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I am submitting herewith a thesis written by Aneta Jovanovska entitled "Transcriptional and Translational Inhibitors Block Serotonergic Phase Advances of the SCN Circadian Pacemaker *In Vitro*" I have examined the final copy of this thesis for form and content and recommend that is accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry and Cellular and Molecular Biology.

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Transcriptional and Translational Inhibitors Block Serotonergic Phase Advances of the SCN Circadian Pacemaker *in Vitro*

A Thesis Presented for the
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Aneta Jovanovska
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DEDICATION

This thesis is dedicated to my parents, who raised me,
Petar Jovanovski and Vera Jovanovska
for all their love, support and devotion.

I would also like to thank two very special friends
Mr. & Mrs. Sam Mars Jr.
who have supported and encouraged me in my education

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ABSTRACT

The mammalian circadian pacemaker is located in the suprachiasmatic nucleus (SCN). Phase shifts of the pacemaker are modulated by various inputs. The input that we investigated is the serotonergic (5HT) input from the raphe nuclei. 5HT phase-advances the SCN pacemaker when applied during mid-subjective day. *In vitro* studies indicate that 5HT phase-advances the mammalian circadian pacemaker through a process that includes stimulation of 5-HT_7 receptors, activation of protein kinase A, and opening of K^+ channels. How these cytoplasmic and membrane events translate into a shift in the molecular core of the circadian oscillator is not known. To further understand this process, we investigated whether serotonergic phase advances require protein synthesis. Using two reversible translational inhibitors, anisomycin and cycloheximide, we show that inhibition of protein synthesis blocks 5HTergic phase shifts. We further show that a transcriptional inhibitor, 5,6-dichloro-1-β-ribobenzimidazole (DRB), also blocks the 5HTergic phase shifts in the SCN circadian pacemaker. These results are similar to those found previously with respect to 5HTergic modulation of the *Aplysia* ocular circadian clock, and suggest that 5HT may phase-shift the SCN pacemaker through increasing transcription and translation of specific proteins.

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LIST OF ABBREVIATIONS

5HT	5-hydroxytryptamine
bHLH	basic-helix-loop-helix
CHX	cycloheximide
cry	cryptochrome gene
DPAT	8-hydroxy-dipropylaminotetralin HBr
DRB	5,6-dichloro-1-β-ribobenzimidazole
GABA	γ-amino butyric acid
IEGs	immediate early genes
JNK	c-Jun NH_2-terminal kinase
MAPK	mitogen-activated protein kinase
NPY	neuropeptide Y
per	period gene
PER	period protein
PKA	protein kinase A
RHT	retinohypothalamic tract
SCN	suprachiasmatic nucleus
TCA	trichloroacetic acid

Chapter I

Background and Significance

The Suprachiasmatic Nucleus and Circadian Rhythms

Circadian rhythms are endogenously driven rhythms in behavior and physiology with a period length of about 24 hours. In addition, circadian rhythms appear to be ubiquitous to all eukaryotes and even in some prokaryotes; their period length is temperature-compensated and varies little at different ambient temperatures; and environmental signals, mainly light and temperature changes, reset the phase to entrain the rhythm to exactly 24 hours (Reppert and Weaver, 2000). Notable examples of circadian rhythms include the sleep-wake cycle, food and water intake, locomotor activity, body temperature, hormonal secretion, and psychomotor performance functions (Rivkees and Hao, 2000). Although these rhythms parallel environmental cycles of light and dark, they are not simply a reaction to environmental fluctuations, but are generated by an endogenous timekeeping mechanism called the circadian clock. This biological clock consists of three components: an entrainment pathway that transmits environmental signals to the timekeeping apparatus; a timekeeping apparatus, or 'oscillator' which operates in the absence of environmental cues and is the core component of the circadian clock; and output pathways by which the pacemaker regulates its various output rhythms (Hardin, 2000; Moore, 1997).

In mammals, a bilaterally paired structure, the suprachiasmatic nuclei (SCN), is considered to be the master circadian clock (Miller et al., 1996; Edery, 2000). The SCN is located above the third ventricle at the base of the optic chiasm in the anterior hypothalamus (Rivkees and Hao, 2000). Synchronization of the multiple, cell-

autonomous circadian clocks within the SCN leads to coordinated circadian outputs that regulate expressed rhythm(Shearman et al., 2000; Tomioka, 2000). Anatomically, the SCN is divided into two main subdivisions: ventrolateral and dorsomedial (Miller et al., 1996). The great majority of SCN neurons contain the inhibitory neurotransmitter γ-aminobutyric acid (GABA), while other peptides are more restricted in their distribution, including arginine-vasopressin, in the dorsomedial SCN, and vasoactive-intestinal peptide, in the ventrolateral part of the SCN (van den Pol, 1991). There are three major input pathways leading to the SCN, all of which terminate in the ventrolateral division of the SCN. Photic information is transported directly from the retina via the retino-hypothalamic tract (Moore, 1997); there is also neuropeptide Y (NPY) input from the intergeniculate leaflet through the geniculo-hypothalamic tract (Moore and Card, 1990; Morin and Moore, 1992); and serotonergic (5-hydroxytryptamine, 5HT) input from the raphe nuclei (Moore et al., 1978; Morin, 1994).

Interacting Molecular Loops in the Mammalian Circadian Clock: Presence of Clock Genes in the SCN

In recent years, there has been extraordinary progress in elucidating the molecular components of the mammalian circadian clock system. The discovery of circadian clock genes in lower organisms (such as fruit flies and fungi), which show many similarities with clock genes in mammals, together with advances in mouse molecular genetics have led to major new discoveries on the molecular and genetic basis of mammalian circadian rhythms.

In its simplest form, the molecular core of the circadian clock in mammals consists of autoregulatory transcriptional and translational negative feedback loops that have both positive and negative elements (Dunlap, 1999). Although not all components of the circadian clock oscillate, interactions among these genes and the proteins encoded by them are essential for the transcription-translation feedback loop to exist. The two positive components of this feedback loop are the *Clock* gene and a heterodimeric binding partner, *Bmal1*, each of which contain bHLH (DNA-binding) and PAS (protein-dimerization) domains. According to the negative feedback loop model, the proteins encoded by these two genes dimerize through the bHLH and/or PAS domains, bind regulatory DNA sequences (E-boxes) and activate transcription of another set of genes. These genes, *per1, per2, per3*, *cry1*, and *cry2* comprise the negative aspect of the feedback loop. The proteins encoded by these genes, translocate to the nucleus following a delay involving translation, phosphorylation, and heterodimerization. Once in the nucleus, they negatively affect the transcription of their own genes by interfering with CLOCK-BMAL1 activity. In time, the level of proteins in the negative feedback loop declines (due to posttranscriptional processes), their negative feedback is reduced, and the CLOCK-BMAL1 complex can begin transcription all over again (Yagita et al., 2000; Shearman et al., 2000; Albrecht et al., 1997; Vitaterna et al., 1994; Oishi et al., 2000; van der Horst et al., 1999; Vitaterna et al., 1999; Kume et al., 1999). This represents one circadian cycle of the clock. The periodic activities of these clock genes are the essence of the feedback loop. A simplified model for the clock mechanism in mammalian SCN pacemaker cells is shown in Figure 1.

