2001

Interactions between DPAT and Optic Chiasm Stimulation in Resetting Circadian Clock Phase

Valerie McMillan

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Abstract

All organisms exhibit daily rhythms in behavior and physiology controlled by endogenous *circadian*, or near 24 h, clocks. In mammals, the circadian clock is in a brain area called the suprachiasmatic nucleus (SCN). Neuronal activity in the SCN exhibits a 24-h pattern that peaks in the day. The circadian clock, located in the SCN, receives modulatory input from other areas of the brain including serotonergic input from the raphe nucleus and glutametergic input via the retinohypothalamic tract. Interactions between these two inputs and their effect on circadian phase were examined using DPAT, a 5-$HT_{1A/7}$ agonist, and optic chiasm stimulation.

For these experiments, we prepared brain slices containing the SCN from rats and placed them in a support chamber. Experimental manipulations were applied to the slices for 10-30 min on day 1 *in vitro*, and neuronal activity recorded on day 2. Under these conditions SCN neuronal activity normally peaks at Zeitgeber time (ZT) 5.81±0.10 (n=5; where ZT 0 is lights-on and ZT 12 is lights-off in the animal colony). At ZT 19, the optic chiasm was stimulated, which releases glutamate onto the SCN via its natural pathway. This treatment advances the SCN clock (peak time = ZT 2.88±0.43, n=4). DPAT application during OCS appears to partially, but not completely, block the advance (peak time = ZT 3.8±0.5, n=5). These results suggest that 5-HT may modulate phase advances induced by OCS at ZT 19.

Introduction

Circadian Rhythms and the Suprachiasmatic Nucleus

For centuries Man has observed organisms and their reactions to the rhythmic environment. Perhaps the most obvious of all environmental rhythms is the 24-h cycle of light and dark. In 1729, astronomer Jean Jacques d'Ortus de Mairan, interested in plants and their adaptations to daily rhythms, placed heliotropes in a dark closet kept at relatively constant temperature for a few days. The leaf movements of the plants were found to continue under these conditions. In the time since de Mairan's experiments, many others have been performed to determine if rhythms persist without the presence of environmental time cues. Human subjects isolated from the 24-h cycle of light and dark are found to exhibit near 24-h rhythms in a variety of physiological parameters including body temperature, urine flow, plasma melatonin, and the sleep-wake cycle (Dijk, 1996). These rhythms continue, but with diminished amplitude and with a period slightly longer than 24 hours (Redfern et al., 1991). Activity rhythms of flying squirrels have also been observed to continue in constant darkness with a period predictably longer than 24-h (DeCoursey, 1960). These rhythms that exhibit near 24-h periodicity under constant conditions are described as circadian rhythms.

Circadian rhythms appear to be ubiquitous, being present in all eukaryotes and even in some prokaryotes. The circadian clock provides daily timing cues to an organism so that it can predict daily rhythmic changes in the environment and adjust its physiology and behavior accordingly. For example, in the evening before sleep onset, body temperature drops in human and then continues to drop

during sleep. These rhythms coincide with the 24-h cycle of light and dark. However, they are more than a reflection of environmental rhythms. The continuation of these circadian rhythms in the absence of photic information and the fact that the period varies from 24 h is strong evidence in favor of there being an internal, self-sustaining clock. This internal clock is thought to have three functionally distinct elements: input pathways that provide information to the circadian pacemaker about environmental changes, a circadian pacemaker that generates near 24-h oscillations, and output pathways by which the pacemaker imposes its rhythms upon the organism (Zlomanczuk and Schwartz, 1997).

The mammalian clock responsible for producing circadian rhythms is located in the suprachiasmatic nuclei (SCN). The SCN are located in the anterior basal hypothalamus, immediately dorsal to the optic chiasm on opposite sides of the third ventricle. A large body of literature supports this as the location of the circadian pacemaker. For example, when specific areas of the hypothalamus including the SCN were destroyed, circadian rhythms in drinking behavior and locomotor activity were eliminated (Stephan and Zucker, 1972). It has been demonstrated that SCN neurons generate a circadian rhythm of electrical activity that peaks during the day (Inouye and Kawamura, 1979). Also, studies have shown that the SCN maintains circadian rhythmicity in an in vitro preparation (Green and Gillette, 1982). Overall, these experiments and others point to the SCN as the location of the circadian clock.

While the circadian pacemaker, located in the SCN, generates 24-h rhythmicity autonomously, it receives modulatory input from other areas of the

brain. The SCN receives four major inputs. Glutamatergic input is received from retinal ganglion cells via the retinohypothalamic tract (RHT). The geniculohypothalamic tract, which projects from the intergeniculate leaflet, releases the neurotransmitters neuropeptide Y and GABA. Fibers project from the raphe nucleus bringing serotonin (5-HT) inputs. Also, melatonin released from the pineal gland serves as a hormonal modulator of the SCN (fig. 1).

