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Dissociation between the circadian rhythm of locomotor activity and the pineal clock in the Japanese newt

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Abstract The circadian locomotor activity rhythm of the Japanese newt has been thought to be driven by a putative brain oscillator(s) subordinate to the pineal clock. The existence of mutual coupling between the pineal clock and the brain oscillator(s) in vivo was examined. We covered the newt's skull with aluminum foil and simultaneously reversed the light-dark cycle, thereby allowing the pineal organ to be exposed to constant darkness while the rest of the animal was exposed to the reversed light-dark cycle. In control animals, whose heads were covered with transparent plastic, the rhythm of synaptic ribbon number in the pineal photoreceptor cells was entrained to the reversed light-dark cycle. Rhythms from newts whose heads were shielded, however, were similar to those observed in the unoperated newts kept under constant darkness. The locomotor activity rhythms of both head-covered animals and control animals were entrained to the reversed light-dark cycle. These data suggest that extrapineal photoreception can entrain the putative brain oscillator(s), but not the pineal clock. Thus, at least in an aspect of photic entrainment, there seems to be little or no mutual coupling between the pineal clock and the putative brain oscillator(s) in the circadian system of the Japanese newt.

Keywords Circadian system · Entrainment · Locomotor activity · Pineal organ · Synaptic ribbon

Abbreviations LD light-dark · DD constant darkness · SCN suprachiasmatic nucleus · SR synaptic ribbon

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Introduction

Many studies have suggested that the circadian systems of non-mammalian vertebrates have at least three sites which are involved in generating rhythmicity: (1) the pineal organ, (2) the eyes, and (3) a region in the hypothalamus (e.g., Gwinner and Brandstaetter 2001; Tosini et al. 2001). The relative contribution of these three sites to overall rhythmicity differ widely among species (Menaker and Tosini 1996). In a urodele amphibian, the Japanese newt (*Cynops pyrrhogaster*), the locomotor activity rhythms slowly dampen out after pinealectomy in constant darkness (DD) showing residual rhythmicity for several days (Chiba et al. 1993). These observations suggest that in this species the pineal organ plays a major role in generating the circadian rhythm of locomotor activity by controlling a subordinate circadian oscillator(s) which is located elsewhere in the brain. The eyes, on the other hand, seem to play only a minor role since bilateral ocular enucleation produces little change in the free-running period (Chiba et al. 1993).

In the presence of a light-dark (LD) cycle, however, both the pineal organ and the eyes appear to provide functional photoreceptive inputs for the entrainment of the putative brain oscillator(s) responsible for driving locomotor activity rhythm. This hypothesis is supported by our previous experimental results: (1) both pinealectomized newts and ocularly enucleated ones are entrained to an LD cycle, and (2) if pinealectomy is performed on ocularly enucleated newts under an LD cycle, the entrained activity rhythm slowly dampens out (Chiba et al. 1993). It is probable that retinally perceived light can entrain the brain oscillator(s) without help from the pineal clock. Light perceived by the pineal organ, on the other hand, could directly entrain the pineal clock, and could then still influence the brain oscillator(s). It is, therefore, possible that to preserve the integrity of a multioscillator circadian system with multiple input pathways, the pineal clock and the brain oscillator(s) are mutually coupled to each other.

The present study is designed to examine whether there is mutual coupling between the pineal clock and the brain oscillator(s) which drives the locomotor activity rhythm in the Japanese newt. To examine this, we covered the skull of newts kept under an LD cycle while simultaneously reversing the LD cycle. In this regime, the pineal is exposed to DD while the rest of the animal is exposed to the reversed LD cycle. The phase of the putative brain oscillator(s) was determined using the locomotor activity rhythm. As for a phase marker of the pineal clock, we used the circadian change in the number of pineal photoreceptor synaptic ribbons (SRs) since this rhythm has been suggested to parallel the circadian rhythm of pineal metabolic activity in many vertebrate species (Vollrath 1973; McNulty 1981; Martinez Soriano et al. 1984; Maitra et al. 1989).

Materials and methods

Animals

Adult Japanese newts, *Cynops pyrrhogaster*, over 50 mm in snout-vent length (4–6 g) were purchased from a commercial supplier (Saitama Experimental Animals Supply; Sugito-cho, Saitama, Japan). Prior to use all animals were maintained in the colony for at least 1 month in aquaria at 22 ± 0.5°C under a controlled LD cycle of 12 h light and 12 h darkness (LD 12:12; lights on at 0700 hours).

