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Control of Pineal Indole Biosynthesis by Changes in Sympathetic Tone Caused by Factors Other Than Environmental Lighting

(serotonin/melatonin/norepinephrine/insulin)

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ABSTRACT **The melatonin (5-methoxy-*N*-acetylserotonin) content and *N*-acetyltransferase activity of rat pineal increase rapidly in response to physical immobilization or insulin-induced hypoglycemia. Carbohydrate consumption, which causes insulin release without hypoglycemia, does not elicit these pineal responses. Prior treatment with propranolol, a β-adrenergic blocking agent, inhibits the *N*-acetyltransferase responses to hypoglycemia and immobilization, indicating that these changes result from stimulation of pineal β-receptors by a catecholamine, presumably norepinephrine released from pineal sympathetic nerve terminals. Prior destruction of those terminals with 6-hydroxydopamine does not block, but actually potentiates, the increase in melatonin content and *N*-acetyltransferase activity after induced hypoglycemia or immobilization. This finding probably reflects an action of circulating catecholamines, secreted from the adrenal medullae or surviving sympathetic nerve terminals, on supersensitive pineal cells. These observations indicate that factors other than changes in environmental lighting, which modify sympathetic nervous tone, can also influence pineal function.**

Environmental lighting, acting by way of the retinae (1), the inferior accessory optic tracts (2), and postganglionic sympathetic neurons arising from the superior cervical ganglia (1), constitutes probably the major factor controlling the rate at which mammalian pineal organ synthesizes the methoxyindole, melatonin. Exposure to light inhibits pineal sympathetic tone in rats (3), causing a decline in the activities of the pineal enzymes hydroxyindole-*O*-methyltransferase (EC 2.1.1.4) (4) and serotonin-*N*-acetyltransferase (EC 2.3.1.5) (5), and in the concentration of melatonin (6). Pineal serotonin (7) and norepinephrine (8) concentrations also exhibit light-dependent daily rhythms. In darkness, sympathetic nerve terminals within the pineal presumably liberate more norepinephrine; this catecholamine then interacts with pinealocyte β-receptors (9), increasing adenylate cyclase activity (10), enhancing the formation of cyclic 3′:5′-AMP, and thereby accelerating melatonin biosynthesis (11).

Although environmental lighting constitutes the major factor controlling pineal sympathetic tone and indole biosynthesis, it apparently is not the only factor, inasmuch as significant daily rhythms in hydroxyindole-*O*-methyltransferase (12) and *N*-acetyltransferase (5) activities and melatonin content (13) persist in the pineals of blinded rats and animals kept under constant darkness.

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Studies described in this report show that, in addition to environmental lighting, other experimental manipulations known to modify sympathetic tone elsewhere in the body can also modify pineal indole biosynthesis. Comparable alterations in sympathetic tone that occur in response to activity or feeding cycles may be instrumental in generating the pineal rhythms that persist in the absence of light–dark cycles.

MATERIALS AND METHODS

Adult male Sprague–Dawley rats (Charles River Laboratories, Wilmington, Mass.) weighing 100–250 g were exposed to light (50 candela/m^2 "Vita-Lite," Duro-Test Mfg. Co. North Bergen, N.J.) between 9 a.m. and 9 p.m. daily, and given free access to Big Red Laboratory Chow and water. For 2 days before each experiment, all of the animals to be used were exposed to continuous illumination, a treatment that suppresses indole rhythms (14).

Some animals were subjected to a partial chemical sympathectomy accomplished through a series of intravenous (tail vein) injections of 6-hydroxydopamine (15). Animals received two injections (34 mg/kg each, in 0.001 N HCl) 12 days before autopsy, and two additional injections (68 mg/kg each) 1 week later.

At 9 p.m. on the evening before an experiment, animals were transferred to clean cages and deprived of food. At noon the next day, groups of seven or eight animals were given one of the following treatments: free access to an agar-based high-carbohydrate diet (16), a subcutaneous injection of insulin (2 U/kg), or immobilization, accomplished by securing each animal to a fiberboard stock with adhesive tape. Control animals, similarly exposed to continuous light and starved, remained undisturbed in their cages and received no further treatment. After 2 hr the animals were decapitated. Blood from the cervical wound was collected in heparinized tubes and centrifuged; the plasma was separated and assayed for glucose with orthotoluidine reagent (Sigma Chemical Co., St. Louis, Mo.) (17).

