

Response of the Mouse Circadian System to Serotonin 1A/2/7 Agonists In Vivo: Surprisingly Little

Michael C. Antle,* Malcolm D. Ogilvie,† Gary E. Pickard,† and Ralph E. Mistlberger‡¹

**Department of Psychology, Columbia University, New York, NY 10027, USA,*

†Department of Anatomy and Neurobiology, Colorado State University, Fort Collins, CO 80523, USA,

‡Department of Psychology, Simon Fraser University, Burnaby, BC, Canada, V5A 1S6

Abstract Serotonin (5-HT) is thought to play a role in regulating nonphotic phase shifts and modulating photic phase shifts of the mammalian circadian system, but results with different species (rats vs. hamsters) and techniques (in vivo vs. in vitro; systemic vs. intracerebral drug delivery) have been discordant. Here we examined the effects of the 5-HT_{1A/7} agonist 8-OH-DPAT and the 5-HT_{1/2} agonist quipazine on the circadian system in mice, with some parallel experiments conducted with hamsters for comparative purposes. In mice, neither drug, delivered systemically at a range of circadian phases and doses, induced phase shifts significantly different from vehicle injections. In hamsters, quipazine intraperitoneally (i.p.) did not induce phase shifts, whereas 8-OH-DPAT induced phase shifts after i.p. but not intra-SCN injections. In mice, quipazine modestly increased c-Fos expression in the SCN (site of the circadian pacemaker) during the subjective day, whereas 8-OH-DPAT did not affect SCN c-Fos. In hamsters, both drugs suppressed SCN c-Fos in the subjective day. In both species, both drugs strongly induced c-Fos in the paraventricular nucleus (within-subject positive control). 8-OH-DPAT did not significantly attenuate light-induced phase shifts in mice but did in hamsters (between-species positive control). These results indicate that in the intact mouse in vivo, acute activation of 5-HT_{1A/2/7} receptors in the circadian system is not sufficient to reset the SCN pacemaker or to oppose phase-shifting effects of light. There appear to be significant species differences in the susceptibility of the circadian system to modulation by systemically delivered serotonergics.

Keywords: 8-OH-DPAT; quipazine; phase shift; PRC; fos; suprachiasmatic nucleus; mouse; hamster

The mammalian circadian clock, located in the SCN, is richly innervated by serotonergic afferents from the midbrain raphe nuclei. mRNA coding for the 5-HT_{1A}, 1B, 2A, 2C, and 7 receptor subtypes has been localized within the SCN in one or more studies (Heidmann et al., 1998; Roca et al., 1993; Wright et al., 1995). In addition, cells and/or fibers within the SCN

have been found to be immunoreactive for the 5HT_{1B}, 2A, 2C, 5A, and 7 receptor subtypes in at least one study (Belenky and Pickard, 2001; Moyer and Kennaway, 1999; Duncan et al., 2000; Neumaier et al., 2001). There are compelling reasons to believe that 5-HT plays a role in the regulation of circadian rhythms in mammals. However, the results from different labo-

1. To whom all correspondence should be addressed: Department of Psychology, Simon Fraser University, 8888 University Dr., Burnaby, BC, Canada, V5A 1S6; e-mail: mistlber@sfu.ca.

ratories, using different rodent strains, species, and test paradigms, appear to tell different, in some cases contrary, stories.

A large collection of studies provides strong evidence that 5-HT afferents convey information about behavioral state to the SCN circadian clock. The relationship between behavioral state and serotonergic raphe neuronal activity has been examined using both single-unit electrical recordings (Jacobs and Fornal, 1997, 1999) and microdialysis (Dudley et al., 1998). These studies consistently reveal that single-cell activity and 5-HT release are maximal during behavioral arousal and minimal during sleep in rats and cats (Jacobs and Fornal, 1997, 1999; Mistlberger et al., 2000). During the subjective night (the usual active phase of nocturnal rodents), 5-HT inhibits the transmission of light input to the circadian clock by actions at presynaptic 5-HT_{1B} receptors on retinohypothalamic terminals and at postsynaptic 5-HT_{1A} or 5-HT₇ receptors on SCN neurons (Rea and Pickard, 2000; Smith et al., 2001). Evidence for serotonergic inhibition of light-induced circadian responses has been obtained in rat, Syrian hamster, and mouse species and has been observed at the molecular (attenuation of photic induction of c-Fos in SCN neurons), cellular (attenuation of photic or glutamatergic activation of SCN single units), and behavioral levels (attenuation of photic phase shifts *in vivo*). It has also been established that behavioral arousal can inhibit light-induced phase shifts (Holmes and Mistlberger, 2000; Mistlberger et al., 1997; Mistlberger and Antle, 1998; Ralph and Mrosovsky, 1992) and that this effect can be blocked in Syrian hamsters by a 5-HT_{1A/7} receptor antagonist (Mistlberger and Antle, 1998). In aggregate, these data suggest that at night, 5-HT is a signaling route by which behavioral state can modulate sensitivity of the clock to photic stimulation.

During the subjective day (the usual rest phase in nocturnal rodents), the circadian clock is generally insensitive to light. 5-HT at this time of day can induce large phase-advance shifts of the circadian rhythm of firing rates of rat and Syrian hamster SCN neurons *in vitro*, most likely by actions at 5-HT₇ receptors (Gannon, 2001; Prosser, 2000). Basal c-Fos mRNA expression in the SCN *in vitro* is suppressed by 5-HT agonists at this time (Prosser et al., 1994). *In vivo*, 5-HT_{1A/7} agonists, applied systemically, can induce phase shifts of behavioral rhythms in the rat and hamster. Behavioral arousal during the subjective day is associated with a prominent increase of 5-HT release within the SCN (Dudley et al., 1998; Grossman et al.,

2000) and can induce large phase shifts of circadian rhythms in hamsters (Antle and Mistlberger, 2000; Mrosovsky, 1988). During the late subjective night, there is some evidence that behavioral arousal and nonspecific 5-HT agonists can induce phase-delay shifts, but these effects have generally been reported to be smaller in magnitude and not consistently reproduced across studies (Mistlberger et al., 2000).

These data are consistent with a model of the mammalian circadian system in which 5-HT participates in the signaling of behavioral arousal to the clock, which resets circadian phase in the day and modulates the clock's phase-resetting response to light exposure at night. However, the generality of this model is compromised by results from several laboratories suggesting that in at least some rat strains, 5-HT may convey photic rather than nonphotic information to the clock. In these rat strains, 5-HT, possibly via SCN 5-HT_{2A/2C} receptors, appears to mimic the phase-shifting effects of light on circadian rhythms of behavior, temperature, or melatonin and to induce SCN neuronal c-Fos expression at circadian phases when light also induces c-Fos (Ferguson and Kennaway, 2000; Kalkowski and Wollnik, 1999; Kennaway and Moyer 1998, 1999; Kohler et al., 1999; Moyer et al. 1997; Mullins et al., 1999). In another study, the 5-HT_{1A/7} agonist 8-OH-DPAT was reported to induce SCN c-Fos following *i.c.v.* administration in the midsubjective day (Neumaier et al., 2001), a phase when light does not affect c-Fos expression, and when 8-OH-DPAT was reported to suppress c-Fos in the rat SCN *in vitro* (Prosser et al., 1994) and quipazine was reported to have no effect in the rat SCN *in vivo* (Kennaway and Moyer, 1998).