Glutamate and photic entrainment

Photic entrainment of circadian rhythms occurs through daily, light induced adjustments in the phase and period of the SCN circadian clock. Photic information necessary for entrainment is conveyed to the SCN directly from the retina via the RHT. The excitatory amino acid glutamate is thought to be the neurotransmitter of the RHT. Activation of glutamate receptors results in light-like phase shifts in behavioral rhythms in hamsters (Mintz et al., 1999). In addition, a number of in vitro experiments have been conducted to examine the pathway of photic stimulation to the SCN and the effects of this stimulation. Glutamate has been shown to be released into the SCN when the optic nerves or optic chiasm is electrically stimulated (Liou et al., 1986; Shibata and Moore, 1993). Optic chiasm or optic nerve stimulation phase-shifts the SCN pacemaker in a pattern similar to that produced by light pulses, i.e., phase delays in the early subjective night and phase advances in the late subjective night (Shibata and Moore, 1993; Liou et al., 1986). Application of glutamatergic antagonists has been shown to block these phase shifts (Cahill and Menaker, 1989). These

experiments point to glutamate as the neurotransmitter of photic information and the RHT.

Phase shifts induced by light appear to involve induction of immediate early genes, including c-fos. Light-induced c-fos production occurs mainly in regions of RHT terminals. Also, light-induced production of c-fos and light-induced phase shifts are limited to the subjective night (van Esseveldt et al., 2000; van den Pol and Dudek, 1993; Rea, 1998). This evidence furthers the idea that c-fos expression may represent a portion of the signal transduction pathway responsible for photic regulation of circadian rhythms. Besides the induction of IEG expression, other processes including a calcium-mediated stimulation of nitric oxide synthase have been implicated in the phase shifting effect of light (Ding et al., 1994).

Serotonergic modulation of circadian phase

In addition to receiving information about light changes, the SCN receives other modulatory inputs. However, the role of these inputs is not entirely defined as of yet. One of these inputs is the large serotonergic projection from the midbrain raphe nucleus to the SCN. Several lines of evidence suggest that serotonin alters the phase of the clock. In one study, application of the neurotoxin, 5,7-dihydroxytryptamine (5,7-DHT), chemically destroys the 5-HT system causing changes in activity rhythms of hamsters. DHT treatment induced rapid appearance of advanced activity onset, delayed offset and longer duration of the nocturnal activity phase (Morin and Blanchard, 1991). Also, intra-cerebroventricular administration of either quipazine or 8-hydroxy-

dipropylaminotetralin (DPAT), both 5-HT agonists, in rats cause phase advances of behavioral rhythms in the daytime (Edgar et al., 1993). Similarly, in vitro application of quipazine and (DPAT) can shift neuronal activity in isolated brain slices containing the SCN when applied during the subjective day (Prosser et al., 1993). These observations suggest that 5-HT may serve as an important regulator of the circadian clock.

Modulation of photic input by serotonin

While 5-HT directly modulates circadian phase, there is also evidence that it modulates the response of the SCN oscillator to light during the subjective night. Selective destruction of 5-HT afferents to the SCN via application of 5,7-DHT accelerates adjustments in response to shifts in the light-dark cycle (Morin and Blanchard, 1991). Also, the 5-HT antagonist, NAN-190, has been shown to increase photic phase shifts during late subjective night in vivo (Rea et al., 1995). 5-HT, the 5-$HT_{1A/7}$ agonist, DPAT, and the 5-HT_{1B} agonist, TFMPP, have also been shown to inhibit light-stimulated c-fos production in the SCN (Selim.M. et al., 1993). These results reveal a possible role of 5-HT as an inhibitor of retinal input to the SCN.

Other studies have investigated 5-HT modulation of photic input at the cellular level. It was reported that 5-HT and DPAT inhibit glutamate-induced increases in intercellular free calcium in isolated SCN neurons caused by co-application of glutamate. The 5-$HT_{7/2/1c}$ antagonist, retanserin, blocked this inhibition. This supports the idea that 5-HT modulates the effects of glutamate on individual SCN neurons via 5-HT_7 receptors (Quintero and McMahon, 1999).

However, it has also been reported (Flett and Colwell, 1999) that increases in calcium transients due to glutamate bath applications were not blocked by 5-HT, while increases in calcium transients produced by synaptic stimulation were inhibited by 5-HT. Similarly, excitatory post-synaptic currents evoked by RHT stimulation were inhibited by 5-HT agonists but current induced by exogenously applied glutamate or NMDA was not (Jiang et al., 2000). These results suggest a presynaptic action of 5-HT to inhibit the release of glutamate. In order to further address this hypothesis, we investigated whether DPAT alters the phase shift in SCN neuronal activity caused by optic chiasm stimulation during late subjective night in vitro.

Materials and Methods

Brain Slice Preparation

Coronal brain slices (500 μm) 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 constant light 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 Balance Salt Solution (EBSS; Sigma) supplemented with glucose and bicarbonate brought to pH 7.4 (Prosser, 1998).

Single Unit Recordings and Data Analysis

The spontaneous activity of individual SCN neurons was recorded on day two in vitro using glass capillary microelectrodes filled with 3 M NaCl. Each neuron whose signal was greater than twice that of the background electrical noise was recorded for 5 minutes, and the data were stored for later determination of firing rate using a Data*Wave* system. As depicted in fig. 2, The firing rates of the individual neurons were then used to calculate two-hour running averages lagged by one hour to obtain a measure of population neuronal activity. As described previously (Prosser et al., 1993), the time of peak neuronal activity was defined as the symmetrically highest point in the resulting curve, estimated to the nearest quarter hour. Phase shifts were calculated as the difference in time-of-peak in treated slices vs. the mean time-of-peak in untreated slices. Student's *t*-tests and ANOVAs were used to test for significant differences between the means.