Pineal *N*-acetyltransferase activity, measured by a modification of the method of Deguchi and Axelrod (18), and pineal melatonin content were used as indices of pineal biosynthetic activity (19). Pineal glands were quickly removed, chilled, and homogenized in a mixture containing 3.5 μmol of potassium phosphate (pH 6.5) and 0.17 μmol of tryptamine. 2 nmol of [^{14}C]acetyl coenzyme A (New England Nuclear, Boston, Mass.; specific activity 49.8 Ci/mol) was added to yield a

TABLE 1. *Pineal N-acetyltransferase response to insulin-induced hypoglycemia or physical immobilization*

Treatment	Intact animals		6-Hydroxydopamine-treated animals	
	N-Acetyltransferase (pmol per pineal per 15 min)	Plasma glucose (mg 100 ml)	*N*-Acetyltransferase (pmol per pineal per 15 min)	Plasma glucose (mg 100 ml)
Control	0.1 ± 0.1 (6)	110 ± 3	1.3 ± 0.1 (8)	84 ± 2
Insulin	35.0 ± 9.3* (7)	40 ± 3*	278.5 ± 45.2*,† (8)	40 ± 4*
Carbohydrate diet	0.5 ± 0.2 (7)	171 ± 6*	4.2 ± 1.0†,‡ (8)	163 ± 5*
Immobilization	11.7 ± 1.4* (7)	124 ± 6	310.9 ± 52.8*,† (7)	147 ± 6*

Rats were exposed to constant light for 2 days and deprived of food for 15 hr before the experiment. Groups of intact and 6-hydroxydopamine-treated animals were then given free access to an agar-based high-carbohydrate diet, injected subcutaneously with insulin (2 U/kg), or rendered immobile by being secured to a fiberboard stock with adhesive tape. They were killed 2 hr later. Control animals, similarly exposed to constant light and starved, remained undisturbed in their cages. The results are expressed as mean ± standard error of the mean. Figure in parentheses indicates the number of animals in that experimental group.

* $P < 0.005$ differs from corresponding control animals.

† $P < 0.005$ differs from corresponding intact animals.

‡ $P < 0.05$ differs from corresponding control animals.

final volume of 70 μl. After incubation at 37° for 15 min, the reaction was stopped by addition of 1.0 ml of 0.5 M borate buffer (pH 10), and *N*-[^{14}C]acetyltryptamine was extracted into 6 ml of toluene–isoamyl alcohol 97:3. After centrifugation, 4 ml of the organic phase was transferred to a scintillation vial and evaporated. The residue was dissolved in 1 ml of absolute ethanol; 10 ml of toluene-based phosphor was added, and the radioactivity was measured in a liquid scintillation spectrometer.

The melatonin content of aqueous pineal homogenates was estimated with a quantitative melatonin bioassay based on the dermal melanophore response of larval anurans to melatonin in their bathing medium (20). A calibration curve was plotted relating melatonin concentration to the extent of nucleocentric aggregation of melanin granules found within the dermal melanophores of tadpoles exposed to known concentrations of authentic melatonin.

RESULTS

Administration of insulin to intact rats caused both profound hypoglycemia and a more than 300-fold increase in pineal *N*-acetyltransferase activity (Table 1). That the increase after insulin administration was caused by the hypoglycemia and not by a direct action of the hormone on the pineal was indicated by the failure of carbohydrate consumption, a potent nonhypoglycemic stimulus to insulin secretion, to elevate pineal *N*-acetyltransferase activity (Table 1). In another set of experimental animals exhibiting hypoglycemia and enhanced *N*-acetyltransferase activity after insulin treatment, pinal melatonin content was more than 3-times greater than in control animals (Table 2). The stress of physical immobilization caused a 100-fold increase in pineal *N*-acetyltransferase (Table 1) and a 6-fold increase in melatonin content (Table 3).

The basal level of *N*-acetyltransferase activity was significantly higher in rats previously treated with 6-hydroxydopamine than in intact animals (Table 1). Chemical denervation of the pineal markedly potentiated the *N*-acetyltransferase responses to both hypoglycemia and immobilization (Table 1).

Prior treatment of intact rats with the β-adrenergic blocking agent propranolol (20 mg/kg) 30 min before insulin administration blocked the rise in pineal *N*-acetyltransferase activity and the increase in melatonin content (Table 2). Prior treat-

TABLE 2. *Effect of β-adrenergic blockade on pineal N-acetyltransferase and melatonin responses to insulin-induced hypoglycemia*

Treatment	*N*-Acetyltransferase (pmol per pineal per 15 min)	Plasma glucose (mg/100 ml)	Pineal melatonin content (ng per pineal)	Plasma glucose (mg/100 ml)
Control	2.7 ± 0.5 (7)	87 ± 3	0.35 ± 0.03 (7)	85 ± 4
Propranolol + insulin	6.1 ± 1.4†,‡ (8)	14 ± 3*	0.42 ± 0.03†,§ (7)	41 ± 8*
Saline + insulin	75.7 ± 17.4* (7)	27 ± 3*	1.22 ± 0.16* (7)	29 ± 3*

Propranolol (20 mg/kg body weight) in 0.5 ml of saline solution (0.9 % NaCl) or 0.5 ml of saline (0.9 % NaCl) alone was injected 30 min before the injection of insulin (2 U/kg). Pineal *N*-acetyltransferase activity, pineal melatonin content, and plasma glucose were measured 2 hr after the insulin injection. (The studies on pineal *N*-acetyltransferase activity were made with 100-g rats; those on melatonin content were made with 250-g rats.) Figure in parentheses indicates the number of animals in that experimental group. Results expressed as mean ± standard error of the mean.