Another group of studies challenges the hypothesized role of 5-HT as a mediator of arousal-induced phase shifts. There is one report that 5-HT_{1A/7} agonists applied directly to the hamster SCN *in vivo* in the subjective day fail to induce significant phase shifts (Mintz et al., 1997), although another study did report very small phase shifts in response to bilateral, peri-SCN injections of 8-OH-DPAT (Challet et al., 1998). A third study used the 5-HT_{1A} autoreceptor antagonist WAY100635 to significantly increase endogenous 5-HT release in the hamster SCN but found no evidence for phase shifts or potentiation of arousal-induced phase shifts (Antle et al., 2000). Single or multiple applications of 5-HT_{1A/7} receptor antagonists, delivered systemically or intra-SCN, failed to attenuate arousal-induced phase shifts in hamsters (Antle et al., 1998). Although neurotoxic ablation of 5-HT afferents

to the SCN impairs entrainment to scheduled locomotor activity in mice (Bradbury et al., 1997; Marchant et al., 1997), similar lesions in hamsters do not appear to prevent phase shifts to acute bouts of wheel running (Bobrzynska et al., 1996b; Meyer-Bernstein and Morin, 1998), although they do attenuate shifts induced by triazolam injections, which are thought to be dependent on the expression of locomotor activity (Meyer-Bernstein and Morin, 1998; Mrosovsky and Salmon, 1990; Van Reeth and Turek, 1989). Activity in response to novel wheels was not attenuated in these studies. Other studies (Cutrera et al., 1994; Penev et al., 1995a) claiming to have eliminated nonphotic phase shifts with 5-HT lesions did not report sufficiently on activity levels, which may have been attenuated due to the lesion, thereby eliminating the shift, not because of the role of 5-HT in mediating the shift but rather because activity did not occur with sufficient intensity. Finally, application of 8-OH-DPAT directly to the median or dorsal raphe mimics the phase-shifting effects of systemic injections (Mintz et al., 1997). As 8-OH-DPAT inhibits release of endogenous 5-HT by activating raphe 5-HT_{1A} somatodendritic autoreceptors, this result implies that phase resetting by serotonergics delivered systemically in vivo is mediated by acute suppression of endogenous serotonergic tone rather than activation of postsynaptic SCN 5-HT receptors (Mintz et al., 1997). Consistent with this model, attenuation of the phase-resetting response to 8-OH-DPAT with age in hamsters (Penev et al., 1995b) is paralleled by loss of 5-HT₇ receptors in the raphe but not in the SCN (Duncan et al., 1999). Also, destruction of 5-HT fibers in the SCN by 5,7-DHT abolishes the phase-resetting response to 8-OH-DPAT in hamsters (Schuhler et al., 1998).

Clearly, several issues need to be resolved before a general model of 5-HT function within the SCN can be constructed. These issues converge on the fundamental role of 5-HT within the circadian system. Under physiological conditions, does 5-HT transmission mediate nonphotic effects on the clock in the subjective day, and does it modulate or mediate photic effects on the clock in the subjective night? If any of these, by what pathways and receptor subtypes? And do these roles or mechanisms vary with species?

With these organizing questions in mind, the present study had two aims. The first aim was to characterize the effects of 8-OH-DPAT and quipazine on circadian rhythms in the mouse. A range of circadian responses to these drugs has been demonstrated in

Syrian hamsters and various rat strains, but mouse studies are lacking, despite the advantages of this species for characterizing the functional roles of 5-HT receptor subtypes, using specific receptor knockout models (e.g., Sollars et al., forthcoming). Previous studies in the mouse suggested that 5-HT contributes to nonphotic entrainment (Edgar et al., 1997; Marchant et al., 1997). However, although 5,7-DHT lesions of 5-HT input to the SCN did prevent entrainment to scheduled daily treadmill running, weak effects on period and phase were still evident in the free-running rhythms (Marchant et al., 1997). Other studies have suggested that the circadian system of the mouse is sensitive to nonphotic manipulations such as phase shifting to activity induced with morphine (Marchant and Mistlberger, 1995) or suppression of clock genes with NPY (Fukuhara et al., 2001). Triazolam does not phase shift one mouse strain (C57BL) but has been reported to produce phase shifts in another strain (SK; Ebihara et al., 1988). However, this finding may be more related to how different strains respond to triazolam rather than the strain's sensitivity to nonphotic zeitgebers, as that study predated the observation that triazolam-induced shifts are due to induced activity.

The second aim was to compare the effects of 8-OH-DPAT and quipazine on c-Fos expression in the mouse and hamster SCN. In hamsters, behavioral arousal in the subjective day suppresses SCN c-Fos expression at those phases when arousal induces large phase-advance shifts (Antle and Mistlberger, 2000; Mikkelsen et al., 1998). If activation of SCN 5-HT_{1/7} receptors mediates phase shifts to behavioral arousal, then 5-HT_{1/7} agonists may also suppress SCN Fos expression in vivo. If this role has species generality, then suppression should be evident in both hamsters and mice. As quipazine has been reported to mimic the effects of light on SCN c-Fos expression in the rat in the subjective night (Kennaway and Moyer, 1999; Kohler et al., 1999), separate groups of mice and hamsters were also tested with this drug at selected phases in the day and the night.

MATERIALS AND METHOD

Animals and Housing

For Experiments 1 and 2, mice (6-week-old male C57BL/6J, Charles River, St. Croix, PQ, Canada) and hamsters (9-week-old male *Mesocricetus auratus*,

Lak:LVG [Syr]; Charles River, PQ) were housed individually in wire-bottom polypropylene cages ($47 \times 26 \times 20$ cm) equipped with running wheels and contact drinkometers. Food and water were available ad libitum. The room was temperature and humidity controlled. Mouse experiments were conducted in constant dark (DD). An infrared viewer was used for general animal maintenance and experimental procedures. Hamster experiments were conducted in LD and DD, using the Aschoff type II procedure for measuring phase shifts (Mrosovsky, 1996). Wheel rotation was detected by closure of a microswitch connected to a microcomputer. Rotations were summed into 10-min bins and transferred to a Macintosh computer for analysis with Circadia.

For Experiment 3, wild-type mice (male C57BL/6J) were obtained from Jackson Laboratory (Bar Harbor, MA), and 5-HT_{1A} receptor knockout (KO) mice on the C57BL/6 genetic background (Parks et al., 1998) were generously provided by Dr. Miklos Toth (Cornell University Medical College). Hamsters (male *Mesocricetus auratus*, Lak:LVG [Syr]) were obtained from Charles River (Kingston, NY). Housing and data collection procedures were as described above, except that the mice were housed in smaller cages ($33 \times 15 \times 13$ cm).

Surgery and Placement Histology

Hamsters ($n = 9$) were deeply anesthetized (80 mg/kg sodium pentobarbital), placed in a stereotaxic frame, and implanted with a 22 G guide cannula (Plastics One, Roanoke, VA) using sterile technique. Surgical coordinates (AP = 0 mm, ML = +0.3 mm from bregma, DV = 7.0 mm below skull surface, with tooth bar set at 2 mm below the interaural level) were such that the tip of the guide cannula was 1 mm above the dorsal margin of the SCN. The injection cannulas were cut so that they would project 1 mm beyond the end of the guide cannula.

