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Resetting the Circadian Clock in Cultured *Xenopus* Eyecups: Regulation of Retinal Melatonin Rhythms by Light and D_2 Dopamine Receptors

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A circadian oscillator is located within the eye of *Xenopus laevis*. This oscillator regulates retinal melatonin synthesis, stimulating it at night. The primary goal of the studies reported here was to define input pathways to this circadian oscillator as a step toward identification of circadian clock mechanisms. A flow-through superfusion culture system was developed to monitor circadian rhythms of melatonin release from individual eyecups. This system was used to determine the effects of light and dopaminergic agents on melatonin production and on the phase of the circadian oscillator. Six hour light pulses suppressed melatonin production and reset the phase of the free-running melatonin rhythm. Light pulses caused phase delays when applied during the early subjective night, phase advances when applied during the late subjective night, and no phase shift when applied during the subjective day. Dopamine receptor agonists mimicked light in suppressing melatonin release and resetting the phase of the circadian rhythm. The phase–response relationship for phase shifts induced by quinpirole, a D_2 dopamine receptor agonist, was similar to that for phase shifts induced by light. Pharmacological analysis with selective catecholamine receptor agonists and antagonists indicated that there are pathways to the melatonin-generating system and the circadian oscillator that include D_2 dopamine receptors. A D_2 receptor antagonist, eticlopride, completely blocked the effects of dopamine on melatonin release and on circadian phase. However, eticlopride did not alter similar effects induced by light, indicating that dopamine-independent pathways exist for light input to these systems. The effects of light and quinpirole on melatonin release and circadian phase were not additive, indicating that the pathways converge. These pathways to the circadian oscillator in the retina present new avenues for pursuit of cellular circadian clock mechanisms.

Circadian clocks are systems of biological oscillators that can generate rhythms with a period of approximately 24 hr in the absence of external timing cues (Pittendrigh, 1981a). The cellular mechanisms of circadian rhythm generation are unknown. Circadian oscillators can be entrained by cyclic environmental stimuli or by coupling signals from other oscillators; light is the most common environmental entraining stimulus (Pittendrigh, 1981a,b). An entraining stimulus, when applied as a short pulse, resets the phase of a free-running circadian oscillator. The direction and magnitude of the resulting phase shift depend on the circadian phase when the pulse is applied (Pittendrigh, 1981b). Because entraining stimuli alter the timing of a circadian oscillator, the input pathways that mediate entrainment must impinge upon the oscillator mechanism itself. Therefore, one approach to mechanistic analysis of circadian systems is to identify and investigate entrainment pathways, following them inward toward the oscillator (Menaker et al., 1978; Eskin, 1979). This general approach has been applied to a few neural and endocrine circadian oscillator systems *in vitro*, including molluscan eyes (Corrent et al., 1982; Eskin et al., 1982, 1984; McMahon and Block, 1987), avian pineal cells (Robertson and Takahashi, 1988; Zatz et al., 1988; Takahashi et al., 1989), and rodent hypothalamic slices containing the suprachiasmatic nucleus (Prosser and Gillette, 1989; Prosser et al., 1990).

A circadian oscillator resides within the eye of the African clawed frog, *Xenopus laevis* (Besharse and Iuvone, 1983). This oscillator regulates the activity of retinal serotonin *N*-acetyltransferase (NAT), the penultimate enzyme in the melatonin biosynthetic pathway. Regulation by the ocular circadian oscillator results in increased NAT activity during the night and decreased activity during the day. This rhythmicity persists in constant darkness, both *in vivo* (Iuvone and Besharse, 1983) and *in vitro* (Besharse and Iuvone, 1983). Rhythmic NAT activity is reflected in rhythmic release of melatonin, which modulates several other rhythmic retinal processes (reviewed by Besharse et al., 1988; Cahill et al., 1991). Although NAT has not been localized to a specific retinal cell type, there is evidence for melatonin synthesis in photoreceptors and in a class of bipolar cells (Wiechmann et al., 1985; reviewed by Cahill et al., 1991).

Light has two distinct classes of effects on retinal rhythms of NAT activity and melatonin production. Light causes acute suppression of retinal NAT activity and melatonin content and release (Binkley et al., 1979; Hamm and Menaker, 1980; Iuvone and Besharse, 1983), and it also affects the circadian oscillator that underlies rhythmicity in NAT activity and melatonin production. The oscillator is entrained by light cycles, and its phase can be reset by shifted light cycles or by single pulses of light; these effects are reflected in the timing of NAT and melatonin rhythms (Besharse and Iuvone, 1983; Underwood et al., 1988, 1990; Cahill et al., 1991). All of these effects of light can be

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observed in cultured *Xenopus* eyecups (Besharse and Iuvone, 1983; Iuvone and Besharse, 1983; Cahill et al., 1991).

It has been proposed that retinal dopamine, acting primarily through D_2 receptors, mediates adaptive effects of light on several rhythmic retinal processes (reviewed by Besharse et al., 1988). Dopamine is synthesized in a class of retinal amacrine cells and, in some species, interplexiform cells (reviewed by Ehinger, 1982). Light causes increases in retinal dopamine synthesis, release, and turnover (Iuvone et al., 1978; Parkinson and Rando, 1983; Godley and Wurtman, 1988; Boatright et al., 1989). Dopamine and D_2 dopamine receptor agonists mimic light in modulating a number of retinal phenomena, including retinomotor movements of cone photoreceptors (Pierce and Besharse, 1985) and pigment granules in the retinal pigment epithelium (Dearry and Burnside, 1988), retinal NAT activity (Iuvone, 1986; Iuvone and Besharse, 1986b) and melatonin content (Zawilska and Iuvone, 1989), the balance of rod and cone photoreceptor input to horizontal cells (Witkovsky et al., 1988, 1989), and photoreceptor cAMP levels (Cohen and Blazynski, 1990). Furthermore, the effects of light on some of these processes are blocked by D_2 receptor antagonists, suggesting that these light responses are mediated by endogenous retinal dopamine (Pierce and Besharse, 1985; Witkovsky et al., 1988, 1989). In addition, evidence has been presented that dopamine acts through D_1 dopamine receptors in teleost retina to mediate horizontal cell responses to prolonged darkness (Yang et al., 1988). The roles of dopamine and dopamine receptors in regulation of the ocular circadian oscillator have not been investigated previously.

We have recently developed an organ culture system with which circadian rhythms of melatonin release from individual *Xenopus* eyecups can be monitored for several days (Cahill and Besharse, 1990; Cahill et al., 1991). This system enables analysis of the effects of putative entraining agents on the ocular circadian oscillator. In the experiments reported here, the effects of pulses of light and dopaminergic agents, both on retinal melatonin synthesis and on the phase of the circadian oscillator, were determined by monitoring free-running circadian rhythms of melatonin release from cultured eyecups. This paradigm was also used to classify the receptors mediating dopaminergic effects pharmacologically and to test the proposition that endogenous dopamine mediates the effects of light on retinal circadian rhythms.

Materials and Methods

Animals. Adult, male *Xenopus laevis* (length, 5–6.5 cm) were obtained from Nasco (Fort Atkinson, WI) and maintained under a light cycle of 12 hr light, 12 hr darkness for at least 2 weeks prior to use in experiments.

Eyecup culture. For all experiments, eyecups were prepared 1–3 hr before the end of the light period by procedures described previously (Besharse and Dunis, 1983). Eyecups were maintained individually in flow-through superfusion chambers (Cahill et al., 1991) modified from a design used for culture of pineal glands by Takahashi et al. (1980). Culture medium was delivered (1 ml/hr) to the superfusion chambers from a multichannel syringe pump via Teflon tubing. Each eyecup was suspended in a wire-mesh basket inside a chamber made from a 5 cc glass syringe barrel. Two stainless-steel tubes penetrated a plastic plug into the chamber: one delivered culture medium directly into the eyecup; the other delivered a humidified gas mixture of 95% O_2/5% CO_2 (50–60 ml/min) into the chamber. As drops of culture medium overflowed the eyecup, they were expelled from the chamber by the gas and delivered via flexible tubing to a fraction collector. This design maximized the rate of superfusate turnover. Ten chambers were installed in each of two transparent Plexiglas water jackets. A constant temperature (21°C) was maintained by a circulating water bath. The water-jacketed culture chambers were enclosed in light-tight cabinets equipped with front panels that could be removed for light exposure.

The defined culture medium used in these experiments was modified from a previously described formulation of balanced salts and amino acids (Besharse and Dunis, 1983). The composition of the base medium was 82.5 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 35 mM $NaHCO_3$, 1 mM NaH_2PO_4, 5 mM dextrose, 5 μg/ml phenol red (omitted in experiments involving light), a mixture of 14 amino acids that was described previously (Besharse and Dunis, 1983), 0.1 mM ascorbic acid, 100 U/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate. The pH of the medium equilibrated with 95% O_2/5% CO_2 was 7.4.