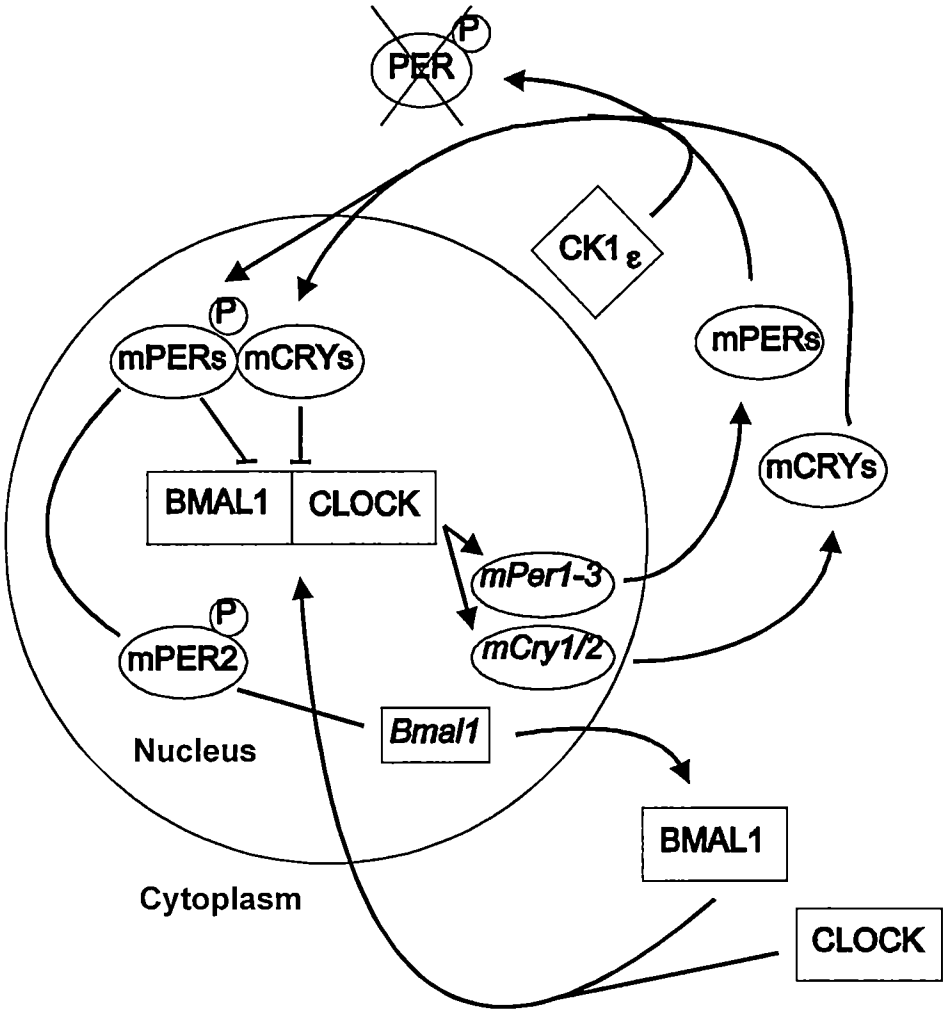


Figure 1. The circadian oscillator mechanisms in mammals: Simplified model for the clock mechanism in SCN pacemaker cells. Arrows indicate positive regulation and lines ending in bars denote negative regulation. Gene symbols are as described in the text.

Gene Induction as the Basis of Resetting the SCN Clock

The function of light in circadian rhythms is to maintain synchrony of the circadian pacemaker with the environment. It accomplishes this through specific pathways and cellular mechanisms. The effect of light is dependent on time of day, with light presented during early night delaying the clock, and light encountered during late night advancing it (Moore, 1997). Environmental light signals are relayed to the SCN via the RHT (Cermakian and Sassone-Corsi, 2000). Activation of ionotropic glutamate receptors on SCN cells initiates a cascade of events leading to a phase shift of the clock. Light pulses that reset the clock also induce the expression of a number of immediate-early genes (IEGs), including *c-fos* and *jun-B* in the retinorecipient part of the SCN (Beaule and Amir, 1999; Guido et al., 1999; Edelstein et al., 2000), as well as clock genes such as *mPer1* and *mPer2* (Shigeyoshi et al., 1997; Zylka et al., 1998; Miyake et al., 2000; Albrecht et al., 1997; Shearman et al., 1997). In addition, the degree of induction of *mPer1* and *mPer2* gene expression is dependent on the phase of the clock and correlated with the size of the phase shift (van Esseveldt et al., 2000). While *mPer1* is induced by light throughout circadian night (parallel to IEG induction), *mPer2* is induced only by light pulses delivered in the earlier part of the night (Miyake et al., 2000). *mPer3* does not appear to be light-regulated (Zylka et al., 1998). Therefore, the transcriptional responses of the SCN to light, which are part of the input to the oscillator, vary with circadian phase.

Conversely, recent findings demonstrate that several other stimuli, such as melatonin secretion, locomotor activity, NPY and 5HT, phase-shift the circadian clock during the subjective day rather than at night. Collectively referred to as 'non-photic

stimuli, these inputs may modulate circadian clock phase by having opposite effects on clock genes relative to light. For example, confinement of hamsters to a running wheel that generally elicits considerable activity and arousal and phase-shifts the clock, results in an acute down-regulation of *Per1* and *Per2* mRNA levels in the SCN (Maywood et al., 1999). In addition, it has been reported that systemic administration of a serotonin agonist also down regulates *hPer1*/h*Per2 m*RNA levels in the hamster SCN (Horikawa et al., 2000). Thus, the *period* genes are a common target for both photic and nonphotic resetting cues. Their sensitivity to nonphotic resetting supports their proposed role as core elements of the circadian oscillator. Moreover, these studies provide a possible explanation at the molecular level for the effects of photic and nonphotic cues on the clock.

Effects of Protein Synthesis Inhibitors on Circadian Pacemakers

Experimental work in a wide variety of organisms suggests that protein synthesis is required for the function of the circadian oscillator. This conclusion is based upon two types of results: the identification of specific genes critical for circadian rhythm generation as outlined above, and the application of protein synthesis inhibitors that cause phase shifts or period changes in circadian rhythms. The range concentration for inducing phase shifts is the same as that which inhibits protein synthesis (Nakashima et al., 1981; Dunlap and Feldman, 1988). Among vertebrates, protein synthesis inhibitors phase shift circadian melatonin rhythms in chick pineal cells (Takahashi et al., 1989), and circadian activity rhythms in hamsters (Takahashi and Turek, 1987). Among invertebrates, translation inhibitors cause large phase shifts and change the period of the *Aplysia* ocular rhythm (Jacklet, 1980; Yeung and Eskin, 1988; Lotshaw and Jacklet, 1986). In all these

cases, data suggest that about half of the circadian cycle is sensitive to inhibitors of translation, with maximal sensitivity occurring from about circadian time CT 18 to CT 6.

In addition to experiments using short pulses of protein synthesis inhibitors (< 6h), longer treatments with these inhibitors can arrest or stop the motion of the molluscan circadian pacemaker (Khalsa et al., 1992). In the mollusc *Bulla*, long-duration application of protein synthesis inhibitor cycloheximide (CHX) delays the phase of the subsequent rhythm, and the size of the delay is equal to how long the CHX treatment extends past subjective dawn. These experiments suggest that the motion of the circadian pacemaker is arrested during these long treatments and that the critical period for protein synthesis is near subjective dawn.

Similar studies have been done in mammals to study the effect of protein synthesis inhibitors on the SCN circadian pacemaker. In hamsters, injections of CHX or another reversible translational inhibitor, anisomycin, between CT 14 and CT 4 consistently caused phase delays of ~ 1 h in the activity rhythm, whereas injections at phases between CT 6 and CT 10 caused phase advances of a similar magnitude (Inouye et al., 1988). These results again suggest that proteins critical for mammalian circadian pacemaker activity are synthesized near subjective dawn or early subjective day. This is approximately the phase when *Per1* and *Per2* mRNA levels are reaching their maximum levels and PER1 and PER2 proteins are beginning to be synthesized.

In other experiments, pulses of protein synthesis inhibitors have been shown to block the induction of phase-shifts by light. For example, in the fungus *Neurospora*, treatment with CHX inhibits light-induced phase shifts (Johnson and Nakashima, 1990). Another invertebrate model used to study circadian rhythms is the mollusc, *Aplysia*. The

isolated eye of *Aplysia* exhibits a circadian rhythm in optic nerve firing (Jacklet, 1969). In *Aplysia* light pulses that phase-shift the ocular circadian pacemaker have been shown to regulate the expression of at least 11 proteins. Both the phase shifts by light and the protein induction can be reversed by protein synthesis inhibitors (Raju et al., 1990; Lotshaw and Jacklet, 1986; Jacklet, 1980). Similarly, serotonin, which can also phase shift the *Aplysia* ocular rhythm, can induce or suppress the expression of some of these proteins (Yeung and Eskin, 1987; Corrent et al., 1982; Koumenis et al., 1995; Zwartjes and Eskin, 2000). Because light and serotonin have opposite phase-shifting effects on the *Aplysia* circadian system, proteins that are affected in opposite directions by these entraining agents could be involved in resetting the *Aplysia* circadian oscillator. Several proteins have been identified that fall into this category and their potential involvement in circadian rhythm generation is undoubtedly being investigated further.

