Regulation of Pineal Rhythms in Chickens: Effects of Blinding, Constant Light, Constant Dark, and Superior Cervical Ganglionectomy

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ABSTRACT. Pineal serotonin N-acetyltransferase activity and melatonin content exhibit marked daily changes in chickens; peak values occur during the period of low locomotor activity which coincides with dark in a 24-hour light-dark cycle. The photic and neural regulation of these daily changes were studied by measuring pineal serotonin N-acetyltransferase activity, hydroxyindole-O-methyltransferase (HIOMT) activity, and melatonin content in experiments in which chickens were subjected to light-dark cycles, constant light, and constant dark and were surgically blinded or superior cervical ganglionectomized. It was found that: 1) The daily changes in N-acetyltransferase activity and melatonin content appear to persist in constant dark, and they disappear in constant light. 2) The eyes are not necessary for photic control of the daily changes, and the effect of constant light on N-acetyltransferase activity and melatonin content may be non-visual, that is, the eyes not being necessary. 3) The occurrence of the daily change in N-acetyltransferase activity and melatonin content does not require the superior cervical ganglia; the persistence of the changes in constant dark, however, may require the ganglia. 4) HIOMT activity was lower in constant light than in light-dark cycles and lower still in constant dark than in constant light. Neither the presence of the eyes nor the superior cervical ganglia affected HIOMT activity, as previously reported. (*Endocrinology* **97**: 1373, 1975)

DAILY rhythms have been demonstrated in the pineal glands of birds and mammals. Three to five-fold changes occur in avian pineal serotonin content (1,2), serotonin N-acetyltransferase activity (3), and melatonin content (3,4). It is probable that the rhythm in melatonin content is a direct consequence of a rhythm in pineal N-acetyltransferase activity. In rats (5) and chickens (3) pineal N-acetyltransferase activity and melatonin content exhibit peaks during the dark portion of a light-dark cycle. At the comparable time pineal serotonin content is at its nadir in rats (6), pigeons (1), and quail (2) though this is not so in chickens (7).

In this paper we report work with chickens in which three pineal variables are measured: N-acetyltransferase activity, hydroxyindole-O-methyltransferase (HIOMT) activity, and melatonin content. The experiments were designed to provide further information about the photic and rhythmic regulation of the production of melatonin.

In our previous work (3,8,9) we concluded that the daily changes in pineal N-acetyltransferase activity and melatonin content had properties of circadian rhythms: N-acetyltransferase activity and melatonin content rhythms had a refractory period during which dark treatment did not result in an increase in enzyme activity (8,10); furthermore, N-acetyltransferase activity rhythms persisted in constant dark with a period length close to 24 hours (9). In the experiments reported here chickens were subjected to constant light (LL) and constant

Received May 2, 1975.

S. Binkley was supported by NIH postdoctoral fellowship FO2 HD52858-01; S. MacBride was supported by NDEA predoctoral fellowship 70-03174.1; and the work was supported by NIH grant NS-08554 to C. Ralph and NSF grant GB-43215 to S. Binkley.

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dark (DD) to see whether the pineal enzyme activities and melatonin content responded as other circadian rhythms do (which often disappear in LL and persist in DD).

Furthermore, in our previous report (8) we concluded that the eyes were not necessary for the rapid decreases in pineal N-acetyltransferase activity and melatonin content which occur if animals are subjected to unexpected light during the dark period. In the experiments reported here, we again studied the possibility of non-visual light perception in the pineal melatonin pathway using responses involving light-dark (LD) and LL.

Lastly, our previous experiments had shown that chickens, unlike rats (10–12), are unresponsive to isoproterenol and norepinephrine; that is, isoproterenol will increase pineal N-acetyltransferase activity in rats *in vivo*, and norepinephrine will do the same *in vitro*, but these compounds do not have the same effect on chickens (9,13). In rats, noradrenergic regulation of the pineal gland takes place via the superior cervical ganglion since removal of the ganglia prevents rhythmicity (12). Thus, in the experiments reported here, we removed the superior cervical ganglia from chickens and studied their pineal rhythms in LD and DD to ascertain whether the ganglia are necessary for regulation of the daily changes.

