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The Adrenal Medulla May Mediate the Increase in Pineal Melatonin Synthesis Induced by Stress, but not that Caused by Exposure to Darkness

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With 4 Figures

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Summary

As previously shown (*Lynch et al.:* Proc. Nat. Acad. Sci. [U.S.A.] *70*, 1704—1707 [1973]), the activity of the enzyme serotonin-N-acetyltransferase (NAT) in the rat pineal increases when the animal is placed in darkness or is subjected to the stress of physical immobilization; partial sympathetic denervation (*i.e.*, pretreatment of the animal with intravenous 6-hydroxydopamine [6-OHDA]) does not block either response. The present studies explored the roles of the pineal sympathetic nerves and the adrenal medullas in mediating these responses. The stress-induced increase in pineal NAT activity was blocked by bilateral adrenalectomy, but not by bilateral superior cervical ganglionectomy or by treatment with 6-OHDA (both of which potentiate the NAT response in normal rats and restore it in adrenalectomized ones). The increase in pineal melatonin content caused by immobilization was also blocked by adrenalectomy, but potentiated by pineal sympathetic denervation. In contrast, bilateral adrenalectomy did not affect the darkness-induced rise in pineal NAT activity, although pineal sympathetic denervation (by bilateral superior cervical ganglionectomy) did block this response. 6-OHDA pretreatment neither blocked the response to darkness nor restored it in ganglionectomized animals; thus, this treatment apparently fails to produce a complete pineal denervation. The pineal response to stress has previously been shown to be blocked by β-adrenergic blocking agents. The present studies demonstrate that α-adrenergic blockade (with phenoxybenzamine) potentiates this response in intact animals and restores it in adrenalectomized rats (possibly by acting presynaptically on

receptors on pineal sympathetic terminals and thereby augmenting norepinephrine release). These observations show that the rat pineal organ normally receives information from two "channels", *i.e.*, trans-synaptically (from pineal sympathetic nerves) and via the circulation (from the adrenal medullas and, perhaps, from distant sympathetic nerves).

Introduction

Abundant evidence shows that environmental lighting constitutes the major factor controlling the synthesis of melatonin in the rat pineal gland (*Wurtman et al.*, 1968). Exposure to continuous illumination suppresses melatonin synthesis (*Wurtman et al.*, 1963) and decreases the flow of nerve impulses to the pineal via its sympathetic nerves (*Taylor* and *Wilson*, 1970); conversely, the daily onset of darkness corresponds to major increases in the activities of the two enzymes catalyzing melatonin synthesis from serotonin (serotonin-N-acetyltransferase [NAT] and hydroxyindole-O-methyltransferase [HIOMT]). Darkness is also associated with a decrease in pineal serotonin content and an increase in pineal melatonin (*Quay*, 1963; *Axelrod et al.*, 1965; *Klein* and *Weller*, 1970; *Lynch*, 1971). *Cardinali et al.* (1972) and *Minneman et al.* (1974) have described the spectral and intensity characteristics of the photic inhibition of melatonin synthesis. In addition, the anatomical and molecular components of the pathways mediating the pineal's response have been shown to include the retinas (*Wurtman et al.*, 1964), the accessory optic tracts (*Moore et al.*, 1967), the sympathetic nerves originating in the superior cervical ganglia (*Wurtman et al.*, 1964), norepinephrine (*Axelrod et al.*, 1969), the β-adrenergic receptors on the pineal parenchymal cells (*Wurtman et al.*, 1971), and cyclic AMP (*Shein* and *Wurtman*, 1969).

Although environmental lighting may act as the primary control of pineal sympathetic tone and indole biosynthesis, it apparently is not the only factor: studies have shown that significant daily rhythms in HIOMT (*Nagle et al.*, 1972) and NAT (*Klein* and *Weller*, 1970) activities and in melatonin content (*Ralph et al.*, 1971) persist in the pineals of animals either blinded or maintained under constant darkness. Moreover, recent studies in our laboratory on the effects of adrenergic drugs on melatonin synthesis led to the intriguing incidental observation that, while the subcutaneous injection of L-dopa in saline suspension caused a marked increase in pineal melatonin content, the administration of a saline solution *alone* could also elicit a slight increase in pineal melatonin content (*Lynch et al.*, 1973 a).