When eyecups are cultured in this base medium, melatonin release is too low to be measured conveniently, but it can be increased up to 100-fold by treatments that increase retinal serotonin levels (Cahill and Besharse, 1990). Therefore, the serotonin precursor 5-hydroxy-L-tryptophan (5HTrp; 100 μM) was added to the culture medium for all experiments, except where noted. This treatment has no apparent effect on the timing function of the circadian oscillator, even when 5HTrp is applied in a cyclic fashion (Cahill et al., 1991).

Light and drug pulses. For light pulses, the front panel of the culture cabinet was replaced with a translucent Plexiglas diffusing screen, and mirrors were placed above and behind the superfusion chambers to distribute the light. Two white light sources were used in different experiments, either a projector (Oriel, Stratford, CT) with a 100 W tungsten quartz-halogen lamp, or a 20 W fluorescent lamp (Bright Stick, General Electric, Cleveland, OH) attached directly to the diffusing screen. Light intensities at the eyecups were estimated by placing a fiber optic probe inside a superfusion chamber and coupling it to an irradiance detector probe of an IL700 radiometer (International Light, Newburyport, MA). Taking into account the attenuation and directional sensitivity of the fiber optic probe, irradiance at the eyecups by the unattenuated quartz-halogen source was estimated to be approximately 10^{-5} W/cm^2 (lower and upper measurements, 6×10^{-6} and 2.7×10^{-5} W/cm^2). In some experiments, this light was attenuated by neutral density filters. Irradiance by the fluorescent source was approximately 10^{-4} W/cm^2 (lower and upper measurements, 3.2×10^{-5} and 2.4×10^{-4} W/cm^2).

Melatonin measurement. The melatonin content of unextracted superfusate samples (100 μl per assay tube) was determined by the radioimmunoassay (RIA) method of Rollag and Niswender (1976) as modified by Takahashi et al. (1980). We have previously validated this assay for measurement of melatonin in culture medium (Cahill and Besharse, 1989, 1990). None of the drugs used in these experiments interfered with the assay (data not shown).

Experimental design and analysis. Eyecups were maintained in flow-through superfusion culture in constant darkness for 5 d, starting at the entrained animals' dark onset time. Experimental eyecups were exposed to pulses of light and/or drugs at times specified relative to the animals' previous light cycle [light onset = *Zeitgeber* time 0 (ZT 0); dark onset = ZT 12), while control eyecups were kept in constant darkness in control medium throughout. In most experiments, paired experimental and control eyecups from an individual animal were compared, to reduce the effects of interindividual variability on measured changes in melatonin levels and circadian phase. In order to test multiple drugs simultaneously in some experiments with selective agonists, eyecups were distributed without pairing among four or five groups, and treatment effects were determined relative to control group means.

Superfusate samples were collected at 2 hr intervals (60 samples per eyecup over 5 d), and a single determination of melatonin content in each sample was made by RIA. In order to facilitate comparison of rhythms from eyecups producing different overall levels of melatonin, melatonin release was normalized relative to the 5 d average release rate for each eyecup. These normalized values were plotted as a function of time in culture.

For measurement of circadian period and phase shifts, rhythms were smoothed by a three-point running average to minimize the effects of random measurement error on phase determination (the individual records illustrated here are not smoothed). The interpolated times of half-rise and half-fall of melatonin rhythm peaks were measured from the smoothed records. These phase reference points were used to make multiple estimates of circadian period and phase for each eyecup. The circadian period of each eyecup was defined as the average of the time intervals between successive half-rises and the intervals between successive half-falls, starting at the first half-fall point (up to seven measurements per eyecup). The phase shift for each experimental eyecup was determined with respect to the phase of an untreated control and

Table 1. Amplitude characteristics of the circadian rhythm of melatonin release from eyecups cultured in constant darkness

Measurement	Mean	Range (min–max)	SD	Coefficient of variation (%)[a]	N[b]
Melatonin release (pg/hr)					
Peak 1 maximum	790	350–1700	280	36	117
Trough 1 minimum	190	75–400	70	36	117
Trough 4 minimum	190	15–410	90	46	97
Peak 5 maximum	420	53–1230	220	53	97
Relative amplitude					
Cycle 1: peak 1/trough 1	4.2	2.3–7.1	1.0	23	117
Cycle 5: peak 5/trough 4	2.3	1.4–3.8	0.5	22	97
Change: Cycle 1 to Cycle 5 (%)					
Peak melatonin release	−45	(−)90–(+)40	25	57	96
Trough melatonin release	+3	(−)90–(+)140	43	1480	96
Relative amplitude	−43	(−)73–(+)16	20	48	96

[a] (SD/Mean) × 100.

[b] Data are from *N* untreated control eyecups from 15 experiments.

was defined as the average of the differences from control in the timing of half-rises and half-falls of the last three circadian peaks of the record (up to six measurements per experimental–control pair). Low-amplitude peaks or missed measurements in some rhythms made phase determination at some phase reference points impossible. At least three measurements were averaged for each experimental eyecup; in 90% of the cases reported here, phase shifts were determined from measurements at five or six phase reference points.

The acute effects of 6 hr light and drug pulses on the production of melatonin are expressed as percentage decrease in the normalized rate of melatonin released during the period 2–6 hr after start of the treatment. The acute effects of 3 hr light pulses were determined from melatonin released during the period 2–4 hr after the beginning of the pulse.

Chemicals. Culture medium salts were obtained from Sigma (St. Louis, MO), and all other medium components were from Gibco (Grand Island, NY). For the radioimmunoassay, the antiserum (R1055) was obtained from Dr. G. D. Niswender of Colorado State University, and the ^{125}I-melatonin analog was from Hazelton Biotechnologies (Vienna, VA). Quinpirole-HCl (LY 171555) was donated by Lilly Research Laboratories (Indianapolis, IN); SKF 38393, by Smith Kline and French Laboratories (Philadelphia, PA); and SCH 23390, by Schering (Bloomfield, NJ). Eticlopride was obtained from Research Biochemicals (Natick, MA). All other drugs were obtained from Sigma.

Results

As reported previously (Cahill and Besharse, 1990; Cahill et al., 1991), eyecups cultured in medium containing 100 μM 5HTrp express robust circadian rhythms of melatonin release in constant darkness, with increased melatonin production during the subjective night. All experiments shown here were terminated after 5 d in culture, but in the one experiment that was prolonged through 7 d, all 19 of the eyecups expressed rhythmicity through the seventh circadian cycle (data not shown). The average free-running period of melatonin rhythms from untreated eyecups in constant darkness was 22.9 hr (SD = 0.5 hr; range, 21.2–24.3 hr; 95 control eyecups from 15 experiments).

Melatonin release levels and rhythm amplitudes determined from untreated eyecups during the first and fifth circadian cycles in constant darkness are summarized in Table 1. Absolute peak and trough levels of melatonin release were highly variable among individuals. The coefficients of variation for peak-to-trough relative amplitudes were 30–60% lower than those for the absolute peak and trough values. Normalized measurements were therefore used to determine the effects of experimental treatments on melatonin rhythms.

The amplitude of melatonin rhythms recorded from most eyecups damped progressively through time in culture. This primarily reflected a decrease in peak melatonin production; there was no consistent change in trough melatonin levels (Table 1). This damping probably was a result of multiple factors. An overall reduction in melatonin levels resulted when a retina detached from the pigment epithelium. Retinal detachment was obvious at a gross level in a small percentage of cultured eyecups, and may have also occurred to some extent in others. We have found that isolated retinas in culture express circadian rhythms of melatonin release with relative peak-to-trough amplitudes comparable to those of eyecups, but that the absolute levels of melatonin release are reduced 70–90% (G. M. Cahill and J. C. Besharse, unpublished observations). These data suggest that maximal melatonin production requires interaction of the retina with the retinal pigment epithelium. In addition to the reduction of melatonin release resulting from loss of the integrity of the eyecups, the observed damping of melatonin rhythms may have resulted in part from damping of the underlying circadian oscillator or from dissociation of multiple constituent oscillators within the retina.

Effects of light pulses

It has been shown previously that exposure to light prevents the nocturnal rise in NAT activity in cultured *Xenopus* eyecups (Iuvone and Besharse, 1983), and that the phase of the NAT rhythm can be reset *in vitro* by a shifted light cycle (Besharse and Iuvone, 1983). We have confirmed and extended these findings by measuring the effects of 6 hr pulses of white light (approximate intensity, 10^{-5} W/cm^2) on melatonin release rhythms from eyecups kept otherwise in constant darkness. Examples of rhythms of individual experimental–control eyecup pairs are shown in Figure 1. The experimental eyecups were exposed to light during the subjective day (Fig. 1*A*), the early subjective night (Fig. 1*B*), or the late subjective night (Fig. 1*C*). Melatonin release was suppressed acutely during light pulses, regardless of the circadian phase during which the pulse was applied (Fig. 1*A–C*).