A necessary role for transcription in circadian pacemaker functioning has also been investigated. Many of these studies have used the reversible RNA synthesis inhibitor, 5,6-dichloro-1-ß-ribobenzimidazole (DRB). Pulses of DRB cause phase-dependent delays when applied between CT 20 and CT 10, and have no effect from CT 10 to CT 20 in the *Aplysia* eye (Raju et al., 1991; Koumenis et al., 1996). These DRB experiments suggest that a critical period for transcription of specific genes involved in the generation of circadian rhythms occurs from CT 20 to CT 10 in *Aplysia* ocular system. In *Bulla*, the data suggests that the sensitive phase for transcription extends throughout most of the subjective day (Khalsa et al., 1996). Thus, a common feature of both circadian systems is a phase-dependent requirement for transcription. Similar studies have not been done in mammals. Taken together, these studies are consistent with the

hypothesis that circadian transcription and translation of specific circadian clock genes is necessary for on-going functioning of the circadian clock. However, as in the case of translation, the precise role of transcription in the generation of the rhythm is still not clear. Transcription could reside either outside the oscillator loop, or be a part of the oscillator mechanism, or both. The latter, if true, would result in oscillating message levels as are found in the *per* gene in *Drosophila* (Hardin et al., 1990). The determination of the precise role of transcription in circadian timing (as of that of translation) will require the identification and study of the mRNAs important in the timing of the oscillator.

Serotonin Phase Resetting of SCN Pacemaker

The SCN in mammals receives robust serotonergic innervation from the midbrain raphe (Moore et al., 1978). Data show that raphe unit activity is highest during waking and lowest during the deepest stages of sleep (McGinty, 1976). The persistence of rhythms in 5HT content in the SCN in constant conditions suggests that the release of this major afferent neurotransmitter in the SCN is under circadian control. In contrast, 5HT content in the entire anterior hypothalamus seems to be driven primarily by the light/dark cycle (Ferraro and Steger, 1990).

The functional significance of the input from the raphe to the circadian system is controversial. To elucidate the role of 5HT function, many investigators have used the chemical and electrical destruction of the 5HT system (Levine et al., 1986; Honma et al., 1979). These treatments generally did not eliminate circadian rhythms, but the rhythms became more irregular with lower overall amplitude. Nonetheless, these experiment show

that 5HTergic input to the SCN has some sort of modulatory effect on the circadian pacemaker.

To further study the function of this projection in the mammalian circadian system, several labs switched to *in vitro* studies. The SCN circadian clock continues to function *in vitro*, generating 24 h rhythms in spontaneous neuronal activity. Prosser et al. (1990) found that quipazine (a non-specific 5HT agonist) could reset the phase of the rat SCN pacemaker *in vitro*. Treatments with both 5HT and quipazine advanced the phase of the clock when applied during the mid- subjective day and delayed the clock when applied during mid-subjective night. Similar results were obtained by other labs using a variety of 5HTergic agonists (Medanic and Gillette, 1992; Shibata et al., 1992b). *In vivo* studies using 5HT and 5HTergic agonists in both rats and hamsters generate similar results to those seen *in vitro*, i.e., daytime phase advances and small or no phase delays at night (Cutrera et al., 1994; Tominaga et al., 1992; Cutrera et al., 1996; Edgar et al., 1993).

Signal Transduction Processes Associated With Serotonergic Phase Shifts

Several *in vitro* studies have investigated the signal transduction processes through which 5HT phase-shifts the circadian clock (Prosser, 2000; Prosser et al., 1994a). Serotonin appears to phase-advance the mammalian circadian pacemaker through a process that includes stimulation of $5HT_7$ receptors, causing activation of adenylate cyclase and an increase in cyclic AMP, activation of protein kinase A, and opening of K^+ channels (see Figure 2). 5HT has also been shown to decrease *c-fos* mRNA in the SCN *in vitro* when applied during the subjective day (Prosser et al., 1994b), and to decrease *Per1* and *Per2* mRNA levels *in vivo* when applied during the day (Horikawa et al., 2000).

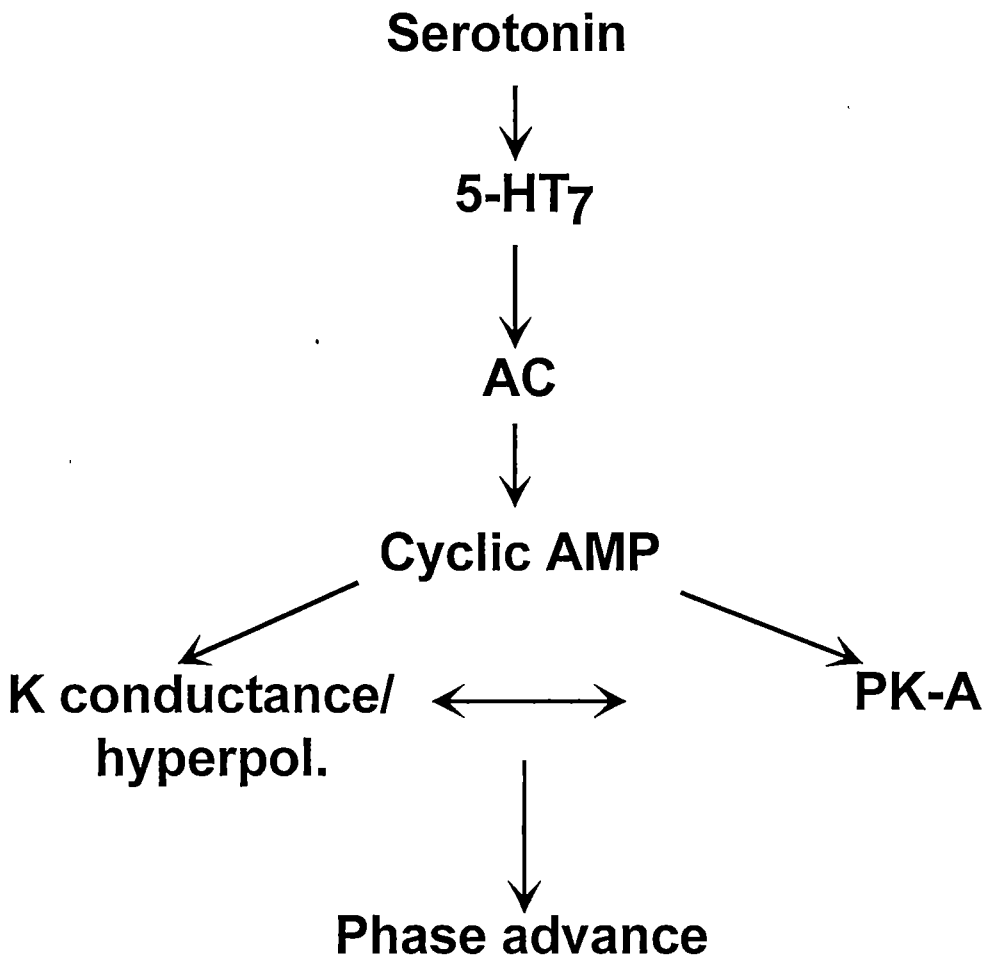


Figure 2. Working model of 5HTergic resetting of the mammalian circadian clock: Previous research suggests that 5HT advances the SCN clock through stimulating $5HT_7$ receptors, activating adenylate cyclase (AC), increasing cAMP levels and activating protein kinase-A (PK-A). Also involved in the phase advances is opening of K^+ channels. Here we are exploring other possible down-stream events linking 5HT stimulation to the core oscillatory loop of transcription/translation negative feedback, specifically whether transcription and/or translation are necessary steps in this signal transduction pathway.

Whether either of these changes are necessary for 5HTergic phase shifts is not known. Undoubtedly, many additional biochemical pathways are involved in 5HTergic advances of the mammalian circadian pacemaker.