A series of studies were thus initiated to test the possibility, first, that changes in sympathetic nervous activity induced by factors *other* than light and darkness might affect melatonin biosynthesis in the pineal and, second, that pineal function might be influenced independently of its direct sympathetic input. To this end, we examined melatonin biosynthesis in rats subjected to an experimental stress—*i.e.*, either insulin-induced hypoglycemia or physical immobilization. In view of the observation that prior denervation of the rat pineal caused it to become supersensitive to L-dopa and to exogenous catecholamines (*Deguchi* and *Axelrod*, 1972), we destroyed the sympathetic neurons of some of our animals by pretreating them with 6-hydroxydopamine (6-OHDA). Both procedures for inducing stress stimulated pineal biosynthetic activity in intact animals, as evidenced by increases in NAT activity and melatonin content; moreover, 6-OHDA pretreatment potentiated the responses (*Lynch et al.*, 1973 b).

We undertook the present series of studies in order to examine these phenomena in animals whose pineals were surgically denervated or whose adrenals were removed (thereby eliminating a major source of circulating catecholamines). Our observations indicated that, while environmental lighting's effect on the pineal is mediated by the pineal's sympathetic nerves, the increase in pineal melatonin synthesis caused by stress is largely the result of its responses to circulating catecholamines, most of which originate in secretion from the adrenal medulla.

Materials and Methods

Intact and surgically prepared Sprague-Dawley male rats weighing 160—200 g were obtained from the Zivic-Miller Laboratories, Inc., Allison Park, PA. Unless otherwise indicated, all rats were maintained under diurnal lighting conditions (lights on from 9 a.m. to 9 p.m.). Illumination, measuring 300 μw/cm^2, was supplied by "Vita-Lite" fluorescent tubes (Duro-Test Manufacturing Co., North Bergen, NJ). Big Red Laboratory Animal Diet and water were available *ad libitum*. All adrenalectomized animals were given drinking water containing 1 % NaCl. Those that were treated with 6-OHDA and/or subjected to physical immobilization were injected subcutaneously with 1 mg of corticosterone in sesame oil daily prior to the experiment.

Some animals were subjected to a partial chemical sympathectomy accomplished through a series of intravenous (tail vein) injections of 6-OHDA (*Thoenen* and *Tranzer*, 1968). Each treated animal received two injections (34 mg/kg each, in 0.001 N HCl) 12 days before the experiment and two additional injections (68 mg/kg each) 1 week later.

All experiments involving physical immobilization were performed between 10 a.m. and 2 p.m. Immobilization was accomplished by securing each animal to a fiberboard stock with adhesive tape; control animals remained undisturbed in their cages. After 2 hours, all of the animals were killed by decapitation and their pineal glands quickly removed. Pineals to be assayed for melatonin content were frozen in 1.0 ml of deionized water and bioassayed within 3 days. Pineals to be assayed for NAT activity were initially frozen on dry ice and assayed within 14 hours.

Pineal melatonin content was estimated by a quantitative bioassay based on the response of dermal melanophores in larval anurans to melatonin present in their bathing medium (*Ralph* and *Lynch*, 1970). Light-adapted *Rana pipiens* larvae were placed in dilute pineal homogenates. The melatonin content was then estimated by comparing the extent of nucleocentric melanin aggregation that resulted in the dermal melanophores of these animals with that observed in tadpoles similarly exposed to known concentrations of authentic melatonin.

We determined NAT activity by measuring the transfer of a ^{14}C-acetyl moiety from acetyl coenzyme A to tryptamine. Each pineal was homogenized in a mixture containing 3.5 μmoles of potassium phosphate (pH 6.5) and 0.17 μmole of tryptamine. Two nmoles of ^{14}C-acetyl coenzyme A (New England Nuclear, Boston, MA; specific activity, 49.8 mCi/mmole) were added to yield a final volume of 70 μl. After incubation at 37 °C for 15 min, the reaction was stopped by the addition of 1.0 ml of 0.5 M borate buffer (pH 10.0) and the ^{14}C-N-acetyltryptamine was extracted into 6 ml of toluene : isoamyl alcohol (97 : 3). After centrifuging the sample, we transferred 4 ml of the organic phase to a scintillation vial for evaporation. The residue was dissolved in 1 ml of absolute ethanol; 10 ml of toluene-based phosphor were added, and the radioactivity was measured in a liquid scintillation spectrometer.

Results

Effect of Adrenalectomy, with and without Pineal Denervation, on the Increase in Pineal Biosynthetic Activity Induced by Physical Immobilization

NAT activity (*Klein* and *Weller*, 1970) and melatonin content (*Quay*, 1963; *Lynch*, 1971) are lowest during the light phase among rats exposed to a diurnal lighting schedule. In accordance with the results of *Lynch et al.* (1973 b), the NAT activities and melatonin contents of rat pineals rose manyfold when the intact animals were stressed for 2 hours by immobilization in a lit environment (Fig. 1; Table 1); these responses were markedly potentiated in animals pretreated with systemic 6-OHDA. Prior surgical denervation of the pineal by bilateral superior cervical ganglionectomy also potentiated

these responses (Fig. 1; Table 1). Animals subjected to bilateral adrenalectomy failed to display increases in pineal NAT following physical immobilization; however, if such animals had also been subjected to either ganglionectomy or pretreatment with 6-OHDA, pineal NAT did respond to immobilization (Fig. 2). Pineal melatonin content did not increase, however, in immobilized rats that had been subjected to both adrenalectomy and ganglionectomy (Table 1).

Table 1. *Effect of physical immobilization on pineal melatonin content following surgical denervation of the pineal or denervation and adrenalectomy*

Surgical preparation	Pineal melatonin content (ng/pineal)	
	Control	Immobilized
None	(5) 0.10 ± 0.06	(5) 1.09 ± 0.24*
Ganglionectomy	(5) 0.21 ± 0.05	(4) 1.89 ± 0.39*†
Ganglionectomy + Adrenalectomy	(5) 0.21 ± 0.02	(4) 0.20 ± 0.10††

Groups of intact and surgically prepared animals were rendered immobile by being secured individually to fiberboard stocks and were then killed 2 hours later. Control animals remained undisturbed in their cages. The results are expressed as the mean ± S.E.M. Each 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 intact immobilized animals.

†† Not significantly different from corresponding control animals.

Pretreatment with the α-adrenergic blocking agent, phenoxybenzamine, increased pineal NAT activity (as also reported by *Deguchi* and *Axelrod* [1972]), potentiated the pineal NAT response to physical immobilization in intact animals (Fig. 2), and restored the NAT response to immobilization in adrenalectomized rats (Fig. 2). (We previously observed [*Lynch et al.*, 1973 b] that pretreatment with propranolol, the β-adrenergic blocking agent, blocks the increase in pineal NAT activity and has no effect on the immobilization-induced increase in pineal melatonin content.)

Effect of Adrenalectomy or Pineal Denervation on the Increase in Pineal NAT Activity Induced by Exposure to Darkness

Untreated rats exposed to a diurnal lighting schedule exhibit a 15- to 40-fold daily variation in pineal NAT activity (*Klein* and *Weller*, 1970). The amplitude of this daily variation was increased in

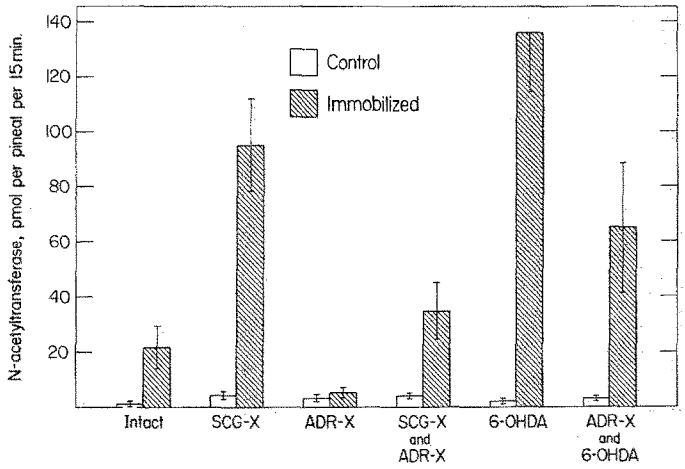

Fig. 1. Effect of physical immobilization on rat pineal NAT activity following various surgical and/or pharmacological treatments. Groups of 4—9 rats were rendered immobile by being secured individually to fiberboard stocks with adhesive tape. They were killed 2 hours later. Control animals remained undisturbed in their cages and were killed at the same time as the corresponding experimental group. Vertical lines indicate S.E.M.

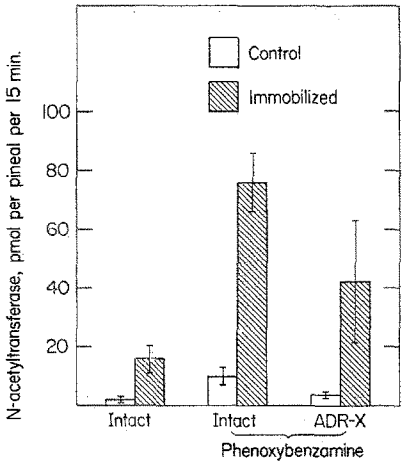

Fig. 2. Effect of physical immobilization on pineal NAT activity in intact and adrenalectomized rats following pretreatment with phenoxybenzamine. Groups of 7 rats were maintained under constant light for 72 hours. Phenoxybenzamine (20 mg/kg body weight) in 0.5 ml saline solution was injected subcutaneously into all rats 30 min before subgroups were immobilized for 2 hours. Vertical lines indicate S.E.M.

rats pretreated with systemic 6-OHDA, and ganglionectomy abolished the anticipated rise in NAT activity after the onset of darkness (Fig. 3). Furthermore, pretreatment with 6-OHDA did not restore the nocturnal rise to ganglionectomized rats (Fig. 3).

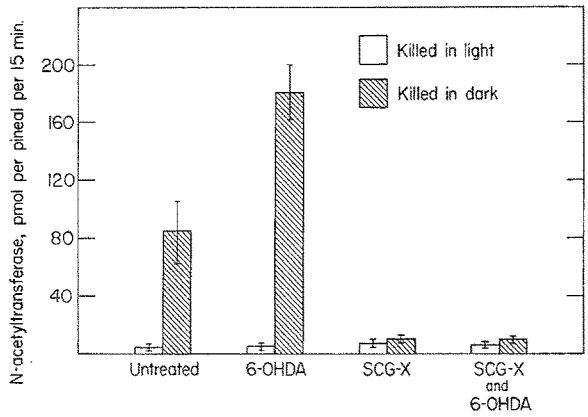

Fig. 3. Effect of environmental illumination on pineal NAT activity following various surgical and/or pharmacological treatments. Groups of 5—6 rats were maintained under a diurnal lighting schedule with lights on from 9 a.m. to 9 p.m. Subgroups were killed either at midnight (3 hours after onset of darkness) or at noon (3 hours after onset of light). Vertical lines indicate S.E.M.

After 10 days of continuous exposure to illumination and then 3 hours of darkness, rats exhibited an increase in pineal NAT activity that was several-fold greater than that observed in animals experiencing daily photoperiods (Fig. 4). Adrenalectomy, which blocks the pineal response to immobilization (Fig. 1), did not block the increase in NAT activity occurring when rats are placed in darkness (Fig. 4).

Discussion

The two increases in pineal NAT activity—one caused by immobilizing rats and the other caused by exposing them to darkness—appear to derive from different mechanisms. The enzyme's response to immobilization is abolished by bilateral adrenalectomy, but not by the surgical or chemical denervation of the pineal (Fig. 1). (The immobilization-induced increase in pineal melatonin content is similarly blocked by adrenalectomy, but not by pineal denervation [Table 1].) In contrast, bilateral superior cervical ganglionectomy blocks the pineal response to darkness, while adrenalectomy has no effect on this phenomenon (Fig. 4). These observations suggest that pineal NAT

activity can be physiologically enhanced (and pineal β-receptors activated) *either* by the release of norepinephrine from sympathetic terminals within the pineal or by circulating catecholamines (liberated from the adrenal medulla and, perhaps, from sympathetic nerve terminals elsewhere in the body). The stress of immobilization, by increasing the levels of catecholamines in the circulation, stimulates the pineal; the onset of darkness apparently does not cause a sufficient rise in circulating catecholamines to do so, and thus the photic control of pineal function requires intact pineal sympathetic innervation.

Using histochemical fluorescence as a criterion, *Eränkö* and *Eränkö* (1971) failed to detect catecholamines in the pineals of rats treated neonatally with 6-OHDA; similarly, we found no chemically assayable norepinephrine in pooled pineals of 10 adult rats treated as described above with 6-OHDA. Nevertheless, we suspect that total sympathetic denervation was not attained in our animals treated with 6-OHDA. The observations that such treatment potentiates the pineal NAT response to darkness (which requires pineal sympathetic innervation) and that ganglionectomy (or pretreatment with 6-OHDA in combination with ganglionectomy) completely abolishes this response (Fig. 3) both suggest that the 6-OHDA treatment alone, while rendering the pineal supersensitive to adrenergic stimulation, does not totally eliminate its sympathetic input.

Beta-adrenergic receptors mediate the effects of norepinephrine on pineal biosynthetic activity (*Wurtman et al.*, 1971), and propranolol,

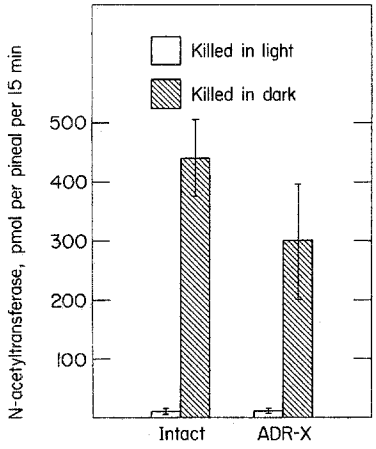

Fig. 4. Effect of environmental illumination on pineal NAT activity following adrenalectomy. Groups of 6—7 rats were maintained in constant light for 10 days. Subgroups were then placed in total darkness for 3 hours. Vertical lines indicate S.E.M.

a β-adrenergic blocking agent, blocks the pineal NAT response to physical immobilization (*Lynch et al.*, 1973 b). The stimulation of pineal NAT *in vivo* by the α-adrenergic blocking agent, phenoxybenzamine, has been reported by *Deguchi* and *Axelrod* (1972) and is confirmed by these studies (Fig. 2). *Klein* and *Weller* (1973) reported a similar effect *in vitro* of the α-adrenergic blocking agent, phentolamine. Just how β-adrenergic stimulation of pineal cells is influenced by α-adrenergic blockade remains to be explained. Pineal parenchymal cell membranes could contain both α- and β-adrenergic receptors. Alternatively, phenoxybenzamine could be acting *pre*synaptically, on sympathetic nerve terminals, to increase the release of norepinephrine (*Kirpekar* and *Puig*, 1971; *Wennmalm*, 1971; *Enero et al.*, 1972). This possibility might explain the drug's ability to restore the NAT response in stressed adrenalectomized rats; it would allow the intact pineal sympathetic nerves to release more of their transmitter. In any event, the *in vivo* observations reported here may help to extend the utility of the pineal gland as a model for studying adrenergic receptors and their control of the diverse physiological functions that are affected by sympathetic nerves (*Axelrod*, 1974).

The adaptive significance of the dual modes of pineal control suggested by these studies remains to be elucidated. The relative contributions of these two "channels"—*i.e.*, the pineal sympathetic nerves and the adrenal medulla (via the bloodstream)—to the net secretory activity of the pineal may vary from one mammal to another and may correlate with the ecological niches occupied by various species. For example, it is possible that among animals living in the earth's temperate zones, where there are marked annual variations in the length of the daily photoperiod, the pineal serves to synchronize cyclic reproductive functions with seasonal variables. In the tropics, where marked rhythmic changes in the length of the photoperiod do not occur, other environmental variables may control pineal function by influencing plasma catecholamine concentrations.

Melatonin secretion from the pineal may have physiologic consequences not hitherto considered. If, as the present studies suggest, nonspecific stress increases melatonin synthesis and secretion, this hormone may participate in mechanisms of adaption that remain to be characterized.

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observations were first presented at the IX Midwest Conference on Endocrinology and Metabolism held at the University of Missouri, Columbia, MO, in 1973 (*Lynch et al.*, 1975).

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