Locomotor activity recording

During the locomotor activity recording each animal was housed in a plastic recording chamber (17 cm long×8.5 cm wide×4 cm high) placed inside a light-tight container. The recording chamber had an air pipe which extended outside of the light-tight container. Water was continuously circulated through the chamber from an external water tank with a gravel filter. The depth of water in the chamber was maintained at 1.5 cm. The container had four white light-emitting diode lamps (NSPW510BS; Nichia; Anan, Tokushima, Japan) on its ceiling and the photoperiod was controlled by an automatic timer. Light intensity was adjusted to 50 lx at the level of the water surface. The room and water temperature were kept at 22 ± 0.5°C.

Locomotor activity (counts per 1-min intervals) of each newt was detected by an infrared photoswitch and recorded on a data collection computer. The beam of the photoswitch was adjusted so that it was located just below the surface of the water along the longitudinal axis of the recording chamber. Signals were generated when the newts moved and interrupted the beam. For visual display, the time series of locomotor data were double-plotted in the form of actograms.

Head-covering

The covering treatment was done under anesthesia in a solution of MS222 (3 g l^{-1}; Sankyo; Tokyo, Japan). After removing a portion of skin from the top of the skull, a small piece of aluminum foil (3 mm×4 mm) was placed directly over the exposed area using a surgical adhesive (Aron Alpha A; Sankyo). The placement of the foil ensured that the diencephalic roof containing the pineal organ would be centered under the covering. During sham operations, a piece of transparent plastic sheet was placed over the same location on the skull.

Electron microscopy and measurement of pineal SR numbers

The newts were decapitated under anesthesia in a solution of MS222 (3 g l^{-1}), and the brains of the newts were removed immediately, quickly dissected, and then fixed in 2.5% glutaraldehyde, 2.0% paraformaldehyde in 0.1 mol l^{-1} cacodylate buffer (pH 7.3) at 4°C. Following primary fixation for 24 h, the brains were washed in cacodylate buffer, trimmed, and post-fixed with 1.5% osmium tetroxide in cacodylate buffer for 2 h. The tissue was washed in distilled water, dehydrated in a series of increasing concentrations of ethanol, infiltrated with propylene oxide, and embedded in Epon 812. All blocks of the brains were oriented to obtain sagittal sections, and were cut close to the median plane using an ultramicrotome. Ultra-thin sections were mounted on a 300-mesh copper grid, contrasted with uranyl acetate followed by lead citrate, and examined with an electron microscope (H-300, Hitachi). For quantitative assessment, the number of SRs lying inside of an aperture comprised of three grids was counted. Data were expressed as the number of SRs per unit area (9,600 μm^2). Results were statistically analyzed using a one-way analysis of variance (ANOVA) followed by a Duncan's new multiple range test.

Experimental procedures

Eighteen newts were used for locomotor activity recording. Each newt was initially placed in a recording chamber under LD 12:12 (lights on at 0700 hours) for at least 10 days and then the LD cycle was reversed by inserting one 24-h light period (0700–0700 hours) instead of 12-h period. At 1900 hours on the day of the LD cycle reversal (i.e., in the middle of the inserted 24-h light period), the newts were either given a foil head covering ($n = 9$) or their skulls were covered with a piece of transparent plastic ($n = 9$). Activity recording was continued for at least another 2 weeks under the reversed LD cycle (DL 12:12; lights on at 1900 hours).

A total of 168 newts were used to examine the daily changes in the pineal photoreceptor SR number. Newts were divided into four groups, each consisting of 42 animals. Newts were kept under LD 12:12 for at least 10 days. Group 1 (unoperated newts) was used to examine the reentrainment of the pineal SR rhythm to DL 12:12. The LD cycle reversal was performed the same way as described above. Group 2 was used to confirm the existence of the circadian rhythm of pineal SR number in DD as previously reported (Kikuchi and Aoki 1985). Newts in group 3 were used to examine the effect of head-covering on the reentrainment of the pineal SR rhythm to DL 12:12. The coverings were performed at 1900 hours on the day of the LD cycle reversal as described above. Newts in group 4 were subjected to a sham operation using a piece of transparent plastic instead of the foil. On the 7th day of DL 12:12 (groups 1, 3, and 4) or DD (group 2), seven animals from each group were sacrificed by decapitation at 1000, 1400, 1800, 2200, 0200 and 0600 hours, and the numbers of pineal SR were measured. Dissection and fixation during the dark period were carried out under dim red light.