In the *Aplysia* ocular system, 5HT also induces phase advances in the subjective day through activation of adenylate cyclase, an increase in cAMP, and an increase in K^+ channel conductance (Koumenis and Eskin, 1992; Eskin et al., 1982). Protein synthesis has also been implicated in the 5HT-entrainment pathway in *Aplysia* (Eskin et al., 1984) since the 5HTergic-phase advances are blocked by protein synthesis inhibitors (Eskin et al., 1989; Yeung and Eskin, 1988), and 5HT induces production of several proteins in the *Aplysia* eye (Zwartjes and Eskin, 2000; Koumenis et al., 1995). Anisomycin also blocked phase shifts induced by cAMP analogues (Eskin et al., 1982). This last result suggests that protein synthesis occurs after the cAMP step in the 5HT-entrainment pathway. In addition, 5HTergic phase shifts in the *Aplysia* circadian system are also blocked by application of DRB (Koumenis et al., 1996).

Rationale For This Study

The potential involvement of transcription and translation in 5HTergic phase shifts of the *Aplysia* circadian clock led us to hypothesize that 5HTergic phase advances of the mammalian circadian clock may also involve transcriptional and translational processes. To begin addressing this hypothesis, we investigated whether transcriptional and translational inhibitors block the phase shifts induced by 5HT in the SCN circadian pacemaker *in vitro*. Since the 5HT agonist (+) DPAT has been shown to mimic the daytime phase-shifts of 5HT, and it is selective for the $5HT_{1A}$ and $5HT_7$ receptors (the

later likely being the receptor involved in 5HT phase-advances) many of our experiments used (+) DPAT instead of 5HT.

Chapter II

Materials and Methods

Brain Slice Preparation

Coronal brain slices (500u) containing the SCN were prepared during the daytime from adult, male Sprague-Dawley rats housed in a 12:12 light-dark cycle. Slices were maintained in a Hatton-style interface brain-slice chamber, where they were perfused continuously with warm (37°C), oxygenated (95% O_2 / 5% CO_2) Earle's Balanced Salt Solution (EBSS; Sigma) supplemented with glucose and bicarbonate, and brought to pH 7.4 (Prosser, 1998).

Single Unit Recordings and Data Analysis

The spontaneous activity of single SCN neurons was recorded on day 2 *in vitro* using a glass microelectrode filled with 3M NaCl. Activity of each neuron was recorded for 5 min and the data stored using a Data*Wave* system. Throughout each hour of the recording process, between 4-6 SCN cells were recorded. The individual firing rates were then used to calculate two-hour running averages to obtain a measure of population neuronal activity, as described previously (Prosser, 1998). The time-of-peak was defined as the symmetrically highest point in the resulting curve. Phase shifts were calculated as the difference in time-of-peak in drug-treated slices vs. untreated slices. Student's *t*-test and ANOVAs were used to test for significant differences between treatment and control experiments.

Experimental Treatments

All drugs were bath applied on day 1 *in vitro* for 1 hour. During drug application, the normal perfusion was stopped and the medium in the chamber was replaced with

medium containing the appropriate test compound using a glass pipette. At the end of the hour, the treated medium was exchanged with normal medium and perfusion was resumed. For blocking experiments, the perfusion medium was first replaced with medium containing the blocking agent. After 15 min this solution was replaced for 1 h with medium containing both compounds. This was followed by another 15min treatment with medium containing the blocking agent, after which the treated medium was exchanged with normal medium and the normal perfusion was resumed. Chemicals used in this study include 5-hydroxytryptamine (5HT), 8-hydroxy-dipropylaminotetralin HBr ((+)DPAT), anisomycin and CHX (Sigma/Research Biochemicals) and 5,6-dichloro-1-β-ribobenzimidazole (DRB) (Calbiochem).

Protein Synthesis Measurements

To determine the effect of anisomycin on protein synthesis we measured the incorporation of [^{35}S] methionine/[^{35}S] cysteine into trichloroacetic acid (TCA)-precipitable material (Raju et al., 1990; Yeung and Eskin, 1988). Two groups of SCN brain slices (1 control and 1 experimental) were run in parallel. These brain slices were reduced in size from those used in recording experiments, so they consisted only of the SCN and adjacent optic chiasm. The experimental group was treated with anisomycin for 1 h beginning at zeitgeber time 6 (ZT 6, where ZT 0 = lights-on in the animal colony). Individual slices from each group were removed from the slice chambers at different time points before, during, and after anisomycin treatment. After each slice was removed from the bath, it was weighed, placed in a mixture of [^{35}S] methionine/[^{35}S] cysteine (Trans-^{35}S label, ICN) and DMEM (150uL radioactive label/450uL DMEM) and incubated for 1 h at 37°C. At the end of incubation, slices were homogenized for at least 2min in *RIPA*

buffer on ice and then centrifuged (*RIPA* buffer: *Tris*-HCl 10mM, Triton 0.5ml, Tween 0.5ml, and SDS 0.5ml). Three 10uL aliquots of supernatant were removed from each sample and placed on pieces of filter paper which were dried and then placed in boiling 10% TCA for 3-5min. The filter papers were then dried and placed in vials with 10ml scintillation fluid. Measurements of TCA-precipitable radioactivity were then made. Protein synthesis was expressed as radioactivity (counts per min) divided by wet tissue weight (ug).

Chapter III

Results

Single-Unit Recording Experiments: Serotonergic Phase Shifts

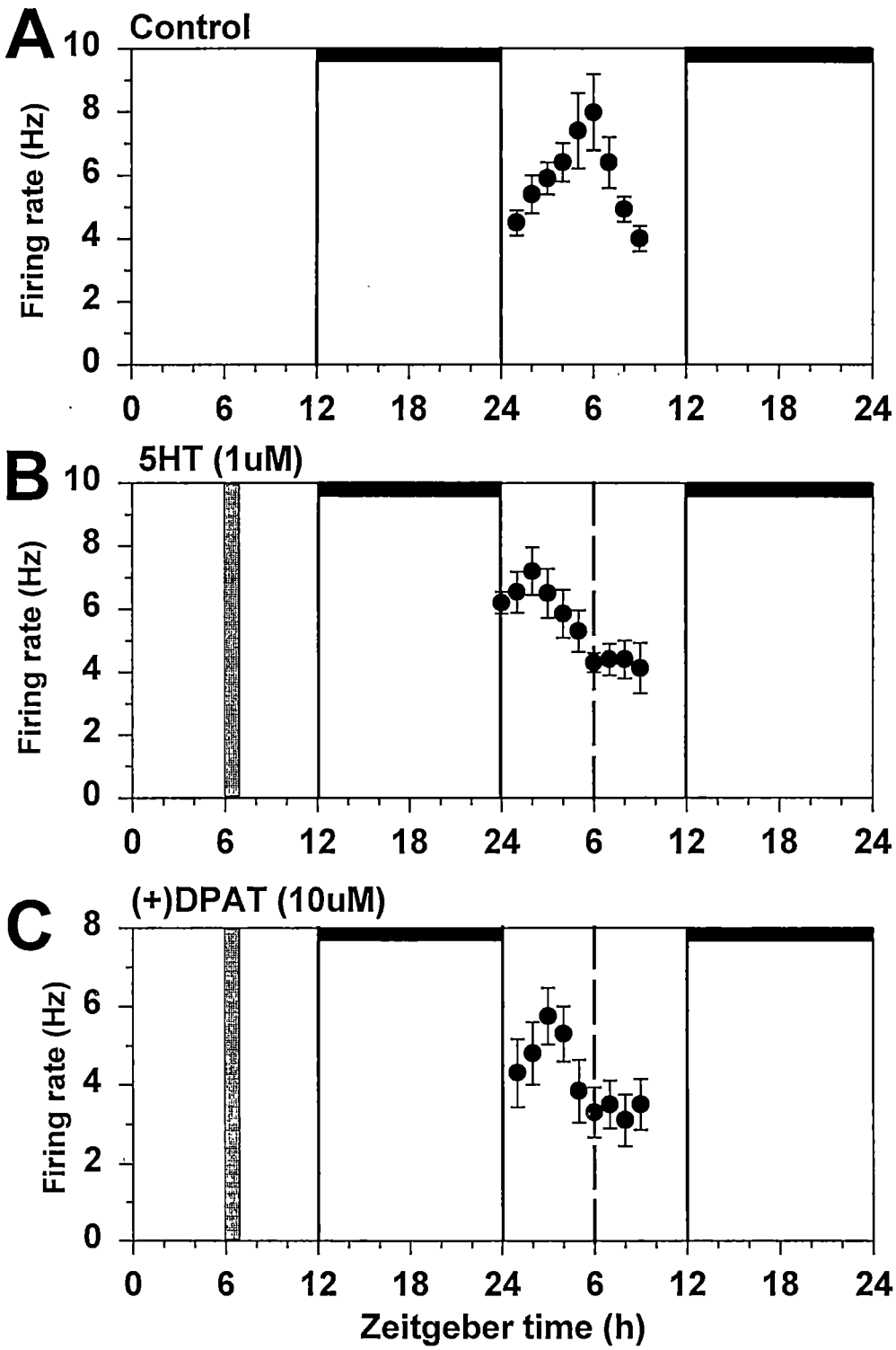
In control experiments, SCN neuronal activity peaked at mid subjective day (Fig.3). The mean (±S.E.M.) time-of-peak for all control experiments was ZT 6.0 ± 0.5 (n=4). Consistent with results obtained from previous studies (Medanic and Gillette, 1992; Shibata et al., 1992b; Prosser et al., 1993), both 5HT and its agonist (+) DPAT significantly advanced the time of peak activity when applied alone at ZT 6 (Fig.3). 5HT (1 and 10 uM) induced mean phase advances of about 4 h, while DPAT induced mean phase advances of about 3 h (10 uM) and 4 h (100 uM) (Table 1).