The effect of a light pulse on the subsequent phase of the melatonin rhythm was dependent on the circadian phase during which the pulse was applied. Phase shifts can be seen by com-

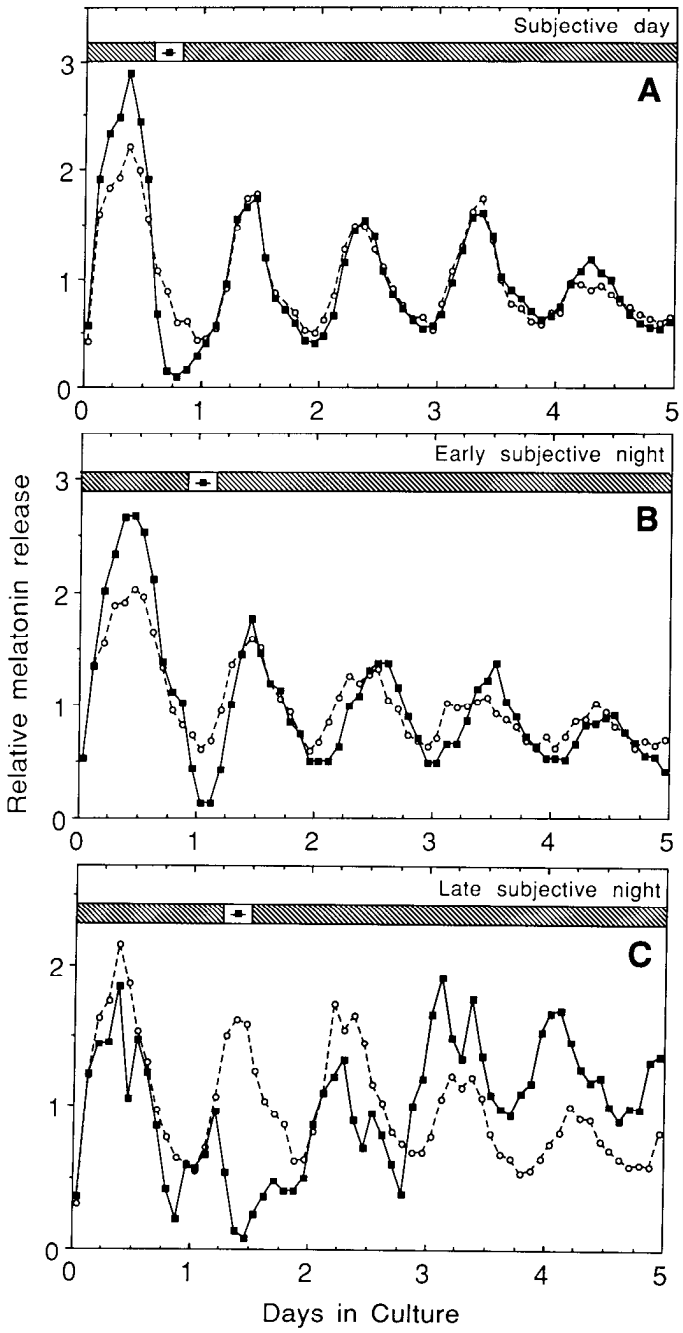

Figure 1. Effects of light pulses on circadian rhythms of melatonin release from eyecups in flow-through superfusion culture. Each panel illustrates rhythms of melatonin release recorded from an experimental–control eyecup pair from a single animal. Control eyecups (*open circles*) were maintained in constant darkness throughout the experiment. Experimental eyecups (*solid squares*) were exposed to a 6 hr pulse of white light ($\approx 10^{-5}$ W/cm^2) during the times indicated by the *open boxes* in the *shaded bars above* the records, and otherwise kept in darkness. *Zeitgeber* times of pulses were *A*, ZT 2–8; *B*, ZT 10–16; and *C*, ZT 18–24. Examples illustrated here are representative of the four or five eyecup pairs tested for each treatment. Melatonin release is normalized relative to the average rate of melatonin release from each eyecup during the entire experiment. Culture and recording of melatonin release began at the time of the animals' normal dark onset.

paring the timing of the last three melatonin cycles recorded from the experimental–control pairs shown in Figure 1; the average phase shift responses to light are summarized in Figure 2. Exposure of eyecups to light during the subjective day (ZT 2–8) produced no change in the timing of circadian rhythms (Figs. 1*A*, 2). A light pulse during the early part of the second subjective night in culture (ZT 10–16) caused a delay in the phase of the rhythm (Figs. 1*B*, 2). When the light pulse was applied during the late subjective night (ZT 18–24) of the second cycle in culture, it caused an advance in the phase of the rhythm (Figs. 1*C*, 2).

The phase shifts produced by light at each half-rise and half-fall point were measured, starting with the first peak after the pulse (the third cycle in culture). The average sizes of light-induced phase shifts remained essentially constant through the end of the culture period (Fig. 2*A*). This suggests that stable shifts in the phase of the underlying oscillator were induced by the light pulses and that these shifts were complete within one cycle. The phase shift for each experimental eyecup was therefore defined as the average of the phase differences measured (with respect to matched controls) at these phase reference points. The group average phase shifts resulting from light are plotted as a function of the circadian phases of the pulses in Figure 2*B*. The phase–response relationship shown in Figure 2*B* is qualitatively similar to that universally found for light-induced phase shifts of circadian clocks (Pittendrigh, 1981b).

Effects of D_2 dopamine receptor agonist pulses

Quinpirole (LY 171555), a selective D_2 dopamine receptor agonist, mimics light in its acute effects on a number of rhythmic retinal phenomena (Pierce and Besharse, 1985; Iuvone, 1986; Dearry and Burnside, 1988; Witkovsky et al., 1989). Therefore, in experiments analogous to those described above for light, we measured the effects of 6 hr pulses of quinpirole (100 nM) on the melatonin rhythms of cultured eyecups maintained in constant darkness. Both the acute effects on melatonin production, and the circadian phase resetting effects of these pulses were essentially identical to those induced by light (cf. Figs. 1, 2 for light; Figs. 3, 4 for quinpirole). Melatonin release was suppressed acutely during quinpirole treatments, regardless of the circadian phase of the treatment (Fig. 3*A–C*).

The circadian phase shifts induced by quinpirole pulses were similar to those induced by light. Quinpirole pulses had no significant effect on circadian phase when applied during the subjective day (ZT 2–8; Figs. 3*A*, 4), caused phase delays when applied during the early subjective night (ZT 10–16; Figs. 3*B*, 4), and caused phase advances when applied during the late subjective night (ZT 18–24; Figs. 3*C*, 4). As with light, these phase shifts appeared to be stable for the duration of the culture period (Fig. 4*A*). The group average phase shifts resulting from quinpirole pulses are shown as a function of the phase of the pulses in Figure 4*B*. This phase–response relationship for quinpirole-induced phase shifts is similar to that for light-induced phase shifts.

Circadian rhythms of melatonin release could also be reset by cycles of quinpirole, in a manner that mimics resetting and entrainment by light cycles (Fig. 5). In this experiment, each eyecup from an animal was exposed to two 12 hr treatments with 100 nM quinpirole during opposite times of day. Quinpirole was applied to one group of eyecups from ZT 20 to ZT 8, and to the other group from ZT 8 to ZT 20. The rhythms of melatonin synthesis in these matched groups were driven to opposite phases after exposure to two opposing cycles of quinpirole. After the quinpirole cycles were stopped, the rhythmic decrease in melatonin levels associated with subjective day time remained in phase with the quinpirole treatments. This phase relationship

