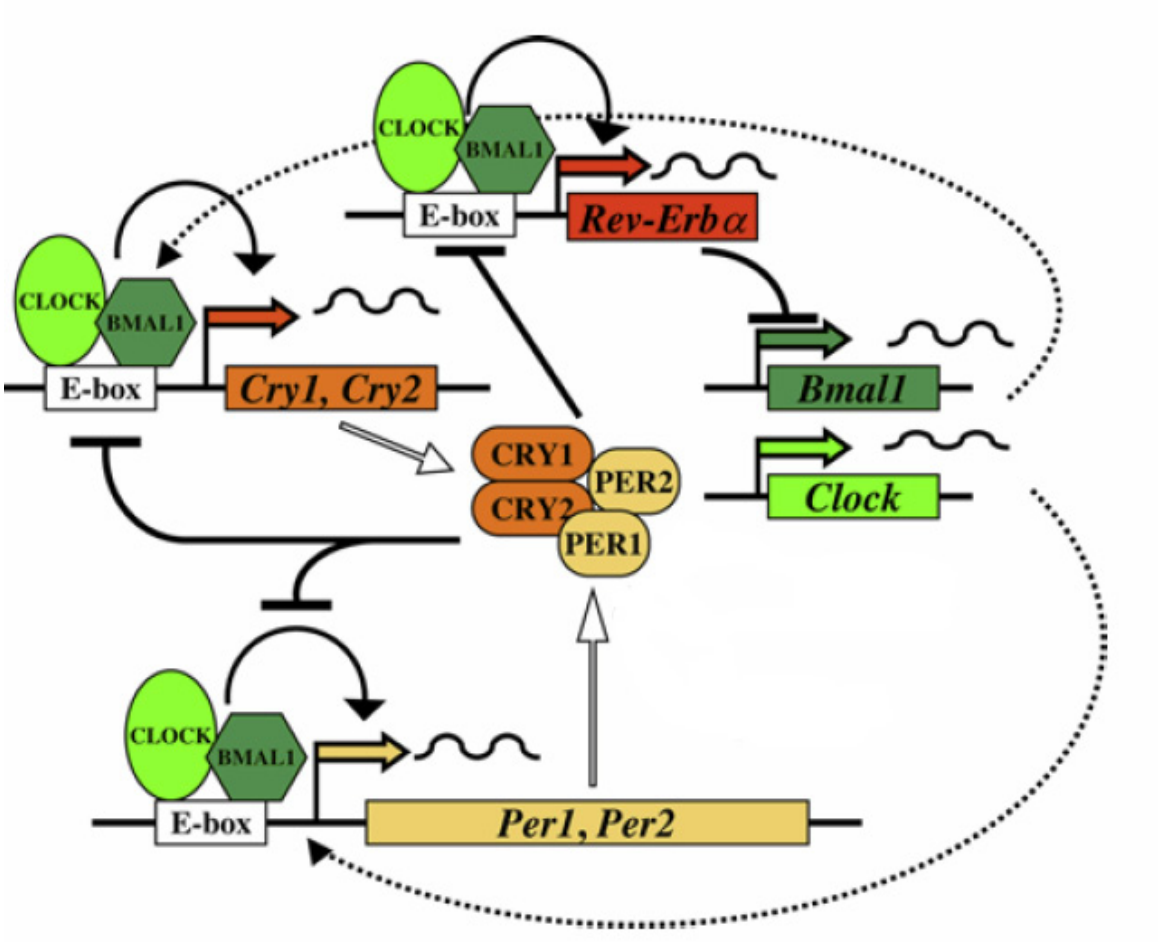

OUTPUT
INPUT
3V
DMH
PVN
Medial sPVZ
Infralimbic Cortex
Basal Forebrain
Ventral
SCN
Raphe
Hypothalamus
GHT
Lateral sPVZ
Dorsal
SCN
RHT
OC

Molecular Basis of the Mammalian Circadian Clock

At the molecular level, the circadian clock involves a cell-autonomous transcription-translation feedback loop comprising a core set of "clock genes" (Lowrey and Takahashi, 2004). In mammals, circadian locomotor output cycles kaput (Clock) and its paralogue neuronal **P**eriod-**A**ryl hydrocarbon nuclear translocator-**S**ingle-minded (PAS) domain protein 2 (Npas2), Brain and muscle aryl hydrocarbon receptor nuclear translocator-like (Bmal1), period homologue 1, 2 (Per1, Per2), and Cryptochrome 1, 2 (Cry1, Cry2) form the primary negative-feedback loop (Fig. 2). During the day, the basic helix-loop-helix transcription factors CLOCK and BMAL1 activate transcription of the Per and Cry genes. The PER and CRY proteins heterodimerize, translocate to the nucleus and inhibit their own transcription (Lee et al., 2001). During the night, the PER-CRY repressor complex is degraded and a new transcription cycle is initiated by CLOCK-BMAL1. The entire transcription-translation loop takes approximately 24 hours to complete. In addition to the primary feedback loop, there is a secondary negative-feedback loop formed by the nuclear hormone receptor, Rev-erba, a direct repressor of Bmal1 transcription. Further stabilization of the primary feedback loop is brought about by auxiliary loops involving the orphan nuclear receptor, RORα, and other clock components Dec1 and Dec2 (Ko and Takahashi, 2006; Ueda et al., 2002).

Figure 2: Simplified model of the mammalian molecular clock (Gachon et al., 2004).

Two interlocking positive and negative loops form the core of the molecular oscillator. During the day, BMAL1 forms a heterodimeric complex with CLOCK that acts via E-BOX DNA regulatory sequences to drive the expression of *Period (Per1, per2)* and *Cryptochrome (Cry1 and Cry2)* genes. PER:CRY complex accumulate during the night translocate to the nucleus and inhibit their own transcription, thus forming the negative loop. Once the PER:CRY complex level goes down after degradation, a new cycle starts all over again. CLOCK and BMAL1 also drive expression of Rev-Erbα, which in turn competes for binding elements on the *Bmal1* promotor, acting to stabilize the loop.



Modified from Gachon et al., 2004

Circadian Clock Resetting By Light – Photic Entrainment

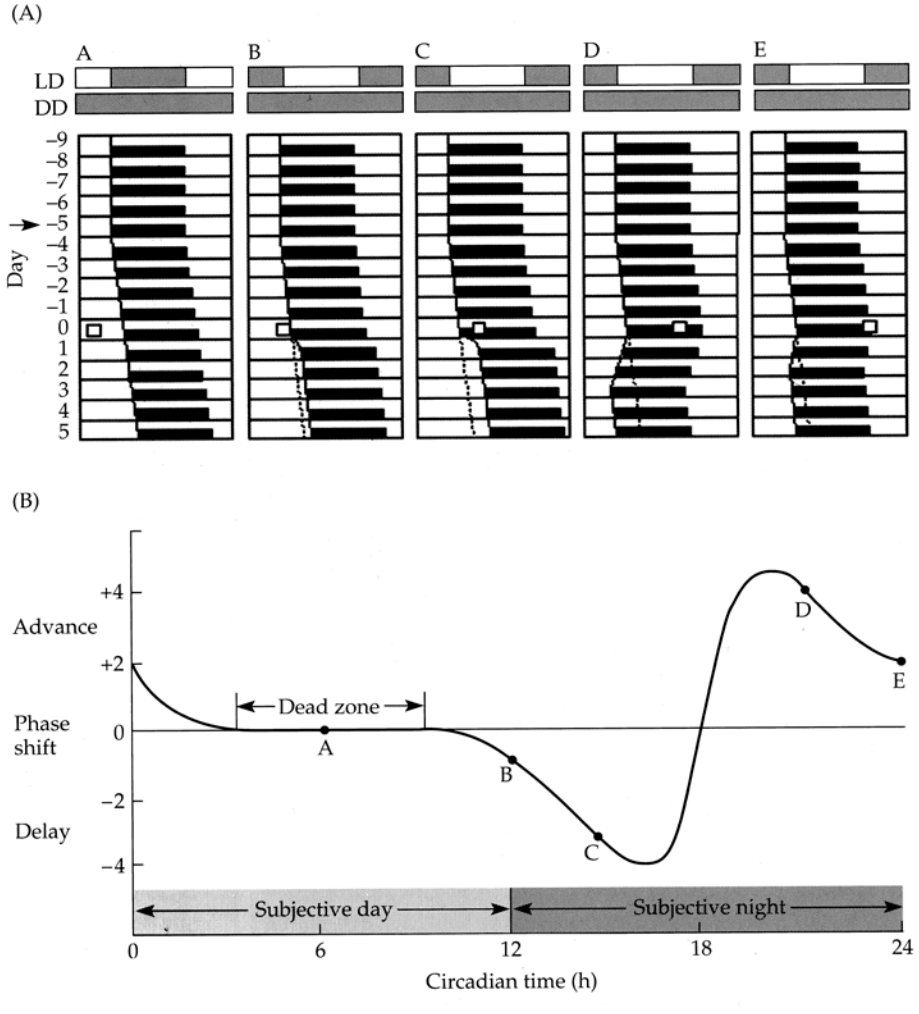
Photic entrainment of circadian rhythms occurs through daily, light-induced adjustments in the phase and period of the SCN pacemaker (Daan, 1976; Decoursey, 1964). In mammals, the photic entrainment process has been characterized mainly by studying the resetting effects of brief light exposure in nocturnal rodents. In experimental animals housed under programmed lighting schedule, the *Zeitgeber time* (ZT) is defined relative to the experimental light:dark (L:D) cycle. By definition, ZT0 is the time when the lights are on and ZT12 is the time when lights are off in the animal housing. When the animals are kept in constant conditions (usually constant darkness), they start to "free-run". This "free running" rhythm often deviates from 24 hr and shows inter-species variation. Under "free-running" conditions, time scale is expressed in *Circadian time* (CT) units (Daan, 1976). One circadian cycle is divided into 24 equally sized circadian units, one unit being defined as the division of the intrinsic "free-running" period (τ) by 24. CT0 is defined as the beginning of the subjective day (the onset of the rest phase in nocturnal rodents) and C12 as the beginning of the subjective night (the onset of the activity phase in nocturnal rodents). Under constant darkness (DD), brief light exposure during the early subjective night causes phase delays, while exposure during the late subjective night causes phase advances of the SCN circadian clock. Light exposure during the subjective day elicits either very minimal phase shift or none at all. The "phase dependence" of the response of the SCN circadian clock to light is depicted in the photic phase response curve (PRC; Fig. 3B) (Daan, 1976; De Coursey, 1960). The quantitative aspects of the photic PRC: the relative amplitude and duration of the phase delay and

advance regions of the PRC curve vary widely among species (Fig. 4) and is also influenced by the photoperiod (Rusak and Zucker, 1979; Travnickova et al., 1996). Typically, animals with intrinsic period τ less than 24 hr show large amplitude phase delays and smaller phase advances. Conversely, animals with τ greater than 24 hr show large phase advances and smaller phase delays.

Figure 3: Photic phase response curves and their derivation (Dunlap et al., 2004).

(A) Light pulse is applied to free-running animal at different circadian phase points to derive the phase response curve. The horizontal lines in A-E, represent the daily activity of the animal maintained in constant darkness over a 15 day period. On day zero, the animals are briefly exposed to light (square, “light pulse”). The time difference between the dotted line extrapolating the trajectory of the animal’s activity prior to the light pulse and the actual onset of activity after the light pulse is known as a phase shift.

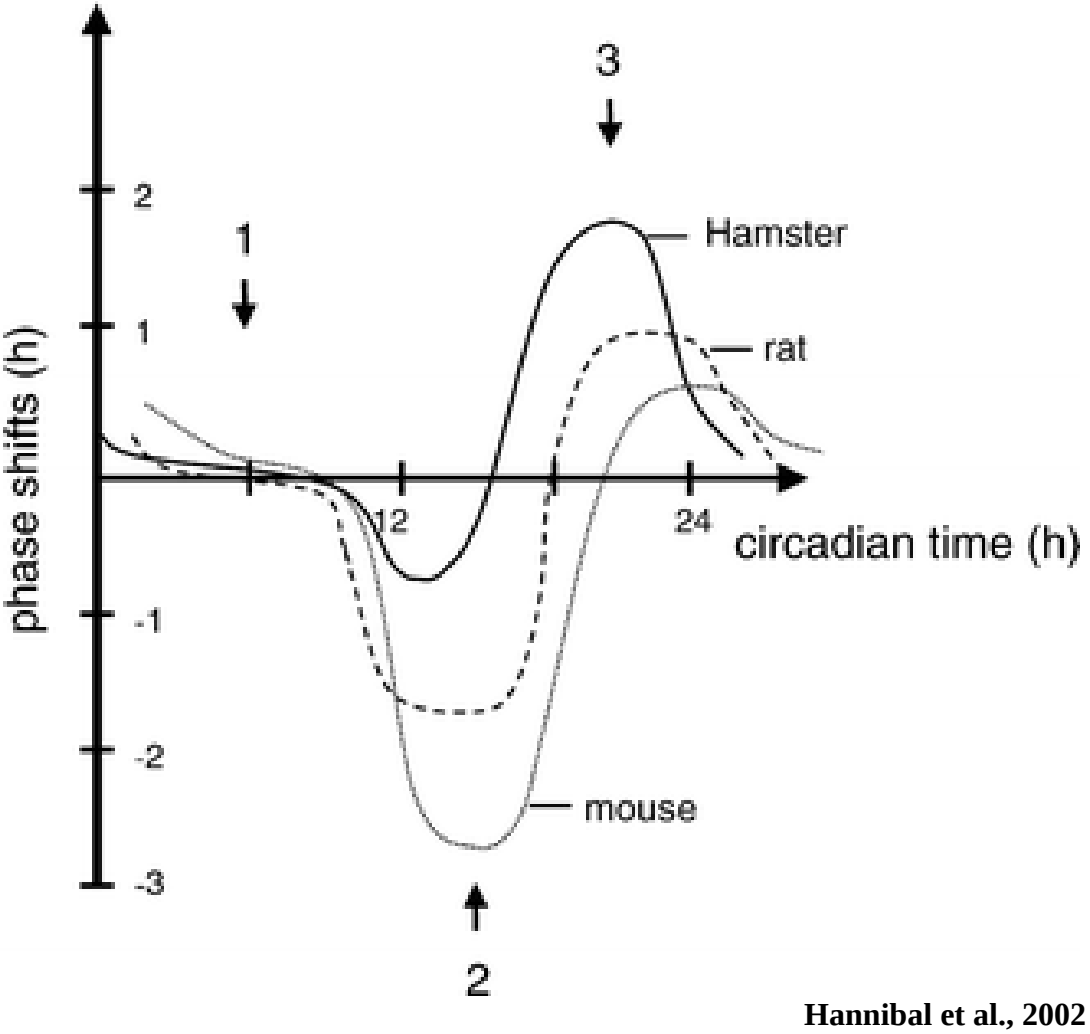
(B) Phase response curve for the animal in A. The sinusoidal phase shift curve plotted, as a function of circadian time, is known as the PRC. Point A represents a time of the animal’s subjective day during the light-insensitive period and is referred to as the “dead zone”. Points C and D are examples of a phase delay and advance, respectively.



Dunlap et al., 2004

Figure 4: Species difference in photic phase response curve (Hannibal et al., 2002).

Depiction of complete photic phase response curve of three different species, mouse, rat, and hamster. Phase delays are plotted in negative direction and phase advances in the positive direction. The horizontal axis represents 24 hr period. Typically, animals with intrinsic period τ less than 24 hr show large amplitude phase delays and smaller phase advances. On the other hand, animals with τ greater than 24 hr show large phase advances and smaller phase delays. Mouse with τ less than 24 hr shows a greater amplitude phase delay and a small amplitude phase advance. Hamster with τ more than 24 hr shows a greater phase advance than phase delay. τ (tau/ "free-running" period) 1, 2 and 3 represent subjective day, subjective early night and subjective late night respectively.



Hannibal et al., 2002

Mechanism of Photic Resetting of the Circadian Clock

Although the phenomenon of photic resetting was discovered in 1960 (De Coursey, 1960), the exact mechanism responsible for the phase dependent photic response of the SCN circadian clock remains elusive. Considerable research done in the last nearly 50 years has improved understanding of this phenomenon. Several signal transduction pathways have been proposed (Fig. 5), including the phosphorylation of transcription factor CREB (Gau et al., 2002; Ginty et al., 1993), the activation of cGMP and Ca^{2+}/calmodulin kinase II (CaMKII) (Gillette, 1996; Ginty et al., 1993; Mathur et al., 1996; Tischkau et al., 2003a; Tischkau et al., 2003b). Several other kinases, including mitogen-activated kinase (MAPK) (Akashi et al., 2008; Akashi and Nishida, 2000; Cao et al., 2008; Dziema et al., 2003; Obrietan et al., 1998) and Ca^{2+}/calmodulin kinase (CaMK) (Wu et al., 2001) have also been implicated in the activation of CREB in the SCN.

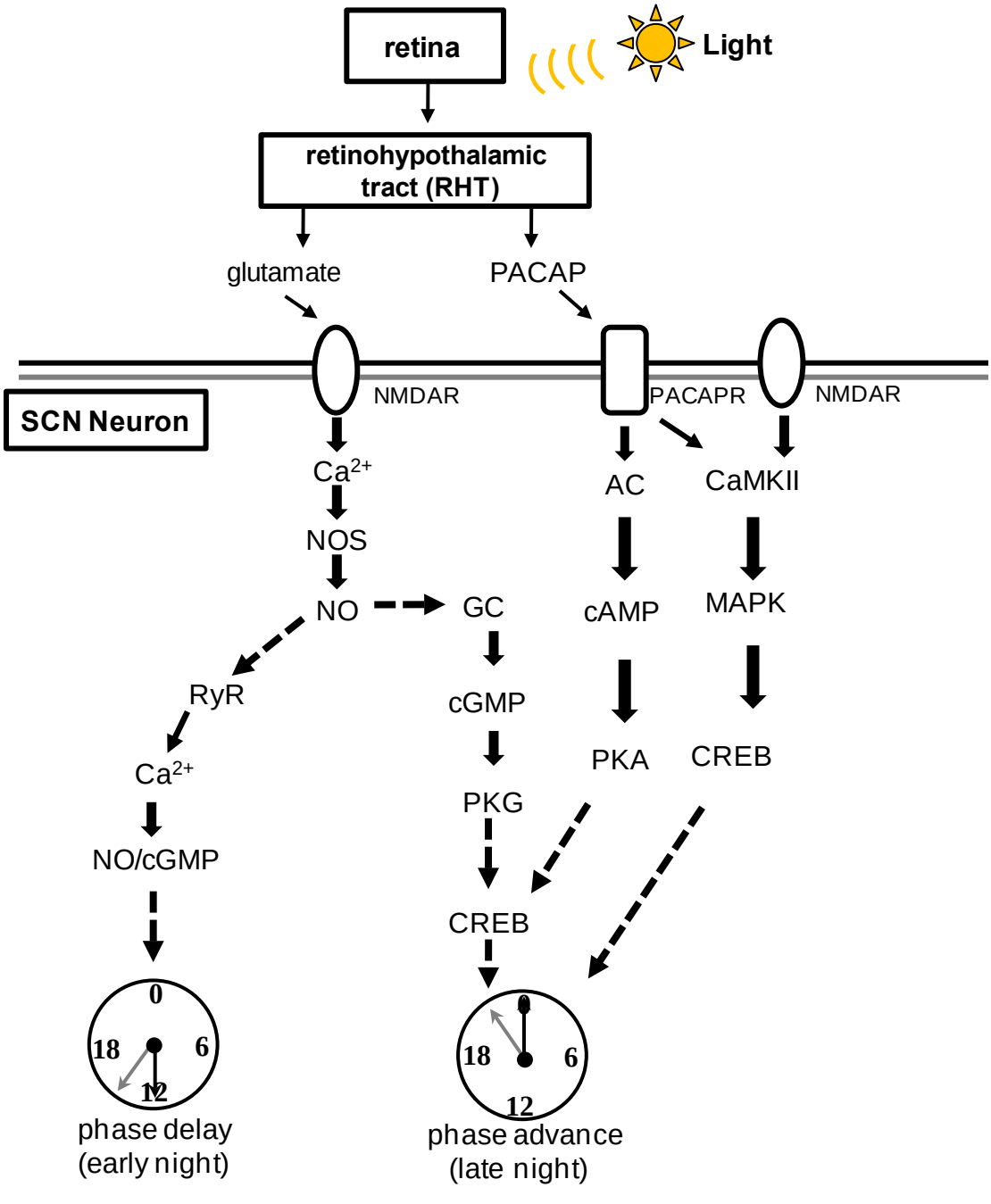
Upon photic stimulation, Glu is released in the RHT initiating a series of signal transduction cascades in the SCN that ultimately result in a phase shift of the circadian clock (Ding et al., 1994; Golombek et al., 2003). Glutamate released from the RHT activates N-methyl-D-aspartate (NMDA), non-NMDA (AMPA/kinate) and metabotropic (mGluR1) receptors in the SCN (Ebling, 1996). Glutamate also induces an increase in intracellular Ca^{2+} levels in the SCN (Colwell, 2001; van den Pol et al., 1992). Although currents evoked by exogenous application of AMPA did not show a diurnal rhythm in their magnitude, there was daily rhythm in the magnitude of AMPA-induced Ca^{2+} transients that peaked during the night (Michel et al., 2002).