Protein Synthesis Inhibition

To examine the need for protein synthesis in 5HTergic phase shifting, two reversible translational inhibitors were used: anisomycin and cycloheximide. We began by measuring the amount and time course of protein synthesis inhibition induced by anisomycin at ZT 6. As shown in Fig.4, 20uM anisomycin decreased incorporation of TCA-precipitable radioactive label by 80% within the first half-hour of its application, and complete recovery of the percentage of total protein synthesis occurred within 30min of drug removal.

Next, we investigated the effects of anisomycin on SCN pacemaker phase in *vitro*. We found that the ability of anisomycin to phase-shift the SCN pacemaker was dependent on the circadian time of drug application. Consistent with previous results

Figure 3. *In vitro* circadian rhythm of neuronal activity. Shown here are the 2h means ±SEM of spontaneous neuronal activity recorded in single experiments A) Control experiment showing a peak in neuronal activity at ZT 6. B) 5-HT (1uM) induces a 4h phase advance when applied at ZT 6. C) Neuronal activity after application of DPAT (10uM) peaks near ZT 3, indicating a phase advance of about 3 h. Horizontal bar: lights-off in the animal colony. Vertical bar: time of drug treatment. Dotted line: mean time-of-peak in control experiments.


A
Control
Firing rate (Hz)
10
8
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4
2
0
0
6
12
18
24
6
12
18
24
B
5HT (1uM)
Firing rate (Hz)
10
8
6
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2
0
0
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12
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24
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24
C
(+)DPAT (10uM)
Firing rate (Hz)
8
6
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12
18
24
6
12
18
24
Zeitgeber time (h)

Table 1. Phase-shifting effects of various drugs bath-applied to SCN brain slices for 1 h at ZT 6.

Treatment	Number of experiments	Phase Shift (h)†
(+) DPAT (10uM)	5	2.7 ± 0.2 *
(+)DPAT (100uM)	3	4.17± 0.4 *
5HT (1uM)	3	3.9 ± 0.6 *
5HT (10uM)	3	4.3 ± 0.2 *
(+)DPAT (10uM)/Anisomycin (20uM)	3	0.25 ± 0.7
(+)DPAT (100uM)/Anisomycin (20uM)	4	1.18 ± 0.5
(+)DPAT (10uM)/Cycloheximide (10uM)	3	0.67 ± 0.3
5HT (1uM)/Anisomycin (20uM)	3	0.5 ± 0.3
5HT (10uM)/Anisomycin(20uM)	3	1.5 ± 0.4
(+)DPAT (10uM)/DRB (10uM)	3	1.17 ± 0.8
(+)DPAT (10uM)/DRB (100uM)	3	0.17 ± 0.2
Anisomycin (20uM)	4	0.68 ± 0.2
Cycloheximide (10uM)	5	2.2 ± 0.7 *
DRB (10uM)	3	0.0 ± 0.0
DRB (100uM)	4	1.32 ± 0.3

† relative to control (ZT 6.0 ± 0.5, n=4)
* $p < 0.05$ vs. control

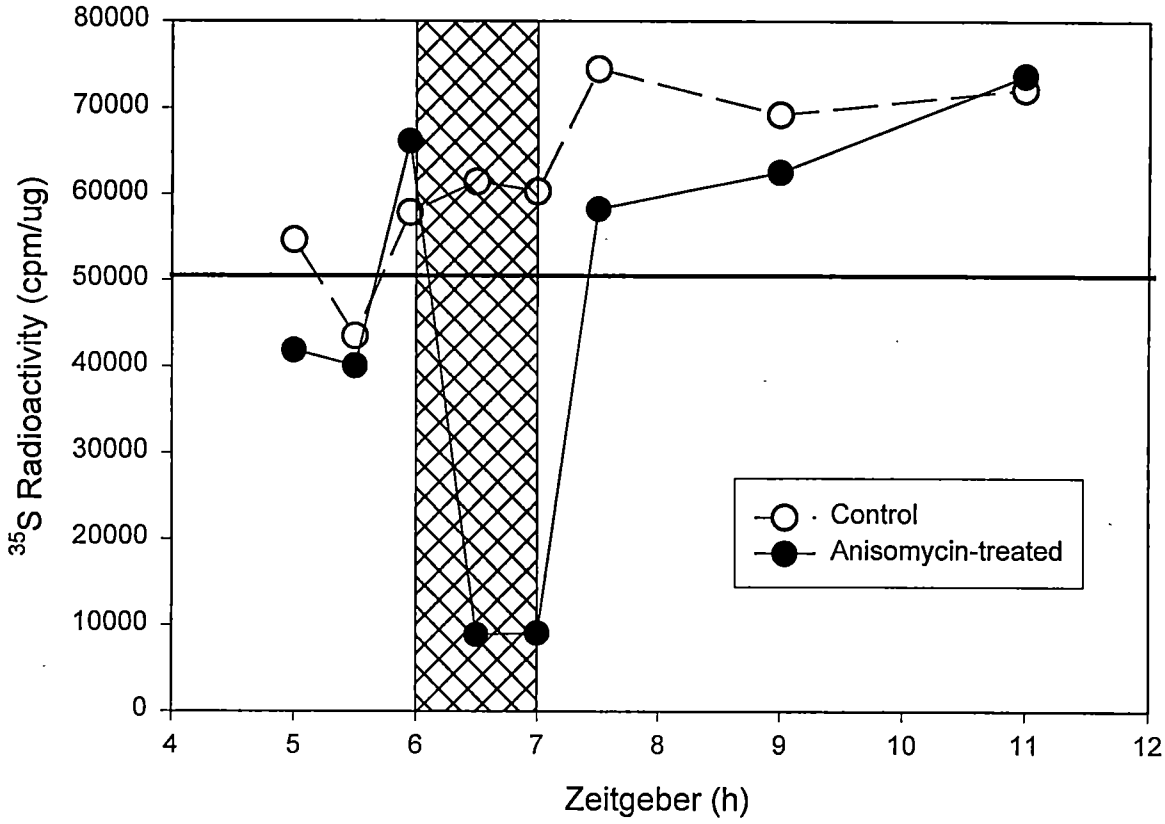


Figure 4. Protein synthesis inhibition and recovery in the SCN following treatment with anisomycin: Incorporation of [^{35}S]-methionine/[^{35}S]-cysteine into TCA precipitable material was assayed at various time points before, during and after treatment with anisomycin. The hatched area indicates the time of anisomycin treatment. Each value represents the mean of 3 replicates of TCA-precipitable radioactivity from a single SCN-containing brain slice (SEM are smaller than the symbols). Horizontal line = mean for all 6 slices assayed prior to anisomycin treatment.

(Shibata et al., 1992a; Inouye et al., 1988; Takahashi and Turek, 1987), anisomycin applied alone at ZT 2 induced a large phase delay (3.2 ± 1.1 h,n=3; $p<0.01$ vs. control), while anisomycin applied at ZT 6 did not induce a significant phase shift (0.68 ± 0.2 h, n=4; $p>0.1$ vs. control).