Following the experiment, these animals were again deeply anesthetized (100 mg/kg sodium pentobarbital) and were perfused transcardially with phosphate-buffered saline (PBS) followed by formalin. Brains were removed and cryoprotected. 50 μ m sections through the anterior hypothalamus were collected on a cryostat and stained with cresyl violet. The site of injection was determined, and distance from the margin of the was SCN measured. A tip within 500 μ m was considered to be a successful implant.

Drugs and Injection

For Experiments 1 and 2, quipazine and ($\pm$) 8-OH-DPAT hydrobromide (Sigma) were dissolved in PBS with heating and filtered (0.2 μ m) prior to use. The final concentration for each dose was such that the volume administered was 4 μ l/g body weight for systemic application in mice and 5- μ l/g for systemic application in hamsters. For central injections in hamsters, the drug was administered in 0.5 μ l PBS.

For Experiment 3, (R)-(+)-8-OH-DPAT hydrobromide (Tocris) was dissolved in 0.9% saline by sonication. The final concentration of each dose was such that the volume administered was 2 μ l/g body weight (mice) or 1 μ l/g body weight (hamsters).

Procedures

Experiment 1: Effects of 5-HT Agonists on Circadian Phase

Mice ($n = 9$) were injected with 8-OH-DPAT (5 mg/kg, subcutaneously [s.c.]) at circadian time (CT) 2, 5, 8, 10, 14, 18, or 22. Injection times were calculated relative to the projected time of activity onset (designated CT12) on the test day. By this convention, CT2 is 10 circadian hours prior to CT12. Six mice received injections at all phases. An additional 3 doses of 8-OH-DPAT (0, 1, and 10 mg/kg) were tested at CT8 and CT22 in some animals. Finally, some animals also received an injection of 10 mg/kg 8-OH-DPAT i.p. to determine if the route of systemic administration mattered. To evaluate phase-shifting effects of quipazine, mice ($n = 32$) received injections of either quipazine (10 mg/kg, s.c.) or PBS vehicle control at CT8, 16, or 23.

Hamsters received 8-OH-DPAT at CT8 either i.p. ($n = 12$, 5 mg/kg) or intra-SCN ($n = 9$, 2.5 μ g in 0.5 μ l). Cannulated hamsters also received 8-OH-DPAT at CT18.5 prior to a light pulse at CT19. The light stimulus was provided by turning on the incandescent light in the home cabinet (10 min, 20 lux). A 3rd group of hamsters ($n = 6$) received quipazine (s.c., 10 mg/kg) and PBS at CT8.

Experiment 2: Effect of 5-HT Agonists on c-Fos Immunoreactivity

Mice received no treatment ($n = 3$) or an injection of 8-OH-DPAT ($n = 4$, 5 mg/kg) or PBS ($n = 4$) at CT8.

Additional groups received quipazine (10 mg/kg) or PBS at CT8, 16, or 22 ($n = 5$ per condition). Hamsters received quipazine (10 mg/kg), 8-OH-DPAT (5 mg/kg), or PBS i.p. at either CT8 or CT13 ($n = 3$ or 4 per condition). All animals were sacrificed for histology 90 min after injections.

Experiment 3: Effects of 5-HT Agonists on Photic Phase Shifts

Wild-type mice were given an i.p. injection of either vehicle ($n = 13$) or 8-OH-DPAT (25 mg/kg, $n = 9$; 42 g/kg, $n = 4$) prior to a light pulse at CT16 (20 lux white light for 10 min). 5-HT_{1A}-KO mice received an i.p. injection of either vehicle ($n = 2$) or 8-OH-DPAT (25 mg/kg, $n = 4$) prior to a light pulse at CT16. Animals were removed from their wheel-running cages in DD with the aid of infrared goggles and placed in a cylindrical white-plastic stimulus chamber (9 cm diameter $\times$ 6 cm deep) inside a light-tight and ventilated light-stimulation apparatus. Light stimulation was provided by a projector module from a Kodak 4200 Carousel projector using a tungsten halogen 300 W lamp (GE:FHS). Light intensity was adjusted with neutral density filters, and illuminance inside the chamber was measured with a Tektronix J1811 Cosine Corrected Illuminance Head attached to a Tektronix J17 photometer/radiometer/colorimeter. The duration of light stimulation was controlled by a manual shutter.

Hamsters received an i.p. injection of either vehicle ($n = 6$) or 8-OH-DPAT (5 mg/kg, $n = 6$) prior to a light pulse at CT19 (20 lux white light for 10 min).

Data Analysis

Phase shifts were measured by fitting separate regression lines to successive onsets of the daily period of wheel-running activity for 7 to 10 days prior to and following injections. Phase shifts were recorded as the displacement in minutes between the 2 lines projected to the day after the injections. Activity onsets were identified by computer algorithm, as the first 10-min bin to exceed 50 wheel counts after an interval of 240 min or more. Regression lines were also fit by computer. The first 2 days after an injection were excluded from the line fits to ensure that the circadian phase was in steady state. The algorithm occasionally identified spurious onsets, which were then deleted. Because of this, separate phase-shift assessments were performed by 2 individuals, 1 of whom was blind to

treatment conditions. These assessments were then averaged. Data were analyzed by ANOVA for repeated or independent samples, as appropriate. Student-Newman-Keuls post hoc tests were used to examine individual differences when a significant main effect was detected. Means shifts are reported in the text $\pm$ standard deviation and are depicted as $\pm$ SEM in figures.

c-Fos Immunocytochemistry and Cell Counting

Animals were perfused transcardially with 50 ml of PBS, followed by 50 ml of 4% paraformaldehyde. Brains were postfixed, cryoprotected, frozen, and sectioned through the hypothalamus at 50 μ m. Each brain provided 3 to 6 sections through the SCN. Sections were rinsed in a 0.3% H₂O₂ in PBSx (0.3% Triton-X-100 in PBS) bath and then in PBSx (3 $\times$ 10-min rinses). The tissue was incubated in 10% normal goat serum (NGS, Vector) in PBSx for 90 min followed by 48 h at 4 $^{\circ}$ C in the primary c-Fos antibody (1:80,000 in 1% NGS-PBSx) raised in rabbits against residues 4 to 17 of human fos (Oncogene Science, c-Fos, AB-5). The tissue was rinsed through 3 $\times$ 10-min PBSx baths and incubated in the secondary goat-anti-rabbit antibody (Vectastain Elite Kit, Vector). Following 3 $\times$ 10-min PBSx baths, the tissue was incubated in an Avidin-Biotin Complex (ABC, Vectastain Elite Kit, Vector) bath for 60 min. Following a final 3 $\times$ 10-min PBSx baths, the tissue was developed with a diaminobenzidine (DAB) reaction for 5 min (0.04% DAB in Tris Buffer + 60 μ l of 8% NiCl and 10 μ l of 30% H₂O₂). The reaction was quenched in PBSx rinses, and tissue was mounted on gelatin-coated slides, dehydrated, and coverslipped.