Materials and Methods

Animals

Day-old chicks (White Leghorn cockerels, *Gallus domesticus*) were obtained from T. Mack and Sons (West Sunbury, Pa.) and were maintained in a LD12:12 lighting regime (lights on, 0600 h EST) except as described below under specific protocols. Food and water were provided *ad libitum*. The chickens were housed in the topmost tier of cages (wire mesh [2.5 × 10 cm] cages in individual compartments [50 × 30 × 43 cm]). The cages were located directly beneath a fluorescent ceiling lamp (40-watt cool-white Westinghouse F40CW; 125–135 foot-candle intensity at the cage tops measured with a Weston photometer model 756).

Killing procedure

Chickens from different treatment groups were killed in groups of 6 in the light or dark by decapitation. Visibility for decapitation in the dark was facilitated by 4-second exposure of the animals to 0.2 foot candles of light from a 7½ watt red lamp (General Electric). Following decapitation, the heads were placed immediately on ice, and the pineal glands were quickly dissected and individually frozen on Dry Ice in coded plastic vials. The vials were shipped on Dry Ice the following day from Pittsburgh to Bethesda where the enzyme assays were performed by persons without prior knowledge of the details of the experiment. After the enzyme assays had been done, samples of the pineal homogenates were diluted and returned to Pittsburgh on Dry Ice where they were assayed for melatonin within a month of sacrifice.

Assays

Enzyme and melatonin assays were done on portions of homogenates of the pineal glands. Each individual gland homogenate was assayed for three variables: 1/10 of each gland homogenate was measured for N-acetyltransferase activity (3,14); 1/20 of each gland homogenate was measured for HIOMT activity (3,15); and 4/10 of each gland homogenate was used to determine melatonin content (3,16).

Surgery

Blinding was done surgically under ether anesthesia by bilateral orbital enucleation. One eye was removed on the 14th day and the other on the 16th day of age (17).

Bilateral superior cervical ganglionectomy and a sham operation were performed in chicks 2–4 days old anesthetized by intraperitoneal injection of Combuthal (thiopental sodium-pentobarbital sodium, Abbott Laboratories). Ganglionectomy resulted in loss of fluorescent fibers in the pineal, eyelid muscle, and meninges, as demonstrated in sections prepared for us by L. Hedlund (19) by the Falck-Owman method (18).

Locomotor activity

For experiments involving constant dark, locomotor activity was individually recorded by means of an apparatus like that described elsewhere (4,20). It has been shown that pineal

rhythms persist in darkness, in phase with the circadian locomotor activity rhythm. Chickens were killed at the midpoints of locomotor activity versus rest, judged by the locomotor activity recordings after 2 weeks.

Protocol

Other details of methods and protocol are specified in the section on results and in the figure captions.

Results

Effects on blinding on chickens kept in LD12:12 (Fig. 1)

Chickens kept for 6 weeks in LD12:12 (after blinding at 2 weeks of age) did not differ in melatonin content or N-acetyltransferase activity from normal animals, when mid-light was compared with mid-dark. HIOMT activity was lower in one of the experiments with blind chickens, but not in the duplicate experiment.

Effects on blinding on chickens kept in LL (Fig. 2)

Following 6 weeks of LD12:12 chickens (normal and blind from 2 weeks of age) were placed in constant light (LL) for two weeks after which they were killed and their pineal glands were assayed at four time points throughout the day. Constant light obliterated the changes in melatonin and N-acetyltransferase activity seen in LD12:12-treated chickens. Blinding did not prevent this effect of LL. LL lowered the values for HIOMT activity.

Effects of superior cervical ganglionectomy on chickens kept in LD12:12 (Fig. 3)

Chickens, superior-cervical-ganglionectomized at 2 weeks of age, and kept for 6 weeks in LD12:12 did not differ from normal or sham-operated ones in melatonin content or N-acetyltransferase activity when mid-light was compared with mid-dark. HIOMT activity was lower in one of the experiments with sham-ganglionectomized chickens, but not in the duplicate experiment.