The increased intracellular Ca^{2+} results in the activation of a plethora of enzymes in the SCN, particularly, CamKII and neuronal nitric oxide synthase (nNOS). Activation

Figure 5: Putative photic signal transduction pathway in the SCN.

Glutamate activation of NMDA receptors in the SCN results in calcium influx that activates NOS and CaMK activity, which in turn modulates other kinases such as PKG and MAPK which lead to the phosphorylation of CREB and induction of clock genes producing phase shifts of the clock. PACAP stimulation activates several signaling pathways via cAMP/PKA and or MAPK pathways. (NOS- Nitric oxide synthase, NO-Nitric Oxide, GC- Guanylyl cyclase, cGMP-cyclic GMP, PKG-Protein kinase G, CREB-cAMP response element binding protein, RyR-ryanodine receptor, AC-Adenylyl Cyclase

cAMP-cyclic AMP, PKA-Protein kinase A, CaMKII- calmodulin-dependent kinase II. See text for details).



Adapted from Hirota & Fukada (2004)

of nNOS leads to the formation of nitric oxide (NO). Several studies have indicated the role of nNOS in circadian photic resetting (Agostino et al., 2004; Ding et al., 1994; Ferreyra et al., 1998; Melo et al., 1997; Watanabe et al., 1994). The pathway downstream of NO diverges and is thought to be responsible for inducing the bi-directional phase shifts during the night. During the late night, NO activates soluble guanylyl cyclase inducing changes in both cGMP levels and cGMP-dependent protein kinase activity (cGK) (Taishi et al., 2001; Tischkau et al., 2003b). Mechanisms downstream of cGK activation are not completely elucidated but, acting through intermediary pathways cGK activation culminates in phosphorylation of CREB and induction of PER protein expression. During early night, there occurs additional mobilization of Ca^{2+} from intracellular stores via the activation of ryanodine receptors (Ding et al., 1998). Recently, Pfeffer et al. showed that dysfunctional ryanodine calcium signaling in the BMAL1 null mutant led to an impaired light-input pathway selectively during early night (Pfeffer et al., 2009). Photic stimulation also releases the neurotransmitter PACAP in the RHT (Hannibal et al., 2000; Liu and Madsen, 1997; Yaka et al., 2002). Multiple signaling pathways are involved in the PACAP-mediated light entrainment of the SCN. Activation of the PACAP receptors by light stimulates the cAMP/PKA signaling pathway resulting in CREB phosphorylation events and phase advance of the clock (Chen et al., 1999; Tischkau et al., 2000). It also modulates gluatamatergic signaling via the ERK/MAPK pathway. NMDA receptor activation also involves CaMKII mediated ERK/MAPK pathway. MAPK stimulation activates the phosphorylation of CREB which results in the induction of clock genes (Butcher et al., 2002; Coogan and Piggins, 2003; Dziema et al.,

2003; Obrietan et al., 1998). The exact downstream targets that induce phase delay during early night remain largely unknown.

Neuronal Plasticity in Circadian Photic Resetting

Circadian rhythms have three defining characteristics: 1) The rhythms are persistent in constant conditions independent of environmental time cues. 2) The rhythms show temperature compensation, the "free-running" period of the organism remains constant over a wide-range of ambient temperatures. 3) The most important feature of circadian rhythms is their ability to be entrained by cycling environmental time cues or *Zeitgebers.* Light is the major environment cue that can reset the circadian clock. The ability of light to reset the clock allows it to maintain temporal alignment with external time cues. A short light pulse given during the sensitive circadian phase can permanently reset the phase of the circadian clock over a period of few days. Light-induced resetting of the circadian clock is a classic example of short-term environmental stimuli inducing long-lasting behavioral adaptations.

Long-term potentiation (LTP), long lasting activity-dependent increase in the size of a synaptic response, was first described by Bliss and Lømo (Bliss and Lomo, 1973). It is now considered to be the cellular mechanism underlying learning and memory. LTP has been divided into an early phase LTP (E-LTP) that lasts for 1-2 hr and involves modifications of pre-existing synapses as a result of increased Ca^{2+} flux through NMDA receptors and accompanying protein phosphorylation events (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999) and a late-phase LTP (L-LTP) that can last as long as 6-8 hrs (Frey et al., 1988). L-LTP requires activation of cAMP-dependent protein kinase

and the transcription factor CREB (Kandel, 2001). It is also dependent on new protein synthesis (Fales et al., 1919; Frey et al., 1988). Tetanic stimulation of the hippocampus that induces L-LTP has been shown to enhance the expression of several proteins (Matsuo et al., 2000; Qian et al., 1993).

Although the exact protein synthesis responsible for the induction and maintenance of L-LTP remains elusive, a few have emerged as the likely candidates, notable among these being: brain-derived neurotrophic factor (BDNF), tissue-type Plasminogen Activator (tPA), Calpain and Cyclin-dependent kinase 5 (Cdk5). BDNF has emerged as a critical secretory protein that regulates synaptic development and plasticity in the CNS (Lu, 2003; Poo, 2001). tPA is another target gene being considered. Seizure, kindling and paradigms that induce L-LTP all cause an enhancement of tPA mRNA expression in the hippocampus (Qian et al., 1993). tPA mutant mice have been shown to exhibit a selective impairment in L-LTP (Calabresi et al., 2000; Frey et al., 1996; Huang et al., 1996). Calpain, a Ca^{2+} dependent cysteine protease has also been implicated in L-LTP process. Calcium influx following titanic stimulation activates calpain that in turn cleaves fodrin, allowing translocation of Glu receptors (Lynch and Baudry, 1984). Several other mechanisms have also been suggested including the degradation of Glu receptor-interacting protein (GRIP) (Lu et al., 2001). Cdk5 is a proline-directed serine/threonine kinase that has been implicated in various neuronal functions, including learning, memory and neurodegenerative disorders (Dhavan and Tsai, 2001). A transient increase in Cdk5 activity was shown to enhance LTP, whereas, a prolonged hyperactivity resulted in learning and memory deficit (Fischer et al., 2005).

The role of the neurotrophin, BDNF has been extensively studied in the past decade. BDNF and its cognate receptor, tyrosine receptor kinase B (TrkB) are expressed in the SCN (Allen and Earnest, 2005; Liang et al., 1998a). Not only is BDNF expressed in SCN, the expression also exhibits a circadian rhythmicity with peak protein levels occurring at night (Liang *et al.*, 1998b). BDNF can also reset the circadian rhythm of SCN neuronal activity in brain slice preparation by modulating NMDA and AMPA currents in the SCN neurons (Michel *et al.*, 2006). Little is known about the potential role in the SCN and circadian resetting of other neuromodulators of plasticity. Drawing similarities in the function of the hippocampus and the SCN, it is reasonable to assume that the neuromodulators implicated in the neuronal plasticity in the hippocampus might play a similar role in the SCN.

Objective of Current Study

The main objective of this study is to investigate the role of modulators of neuronal plasticity in resetting the circadian clock. To achieve this objective, the following specific aims are devised:

1) Investigate the role of extracellular serine protease tPA in the modulation of circadian clock resetting.
2) Investigate the role of Cdk5 and its activators in circadian clock resetting.
3) Investigate the role of cysteine protease, calpain in the photic resetting of the circadian clock.

CHAPTER 2 – METHODS

Animals

All animal treatments conformed to the standards in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the East Carolina University Institutional Animal Care and Use Committee.

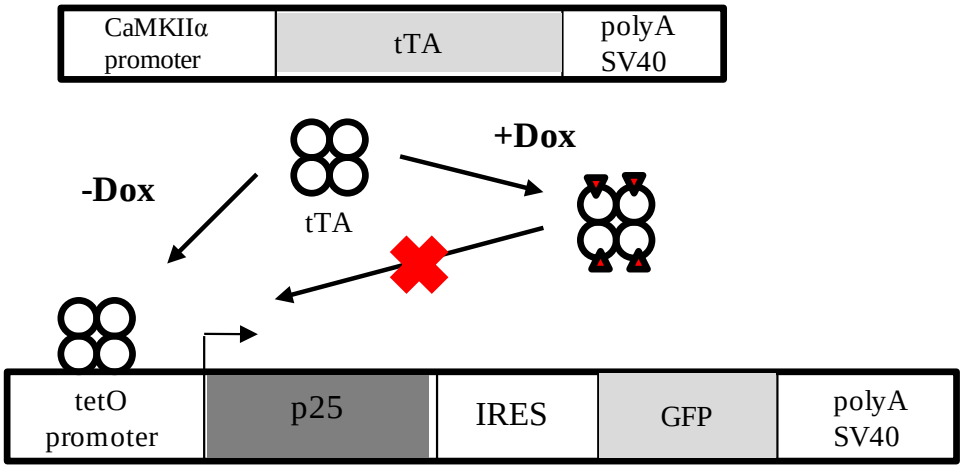
C57BL/6: Adult male mice, 6-8 weeks of age were purchased from either The Jackson Laboratory (Bar Harbor, ME; Stock No. 000664) or from Harlan Laboratories (Indianapolis, IN). Mice were housed under standard conditions with a 12:12 hr Light:Dark cycle (Lights On – 7:30 AM, Lights Off- 7:30 PM) with free access to food and water *ad libitum.* Transgenic Mice: Breeding pairs of C57BL/6-Tg(tetO-CDK5R1/GFP)337Lht/J (Stock No: 005706) and B6.Cg-Tg(Camk2a-tTA)1Mmay/DboJ (Stock No: 007004) were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were conceived and raised on a doxycycline containing diet (1mg/g, Bio-Serv, Frenchtown, NJ) to keep the transgene repressed. The animals were switched to a normal diet for 2 weeks to induce the expression of the transgene. All transgenic transgenes were heterozygous. Wild type littermates were used as controls. The bi-transgenic mice will be referred to as CK-p25. In the inducible CK-p25 mice, the expression of green fluorescent protein (GFP) tagged p25 is mediated by the CaMKIIα promoter regulated tet-off system. p25 expression is repressed in the presence of tetracycline analog, doxycyline. Robust p25 expression and elevated hippocampal Cdk5 activity is observed after 1-2 weeks of induction (Fischer et al., 2005). A schematic of the tetracycline-inducible p25 transgene is given in Fig. 6.

Genotyping

Routine genotyping of transgenic mice offspring was done from tail snips using REDExtract-N-Amp Tissue PCR Kit (Sigma, St. Louis, MO). DNA extraction was done by incubating the tail snips in a 4:1 mixture of extraction solution and tissue preparation solution from the kit first at 55°C for 10 min and then at 95°C for 3 min. 100 µl of neutralization solution B was added to sample and mixed by gentle vortexing. The neutralized tissue extract was immediately used for PCR reaction. The PCR reaction sample contained 10 µl of REDExtract-N-Amp PCR reaction mix, 4 µl of tissue extract, 2 µl of forward and reverse primers (final concentration of 0.1M) and 2 µl of PCR grade water. PCR was performed on tail genomic DNA for 35 cycles of 95°C at 1 min, 55°C at 1 min, and 72°C at 1 min on Eppendorf MasterCycler gradient (Eppendorf, Westbury, NY). The following PCR primers were used: 5'-AAGTTCATCTGCACCACCG-3', 5'- TCCTTGAAGAAGATGGTGCG-3' forward and reverse primers for p25-GFP transgene and 5′-GTGATTAACAGCGCATTAGAGC-3′ and 5′-GAAGGCTGGCTCTGACCTTGGTG-3′ as forward and reverse primers for CAMKII-tTA transgene. 15 µl of the PCR reaction product was loaded on 2.5% agarose gel, electrophoresis was done at 75 V for 40 min. The bands on gel were visualized using Alpha Imager (Alpha Innotech, San Leandro, CA).

Figure 6: Schematic of tetracycline-inducible p25 transgene.

The bi-transgenic mice (CK-p25) contains two trangenes: GFP tagged p25 and CaMKIIα-tTA, tetracycline regulated promoter. In the absence of tetracycline, CK-p25 mice express p25 in a tissue specific manner. The transgene is switched on and off by switching the diet between normal rodent chow and a doxycycline based diet. (tTA-tetracycline transactivator, IRES – internal ribosome entry site, Dox- doxycycline, GFP – green fluorescent protein).


CaMKIIα promoter
tTA
polyA SV40
+Dox
-Dox
tTA
tetO promoter
p25
IRES
GFP
polyA SV40

Western blotting

On the day of the experiment, mice, having been entrained to a 12 hour L:D cycle for 10 days, were given a light pulse (~ 100 lux) starting at *Zeitgeber Time* (ZT) 16. At 20 minute intervals, animals were deeply anesthetized (ketamine/xylazine, 18/2 mg/kg, *i.p*) and decapitated with a guillotine. Control animals received no light pulse and their SCNs were collected under dim red-light (less than 5 lux). The brain was dissected out and SCN-containing coronal hypothalamic brain sections (500 µm) were prepared using a customized tissue slicer. The sections were immediately frozen on to a glass slide over dry ice. A stainless steel needle (400 µm inner diameter) was then used to punch out both SCN from each brain section. For each experimental condition, SCNs from 4 animals were collected, pooled and stored at -80°C until use. SCN tissues were homogenized in a small volume of modified radio immunoprecipitation assay buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 µg/ml each Aprotinin, Leupeptin, Pepstatin, 1 mM Na_3VO_4, and 1 mM NaF) centrifuged at 8000 x g for 10min at 4°C, then the supernatant was collected and stored at -80° C until the extracts were processed for Western blotting. Total protein content in the lysate was quantified using a Bio-Rad DC protein assay kit following the manufacturer's protocol. 50 µg of protein lysate was loaded onto 4-12 % bis tris gel, subjected to electrophoresis and transblotted onto PVDF membrane. After transfer, membranes were washed with 0.5 % TBST, blocked with 5% powdered non-fat dry milk in TBST and then incubated overnight at 4°C with the primary antibody, rabbit polyclonal anti-tPA (1:1000). After incubation with primary antibody, membranes were washed with TBST and incubated with horseradish peroxidase-conjugated anti-rabbit secondary (1:5000). The ECL Plus detection

system (Amersham BioSciences) was used for visualization of the signal. Blots were quantified using UNSCAN-IT quantitative densitometry analysis software (Silk Corporation, USA).

Behavioral Experiments

Wheel Running Activity for tPA Study

Male mice (8-10 weeks old), were anesthetized (ketamine/xylazine, 18/2 mg/kg, *i.p.*) and stereotaxically implanted with a guide cannuli (Plastic One, 22-gauge with 28-gauge stylet, the stereotaxic co-ordinates were AP 0.4 mm, LM 0.0 mm, DV 4.5 mm). Post-cannulation, mice were individually housed in polypropylene cages equipped with 4 ½ -inch running wheels for at least 10 days in 12 hour L:D cycle to recover before being put into constant darkness cycle (DD). Wheel running activity was monitored with Vital View 3.11 data acquisition software (Minimitter, Sunriver, OR) in 5- minute bins on an Intel Pentium computer. The injection times (CT16, CT22) were calculated for free running animals by adding 4 hours to the calculated CT12 time derived from a regression fit line of onset of activity, considering 7 days prior to the treatment day. The inhibitor, tPA-STOP™ (200 μM) or saline was injected into the third ventricle of restrained mice over a period of 2 minutes by removing the stylet and inserting a 28-gauge injector attached to a microsyringe. The procedure was performed in the dark with the aid of infrared night-vision goggles (Night Owl, CA). After 10 days in DD following the first injection, the animals were given a second injection, this time the treatment was reversed, mice that received a first injection of tPA-STOP™ were injected with saline the second time and *vice versa*. Raw phase-shifts were calculated as the difference between two

regression fit lines of activity onsets considering 7 days prior to the treatment and 7 days after the treatment (avoiding the first two days following treatment). Raw phase-shifts were converted to circadian hours by multiplying with the factor, $\tau/24$, where τ is the calculated free-running period of the particular animal.

Wheel Running Activity for Cdk5 Study

Adult CK-p25 transgenic mice and their wild type littermates 6-8 weeks old were transferred to light proof chambers, individually housed in polypropylene cages equipped with 4 ½ -inch running wheels for at least 10 days in 12:12 hr L:D cycle before being put into constant darkness cycle (DD). The transgene was turned on and off by switching the doxycycline diet (1g/kg, Bio-Serv, Frenchtown, NJ) to normal diet. The time line for the experiment is given in Fig. 7.

Wheel running activity was monitored with ClockLab data acquisition software (Actimetrics, Wilmette. IL) in 6- minute bins on an Intel Pentium IV computer. “Free-running” mice were exposed to a 20 min, 100-lux light pulse at one of the following circadian time points: CT12, CT22, CT6. At the appropriate CT, the mouse was taken out of the light-proof chamber in complete darkness with the aid of night vision goggles. The mouse was transferred to the light exposure chamber which had lights adjusted to provide ~100 lux at cage level. After light exposure, the mouse was transferred back to the light-proof chamber. Animals were allowed to “free-run” for 10 days between the light pulse treatments. Raw phase-shifts were calculated as the difference between two regression fit lines of activity onsets considering 7 days prior to the treatment and 7 days after the treatment (avoiding the first two days following treatment). Raw phase-shifts were

converted to circadian hours by multiplying with the factor, τ/24, where τ is the calculated free-running period of the particular animal.