Experimental Treatment

All experimental treatments were applied at zeitgeber time (ZT) 19, where ZT 0 is the time of lights-on in the animal colony and ZT 12 is the time of lights-off in the animal colony. For optic chiasm stimulation (OCS) experiments, a bipolar tungsten electrode, insulated except for the tips was placed in the optic chiasm of the brain slice. Current was applied for 10 minutes (5 Hz, 3 msec duration, 10 V). For drug application experiments, 8-hydroxy-dipropylaminotetralin (DPAT; Sigma Chemical Co.) was bath-applied for 30 minutes. For drug application, the normal perfusion was stopped and the medium in the brain slice chamber was replaced with medium containing DPAT (10 μM). At the end of the thirty minutes, the treated medium was replaced with normal medium and the perfusion was resumed. For combination experiments, first the perfusion medium was replaced with medium containing DPAT. After 10 minutes, the optic chiasm was electrically stimulated for 10 minutes. The DPAT-containing medium was left in the chamber for an additional 10 minutes, after which, the treated medium was exchanged with normal medium and the normal perfusion was resumed.

Results

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 5.81 ± 0.1 (n=4). As shown in Fig. 4, stimulation of the optic chiasm at ZT 19 produced a phase advance of approximately 3 hours. The mean phase advance induced by OCS (2.93 ± 0.28, n=4) was significantly different from the controls ($p<0.01$). Application of DPAT at ZT 19 did not significantly phase-shift the circadian pacemaker (0.13 hr ± 0.41, n=4). Co-application of DPAT with OCS produced a slightly smaller phase advance than that induced by OCS alone (2.01 ± 0.5,n=5)(See fig. 4.). While smaller than the phase advance induced by OCS alone, this phase advance was still significantly different from controls ($p<0.05$). These results are summarized in Fig. 5.

Discussion

DPAT application at ZT 19 did not significantly alter the phase of the circadian clock. These results are consistent with previous studies where DPAT was found to have no effect on circadian phase at this time (Shibata et al., 1992). Also consistent with previous results, stimulation of the optic chiasm produced phase advances of approximately 3-h (Shibata and Moore, 1993). While glutamate release in response to OCS was not measured in these experiments, others have shown that OCS induces release of glutamate in the SCN (De Vries et al., 1994; Liou et al., 1986). Furthermore, in other experiments we have shown that OCS during the subjective day does not phase shift the SCN in vitro (mean phase shift -0.35 $\pm$ 0.21 h, n=3, $p>0.05$ vs control). Thus this phase shifting response to OCS is limited to specific phases of the circadian cycle and is consistent with the response being due to the release of glutamate. Furthermore,

This study was designed to determine whether or not DPAT alters the shift in SCN neuronal activity caused by OCS during late subjective night in vitro. In this study, co-application of DPAT was found to slightly reduce the size of the phase delay induced by OCS at ZT 19. While statistical analysis indicates OCS continued to phase advance the circadian pacemaker in the presence of DPAT, the size of the phase shift induced by OCS appears to be reduced by DPAT application.

Previous research demonstrates a role for 5-HT as a modulator of glutamatergic phase shifts. When co-applied with NMDA or glutamate at ZT 14,

DPAT blocks the glutamatergic phase delay in vitro (Forrest and Prosser, 2000). DPAT has also been shown to inhibit light induced phase advances in vivo (Rea et al., 1994). These results, combined with the results from this study, suggest that DPAT plays a role in modulating photic input to the SCN and that this role may be time dependent.

Our results here where DPAT appears to partially block the OCS induced phase advance is somewhat at odds with our earlier experiments showing that, DPAT completely blocks the phase delay induced by bath-application of glutamate at ZT 14. However, the difference in these results could be due to various factors. One possibility is that the inhibitory effects of 5-HT are time-dependent. That is, 5-HT blocks phase delays at ZT 14 but not phase advances at ZT 19. However, since we used different stimuli to induce phase changes at ZT 14 and ZT 19 (glutamate vs. OCS), additional experiments are nedeed where we examine interactions of glutamate and DPAT at ZT 19 and interactions of OCS and DPAT at ZT 14 in order to determine whether the time of treatment or the phase shifting stimulus is the critical factor in the difference between these two sets of data.

In vivo, 5-HT has been shown to block photic phase shifts through presynaptic 5-HT_{1B} receptors as well as through 5-HT_{1A} and 5-HT_7 receptors which are presumably postsynaptic. Since DPAT is selective for the 5-HT_{1A} and 5-HT_7 receptors, our experiments presumably were aimed at investigating postsynaptic inhibition. To test for involvement of other 5-HT receptors, agonists selective for additional 5-HT receptors need to be examined.

Other parameters of this experiment could also affect the degree to which DPAT blocks OCS induced phase advances. For example, this partial blockage of the phase advances could be due to the concentration of DPAT used in this study. It is possible that blockage of OCS-induced phase advances requires a higher concentration of DPAT than that required to block glutamate induced phase delays. Also, the duration of DPAT application could be a factor in modulation of light induced phase advances. The experiments at ZT 14 used an hour long treatment with DPAT, while in these experiments we only applied DPAT for 30 minutes. A longer duration of DPAT application may be needed to completely block these phase advances.

In conclusion, the results from this study suggest that DPAT may inhibit phase advances induced by OCS at ZT 19. Further research examining the interactions between OCS and DPAT will be necessary to determine the degree to which DPAT may block OCS induced advances of circadian clock phase at ZT 19.

