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Control of Circadian Change of Serotonin *N*-Acetyltransferase Activity in the Pineal Organ by the β-Adrenergic Receptor

(diurnal/neural regulation/norepinephrine)

TAKEO DEGUCHI AND JULIUS AXELROD

Laboratory of Clinical Science, National Institute of Mental Health, Bethesda, Maryland 20014

Contributed by Julius Axelrod, July 7, 1972

ABSTRACT **Serotonin *N*-acetyltransferase (EC 2.3.1.5) activity in the rat pineal organ is enhanced 50-fold at night. Rats exposed to light at night or kept in darkness during the daytime do not show any elevation of enzyme activity. Treatment with reserpine, a compound that depletes norepinephrine from nerves, l-propranolol, a β-adrenergic blocking agent, or cycloheximide, an inhibitor of protein synthesis, abolishes the nocturnal increase in serotonin *N*-acetyltransferase activity, indicating that the enzyme activity is modulated by neural release of norepinephrine from sympathetic nerves via β-adrenergic receptors, and that the increase in enzyme activity is due to synthesis of new enzyme molecules. When rats are exposed to light at night or injected with l-propranolol, there is a precipitous fall in serotonin *N*-acetyltransferase activity (half-life 5 min). Cycloheximide administered at night results in a slow fall in enzyme activity (half-life 60 min). When rats are kept in darkness and then exposed to light for 10 min, L-isoproterenol rapidly initiates the elevation of serotonin *N*-acetyltransferase activity to the initial level in 60 min. On the other hand, when the rats are kept in continuous light, L-isoproterenol initiates an increase in serotonin *N*-acetyltransferase activity after a lag phase of 60 min. The results indicate that there are two types of changes in serotonin *N*-acetyltransferase activity; a rapid increase and decrease mediated by the β-adrenergic receptor, and a slow increase and decrease in enzyme activity that appears to represent the turnover of the enzyme.**

Serotonin content (1), melatonin content (2), and activities of melatonin-synthesizing enzymes, hydroxyindole *O*-methyltransferase (EC 2.1.1.4) (3), and serotonin *N*-acetyltransferase (EC 2.3.1.5) (4) in the rat pineal organ show circadian changes mediated by sympathetic nerves whose cell bodies are in the superior cervical ganglion. The diurnal rhythms of serotonin and melatonin have been postulated to be driven by *N*-acetyltransferase activity (4).

In pineal-organ culture, the synthesis of melatonin from tryptophan is markedly stimulated by norepinephrine, monoamine oxidase inhibitors, and dibutyryl adenosine 3′:5′-cyclic monophosphate (5, 6). Norepinephrine or dibutyryl adenosine 3′:5′-cyclic monophosphate induces *N*-acetyltransferase in organ culture of rat pineal organ (7). Recent studies have shown that 3-(3,4-dihydroxyphenyl)-L-alanine (L-DOPA), catecholamines, or monoamine oxidase inhibitors induce *N*-acetyltransferase activity in rat pineal organ *in vivo* that is blocked by prior treatment with a β-adrenergic blocking agent (8). Electrical stimulation of the sympathetic nerve that innervates the rat pineal organ also causes an increase in *N*-acetyltransferase activity (9). These observations suggest that the neurotransmitter released from sympathetic nerves regulates the activity of *N*-acetyltransferase in the pineal organ by stimulating the adrenergic β-receptor and adenosine 3′:5′-cyclic monophosphate (cyclic AMP), and that the release of norepinephrine is modulated by environmental light and darkness.

Circadian variations of norepinephrine content (10), cyclic AMP content (11), and *N*-acetyltransferase activity (4), however, are unrelated to each other. The norepinephrine rhythm in the pineal organ is exogenous (10), while that of *N*-acetyltransferase is endogenous (4). Cyclic AMP concentration is 6-fold higher in light than in darkness (11), which is 180° out of phase with the rhythm of *N*-acetyltransferase activity. These discrepant reports motivated us to study the mechanism of diurnal change of *N*-acetyltransferase activity in rat pineal organs. We show that the daily increase and decrease of *N*-acetyltransferase activity is regulated by norepinephrine via the β-adrenergic receptor, and that there are two types of changes in *N*-acetyltransferase activity; a rapid change mediated by the β-adrenergic receptor and a slow change presumably due to the turnover of enzyme protein.