Immunohistochemistry

Under deep anesthesia with ketamine/xylazine (0.1 ml/10g body weight; (18mg/2mg)per kg *i.p*), mice were perfused transcardially with 40 ml of ice-cold phosphate buffered saline (PBS) pH 7.4 followed by 40 ml of 4% paraformaldehyde fixative. The brain was dissected out and post-fixed in the same fixative for 24 hr at 4°C. Coronal sections were cut using a vibratome (Vibratome 1000 Plus, St. Louis, MO) at a thickness of 30µm. The sections were washed with PBS for 10 min. After the wash, the sections were blocked with 5% Normal Horse Serum (NHS) in PBS containing 0.3% Triton X-100 (PBST) for 1 hour. Following blocking the sections were incubated in primary antibody in PBST containing 1% NHS overnight at 4°C. After incubation with primary antibody, the sections were washed with PBS for 3x10 min. The sections were then incubated with Anti-Rabbit ImmPRESS™ reagent (Vector Laboratories, Burlingame, CA) for 1 hour at 22°C. Next, the sections were washed with PBST for 3x10 min. For visualization, the sections were treated with 3, 3'-diaminobenzidine (DAB) substrate and 0.03% hydrogen peroxide. Once the desired staining intensity has developed (4-7 min), the reaction was stopped by transferring the solution to PBS. The sections were then mounted on Superfrost Plus microscopic slides (Fisher Scientific, Pittsburgh, PA) and allowed to dry overnight. Sections were cleared in CitriSolv (Fisher Scientific, Pittsburgh, PA) 2x10 min each and coverslipped with DPX Mountant (Sigma-Aldrich, St. Louis, MO). Images were captured using Olympus DP20 digital camera on

an Olympus BX51 microscope (Olympus America, Center Valley, PA). Negative control (no primary antibody) was included in the staining procedure to validate the specificity of staining. List of primary antibodies used is given in Table 1.

Immunofluorescence

Immunofluorescence staining was done in the same way as described under immunohistochemistry. The only difference was fluorescent labeled secondary antibodies were used. Confocal microscopy was done using Zeiss LSM510 confocal microscope.

Electron Microscopy

Brains from transgenic animals with and without the induction of the transgene were collected as described under immunohistochemistry method. They were postfixed in 2% glutaraldehyde fixative for 2 hrs, trimmed to 10 mm^2 cube containing the SCN using a sharp scalpel and further postfixed in 4% paraformaldehyde fixative for 24 hr at 4°C. The cubes were trimmed further to 5 mm^2 cubes, washed in 0.1M cacodylate buffer 3 times 10 min each. After the wash they were fixed in 1% osmium tetraoxide for 1 hr rinsed with 0.1 M phosphate buffer for 10 min and stained en-block with 1% uranyl acetate. The SCN cubes were then dehydrated in graded series of ethanol (25, 50, 75, 95, 100 and 100%) for 15 min each. Embedding was done in increasing concentrations of Spurr's media in ethanol – 30% for 30 min, 70% for 1 hr, 100% for 2 hr, 100% for 30 min and finally in 100% media in a flat tissue tear-away container. The block was trimmed to 2 mm^2 cube and ultrathin silver-gray sections (80nm) were cut using Leica EM UC6 ultramicrotome (Leica, Germany), collected on 200 mesh copper grids.

Table 1: Primary antibodies used for immunohistochemistry, immunofluoresece and Western blotting.

Primary Antibody	Concentration
tPA **(American Diagnostica)**	1:500
Cdk5 (C-18) **(Santacruz Biotechnology)**	1:500
P35 (C-19) **(Santacruz Biotechnology)**	1:500
Calpain-1 (domain IV) **(Abcam)**	1:1000
p-ERK (anti-phospho42/44 MAPK) **(Cell Signaling Technology)**	1:400
GRP (FL-148) **(Santacruz Biotechnology)**	1:500

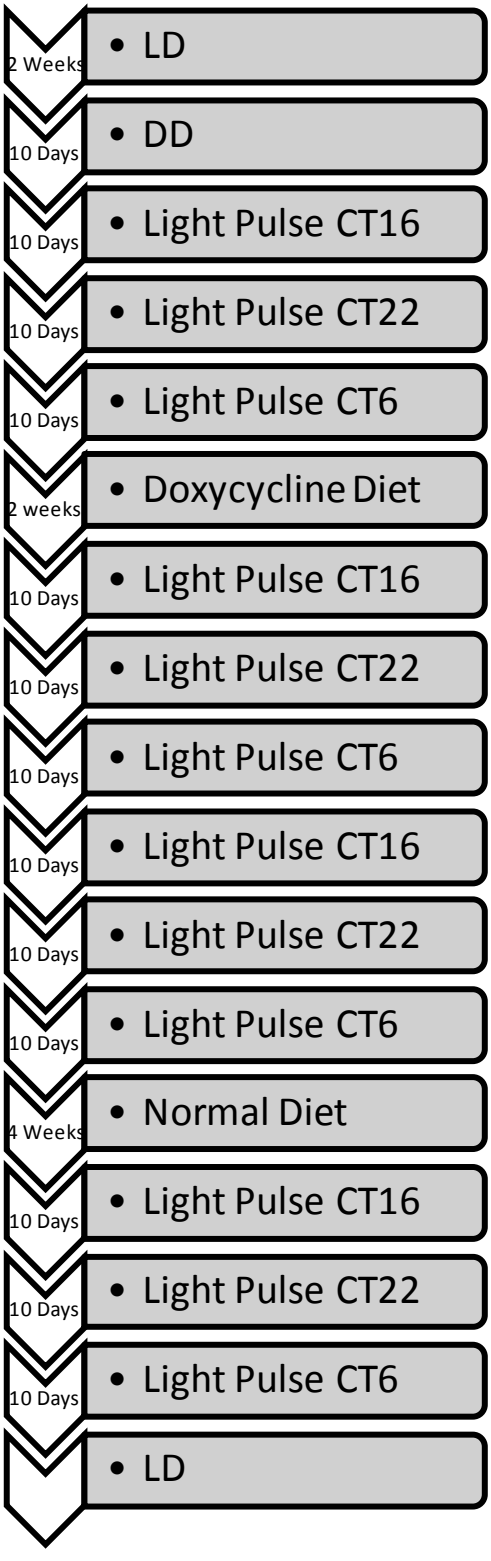
Sections were viewed with a JEOL 1200 EX electron microscope (Japan Electron Optics Laboratory Co., Japan) operated at 60 kV accelerating voltage. Images were captured using a SIS MegaView III CCD camera. Synaptic counting was done as described by (Knott et al., 2002). Briefly, serial electron micrographs were arranged in sequence and sampling rectangle was drawn on each micrograph. Synapses were counted within this sample rectangle. A synapse was identified as a region of synaptic density with at least 3 identifiable vesicles. Synapses that crossed the right and lower sides of the sampling rectangle were included in the count, those that crossed the left and upper sides were excluded.

Kinase Activity Assay for Cdk5

The kinase activity assay was performed according to Glass and Krebs (1982) using the Cdk5-specific peptide substrate (Peninsula Labs). The animals were exposed to a light pulse at ZT12 (100 lux, 15 min) and the SCN was rapidly dissected and snap frozen in liquid nitrogen and stored at -80°C until further processed. The SCN tissue was sonicated for 10 sec in 50 µl buffer containing glycerophosphate 10 mM and a cocktail of protease inhibitors (phenylmethylsulfonyl fluoride (100 µM), phenanthroline (10 mM), leupeptin (2 µM), pepstatin (0.2 µM), and aprotinin (1%) (Sigma)), BSA (0.1 mg/ml), KT5720 (protein kinase A inhibitor, 0.1 µM), Tris (20 mM), pH 7.4, Mg^{2+} acetate (20 mM), and isobutylmethylxanthine (IBMX, 100 µM), and then centrifuged at 2000 × g for 3 min at 4°C. A 30 µl aliquot of the supernatant was taken and mixed with 15 µl of buffer containing the peptide substrate (400 µg/ml), [γ-^{32}P]ATP (1 µCi/tube), and cold ATP (14 µM), and then incubated for 2 min at 37°C. The reaction was stopped by

Figure 7: Timeline of light pulse experiment for CK-p25 mice.

Heterozygous CK-p25 mice, 6-8 weeks old, were entrained with 12:12 hr Light:Dark cycle. After 2 weeks of entrainment, the mice were released to constant darkness. Mice were kept in constant darkness until the end of the experiment. Mice were allowed to "free-run" for 10 days after every light pulse treatment. Normal rodent chow was replaced with doxycyline based diet for 2 weeks to induce p25 gene expression. To repress the gene expression, doxycycline diet was switched to normal rodent chow for 4 weeks. The experiment was terminated by putting the mice back to Light:Dark cycle.


2 Weeks
• LD
10 Days
• DD
10 Days
• Light Pulse CT16
10 Days
• Light Pulse CT22
10 Days
• Light Pulse CT6
2 weeks
• Doxycycline Diet
10 Days
• Light Pulse CT16
10 Days
• Light Pulse CT22
10 Days
• Light Pulse CT6
10 Days
• Light Pulse CT16
10 Days
• Light Pulse CT22
10 Days
• Light Pulse CT6
4 Weeks
• Normal Diet
10 Days
• Light Pulse CT16
10 Days
• Light Pulse CT22
10 Days
• Light Pulse CT6
• LD

cooling on ice and adding 10 µl of 1N HCl. A 35 µl aliquot of the reaction product was placed on filter paper (Whatman P81 disks) and air-dried. The filter paper was washed with 0.5% orthophosphoric acid until no more radioactivity appeared in the effluent. The dried filter paper was placed in a scintillation vial with 5 ml scintillation fluid and counted for 2 min. Activity is expressed as a percentage of the average difference with and without light exposure.

Calpain Activity Assay

One day before the experiment, C57BL/6 mice, previously stably entrained to a 12:12 hr L:D cycle were transferred to light proof chambers with the same light cycle, housed individually in polypropylene cages with free access to food and water *ad libitum.* Mice were divided into 6 groups – control and experimental groups for each of the three circadian time points – ZT16, ZT22 and ZT7. On the day of the experiment, mice were given a light pulse (~ 100 lux) for 20 min duration at each circadian time point mentioned above. Control mice received no light exposure. Animals were deeply anesthetized (ketamine/xylazine, 18/2 mg/kg, *i.p*) and decapitated with a guillotine. The SCN of control animal was collected under dim red-light (less than 5 lux). The brain was dissected out and SCN-containing coronal hypothalamic brain sections (500 µm) were prepared using a customized tissue slicer. The sections were immediately frozen on to a glass slide over dry ice. A stainless steel needle (400 µm inner diameter) was then used to punch out both SCN from each brain section. Cerebellum was also collected from each animal. For each experimental condition, SCN and cerebellum from 4 animals were collected, and stored at -80°C until use. The tissue was homogenized in sample buffer

(Tris-HCl 20 mM pH 7.5, EDTA 1mM, EGTA 1mM, DTT 1mM). The homogenate was centrifuged at 12,000 x g for 10 min and the supernatant was collected. Total protein content in the samples was measured using Bio-Rad DC protein assay following manufacture's protocol. Calpain activity was measured using Calpain Glo-Protease Assay kit (Promega, Madison, WI) according to the manufacture's protocol. In this assay, activity is measured with luminescence, The luminescence was measured using 20/20n luminometer (Turner BioSystems, Sunnyvale, CA). The relative luminescence was averaged over 10 sec, background-subtracted, and normalized to the amount of protein in the sample.

Dye-Quenched Fluorescent In-Situ Zymography

One day before the experiment, C57BL/6 mice, previously stably entrained to a 12:12 hr L:D cycle were transferred to light proof chambers with the same light cycle, housed individually in polypropylene cages with free access to food and water *ad libitum.* On the day of the experiment, mice were given a light pulse (~ 100 lux) starting at ZT16. At 20 minute intervals, animals were deeply anesthetized (ketamine/xylazine, 18/2 mg/kg, *i.p*) and decapitated with a guillotine. The brain was dissected out and post-fixed in ethanol:methanol fixative (3:1 by volume) for 6 hr at 4°C. After post-fixation, brains were washed in 99% ethanol (4°C) two times 10 min each. It was then trimmed to a 5 mm block and infiltrated with a mixture of Steedman's/Polyester wax (Electron Microscopy Sciences, Hatfield, PA) with increasing concentrations of the wax (50%, 75%, 95%, 100%) for 1 hr each in an oven at 37°C. The brain block was left in pure wax overnight at 37°C. Finally, the specimens were embedded in 100% polyester wax in flat

peel-away tissue embedding containers (VWR, West Chester, PA). Blocks were cut into 10 µm thick sections using HM340E rotary microtome (Microm, Germany). During cutting, the block was cooled by down-draft from dry ice in a funnel positioned 6 inches above the cutting block. Ribbon sections obtained were floated in a water bath at 39°C. The sections were mounted on Superfrost Plus microscopic slides (Fisher Scientific, Pittsburgh, PA) and air dried for 30 min. The sections were then dewaxed briefly in graded concentrations of ethanol (50%, 75%, 95%, 100%) for 1 min each. After dewaxing, sections were dried overnight at 4°C. The sections were again dewaxed in absolute alcohol at 37°C for 5 and 10 min each. The slides were air dried for 10 min between each alcohol dewaxing. Following dewaxing, the sections were hydrated in graded ethanol (95%, 75%,50%, distilled water) for 3 min each. The in-situ zymography protocol was adapted from Gawlak et al. 2009, Fredericks and Mooke, 2004 and Sappino et al. 1993. A dye-quenched fluorescent casein substrate, DQ Casein (EnzChek Protease Assay Kit, Red fluorescence, Molecular Probes/Invitrogen, Eugene, OR) was used. The substrate is heavily labeled with pH-insensitive red-fluorescent BODIPY FL which shows an increase in fluorescence once cleaved by the protease. The specimens were pre-incubated in PBS pH 7.4 at 37°C for 30 min. They were then overlaid with the fluorescent substrate, DQ casein diluted 1:100 in 1% low gelling point agarose in PBS with 0.9 mM Ca^{2+} and 1mM Mg^{2+} and 20 µl of a 4mg/ml solution of purified human plasminogen (American Diagnostica, Stamford, CT). 20 µl of the overlay mixture was applied to each section, and was spread evenly under 40 x 32 mm glass coverslip. Slides were incubated at 37°C in a humidified oven. The incubation was carried out for 3 hr. The specificity of protease activity was tested by incubating the sections with an overlay

mixture that did not contain plasminogen and one which contained tPA-STOP™. The zymograms were observed under a Leica DM4000b epi-fluorescent microscope (Leica, Germany). Pictures were taken using DFC 420C cooled CCD camera (Leica, Germany). Images were obtained using Leica Application Suite 3.3 (Leica, Germany). The exposure settings were 1.5s, gamma=1 and gain=1. For quantitation of fluorescence density, higher magnification images (400x) were captured for each SCN. Quantitation was done using NIH ImageJ (1.43e, NIH, Bethesda, MD). The images were converted to 8-bit gray-scale images (0= black;255=white). To account for background fluorescence, images were also captured from the adjacent area of the SCN.

Gray-scale density correction was done with the following formula (Clemens et al., 2005):

$$C = 255\ x\ \frac{(\sigma - \sigma_{min})}{(\sigma_{max} - \sigma_{min})}$$

where C = corrected gray-scale density, σ = average of gray-scale values within the SCN, σ_{min} = average of gray-scale values in the adjacent SCN region and σ_{max} = maximum gray-scale value.

Data Analysis and Statistics

Statistical analysis was done using the SPSS software (Version 16.0; SPSS Inc., Chicago, Illinois). Results are expressed as mean±S.E.M. Paired t-test was used to compare two groups. Differences between groups were determined by one way ANOVA and Tukey's HSD post-hoc test where appropriate. $p \leq 0.05$ was considered to be significant. Graphs were plotted using SigmaPlot (version 11.0; Systat Software, Inc.,

San Jose, CA). Images were processed with Photoshop CS4 (Abode Systems Inc., San Jose, CA). Image analysis was done using NIH ImageJ (version 1.43e; NIH, Bethesda, MD).

CHAPTER 3 - THE ROLE OF EXTRACELLULAR SERINE PROTEASE TISSUE-TYPE PLASMINOGEN ACTIVATOR IN THE MODULATION OF NEURONAL PLASTICITY IN CIRCADIAN CLOCK RESETTING

Summary

Tissue-type plasminogen activator (tPA) is an extracellular serine protease that has been shown to modulate neural plasticity in many brain regions including the hippocampus, amygdala and cerebellum. The present investigation focused on identifying the role of tPA in light-induced circadian rhythm resetting in a mouse model.

Light exposure at night, the time when light is capable of resetting the circadian clock, induced transient elevation in the expression of tPA in the mouse SCN. Not only was the expression of tPA in the SCN elevated by light, there was also a corresponding increase in the proteolytic activity of tPA in the SCN. Furthermore, light-induced phase delay of the circadian rhythm of mouse wheel running activity was markedly reduced after intra-cerebroventricular injection of tPA-STOP™, an inhibitor of tPA. These results strongly suggest a potential role for tPA in modulating of light-induced resetting of the circadian clock.

Introduction

Circadian rhythm resetting is an important behavioral adaptation to a change in environment. Neuroplasticity often underlies behavioral adaptations to environmental changes (Bailey and Kandel, 1993; Schacher and Montarolo, 1991). Neurotrophins, including nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) along with their respective receptors are located within the SCN and have been found to modulate photic resetting (Bina and Rusak, 1996; Bina et al., 1997; Liang et al., 2000; Michel et al., 2006).

A mechanical change in synaptic morphology has been hypothesized for the acquisition, consolidation and retention of long-term memory (Lamprecht and LeDoux, 2004). Inter-cellular membrane interactions, extracellular matrix (ECM), intracellular cytoskeleton all contribute to the synaptic cytoarchitecture. Extracellular proteolysis is likely to be a major player in the modulation of inter-cellular adhesions (Neuman et al., 1989; Shiosaka and Yoshida, 2000). The dynamic remodeling requires rapid changes in the ECM and receptor signaling (Dityatev and Schachner, 2003). Extracellular proteolytic enzymes, in particular, serine proteases, are likely candidates for the plastic remodeling process (Shiosaka, 2004; Shiosaka and Yoshida, 2000; Vassalli et al., 1991). Among the serine proteases, tPA has been implicated in numerous aspects of synaptic plasticity.

tPA is a serine protease with a molecular weight of approximately 70kDA. It contains domains of kringle, epidermal growth factor, serine protease and fibronectin type I (Lunen and Collen, 1993). It was originally identified as a fibrinolytic protease that

converted the inactive zymogen, plasminogen into the active protease, plasmin (Lunen and Collen, 1993). tPA is released by both neurons and glia (Teesalu *et al.*, 2002; Tsirka, 2002) and is expressed in many regions of the brain, including the hippocampus, amygdala, hypothalamus and cerebellum (Baranes et al., 1998; Pawlak et al., 2003; Sappino et al., 1993; Seeds et al., 1995). Overwhelming evidence suggests a role for tPA in synaptic plasticity, especially characterized is its effect on hippocampal long-term potentiation (LTP). tPA has been identified as one of the brain activity dependent (BAD), immediate-early genes induced in the hippocampal neurons during seizures, kindling and LTP (Qian *et al.*, 1993). A previous study suggested that the expression of BAD genes were induced in the SCN in response to a light stimulus at night (Kornhauser *et al.*, 1992). A more recent study showed that tPA can modulate Glu-induced phase shifting in the SCN brain slice (Mou and Prosser, 2006). These observations led us to explore the role of tPA in resetting the circadian clock.

Results

Light Induces tPA Expression in the SCN

To examine the role of tPA in light entrainment, we investigated whether light can induce the expression of tPA in the SCN. Figure 8 describes the expression of tPA following light exposure at ZT16. tPA expression in the SCN is rapidly and transiently induced in response to light exposure at night. Western blot analysis showed that tPA protein level in the SCN started to increase after 40 minutes of light exposure at ZT16, and reached a peak level after 60 minutes of light exposure. The tPA level began to decline after 80 minutes, and returned to half maximal level after 120 minutes following light exposure. The expression was significantly increased following 60 minutes of light exposure.