Results

Figure 1 shows locomotor activity records from representative newts which were subjected to the foil head-covering (Fig. 1a, b) and the sham operation (c, d) followed by the reversal of LD 12:12 to DL 12:12. The locomotor activity rhythms of all the newts in both groups were reentrained to DL 12:12 after a few transient periods. Anticipatory activity before light onset of the reversed LD cycle was distinct although the amounts of activity markedly decreased either because of the slight surgical damage and/or masking effect of light.

The 24-h profiles of the pineal SR number of newts from the four different groups are shown in Fig. 2.

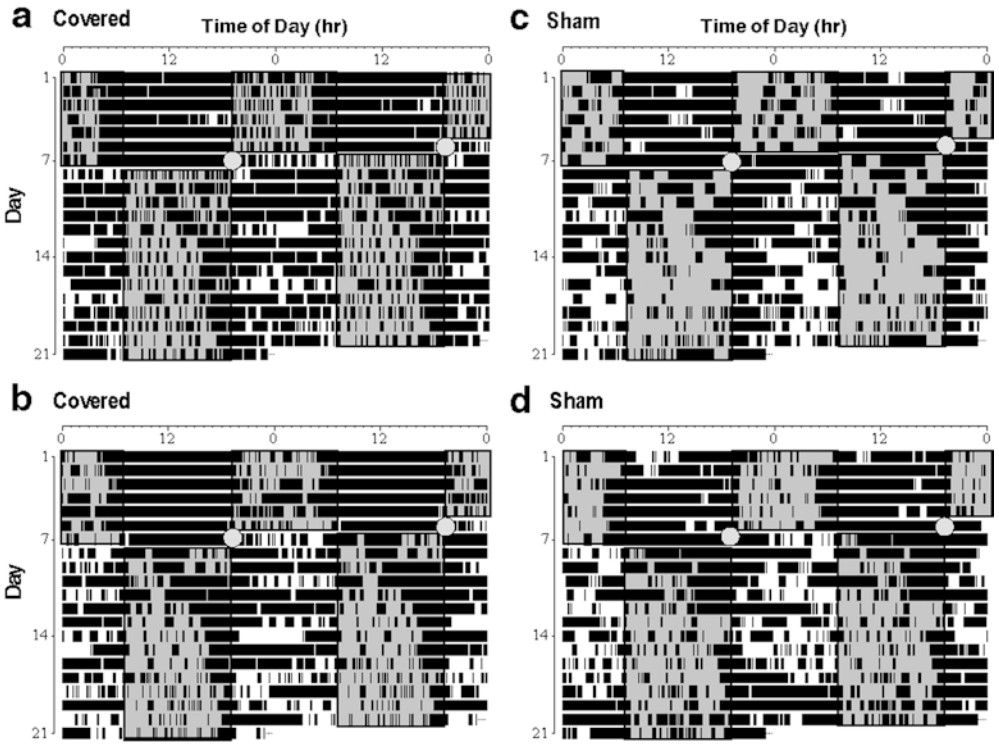

Fig. 1 Double-plotted locomotor activity records of two representative newts subjected to head-covering with aluminum foil (**a**, **b**) and control operation with transparent plastic (**c**, **d**), followed by the reversal of light-dark (LD) cycle. Surgeries were performed at 1900 hours on day 7 (*small circles*). *Shadowed areas* indicate dark phase

Figure 2a shows the profile from the unoperated animals which were first entrained to LD 12:12 and then exposed to DL 12:12 for 7 days. This profile displays a rhythm with a peak at 1800 hours (11 h after the onset of darkness of DL 12:12). Figure 2b shows the pineal SR number of unoperated animals which were first entrained to LD 12:12 and then exposed to DD for 7 days. The pineal SR number peaked at 0200 hours (7 h after dark onset of the initial LD cycle). Figure 2c shows that the profile of the circadian rhythm of SR number from newts first entrained to LD 12:12 and which, after head-covering, were exposed to DL 12:12 for 7 days. The peak in SR number occurs at 0200 hours (7 h after the light onset of the reversed LD cycle). Figure 2d shows the profile of SR number from animals first entrained to LD 12:12 and which, after a sham operation with transparent plastic, were exposed to DL 12:12 for 7 days. The profile has a peak at 1400 hours (7 h after the dark onset of the reversed LD cycle).

Discussion

The pineal organs of some birds, lizards and fish are directly photosensitive and contain circadian oscillators as confirmed by the demonstration that isolated pineals, when cultured in vitro, can maintain circadian rhythms of melatonin synthesis for many days and that those rhythms can be entrained to an LD cycle (Takahashi et al. 1980; Menaker and Wisner 1983; Falcon et al. 1987; Barrett and Takahashi 1995; Bolliet et al. 1996). Although direct evidence is scant in amphibians, our previous lesion experiments (Chiba et al. 1993) strongly suggest that the pineal organ of the Japanese newt also contains a photoreceptive circadian oscillator responsible for the generation and entrainment of the circadian locomotor activity rhythm.