Next, we investigated the effect of co-applying anisomycin with (+) DPAT. 20uM anisomycin blocked the phase advances induced by both 10uM and 100 uM (+) DPAT (Fig. 5; Table 1). Anisomycin also blocked the phase shifts induced by 1 and 10 uM 5HT (Table 1).

To further investigate the effects of inhibiting protein synthesis, we tested the effects of a second translational inhibitor, cycloheximide. Cycloheximide blocked phase advances induced by 10 uM (+) DPAT at ZT 6 (Fig. 5; Table 1). Interestingly, when applied alone at ZT 6 cycloheximide induced a significant phase advance (Table 1).

Transcriptional Inhibition

Next we investigated the effects of transcriptional inhibition on the SCN pacemaker. As seen in Fig.6, DRB (100uM) application at ZT 6 induced a small phase advance. Overall, treatment of SCN slices with DRB (100uM) at ZT 6 induced a mean phase advance of 1.3 h (Table 1) which was not significantly different from control experiments. DRB application during late subjective day and early subjective night induced similar small phase advances. However, DRB induced large phase advances when applied during mid subjective night (ZT 18-19), and large phase delays during late subjective night (ZT 21; Fig. 6). The full phase response curve for DRB is shown in Fig. 7.

Figure 5. (+)DPAT-induced phase advances of the SCN neuronal activity rhythm are blocked by transcriptional and translational inhibitors. Shown are the 2 h means ± S.E.M. of neuronal activity from three different experiments. A) Co-application of anisomycin (20uM) with (+) DPAT at ZT 6 completely blocked the (+) DPAT-induced phase advance. B) Co-application of cycloheximide (10uM) blocked the phase-advance induced by (+) DPAT. C) Co-application of DRB (10uM) with (+) DPAT at ZT 6 also blocked the (+) DPAT-induced phase advance at ZT 6. See Fig. 3 for additional details.

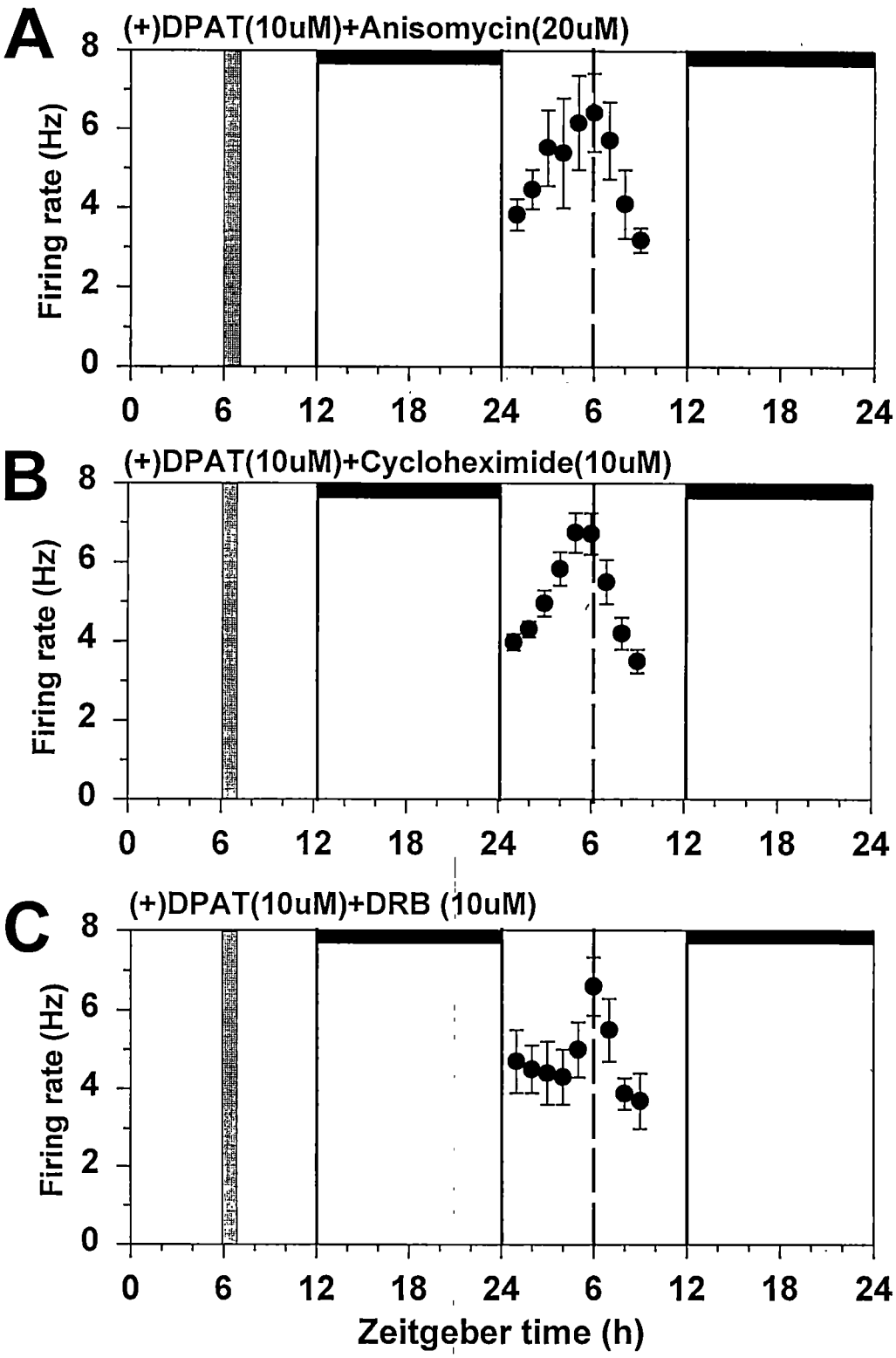
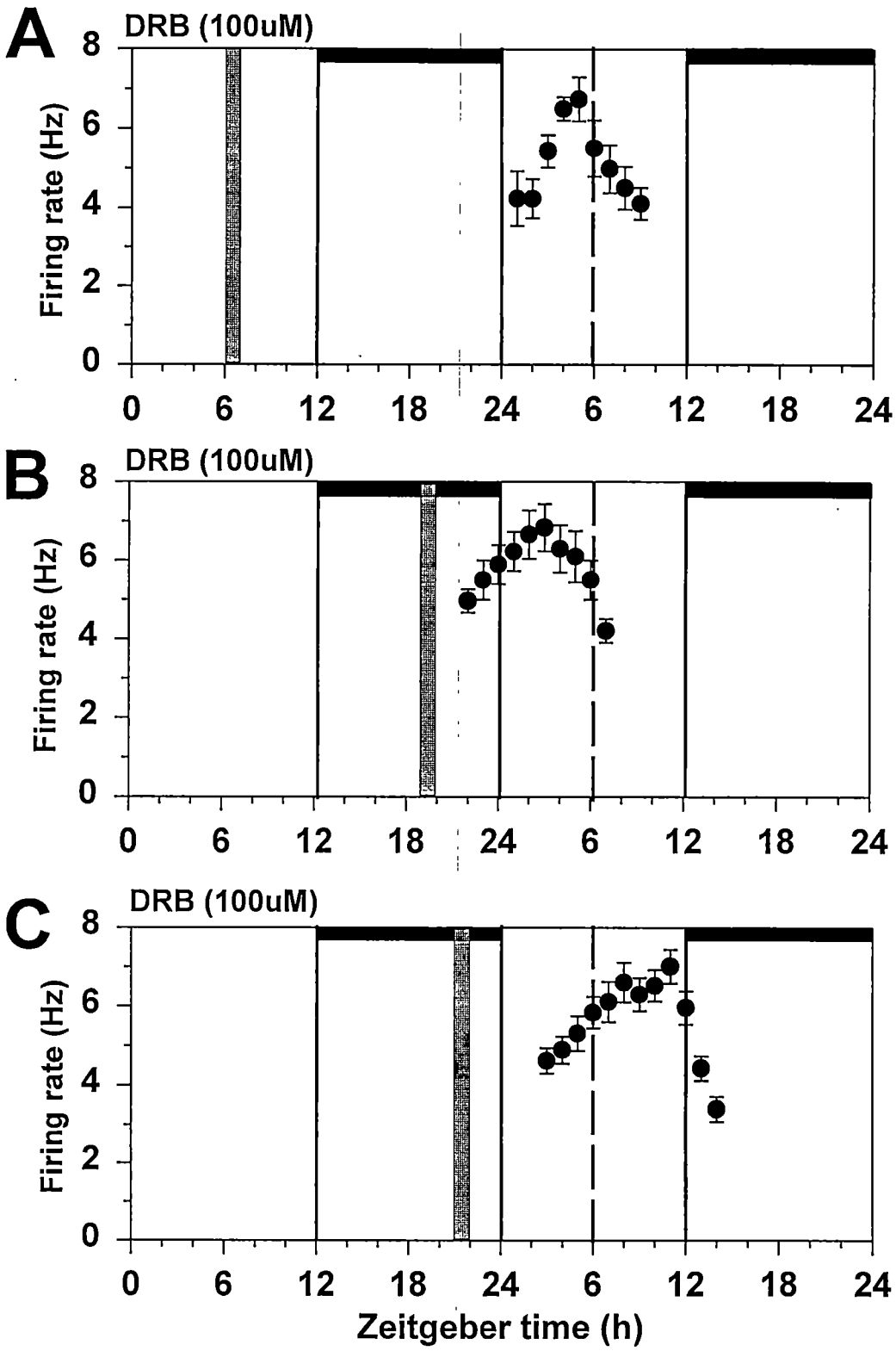