Figure Legends

Fig. 1. Modulatory inputs to the SCN. This figure depicts the major inputs to the SCN and their corresponding neurotransmitters or hormone.

Fig. 2. Schematic illustrating the procedure for data analysis. A) Plotted are the firing rates of individual neurons recorded on day 2 *in vitro* (open circles). B) Firing rates are replotted from (A), together with the running 2 h means ± SEM (closed circles). C) Plotted are the 2 h means ± SEM from (B), together with a regression line fitted to the data.

Fig. 3. Control Experiment. Shown is the rhythm in neuronal activity obtained from a single control experiment in which no pharmacological agents were applied. The horizontal black bars from ZT 12 to ZT 24 depict lights out. The absences of the bars show lights on. Plotted are the 2h means ± S.E.M. of neuronal activity recorded from SCN *in vitro*. Neuronal activity peaks at ZT 6 in the middle of subjective day (ZT≅6). The vertical dashed line shows the mean time of peak for all controls.

Fig. 4. The effects of optic chiasm stimulation and DPAT on the circadian rhythm at ZT19. Shown are individual experiments involving OCS and DPAT. A) At ZT 19, OCS results in a phase advance of approximately 3 hours. B)

DPAT does not exhibit a shift in the rhythm. C) The application and DPAT with OCS decrease the OCS phase advance.

Fig. 5. Summary of phase shifting experiments at ZT19. The histogram bars depict the mean phase-shift ± S.E.M. for each experimental treatment done at ZT19. The numbers above and below the bars indicate the number of experiments. The asterisk denotes that the phase shifts were significantly different from controls ($p<.05$).