Light Induces tPA Proteolytic Activity in the SCN

Figure 9 show representative photomicrographs of in-situ zymography for tPA proteolytic activity in the SCN. The proteolytic activity was found to be significantly increased following 40 minutes of light exposure. The specificity of the reaction was tested by omitting plasminogen in the overlay mixture (Figure 9 C) and by adding tPA-STOP™, an inhibitor of tPA (Figure 9 D). In both the conditions the proteolytic activity was found to be decreased. Quantitation of fluorescence show that the proteolytic activity increased significantly, showing almost a 2-fold increase following 40 min light exposure at ZT16. The specificity of the reaction was tested by incubating with tPA-STOP™. In

the presence of the inhibitor, the proteolytic activity was significantly decreased versus 40 min light exposure (Figure 9 E).

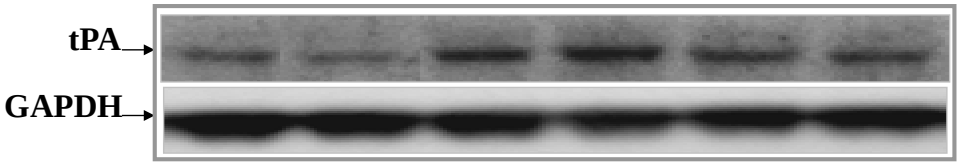
tPA Modulates Light-induced Phase Shifts *in vivo*

To determine whether tPA could modulate light-induced phase shift *in vivo*, we monitored the circadian rhythm of mouse wheel running in constant darkness. A representative actogram in Figure 10 shows that in tPA-STOP™ infused mice the light-induced phase delay was significantly reduced (0.3 hrs ± 0.22, n=12) compared to control mice that received saline infusion (1.3 ± 0.25 hrs, n=12)**.**

Figure 8: Light induces tPA protein expression in the SCN.

A: Representative Western blot showing the expression of tPA after different durations of light exposure at ZT16. B: Bar graph showing fold change in tPA expression levels normalized to the control. tPA expression level was maximally induced 1 hour after light exposure. An increase in expression level was first observed at 40 min. The levels started to decline after 60 min. An increased expression was seen up to 120 min. following the light exposure. Values are presented as mean±S.E.M., n=5 *$p<0.05$.

A


tPA
GAPDH

B

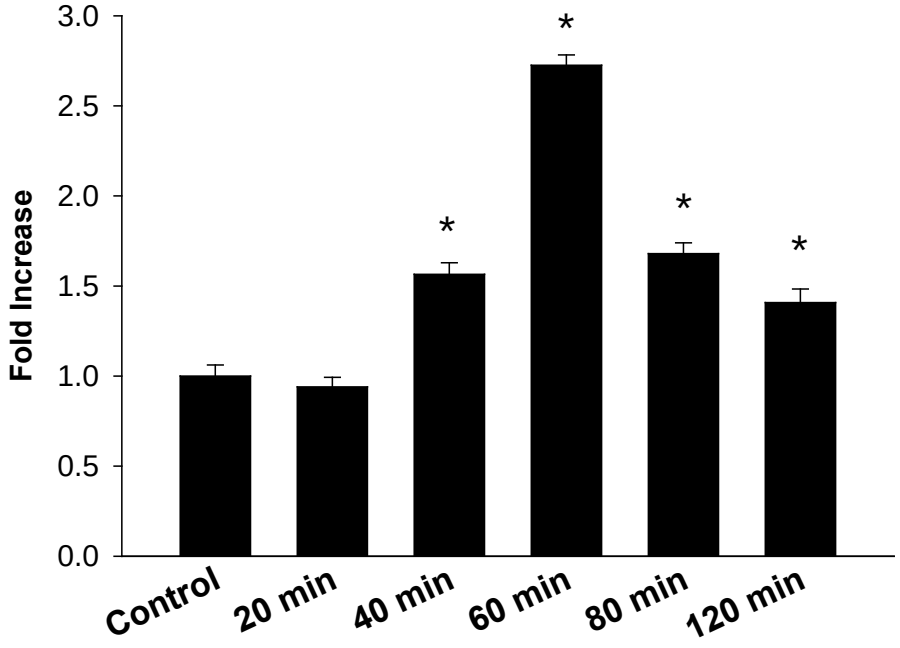
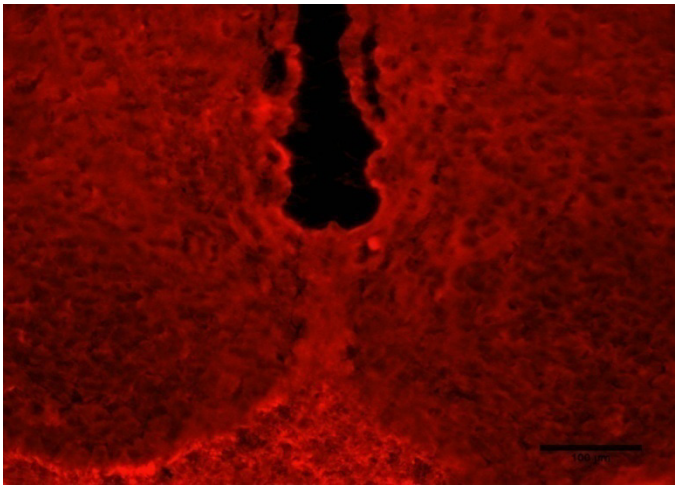

Fold Increase
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
*
Control
20 min
40 min
60 min
80 min
120 min

Figure 9 A-E: Light induces tPA proteolytic activity in the SCN.

The photomicrographs depict dye-quenched fluorescent in-situ zymography for tPA. A) shows the proteolytic activity in the SCN of the control animal (no light exposure). B) is the proteolytic activity in the SCN measured following 40 min of light exposure. The specificity of the reaction by tested by omitting plasminogen in the overlay mixture (C) by co-incubation with tPA-STOP™ (5μM) (D) F) represents the quantitation of fluorescence, a measure of the proteolytic activity. Following 40 min of light exposure, the proteolytic activity significantly increased showing almost two-fold increase versus control. Incubation with tPA-STOP™ significantly decreased the proteolytic activity. Values are mean±S.E.M., n=4 *$p<0.05$, #$p<0.05$. Scale bar =100 μm.

A



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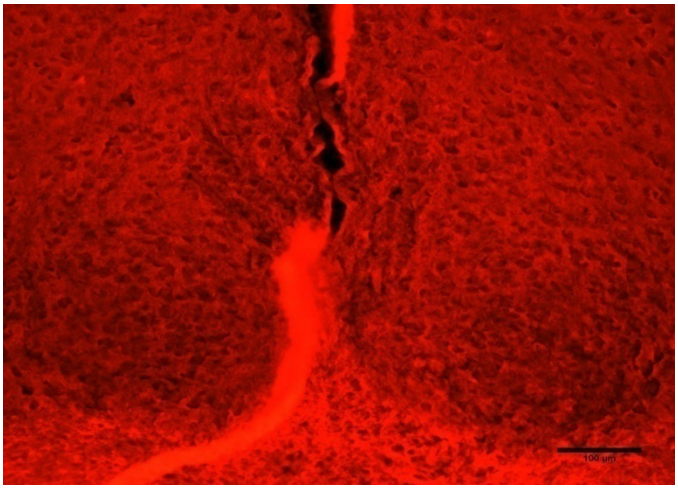
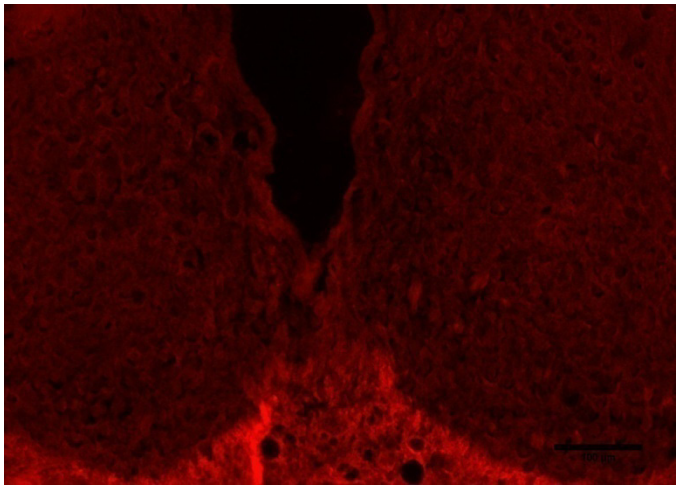
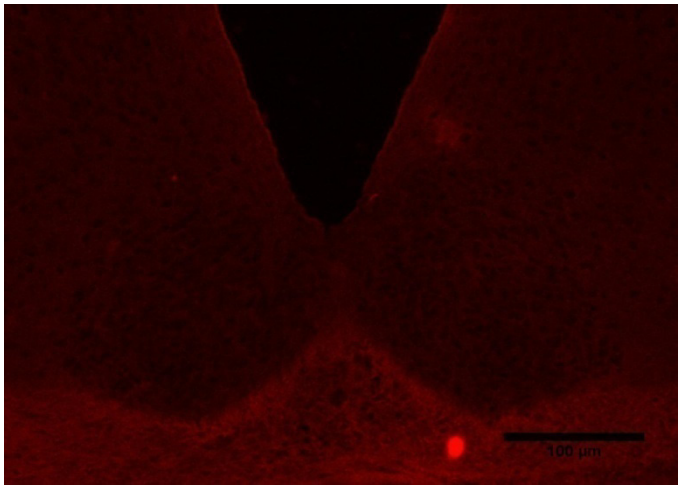


C



D


100 µm

E

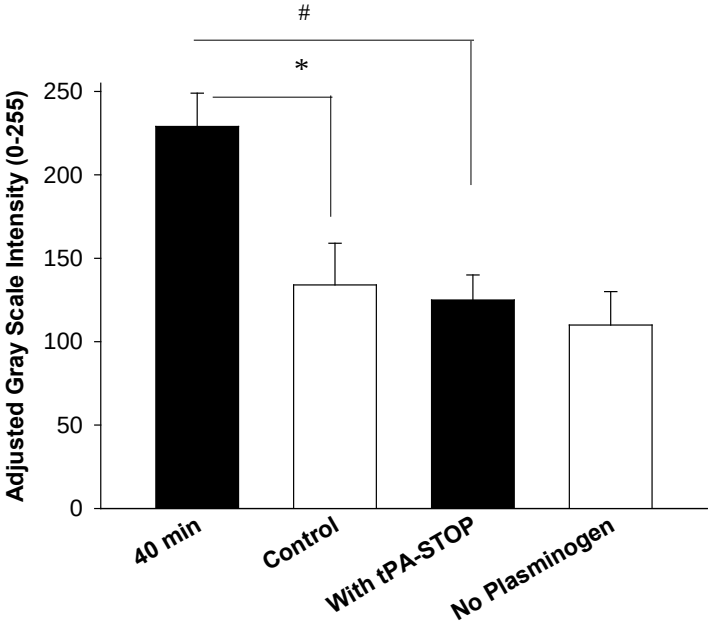
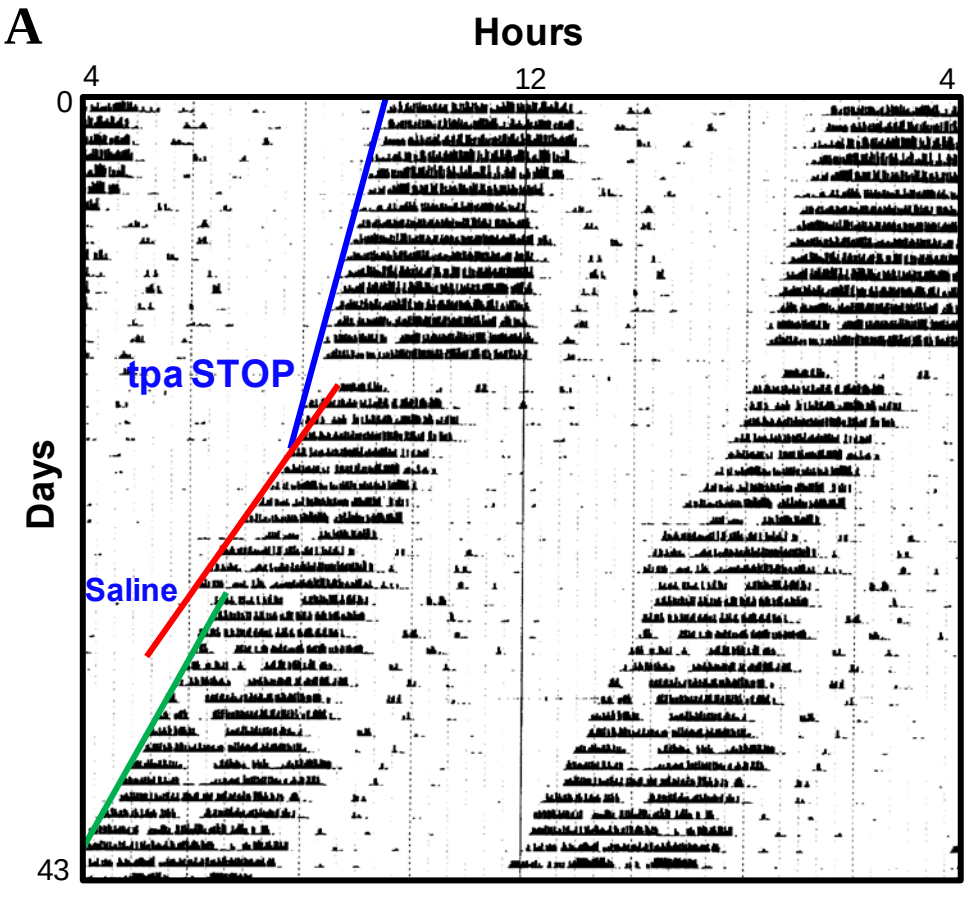
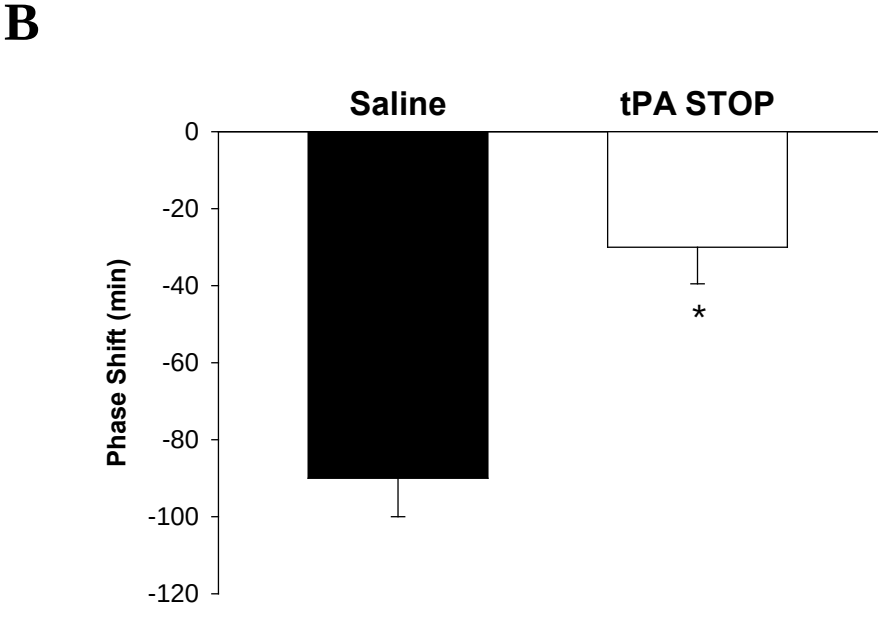

#
*
Adjusted Gray Scale Intensity (0-255)
250
200
150
100
50
0
40 min
Control
With tPA-STOP
No Plasminogen

Figure 10 A-B: tPA-STOP™ attenuates light-induced phase shifts.

(A) A representative double-plotted actogram showing 43 days of activity demonstrates that intracerebroventricular injection of tPA-STOP™ into the 3rd ventricle reduces the light-induced delay of running activity when compared to the control animals injected with saline. The red, blue and green colored lines represent the regression fit lines of activity onset after tPA-STOP™ treatment, before tPA-STOP™ treatment and after saline treatment (B) The bar graph represents the mean phase delays after injection of tPA-STOP™ and following injection of saline. Light-induced phase delay after injection of tPA-STOP was decreased (26±8 min.) compared to that following injection of saline (90±10 min.). Values are presented as mean±S.E.M n=16 *$p<0.05$.


A
Hours
4
12
4
0
Days
43
tpa STOP
Saline


B
Saline
tPA STOP
0
-20
-40
-60
-80
-100
-120
Phase Shift (min)
*

Discussion

The main goal of this study was to explore the potential role of the extracellular protease tPA in the photic resetting of circadian rhythm. The tPA/plasmin system consists of an inactive zymogen, plasminogen that is converted to plasmin, a broad spectrum serine protease, by tPA (Collen, 1999). The tPA/plasmin system has been implicated in various physiological processes, such as learning and memory in the hippocampus, stress responses in the amygdala and motor learning tasks in the cerebellum (Madani et al., 1999; Pawlak et al., 2003; Seeds et al., 1995). Several recent studies have revealed a critical role of tPA in the maintenance of LTP in the hippocampus. A selective reduction of LTP was seen in hippocampal slices from tPA deficient mice, suggesting a potential role of tPA in synaptic facilitation (Calabresi et al., 2000; Frey et al., 1996; Huang et al., 1996). LTP was also shown to be enhanced in hippocampal slices from mice overexpressing tPA (Madani *et al.*, 1999). Furthermore, mice over expressing tPA showed improved learning capacity (Madani *et al.*, 1999), whereas, the mutant mice with a genetic deletion of tPA showed impairment in spatial learning tasks (Huang *et al.*, 1996).

In the first set of experiments, we showed that light could induce transient expression of tPA protein in the mouse SCN. An increased expression was seen after 40 min of light exposure. The tPA level peaked after one hour and remained elevated for up to 2 hours following light exposure. Not only was the expression level of tPA increased, there was also a corresponding increase in the proteolytic activity of tPA in the SCN

following light exposure as demonstrated in in-situ zymography. A similar light-induced expression of polysialic acid and polysialylated neural cell adhesion molecule have been observed in the SCN. Polysialylated neural cell adhesion molecule is an important mediator of plasticity in cell interactions and has been shown to play a critical role in the photic regulation of circadian rhythm (Glass *et al.*, 2000; Prosser *et al.*, 2003). Glu-induced tPA mRNA translation has been demonstrated in hippocampal neurons (Shin *et al.*, 2004). In the hippocampus and cerebellum, transcription of tPA mRNA is induced after synaptic activation (Amir and Stewart, 1998; Qian et al., 1993; Seeds et al., 2003). It has been suggested that there is an immediate site-specific synthesis and release of tPA which is followed by an NMDA receptor mediated upregulation of tPA mRNA (Qian *et al.*, 1993; Shin *et al.*, 2004). An analogous mechanism might exist in the SCN. The exact mechanism by which tPA expression in the SCN is induced after photic stimulation needs to be investigated further.

The inhibition of light-induced phase delay of circadian wheel running activity by tPA-STOP™ suggests that tPA can modulate circadian rhythm resetting. tPA is a broad spectrum extracellular protease that converts the inactive zymogen, plasminogen to its active form, plasmin, through proteolytic cleavage. Although the primary substrate of tPA is plasminogen, tPA has been shown to mediate the proteolysis of other ECM proteins (Hoffman *et al.*, 1998; Nakagami *et al.*, 2000; Wu *et al.*, 2000). Upon synaptic activation in the hippocampus, plasmin converts the immature precursor form of BDNF, proBDNF, to its mature form, mBDNF (Pang *et al.*, 2004). Previous studies have demonstrated that BDNF in the SCN could enhance Glu release and potentiate NMDA and AMPA mediated currents, through pre-synaptic and post-synaptic mechanisms,

respectively (Michel *et al.*, 2006). Plasmin directly interacts with the NMDA receptor, cleaving an amino terminal of the NR2A subunit and augmenting NMDA receptor responses (Yuan *et al.*, 2009). tPA also directly binds to the low-density lipoprotein receptor (LRP) independent of its protease activity (Orth *et al.*, 1994). tPA is rapidly endocytosed by LRP after binding to the receptor initiating a series of intracellular signaling events (Zhuo *et al.*, 2000). A growing body of evidence suggest that tPA can directly cleave the NR1 subunit and thereby increase Ca^{2+} influx (Benchenane et al., 2007; Fernandez-Monreal et al., 2004; Nicole et al., 2001). Thus, tPA has a dual influence on the NMDA receptor: an indirect potentiation of Ca^{2+} influx via LDL receptor, and a plasmin mediated enhancement of NMDA receptor function. tPA may modulate photic resetting through one or more of these mechanisms.