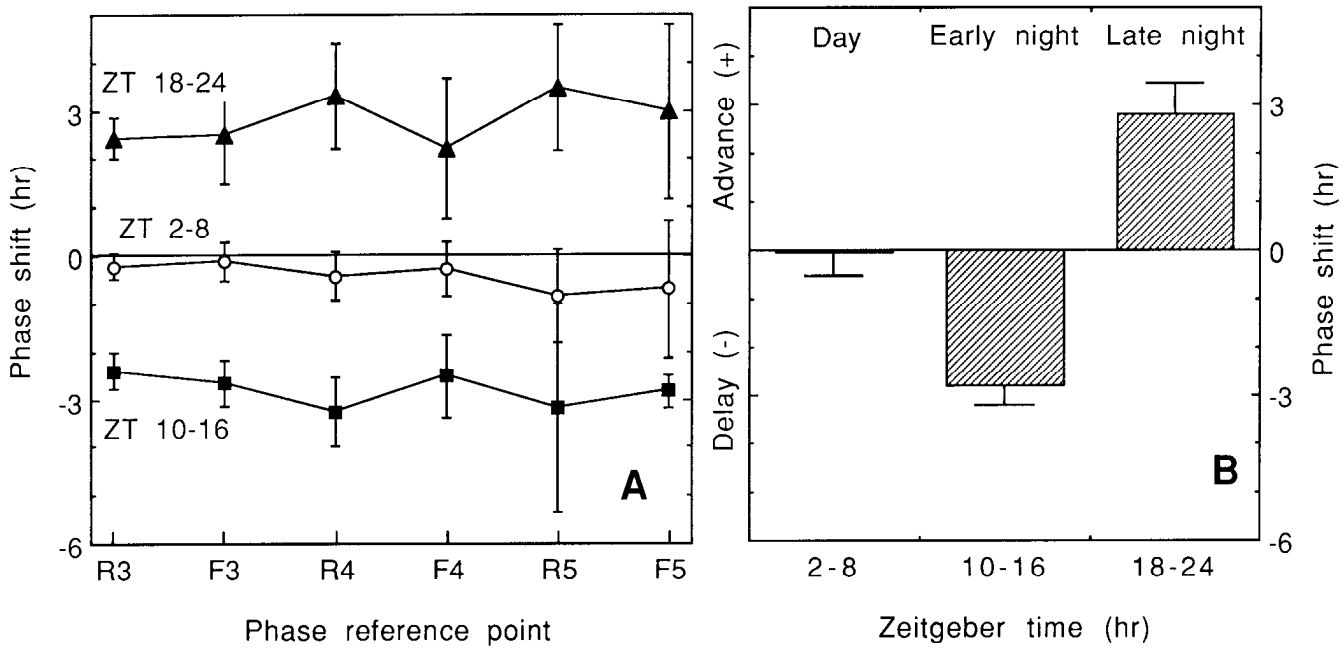

Figure 2. Effects of light pulses on the phase of circadian rhythms of melatonin release from cultured eyecups: summary of data from experiments illustrated in Figure 1. *A,* Phase differences in experimental–control eyecup pairs, measured at six phase reference points, after light pulses during the first subjective day (*open circles*), the second early subjective night (*solid squares*), and the second late subjective night (*solid triangles*) in culture. Phase advances are plotted as positive values; delays, as negative values. The phase reference points are the times of half-rises (*R*) and half-falls (*F*) of the third, fourth, and fifth peaks in culture. Data plotted are means ± SEM for three to five measurements at each phase reference point (phases were not measurable from some low-amplitude peaks); four or five eyecups were tested for each treatment. *B,* Average overall phase shifts induced by light pulses, as a function of the circadian phase when the pulse was delivered. For each experimental–control eyecup pair, the overall phase shift is defined as the average of the phase differences measured at the phase reference points described in *A. Error bars* indicate SEM determined from variation between eyecup pairs. $N = 4$ pairs for *ZT 2–8*; $N = 5$ pairs for *ZT 10–16* and *ZT 18–24.*

between melatonin rhythms and quinpirole cycles is similar to that between retinal NAT rhythms and entraining light cycles (Besharse and Iuvone, 1983; Iuvone and Besharse, 1983).

Receptor pharmacology

Two classes of dopamine receptors, D_1 and D_2, can be distinguished on the basis of the efficacy of selective agonists and antagonists (Stoof and Kebabian, 1984). The D_2 class has recently been shown to comprise multiple molecular forms with distinct functions (Todd et al., 1989; Sokoloff et al., 1990). In order to make a preliminary determination of the receptor type that mediated the effects of quinpirole on melatonin rhythms, the effects of selective receptor agonists and antagonists were compared.

The effects of selective catecholamine agonists on circadian phase and melatonin release are summarized in Table 2. Drugs were applied during the early subjective night of the second cycle in culture (ZT 10–16), as in Figure 3*B*. In order to compare effects of multiple drug treatments within an experiment, eyecups were distributed without pairing among four or five groups, and phase shifts and acute suppression of melatonin release were determined with respect to average values from an untreated control group in each experiment.

The most potent effectors of melatonin suppression and circadian phase delays were quinpirole and dopamine, which both were effective at submicromolar concentrations (Table 2). These data are consistent with a primary role for D_2 receptors in both effects. The D_1-selective dopamine agonist SKF 38393 was at least 100-fold less potent, but did mimic the effects of light and dopamine at 50 μM, a concentration at which it can cross-react with D_2 receptors (Munemura et al., 1980). The α_1- and α_2-adrenergic receptor agonists phenylephrine and clonidine, respectively, were ineffective, while the β-adrenergic agonist isoproterenol had a relatively weak effect on melatonin release at a concentration of 1 μM. Because of the high concentration of isoproterenol necessary for this effect, it is unlikely that β-adrenergic receptors are major mediators of dopamine effects, but some role for these receptors cannot be ruled out.

Paired experimental and control eyecups were used to measure the effects of selective dopamine receptor antagonists on dopamine-induced phase shifts and melatonin suppression. Examples of these effects are illustrated in Figure 6, and the data are summarized in Table 3. A selective D_2 dopamine receptor antagonist, eticlopride (50 μM), blocked the phase delay and the decrease in melatonin release that were induced by 500 nM dopamine delivered during the early subjective night (Fig. 6*A,B*). A higher concentration of the D_1 receptor antagonist SCH 23390 (100 μM) did not block either effect of dopamine (Fig. 6*C*). Neither antagonist had a significant effect when applied alone (Table 3). These data indicate that the effects of dopamine were mediated by receptors of a D_2 class.

Light and endogenous dopamine

The results above indicate that activation of retinal D_2 receptors mimics light in suppressing melatonin production and resetting the phase of the circadian oscillator that drives melatonin rhythms. Previous reports indicated that light causes increases in release and turnover of endogenous retinal dopamine in *Xenopus* eyecups in static culture (Iuvone et al., 1987; Boatright et al., 1989). These data suggest that endogenous retinal dopamine

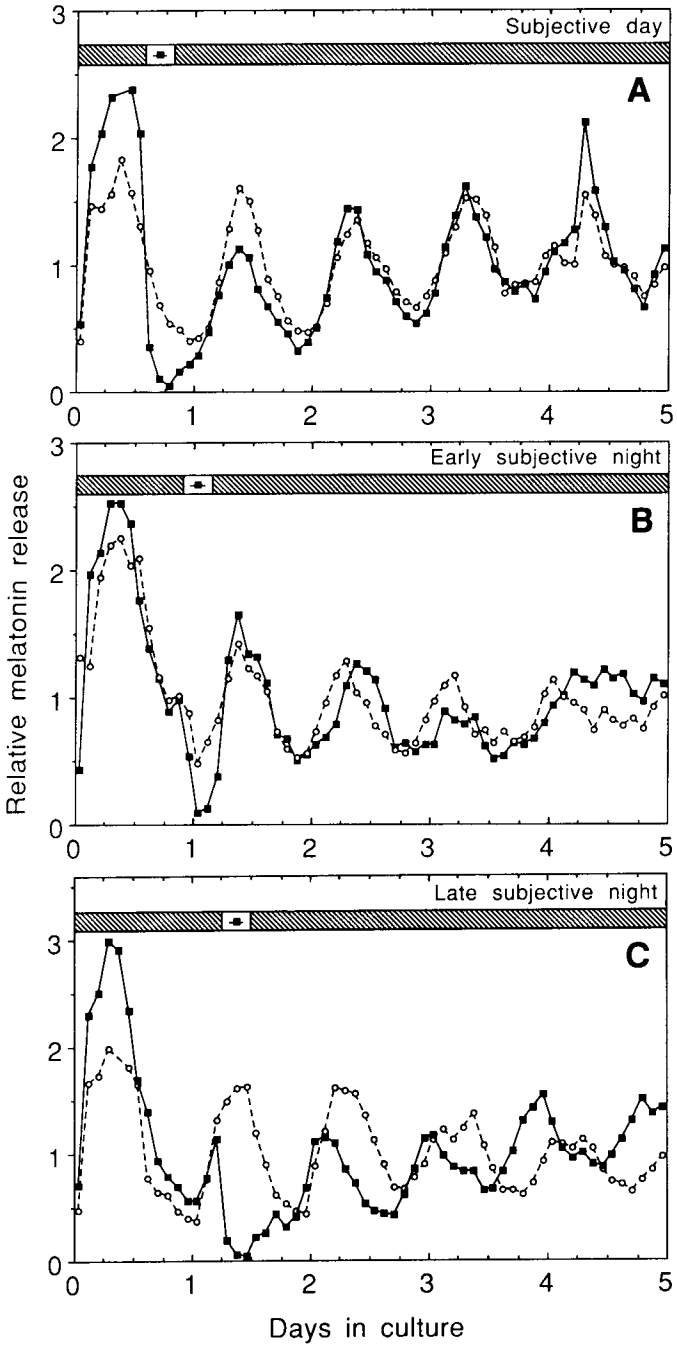

Figure 3. Effects of pulses of quinpirole, a D_2 dopamine receptor agonist, on circadian rhythms of melatonin release by cultured eyecups. Each panel illustrates rhythms of melatonin release from an experimental–control eyecup pair from a single animal. All eyecups were maintained in constant darkness throughout the experiments. Experimental eyecups (*solid squares*) were exposed to 100 nM quinpirole-HCl during the times indicated by the *open boxes* in the *shaded bars above* the records. Control eyecups (*open circles*) were cultured in control medium throughout. *Zeitgeber* times of pulses were *A*, ZT 2–8; *B*, ZT 10–16; and *C*, ZT 18–24. Examples illustrated here are representative of five or six eyecup pairs per treatment. Conventions and data analysis were as described for Figure 1.

might either mediate or modulate the effects of light on melatonin release and/or circadian phase. In order to determine whether endogenous dopamine has a role in these light responses, we tested whether the antagonist eticlopride could block responses to light pulses.