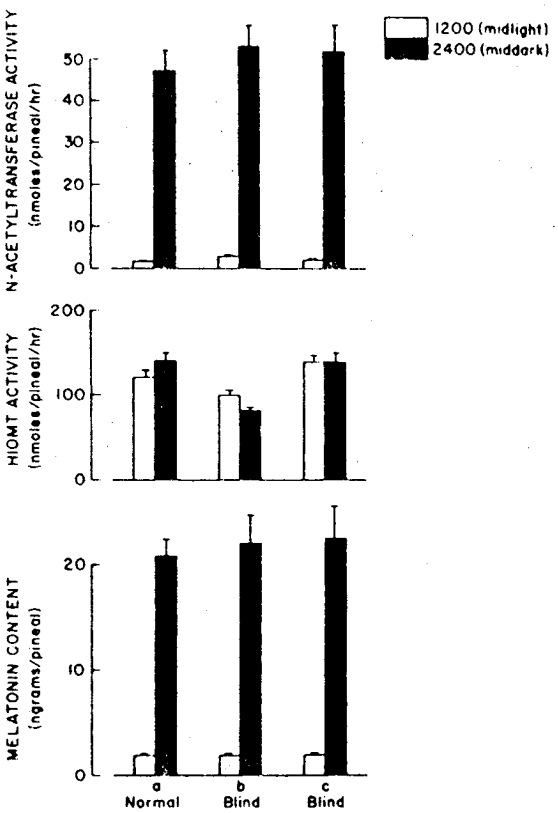

FIG. 1. Effects of blinding on chickens in LD12:12. Chickens were kept in LD12:12 to 8 weeks of age. Blinded birds were operated on at 2 weeks of age and kept in a separate experimental room on the same light-dark cycle as the unoperated (normal) chickens. At 8 weeks of age the chickens were killed at mid-light and mid-dark and their pineal glands were assayed for enzyme activities and melatonin content ($P < .001$, mid-light versus mid-dark). Data are expressed as the mean for 6 chickens + one standard error. Duplicate experiments for blind birds are shown.

Effects of superior cervical ganglionectomy on chickens kept in DD (Fig. 3)

In other experiments, chickens were superior cervical ganglionectomized or sham-operated on days 2–4 of age. These birds were raised for 4 weeks in LD12:12 followed by DD for 2 weeks before killing at 6 weeks of age. They were killed during activity or rest periods, as judged

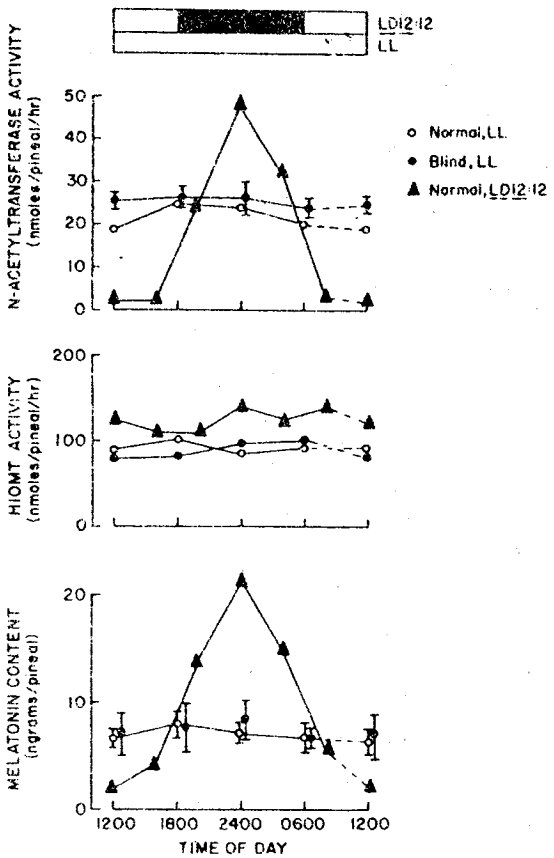

FIG. 2 Effects of blinding on chickens in LL. Chickens were kept in LD12:12 for 6 weeks followed by 2 weeks of constant light (LL). Half of the chickens were blinded at 2 weeks of age and transferred to a separate experimental room with the same lighting as for the unoperated (normal) chickens. At 8 weeks of age the chickens were killed at four times during a single 24-hour period and their pineal glands were assayed for enzyme activities and melatonin content. Data are expressed as the mean for 6 chickens ± one standard error. Error bars are not shown where they did not exceed the size of the plotted symbols. The data (triangles) for the light-dark cycle (diagrammed at the top of the figure) are from Binkley *et al.* (3) and are shown for comparison without their standard errors. The points connected by dashed lines are duplicate 1200 h points. The bars at the top of the figure represent the lighting schedule.

from records of their locomotor activity. As is typical in continuous darkness (20), the circadian variations in enzyme activity and melatonin content were damped. Ganglionectomy in this experiment appears to have obliterated the difference seen between activity and rest in the pineal melatonin and N-acetyltransferase activity.