* $P < 0.005$ differs from control.

† $P < 0.005$ differs from saline + insulin.

‡ $P < 0.05$ differs from control.

§ Not statistically different from control.

TABLE 3. *Effect of β-adrenergic blockade on pineal N-acetyltransferase and melatonin responses to immobilization*

Treatment	*N*-Acetyltransferase (pmol per pineal per 15 min)	Pineal melatonin content (ng per pineal)
Control	0.5 ± 0.2 (8)	0.25 ± 0.01 (8)
Propranolol + immobilization	5.6 ± 1.3* (7)	1.42 ± 0.26‡ (8)
Saline + immobilization	35.0 ± 8.4*,† (6)	1.65 ± 0.29‡,§ (8)

Propranolol (20 mg/kg body weight) in 0.5 ml of saline solution (0.9 % NaCl) or 0.5 ml of saline alone (0.9 % NaCl) was injected 30 min before the animals were immobilized. Pineal *N*-acetyltransferase activity or pineal melatonin content was measured after 2 hr of immobilization. The results are expressed as mean ± standard error of the mean. Figure in parentheses indicates the number of animals in that experimental group.

* $P < 0.01$ differs from control.

† $P < 0.01$ differs from propranolol + immobilization.

‡ $P < 0.005$ differs from control.

§ Not significantly different from propranolol + immobilization.

ment with propranolol blocked the increase in enzyme activity caused by immobilization, but did not suppress the accumulation of melatonin in the pineal (Table 3).

DISCUSSION

These studies show that other factors, in addition to environmental illumination, that influence sympathetic nervous activity elsewhere in the body can also modify pineal indole metabolism. Both insulin-induced hypoglycemia and physical immobilization rapidly increase pineal *N*-acetyltransferase activity and elevate pineal melatonin content. The failure of carbohydrate ingestion to reproduce the pineal effects of insulin and the ability of prior treatment with propranolol to block these effects indicate that insulin is not acting directly on the pineal; rather, its effect is mediated by catecholamines. The blockade of the effect of immobilization on pineal *N*-acetyltransferase by propranolol similarly suggests that this effect is also mediated by catecholamines and not, for example, by adrenocortical steroid hormones.

The catecholamines that mediate the pineal responses to insulin or immobilization in intact animals could theoretically derive from three sources: sympathetic nerves terminating on pineal cells, sympathetic nerves terminating elsewhere in the body, or the adrenal medulla. Norepinephrine or epinephrine released by the latter two sources would reach the pineal by way of its blood supply. The persistence of pineal responses to hypoglycemia or immobilization among animals first treated with 6-hydroxydopamine, which destroys sympathetic nerve terminals in the pineal (21) and at most other sites in the body, indicates that circulating *endogenous* catecholamines can control pineal indole metabolism. [Deguchi and Axelrod (19) have shown that *exogenous* circulating catecholamines, or those formed from administered L-dopa, can elevate pineal *N*-acetyltransferase activity.] The source of the circulating catecholamines in the animals previously treated with 6-hydroxydopamine could be either the adrenal medulla, an organ in which catecholamine synthesis may be enhanced after 6-hydroxydopamine administration (22), or surviving sympathetic nerve terminals, possibly those located within blood vessels (23). The potentiation of *N*-acetyltransferase responses to hypoglycemia or immobilization in animals treated with 6-hydroxydopamine could reflect enhanced secretion of adrenal or neuronal catecholamines into the circulation, or the development of denervation supersensitivity in the pinealocytes (19), or both.

Prior treatment with propranolol substantially blocked the immobilization-induced increase in *N*-acetyltransferase activity, but failed to block the rise in pineal melatonin content (Table 3). This dissociation suggests that pineal melatonin may not, under some conditions, accurately reflect the rate of melatonin biosynthesis. Melatonin content could, for example, increase if changes in pineal blood flow or intracellular processes suppressed melatonin secretion.

The present observations indicate that in the absence of rhythmic changes in environmental illumination, other factors that occur cyclically (e.g., sleeping, eating, locomotor activity) might, by varying sympathetic tone, generate continuing rhythms in pineal function.

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