For analysis, digital images were captured maintaining the same light column and were flat field corrected using MCID (Imaging Research, Inc., St. Catherine's, ON, Canada). An object-counting routine in MCID was used to estimate the number of stained nuclei. Using the 3rd ventricle and optic chiasm as guides for placement, a standard-sized oval encompassing the SCN was placed over each SCN, and the mean number of cells within the oval per section through the SCN was obtained. Six sections were examined through the SCN of hamsters, and 3 sections were examined through the SCN of mice. FOS-ir cells were counted in both nuclei, providing a bilateral total for each section. For analysis, the mean number per section was determined for each animal. For analysis of the paraventricular nucleus (PVN), a single

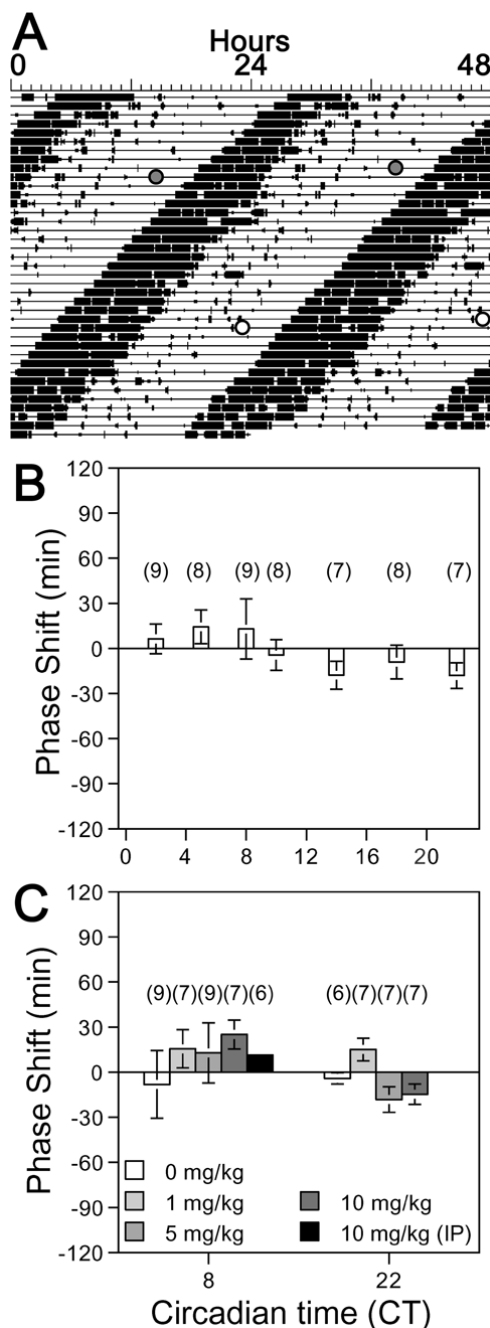


Figure 1. (A) Double-plotted actogram of wheel-running activity of a representative mouse receiving 5 mg/kg 8-OH-DPAT (gray circle) and phosphate-buffered saline (white circle) at CT8. Each horizontal line represents two 24-h periods. The last 24 h of each line are replotted as the first 24 h of the subsequent line. Vertical deflections represent a 10-min period during which the animal was active as monitored by revolution of their running wheel. (B) Phase-response curve to 8-OH-DPAT (5 mg/kg) at CT2, 5, 8, 10, 14, 18, and 22. (C) Dose-response curve to 8-OH-DPAT administered at CT8 and 22. All injections were administered subcutaneously (s.c.) except for one manipulation at CT8 in which the drug was given intraperitoneally (i.p.) as well. All means are presented $\pm$ SEM. Numbers in brackets represent the number of data points contributing to the corresponding mean.

representative section was used as this was all that was needed to confirm, by within-subject positive control, that the drugs had significant neuronal effects at the doses used.

RESULTS

Experiment 1: Phase Shifts in Mice and Hamsters

In mice, group mean phase shifts to 8-OH-DPAT (5 mg/kg) and PBS did not exceed 20 min at any circadian phase (Fig. 1). There was no significant main effect of phase of injection ($F_{6,41} = 1.294$, $P > 0.05$) and no significant difference between drug and PBS at any phase or dose (Fig. 1 B,C). There was a significant dose effect at CT22 ($F_{3,15} = 4.05$, $P < 0.05$), but this was due to a group mean advance (only 16 ± 20 min) in response to 1 mg/kg 8-OH-DPAT, compared to group mean delays (only 18 ± 23 and 14 ± 18 min) to the 5 and 10 mg/kg doses, respectively. There was no difference between s.c. and i.p. injections at CT8 ($t_5 = 1.00$, $P > 0.05$; Fig. 1C).

Mean shifts to quipazine did not exceed 12 min at any of the 3 phases tested, and there was no significant main effect of drug ($F_{1,26} = 1.396$, $P > 0.05$) or Drug $\times$ Phase interaction ($F_{2,26} = 0.736$, $P > 0.05$; Fig. 2). There was a significant main effect of injection phase ($F_{2,26} = 4.535$, $P < 0.05$) primarily due to 3 phase delay shifts in the 40-min range to PBS at CT22 (Fig. 2C).

In hamsters, 8-OH-DPAT (5 mg/kg) injected i.p. at CT8 resulted in a group mean phase advance of 70 ± 46 min, which was significantly greater than that to PBS (0 ± 12 min, $t_{14} = 3.59$, $P < 0.01$; Fig. 3 A,E). However, 8-OH-DPAT (2.5 μ g) injected directly into the peri-SCN area did not induce significant shifts at CT8 ($t_8 = 0.16$, $P > 0.05$; e.g., Fig. 3 B,E), but injections at CT18.5 did significantly attenuate phase shifts to light pulses at CT19 ($t_7 = 3.86$, $P < 0.01$; Fig. 3E). Quipazine (10 mg/kg) injected s.c. at CT8 also did not induce phase shifts different from saline (Fig. 3 C-E).

Experiment 2: Fos Immunoreactivity in Mice and Hamsters

In the mouse, 8-OH-DPAT at CT8 did not affect c-Fos immunoreactivity in the SCN ($F_{2,8} = 0.493$, $P < 0.05$) but strongly induced c-Fos in the PVN by comparison with both PBS and no-treatment controls ($F_{2,8} = 12.3$, $P < 0.05$; Fig. 4).

