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Amanda Beth Wehner
University of Tennessee - Knoxville

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Fluoxetine pretreatment blocks DPAT-induced phase shifts, *in vitro*

Amanda Wehner

Introduction

Circadian rhythms are the manifestations of the roughly twenty-four hour cycle of behavioral and physiological systems controlled by the internal clock. In mammals, this internal clock is located in the suprachiasmatic nucleus (SCN). The SCN is a bilateral nucleus in the hypothalamus, consisting of only about 20,000 neurons. It lies just above the optic chiasm and on either side of the third ventricle. SCN cells have been shown to demonstrate rhythmic firing, glucose uptake, and neurotransmitter release. The overall activity of the organism is influenced by the physiological factors controlled by the SCN (Herzog and Swartz 2002).

SCN activity does not function on an exactly twenty-four hour cycle; external and physiological factors are constantly resetting it. These constant adjustments are referred to as phase shifts. Light input has the strongest impact on SCN rhythmicity. Light input to the retina reaches the SCN through the optic nerve. The retino-hypothalamic pathway neurons synapse directly on SCN neurons (Moore 1995). Glutamate is the main neurotransmitter of this pathway. The presence of light stimulates the release of glutamate into the SCN, causing the neurons to produce more activity (Rea 1998). However, light input can be modulated by other SCN afferents.

One of these afferents originates in the midbrain raphe nucleus, widely recognized for its involvement in the regulation of brain arousal and consciousness. Serotonin is the main neurotransmitter utilized by this pathway. Activity, through the release of 5-HT, can modify light-induced-entrainment during the night. During the day, addition of serotonin or its agonists to the SCN can induce phase advances on their own (Mistlberger, et al. 2000, Morin 1999, and Prosser 2003, and Weber, et al. 1998).

Serotonin acts through many different types of receptors in the SCN and other circadian rhythms related areas; such as type-1A, 1B, 1D, 2A, and 7 receptors (Barnes and Sharp 1999, Roca, et al. 1993, and Sumner, et al. 1992). DPAT, a serotonin agonist, has been shown to cause phase shifts in *in vitro* SCN experiments (Edgar, et al. 1993). The 5-HT₇ receptor is known to affect the regulation of circadian rhythms (Lovenberg, et al. 1993), and has been found to exist in relatively high densities in the limbic areas, including the hypothalamus (Waeber and Moskowitz 1995). Ligands, which bind exclusively to the 5-HT₇ receptor were shown to advance the mean neuronal activity in the rat SCN (Lovenberg, et al. 1993). Sprouse, et al. showed that the phase resetting actions of DPAT are sensitive to 5-HT₇ receptor antagonists, suggesting this receptor is the site of DPAT-binding (2004).

While serotonin and its agonists have been shown to induce daytime phase advances when applied to the SCN *in vitro*, these effects are not always seen when applied *in vivo* (Antle, et al. 2003, Horikawa and Shibaka 2004, and Bobrzynska, et al. 1996). Differing levels of endogenous serotonin present in the SCN *in vivo* versus *in vitro* may cause this discrepancy. The severing of the serotonin afferents during slice preparation may lower the level of serotonin present in the SCN, leading to an increase in 5-HT receptor expression or sensitivity. Thus, acute administration of 5-HT or 5-HT-agonist could cause a phase advance *in vitro* while the same treatment would have no effect *in vivo*.

Fluoxetine is a selective serotonin reuptake inhibitor. Studies have shown that treatment with fluoxetine causes decreases in light-induced phase delays if serotonin is not already present in the SCN. If serotonin is already present due to sleep deprivation,

no further decrease in light-induced phase delay is seen, suggesting fluoxetine works through the serotonin system (Challet, et al. 2001). Sleight and colleagues showed that treatment of rats with fluoxetine causes a significant reduction in the number of 5-HT₇ receptor binding sites in the hypothalamus, suggesting that chronic treatment of fluoxetine causes the receptor to be down-regulated in the hypothalamus of the rat (Sleight, et al. 1995). Pretreatment with fluoxetine should raise the effective level of serotonin in the slice by blocking the reuptake of released serotonin (Mullins, et al. 1999). Duncan, et al. also showed that chronic antidepressant treatment reduced agonist-induced phase advances, as with DPAT (1999). In light of these data, we hypothesized that, by increasing the effective serotonin levels, in the SCN, fluoxetine treatment of the SCN brain slices would block serotonergic phase shifts. If fluoxetine treatment blocks the *in vitro* DPAT-induced phase advances, it provides support for the theory that the difference between endogenous levels of serotonin *in vivo* and *in vitro* is a possible cause for the different responses to acute serotonin administrations.