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Fig. 1

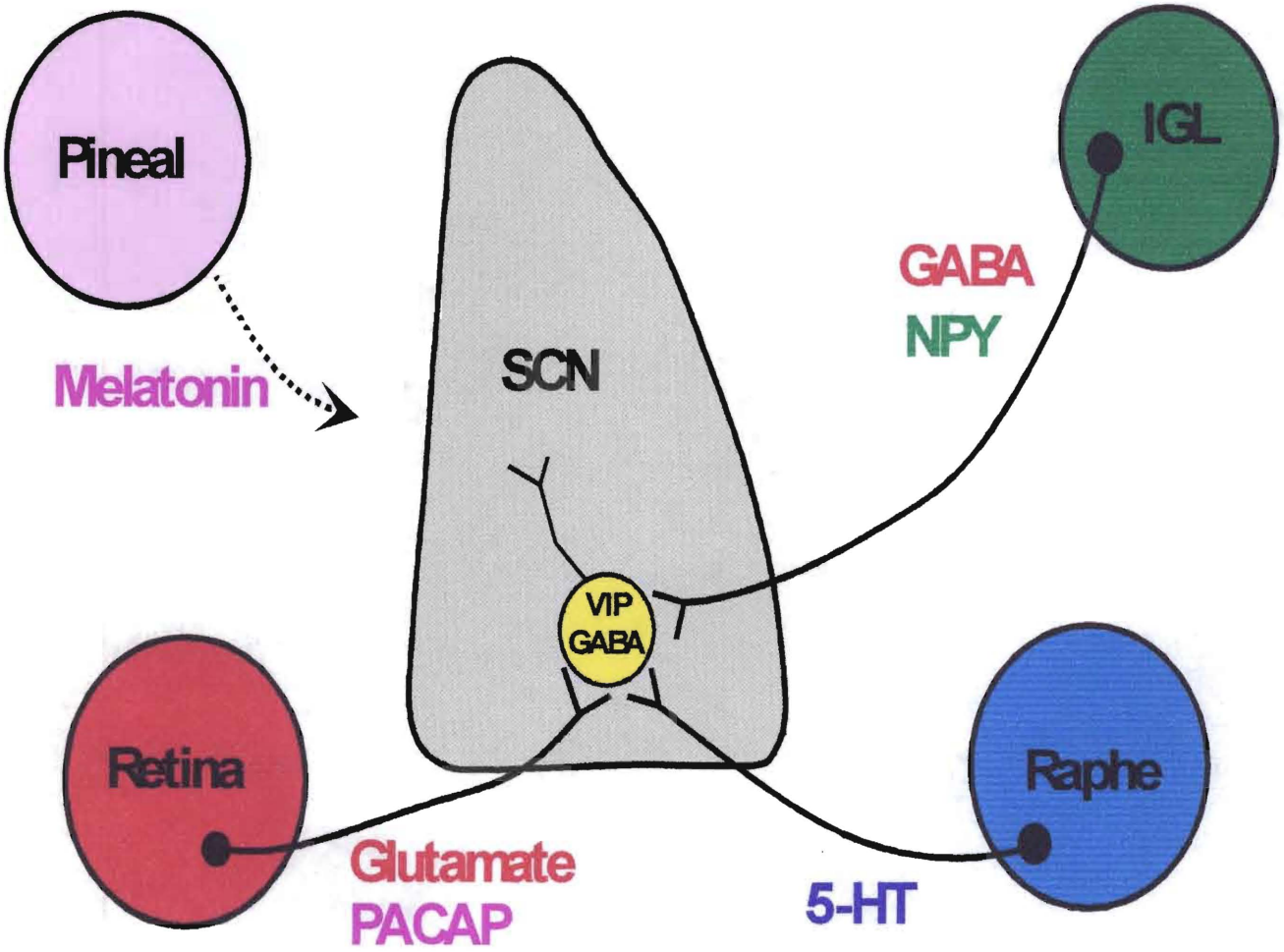

Pineal
Melatonin
SCN
IGL
GABA
NPY
VIP
GABA
Retina
Glutamate
PACAP
Raphe
5-HT

Fig. 2