Six hour pulses of white light ($\approx 10^{-5}$ W/cm^2) were delivered at different phases of the circadian cycle. These pulses were subsaturating for induction of phase shifts, but maximally suppressed melatonin release (controls for this experiment are illustrated in Figs. 1, 2). Eticlopride (50 μM) was added to the culture medium during the period from 1 hr before to 1 hr after the light pulse. Eticlopride had no effect on light-induced circadian phase shifts or on melatonin suppression at any time tested (Table 4). Eticlopride was also ineffective in experiments with lower light intensities. A 3 hr pulse of incandescent white light ($\approx 10^{-8}$ W/cm^2) delivered at ZT 12–15 caused a phase delay of 1.6 ± 0.4 hr and suppressed melatonin release acutely by 54 ± 7% (mean ± SEM; $n = 5$). In the presence of 50 μM eticlopride, the phase delay was 1.2 ± 0.5 hr, and melatonin was suppressed by 63 ± 8% (mean ± SEM; $n = 5$). All responses to this dim light were submaximal [when compared with responses to 3 hr pulses of bright ($\approx 10^{-4}$ W/cm^2) fluorescent light (data not shown)], but eticlopride still had no effect. These data indicate that dopamine-independent pathways exist for the effects of light on the circadian oscillator and on melatonin production.

It is possible that our standard culture conditions inhibit retinal dopamine release, obscuring a role for endogenous dopamine in light effects. Melatonin inhibits the release of retinal dopamine (Dubocovich, 1983; Boatright and Iuvone, 1989), and culture of eyecups in the presence of 5HTrp, as in all of the above experiments, increases retinal melatonin production as much as 100-fold (Cahill and Besharse, 1990). Therefore, in another experiment, 5HTrp was omitted from the culture medium for the first cycle in culture, through the time of a 6 hr light pulse ($\approx 10^{-5}$ W/cm^2) in the early subjective night of the second cycle in culture (ZT 10–16). After this time, 5HTrp was added to the medium to enable measurement of phase shifts. The subsequent rhythms of melatonin release were similar in period and amplitude to those produced when 5HTrp was present from the beginning of the experiment. We have previously shown that cyclic addition of 5HTrp to the culture medium, in a paradigm analogous to that shown for quinpirole in Figure 5, has no effect on circadian phase (Cahill et al., 1991). Eticlopride (50 μM) was added to the culture medium during the period from 1 hr before until 1 hr after the light pulse. Representative records of melatonin release by experimental–control eyecup pairs from this experiment are shown in Figure 7. Eticlopride had no effect on the phase shift produced by light. Light alone caused a phase delay of 2.0 ± 0.2 hr (mean ± SEM; $n = 3$), while light in presence of eticlopride caused a phase delay of 2.3 ± 0.2 hr (mean ± SEM; $n = 5$). Thus, we could find no direct evidence for a role for endogenous dopamine in responses of the ocular circadian oscillator to light. Melatonin release in the absence of 5HTrp was too low for accurate measurement of the effect of eticlopride on acute melatonin suppression.

Interaction of light and dopamine receptor–mediated pathways

The apparent dopamine independence of the responses of the ocular circadian oscillator and melatonin-generating system to light suggests that the effects of light and dopamine receptor agonists are mediated by pathways that are, to some extent, separate. To determine the extent of this separation, we tested whether the effects of light and quinpirole, applied together, are additive. Additivity of the responses would be predicted if the light and dopamine pathways are entirely independent. If the pathways converge at, or prior to, a point where the response can be saturated by either treatment, the effects would not be

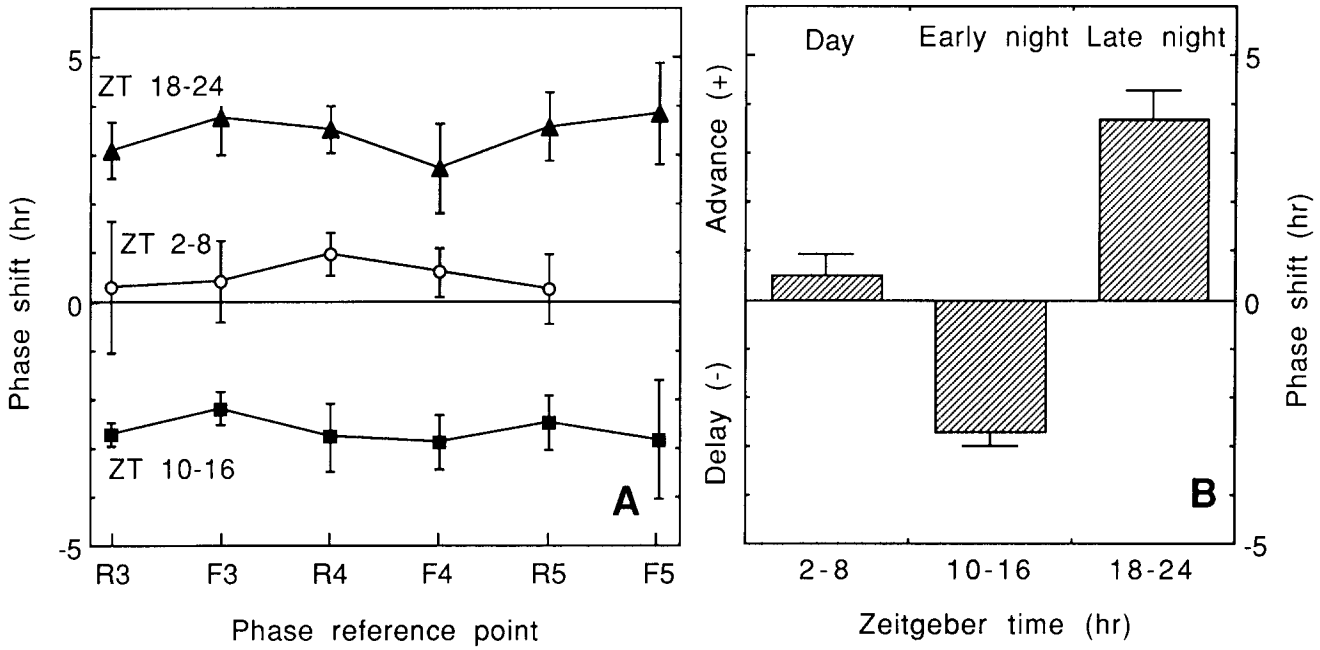

Figure 4. Effects of quinpirole pulses on the phase of circadian rhythms of melatonin release from cultured eyecups: summary of data from experiments illustrated in Figure 3. *A,* Phase differences in experimental–control eyecup pairs, measured at six phase reference points, after quinpirole pulses during the first subjective day (*open circles*), the second early subjective night (*solid squares*), and the second late subjective night (*solid triangles*) in culture. Data plotted are means ± SEM for three to six measurements from the five or six eyecup pairs in each treatment (phases were not measurable from some low-amplitude peaks). *B,* Overall phase shifts (mean ± SEM) induced by quinpirole. $N = 5$ pairs for *ZT 2–8*; $N = 6$ pairs for *ZT 10–16* and *ZT 18–24*. Conventions and data analysis were as described for Figure 2.

additive. For this experiment, a brighter, fluorescent white light source was used (intensity, $\approx 10^{-4}$ W/cm^2), because additivity of subsaturating treatments cannot be interpreted as evidence for nonconvergent pathways. A 6 hr pulse of bright fluorescent light at ZT 10–16 produced a phase delay of 4.5 ± 0.3 hr (a 70% increase over the response to a light pulse of 10^{-5} W/cm^2) and suppressed melatonin production by 80 ± 7% (mean ± SEM; $n = 5$ experimental–control pairs). Simultaneous treatment with light and 200 nM quinpirole produced no increase in the responses, causing a phase delay of 4.3 ± 0.4 hr and sup-

Table 2. Effects of catecholamine agonists on circadian phase and melatonin release

Receptor specificity	Agonist	Experiment number	Concentration (M)	Phase shift (hr, mean ± SEM)	Acute inhibition (%, mean ± SEM)
Dopaminergics					
D_1, D_2	Dopamine	1	1×10^{-7}	−2.4 ± 0.4**	73 ± 6**
		2	5×10^{-7}	−3.9 ± 0.4**	91 ± 1**
		3	1×10^{-6}	−4.6 ± 0.9**	95 ± 2**
D_2	Quinpirole	1	1×10^{-8}	−1.5 ± 0.5*	56 ± 4**
		4	1×10^{-7}	−4.4 ± 0.7**	88 ± 1**
		4	1×10^{-6}	−5.4 ± 0.3**	78 ± 2**
D_1	SKF 38393	1	1×10^{-6}	−0.5 ± 0.4	6 ± 4
		5	5×10^{-5}	−3.7 ± 0.4**	79 ± 3**
Adrenergics					
α_1	Phenylephrine	3	1×10^{-6}	−0.8 ± 0.3	7 ± 16
α_2	Clonidine	3	1×10^{-6}	+0.2 ± 0.5	9 ± 10
β	Isoproterenol	4	1×10^{-7}	−0.1 ± 1.0	24 ± 8
		3	1×10^{-6}	−0.8 ± 0.2	44 ± 3*

Eyecups were cultured in constant darkness, and drugs were delivered in the superfusion medium for 6 hr, starting at 22 hr of culture (ZT 10–16). Phase shifts were measured with respect to average phases of untreated controls. Acute inhibition was determined from normalized melatonin release during the period 2–6 hr after the start of the drug pulse. Each experiment (denoted by an experiment number) included four or five treatment groups; some treatment groups not shown here (repetitions and nonagonist drugs) were included in the analysis of variance; $N = 4$–5 eyecups per treatment.

