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Retinally perceived light is not essential for photic regulation of pineal melatonin rhythms in the pigeon: studies with microdialysis

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Abstract Using in vivo microdialysis, effects of retinally perceived light on pineal melatonin release and its rhythmicity was examined in the pigeon. In the first experiment, light-induced suppression of pineal melatonin release was studied. Although light given to the whole body during the dark strongly suppressed pineal melatonin release to a daytime level, light exclusively delivered to the eyes did not remarkably inhibit melatonin release. In the second experiment, in order to determine whether retinally perceived light has phase-shifting effects on pineal melatonin rhythms, pigeons were given a single light pulse of 2 h at circadian time (CT) 18 and the phases of the second cycle after the light pulse were compared with those of control pigeons without the light pulse. In this experiment, phase advances of pineal melatonin rhythms were observed when the light was given to the whole body but not when only the eyes were illuminated. In a third experiment, after entrainment to light-dark 12:12 (LD 12:12) cycles, birds whose heads were covered with black tapes were transferred into constant light (LL) conditions and only the eyes were exposed to new LD cycles for 7 days (the phase was advanced by 6 h from the previous cycles) using a patching protocol. This procedure, however, could not entrain pineal melatonin rhythms to the retinal LD cycles. These results indicate that the eyes are not essential for photic regulation of pineal melatonin release and its rhythmicity in the pigeon.

Key words Circadian rhythm · Pineal · Melatonin Microdialysis · Pigeon

Abbreviations *CT* circadian time · *LD* light-dark *LL* constant light · *SCN* suprachiasmatic nucleus *LLdim* constant dim light · *NE* norepinephrine *SCG* superior cervical ganglia · *WB* whole body *E* eye · *EX* extraretina · *C* control

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Introduction

Many lines of evidence have suggested that avian circadian rhythms are regulated by a system of multiple oscillatory components of the pineal gland, the eyes and the anterior hypothalamus (the suprachiasmatic nucleus (SCN) or structures functionally similar to them) (Cassone and Menaker 1984; Ebihara et al. 1987; Gwinner 1989; Oshima et al. 1989; Underwood and Siopes 1984). Melatonin which is rhythmically synthesized in both the pineal gland and the eyes (not all species) appears to be involved in the regulatory mechanisms of the avian circadian system. In pigeons, for example, the phase of the circadian rhythm of locomotor activity is closely related to the phase of circulating melatonin (phase relationships among them are maintained 180° out of phase) in constant dim light (LLdim) conditions (Oshima et al. 1987). Moreover free-running locomotor activity and body temperature rhythms of pigeons are abolished by a combination of pinealectomy and ocular enucleation (Ebihara et al. 1984), and the rhythms can be restored by cyclic melatonin infusion in constant conditions (Oshima et al. 1989; Chabot and Menaker 1992). These results indicate that the pineal gland and the eyes and their hormone, melatonin, are important in the control of avian circadian rhythms.

It is known that pineal melatonin synthesis is controlled by endogenous circadian pacemakers; it shows a 24-h rhythm with a nocturnal rise under light-dark cycles (LD) and the rhythm persists in constant darkness. In addition to regulation by circadian pacemakers, melatonin synthesis is directly affected by light; it is inhibited by acute exposure to light at night when melatonin levels are high. In mammals the perception of light by the retina is essential for the regulation of pineal melatonin synthesis (Reiter 1991). Light information received by the retina is delivered to the SCN, the circadian pacemaker of mammals, through the retino-hypothalamic tract, and then transmitted to the pineal gland by norepinephrine (NE) via the superior cervical ganglia (SCG). In birds,

however, the pineal gland is photosensitive and photic regulation of pineal melatonin synthesis can be mediated by its own photoreceptors (Takahashi et al. 1989). In addition to photoreception by the pineal gland, the neural routes similar to mammals conveying light information from the retina to the pineal gland are thought to exist in birds (Cassone 1991). It has been demonstrated in Japanese quails that retinally perceived light can entrain pineal melatonin rhythmicity (Barrett and Underwood 1991).