MATERIALS AND METHODS

Drugs. [1-^{14}C]Acetyl coenzyme A (49.8 Ci/mol) was purchased from New England Nuclear Corp., Boston, Mass. l-Propranolol was kindly supplied by Ayerst Laboratories, New York, N.Y. Phenoxybenzamine was donated by Smith, Kline, and French Laboratories, Philadelphia, Pa. Other chemicals were obtained from commercial sources.

Animals. Osborne–Mendel female rats weighing 180–200 g were supplied by the National Institutes of Health and were kept under diurnal lighting conditions with light on from 6 a.m. to 6 p.m. for at least 5 days after they were supplied. When indicated, rats were transferred to a plastic cage covered with glass plate and brought out of darkness at 10 p.m. and exposed to fluorescent light 110–150 ft-candles. All drugs were dissolved in 0.9% NaCl and injected subcutaneously or into the tail vein of rats, as will be described for each experiment. Groups of five rats were used in each experiment. Bilateral ganglionectomy or decentralization of the superior cervical ganglion was performed under ether anesthesia. Ptosis was used to monitor the success of the operation. The operated rats were kept under diurnal lighting conditions for 6 days.

TABLE 1. *Effect of light and darkness on N-acetyltransferase activity of the pineal organ*

Time pineal organ removed	Lighting conditions (hr)	*N*-Acetyltransferase pmol/pineal per 10 min
10 a.m.	Light 4	5 ± 1
7 p.m.	Darkness 1	24 ± 6
10 p.m.	Darkness 4	265 ± 61
10 p.m.	Light 16	6 ± 2
4 p.m.	Darkness 6	4 ± 1

Rats were kept under the lighting conditions described and killed at the time indicated. The results are expressed as mean ± standard error of the mean.

Assay of N-Acetyltransferase Activity. Rats were killed by decapitation in a dim red light (General Electric red bulb, 25 W, at a distance of 91 cm (3 ft.). *N*-Acetyltransferase activity was assayed by the method of Deguchi and Axelrod (12). A pineal organ was quickly removed, chilled, and homogenized in 70 μl of a reaction mixture containing 2.5 μmol of potassium phosphate (pH 6.5), 0.1 μmol of tryptamine, and 4 nmol of [*1*-^{14}C]acetyl coenzyme A in a small glass homogenizer. After incubation at 37°C for 10 min, the radioactive *N*-acetyltryptamine formed was extracted into toluene–isoamyl alcohol (97:3) at pH 10, and radioactivity was measured (8).

RESULTS

Effect of light or drugs on nocturnal increase in *N*-acetyltransferase

N-Acetyltransferase activity increased 50-fold after rats were kept 4 hr in darkness at 10 p.m. (Table 1). In the first hour after the onset of darkness, there was relatively little increase in enzyme activity. Thereafter, *N*-acetyltransferase activity gradually increased to reach the maximum level 3 hr after the onset of darkness. The rats kept in continuous lighting until 10 p.m. or rats kept in darkness for 6 hr during daytime (from 10 a.m. to 4 p.m.) showed no elevation of *N*-acetyltransferase activity. This result indicates that both darkness and the proper setting of an internal clock are necessary for the nocturnal increase in *N*-acetyltransferase activity in rat pineal organs.

Ganglionectomy or decentralization of the superior cervical ganglion completely blocked the nighttime increase in *N*-acetyltransferase activity (Table 2), in confirmation of the finding by Klein *et al.* (13). Reserpine, a compound that depletes both catecholamines and serotonin in nerves, 1-propranolol, a β-adrenergic blocking agent, or cycloheximide injected in rats immediately before the onset of darkness blocked the nighttime increase in enzyme activity, whereas treatment with phenoxybenzamine, an α-adrenergic blocking agent, or actinomycin D, inhibitor of RNA synthesis, had no effect. *p*-Chlorophenylalanine, a drug that depletes serotonin in brain and pineal (14, 15), did not prevent the nighttime increase of enzyme activity. These observations indicate that the nocturnal increase in *N*-acetyltransferase activity is induced by norepinephrine released from sympathetic nerves via β-adrenergic receptor, and that the increase in enzyme activity is due to synthesis of new enzyme molecules.