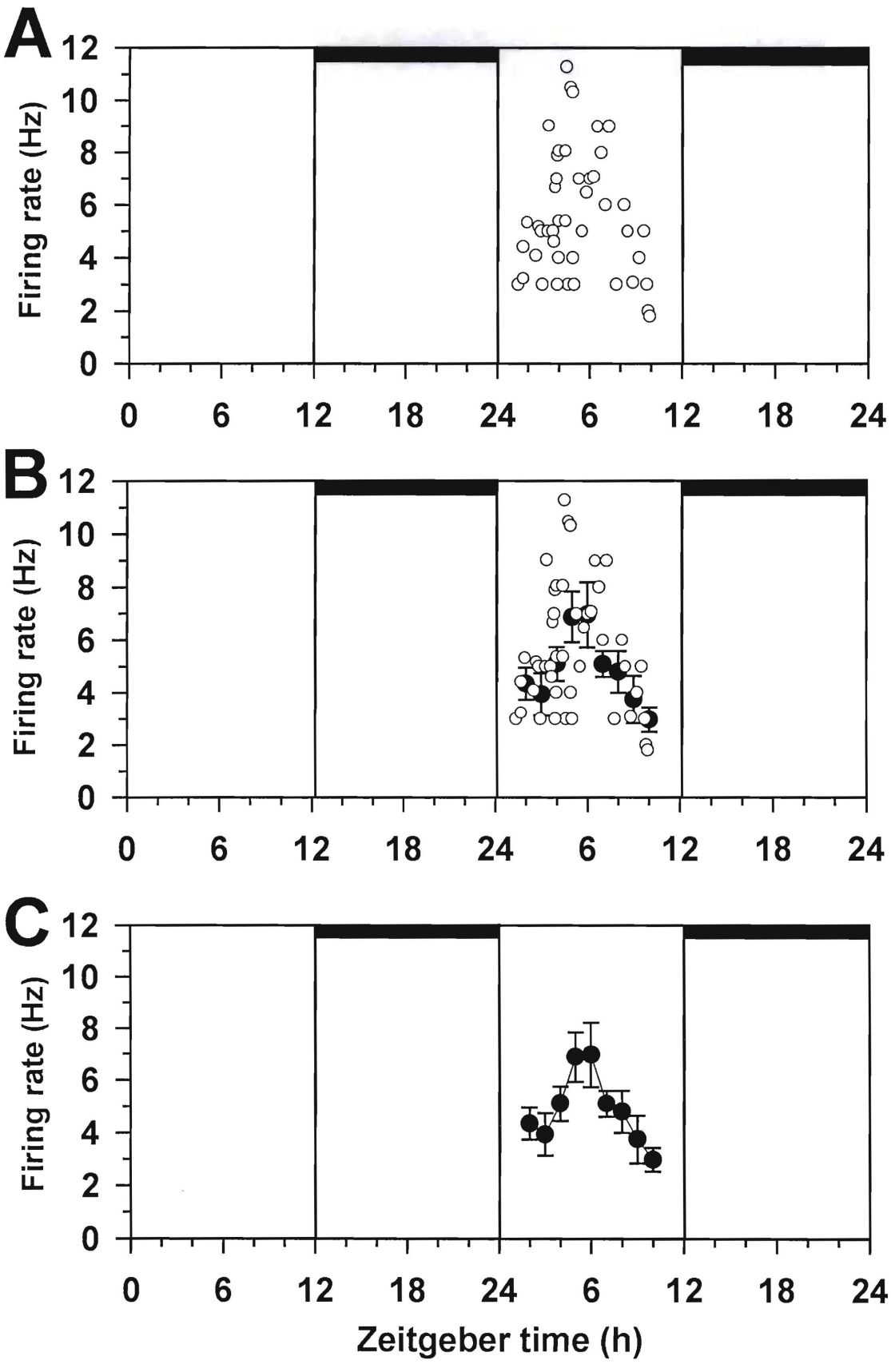


Fig. 3
s062300 - Control Experiment

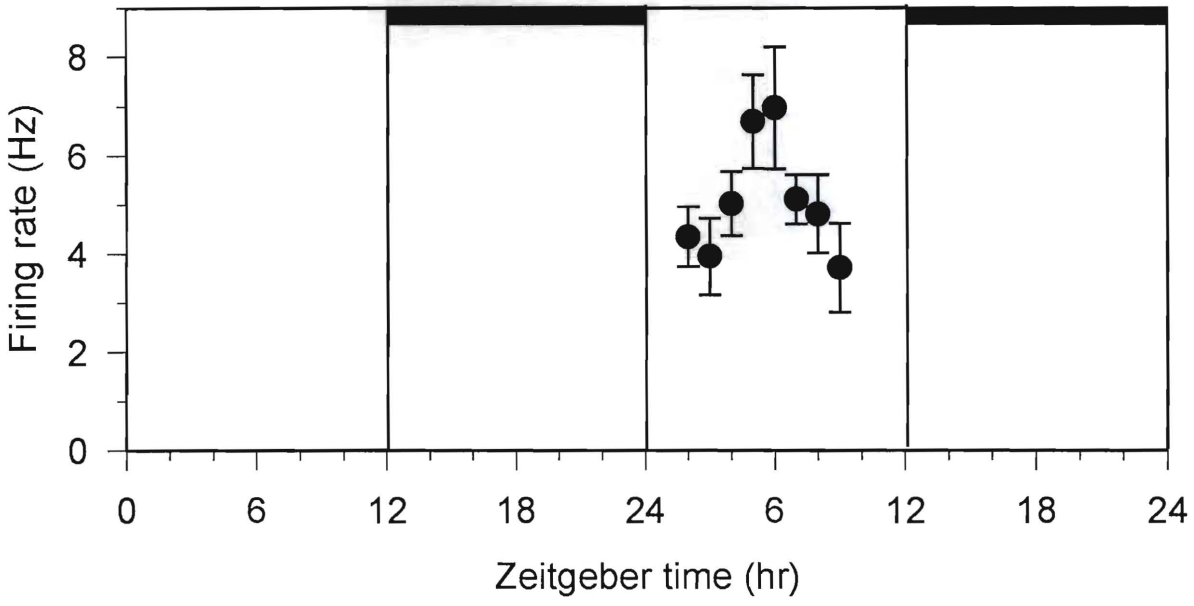


Fig. 4

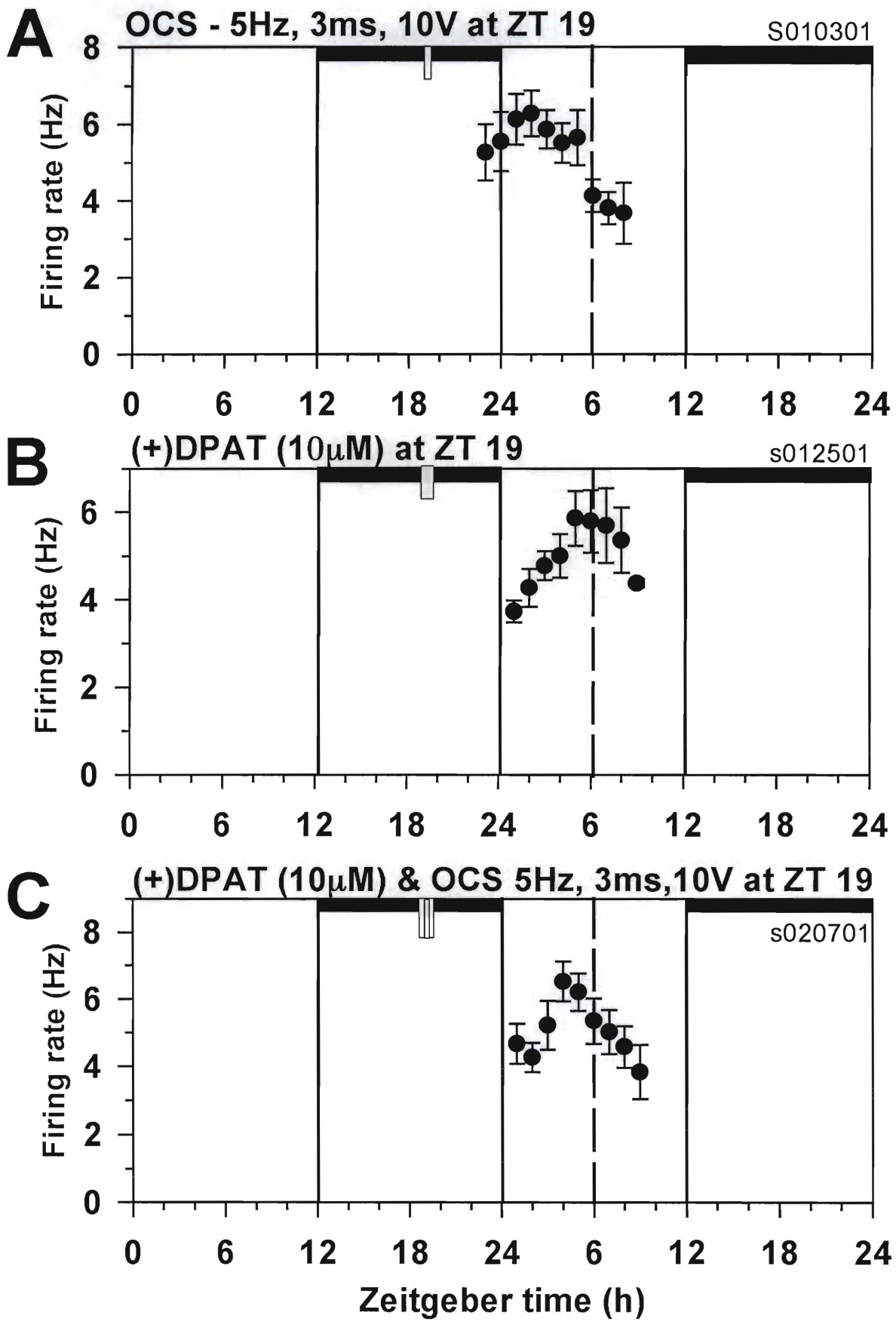
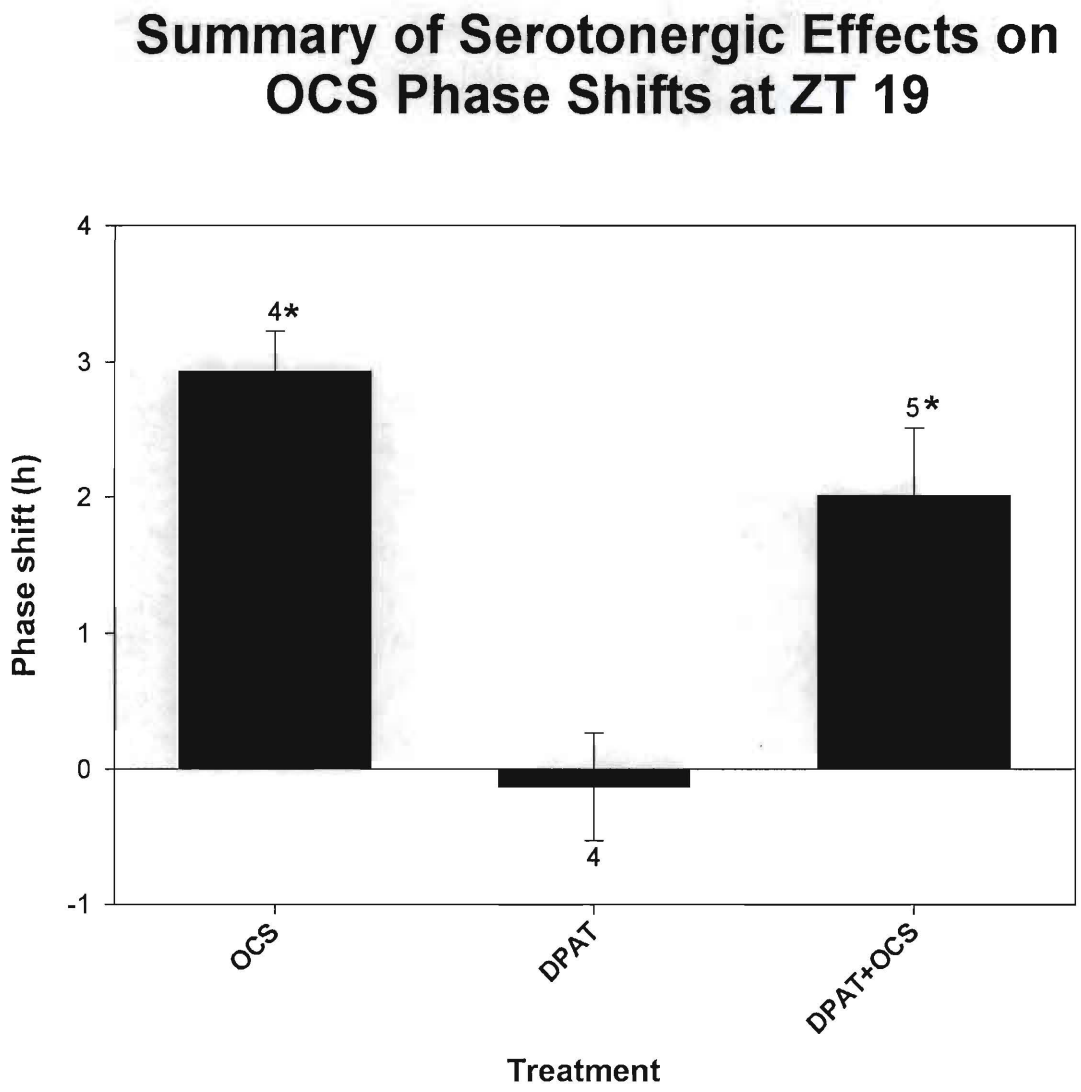