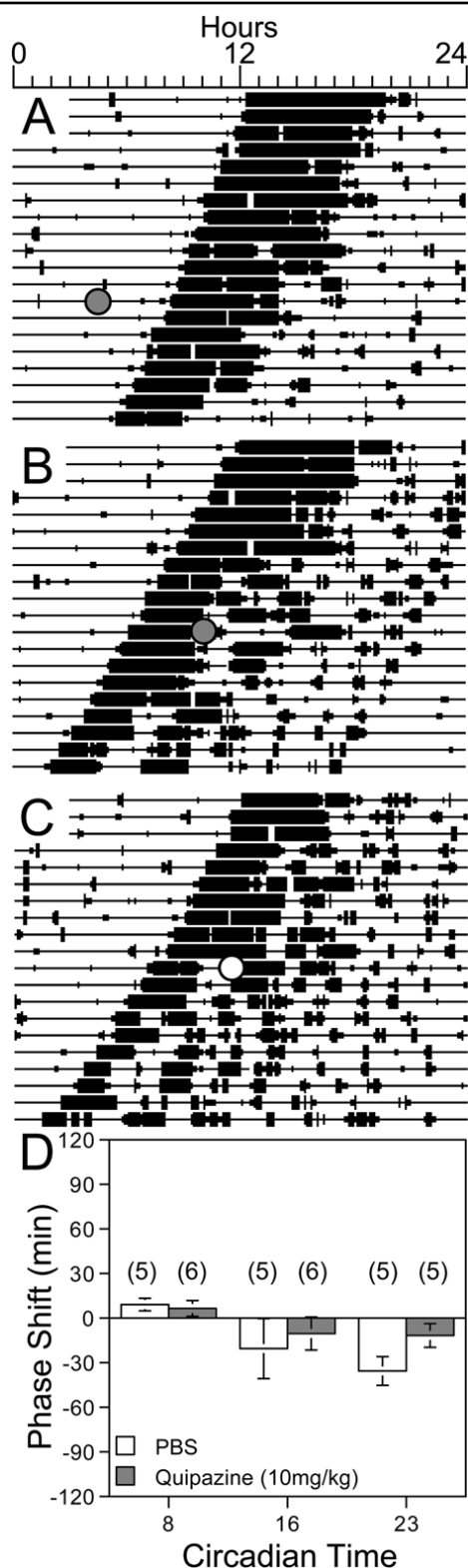


Figure 2. Single-plotted actograms of wheel-running activity from mice receiving quipazine (10 mg/kg) at CT8 (A), quipazine at CT16 (B), or phosphate-buffered saline (PBS) at CT16 (C). (D) Group mean ($\pm$ SEM) phase shifts to quipazine and PBS when administered at CT8, 16, or 23. Numbers in brackets represent the number of data points contributing to the corresponding mean.

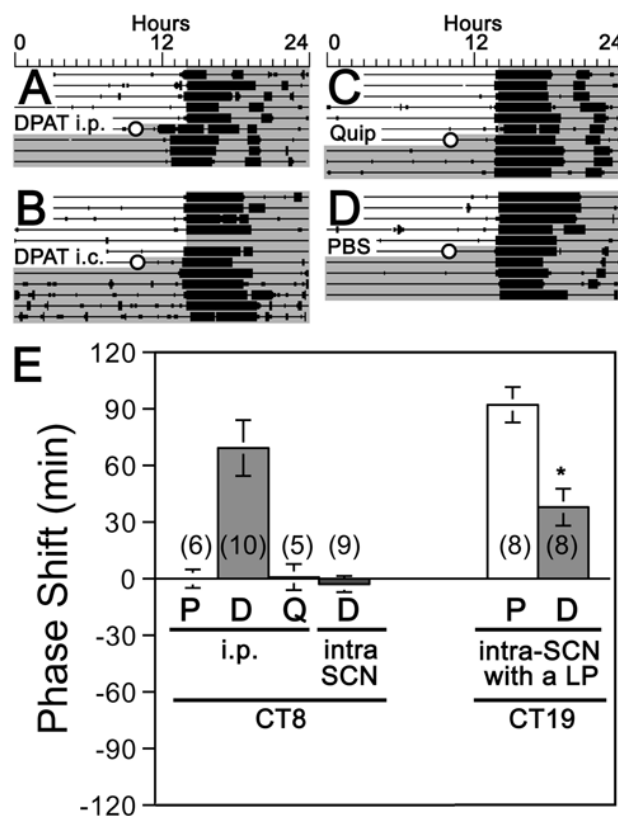


Figure 3. Single-plotted actograms of wheel-running activity from hamsters receiving (A) 8-OH-DPAT intraperitoneally (i.p.) (5 mg/kg), (B) 8-OH-DPAT intra-SCN (2.5 μ g in 0.5 μ l), (C) quipazine i.p. (10 mg/kg), or (D) phosphate-buffered saline (PBS). In all cases, hamsters were housed in a 14:10 LD cycle until the day of the manipulation. The lights were turned off when the manipulation was performed, and the lights remained off for 3 to 5 days. Group means ($\pm$ SEM) are illustrated in panel E. Numbers in brackets represent number of data points contributing to the corresponding mean. P = PBS; D = 8-OH-DPAT; Q = quipazine; LP = 10 min 20 lux light pulse. * $P < 0.01$.

The quipazine experiment yielded significant main effects of phase ($F_{2,14} = 12.29$, $P < 0.05$) and drug ($F_{1,14} = 5.53$, $P < 0.05$), as well as a significant interaction ($F_{2,14} = 7.38$, $P < 0.05$) on c-Fos expression in the SCN (Fig. 5). Post hoc tests revealed that FOS-ir was higher during the subjective day (CT8) than in the subjective night (CT16 and 23) and that quipazine increased SCN c-Fos at CT8 but had no effect at CT16 or 23 (Fig. 6). Quipazine strongly induced FOS in the PVN ($F_{1,15} = 6.913$, $P < 0.05$), but there was no main effect of phase ($F_{2,15} = 1.184$, $P > 0.05$), nor was there a significant Phase $\times$ Drug interaction ($F_{2,15} = 0.705$, $P > 0.05$).

In the hamster, both 8-OH-DPAT and quipazine significantly attenuated c-Fos expression in the SCN at CT8 when compared with the effects of PBS ($t_8 =$

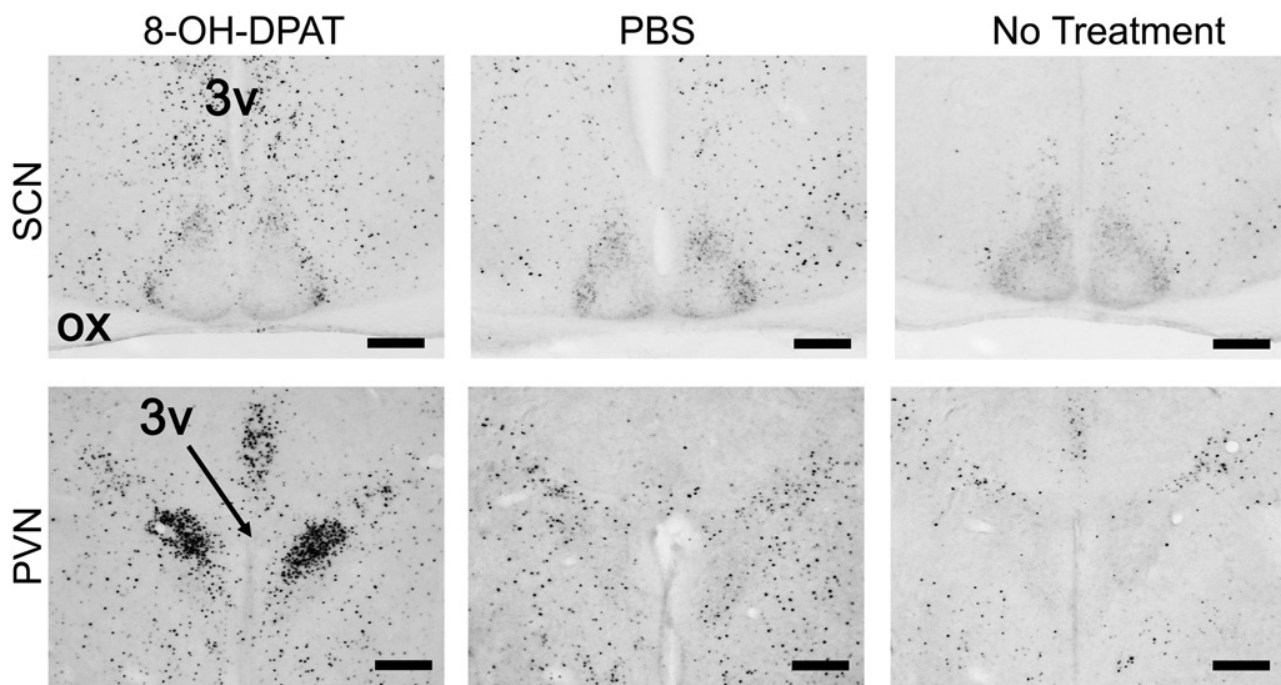


Figure 4. FOS-ir in the mouse SCN and paraventricular nucleus following 8-OH-DPAT (5 mg/kg s.c.), PBS, or no treatment. Scale bars = 200 μm.