Rapid and slow decrease in *N*-acetyltransferase activity

When the rats were brought out of darkness to light at 10 p.m., *N*-acetyltransferase activity disappeared very rapidly (Fig. 1). In the first 5 min, there was almost no change in enzyme activity. In the second 5 min, however, *N*-acetyltransferase activity rapidly decreased to less than 10% of the initial activity and remained at this level as long as the rats were kept in the light. A similar observation was reported by Klein that light causes a rapid disappearance of enzyme activity with a halving time of 3.5 min (16). When the rats were returned to darkness after 10 min in the light, *N*-acetyltransferase activity gradually returned to the initial level after 3 hr.

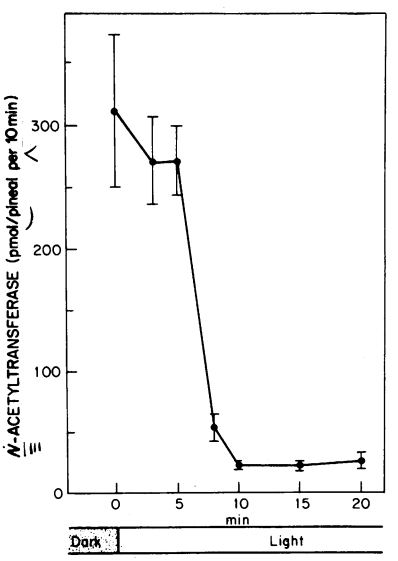

FIG. 1. Light-induced decrease in *N*-acetyltransferase activity. Rats were brought out of darkness into light at 10 p.m. *Vertical bar* in each case indicates standard error of the mean.

TABLE 2. *Effect of various treatments on blocking of the nocturnal increase in pineal N-acetyltransferase*

Treatment	*N*-Acetyltransferase pmol/pineal per 10 min
None	429 ± 70
Ganglionectomy	12 ± 2
Decentralization	5 ± 1
Reserpine (2.5 mg/kg)	3 ± 1
1-Propranolol (20 mg/kg)	15 ± 4
Phenoxybenzamine (20 mg/kg)	532 ± 54
Cycloheximide (20 mg/kg)	2 ± 1
Actinomycin D (1 mg/kg)	337 ± 25
p-Chlorophenylalanine (300 mg/kg)	469 ± 76

Drugs were injected subcutaneously in rats 5 to 10 min before the onset of darkness (6 p.m.), at the doses indicated. *p*-Chlorophenylalanine was injected 24 hr and 10 min before the onset of darkness. Rats were killed at 10 p.m. Results are expressed as mean ± standard error of the mean.

Various drugs were tested for their capability of preventing the light-induced decrease in *N*-acetyltransferase activity. L-Isoproterenol injected intravenously before the rats were exposed to light completely prevented the light-induced decrease in enzyme activity (Table 3).

Various drugs were injected in rats under a dim red light at 10 p.m. and thereafter the rats were kept in darkness. 1-Propranolol, a β-adrenergic blocking agent, rapidly caused the decrease of *N*-acetyltransferase activity to less than 15% of the initial activity within 10 min (Fig. 2), a similar pattern to that induced by light (Fig. 1). Cycloheximide administered at a dose that immediately inhibits protein synthesis *in vivo* (17) gradually causes the decrease of *N*-acetyltransferase activity with a halving time of 60 min (Fig. 2). This result indicates that there are two types of inactivation processes for the enzyme, a rapid decrease induced by light or β-adrenergic blocking agent and a slow decrease induced by inhibition of protein synthesis.