* $P < 0.05$, ** $P < 0.01$; significantly different from untreated controls (ANOVA, Dunnett's t test).

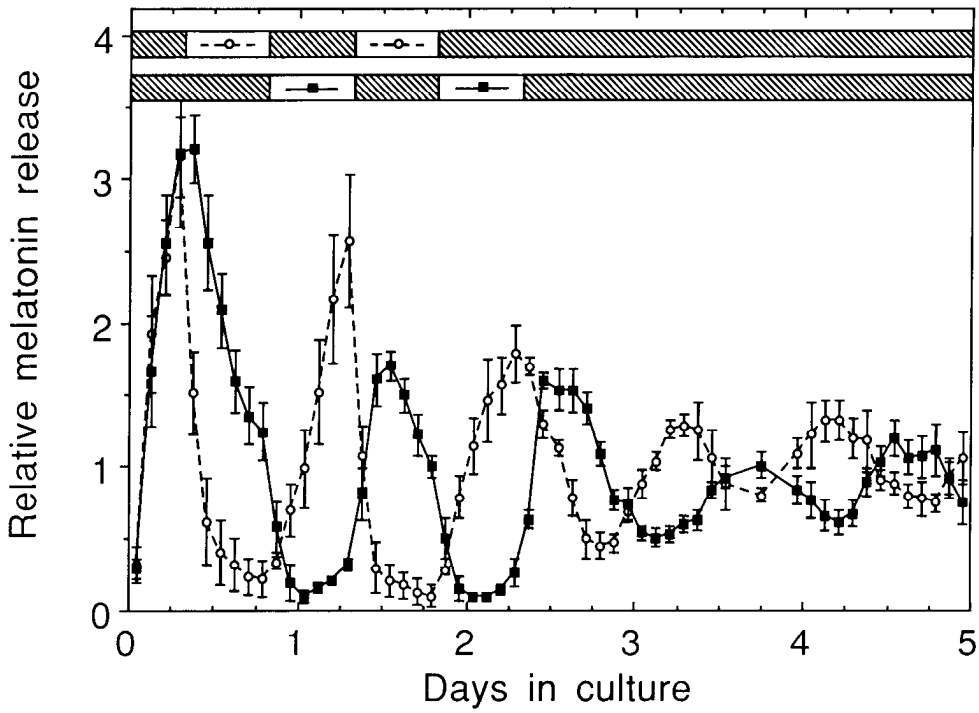

Figure 5. Effects of alternating cyclic quinpirole treatments on circadian rhythms of melatonin release in cultured eyecups. Plotted are means ± SEM of normalized melatonin release from eyecups cultured individually in constant darkness, starting at the normal time of dark onset. Five left–right pairs were split between the groups. Quinpirole-HCl (100 nM) was added to the superfusion medium during the 12 hr periods indicated by *open boxes* in the *shaded bars above* records: *open circles,* ZT 20–8; *solid squares,* ZT 8–20. Culture conditions were constant, without quinpirole, during the last 64 hr of the experiment.

pressing melatonin by 86 ± 0.5% (mean ± SEM; $n = 5$ experimental–control pairs). Thus, the pathways for light and dopamine receptor effects apparently converge, either at the circadian oscillator and at the melatonin-generating system, or somewhere in the input pathways to these systems.

Discussion

Continuous monitoring of circadian rhythms of melatonin release from cultured eyecups enables precise measurement of the effects of experimental manipulations on melatonin levels as well as on the phase of the ocular circadian oscillator. The ability to measure long-term changes in circadian phase is important for analysis of the mechanisms of circadian rhythm generation and entrainment, which are unambiguously revealed only in the timing of rhythms. Acute effects of experimental treatments on *levels* of a rhythmic variable, such as melatonin production, may be mediated entirely independently of the oscillator.

The *Xenopus* eyecup is the only vertebrate retinal preparation currently available for this type of investigation of circadian mechanisms. A variety of retinal processes in many species are controlled by circadian oscillators, but localization of the controlling oscillator in the eye has been demonstrated in only a few cases (reviewed by Besharse et al., 1988; Cahill et al., 1991). There is strong evidence for ocular localization of a circadian clock controlling retinal melatonin rhythms in Japanese quail (Underwood et al., 1988, 1990) and some evidence for ocular control of circadian rhythms of rod photoreceptor disk shedding in rat retina (Teirstein et al., 1980) and behavioral photic sensitivity in the rat (Terman and Terman, 1985). *In vitro* preparations for analysis of cellular circadian oscillator mechanisms in these systems have not yet been developed.

In this study, light pulses reset the phase of *Xenopus* retinal melatonin rhythms in a phase-dependent manner, causing phase delays when presented in the early subjective night, phase advances when presented in the late subjective night, and no phase shift when presented during the subjective day. The sizes of the circadian phase shifts produced by light remained constant through at least three circadian cycles after the pulses. This strongly suggests that the measured phase shifts reflect stable resetting of the underlying circadian oscillator, although transient effects on the oscillator or on coupling of the overt rhythm to the oscillator cannot be completely ruled out with experi-

Table 3. Effects of selective dopamine receptor antagonists on dopamine-induced phase shifts and melatonin suppression

Experiment number	Treatment	Phase shift (hr, mean ± SEM)	Acute inhibition (%, mean ± SEM)
1	Dopamine (500 nM)	−3.6 ± 0.3	88 ± 1
	Dopamine + eticlopride (50 μM)	−0.3 ± 0.2**	30 ± 5**
2	Dopamine (500 nM)	−2.8 ± 0.3	85 ± 1
	Dopamine + SCH23390 (100 μM)	−2.5 ± 0.4	73 ± 4
3	Eticlopride only (50 μM)	+0.1 ± 0.3	7 ± 18
	SCH 23390 only (100 μM)	+0.4 ± 0.5	11 ± 9

Drugs were added to superfusion medium for 6 hr, starting 22 hr after lights off (see Fig. 2). Phase shifts and acute effects determined with respect to paired, untreated controls. $N = 5$ eyecup pairs per treatment.