Comparisons of overall levels of enzyme activities and melatonin content

Constant dark (Fig. 3) reduced the overall levels of N-acetyltransferase activity, HIOMT activity, and melatonin content in the pineal gland compared with treatment with LD12:12 (Fig. 1).

Constant light treatment (Fig. 2) produced values for pineal N-acetyltransferase activity and melatonin content that were intermediate between light-time values and dark-time values in LD12:12. Constant light lowered HIOMT activity compared with LD12:12 but not as far as did DD (Fig. 3).

Discussion

The following conclusions can be made concerning changes in N-acetyltransferase activity and melatonin content in the pineal glands of chickens:

(a) In DD, both are greater in the phase of higher locomotor activity. The amplitude of this change is less than in LD12:12, and the absolute amounts of enzyme activity and melatonin content are less than those observed in LD12:12.

(b) In LL daily changes are not apparent. The absolute amounts of N-acetyltransferase activity and melatonin content are intermediate between the amounts observed at mid-dark and mid-light in LD12:12.

(c) Blinding does not block the response to LL nor does it change the mid-light and mid-dark values in LD12:12.

(d) Superior cervical ganglionectomy deprives the pineal gland in chickens of its sympathetic innervation (as demonstrated by loss of fluorescence in Falck-Owman preparations [19]), but does not change the mid-light versus mid-dark values in LD12:12.

(e) Superior cervical ganglionectomy does result in the loss of the activity-rest difference in constant dark.