CHAPTER 4 - THE ROLE OF CYCLIN-DEPENDENT KINASE 5 AND ITS ACTIVATORS IN CIRCADIAN CLOCK RESETTING BY LIGHT

Summary

Cyclin-dependent kinase 5 (Cdk5) is a unique member of the cyclin-dependent kinase family that is activated by non-cyclin activators mainly, p35 and p25. Recent studies have implicated Cdk5 and its activators in various neurological processes, including but not limited to learning and memory, drug addiction and neurodegenerative disorders. Cdk5 is a signal transduction molecule downstream to NMDA receptors in the Glu pathway. Since Glu is the principal neurotransmitter of the RHT (Ding et al., 1994), we sought to investigate the role of Cdk5 in the light entrainment of the circadian clock.

SCN neurons expressing the Cdk5 activator, p25 co-localized with phosphorylated-extracellular signal-related kinase (p-ERK) and Gastrin Releasing Peptide (GRP). Cdk5 activators showed a differential response to light. At ZT16, the levels were significantly decreased whereas, at ZT22, it was significantly elevated. At ZT6, there was no significant change in the levels. A similar response was seen in the activity of Cdk5. The kinase activity was significantly decreased at ZT16, significantly increased at ZT22, and unchanged at ZT6. These results were confirmed in a bi-transgenic mouse model which overexpressed the activator, p25. In the bi-transgenic animal, light-induced phase delay in circadian wheel running activity at CT16 was significantly increased whereas light-induced phase advance was significantly attenuated. Moreover, the bi-transgenic animals exhibited an increased Cdk5 kinase activity in the SCN following overexpression of p25, which returned to basal levels once the overexpression was repressed. The bi-transgenic animals also demonstrated changes in synapse density in the SCN. The synaptic density in the SCN was significantly increased following overexpression of p25. Once the overexpression was turned off the synaptic

count also returned to basal levels. Taken together, these results strongly suggest that Cdk5 along with its activators play a role in the modulation of circadian photic resetting.

Introduction

Cdk5 was first discovered in the bovine brain and because it had a sequence homology to human CDC2, it was referred to as neuronal CDC2-like kinase (NCLK) (Lew et al., 1992; Meyerson et al., 1992). Cdk5 is a proline-directed kinase that phosphorylates serines and threonines immediately upstream of a proline residue. Like other cyclin-dependent kinases, Cdk5 by itself has no enzymatic activity. It requires association with a regulatory unit for activation. Cdk5 is activated by p35 and p39. It can also be activated by p25 and p29, the calpain-mediated cleavage products of p35 and p39. Although, Cdk5 is expressed in many tissues, the activators are expressed mainly in post-mitotic neurons, thus Cdk5 kinase activity is seen highest in the central nervous system (Paglini and Caceres, 2001; Paglini et al., 2001). Cdk5 is a pleotropic kinase and more than two dozen substrates have been identified (Dhavan and Tsai, 2001; Smith, 2003).

Cdk5 has been implicated in various neural functions. It is involved in regulation of cytoskeletal elements (Hallows et al., 2003; Smith, 2003), axon guidance (Nikolic et al., 1996), membrane transport (Barclay et al., 2004; Paglini and Caceres, 2001; Paglini et al., 2001; Shea et al., 2004), and synaptic function (Cheng and Ip, 2003).

In the past few years, Cdk5 has gained more attention for its role in synaptic plasticity, learning and memory. A role of Cdk5 in dendritic spine formation has been reported in hippocampal neurons (Cheung et al., 2007; Cheung and Ip, 2007). BDNF induced dendritic growth was abolished in a TrkB mutant mouse model lacking Cdk5 phosphorylation site (Cheung et al., 2007). Cdk5 also modulates neuronal secretion at the synapse (Cheng and Ip, 2003). Several lines of evidence point to role of Cdk5 in both short-term and long-term potentiation. Its role in synaptic plasticity and learning was first

studied using the inhibitors, roscovitine and butyrolactone I. The inhibitors suppressed LTP induction in hippocampal CA1 area (Li et al., 2001). Long-term depression and spatial learning has been shown to be impaired in p35 null mutant mouse model (Ohshima et al., 2005). The role of Cdk5 in synaptic plasticity and spatial learning is further supported by the observation that transgenic mice that overexpress p25 show enhanced LTP and spatial learning (Angelo et al., 2003; Fischer et al., 2005; Ris et al., 2005). Thus Cdk5 has a multifaceted role in synaptic plasticity. The potential role of Cdk5 in light entrainment also stems from the fact that glutamate has been shown to induce transient activation of Cdk5 ((Wei et al., 2005). The present study was envisaged to investigate the role of Cdk5 in light entrainment by employing the bi-transgenic mice that conditionally overexpress the Cdk5 activator, p25.

Results

Regional Localization of Cdk5 Activator, p25 in the SCN

Figure 11 A shows the distribution of p25 immunoreactivity in the SCN. p25 positive immunoreactivity is seen in both ventro-medial and dorso-medial regions of the SCN, more towards the dorsal region. Since the p25 transgene is GFP tagged, it can be visualized without immunolabelling. Figure 11 B shows that p25 expressing neurons in the SCN co-localize with neurons expressing p-ERK. Figure 11 C shows that p25 expressing neurons in the SCN are in close approximation to GRP fibers in the "cap" region of the SCN.

Effect of Light on Cdk5 and its Activators in the SCN

Figure 12 describes the effect of light on Cdk5 activator levels in the SCN at ZT16, ZT22 and ZT6. At ZT16 (Figure 12 A) both p35 and p25 levels were decreased (p35- $p<0.032$, p25-$p<0.021$) compared to control which received no light pulse. At ZT22 (Figure 12 B) both p35 and p25 levels were increased in the SCN following a 20 min light pulse (p35-$p<0.004$, p25-$p<0.04$). At ZT6 (Figure 12 C) there was no change in p35 or p25 levels in the SCN. In contrast to the light exposure at night, light pulse at ZT6 did not induce change in p35 or p25 levels in the SCN. Furthermore, light exposure at night induced changes of p25 and p35 levels only in the SCN, but not in the cerebellum.

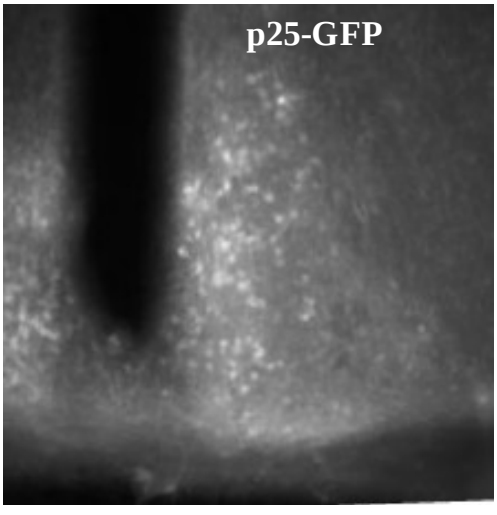
Effect of Light on Cdk5 Kinase Activity in the SCN

The Western blot analysis showed that light exposure at night could induce changes in the expression of both p25 and p35 in the SCN. Figure 13 describes the effect of light on Cdk5 kinase activity in the SCN. After a 20 min light pulse (~100 lux), the kinase activity was increased at ZT22. The activity decreased at ZT16, whereas, at ZT6 there was no change in the kinase activity.

Figure 11 A-C: Co-localization of Cdk5 activator, p25 in the SCN.

Representative photomicrographs show (A) distribution of neurons expressing p25 neurons in the SCN. p25-GFP immunoreactivity is seen in the dorsomedial region of the SCN (B) p25 expressing neurons are seen to co-localize with p-ERK expressing neurons (C) p25 expressing neurons are seen in close approximation to GRP fibers. (Red – pERK in 11 B, GRP in 11 C, Green – p25-GFP, white arrows point to co-localization).

A



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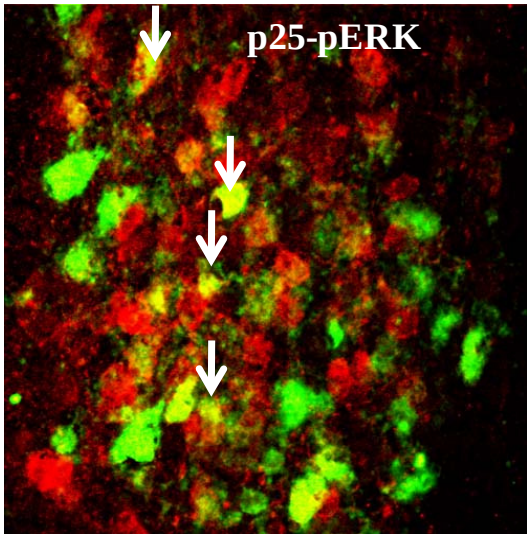

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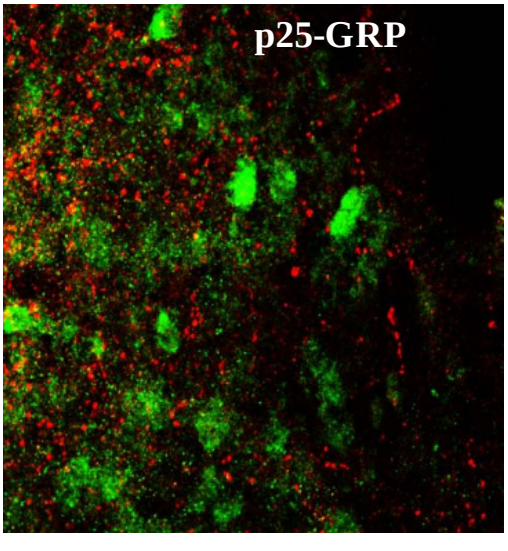
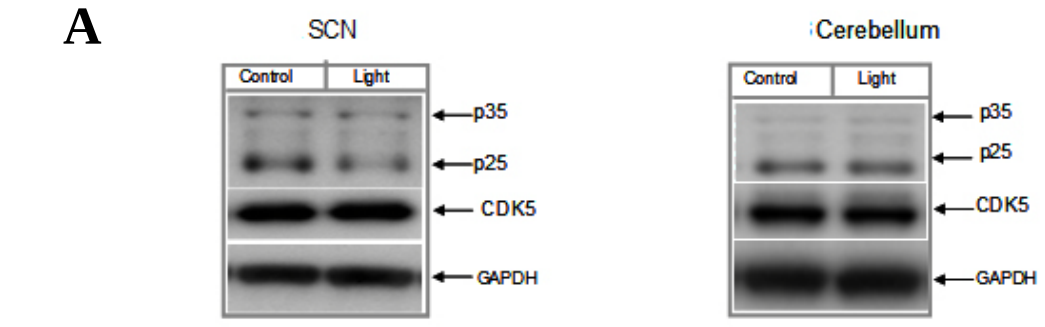
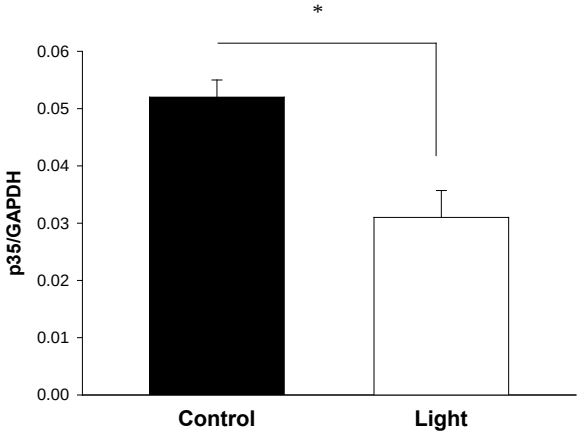


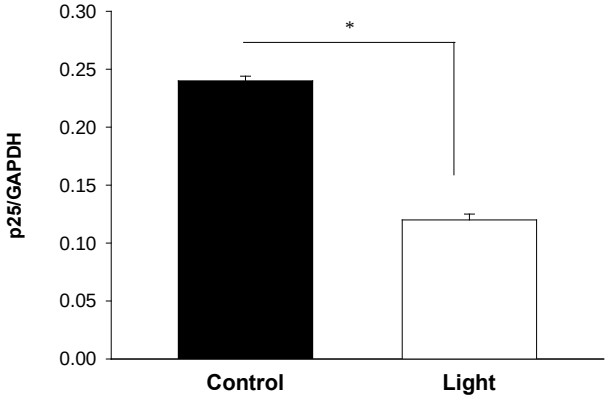
Figure 12 A-C: Effect of light on Cdk5 activators in the SCN.

A- Cdk5 activator levels in the SCN and Cerebellum at ZT16. B- Cdk5 activator levels in the SCN and Cerebellum at ZT22. C- Cdk5 activator levels in the SCN and Cerebellum at ZT6. In all the figures upper panel shows representative Western blot bands for Cdk5, p25, p35 and GAPDH in the control and light treated group. Lower panel depicts the relative densitometric ratio normalized to GAPDH. At ZT16, p35 and p25 levels were decreased in the SCN following light exposure. There was no change in the total Cdk5 level . In the cerebellum, no change was seen in the levels of Cdk5, p35 and p25 after light exposure. At ZT22, p35 and p25 levels in the SCN increased following light exposure, whereas total Cdk5 in the SCN and p35, p25, Cdk5 levels in the cerebellum remain unchanged after the light exposure. At ZT6, there was no change in the levels of p35, p25 or Cdk5 in both the SCN and cerebellum. Values are presented as mean±S.E.M., n=6, *$p<0.05$.





SCN



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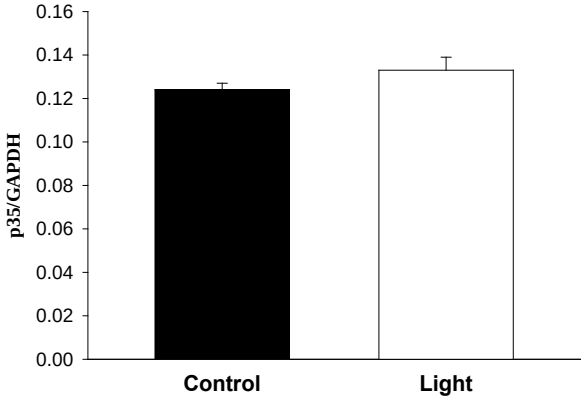
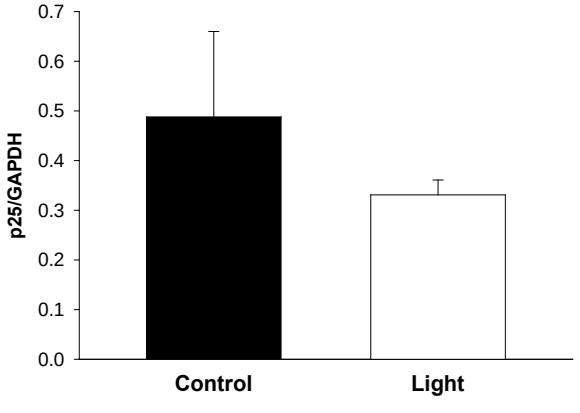


CEREBELLUM



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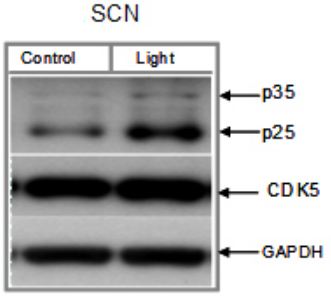
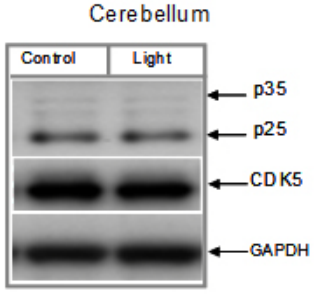
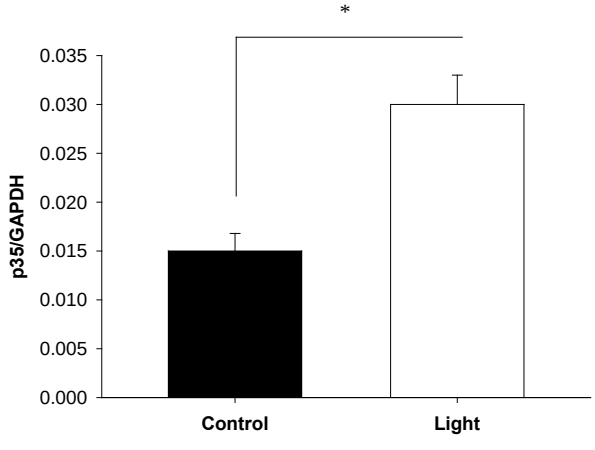
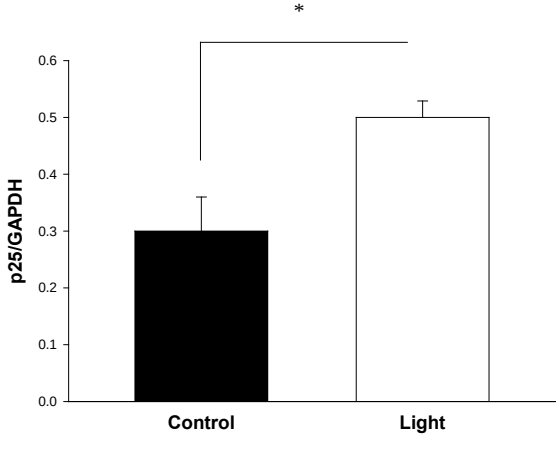
B



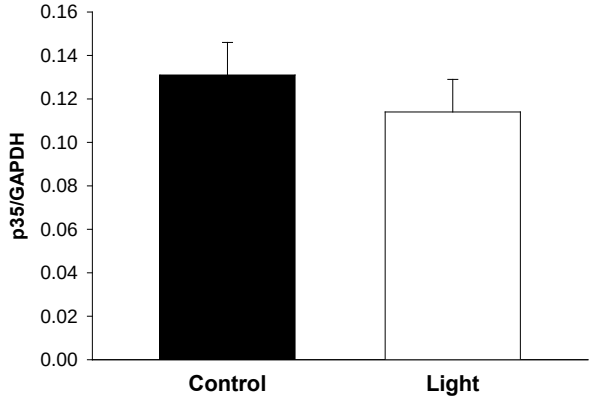




SCN



SCN



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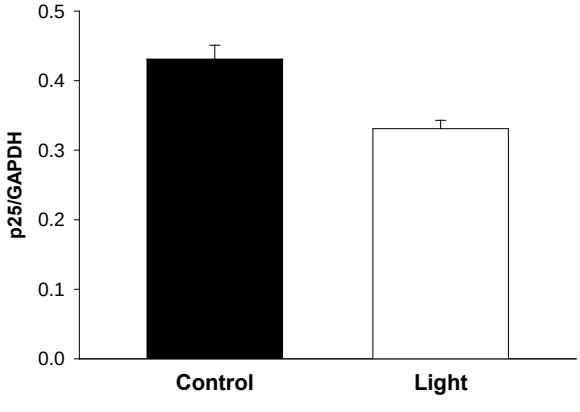