In order to understand avian circadian organization it is essential to explore photic inputs to the circadian system. Therefore, in the present study we examined the role of the eyes in the photic regulation of pineal melatonin release and its rhythmicity in the pigeon because the pineal gland and melatonin play a major role in the avian circadian system.

Materials and methods

Adult pigeons were captured in Nagoya city and kept in an outdoor aviary before being housed in individual cages (26×36×30 cm). During the experiments these cages were kept inside a light-tight box. The box was placed in a room where the temperature was held at 24±1 °C. Food and water were available ad libitum throughout the experiments. Illumination was supplied by warm white fluorescent lamps for the light phase of LD cycles (LD 12:12, lights on at 0600 hours), and small incandescent lamps covered with black tapes for the dark phase of LD cycles, except for experiment 3. In experiments 1 and 2, light intensities measured at the level of the bird's head were about 300 lx in the light phase of LD cycles and 0.1 lx in the dark phase and LLdim. In experiment 3, light intensities for the light phase of LD cycles and continuous light condition (LL) were 100–150 lx. The dark phase of LD cycles was complete darkness (0 lx).

In vivo microdialysis

Under sodium pentobarbital anesthesia (25 mg · kg^{-1}, i.v.) a guide cannula for a microdialysis probe was implanted on the surface of the pineal gland with the aid of a microscope (Hasegawa and Ebihara 1992). The cannula was then fixed to the skull by dental cement with two anchor screws. After a few days of postoperative recovery, a dummy cannula was replaced with a dialysis probe for sampling and a few hours after the implantation of the probe sampling was started. The tip of the probe consisted of a cellulose acetate membrane (1.7 mm in length, 0.25 mm o.d., 10 μm membrane thickness, 5000 Da cut off). Ringer solution (NaCl 147 m*M*, $CaCl_2$ 2.3 m*M*, KCl 4 m*M*, pH 6.5) was continuously perfused by an infusion pump through the probe at a rate of 2.0 μl · min^{-1}. Dialysis samples were collected into microtubes of a fraction collector and cooled at 4 °C. Then they were kept in a freezer at −20 °C until assayed for melatonin. After sampling birds were killed an overdose of pentobarbital (100 mg · kg^{-1}, i.v.) for an autopsy of examine the position of the inserted probe.

Experiment 1

This experiment was performed to examine effects of acute exposure to light during the dark on pineal melatonin release. Twenty-eight pigeons were used in this experiment. The pigeons were kept under LD12:12 cycles for at least 3 days before the start of sampling. They were then surgically implanted with a guide cannula for a microdialysis probe. Two days after the surgery, pigeons were gently restrained and sampling was started at 1500–1600 hours and ended around 0500 hours. Light (halogen light, 450 μW · cm^{-2}) was applied for 2 h from about midnight (2330 hours). Twenty-eight pigeons were divided into four groups depending on the experimental treatments: (1) a whole body was exposed to light (WB; n=5); (2) Only the eyes were exposed to light (E; n=10). In this case, black rubber caps connected to the light source by light-conducting fibers were attached to the eyes during the light pulse; (3) The eyes of pigeons were covered with black rubber caps and exposed to light (EX; n=8). In this case, only the extraretinal photoreceptor(s) were illuminated; (4) Pigeons were not exposed to light. However, rubber rings were applied (the center of the rubber cap was cut off to prevent the eyes from being exposed to complete darkness) to their eyes instead of the light pulse (C; n=5). Dialysis samples were collected every hour from the start to 2100 hours and every 30 min from 2100 hours to the end of the experiment.

Experiment 2

This experiment was performed to examine the effects of a light pulse on the phase of pineal melatonin rhythmicity. Seventeen pigeons were entrained to LD12:12 cycles (lights on at 0600 hours) for at least 1 week before the surgery. Two days after the surgery, ten pigeons were given a single light pulse (halogen light, 450 μW · cm^{-2}) of 2 h from 0030 to 0230 hours (CT 18: light on is CT 0). During the light pulse, pigeons were held in the restrainer. After the light pulse they were transferred into LLdim (0.1 lx). Five out of ten birds were given the light pulse to the whole body and the other five birds received a pulse only to the eyes. The method for applying the light exposure was the same as experiment 1 (the same abbreviations as experiment 1 in terms of the treatment apply). Seven control birds were also held in the restrainer for 2 h but were not given the light pulse. Sampling was started 26–33 h after the light pulse and the phase of the second cycle after the light pulse was compared with the control group. Dialysis samples were collected every 1 h.

Experiment 3

After entrainment to LD12:12 cycles (lights on at 0600 hours) for 2 weeks, nine pigeons were implanted with a guide cannula for a microdialysis probe. Two days after the surgery, they were transferred into LL (100–150 lx) at 0600 hours. The heads of these pigeons covered with dental cement were permanently stuck with black tapes. This procedure was used to prevent pineal melatonin from being suppressed by LL. At 1200 hours on the day when LL began, both eyes were covered with bandaids (25 mm in diameter) pasted non-adhesive side down with black tape, and then the patches were removed from the eyes after 12 h (0000 hours). Patches were reapplied 12 h later. These procedures were continued for 7 days. Thereby only the eyes of pigeons were directly exposed to LD12:12 (the start of L phase was 0000 hours) cycles and other photoreceptor(s) were kept under constant conditions, i.e., the phase of the light cycles for the eyes was advanced by 6 h from the previous cycles. To increase adhesion of bandaids to the bird, feathers around the eyes were cut away and rubber cement was applied to both the patch and the skin of the bird. On day 7, sampling was started from 0000 hours and continued for 34 h to obtain an individual rhythm of pineal melatonin release. Dialysis samples were collected every 2 h.

Melatonin radioimmunoassay

Melatonin content in samples of 50 or 100 μl (50-μl samples were used in experiment 1) was measured by a double-antibody radioimmunoassay (RIA) (Oshima et al. 1987). Into each assay tube was dispensed 100-μl samples or 50-μl samples plus 50 μl phosphate-buffered saline gel (PBS gel: 0.01 *M* sodium phosphate buffer, 0.14 *M* NaCl, 0.1% gelatin, pH 7.4). Then, 50 μl (3000 cpm) ^{3}H-labeled melatonin (81Ci · $mmol^{-1}$, Amersham) and 50 μl antiserum (1:30000 dilution in the PBS containing 0.05 *M* EDTA and 1% nor-

mal rabbit serum) were added to each assay tube. After incubation at 4 °C for 48 h, 200 µl goat anti-rabbit γ-globulin serum (Nihon Shibayagi-Center, 1:150 dilution of PBS containing 0.05 *M* EDTA) was added to each tube. Following incubation at 4 °C for 48 h, bound melatonin was separated by centrifugation (2000 *g*, 30 min, 4 °C) and the radioactivity of the precipitate was counted. The inhibition curve was linearized using a logit transformation and the slope was determined for least-square fit. The intra- and interassay coefficients of variation for pooled nighttime plasma containing 24.54±0.88 pg per tube (n=11) were 9.87% and 11.90%, respectively. The limits of sensitivity varied from 1.0 to 1.5 pg per tube among assays.

Statistical methods

Statistical analyses were performed using a one-way analysis of variance (one-way ANOVA) and Fisher PLSD test for determination of significant differences among groups. A least-squares spectrum method was used for the calculation of the acrophase (the maximum of the best least-squares fit cosine wave). Significant differences in the acrophase were determined by Duncan's new multiple range test.

Results

Experiment 1

In the WB group, a 2-h light pulse strongly suppressed pineal melatonin release down to a daytime level and the suppression continued through the light pulse (Fig. 1A). The mean value of each time point was significantly lower than that of the C group (P<0.05) (Fig. 2). However, in the E group, although the mean values were lower than the C group, no significant differences between the E and C group were obtained at any time point (P>0.05). In the E group, some birds (n=6) showed a weak inhibition (Fig. 1C) but other birds (n=4) did not (Fig. 1B). In the former case, however, the suppression did not always continue until the end of the light pulse and melatonin levels began to recover during the light pulse. The EX group showd the same inhibition pattern as the WB group and no significant differences in the mean values between the WB and EX group (P>0.05) were observed. Differences between the EX and C group were significant at all time points (P<0.05) (Fig. 2).

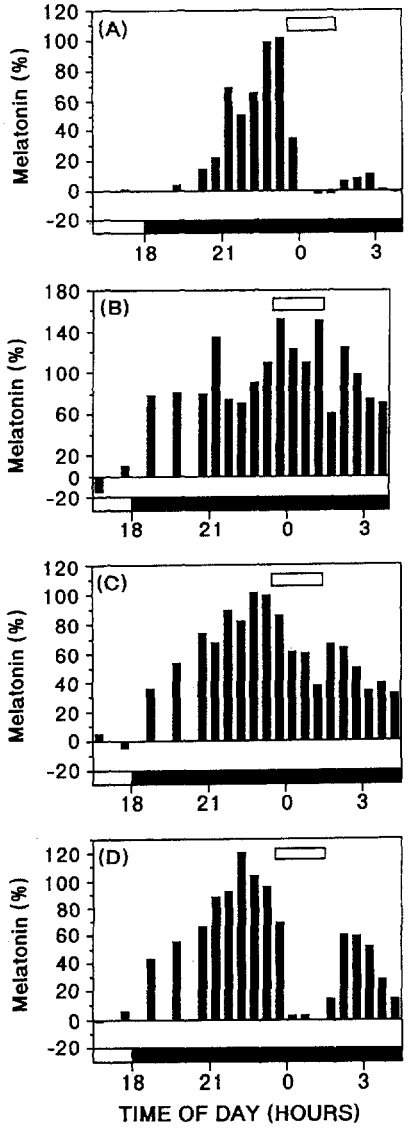

Fig. 1A–D Effects of light exposure on pineal melatonin release in pigeons. Pigeons were kept under LD 12:12 (lights on at 0600 hours) and exposed to the light of a halogen lamp (450 µW · cm^{-2}) for 2 h (2330–0130 hours). Pigeon (**A**) was given the light to the whole body, pigeons (**B**) and (**C**) were given the light only to the eyes; (**B**) melatonin release was not inhibited, (**C**) melatonin release was weakly suppressed. Pigeon (**D**) was given the light to the whole body except the eyes. The difference between the mean of two samples just before the 2-h light pulse and that of the last two to three samples during the day (1500–1800 hours) is defined as 100% in each bird. Sampling intervals are 1 h from the start to 2100 hours and 0.5 h from 2100 hours to the end. Duration of light exposure is shown by a *white square* in each graph. *Horizontal black bars* and *white bars* at the bottom indicate the dark and the light, respectively

Experiment 2

Figure 3 shows the time schedules of the experiment. A 2-h single light pulse at CT 18 induced phase advances when the light was given to the whole body but did not induce phase shifts when only the eyes were illuminated (Fig. 4). Mean acrophases of melatonin rhythms determined by the least-square method were 23:08±15 (control group; C), 21:19±43 (light to the whole body; WB) and 00:12±47 (light to the eyes; E). Significant differences in the acrophase of melatonin rhythms were observed between the C and WB group (1.82 h, P<0.05), but not between the C and E group (1.07 h, P>0.05).

Experiment 3

Figure 5 shows the time schedules of treatments for the experiment. Pineal melatonin rhythms on the 7th day of patching did not entrain to the LD cycles given to the eyes (Fig. 6). In many pigeons (n=7), melatonin levels

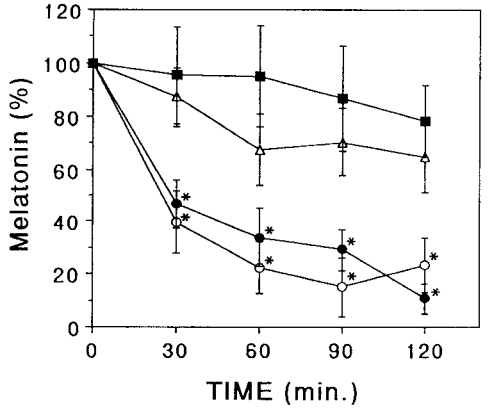

Fig. 2 Time-course of pineal melatonin suppression by acute light exposure (450 μW · cm^{-2}, 2 h) during the dark. Pigeons were kept under LD 12:12 (lights on at 0600 hours) and they were exposed to 2 h light (2330–0130 hours) during the dark. Four groups (*WB:* the whole body was illuminated [○], *E:* only the eyes were illuminated [△], *EX:* the whole body except the eyes was illuminated [●], *C:* no light exposure [■]) are shown. The difference between the mean of two samples just before the 2-h light pulse and that of the last two to three samples during the day (1500–1800 hours) is defined as 100% in each bird. Group mean of the percentage and SEM (n=5–10) are shown. A *horizontal axis* shows the time after the light pulse (min). *P<0.05 vs *C* at each point

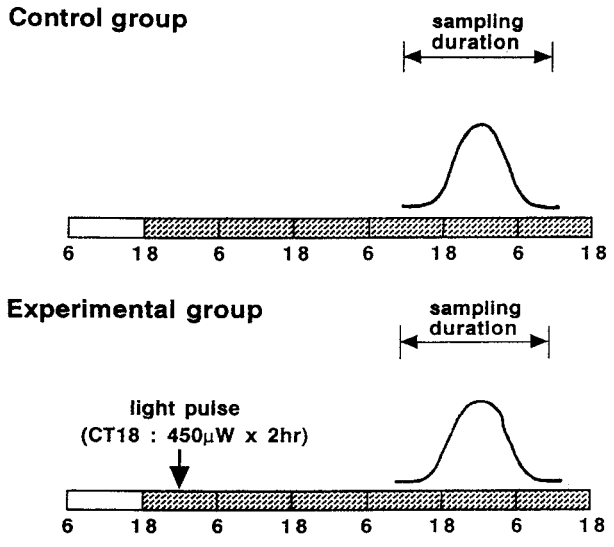

Fig. 3 Time schedules of treatments for the study of effects of a 2-h single light pulse (450 μW · cm^{-2}) at CT 18 on the phase of pineal melatonin rhythms (experiment 2). *Open bars* represent the light period and *shaded bars* the dark period

increased around 1800 hours and high levels of melatonin continued at least for 12 h. The high levels of melatonin were kept even after receiving the light from the eyes. In some birds (n=2), however, melatonin increased much earlier than 1800 hours. Thus, the phases of pineal melatonin rhythms were not identical among the pigeons.

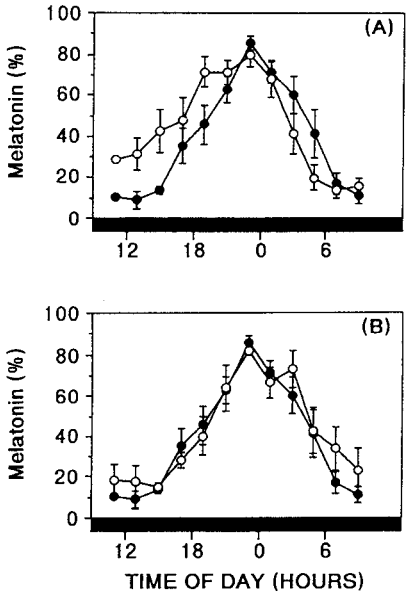

Fig. 4A, B Circadian rhythms of pineal melatonin on the 2nd cycle after a 2-h single light pulse (450 μW · cm^{-2}) at CT 18. Pigeons were transferred into LLdim after entrainment to LD12:12 (lights on at 0600 hours) and given a light pulse to the whole body (**A**) or only to the eyes (**B**) at CT 18 in the first day of LLdim. In both figures, the groups receiving the light pulse were shown by *open circles. Closed circles* are for a control group which did not receive the light pulse. The difference between the peak and nadir of melatonin rhythm is defined as 100% in each bird and the percentage at each time was obtained. Group mean and SEM are shown. *Horizontal black bars* at the bottom indicate the dark period

Discussion

In the WB group a light pulse rapidly suppressed pineal melatonin release to the daytime level as has been demonstrated in previous studies (Yamada et al. 1988; Takahashi et al. 1989). This suppression was strong and continued until the end of the light pulse. In the E group, however, the same light pulse did not dramatically suppress nocturnal melatonin release. Only weak suppression (about 50%) was observed in six out of ten pigeons and no suppression was observed in the other four pigeons. Because we used relatively high intensity of a halogen light (450 μW · cm^{-2}) which was sufficient for pineal melatonin suppression in the WB group, it is suggested that light perceived by the eyes may not be essential for photic inhibition of pineal melatonin release in the pigeon. Actually light perceived by extraretinal photoreceptor(s) (the EX group) strongly suppressed pineal melatonin release as seen in the pigeons of the WB group. These results are consistent with the result of experiment 3 that retinally perceived light did not suppress pineal melatonin release.

It is thought that chicken pineal is innervated by sympathetic nerves from the SCG to which light information is transmitted via a multisynaptic neural pathway from the eyes (Cassone 1991). NE, which is released from the

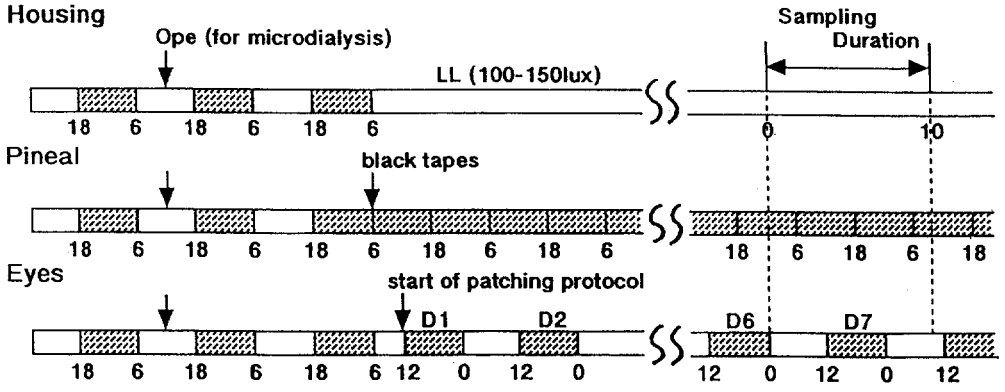

Fig. 5 Time schedules of treatments for experiment 3 examining effects of retinal LD cycles on pineal melatonin rhythms. *Open bars* represent the light period and *shaded bars* the dark period. *Ope* operation for in vivo microdialysis; *D1* the dark of the 1st day of the patching protocol; *D2* the dark of the 2nd day of the patching protocol

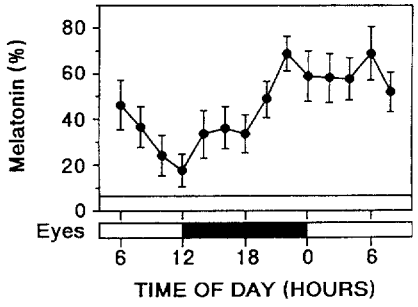

Fig. 6 The pineal melatonin rhythm on day 7 of the patching protocol. The difference between the peak and nadir of melatonin rhythms is defined as 100% in each bird and the values are shown as the percentage at each time. The mean of the percentage and SEM (n=9) are shown. Sampling intervals are 2 h. The LD cycle to the eyes during the patching protocol is represented by *black* (the dark period) and *white bars* (the light period) at the bottom

sympathetic nerves, strongly suppress pineal melatonin release in cultured chicken pineal although it does not have phase-shifting effects on pineal melatonin rhythms (Zatz and Mullen 1988), and periodic infusion of NE can restore plasma melatonin rhythms in the SCG-removed chickens which lost plasma melatonin rhythms (Cassone and Menaker 1983). These results indicate that photic information for pineal melatonin suppression from the eyes are mediated by NE released from the sympathetic nerves of the multisynaptic pathway. In our preliminary study, however, pigeon pineal melatonin did not respond significantly to NE (1 m*M* contained in perfusing solution) infused for 2 hours during the dark (the data are not shown). Neural regulation for melatonin synthesis, therefore, may be different between pigeons and chickens. Lower levels of pineal NE content in the pigeon (166.1±27.8 pg per gland: n=5) than the chicken [98.8±6.3 ng per gland, data from Cassone and Menaker (1983)] may support this possibility.

We also investigated whether retinally perceived light has phase-shifting effects on pineal melatonin rhythms. In this experiment, a light pulse at CT18 was tested. This is because 6-h light pulses beginning late in the subjective night (CT18–24) advance the phase of the melatonin rhythm in cultured chicken pineal (Takahashi et al. 1989) and a single 2-h light pulse (450 $\mu W \cdot cm^{-2}$, halogen light) at CT18 can induce phase advances of locomotor activity rhythms in pigeons in LLdim ($\Delta\Phi$=1.59±0.55 h, mean±SEM, n=7) (Hasegawa et al., unpublished observations). Although a light pulse at CT18 could induce phase advances of pineal melatonin release in the WB group, the same light pulse delivered only to the eyes had no effects on the phase of melatonin rhythms. These results suggest that retinally perceived light does not have essential roles in inducing the phase shift of pineal melatonin rhythms in pigeons.

The above suggestion is supported by the result of experiment 3 that pineal melatonin rhythm did not entrain to retinal LD cycles. If retinally perceived light has entraining effects, then it would be expected that the phase of pineal melatonin rhythms would move forward and the level of melatonin would rise during the D phase of retinal LD cycles and decrease during the L phase. In most of the pigeons examined, however, the phase of pineal melatonin rhythms did not drift away from the LD cycles prior to the patching. Our interpretation of this result is that pineal melatonin rhythms were free-running under the patching condition because in two pigeons the phase of melatonin shifted away from the LD cycles prior to the patching. In Japanese quails, the same treatment of retinal patching for 7 days can entrain pineal melatonin rhythms to an LD 12:12 cycle which is 180° out of phase with the LD 12:12 cycle experienced prior to the patching (Barrett and Underwood 1991). The present study used 6-h phase advances of LD cycles perceived by the eye instead of 12-h phase shifts. Therefore it is unlikely that 7 days of patching are not long enough for re-entrainment to the LD cycles. Thus, it is reasonable to conclude that retinally perceived light does not contribute to photic entrainment of pineal melatonin rhythms in pigeons; even if this were the case, the role of the eye is not essential.

Barrett and Underwood (1992) have demonstrated that retinally perceived light can entrain pineal melatonin rhythms in Japanese quails and bilateral removal of the SCG does not prevent the entrainment of the melatonin rhythms. These results indicate that the pathway from the eyes to the pineal gland for entrainment of pineal melatonin rhythm is present in Japanese quails which does not involve the SCG. It is not certain whether the path-

ways for photic inhibition of pineal melatonin and entrainment of its rhythmicity are different in birds, but if the two pathways exist, neither route is essential for photic regulation of pineal melatonin in the pigeon. Thus, it is likely that a system of photic regulation of pineal melatonin rhythms is dramatically different among species.

In cultured chicken pineal, melatonin rhythms can entrain and respond to environmental light conditions indicating that the pineal gland itself has photoreceptor(s) for suppression of melatonin and entrainment of its rhythmicity to light conditions (Takahashi et al. 1989). Also in the pigeon, it has been demonstrated that melatonin release from cultured pineal cells shows daily changes with higher levels during the dark and lower levels during the day (Taniguchi et al. 1993). Therefore, it is reasonable to assume that the photoreceptors within the pineal itself are responsible for photic regulation of pineal melatonin in the pigeon. In addition to the pineal gland, encephalic photoreceptors are known to be involved in the avian circadian and photoperiodic systems (Menaker and Keatts 1968; Menaker et al. 1970). Therefore, we can not exclude the possibility that encephalic photoreceptors contribute to photic response of pineal melatonin in birds. In the present study, weak suppression of pineal melatonin release observed in the E group may be due to light which penetrates the eyes and is perceived by encephalic photoreceptors.

In mammals, the pineal is richly innervated by sympathetic nerves which originate in the SCG and pineal melatonin rhythmicity is controlled by NE (Reiter 1991). Photic information received by the eyes is conveyed by this route. It is likely that the progressive increase of the sympathetic innervation occurs in the process of vertebrate evolution. For example, in amphibia only few sympathetic nerve fibers are observed (Owman et al. 1970), whereas reptiles possess more (Collin 1979). This innervation exists in all birds examined, but the extent of the innervation differs among species (Sato and Wake 1983). Thus, it is conceivable that the effects of retinally perceived light on pineal melatonin are variable among avian species.

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