Our previous experiments demonstrated that the number of SRs observed in the newt pineal photoreceptor basal process is low during the day and usually reaches a peak during the latter half of the dark phase under LD cycles of 12 h light and 12 h darkness (Kikuchi and Aoki 1985; Kikuchi et al. 2000), and that this rhythm persists in DD for several days in vivo (Kikuchi and Aoki 1985). The SRs are likely to be involved in the transmission of light information from the photoreceptor cells to the ganglion cells. Because ganglion cell axons connect the pineal organ with the brain, changes in the number of pineal SR may play a role in circadian organization by regulating the output from the pineal organ to the rest of the circadian system.

In the present study, the profiles of SR numbers from control animals, both untreated and sham operated (Fig. 2a, d), clearly showed that the SR rhythms were entrained to the reversed LD cycle with a peak during the latter half of the dark period of DL 12:12. Considering the time intervals were 4 h, the profile in Fig. 2d is similar to that of Fig. 2a. This suggests that the control operation did not affect the reentrainment of pineal SR rhythms to the reversed LD cycle. In the newts exposed to DD for 7 days (Fig. 2b) the pineal SR number peaked at 7 h after dark onset of the initial LD cycle, suggesting that the rhythm of SR were freerunning after being transferred to DD. Some phase differences among the circadian rhythms of SR number from individual newts may be produced as a result of the variability in their

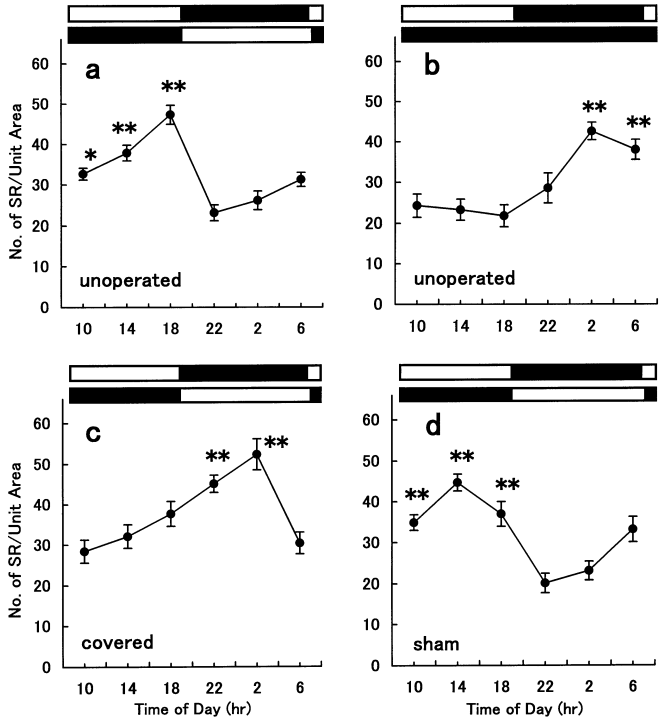

Fig. 2 24-h profiles of synaptic ribbon numbers (mean ± SE) per unit area from **a** the unoperated newts which were exposed to the reversed LD cycle for 7 days, **b** the unoperated newts which were exposed to constant darkness (DD) for 7 days, **c** the newts whose heads were covered with aluminum foil and exposed to the reversed LD cycle for 7 days, and **d** the control animals whose head covered with transparent plastic and exposed to the reversed LD cycle for 7 days. $n = 7$ for each time point of each profile. The *solid* and *open bars* at the top of each figure represent the dark and light phase, respectively, of the light regimes given (*top bar*, initial one; *bottom bar*, the following one). $^{**}P < 0.001$; $^{*}P < 0.01$, respectively, versus the lowest value of each profile

free-running periods. These differences, however, are small because both a peak and trough are still evident in the combined profiles.