CEREBELLUM

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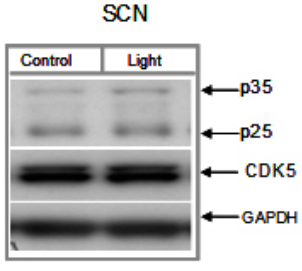
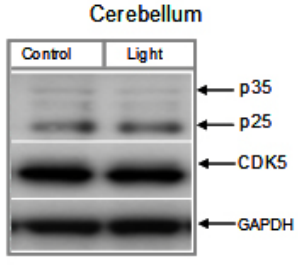
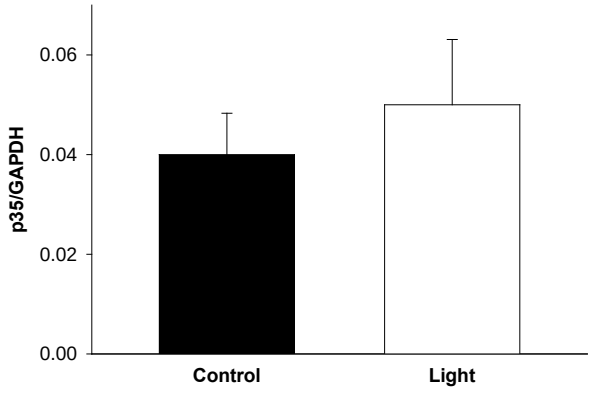
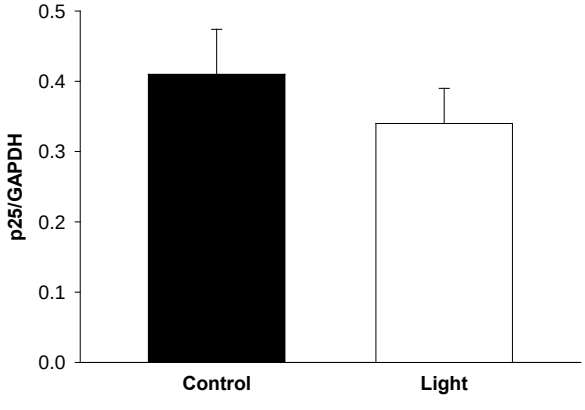






SCN



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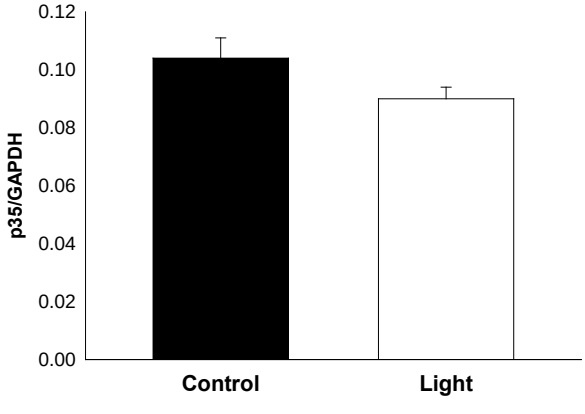
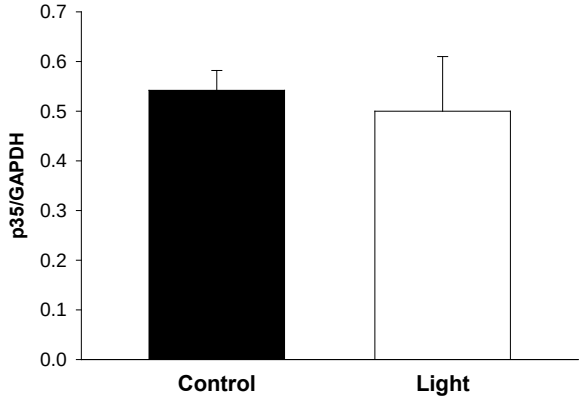


CEREBELLUM



CEREBELLUM

Figure 13: Effect of light on Cdk5 kinase activity in the SCN.

Cdk5 kinase activity was measured using Cdk5 specific substrate. The average activity of the control sample was taken as 1. The kinase activity was measured after light pulse at the different circadian time points, ZT16, ZT22, ZT6. The activity measured was normalized to basal activity and is expressed as a ratio of the basal activity. Cdk5 kinase activity was significantly increased after light pulse at ZT22, at ZT16, the activity was significantly reduced, at ZT6 there was no significant change in the kinase activity. Values are presented as mean±S.E.M., n=12 *$p<0.05$, #$p<0.05$.

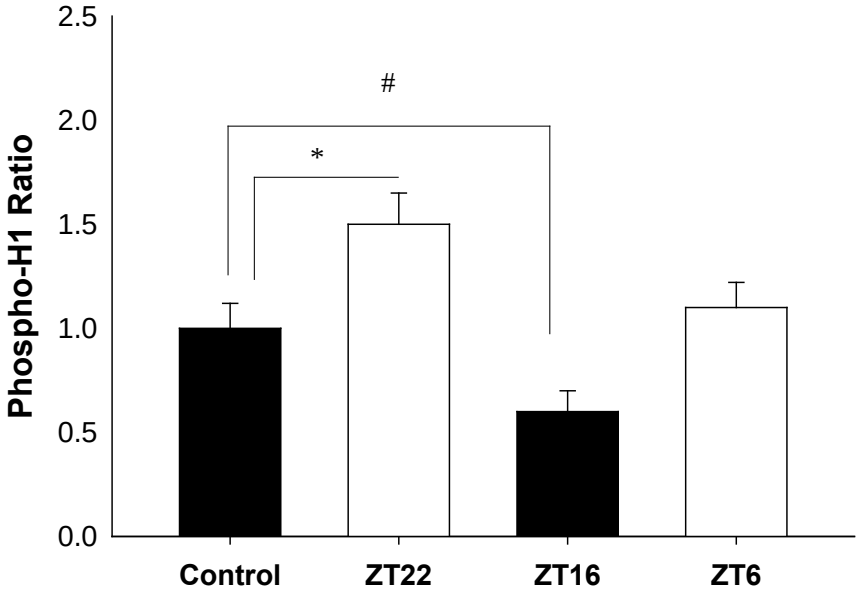
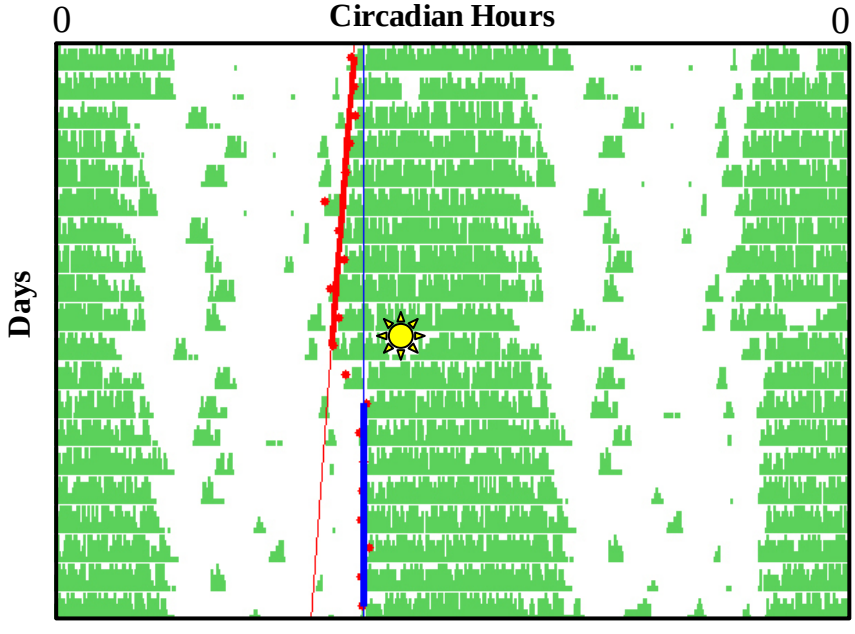

Phospho-H1 Ratio
2.5
2.0
1.5
1.0
0.5
0.0
#
*
Control
ZT22
ZT16
ZT6

Figure 14 A-E: Effect of p25 overexpression on light-induced phase shifts in the CK-p25 mice.

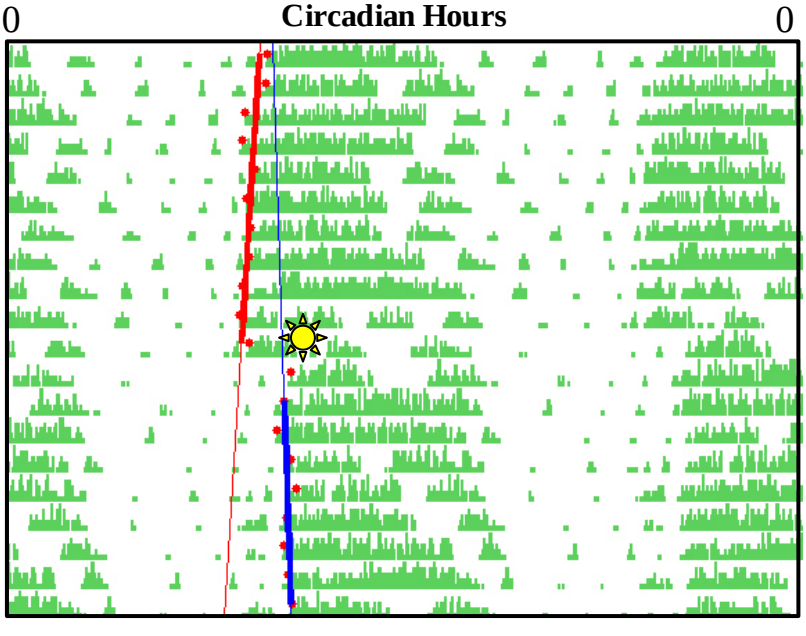
Figure 14 A-E are representative double-plotted actograms of wheel-running activity. Each green line represents two consecutive days of activity. Red solid line represents the regression fit line of activity onsets of 7 days before the light pulse. Blue solid line represents the regression fit line of activity onsets of 7 days (excluding the first two days) after the light pulse. Yellow star indicates the time of light pulse. X axis represents time in hrs, Y axis represents days. The difference in X axis between the lines on the third day after light pulse is the calculated phase shift. Mice were free-running for 10 days before the experiment. A 15 min light pulse of 50 lux was given at CT16 (A, B) and CT22 (C, D). Subsequent to the light pulse, animals were maintained in DD. p25 overexpression was induced by switching the doxycyline based diet to normal rodent chow for 2 weeks. Figure 14 E is the quantification of light-induced phase shifts. Values are presented as mean±S.E.M. n=12/group. When p25 was overexpressed the phase delay at CT16 was significantly increased (No overexpression – -1.28±.0.21 hr, Overexpression -2.27±0.22 hr, p=0.03). At CT22 the phase advance was significantly attenuated (No overexpression – 0.94±0.15 hr, Overexpression – 0.21±0.12 hr, p=0.002). The phase shifts returned to basal levels once the overexpression was turned off for 4 wks.

A



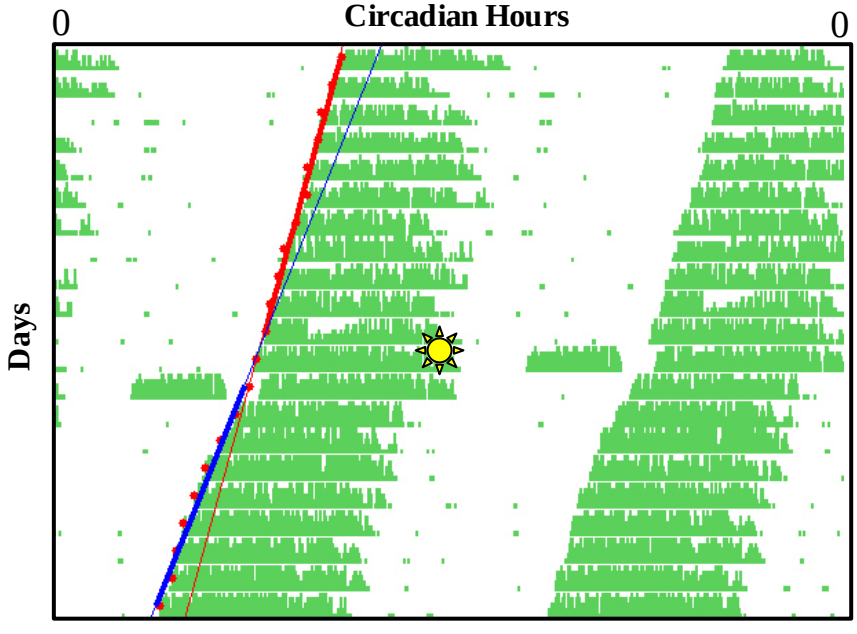
No overexpression of p25

B



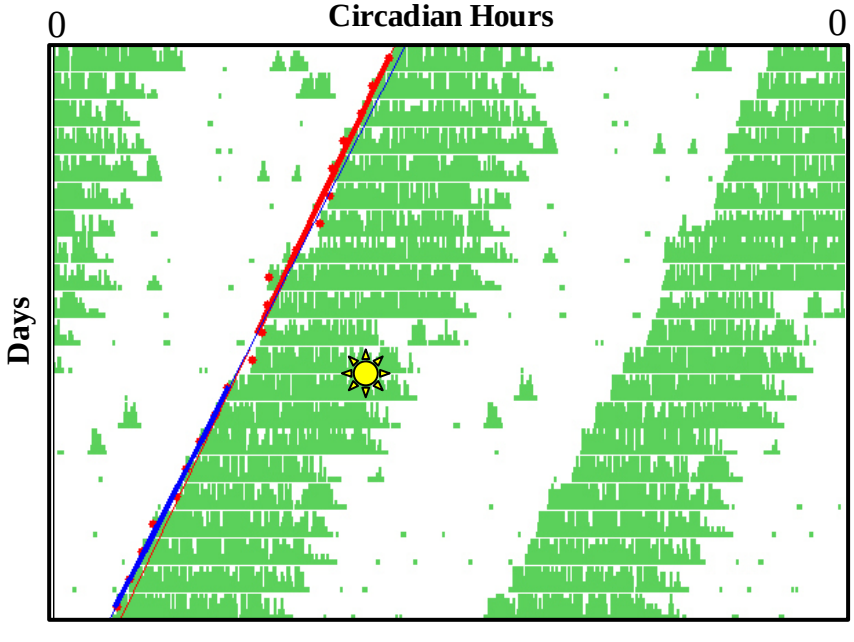
After overexpression of p25

C



No overexpression of p25

D



After overexpression of p25

E

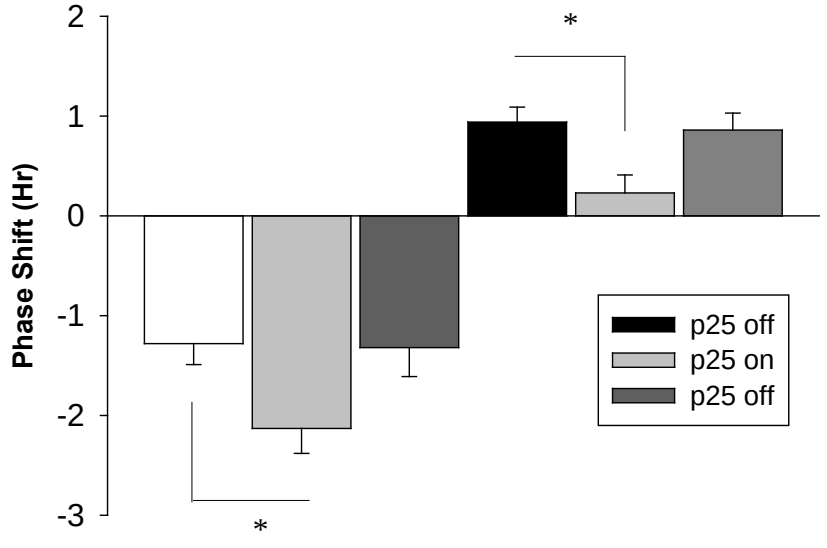


Figure 15: Effect of p25 overexpression on Cdk5 kinase activity.

Cdk5 kinase activity was measured using Cdk5 specific substrate. The average activity of the control sample was taken as 1 The kinase activity was measured after overexpression for 2 wks and 4 weeks after overexpression was turned off . The activity measured was normalized to basal activity and is expressed as a ratio of the basal activity. Cdk5 kinase activity was significantly increased after p25 overexpression, it returned to basal values when expression returned to normal. Values are presented as mean±S.E.M., n=12, *$p<0.05$, #$p<0.05$.

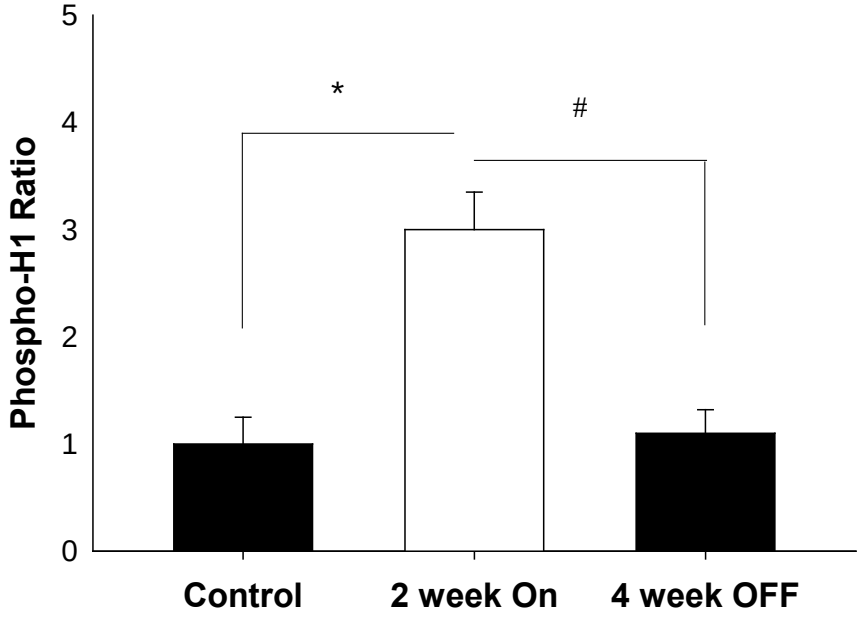
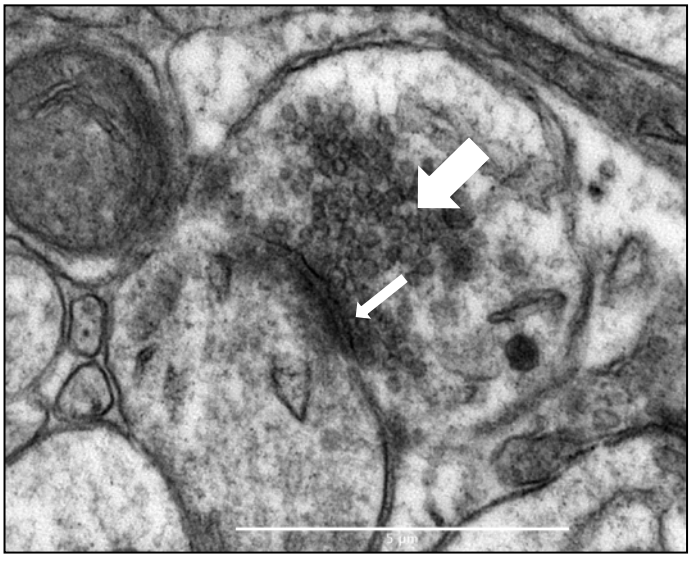

Phospho-H1 Ratio
5
4
3
2
1
0
*
#
Control
2 week On
4 week OFF

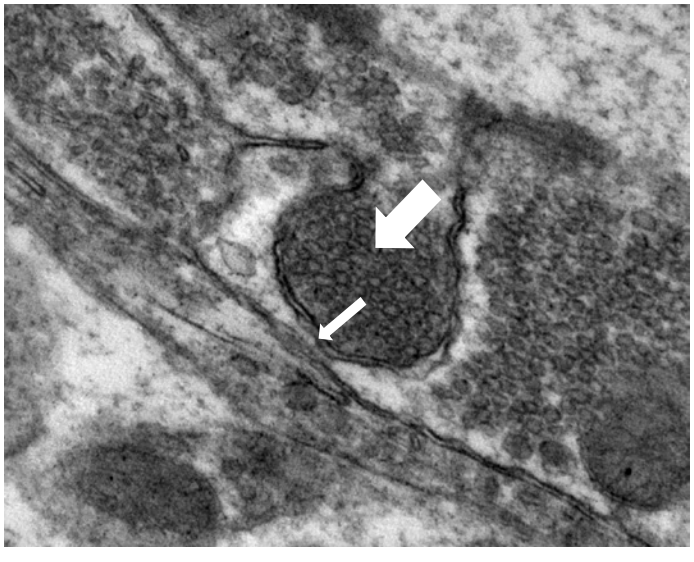
Figure 16 A-C: Effect of p25 overexpression on synaptic density in the SCN.