A
(+)DPAT(10uM)+Anisomycin(20uM)
Firing rate (Hz)
0
2
4
6
8
0
6
12
18
24
6
12
18
24
B
(+)DPAT(10uM)+Cycloheximide(10uM)
Firing rate (Hz)
C
(+)DPAT(10uM)+DRB (10uM)
Firing rate (Hz)
Zeitgeber time (h)

Figure 6. Phase-shifting effects of the transcriptional inhibitor, DRB. Neuronal activity recorded in individual experiments after DRB treatment at (A) ZT 6, (B) ZT 19, and (C) ZT 21. DRB treatment at ZT 19 induced a large phase advance, while treatment at ZT 21 induced a large phase delay in the time of peak activity. See Fig. 3 for additional details.


A
DRB (100uM)
Firing rate (Hz)
0
2
4
6
8
0
6
12
18
24
6
12
18
24
B
DRB (100uM)
Firing rate (Hz)
0
2
4
6
8
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12
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C
DRB (100uM)
Firing rate (Hz)
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Zeitgeber time (h)

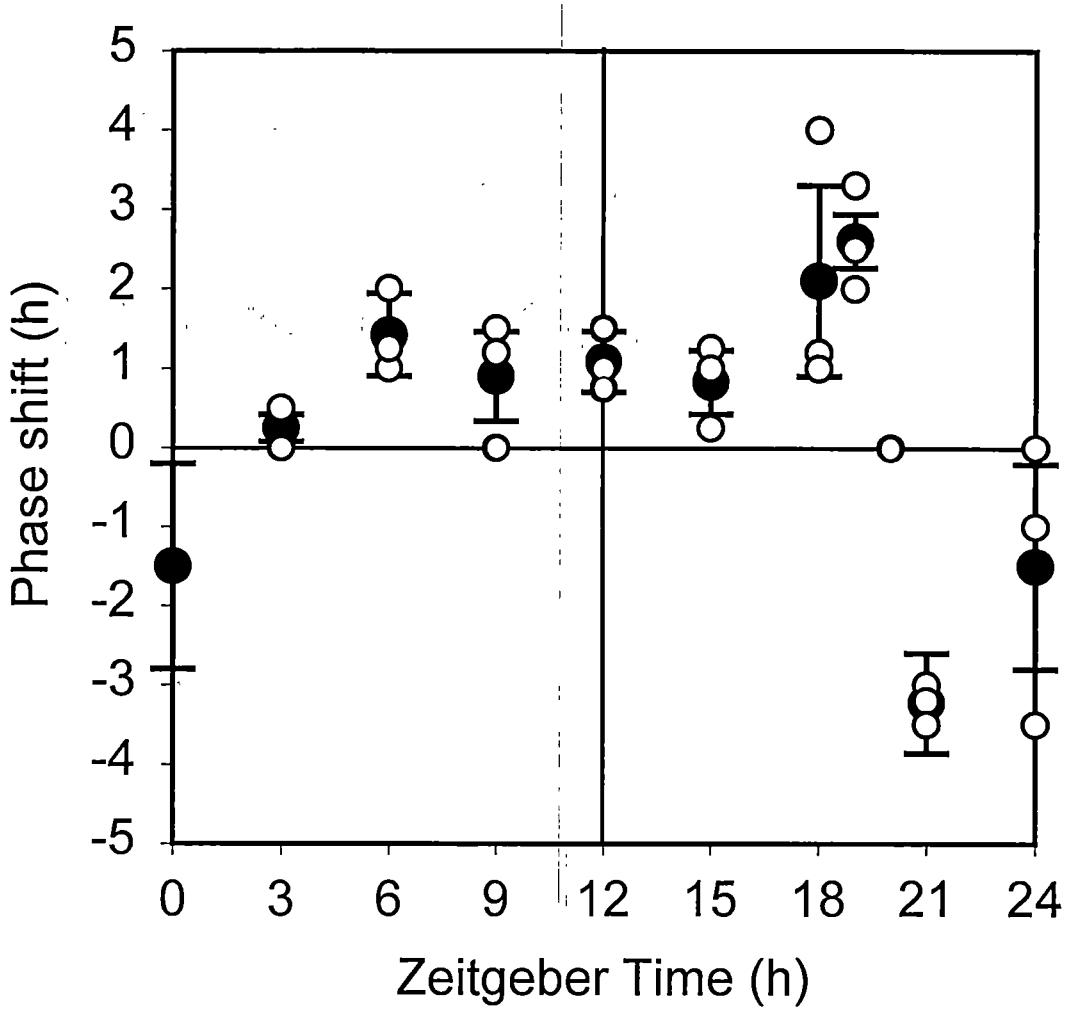

Figure 7. Phase response curve for DRB (100uM). The phase-shifts induced by DRB application at different time points *in vitro* are shown as mean phase-shift ± SEM (closed circles). The results of individual experiments are shown in open circles. Advances are plotted as positive values, while delays are plotted as negative values.

Finally, we investigated the effect of co-applying DRB with (+) DPAT. As shown in Fig. 5, 100 uM DRB completely blocked the phase advances induced by (+) DPAT at ZT 6. Because this concentration of DRB induced a small (but non-significant) phase advance when applied alone at this time, we also investigated the effects of a lower concentration of DRB. 10 uM DRB, which induced no phase shifts when applied alone, decreased the (+) DPAT-induced phase advance such that it was no longer significantly different from controls. The results of these experiments are summarized in Table 1.

Chapter IV

Discussion and Future Directions

The aim of these experiments was to determine the effects of transcription and translation inhibitors on 5HTergic phase shifts. We found that two inhibitors of protein synthesis, anisomycin and cycloheximide, both blocked 5HTergic phase shifts when applied at ZT 6 *in vitro*, and that the reversible transcription inhibitor, DRB, also blocked (+) DPAT-induced phase shifts at ZT 6.

Consistent with earlier studies (Takahashi and Turek, 1987), we found that anisomycin had no effect on circadian pacemaker phase when applied alone at ZT 6, although it did induce substantial phase delays when applied a few hours earlier, at ZT 2. The inability of anisomycin to phase-shift the SCN pacemaker at ZT 6 was not due to a lack of effect on translation, since anisomycin inhibited protein synthesis when applied at ZT 6. Inhibition of protein synthesis occurred very rapidly after drug application (80% inhibition within ½ h), and recovery was also rapid, suggesting that any immediate effects of this treatment are well-circumscribed. The phase delays induced by anisomycin at ZT 2 add to the evidence that protein(s) critical to the SCN circadian pacemaker are being synthesized near subjective dawn in the SCN.