Methods

Brain slice preparation

Coronal brain slices (500 μ m) containing the SCN were prepared during the daytime from adult, male C57BL/J6 mice, housed in a 12-hour light/dark cycle, where ZT 0 is lights on and ZT 12 is lights off. ZT stands for zeitgeberger time. Slices were prepared between ZT 0-2. Slices were maintained at the interface of a Hatton-style brain slice chamber (Hatton, et al., 1980) in which they were perfused continuously with warm (37°C), oxygenated (95% O₂/5% CO₂), glucose-bicarbonate-supplemented Earle's Balanced Salt Solution (EBSS; Sigma, St. Louis, MO), pH 7.4-7.5.

Experimental Treatments

The drugs used in phase-shifting experiments were applied during day 1 *in vitro* by stopping the perfusion and replacing the medium in the slice chamber with medium containing the test compound. Following the treatment period, normal medium was replaced in the slice chamber and perfusion was restored. Fluoxetine was applied for 1 hour from ZT5-6 and (+) 8-hydroxy-2-(di-n-propylamino) tetralin [DPAT] was applied for 10 minutes starting at ZT 6.

Single unit recordings and data analysis

Single-unit recordings commenced early on day 2 *in vitro*. For the treatment slices, recording began approximately 18 hours following treatment. The procedure followed was described previously (Prosser, et al., 1993). The spontaneous activity of single SCN neurons was recorded using glass capillary microelectrodes filled with 3M NaCl. Each neuron was recorded for approximately 5 minutes, and the data was stored for later analysis using a DataWave system (DataWave Technologies, Longmont, CO). In general, four to seven cells were recorded each hour. These firing rates were then used to calculate 2 hour running averages, lagged by 1 hour, to obtain a measure of population neuronal activity. The time of peak neuronal activity was assessed by visually estimating, to the nearest quarter hour, the time of symmetrically highest activity. Phase shifts were calculated as the difference in time of peak of untreated slices vs. drug-treated slices.

Results

Control experiments

The SCN neuronal activity recordings obtained from untreated mouse slices on the 2nd day *in vitro* demonstrated a peak in activity near mid-subjective day. The mean of

peak activity was ZT 6.0 +/- 0.71 (n=3). An example of an untreated slice's neuronal rhythm is shown in Figure 1.

DPAT treatment causes phase advances in vitro

One set of experiments consisted of brain slices which were treated for 10 minutes at ZT 6 on day 1 *in vitro* with 10 μM DPAT, shown in figure 2. These slices exhibited a mean time of peak activity at ZT 2.85 +/- 0.17 (n=5), indicating a phase advance of 3.15 hours, which is statistically significant.