Immediate and delayed increase of *N*-acetyltransferase activity

Rats were kept in darkness from 6 p.m. to 10 p.m., exposed to light for 10 min, then injected with L-isoproterenol. *N*-Acetyltransferase activity was rapidly increased to the initial level 60 min after injection of L-isoproterenol and remained at this level for at least 2 hr (Fig. 3). On the other hand, when the rats were kept under light for 16 hr until 10 p.m., and then injected with L-isoproterenol, there was almost no increase in *N*-acetyltransferase activity for 60 min. Between 1 and 3 hr after injection of L-isoproterenol, *N*-acetyltransferase activity gradually increased. Cycloheximide administered just before isoproterenol blocked both immediate and delayed increases of the enzyme activity that is induced by L-isoproterenol.

DISCUSSION

Our experiments show that the nocturnal increase in *N*-acetyltransferase activity of the pineal organ is caused by the absence of light, which stimulates the release of norepinephrine from sympathetic nerves. If the rats are placed in darkness before 6 p.m., there is no elevation of enzyme activity, indicating that there is an endogenous clock that controls the neuronally mediated increase in *N*-acetyltransferase activity. The nighttime elevation of enzyme activity is prevented by ganglionectomy or decentralization of the superior cervical ganglion (13) and is also blocked by reserpine, 1-propranolol, or cycloheximide. These observations indicate that the nocturnal increase in *N*-acetyltransferase activity is induced by release of the neurotransmitter norepinephrine, which activates the β-adrenergic receptors on the pineal-organ cell, which in turn initiates synthesis of the enzyme molecules, presumably by the adenyl cyclase system.

TABLE 3. *Blockade of light-induced decrease in pineal N-acetyltransferase by* L-*isoproterenol*

Time of exposure to light (min)	*N*-Acetyltransferase pmol/pineal per 10 min	
	No treatment	L-Isoproterenol
0	360 ± 29	350 ± 74
10	29 ± 7	342 ± 58
30	28 ± 3	384 ± 76

At 10 p.m., the whole body of the rat except tail was covered with a bag made of thick black cloth under red dim light, and L-isoproterenol (15 mg/kg) was rapidly injected into the tail vein under a surgery lamp. Immediately after injection the surgery lamp was turned off and the rats were quickly returned to darkness. After 5–10 min in darkness, the rats were exposed to light for 10 or 30 min. The results are expressed as mean ± standard error of the mean.

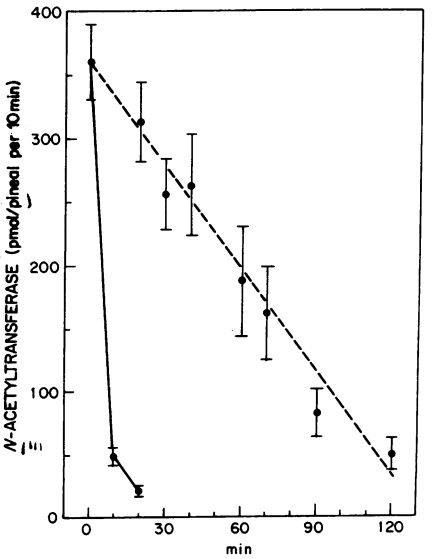

FIG. 2. Effect of 1-propranolol or cycloheximide on *N*-acetyltransferase activity in darkness. Rats were kept in darkness from 6 p.m. to 10 p.m. and received subcutaneous injection of either 1-propranolol (20 mg/kg) or cycloheximide (20 mg/kg) under a dim red light, then immediately returned to darkness until they were killed. (——), 1-propranolol; (– – –), cycloheximide. *Vertical bar* indicates standard error of the mean.