In addition to inducing phase shifts when applied alone at ZT 2, anisomycin also blocked phase advances induced by (+) DPAT and 5HT at ZT 6. It is important to emphasize that anisomycin did not phase-shift the SCN pacemaker when applied by itself at ZT 6, so the inhibition of 5HTergic phase shifts at this time cannot be attributable to an inhibitory interaction between two phase-shifting stimuli. On the other hand, whether the inhibition is due to a general reduction in protein synthesis in the SCN or to the inhibition

of specific protein(s) being synthesized in response to 5HTergic stimulation cannot be determined at this point. To address this question, we would first need to investigate whether 5HT increases the synthesis of specific proteins in the SCN when applied at ZT 6. Similar experiments investigating the ocular circadian clock in *Aplysia* have shown that 5HT alters the synthesis of at least nine proteins (Koumenis et al., 1995). It should also be pointed out that while anisomycin has proven to be remarkably free of side effects on neuronal functions such as resting potentials, action potentials, and synaptic transmission (Lotshaw and Jacklet, 1986; Jacklet, 1980), it has been shown to activate at least two protein kinases, p38 mitogen-activated protein kinase (MAPK) and c-Jun NH_2-terminal kinase (JNK) (Torocsik and Szeberenyi, 2000a,b). Thus, it is possible that the inhibition of 5HTergic phase shifts could be due to mechanisms other than inhibition of protein synthesis.

The results with cycloheximide, a second protein synthesis inhibitor with distinct mechanisms of action, generally support the results with anisomycin, since no phase shift was seen when cycloheximide was co-applied with (+) DPAT at ZT 6. However, the results with cycloheximide are complicated by the fact that cycloheximide applied alone induced a phase advance at ZT 6. Two questions arise from these results: why does cycloheximide induce a phase advance when anisomycin does not, and why does the co-application of (+) DPAT + cycloheximide result in no shift? As to the first question, perhaps it has to do with side effects of cycloheximide that are not shared with anisomycin, such as the ability to affect membrane permeability and transport (Dunlap and Feldman, 1988). The effects of altering membrane permeability on the SCN circadian pacemaker have not been explored, but evidence suggests that these types of

manipulations may affect the circadian pacemaker in other organisms (Cote and Brody, 1987a,b; Mattern and Callaway, 1987). With respect to the lack of phase shift when (+) DPAT and cycloheximide are co-applied, this does not appear to be a simple case of additivity, since that would mean an even larger (~ 5 h) advance. This is the case even if one assumes that the cycloheximide-induced phase advance is completed prior to (+) DPAT being applied. Assuming a full shift in response to cycloheximide, that would mean that (+) DPAT was added around ZT 8-9. This is still a time when DPAT induces phase advances (Shibata et al., 1992b), so one would still predict a large phase advance. An alternative hypothesis is that the mechanisms through which cycloheximide induces phase advances are blocked by subsequent addition of (+) DPAT, while cycloheximide concurrently blocks the phase advances normally induced by (+) DPAT. Speculation concerning cycloheximide-induced phase advances would require more detailed knowledge of cycloheximide's actions in the SCN.

Our results indicate that the reversible transcription inhibitor, DRB, also blocks the phase advances induced by (+) DPAT. Both 10uM and 100 uM DRB were effective at blocking (+) DPAT-induced phase advances, although a small, non-significant phase advance was still seen with the smaller concentration of DRB. The small phase-advance seen with the co-application of DRB at the lower concentration may be because 10 uM is near the low end of the effective concentration range for transcriptional inhibition by DRB (Tamm, 1983; Chodosh et al., 1989; Zandomeni et al., 1982). The inhibition of 5HTergic phase shifts we observed with DRB is similar to that seen in *Aplysia*, where DRB also blocked 5HT-induced phase shifts. These results also are consistent with the

hypothesis that 5HTergic phase shifts of the SCN circadian pacemaker require ongoing transcription.

Again, these results cannot discriminate between whether the blocking by DRB is due to general transcriptional inhibition or whether it is due to DRB inhibiting the transcription of specific genes in the SCN. DRB has been shown to inhibit transcription of mRNA precursors at or close to the site of initiation of transcription *in vitro* and to cause premature termination (Zandomeni et al., 1983; Chodosh et al., 1989; Zandomeni et al., 1982). Thus, it should block most, if not all, ongoing gene transcription. In *Aplysia*, a number of genes appear to be transcribed in response to 5HT (Yeung and Eskin, 1987; Raju et al., 1990; Koumenis and Eskin, 1992). Future experiments could determine whether similar transcriptional activation occurs in response to serotonergic stimulation in the SCN.

The phase-shifting effects of DRB applied alone are also quite interesting. DRB induced small, but non-significant phase advances during most of the subjective day and into early subjective night. However, during mid-subjective night large advances, up to 2.5 h at ZT 19, were seen, followed by a rapid switch to large phase delays at ZT 21. No phase shift was observed with DRB application at ZT 20. This dramatic switch from phase advances to phase delays around ZT 20 suggests that at this time there is a rapid onset of transcription of genes critical for circadian clock functioning. Since this is approximately when *mPer1* and *mPer2* mRNA levels begin to rise in the SCN (Hastings et al., 1999; Field et al., 2000), it is possible that the phase delays induced by DRB during late subjective night are due to its inhibition of *Per1* and *Per2* transcription.

The pattern of phase shifts induced by DRB in the SCN is very different from that seen in *Aplysia.* In that system, DRB induces large phase delays throughout the subjective day (Koumenis et al., 1996). This period of phase delay between CT 20–10 is coincident or even slightly after the time of maximal phase delays induced by protein synthesis inhibitors in *Aplysia* (Yeung and Eskin, 1987). This suggests there is a very long (~18 h) delay between transcritpion and translation of proteins critical for the *Aplysia* circadian pacemaker. Determining what genes and their protein products are critical for *Aplysia* circadian pacemaker functioning may provide some explanation for these intriguing results. Conversely, the time when DRB induces phase delays in the SCN is slightly ahead of when protein synthesis inhibitors induce maximal phase delays (Yeung and Eskin, 1988; Lotshaw and Jacklet, 1986). Thus, the results seen in the SCN appear to fit better with the general pattern of gene transcription slightly preceeding translation.

Previous research points to 5HTergic phase advances of the SCN pacemaker involving stimulation of $5HT_7$ receptors, activation of adenylate cyclase and protein kinase A, and an increase in K^+ conductance. The results here suggest that the transcription and translation of specific protein(s) may also be involved. What these proteins may be is not known. Horikawa et al. (2000) recently showed, using *in situ* hybridization, that 5HTergic stimulation at ZT 6 *in vivo* decreases *hPer1* and *hPer2* mRNA expression in the SCN for approximately 2 h. An effect on PER protein levels was not seen. It has not been determined, however, if this inhibition is critical for 5HTergic phase shifts. In light of those and our results, however, one could speculate that (+) DPAT increases the transcription of a gene(s) whose protein product(s) down-

regulates *Per* mRNA levels. Then again, it is also possible that the protein(s) critical for 5HTergic phase shifts are transcribed and translated some hours after drug application. The time course for these events needs to be investigated further.

In order to focus on specific protein changes in response to serotonergic treatments in the SCN, several techniques such as 2-dimensional sodium dodecyl sulphate polyacrylamine gel electrophoresis (2-D SDS-PAGE) and Western blots could be utilized. With the Western blots, we will also look for increases or decrease in specific clock-related proteins in the SCN such as PER, CLOCK, BMAL. With the help of the 2-D SDS-PAGE technique, we would look more broadly at proteins whose levels are increased or decreased in the SCN in response to either 5HT or (+) DPAT treatment at ZT 6. If we do not find protein changes in response to 5HTergic stimulation, we can conclude that either the proteins increased in response to 5HT are not being detected by the assays, or that the transcriptional and translational inhibitors blocked the phase shifts by disrupting ongoing synthesis of proteins critical for circadian functioning. Also, it would suggest that 5HT does not phase-shift the clock through increasing the synthesis of specific proteins in the SCN. Thus, with the help of these techniques we could draw conclusions regarding the role of protein synthesis in serotonergic phase shifts of the mammalian circadian clock.

In conclusion, our study indicates that both transcriptional and translational inhibitors block 5HTergic phase advances *in vitro*. These results are similar to those found in the *Aplysia* circadian pacemaker. Further, these results are consistent with transcription and translation of new proteins being important for 5HTergic phase shifts of the SCN circadian pacemaker *in vitro*. Additional experiments will be necessary to

determine whether the synthesis of specific proteins is altered by 5HT, and to identify how changes in these proteins affect the mammalian circadian clock.

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