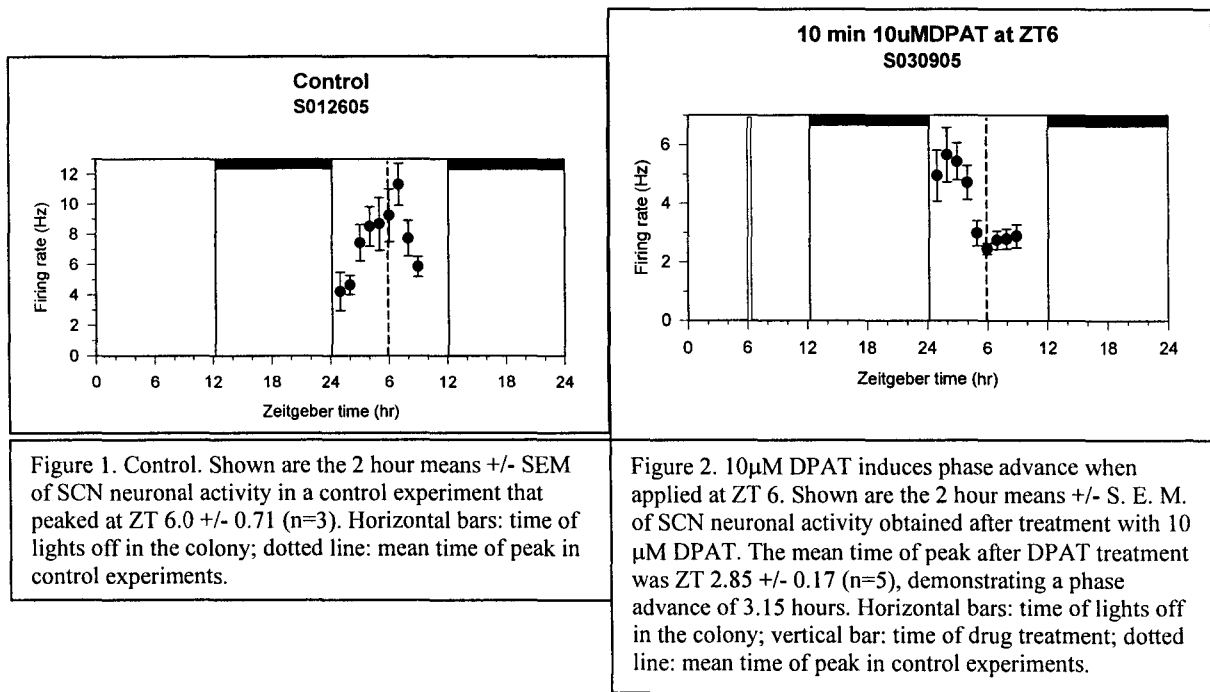


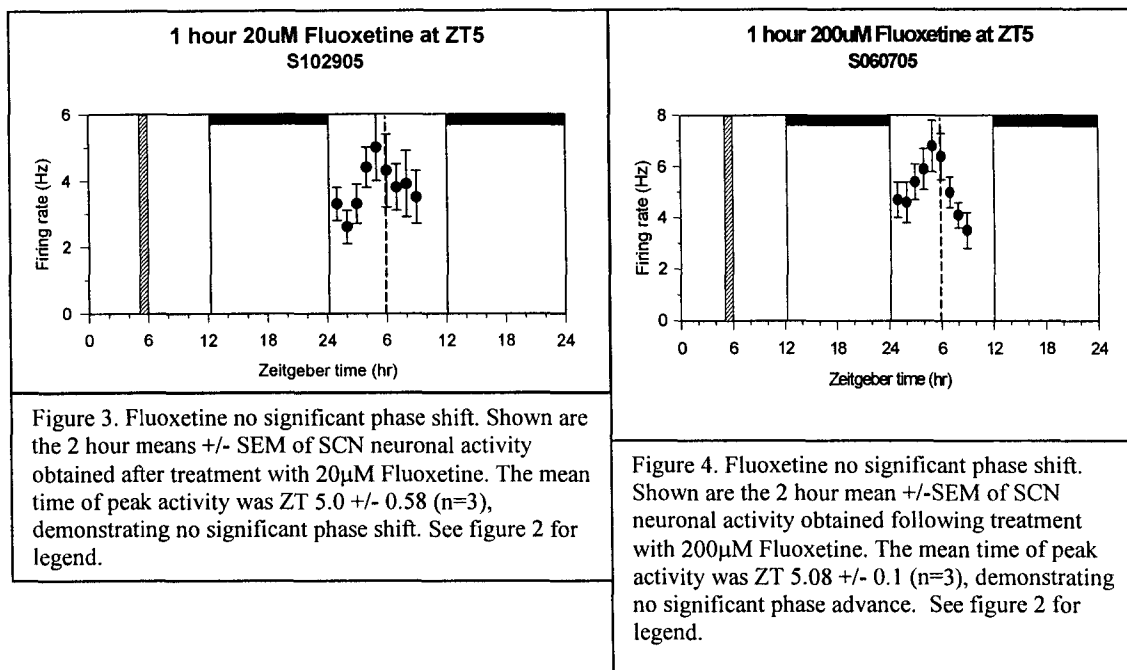
Figure 1. Control. Shown are the 2 hour means +/- SEM of SCN neuronal activity in a control experiment that peaked at ZT 6.0 +/- 0.71 (n=3). Horizontal bars: time of lights off in the colony; dotted line: mean time of peak in control experiments.