SCN samples from animals with and without overexpression of p25 were subjected to electron microscopic examination for synaptic morphology. A & B are representative electron microscopic images. Arrows point to electron-dense post synaptic density(thin arrow) and synaptic vesicles (thick arrow). The number of synapses was significantly increased following 2 weeks of overexpression of p25 in the CK-p25 mice. The number of synapses returned to basal levels once the overexpression was turned off for 4 wks. Values are presented as mean±S.E.M., n=12, *$p<0.05$ Scale bar = 0.5 µm.

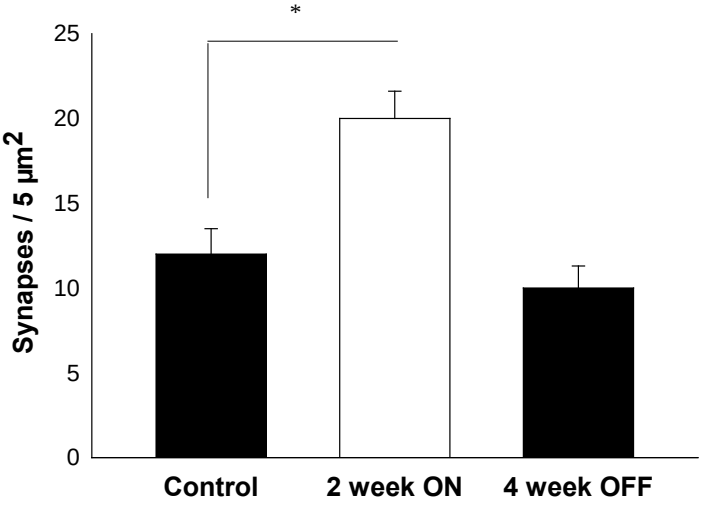
A



B



C



Discussion

Multiple lines of evidence have implicated Cdk5 in synaptic plasticity, learning and memory. The overall objective of this investigation was to identify the role of Cdk5 in light-induced circadian clock resetting.

Cdk5 activator, p25 distribution was observed in the dorsomedial and dorsolateral regions of the SCN. Co-localization of neurons expressing p25 with p-ERK and GRP was also demonstrated. GRP has been considered as the candidate for intra-SCN communication of photic input from the retino-recipient calbindinB (CalB) cells found in the ventral "core" region of the SCN. Light induces c-Fos and *Per1* mRNA in GRP expressing neurons in the SCN (Aida et al., 2002; Antle et al., 2005; Earnest et al., 1993; Karatsoreos et al., 2006; Kawamoto et al., 2003; Romijn et al., 1996). GRP application can produce phase shifts of the circadian clock (Gamble et al., 2007; Kallingal and Mintz, 2006, 2007; McArthur et al., 2000). GRP induces this phase shift via activation of CREB (Gamble et al., 2007). CREB activation is an essential process in light-induced and glutamate-induced phase shifts in the SCN (Ding et al., 1997; Tischkau et al., 2003a). Furthermore, GRP-induced phase shifts and Per induction requires the MAPK pathway (Antle et al., 2005; Moody and Merali, 2004). Our finding that Cdk5 activator p25 co-localizes with GRP and p-ERK suggests that Cdk5/p25 may be a modulator of this pathway. Further studies will be required to elucidate the exact function of Cdk5/p25 in this pathway.

Immunoblot and kinase assay studies demonstrated that light produces a differential response in Cdk5 activator levels. The activator levels were decreased at ZT16 and increased at ZT22. Consistent with this finding, we found that the kinase

activity was decreased at ZT16 whereas, at ZT22 it was increased. A similar finding was observed in the light-induced phase shifts of circadian wheel running activity in the bi-transgenic animal with conditional p25 overexpression. While transient Cdk5 hyperactivation augmented the phase delaying effect of light at CT16, the phase advance at CT22 was attenuated. Taken together, it is apparent that Cdk5 might be modulating the integration of photic signaling within the SCN. The only other reported occurrence of impairment in phase-advancing photic signaling is in PACAP null mutant mice (Beaule et al., 2009; Kawaguchi et al., 2003). Deficits observed in the PACAP null mutant mice has been attributed to impairments in the animal's ability to integrate stimulus intensity and/or duration (Beaule et al., 2009; Nelson and Takahashi, 1991). Considering our histological observation that Cdk5 activator co-localizes with cells implicated in intra-SCN photic signaling pathway, it is a possibility that Cdk5 modulates the sensitivity of the circadian system to light. Further studies will be required to test this hypothesis.

Electron microscopy revealed an increase in the number of synapses in the SCN following p25 overexpression. Synaptic morphogenesis is an integral part of neural plasticity in other regions of the brain. This is the first report of an alteration of synaptic morphology within the SCN. Structural plasticity has been proposed as a mechanism involved in light entrainment (Becquet et al., 2008; Bosler et al., 2009; Glass et al., 2003; Guldner et al., 1997).

Collectively, our results indicate a role of Cdk5 in light entrainment, possibly by modulating the light sensitivity of SCN and/or altering synaptic morphology. Cdk5 is a pleotropic kinase with more than two dozen substrates, it is thus a possibility that both the

mechanisms mentioned above or even additional mechanisms are involved in the modulation of circadian photic resetting.

CHAPTER 5 - THE ROLE OF CYSTEINE PROTEASE CALPAIN IN THE PHOTIC RESETTING OF CIRCADIAN CLCOK

Summary

Calpain is a cysteine protease that is activated by calcium. Calpain has been implicated in neuronal processes, including plasticity, learning and memory in the hippocampus. Calpain regulates Cdk5 activity by proteolytic cleavage of Cdk5 activators, p25 and p35. Studies have indicated that Cdk5 activators also play a role in synaptic plasticity, learning and memory. This study was designed to explore the role of calpain in circadian photic resetting.

Calpain-1 immunoreactivity was distributed in the entire SCN from the rostral to caudal ends. A denser immunoreactivity was seen in the mid-caudal regions of the SCN. Basal expression levels of calpain-1 did not change between the various circadian time points, ZT16, ZT22, ZT6. The expression levels also showed no significant change after a light pulse at ZT16, ZT22 and ZT6. In contrast to expression levels, basal calpain activity levels exhibited a trend, the highest activity was seen at ZT16 and the lowest at ZT22. Light induced calpain activity in the SCN. Calpain activity levels were significantly increased at ZT16 and ZT22. Light pulse did not produce any significant change in calpain activity at ZT6. Our data suggest a potential role for calpain in circadian photic resetting but future studies are needed to ascertain the exact role of calpain.

Introduction

Calpain is a neutral, calcium activated, intracellular cysteine protease expressed both in the cytosol and the synaptic terminal in neurons (Mellgren et al., 1989; Pontremoli et al., 1989; Tomimatsu et al., 2002). There are at least 14 different types of mammalian calpains. Two isoforms of calpain are ubiquitously present in the brain: μ-calpain, and m-calpain (also referred to as calpain I and calpain II, respectively). The other forms of calpain are tissue specific and not very well understood (Bevers and Neumar, 2008; Saez et al., 2006). Both calpain I and II have similar substrate specificity but differ in their sensitivity to Ca^{2+}. Calpain I requires 3-50 μM calcium for activity, whereas calpain II requires 0.4-0.8 mM Ca^{2+} for half-maximal activity (Goll et al., 2003; Mellgren et al., 1989). Both the calpains contain a subclass-specific 80 kDa catalytic subunit containing domains I-IV and a common small 30 kDa regulatory subunit containing domains V and VI. Domain II contains the catalytic activity and interacts with substrates and the endogenous calpain inhibitor, calpastatin. Calpain undergoes autolytic cleavage on domain I (Carafoli and Molinari, 1998; Nakagawa et al., 2001; Ono et al., 1998; Sorimachi et al., 1997).

In the nervous system calpains have been implicated in various neuronal functions, including learning, memory and neurotoxicity. Calpain substrates include synaptic proteins such as membrane receptors, cytoskeletal proteins, post synaptic density proteins and other intracellular mediators of synaptic function (Croall and DeMartino, 1991; Goll et al., 2003; Guttmann et al., 2001; Lee et al., 2000; Lu et al., 2000). Calpain has been implicated in the induction and maintenance of LTP. Inhibition of calpain

activity reduces induction of LTP in the hippocampal CA1 area (Oliver et al., 1989; Staubli et al., 1988).

Recent studies have implicated the role of Cdk5 in synaptic plasticity, learning and memory (Fischer et al., 2005; Fischer et al., 2002, 2003a; Fischer et al., 2003b; Seeburg et al., 2008). Several studies have also indicated a role of Cdk5 activators, p35, and p25 in learning and plasticity. p35 homozygous and heterozygous knockout mice display impaired contextual fear-conditioned memory (Fischer et al., 2005). p35-knockout mice also exhibit a lower threshold for theta-burst rhythm-induced LTP (Wei et al., 2005). Transient and low-level expression of p25 has been shown to improve plasticity and performance in learning tasks in mice that overexpress p25 (Angelo et al., 2003; Fischer et al., 2005; Fischer et al., 2007). p25 is the calpain mediated proteolytic cleavage product formed from p35 (Patrick et al., 1999). Calpain also cleaves the other cdk5 activator, p39 to p29 (Patzke and Tsai, 2002). Since circadian photic resetting mainly involves the glutamatergic signaling pathway, we hypothesized that the calcium activated protease, calpain might play a role in regulating the activity of Cdk5 which in turn can modulate light-induced circadian clock resetting. In this study, we sought to investigate whether light can induce calpain expression level and or its activity in the SCN.

Results

Regional Distribution of Calpain in the SCN

Figure 17 A-L show staining for calpain-1 immunoreactivity in the SCN from the rostral to caudal ends. Calpain-1 immunoreactivity was observed in the entire SCN at all rostrocaudal levels. A denser immunoreactivity for calpain was seen in the mid-caudal regions of the SCN.

Effect of Light on Calpain-1 Expression Levels in the SCN

Figure 18 A-C describes the effect of light on calpain-1 expression levels in the SCN. Animals were given a 20 min light pulse of 100 lux at circadian time points ZT16, ZT22 and ZT6. Control animals received no light pulse. There was no significant change in the expression levels of calpain-1 in the SCN after light treatment at any of the three circadian time points. There was also no significant difference in the basal expression level of calpain-1 at the three circadian time points.

Effect of Light on Calpain Activity in the SCN

Figure 19 A & B depict the effect of calpain activity in the SCN. Animals were given a 20 min light pulse of 100 lux at circadian time points ZT16, ZT22 and ZT6. Control animals received no light pulse. Calpain activity was measured using a luminescent based

assay. Basal levels of calpain activity showed a trend. The activity was the highest at ZT16 and the lowest at ZT6 (A). The activity was significantly increased at both ZT16 and ZT22 after light treatment (B).

Figure 17: Distribution of calpain immunoreactivity in the SCN.

Representative photomicrographs of serial sections from the SCN arranged from the rostral to caudal ends. Each section is 30µm thick. Immunoreactivity for Calpain-1 is seen in the entire SCN region throughout the rostrocaudal extent. Denser immunoreactivity is seen in the mid-caudal sections. OC - optic chiasm, 3V- third ventricle. Magnification for all images x100.

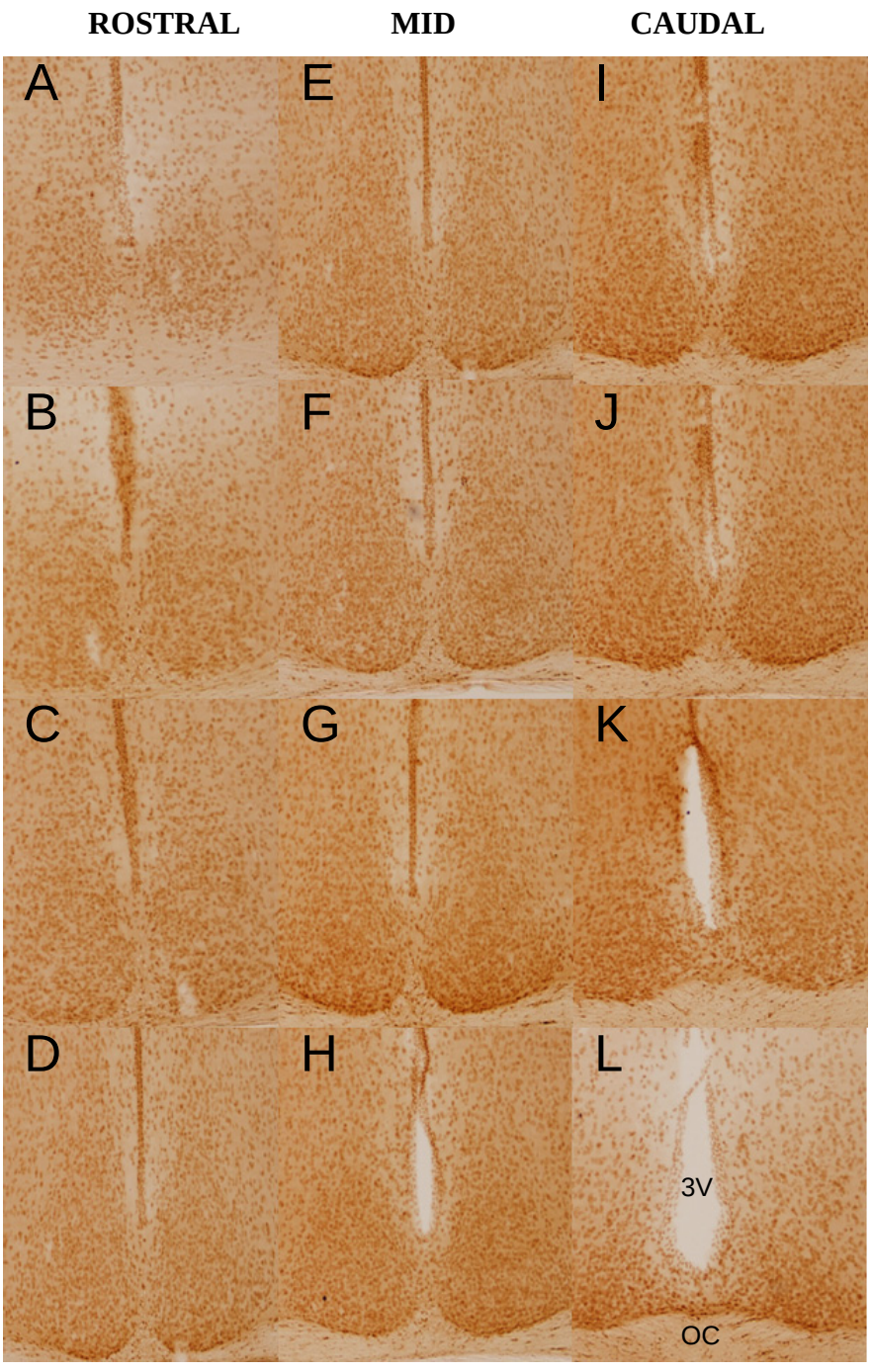
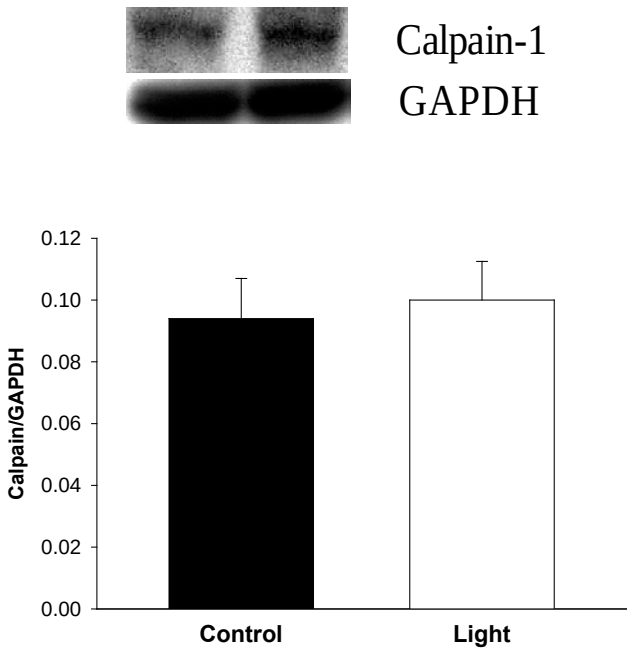

ROSTRAL
MID
CAUDAL
A
E
I
B
F
J
C
G
K
D
H
L
3V
OC

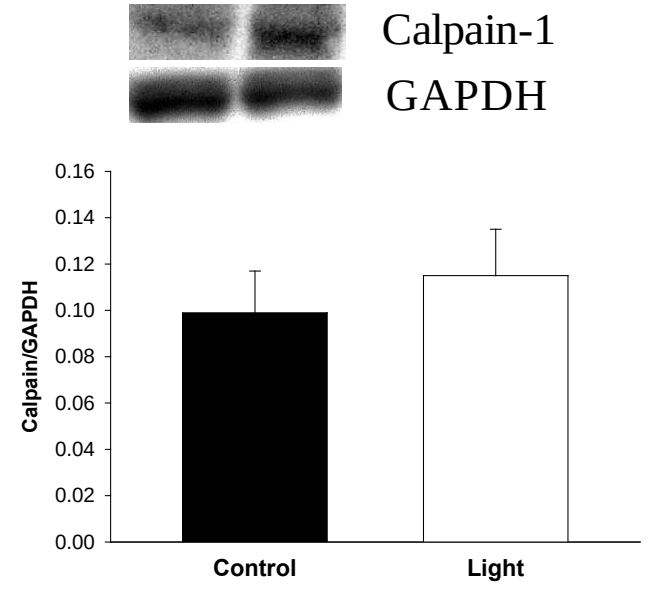
Figure 18 A-C: Effect of light on calpain expression in the SCN.

Calpain expression levels at (A) ZT16 (B) ZT22 and (C) ZT6 following a 20 min light pulse of 100 lux intensity. Upper panel in all figures are representative Western blot bands for calpain-1 and GAPDH. Lower panel bar graphs represent relative densitometric ratio of calpain-1 normalized to GAPDH. At all time points, calpain-1 expression did not show any change. No change in expression of calpain-1 was observed following light exposure at the three points. Values are represented as mean±S.E.M, n=3/group.