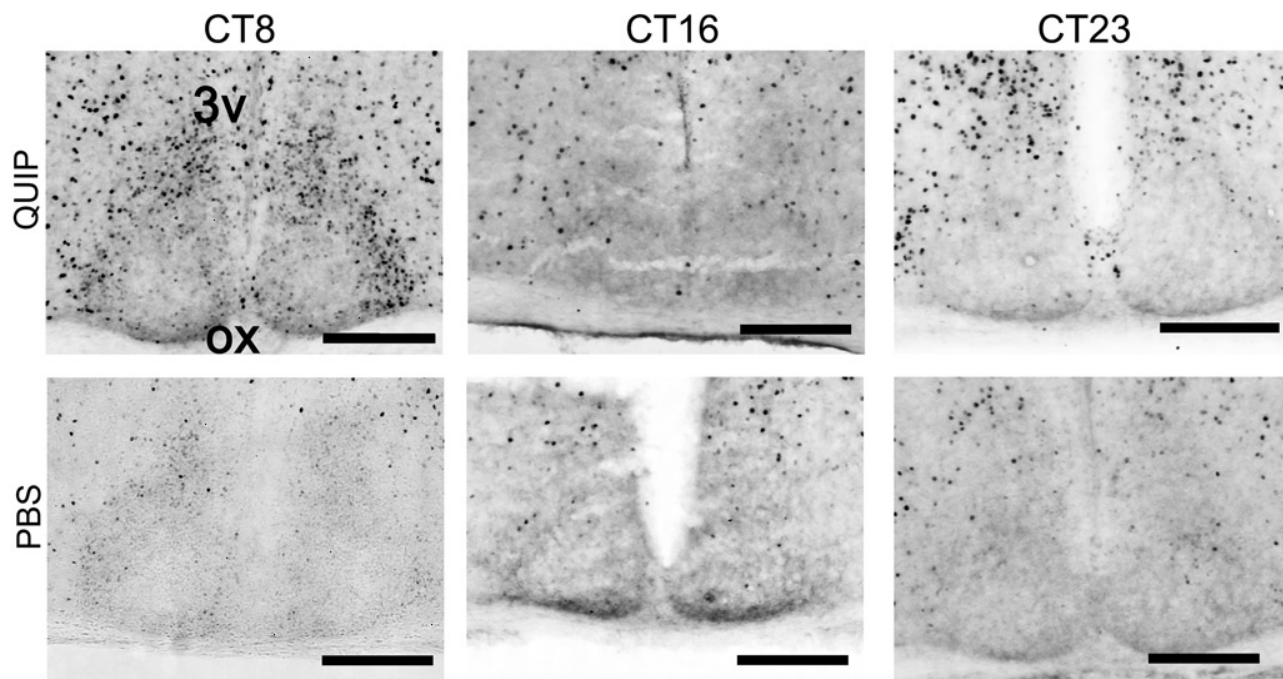


Figure 5. FOS-ir in the mouse SCN and paraventricular nucleus (PVN) following quipazine (10 mg/kg s.c.) or phosphate-buffered saline (PBS) at CT8, 16, or 23. Scale bar = 200 μm.

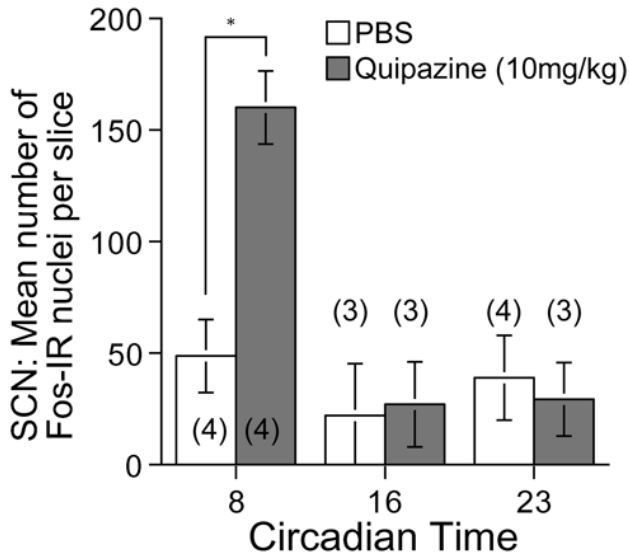


Figure 6. Group mean ($\pm$ SEM) FOS-ir cell counts in the mouse SCN following quipazine (10 mg/kg s.c.) or phosphate-buffered saline (PBS) at CT8, 16, or 23. Numbers in brackets represent the number of data points contributing to the corresponding mean. * $P < 0.05$.

4.48, $P < 0.01$; $t_7 = 5.73$, $P < 0.001$, respectively; Fig. 7), but neither affected c-Fos expression at CT13. Both drugs induced c-Fos strongly in the PVN at both CT8 and 13.

Experiment 3: Effects of 8-OH-DPAT on Photic Shifts in Mice

Light-induced phase shifts were not attenuated by 8-OH-DPAT in wild-type mice at either dose tested ($F_{2,23} = 0.177$, $P > 0.05$; Fig. 8) or in 5-HT_{1A}-KO mice at the single dose tested ($t_4 = 0.418$, $P > 0.05$). However, 8-OH-DPAT did significantly attenuate light-induced phase advances in hamsters ($t_{10} = 2.97$, $P < 0.05$; Fig. 8).

DISCUSSION

Phase Shifts

The results of this study add further to the evidence for significant strain and species differences in the response of the rodent circadian system to serotonergics. In mice, quipazine and 8-OH-DPAT had little effect on circadian phase when applied during the subjective day or night at several doses.