In the newts whose heads were covered with foil and kept in a reversed LD cycle for 7 days (Fig. 2c), on the other hand, the profile is approximately 180° out of phase with the profiles of SR number from unoperated and sham-operated newts (Fig. 2a, d); the peak in SR number occurs at 0200 hours during the light period of the reversed LD cycle. Instead, the profile is similar to that in Fig. 2b; in both profiles the peak occurred at 7 h after the dark onset of the previously experienced LD cycle (LD 12:12). This suggests that in the head-covered animals the pineal SR rhythms were not entrained to the reversed LD cycle and that the rhythms were likely freerunning after the covering. These observations suggest that (1) light perceived by a structure under the covered area (which includes the pineal organ) is responsible for the photic entrainment of the circadian rhythm of the pineal SR number, and that (2) the foil covering reduced the amount of light penetrating the skull to subthreshold levels for entrainment of the pineal SR rhythm. Interestingly, the locomotor activity rhythms were entrained to the reversed LD cycle in both the foil-covered and control animals (Fig. 1). The existence of a transient period lasting for several days and anticipatory activity before light onset can be observed in the locomotor activity rhythms, suggesting that the reversed LD cycle entrained the circadian oscillator responsible for generating the locomotor activity rhythm. Taken together, the present data suggest that extrapineal photoreception entrains the putative brain oscillator(s) which drive the locomotor activity rhythm, but not the pineal clock. Probably, the pineal clock is entrained exclusively by light perceived by the pineal organ itself.

The lack of entrainment of the pineal clock to a retinally perceived light cycle indicate that in the circadian system of the newt the pineal clock affects the brain oscillator(s) but inputs from the brain oscillator(s), if any, would not mediate photic entrainment. In mammals, pineal organ is not photosensitive and is under control of suprachiasmatic nuclei (SCN) of the hypothalamus, a central circadian pacemaker (Moore and Klein 1974). Retinally perceived light entrains the SCN via the retino-hypothalamic pathway, and then SCN phases the pineal rhythm through a polysynaptic sympathetic pathway via the superior cervical ganglia (SCG) (Klein 1985). In non-mammalian species, however, the region that is homologous to mammalian SCN has not been clearly demonstrated, although the existence of retino-hypothalamic connections has been reported in many species including amphibians (Fritzsch 1980; Norgren and Silver 1989; Tilgner et al. 1990; Tuinhof et al. 1994). Furthermore, the sympathetic innervation of the pineal organ, if any, does not seem to play a critical role in the photic entrainment by the eyes. In the Japanese quail, for example, the pineal melatonin rhythm is entrained by retinally perceived light even after removal of SCG (Barret and Underwood 1992), and in the pigeon, the pineal rhythm is not entrained by retinally perceived light (Hasegawa et al. 1994).

Considering the fact that the newt's pineal organ plays a major role in generating circadian rhythms of locomotor activity, it is interesting that locomotor activity rhythm in the foil-covered newts were entrained to the reversed LD cycle even though their pineal clocks appeared to be freerunning. The entraining effects of photic input to the brain oscillator(s) from the retina may be stronger than the synchronizing effects of the hormonal and/or neuronal input from the freerunning pineal clock. In the newts, data are still too scant to determine the pathway by which the eyes contribute to the entrainment of the locomotor activity rhythm. The eyes of the newts may control the phase of the putative brain oscillator(s) by mediating photic information through periodic melatonin secretion (Oshima et al. 1989) and/or a neuronal output (Underwood et al. 1990; Minutini et al. 1994; Underwood 1994). In the latter case, retinally perceived light may directly entrain brain oscillator(s) which is possibly located in the hypothalamus. It is, however, also possible that photic information

is transmitted via indirect neural pathways to other brain regions that influence locomotor activity and that might contain circadian oscillators, including the mesencephalic tectum or the cerebellum.

As shown in most non-mammalian vertebrates (reviewed in Foster et al. 1994), *Cynops* may possess extraretinal-extrapineal deep brain photoreceptors which impinge upon the circadian system. However, we have previously reported that locomotor activity rhythm of the pinealectomized-enucleated newts was no longer entrained to a LD cycle of the same light intensity used in the present experiments (Chiba at al. 1993). Therefore, at least in our experimental conditions, whether or not the foil covering reduces the amount of light reaching the extrapineal-extraretinal photoreceptors, these structures would not be essential for entraining the locomotor rhythms. We cannot, however, exclude the possibility that in normal situations the pineal organ and the brain oscillators are kept coupled by sharing photic input from the extraretinal-extrapineal photoreceptors.

In the present study, it was suggested that the LD cycle perceived by the eyes entrains the putative brain oscillator(s) but not the pineal clock. Therefore, it seems that at least in an aspect of photic entrainment there is little or no mutual coupling between the pineal clock and the putative brain oscillator(s) responsible for locomotor activity rhythm. Under natural conditions, however, the loss of the mutual coupling between the pineal clock and the brain oscillator(s) would not produce any internal desynchronization within the overall circadian system. Normally, the photic information perceived by both the eyes and the pineal organ would not be in conflict with each other and thus both the brain oscillator(s) and the pineal clock would be kept in phase.

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