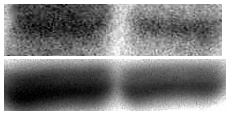
A


Calpain-1
GAPDH
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Calpain/GAPDH
Control
Light

B


Calpain-1
GAPDH
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Calpain/GAPDH
Control
Light

C



Calpain-1

GAPDH

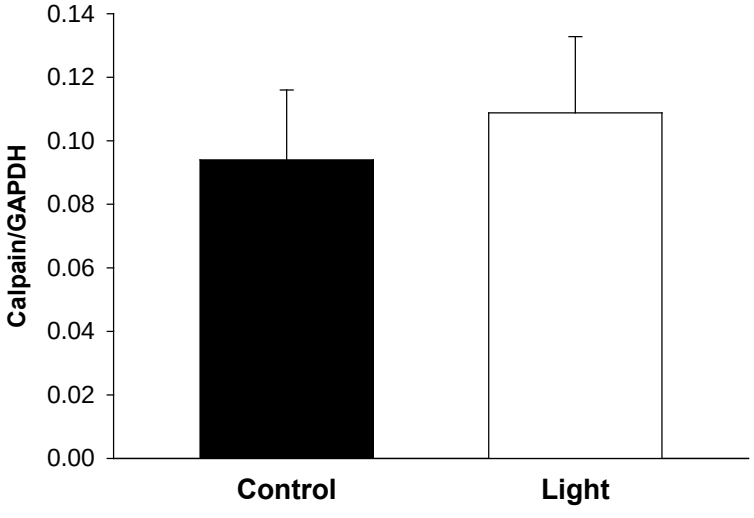
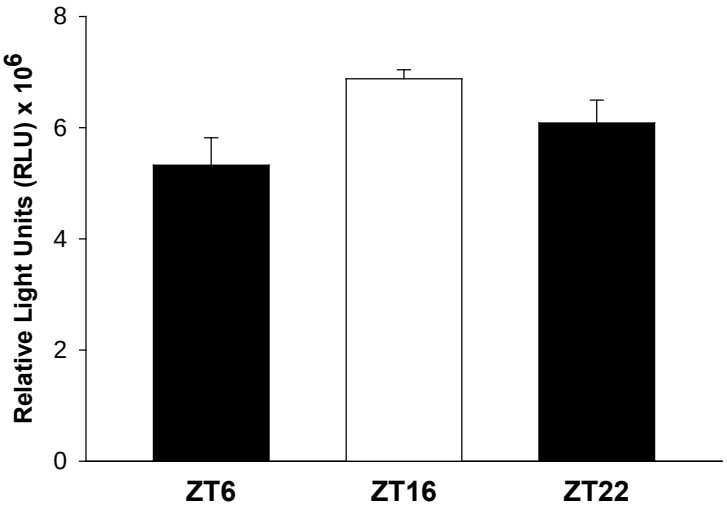


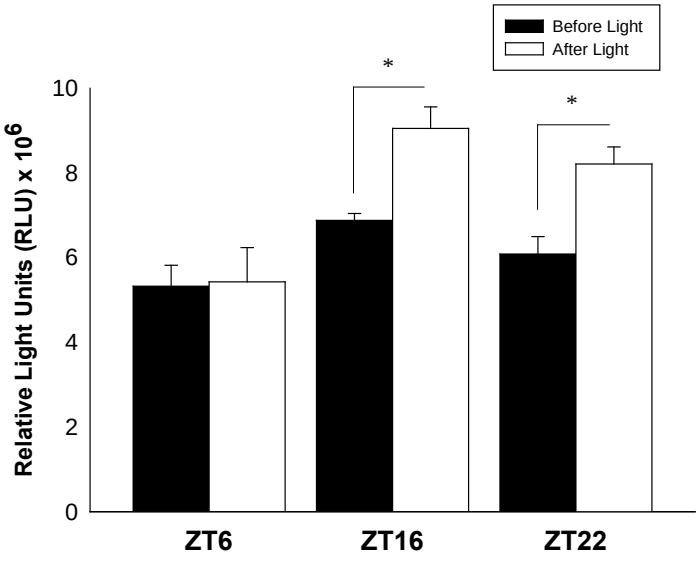
Figure 19 A-B: Effect of light on calpain activity in the SCN.

Calpain activity was measured using a luminescent assay based on a proluminescent calpain substrate. Values are represented as relative light units. A - calpain activity in the SCN at circadian time points ZT16, ZT22 and ZT6. B - Calpain activity in the SCN following a light pulse at ZT16, ZT22 and ZT6. Basal calpain activity was the greatest at ZT16 and the lowest at ZT6. At ZT16 and ZT22 calpain activity increased following light exposure. At ZT6 there was no change in calpain activity following light exposure. Values are presented as mean±S.E.M., n=3/group, *$p<0.05$.

A


Relative Light Units (RLU) x 10^6
8
6
4
2
0
ZT6
ZT16
ZT22

B


Before Light
After Light
*
*
Relative Light Units (RLU) x 10^6
10
8
6
4
2
0
ZT6
ZT16
ZT22

Discussion

Light-induced phase shifts of the circadian clock are initiated with the release of glutamate in the RHT. Glutamate release mainly stimulates NMDA receptors in the SCN causing an increased Ca^{2+} influx that initiates a cascade of intracellular signaling events and gene expression ending in phase shift of the circadian clock. Circadian variations in the magnitude of NMDA-induced Ca^{2+} events have been proposed in the SCN neurons (Colwell, 2001; Ikeda et al., 2003; Pennartz et al., 2002). Circadian rhythms in cytosolic Ca^{2+} have also been observed in cultured SCN neurons (Ikeda et al., 2003). Ryanodine receptors contribute to the amplification of cytosolic Ca^{2+} via mobilization of Ca^{2+} from internal stores. This Ca^{2+} induced Ca^{2+} release has been proposed as the mechanism for light-induced phase delays during early subjective night (Ding et al., 1998). Thus, the evidence indicates that Ca^{2+} plays a central role in circadian photic resetting. The goal of this study was to explore the role of calpain, a calcium-dependent protease in the photic signaling pathway.

Calpain-1 immunoreactivity was distributed in the entire SCN. This was the expected finding since both calpain-1 and calpain-2 are ubiquitous. The interesting finding was that the immunoreactivity was denser in the mid-caudal region of SCN. The canonical clock proteins, mPER1 and mPER2 while expressed uniformly in the rostral SCN, are concentrated in the shell regions of mid-SCN (Yan and Silver, 2004). It is possible that the pattern seen with calpain-1 distribution might be of functional significance in circadian photic signaling.

The basal expression levels of calpain-1 did not show a change at any of the three circadian points. Also, no significant change in calpain-1 expression was observed after

light pulse treatment. Calpain-1 does not appear to have a circadian expression profile. Calpain being a Ca^{2+} dependent protease, its activity might be regulated by the cytosolic Ca^{2+} rhythms in the SCN. Moreover, the antibody used for Western blotting (Calpain-1, domain IV, Abcam, Cambridge, MA) detected both activated and latent forms of calpain. This proposed hypothesis is supported by our calpain activity data. We found a trend in calpain activity levels in the SCN. The activity was highest at ZT16 and the lowest at ZT22. A complete circadian profile of calpain activity will be able to confirm this trend. We have demonstrated that light significantly induces calpain activity at both ZT16 and ZT22, the time period when SCN is sensitive to light. There was no change in calpain activity following the light pulse at ZT6, the time when the SCN is insensitive to light. Collectively, our results indicate that calpain might have a role in the modulation of light-induced phase shifts of circadian clock. The exact mechanism warrants future studies.

CHAPTER 6 – SUMMARY

It is nearly half a century now, since the phenomenon of light-induced photic resetting in mammals was first discovered. In the last 5 decades our understanding of circadian rhythms have grown tremendously; many “clock genes”, several signal transduction pathways, and presence of “peripheral clocks” have been identified but, the enigma of bi-directional response of the circadian clock to light remains unsolved. The overall goal of this investigation was to explore the potential role of some known modulators of neural plasticity in the light-induced resetting of the circadian clock. To achieve this goal the investigation had the following specific aims: (1) Investigate the role of extracellular serine protease tPA in the modulation of circadian clock resetting. (2) Investigate the role of Cdk5 and its activators in circadian clock resetting. (3) Investigate the role of cysteine protease, calpain in the photic resetting of the circadian clock.

In our first set of experiments (Chapter 3), we demonstrate that light induces the expression of tPA in the SCN. The increased expression is associated with a corresponding increase in its proteolytic activity. This finding is similar to the one seen in the hippocampus where tPA expression was induced in an immediate-early gene fashion following stimuli that evoke kindling, seizure or kainic acid injection (Nagai et al., 1999; Qian et al., 1993; Salles and Strickland, 2002). The finding is further confirmed in our behavioral experiments where we demonstrate that injection of tPA-STOP™, an inhibitor of tPA, can attenuate the light-induced phase delay of circadian wheel running activity. To our knowledge, this is the first observation to report the role of an extracellular protease in circadian photic resetting.

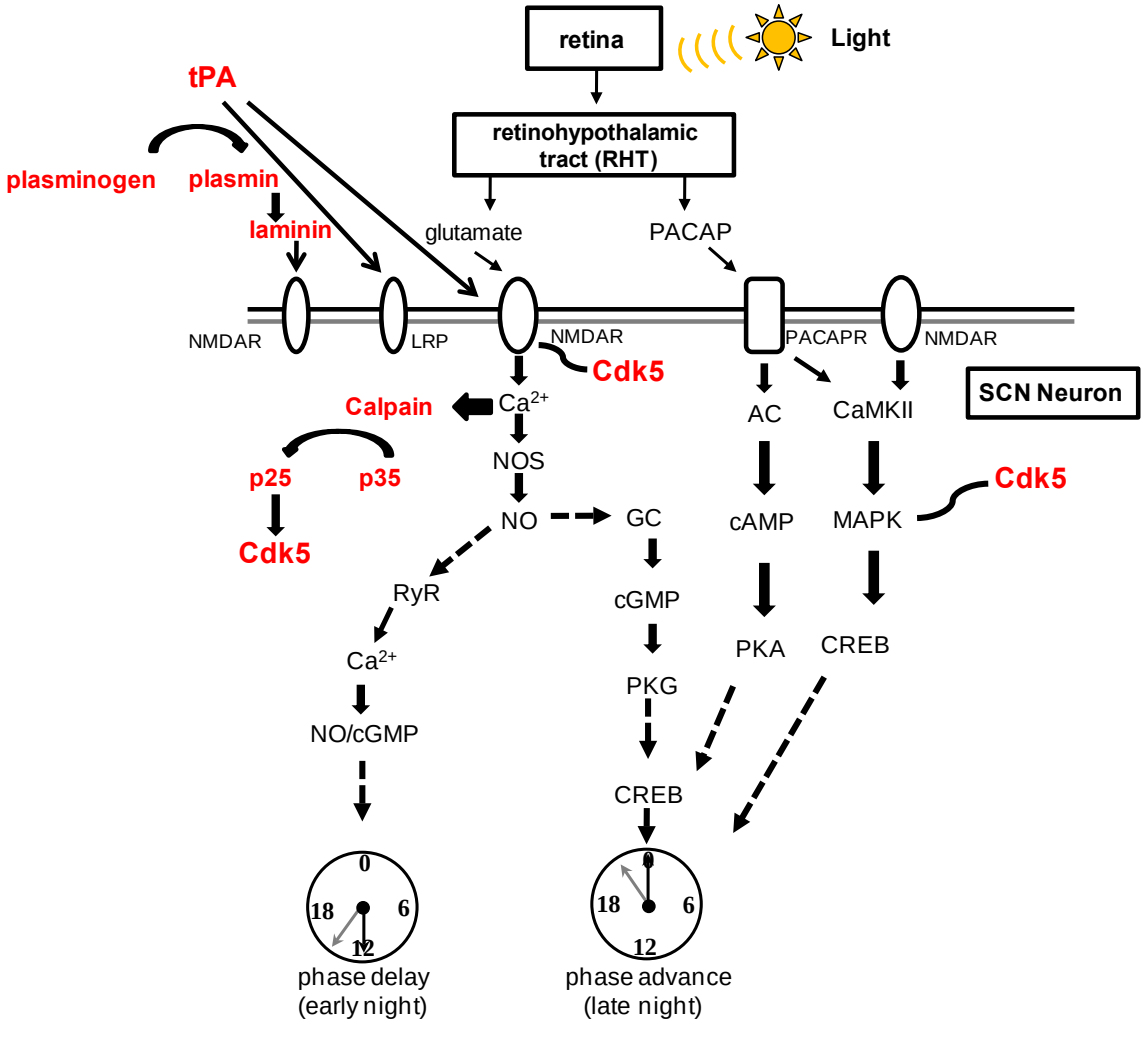
The second series of experiments (Chapter 4) tested the hypothesis that Cdk5 and its activators modulate light-induced resetting of the circadian clock. We provide histological, biochemical, functional and behavioral evidences that prove this hypothesis. This is also the first report of the role of Cdk5 in the SCN.

Thirdly, we provide evidence that the Ca^{2+} dependent protease, calpain might have a role in circadian resetting.

To summarize, we have identified three new candidates, two proteases and a kinase that has a role in modulating circadian photic resetting. We propose a model (Fig. 20) that integrates the various mechanisms by which these modulators of neuronal plasticity might modulation circadian photic resetting. Extracellular serine protease, tPA might mediate its action through (1) plasmin mediated interaction of laminin and NMDA receptor. (2) tPA can also directly cleave the NR1 subunit of NMDA receptor and thereby potentiating its signaling (3) tPA can also bind to the LRP receptor and after being endocytosed potentiate PKA signaling. The cysteine protease, calpain is activated following the calcium influx. Activated calpain can cleave the Cdk5 activator, p35 to p25 which is more stable than p35. Stable p25 produces transient activation of Cdk5 in the SCN. Activated Cdk5 might modulate circadian photic resetting by directly interacting with NMDA receptor signaling or by modulation of MAPK activity. These are just a few suggested mechanisms by which these modulators can integrate circadian photic signaling. Additional work will be required to determine the precise cellular signaling events that underlie the modulation of circadian photic signaling.

Figure 20: Proposed model integrating the modulators of neuronal plasticity in the putative photic signal transduction pathway in the SCN.

Photic signal transduction in the SCN is initiated by the release of glutamate and PACAP in the RHT, activating a series of intra-cellular signaling cascades, induction of clock genes resulting in phase shifts of the circadian clock. Extracellular serine protease tPA might modulate glutamate signaling by (1) plasmin mediated interaction of laminin and NMDA receptors (2) directly interacting with NMDA receptors or (3) direct interaction with LRP receptors. Calcium influx resultant to NMDA receptor activation activates calpain which in turn cleaves the Cdk5 activator, p35 to p25. Stable p25 causes transient activation of Cdk5 which might modulate NMDA receptor mediated signaling by direct interaction with the NMDA receptors or by modulation of MAPK activity. (NOS- Nitric oxide synthase, NO-Nitric Oxide, GC- Guanylyl cyclase, cGMP-cyclic GMP, PKG-Protein kinase G, CREB-cAMP response element binding protein, RyR-ryanodine receptor, AC-Acetyl choline, cAMP-cyclic AMP, PKA-Protein kinase A, CaMKII- calmodulin-dependent kinase II, MAPK-Mitogen activated protein kinase, LRP-Low-density lipoprotein receptor related protein).



Adapted from Hirota & Fukada (2004)

Several recent studies have implicated circadian rhythm disturbances associated with an increased risk of cancer development, metabolic and cardiovascular disturbances. Recently, light therapy has been proposed for some sleep disorders. A better understanding of circadian rhythm resetting may lead to the development of new treatment modalities.

Future Studies

To test the proposed model it is necessary to identify the downstream targets of both tPA and Cdk5. Recent studies from our lab have in fact identified the presence of LRP receptors in the SCN. Electrophysiological studies employing brain slice preparation will help delineate the exact mechanism of action of tPA. Suggested mechanisms indicate that Cdk5 can modulate circadian photic signal transduction pathways involved in both phase delay and phase advance of the clock. Pharmacological modulation of Cdk5 activity as a potential therapy for circadian rhythm disturbances is an interesting research idea that needs further exploration.

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APPENDIX A: RESEARCH STATEMENTS

Responsible Conduct of Research

The ECU Research Ethics Oversight Committee oversees all research compliance activities of the University under the direction of the Vice Chancellor for Research, Economic Development, and Community Engagement. The committee reviews, approves, and resolves conflicts of interest issues and reviews and investigates cases of scientific misconduct for individual faculty and the University. A copy of policy regarding scientific ethics and misconduct (Part VII section VI of the ECU Faculty Manual) is given to each faculty member upon employment. This policy is updated regularly on the ECU web site. The University's "Policy and Procedures on Ethics in Research and Creative Activity" constitutes institutional efforts to promote responsibility by faculty, staff, post doctoral students, and students "to seek honestly and to promulgate ethically the truth in all phases of work." The Policy provides, among other things, a list of principles to which the University subscribes in its research activities, and procedures for reporting, investigating, and determining penalties for unethical research activities (Part 7(VI)).

The Bioethics Center, a joint program of BSOM and PCMH, provides educational opportunities for faculty, students, physicians, and hospital staff and supports the efforts of hospital committees charged to address ethical issues, including those dealing with research. The center sponsors bioethics conferences, workshops, and lectures and is staffed by faculty of the Department of Medical Humanities. The University's "Principles and Policy for the Protection of Human Subjects of Research" establishes

"responsibilities for protecting the rights and welfare of individuals who act as subjects for research conducted by its [principle investigators]." The Policy provides a Statement

of Ethical Principles and implements these Principles through the University Medical Center Institutional Review Board (Part 7(1 V)).

The University and Medical Center Institutional Review Board (UMCIRB) reviews and approves of any research, including clinical trials, involving the use of human subjects. The board protects the rights and welfare of human subjects engaged in research at BSOM, PCMH and its affiliates, and ECU, and in research conducted elsewhere by representatives of the University in connection with their responsibilities.

The University's Policy on "Animal Care and Use in Research and Instruction" becomes another instance of institutional efforts to ensure that "animals used in research and teaching will receive humane treatment at all times," and to comply fully with applicable federal laws and regulations. The Policy provides specific responsibilities required by faculty members who conduct or supervise the conduct of animal experimentation. The Policy, moreover, establishes the Animal Care and Use Committee with the authority formally to monitor the care and use of vertebrate animals (Part 7(V)).

Statement on the Care and Use of Animals in Research

All animal research at East Carolina University meets the requirements and guidelines of the NIH Office of Animal Care and Use. The animal facilities at East Carolina University are managed under the direction of the Department of Comparative Medicine and are AAALAC and IACUC accredited.

APPENDIX B: ANIMAL CARE AND USE COMMITTEE PROTOCOL APPROVAL

EAST CAROLINA UNIVERSITY

Animal Care and Use Committee
East Carolina University
212 Ed Warren Life Sciences Building
Greenville, NC 27834
252-744-2436 office • 252-744-2355 fax

August 1, 2006

Jian Ding, Ph.D.
Department of Physiology
Brody 6N-98
ECU Brody School of Medicine

Dear Dr. Ding:

Your Animal Use Protocol entitled, "Dysregulation of circadian rhythm by HIV protein Tat," (AUP #Q201a) was reviewed by this institution's Animal Care and Use Committee on 8/1/06. The following action was taken by the Committee:

"Approved as submitted"

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure

Animal Care and Use Committee
East Carolina University
212 Ed Warren Life Sciences Building
Greenville, NC 27834
252-744-2436 office • 252-744-2355 fax

August 9, 2006

Jian Ding, Ph.D.
Department of Physiology
Brody 6N-98
ECU Brody School of Medicine

Dear Dr. Ding:

Your Animal Use Protocol entitled, "Role of Circadian Clock Genes in the Cardiovascular System," (AUP #Q233) was reviewed by this institution's Animal Care and Use Committee on 8/8/06. The following action was taken by the Committee:

"Approved as submitted"

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure