Identification of the suprachiasmatic nucleus in birds

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OVER THE PAST FEW DECADES, a considerable number of studies has been done on the histology, physiology, and molecular aspects of the suprachiasmatic nucleus (SCN) in the hypothalamus, and now it is clear that the SCN is the dominant biological clock regulating circadian rhythms in mammals. In birds, two nuclei, the so-called medial SCN (mSCN) and the visual SCN (vSCN), have both been proposed to be the avian SCN. However, it remains an unsettled question which nuclei are homologous to the mammalian SCN. We have identified circadian clock genes in Japanese quail and demonstrated that these genes are expressed in known circadian oscillators, the pineal and the retina. Here, we report that these clock genes are expressed in the mSCN but not in the vSCN in Japanese quail, Java sparrow, chicken, and pigeon. In addition, mSCN lesions eliminated or disorganized circadian rhythms of locomotor activity under constant dim light, but did not eliminate entrainment under light-dark (LD) cycles in pigeon. However, the lesioned birds became completely arrhythmic even under LD after the pineal and the eye were removed. These results indicate that the mSCN is a circadian oscillator in birds.

circadian rhythm; Japanese quail; pigeon; clock gene

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MATERIALS AND METHODS

Animals. Japanese quail (Coturnix coturnix japonica), Java sparrow (Padda oryzivora), chicken (Gallus gallus), and pigeon (Columba livia) were obtained from local dealers and housed in light-tight boxes (55 x 210 x 62 cm) where light cycles were provided. The boxes were placed in a room at a temperature of 24 ± 1°C. For in situ hybridization, birds were maintained under either 12:12 h light-dark (LD 12:12) or 18:6 h LD (18:6) cycles and, in some cases, were transferred from LD to constant darkness (DD). The light was
supplied by fluorescent lamps with a light intensity of ~200 lx measured at the level of the bird's head. For behavioral experiments, birds were maintained under LD 12:12 and, in most cases, transferred to constant dim light (dimLL). In this experiment, an incandescent lamp almost completely covered with black tape was used for the dark phase (0.15 lx) of the LD cycles, with the same light source and intensity being used for dimLL. Food and water were available ad libitum and were replenished at least once a week.

In situ hybridization. Animals were killed by decapitation, and the brain was immediately removed to avoid acute changes in gene expression. In situ hybridization was carried out according to Yoshimura et al. (25). Antisense and sense 45mer oligonucleotide probes (qClock: nucleotides 861–905 of GenBank accession number AB029889; qPer2: 1904–1948 of AB029890; qPer3: 1382–1426 of AB029891) were labeled with [35S]dATP (New England Nuclear) using terminal deoxyribonucleotidyl transferase (GIBCO-BRL). Hybridization was carried out overnight at 42°C. After the glass slides were washed, they were air dried and exposed to Hyperfilm-β max (Amersham) for 4 wk. 14C standards (American Radiolabeled Chemicals) were included in each cassette, and densitometric analysis was carried out using a computed image-analyzing system (MCID, Imaging Research).

In the light-pulse experiment, birds were exposed to a 1-h white light pulse (1,600 lx of fluorescent lamps) at circadian time (CT) 16 on the second day after transfer from LD 12:12 to DD (40 h after transfer to DD; CT0 is defined as the time when lights would have come on under LD). In the eye-patch experiment, the eyes of birds were completely covered with black rubber caps at the offset of the last light phase of LD 12:12 and released into DD. Samples were collected 90 min after the light pulse.

Measurement of locomotor rhythms. Each bird was housed in individual cages (26 × 36 × 30 cm). The floor of the cage moved like a seesaw, and its movement was measured with a microswitch and an event recorder (9, 15). The recordings were examined by visual inspection and periodogram analysis (Circadia software, Behavioral Cybernetics, Cambridge, MA) when visual estimation was not reliable.

Surgery. All surgeries were conducted with pentobarbitonal sodium anesthesia (25 mg/kg). Additionally, for blinding (EX), lidocaine chloride (6 mg per bird) was used for local anesthesia, and antibiotic (Gentocin, Schering) was used to prevent infection. The procedures of pinealectomy (PX) and EX were basically the same as in our previous study (9, 15). Briefly, EX was carried out by removal of one eye. After recovery from the first enucleation, the other eye was removed. A gelatin sponge was used to stop the bleeding. Blinded birds had no difficulty in feeding or drinking and maintained their previous body weights after EX. The pineal was carefully removed by drilling a hole in the skull directly over it, then grasping it with a pair of extra-fine forceps under a stereomicroscope. For mSCN lesions, the head of the bird was held with a stereotaxic instrument, and lesions were made by passing 2 mA direct current for 20 s through an insect-pin electrode insulated except for 0.4 mm at the tip. At the end of the experiment, the birds were perfused with saline followed by 10% formalin, and the brain and pineal were examined histologically using cresyl violet staining to know the extent of the lesion and PX.

Animals used in the present experiment were treated in accordance with the guidelines of Nagoya University.

RESULTS

In situ hybridization. We studied the expression of qClock, qPer2, and qPer3 genes in the hypothalamus in Japanese quail. All three genes were expressed in the mSCN, but no signals were detected in the vSCN (Fig. 1). Of these genes, the signal of qPer2 was the most obvious, but qClock and qPer3 signals were weak in the mSCN. To confirm our findings in Japanese quail, other avian species were examined for the expression of Per2. As in the case of Japanese quail, Per2 signals in the mSCN were observed in Java sparrow (Padda oryzivora), chicken (Gallus gallus), and pigeon (Columba livia; Fig. 2). However, no signals were detected in the vSCN. Previously, we have shown that qPer2 expression in the eye and the pineal is rhythmic, with higher levels during the day and lower levels during the night (25). Therefore, in the next experiment, the temporal change of qPer2 gene expression in the mSCN was examined. Under LD 18:6 cycles, qPer2 expression was the strongest at zeitgeber time (ZT) 4 (ZT0 corresponds to the light onset), became weak but detectable at ZT12, and then undetectable at ZT20 (Fig. 3A). This temporal change in gene expression was also observed under DD (the second day after being transferred to DD; data not shown). We have also shown that qPer2 expression in the pineal and the eye is induced by light (25). To know the effect of light on qPer2 expression in the hypothalamus, Japanese quail were exposed to a 1-h light pulse starting at CT16.
Ninety minutes after the cessation of the light exposure, qPer2 was significantly induced in the mSCN (Fig. 3B), but no signal was detected in the vSCN. If the mSCN is a circadian oscillator in the hypothalamus, it is expected that qPer2 mRNA in this nucleus is induced by light received by extraretinal photoreceptors, because EX does not abolish photic entrainment of circadian rhythms of locomotor activity in birds (10, 13, 15, 23). To study this, the eyes of Japanese quail kept under LD 12:12 cycles were covered with black rubber caps at the onset of the dark phase and then released into DD. At CT16 in the second subjective night, the birds were exposed to 1 h of light. This treatment also induced qPer2 expression in the mSCN (Fig. 3B) but not in the vSCN.

mSCN lesions. Because free-running rhythms of locomotor activity are not very robust in Japanese quail, we used pigeons in the following behavioral studies. Ablation of the mSCN was completed in 9 of 36 lesioned birds, as determined by histological examination (Fig. 4C). Of these completely lesioned birds, the locomotor activity of seven birds was recorded under LD and dimLL. The other two birds were examined only under LD. Complete bilateral mSCN lesions eliminated the free-running rhythm in dimLL. Figure 4A demonstrates one example of an activity record from a bird that became arrhythmic under dimLL. Some birds showed weak circadian rhythms, but the rhythmicity was severely disorganized. Birds with complete mSCN lesions entrained to LD cycles with a significant phase lead (Fig. 4, B and D). Intact birds showed no anticipatory activity in the D phase; however, lesioned birds started activity early in the D phase. In these lesioned birds, the vSCN was undamaged.

mSCN lesions, PX, and EX. As shown in our previous studies, pigeons with PX and EX still can entrain to LD cycles and show residual circadian rhythmicity after transfer from LD to dimLL. Because these results indicate the existence of another oscillator(s) in the avian circadian system, we next examined whether the mSCN is responsible for the residual rhythmicity in PX+EX pigeons (n = 4). Figure 5A is a representative activity record of a bird with complete elimination of the mSCN, pineal, and eye. Although mSCN lesions did not disrupt entrainment, PX and EX led to a permanent arrhythmic state in the lesioned birds. The arrhythmic activity in LD and dimLL was confirmed by periodogram analysis (Fig. 5B). In the other three birds, a similar pattern was observed.

DISCUSSION

Our results presented here provide the most compelling evidence that the mSCN is a circadian oscillator in birds. The first evidence is that clock genes are expressed in the mSCN but not in the vSCN of several avian species. Second, qPer2 gene expression in the mSCN shows circadian rhythmicity with higher levels during the day, which is the same pattern as the other avian circadian oscillators in the pineal and the eye (25). Third, light can induce qPer2 gene expression in the mSCN that can be mediated by nonvisual photoreceptors (i.e., without retinal photoreception). In the mammalian SCN, the photic induction of Per mRNA (Per1 and Per2) is thought to be required for light-induced phase shifts (7), with this characteristic being observed only in the SCN. In addition to this evidence, our behavioral analyses clearly demonstrate the significant role of the mSCN in the avian circadian system. Lesions of the mSCN resulted in a phase lead in LD cycles and arrhythmicity or disorganized rhythms under dimLL. Similar phase lead is observed in blinded or pinealectomized birds (10, 11). The residual rhythmicity in mSCN-lesioned birds disappeared both in LD and in dimLL after PX and EX, suggesting that the pineal and/or eye contribute to sustaining the circadian rhythm.

Fig. 2. Expression of Per2 in various avian species. Representative autoradiographs of Per2 signal at zeitgeber time (ZT) 4 in Japanese quail, Java sparrow, chicken, and pigeon. These results are representative of 3 independent experiments.

Fig. 3. A: temporal changes of qPer2 expression in the mSCN in Japanese quail. Birds were housed in 18:6-h light-dark (LD) cycles. ZT0 corresponds to the light onset. qPer2 expression in the mSCN is high at ZT4, low at ZT12, and undetectable at ZT20. Representative results are shown from 3 series of independent experiments with similar results. B: qPer2 expression was induced by 1 h of light in the mSCN [light(−): no light pulse; eye patch: eyes covered and light pulse; light(+) eyes open and light pulse]. Birds were exposed to a 1-h white light pulse (1,600 lx) at circadian time (CT) 16 on the second day after transfer from 12:12-h LD cycle (LD 12:12) to constant darkness (DD; 40 h after transfer to DD). In the eye-patch group, the eyes of birds were covered with black rubber caps at the offset of the last light phase in LD 12:12 and released into DD. Statistically significant differences were observed (n = 5–7, *Kruskal-Wallis, P = 0.0027, Mann-Whitney U-test, P < 0.01).
rhythmicity after mSCN lesions. In these lesioned birds, the vSCN was undamaged. In addition, we have demonstrated that vSCN lesions do not affect circadian rhythms of locomotor activity in pigeons (9). Therefore, it is reasonable to conclude that the mSCN is homologous to the mammalian SCN and functioning as a circadian oscillator in birds.

Mammalian SCN is known to be divided in a dorso-medial part and a ventrolateral part based on morphological differences (12). Although there exists interspecies variability, the main target of the RHT is the ventrolateral part of the SCN in mammals. In birds, the vSCN receives prominent but the mSCN does very small retinal projection (if any). Therefore, one can assume that the vSCN and the mSCN correspond to the ventrolateral part and the dorsomedial part of mammalian SCN, respectively. However, in both parts of mammalian SCN, circadian expression of Pers was observed, and light exposure can induce the expression of Pers in the ventrolateral part of the SCN. In contrast, no signals of qPer2 as well as other clock genes were detected in the vSCN throughout the day and after light stimulation during subjective night in birds. Therefore, it is likely that the vSCN is not functioning as an avian oscillator. It is of interest to note that in rats, the retinal projection to the lateral hypothalamic area appears at embryonic days 21-22 and develops before the projection to the SCN initiates (22). This projection might be equivalent to that in the vSCN of birds.

It is not clear how light information is conveyed to the mSCN. One possibility is that functional retinal projections to the mSCN exist that have not yet been confirmed. Another possibility is that the retinal projection to the vSCN conveys the light information to the mSCN via unidentified neural connections. However, it is clear from our results that extraretinal photoreceptors mediate light input to the mSCN, because photic induction of qPer2 was observed in birds whose eyes were covered with black rubber caps. The site of the extraretinal photoreceptor for circadian entrainment is not known, but several brain regions such as the lateral septum and the infundibulum are known to contain photoreceptive molecules (17, 24). These regions might connect with the mSCN. Alternatively, the mSCN itself might be photosensitive.

Melatonin produced in the pineal and/or the retina is involved in the control of circadian rhythms both in mammals and in birds. In mammals, melatonin recep-

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**Fig. 4.** Effects of mSCN lesions on circadian rhythms of locomotor activity in pigeon. A: the activity record of a bird that received bilateral mSCN lesions in constant dim light (dimLL). B: the activity record of a bird that received bilateral mSCN lesions in LD 12:12. C: top shows a photomicrograph of an mSCN from an intact bird, and bottom shows that from a lesioned bird. D: activity profiles of intact and lesioned birds in LD cycles. Top shows an intact bird, and bottom shows a lesioned bird. Group means of locomotor activity (±SE) for each hour are shown (n = 9 in both group). Each value was the mean from 10 days. The value of each hour is shown as the percentage of total activity for 24 h. Symbols indicate significantly higher values [Student’s t-test (*P < 0.05, **P < 0.01)].

**Fig. 5.** Activity pattern of a bird with mSCN lesions, pinealectomy, and blinding. A: the locomotor activity record of a bird that received bilateral mSCN lesions, pinealectomy, and blinding in LD 12:12 and dimLL. The vertical lines to the left of the actogram indicate the period that was subjected to periodogram analysis. B: the figures show the results of the periodogram analysis in LD 12:12 (top) and dimLL (bottom).
tors are found in the SCN. However, at present, no consistent data demonstrating melatonin receptors in the mSCN are available (16, 21). Although the presence of melatonin receptors in the mSCN remains to be determined, it is important to know how melatonin is involved in the regulation of circadian rhythms in the mSCN.

**Perspectives**

In birds, “neuroendocrine loop model” has been proposed for circadian systems. This model assumes that the system is composed of multiple oscillators in the pineal, the vSCN, and the eye (2, 5). These components are damped oscillators, thus they must receive circadian input from other components to sustain long-term stability of circadian rhythms. This model is based on the assumption that the vSCN is a circadian oscillator. However, our molecular and behavioral analyses could not support the idea that the vSCN functions as a circadian oscillator. Thus we must reexamine the neuroendocrine loop model, which may represent the common mechanism of the vertebrate circadian system.

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