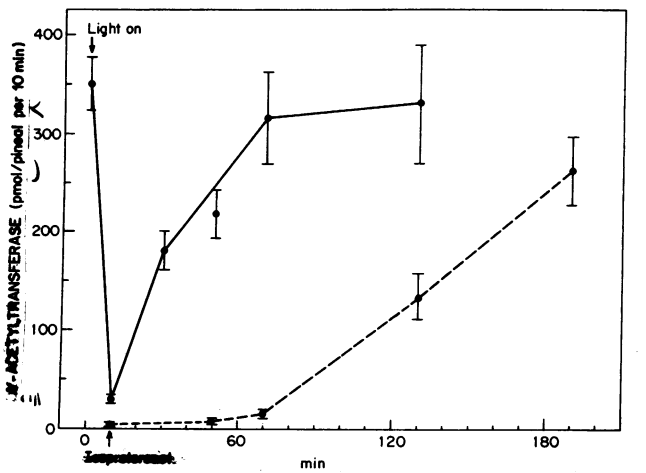

FIG. 3. Effect of L-isoproterenol on *N*-acetyltransferase activity. (——), Rats were kept in darkness from 6 p.m. to 10 p.m. After 10 min under the light, rats received intravenous injections of L-isoproterenol (15 mg/kg) and were kept in the light until killed. (– – –), Rats were kept in light continuously for 16 hr. At 10 p.m., they received an intravenous injection of L-isoproterenol and were killed at various time intervals thereafter. The *vertical bar* indicates standard error of the mean of *N*-acetyltransferase activity.

Exogenously administered catecholamines can induce *N*-acetyltransferase during daytime (8), indicating that the low *N*-acetyltransferase activity during daytime is not due to the lack of responsiveness of β-adrenergic receptor, but is due to the lack of or reduced release of the neurotransmitter. When the rats were kept in darkness during daytime, there appeared to be no release of neurotransmitter, since *N*-acetyltransferase activity was not elevated. The release of neurotransmitter from the sympathetic nerve in the pineal organ is probably under the dual control of light "Zeitgeber" and an endogenous clock.

The *N*-acetyltransferase activity decreases very rapidly when the rats are brought out of darkness into light. The observations that prior treatment with L-isoproterenol prevents the light-induced decrease of *N*-acetyltransferase activity and that 1-propranolol, a β-adrenergic blocker, mimics the effect of light suggest that maintenance of the elevated activity of *N*-acetyltransferase requires the continuous activation of the β-receptor by norepinephrine, and light immediately interrupts the release of the neurotransmitter. Taylor and Wilson have also shown that electrical activity in the rat pineal organ in darkness is markedly depressed by a pulse of light (18).

The sharp decrease in *N*-acetyltransferase activity resulting from either blockade of β-adrenergic receptor or light exposure and the much slower decrease in enzyme activity when cycloheximide is administered suggest that there are two mechanisms for inactivating the enzyme. The slower decrease of enzyme activity after inhibition of protein synthesis presumably represents turnover of the enzyme. The rapid decrease might be due to conversion of an active to inactive form of the enzyme or to a disaggregation of subunits of the enzyme molecule.

There are also two patterns in which *N*-acetyltransferase activity is elevated. When the rats are kept in continuous light up to 10 p.m., L-isoproterenol causes an increase in *N*-acetyltransferase activity only after a lag period of about 60 min. On the other hand, when the rats are kept in darkness until the enzyme reaches its maximum level at 10 p.m. and then are exposed to light for 10 min, L-isoproterenol increases *N*-acetyltransferase activity without any lag period. The delayed elevation of enzyme activity after exposure to long periods of light might represent *de novo* synthesis of new enzyme molecule. The immediate elevation of *N*-acetyltransferase activity might be explained by the following mechanisms: (*a*) an intermediate compound necessary for the synthesis of *N*-acetyltransferase has already been made in the rats kept in darkness; thus, the synthesis of the enzyme is initiated without a lag phase; (*b*) the rapid elevation might be due to the conversion of an inactive to an active form of the enzyme or to an aggregation of subunits of the enzyme molecule. Although the observation that cycloheximide blocks the rapid elevation of enzyme activity does not support this possibility, this inhibitor might have unknown effects on the changes in the enzyme molecule. Recently, Grossman and Boctor postulated that hepatic tyrosine aminotransferase is initially inactivated to an inactive form that can be reactivated, and then is further degraded to an irreversibly inactive form (19). The rapid elevation observed with *N*-acetyltransferase of the pineal organ might be due to a similar mechanism.

N-acetyltransferase in the rat pineal organ appears to be a productive model for the study of neuronal regulation of the metabolism of endorgans. It also could serve as an ideal enzyme for studies on how the synthesis, degradation, activation, and inactivation of protein molecules is modulated by environmental stimuli and excitable membranes.

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