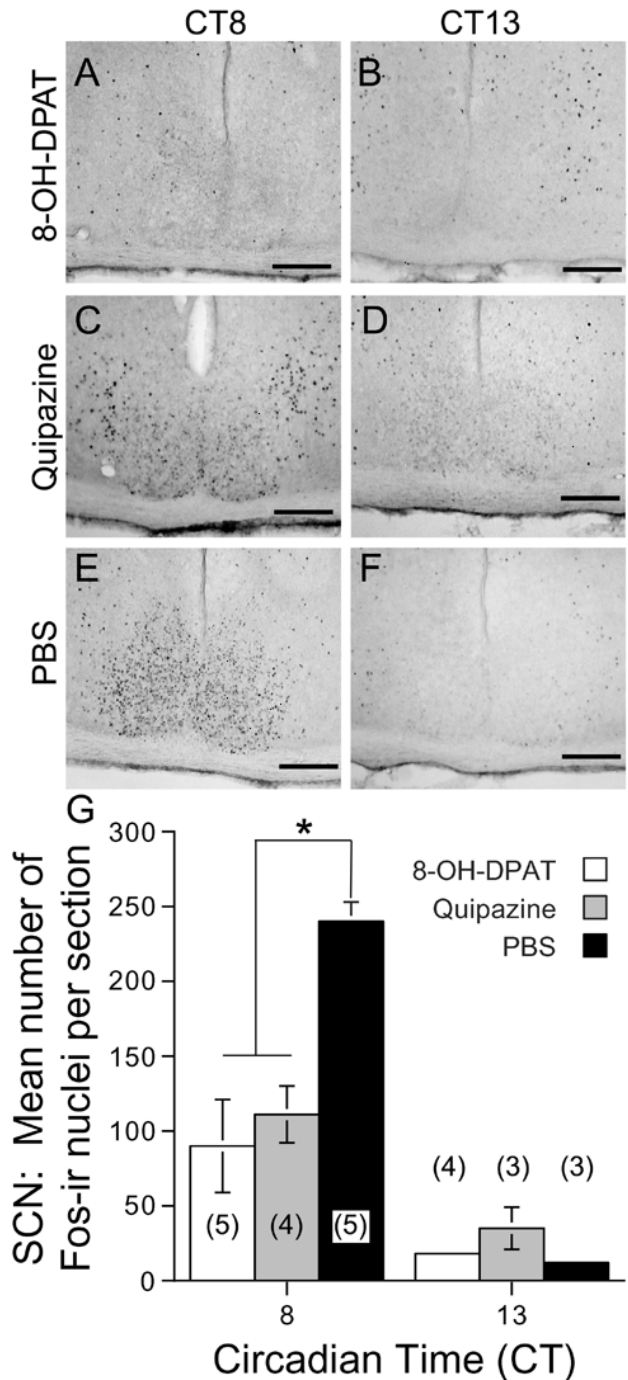


Figure 7. FOS-ir in the hamster SCN following 8-OH-DPAT (A,B) (5 mg/kg, i.p.), quipazine (C,D) (10 mg/kg, i.p.), or phosphate-buffered saline (PBS) (E,F) at CT8 (A,C,E) or CT13 (B,D,F). Scale bar = 200 μ m. (G) Group mean ($\pm$ SEM). FOS-ir cell counts in the hamster SCN following 8-OH-DPAT (5 mg/kg, i.p.), quipazine (10 mg/kg i.p.), or PBS at CT8 or 13. Numbers in brackets represent the number of data points contributing to the corresponding mean. *significantly different from PBS treatment ($P < 0.01$).

Although the phase-response curves obtained appeared to have the general shape characteristic of

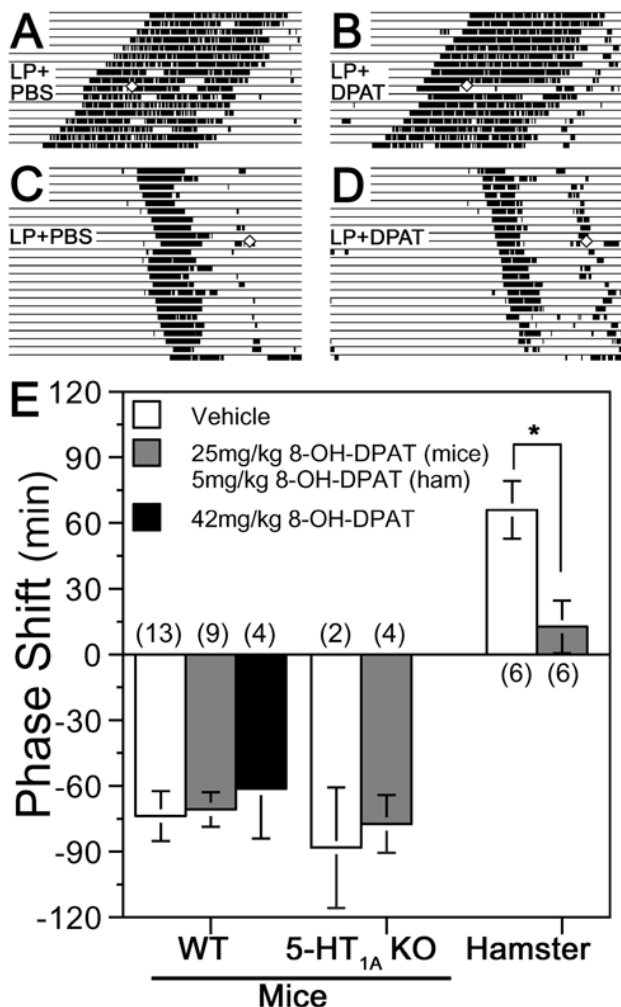


Figure 8. (A,B) Actograms of wheel-running activity from mice treated with vehicle (A) or 8-OH-DPAT (B) (25 mg/kg, i.p.) prior to a light pulse (white diamond) at CT16. (C,D) Actograms of wheel-running activity from hamsters treated with vehicle (C) or 8-OH-DPAT (D) (5 mg/kg, i.p.) prior to a light pulse (white diamond) at CT19. (E) Mean ($\pm$ SEM) phase shifts to light and drug treatments in mice and hamsters. Numbers in brackets represent the number of data points contributing to the corresponding mean. * $P < 0.05$.

those associated with nonphotic stimuli, the amplitudes were extremely small, there were no significant differences from vehicle injections, and we could find no cases by which to illustrate an obvious phase shift. Saline injections late in the subjective day have been reported to produce small phase-advance shifts in hamsters (Hastings et al., 1992); therefore, it is likely that any weak effects observed here are attributable to arousal induced by the injection procedure.

These data differ markedly from those obtained in studies of hamsters, in which phase shifts of 60 min or more are routinely observed following systemic injections of 8-OH-DPAT in the subjective day (Tominaga et al., 1992; present study). However, in hamsters in vivo, single bolus injections of 8-OH-DPAT directly into the SCN area induce shifts that are either very small (Challet et al., 1998) or are undetectable by comparison with saline (Experiment 1; Mintz et al., 1997), even in cases in which the drug at the same dose, through the same cannula, potentially inhibited light-induced phase shifting (Experiment 1). Other work has shown that phase shifts to systemic 8-OH-DPAT can be mimicked by injections into the dorsal raphe nucleus (DRN) (Mintz et al., 1997), which contains serotonergic neurons projecting to the thalamic intergeniculate leaflet (IGL). One interpretation of these data is that systemic 8-OH-DPAT induces phase shifts indirectly by inhibiting DRN 5-HT neurons and thereby disinhibiting neuropeptide Y-containing efferents projecting from the IGL to the SCN (Mintz et al., 1997). This model suggests that the differential response to systemic 8-OH-DPAT in mice and hamsters likely reflects a species difference in functional responses to 8-OH-DPAT in cell groups one or more synapses afferent to the SCN.