A
OCS - 5Hz, 3ms, 10V at ZT 19
S010301
Firing rate (Hz)
0
2
4
6
8
0
6
12
18
24
6
12
18
24
B
(+)DPAT (10μM) at ZT 19
s012501
Firing rate (Hz)
0
2
4
6
0
6
12
18
24
6
12
18
24
C
(+)DPAT (10μM) & OCS 5Hz, 3ms,10V at ZT 19
s020701
Firing rate (Hz)
0
2
4
6
8
0
6
12
18
24
6
12
18
24
Zeitgeber time (h)

Fig. 5


Summary of Serotonergic Effects on OCS Phase Shifts at ZT 19
Phase shift (h)
4
3
2
1
0
-1
4*
5*
4
OCS
DPAT
DPAT+OCS
Treatment

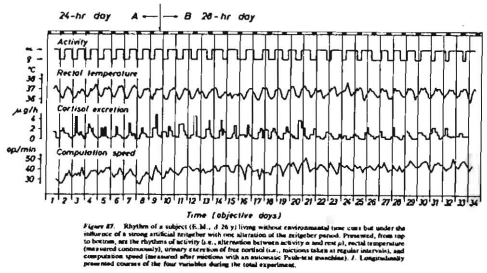
Interactions between DPAT and Optic Chiasm Stimulation in Resetting Circadian Phase

Valerie McMillan

What is time? The shadow of the dial, the striking of the clock, the running of the sand, day and night, summer and winter, months, years, centuries - these are but arbitrary and outward signs, the measure of time, not time itself.