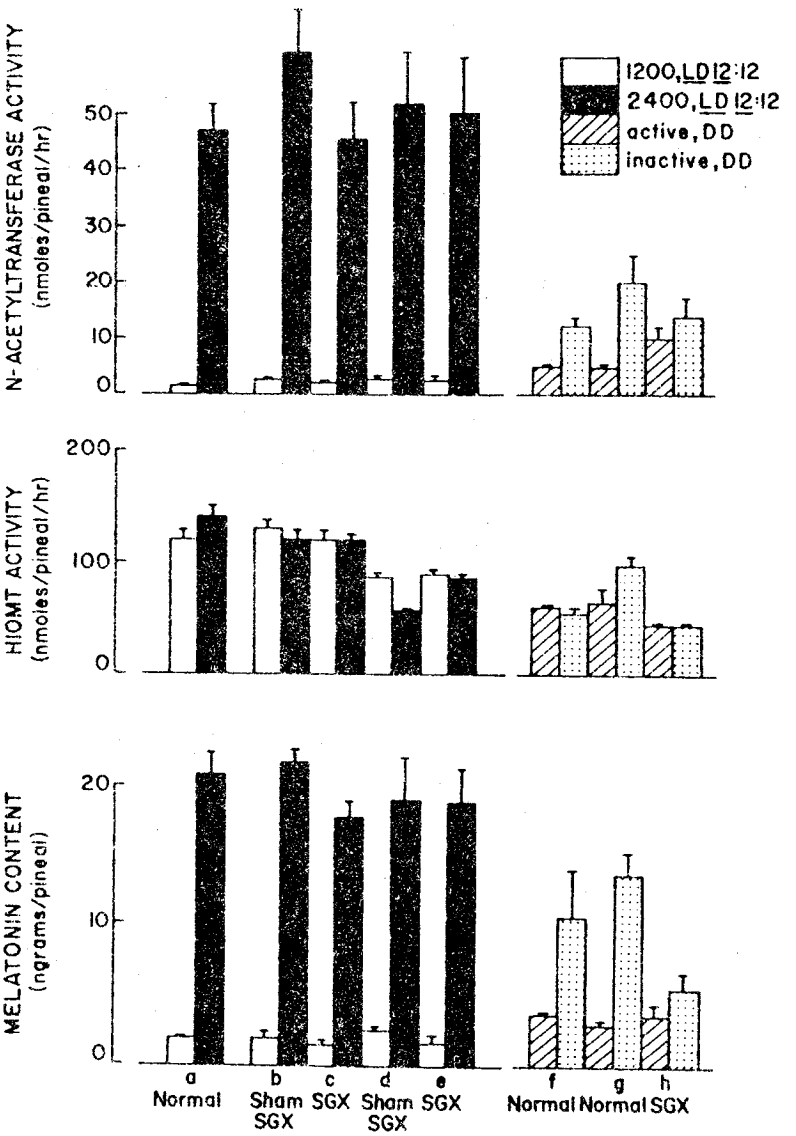

FIG. 3. Effects of superior cervical ganglionectomy on chickens in LD12:12 or DD. At 2–4 days of age, chickens were ganglionectomized (SGX), sham-operated, or left unoperated (normal). Those kept in LD12:12 until they were 6 or 8 weeks of age were killed at mid-light or mid-dark of the LD12:12 cycle. Melatonin content and enzyme activities of their pineal glands were measured. Chickens raised for 4 weeks in LD12:12 and then kept in constant dark (DD) for 2 weeks were killed during the midpoints of their locomotor activity or rest time and their pineal glands were assayed. The data are expressed as means for 6 chickens + one standard error. The data show no statistical effect of ganglionectomy in LD12:12 and a possible effect of ganglionectomy in DD. Inactive (normal) chickens had higher N-acetyltransferase activity and melatonin content than active ones ($P < .01$ and $< .001$, respectively). HIOMT activity was lower in DD than in LD12:12 ($P < .01$). Chickens in a, b, c, f, g were 8 weeks old; chickens in d, e, h were 6 weeks old. Replicate experiments are shown for ganglionectomy in LD12:12 and for normal chickens in DD.

These conclusions substantiate our previous assertion that the daily changes in melatonin content and N-acetyltransferase activity in the pineal gland are manifestations of true circadian rhythms (3,5,9). Sparrow locomotor activity rhythms show similar responses: persistence in DD, synchrony by LD cycles, lowered amplitude in DD, obliteration in LL.

Non-visual light perception is indicated by our experiments. Non-visual light perception has also been found in behavioral studies of rhythms in birds (21,17; sparrows and chickens) but this is the first time that non-visual light perception has been suggested for the absence of a rhythm in LL. A possible slight difference can be seen between the blind and normal birds in LL—while both groups lose rhythmicity, the blind birds are more variable and had slightly greater values for N-acetyltransferase activity in LL. If this small difference is real, it means that the eyes have some role in the response to LL. We have not rigorously eliminated the possibility that the chickens are detecting heat rather than light from the fluorescent lamps.

The superior cervical ganglia may only

be involved in the regulation of the apparent endogenous rhythm (persistence in DD of difference between activity and rest). This result is important because it means that the DD persistent change may be mediated by an anatomical route other than that used to control changes in LD12:12. The finding of no effect of ganglionectomy in LD12:12 is consistent with the finding of lack of beta-adrenergic stimulation of pineal N-acetyltransferase activity in chickens (8).

The non-visual light perception and lack of superior cervical ganglion involvement in the response to LD12:12 are two aspects of the chicken pineal regulation that differ from rats; in rats the responses of pineal N-acetyltransferase activity require the eyes and the superior cervical ganglia (5,12).

HIOMT activity appears in our experiments to be lowered in LL compared with LD12:12; this conflicts with the finding of Axelrod *et al.* (22) who reported an increase; however, their experiment lasted for 5 days of LL and ours lasted for 14 days of LL which may account for the discrepancy in results. We also found lower HIOMT activity in DD than LL, and HIOMT activity was not affected by blinding or ganglionectomy, which agrees with Lauber *et al.* (23). We have not consistently found that HIOMT differs in light versus dark in LD12:12 (3, Figs. 1b, 3d, 3g), which disagrees with Pelham and Ralph (15) and which we have discussed previously (8).

The results of the studies support the previous postulate (3,5) that the rhythmic changes in melatonin content of the pineal gland are regulated primarily by changes in the enzyme N-acetyltransferase. The changes in melatonin content are seen in serum as well and are positively correlated (20) with changes in the pineal gland. Thus, the amounts of melatonin found in serum appear to be regulated by pineal N-acetyltransferase activity. The precise physiological importance of rhythms in circulating melatonin has yet to be determined. In view of the occurrence of similar rhythms in birds and mammals (3,5,13), which are coordinated primarily with the environmental lighting rather than behavioral activity, the physiological function of melatonin is likely to involve measurement of light and dark periods, or photoperiodism.

Acknowledgments

We thank Karla MacBride, Patrick Fruscello, Diane Reilly, Kathy Hall, and Emily Feinberg.

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