Although single bolus injections of 8-OH-DPAT directed at the SCN have little or no phase-resetting effect, intra-SCN injections can induce large phase shifts in vivo if the infusions are prolonged (3 h) and the animals are pretreated with the 5-HT synthesis inhibitor p-CPA to upregulate 5-HT receptor levels (Ehlen et al., 2001). Supersensitivity may also occur in the SCN slice preparation, thereby explaining the characteristic large phase shifts induced by 8-OH-DPAT in vitro. Given the recent report that the mouse SCN also shows large phase shifts to 8-OH-DPAT in vitro (R. Prosser, June 2002, personal communication), we predict that 5-HT receptor upregulation will also permit phase shifts to 8-OH-DPAT in the mouse in vivo. Loss of retinal inputs to the slice may also contribute. Photic neurotransmitters attenuate nonphotic phase shifting, and their absence in vitro may disinhibit responses to nonphotic inputs (Prosser, 2000).

Mice and hamsters in the present study were similar in their lack of phase-shifting response to quipazine administered systemically during the subjective day and night. The drug appears to have neither a photic nor a nonphotic action in these 2 species. This result is consonant with one other study of hamsters (Bobrzynska et al., 1996a) but not with the results from studies of rats, which differ markedly across labs. One rat study has reported phase advances in

response to daytime quipazine (Edgar et al., 1993), another using the same strain has reported phase delays to daytime injections (Kennaway et al., 1996), and several have reported phase-shifting responses at night that mimic the effects of light (Kalkowski and Wollnik, 1999; Kennaway et al., 1996; Kohler et al., 1999; Prosser et al., 1993). The nature of these discrepancies remains unclear, but evidently there can be as much variability within as between species.

c-Fos Immunoreactivity

Additional evidence for species diversity in the response to serotonergics was obtained by measures of FOS-ir. Previous work has demonstrated that exercise and forced arousal in the subjective day significantly attenuate FOS-ir in the hamster SCN (Antle and Mistlberger, 2000; Mikkelsen et al., 1998). Quipazine also suppresses c-Fos expression in the subjective day in the rat SCN in vitro (Prosser et al., 1994) but has been reported to induce expression in the subjective night in rat SCN in vivo, again mimicking effects of light (Kohler et al., 1999; Moyer et al., 1997; Neumaier et al., 2001). Our data support the evidence for species differences but define a different pattern of responses. In the mouse, we did not see a significant attenuation of SCN FOS-ir in the subjective day following either 8-OH-DPAT or quipazine treatment; rather, 8-OH-DPAT had no effect on c-Fos while quipazine resulted in a significant elevation. However, FOS-ir cells in quipazine-treated mice were evident primarily in the dorsal and lateral borders of the SCN, and it is possible that some of these cells were outside of the SCN. Nonetheless, there was no evidence for suppression of c-Fos in the core region of the SCN at any rostro-caudal level. During the subjective night, neither drug had an effect on c-Fos expression, nor did we see evidence of photic-type induction. Both 8-OH-DPAT and quipazine strongly induced c-Fos expression in the PVN at all circadian phases, indicating that the drugs were biologically active at the doses employed and had rapid access to the brain following systemic administration. In the Syrian hamster, we did see significant attenuation of SCN FOS-ir by both drugs applied systemically in the subjective day but, again, no effect at night. Thus, in neither species were photic-type responses evoked, and only in the hamster did the response mimic that associated with arousal-inducing procedures that elicit large phase shifts in the subjective day.

Previous studies have demonstrated that changes in c-Fos expression in the SCN correlate with phase shifts; that is, light at night induces c-Fos and phase shifts, while arousal in the subjective day suppresses c-Fos and induces shifts. In the present study, we saw a dissociation between changes in c-FOS and phase; in hamsters, quipazine i.p. significantly attenuated SCN FOS-ir but had no phase-shifting effect, suggesting that changes in c-Fos expression are a correlate rather than a cause of clock resetting.

Effects on Photic Phase Shifts

A consistent finding in studies of 5-HT and rodent circadian rhythms has been the inhibitory effect of 5-HT_{1A/7} agonists on light-induced responses in the circadian system. In the present study, we successfully used inhibition of light-induced phase shifting in hamsters as a positive control to demonstrate efficacy of 8-OH-DPAT delivered either via peri-SCN cannulas (Experiment 2) or systemically (Experiment 3). Thus, the failure of systemic 8-OH-DPAT to inhibit light-induced phase shifts in wild-type or 5-HT_{1A} KO mice was unexpected. It has recently been shown in the same wild-type mouse strain that 8-OH-DPAT does attenuate excitatory postsynaptic currents generated in the SCN by optic nerve stimulation (Smith et al., 2001). Thus, there is no question that appropriate receptors for 8-OH-DPAT are present within the mouse SCN. There must be other factors present in the intact SCN in vivo that limit the effects of 8-OH-DPAT on photic transmission to SCN clock cells. One possibility is that inhibitory effects of 8-OH-DPAT on SCN neurons receiving RHT input are offset by concurrent disinhibitory effects of 8-OH-DPAT on GABA terminals in the SCN. 5-HT₇ receptors have been described on GABA terminals in the mouse SCN (Belenky and Pickard, 2001), and thus inhibition of GABA release, the major fast neurotransmitter in the SCN, could have mitigated the effects of 8-OH-DPAT at RHT terminals. Analysis of these factors may be facilitated with the use of other mouse KO models, and this may shed light on how photic and nonphotic information is integrated within the circadian pacemaker.

CONCLUSIONS

The results reported here demonstrate that under normal conditions, activation of 5-HT_{1A/2/7} recep-

tors in the SCN is not sufficient to induce either photic or nonphotic-type phase shifting in either mice or hamsters. The clock may be vulnerable to 5-HT mediated shifting only in situations in which there has been a chronic change in 5-HT release or receptor sensitivity somewhere within the circadian system (as presumably occurs following p-CPA treatment and as likely occurs in the deafferented SCN slice preparation). Whether such changes can occur under physiological conditions, possibly in association with pathological states underlying depression or other disorders, remains to be explored.

It might be reasoned that the lack of a significant effect of DPAT or quipazine on the circadian behavior of the mouse is surprising. On the other hand, it is perhaps more surprising that DPAT and quipazine alter the circadian behavior of the hamster or rat. As summarized by Jacobs and Azmitia (1992), "Although 5-HT has been implicated in a vast array of physiological and behavioral processes in vertebrates, it appears to be essential for none of them" (p. 165). The apparent contradiction of this statement is reconciled by the fact that serotonergic neurons exert a tonic modulatory influence on widespread targets. The influence on target neurons is expressed in direct association with the motor activity and behavioral state of the organism. That is, serotonergic neurons express highest activity during arousal and active waking, intermediate activity during quiet waking, and virtually no activity during REM sleep (Jacobs and Azmitia, 1992). It has been suggested that serotonergic neurons may act to facilitate motor systems while suppressing sensory systems (Aghajanian and Vandermaelen, 1986), and this hypothesis has been further developed by Jacobs and Fornal (1995). Data consistent with this hypothesis and, importantly, consistent across rodent species (i.e., mouse, hamster, and rat) are the suppression of RHT sensory input to the SCN by the activation of 5-HT_{1B} receptors on RHT terminals (Pickard et al., 1996; Pickard and Rea, 1997; Pickard et al., 1999; Smith et al., 2001).

The inconsistency of the effect of DPAT observed in the mouse in vivo (this study) and in vitro (Smith et al., 2001; R. Prosser, unpublished data) has, as noted, several possible explanations. A complete understanding of the subcellular distribution of all 5-HT receptor subtypes in the SCN is necessary to assist in interpreting the complex role 5-HT surely must play in modulating SCN neuronal activity.

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