- Henry Wadsworth Longfellow

Examples of Human Rhythms



Circadian Clock

- Provides daily timing cues
- Three distinct elements
  - input pathways
  - circadian pacemaker
  - output pathways
- In mammals, located in the Suprachiasmatic Nucleus (SCN)

Location of the SCN in the Human Brain

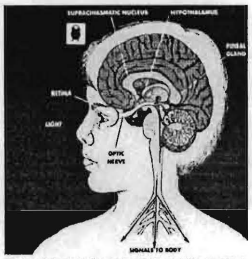
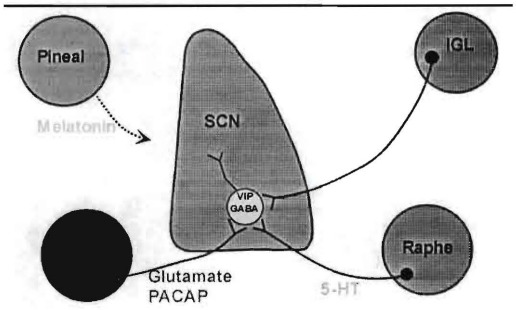


Illustration by Lydia Kibiuk, Copyright © 1995 Lydia Kibiuk.

Primary Inputs to the SCN



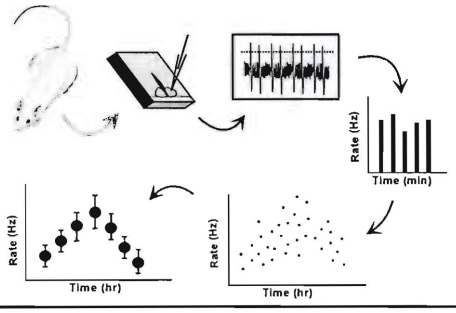
Modulation of Photic Input by 5-HT

- Chemical destruction of 5-HT afferents alters behavior.
- DPAT inhibits c-fos production.
- DPAT inhibits post-synaptic current evoked by RHT stimulation.
- When DPAT is co-applied with glutamate at ZT 14, the glutamatergic phase delay is completely blocked.

Rationale for this Study

- Previous studies suggest a role of 5-HT in the inhibition of glutamate release.
- This study investigated whether or not DPAT will alter the shift in SCN neuronal activity caused by optic chiasm stimulation during late subjective night in vitro.

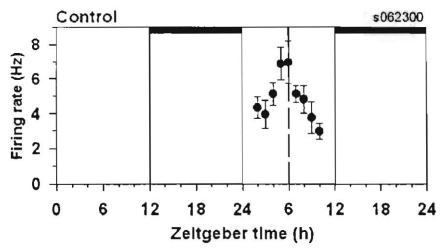
Methods and Materials



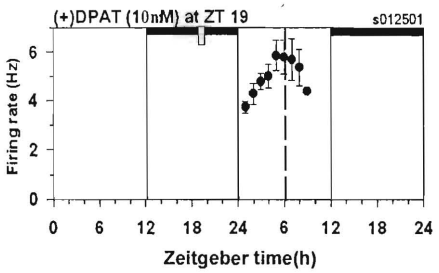
Experimental Treatment

- AT ZT 19:
  - Optic Chiasm Stimulation (OCS)
    - 5Hz, 3 msec, 10 V for 10 min.
  - DPAT
    - 10 μM bath applied for 30 min.
  - OCS and DPAT
    - 10 μM DPAT applied for 10 min.
    - 5Hz, 3 msec, 10 V for 10 min.
    - DPAT remained in chamber for additional 10 min.

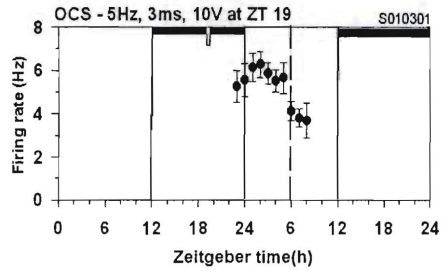
Control Experiment



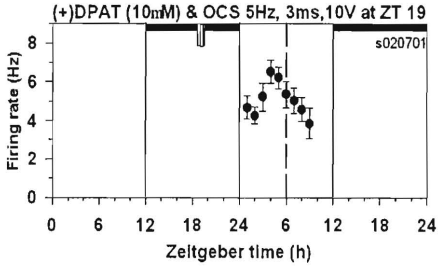
8-OH-DPAT



Optic Chiasm Stimulation



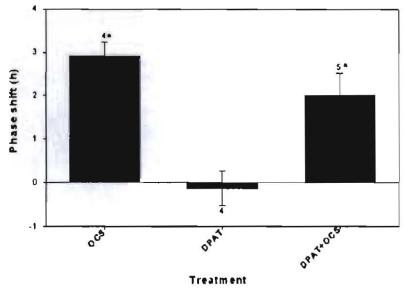
DPAT and OCS



Analysis of Variance

Experiment	Phase Shift	S.E.M	Number of Trials	
DPAT	-0.130	±0.10	n=4	
OCS	2.93	±0.28	n=4	p<0.01
OCS & DPAT	2.01	±0.55	n=5	p<0.01

Phase Shifts



Summary of Results

At ZT 19:

- DPAT does not significantly phase shift the clock.
- OCS induces a phase advance of approximately 3 hours.
- DPAT co-applied with OCS appears to partially block the OCS induced phase advances.

Discussion and Future Directions

- OCS produces phase advances when applied during subjective night.
- DPAT appears to modulate OCS induced phase advances at ZT 19.
- DPAT and OCS interactions will be further investigated.