** $P < 0.01$, significant reduction of dopamine effect (t test).

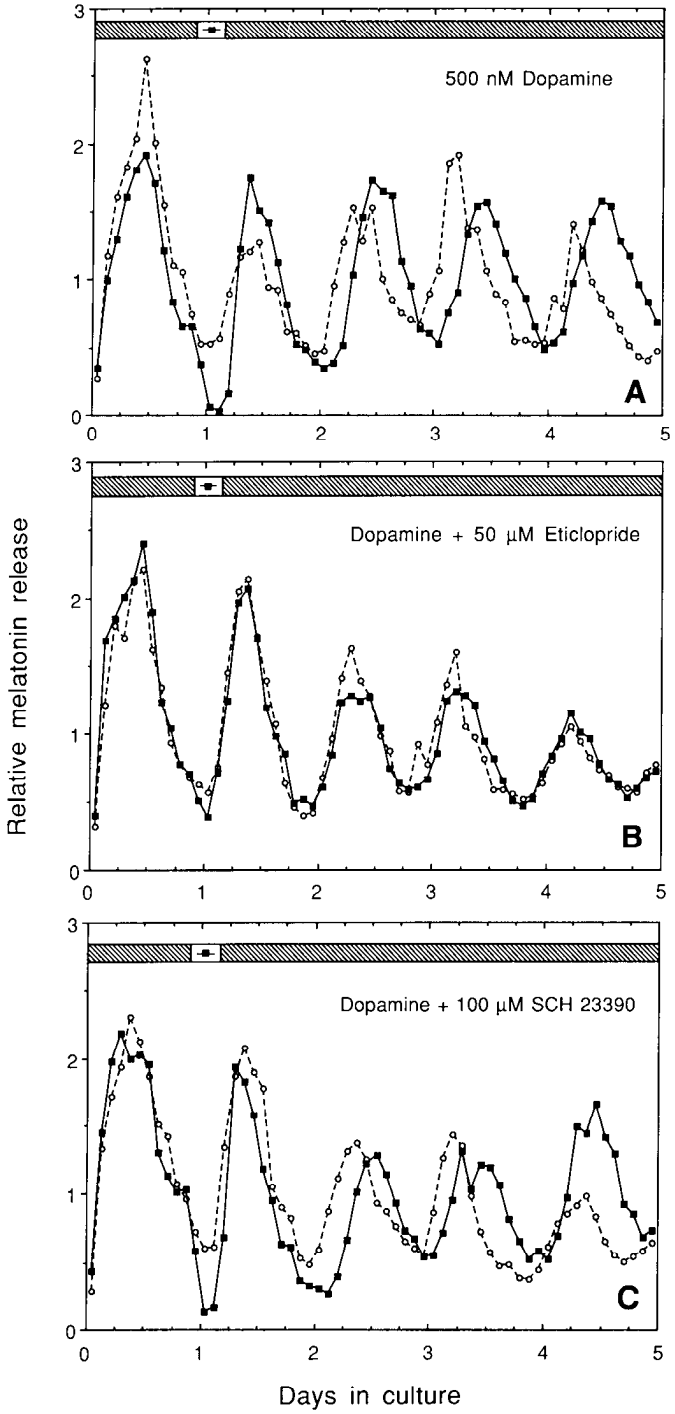

Figure 6. Effects of selective dopamine receptor antagonists on dopamine-induced phase delays and melatonin suppression. Each panel illustrates rhythms of melatonin release by a pair of eyecups from a single animal. Eyecups were cultured in constant darkness; *open circles,* untreated control eyecups; *solid squares,* experimental eyecups. Dopamine (500 nM) was added to experimental culture medium, with or without an antagonist, for 6 hr during the early subjective night (ZT 10–16). *A,* Effect of dopamine alone; *B,* dopamine with 50 μM eticlopride; *C,* dopamine with 100 μM SCH 23390. Records *A* and *B* are from Table 3, Experiment 1; *C* is from Table 3, Experiment 2. Examples illustrated here are representative of responses from five eyecup pairs per treatment (see Table 3). Conventions and data analysis were as described for Figure 1.

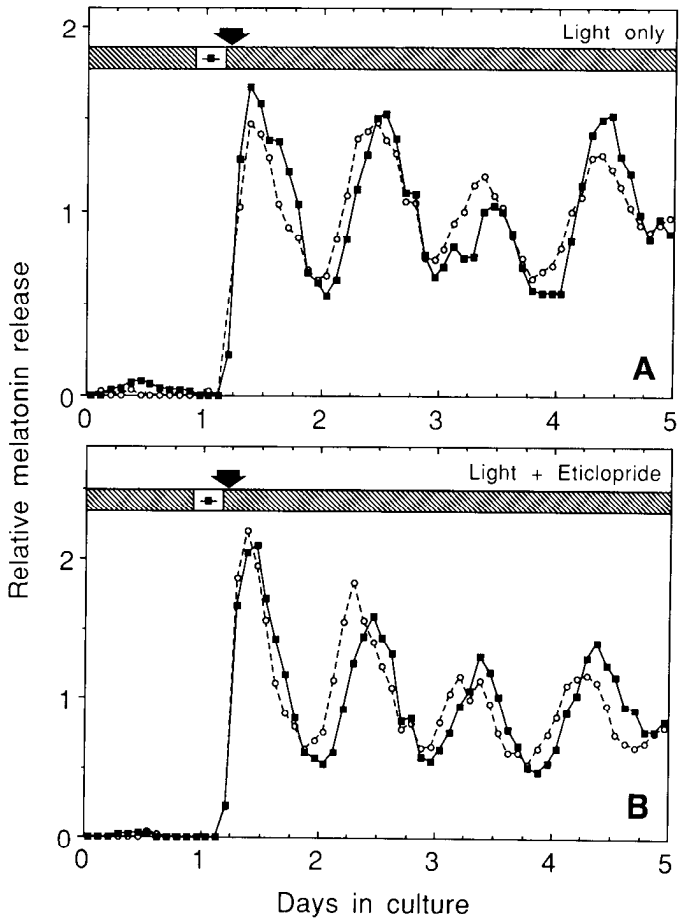

Figure 7. Effect of eticlopride, a D_2 antagonist, on light-induced phase delays. Each panel illustrates rhythms of melatonin release by a pair of eyecups from a single animal. Experimental eyecups (*solid squares*) were exposed to a 6 hr pulse of light (10^{-5} W/cm^2) at the time indicated by the *open boxes* in the *shaded bars above* the records (ZT 10–16). Control eyecups (*open circles*) were maintained in the dark. 5HTrp was omitted from the culture medium for the first 29 hr, then returned at the time indicated by the *arrow. A,* Effects of light alone; *B,* 50 μM eticlopride was added to culture medium of both the dark control and light treated eyecup from 1 hr before to 1 hr after the light pulse. Conventions and data analysis were as described for Figure 1.

ments of this duration. The phase dependence of circadian responsiveness to light found in this preparation is similar to that universally found in light-sensitive circadian oscillator systems, both *in vivo* (Pittendrigh, 1981b) and *in vitro* (Corrent et al., 1982; Block and McMahon, 1984; Robertson and Takahashi, 1988; Zatz et al., 1988), and is consistent with a role for light as a dominant environmental entraining stimulus. Light pulses also acutely suppress release of melatonin by cultured *Xenopus* eyecups at all times in the circadian cycle. This is consistent with previous results in studies of retinal NAT activity and melatonin content (Binkley et al., 1979; Hamm and Menaker, 1980; Iuvone and Besharse, 1983).

D_2 dopamine receptor agonists also affected both the timing of circadian rhythmicity and melatonin release levels, indicating that dopaminergic input pathways exist for both systems. Quinpirole, a selective D_2 agonist, reset the phase of the melatonin rhythm in a phase-dependent manner similar to that of light. The phase shifts produced by quinpirole appear to result from stable effects on the circadian oscillator. This is the first dem-

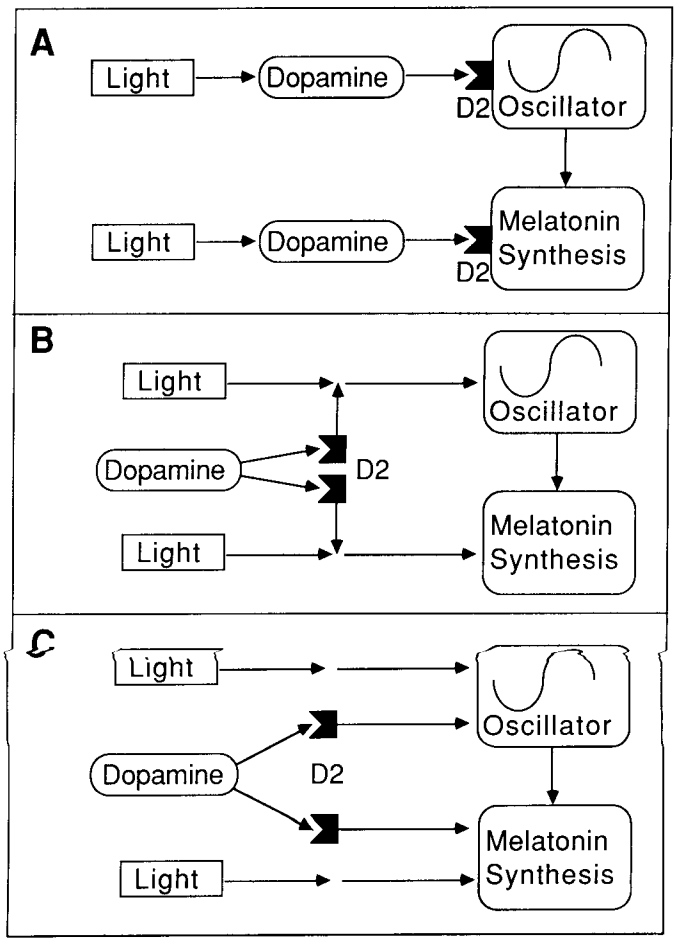

Figure 8. Hypothetical models of interactions of input pathways for effects of light and dopamine on melatonin synthesis and the circadian oscillator. *A*, Dopamine directly mediates effects of light. *B*, Light and dopamine pathways converge, acting through a common final pathway. *C*, Light and dopamine act through separate pathways.

onstration of a dopaminergic input pathway to the ocular circadian clock. Like light, quinpirole also suppressed melatonin levels at all times in the circadian cycle, consistent with previously reported dopaminergic effects on retinal NAT activity (Iuvone, 1986; Iuvone and Besharse, 1986b) and melatonin content (Zawilska and Iuvone, 1989).