Figure 2. 10μM DPAT induces phase advance when applied at ZT 6. Shown are the 2 hour means +/- S. E. M. of SCN neuronal activity obtained after treatment with 10 μM DPAT. The mean time of peak after DPAT treatment was ZT 2.85 +/- 0.17 (n=5), demonstrating a phase advance of 3.15 hours. Horizontal bars: time of lights off in the colony; vertical bar: time of drug treatment; dotted line: mean time of peak in control experiments.

Fluoxetine causes no phase shifts of mouse SCN in vitro

Two series of experiments tested the effects of Fluoxetine treatment on the neuronal activity pattern. In the first series of experiments, the slices were treated with 20 μM Fluoxetine from ZT5-6 on day 1 *in vitro*. They exhibited a mean time of peak activity at ZT 5.0 +/- 0.58 (n=3), which is not a significant phase shift. The results of one of these

experiments are seen in Figure 3. In the second series, the slices were treated with 200 μ M Fluoxetine from ZT5-6 on day 1 *in vitro*. These slices showed a mean time of peak activity at ZT 5.08 \pm 0.1 (n=3), which also does not show a significant phase shift. The results of one of these experiments are shown in Figure 4.



Pretreatment with Fluoxetine blocks DPAT-induced phase shifts

In the next set of experiments, slices were pretreated from ZT 5-6 with 20 μ M Fluoxetine before the 10 minute DPAT treatment. In these experiments, DPAT continued to induce phase shifts, such as is shown in Figure 5. In the final set of experiments, slices were pretreated with 200 μ M Fluoxetine from ZT 5-6, followed by the 10 minute DPAT treatment. These slices demonstrated a mean time of peak activity at ZT 6.08 \pm 0.1 (n=3), showing a complete block of the DPAT-induced phase advance. These experiments are represented in Figure 6.

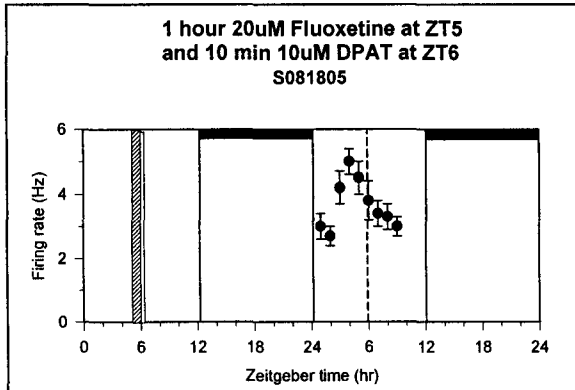


Figure 5. 20 μ M Fluoxetine pretreatment not sufficient to block DPAT-induced phase advance. Shown are the 2 hour means \pm SEM of SCN neuronal activity following treatment with 20 μ M Fluoxetine and 10 μ M DPAT. The mean time of peak activity was ZT 4.0 \pm 0.18 (n=3), showing a phase advance of 2 hours, which is not significantly different from the peak activity following DPAT treatment alone. See figure 2 for legend.

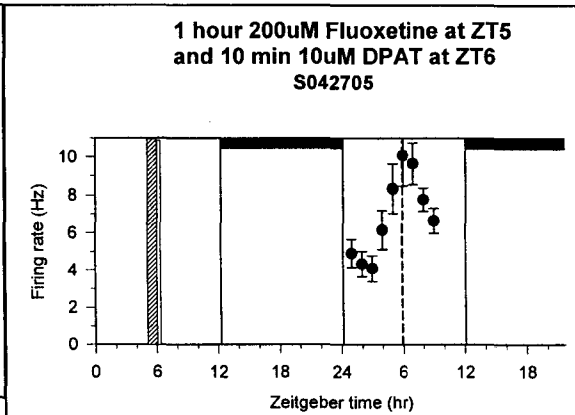


Figure 6. Pretreatment with 200 μ M Fluoxetine blocks DPAT-induced phase shift. Shown are the 2 hour means \pm SEM of SCN neuronal activity following treatment with 200 μ M fluoxetine and 10 μ M DPAT. The mean time of peak activity was ZT 6.08 \pm 0.1 (n=3), showing a phase delay of 0.08 hours. Therefore, this concentration of fluoxetine completely blocked the phase advance induced by DPAT. See figure 2 for legend.