Pharmacological analysis with selective agonists and antagonists indicates that the receptors that mediate dopamine-induced suppression of melatonin and resetting of the circadian oscillator are D_2-like. Recent molecular genetic studies have shown that pharmacologically defined D_2 receptors are a heterogenous class, comprising multiple distinct receptor molecules that interact with different cellular transduction systems. The first of these receptor molecules for which a cDNA sequence was obtained is a member of the G-protein–linked receptor family that can inhibit adenylate cyclase (Bunzow et al., 1988). The second reported D_2-like receptor type (termed D_{2B} by Todd et al., 1989) is genetically distinct, has no effect on cAMP, but can stimulate calcium influx (Todd et al., 1989). The third receptor in this class (termed D_3 by Sokoloff et al., 1990) is coded by another gene, and its transduction mechanism is unknown, but does not include effects on cAMP (Sokoloff et al., 1990).

The variation in responsiveness of D_2 receptor subtypes to currently available receptor ligands (Todd et al., 1989; Sokoloff et al., 1990) is too subtle to allow further pharmacological identification of the receptors mediating dopamine effects on retinal melatonin rhythms. A more productive approach will be to identify the second-messenger systems involved in responses to dopamine receptor agonists. Previous studies have indicated that retinal NAT activity is cAMP dependent and have suggested that suppression of NAT by dopamine results from inhibition of adenylate cyclase activity (Iuvone, 1986; Iuvone and Besharse, 1986a). Manipulations of cAMP levels can reset circadian rhythms in the eye of *Aplysia* (Eskin et al., 1982; Eskin and Takahashi, 1983) and in the rat suprachiasmatic nucleus (Prosser and Gillette, 1989), but not in the avian pineal (Zatz and Mullen, 1988b; Nikaido and Takahashi, 1989). No information is yet available concerning second-messenger effects on the ocular circadian oscillator.

The retinal melatonin-generating system and the ocular circadian oscillator both respond to light as well as to dopamine receptor agonists, suggesting that input pathways to these two systems may share common elements. However, the evidence does not support an intermediary role for melatonin suppression in resetting the circadian oscillator, nor is it consistent with melatonin suppression resulting simply from a shift in the phase of the oscillator. Imposition of cyclic variation in retinal melatonin levels by manipulation of precursor levels has no effect on subsequent timing of the circadian rhythm (Cahill et al., 1991), suggesting that the ocular circadian oscillator is insensitive to melatonin. Conversely, pulses of light and dopamine agonists suppress melatonin release during the subjective day

Table 4. Effects of eticlopride, a D_2 dopamine receptor antagonist, on light-induced phase shifts and melatonin suppression

Time of pulse	Treatment	Phase shift (hr, mean ± SEM)	Acute inhibition (%, mean ± SEM)	*N*
ZT 2–8	Light	0.0 ± 0.5	69 ± 8	4
	Light + eticlopride	−0.3 ± 0.3	78 ± 4	3
ZT 10–16	Light	−2.8 ± 0.4	80 ± 4	5
	Light + eticlopride	−2.6 ± 0.3	79 ± 3	5
ZT 18–24	Light	+2.8 ± 0.7	90 ± 2	5
	Light + eticlopride	+3.0 ± 0.2	88 ± 2	5

The effects of light alone from these experiments were illustrated in Figures 1 and 2. For each time point, light alone ($\approx 10^{-5}$ W/cm^2) and light with eticlopride (50 μM) were tested in the same experiment. Phase shifts and acute effects were determined with respect to paired, untreated controls.

without affecting circadian phase, indicating that a phase shift is not necessary for suppression of melatonin. Thus, input pathways to the two systems appear to be at least partially separate, but the points of divergence are unknown. The same photoreceptor mechanisms and dopamine receptors may initiate both acute and circadian responses, with the pathways splitting near the final steps, or the systems may be entirely separate. Studies of avian pinealocytes indicate that melatonin suppression and circadian phase shifting are mediated by different transduction mechanisms and second-messenger systems (Zatz and Mullen, 1988b,c; Nikaido and Takahashi, 1989; Takahashi et al., 1989), and this may also be true of retinal melatonin rhythms.

Hypothetical models of possible roles for the D_2 dopamine receptor–mediated pathways to the circadian oscillator and the melatonin-generating system are illustrated in Figure 8. In the simplest model (Fig. 8*A*), the effects of light on these systems are actually mediated by endogenous dopamine acting at D_2 receptors. Alternatively, separate light and dopamine pathways may converge and influence the systems through a common final pathway (Fig. 8*B*). A third possibility is that light and dopamine act by separate pathways that converge only at the oscillator and at the melatonin-generating system (Fig. 8*C*). The data presented here are not consistent with the first model in its simplest form and suggest that the organization of the retinal system can best be described by one (or a combination) of the other two models. Despite the similarities in the effects of light and of dopamine receptor agonists on circadian phase and melatonin release in this system, and the previous evidence of light-induced retinal dopamine release in *Xenopus* retina (Iuvone et al., 1987; Boatright et al., 1989), we found no evidence that endogenous dopamine directly mediates light effects on either system. Eticlopride, a D_2 dopamine receptor antagonist, completely blocked the effects of dopamine on melatonin release and circadian phase, but had no effect, in a variety of circumstances, on responses that were induced by subsaturating pulses of white light. These data indicate that pathways that are not mediated by D_2 receptors exist for light-induced melatonin suppression and circadian phase resetting. In previous studies, another D_2 receptor antagonist, spiroperidol, also failed to block suppression of retinal NAT activity by light (Iuvone and Besharse, 1986b), except in the presence of phosphodiesterase inhibitors, which can increase endogenous retinal dopamine content and turnover (Iuvone et al., 1987). The data presented here do not support the model in Figure 8*A*, but the possibility remains that under some conditions endogenous dopamine may be part of pathways for light inputs to the melatonin system or the circadian oscillator. Alternatively, endogenous retinal dopamine might mediate responses of these systems to other environmental or physiological stimuli.

The effects on melatonin rhythms of bright light and quinpirole were not additive, indicating that the pathways mediating these effects converge at some point. Convergence might be at any point in pathways leading up to the circadian oscillator (Fig. 8*B*), or at the level of the oscillator itself (Fig. 8*C*). These cannot be distinguished on the basis of available data, because it is unclear which points in the system were saturated by bright light. If an input pathway was saturated, but the oscillator could be reset further or melatonin suppressed more by other treatments, nonadditivity would indicate that the dopamine and light pathways converge on common input pathways (Fig. 8*B*). However, if the responses were saturated at the levels of the oscillator and/or the melatonin-generating system, additivity data will not distinguish between the models in Figure 8, *B* and *C*. A more concrete understanding of the pathways will be necessary for discrimination between these possibilities.

The pathways that mediate the effects of light and dopamine provide parallel avenues to follow toward the circadian oscillator in the retina. The convergence of these pathways can be used to direct investigations aimed at identifying the cellular locus and molecular mechanisms of circadian rhythm generation. This concept can be used in identification of candidate clock mechanisms. For example, the retinal cell types that generate circadian rhythmicity are unknown, but photoreceptors, which are structurally and functionally related to putative circadian oscillator cells in the pineal (reviewed by Wiechmann, 1986) and contain the final enzyme in melatonin biosynthetic pathway (Wiechmann et al., 1985), are sensitive to light *and* have D_2 receptor binding sites (Brann and Young, 1986), all consistent with a central role in retinal rhythmicity. Furthermore, cells and processes that are affected in the same way by both light and dopamine receptors, and can in turn affect circadian timing, are likely to be closer to the circadian oscillator than processes that are affected by only light or only dopamine.

The dopaminergic input pathway to the *Xenopus* retinal circadian oscillator is the first receptor-mediated input pathway found to mimic light in the phase dependence of its effects on a circadian oscillator *in vitro*. In cultured avian pineal cells, norepinephrine can act through α_2-adrenergic receptors to mimic light in causing acute inhibition of melatonin production (analogous to the acute effect of dopamine in the *Xenopus* eyecup), but it has no effect on the timing of the pineal circadian oscillator (Pratt and Takahashi, 1987; Zatz and Mullen, 1988a; Takahashi et al., 1989). Activation of 5-HT receptors can reset the phase of the circadian oscillators in the *Aplysia* eye (Corrent et al., 1982) and the rat suprachiasmatic nucleus (Prosser et al., 1990), but in both cases the phase–response relationship for 5-HT–induced phase shifts is similar to that for responses to dark pulses, rather than light pulses. Other pharmacological treatments in various model systems also mimic darkness in affecting circadian oscillators. This is true for treatments that increase cAMP levels (Eskin and Takahashi, 1983; Prosser and Gillette, 1989), inhibit protein synthesis (Rothman and Strumwasser, 1976; Takahashi et al., 1989), inhibit Na,K-ATPase (Zatz and Mullen, 1989a,b), or inhibit calcium influx (Khalsa and Block, 1990). Other treatments known to mimic light in resetting neural circadian oscillators *in vitro* include membrane depolarization (McMahon and Block, 1987) and elevated cGMP (Eskin et al., 1984) in molluscan eyes, in which these mechanisms appear to mediate the effects of light on the circadian oscillators. The unique relationship between the light and dopamine pathways in the *Xenopus* retina may provide some advantages in further investigation of mechanisms of circadian rhythm generation and entrainment.

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