Figure 7 summarizes the phase advances seen by all the treatment groups. As seen in the histogram, DPAT alone causes the most robust phase advance and pretreatment with 200 μ M Fluoxetine completely eliminates this phase advance.

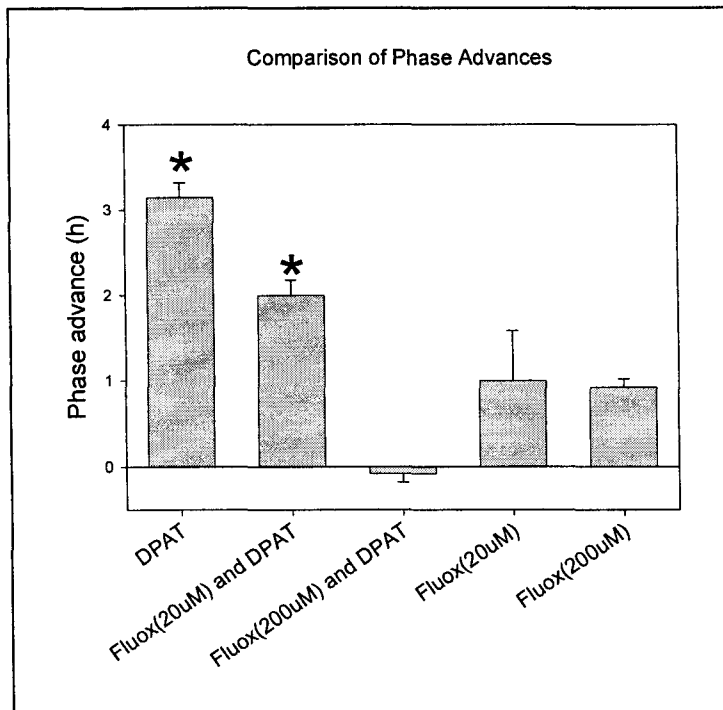


Figure 7. DPAT-induced phase shifts are dose-dependently blocked by fluoxetine. Shown are the mean phase shifts induced by the different treatments. The * shows phase shifts which are significant ($p < 0.05$) vs. the control.

Discussion

The results of these experiments clearly demonstrate that the DPAT-induced phase advance can be blocked by pre-treatment with Fluoxetine in a dose-dependent manner. These results may provide an explanation for the discrepancy between the effects of *in vivo* and *in vitro* treatments with DPAT. Previous studies with *in vivo* injection of DPAT into the SCN have shown no phase advances (Antle, et al. 2003), while work with DPAT treatments *in vitro*, as seen in this experiment, have shown a robust phase advance (Prosser 2000).

It is possible that fluoxetine pretreatment blocks *in vitro* phase advances by DPAT because it produces conditions that more closely mimic the *in vivo* environment. Fluoxetine blocking 5HT reuptake may raise the effective concentration of serotonin present in the slice. This rise in effective serotonin levels may desensitize the clock to serotonin, causing the DPAT treatment not to produce a phase shift. One study provides additional support for this hypothesis. They showed that *in vivo* 5-HT administration to the SCN induces robust phase advances when animals have been pretreated with a serotonin antagonist which reduces 5-HT levels in the SCN (Ehlen, et al. 2001). The fact that lowering the *in vivo* 5-HT levels allows serotonergic phase shifts to be expressed lends credence to the hypothesis that the different effects seen *in vivo* and *in vitro* are due to different levels of endogenous serotonin stimulation in the two conditions.

While these experiments show a block of the DPAT-induced phase advance, they do not show how or at what level the fluoxetine is working to block the phase advance. The fluoxetine induced increase in effective serotonin concentration may be acting directly on the receptor, causing a decrease in sensitivity. However, it may also be acting

further down the signaling pathway. Further studies to show the effects of blocking downstream pathways on the blockage of the phase advance could help to elucidate the mechanism of fluoxetine's effect on the DPAT-induced phase advance. Understanding how fluoxetine is acting to affect circadian rhythms may help to mitigate or explain side effects shown in those using fluoxetine as an anti-depressant. If the mechanism of fluoxetine action can be illuminated, these side effects may be more easily treatable.

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