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EFFECTS OF DARKNESS AND OF DESMETHYLIMIPRAMINE ON PINEAL GLAND CONCENTRATIONS OF ADENOSINE 3′, 5′-MONOPHOSPHATE*

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Abstract—The concentration of cyclic AMP in the pineal glands of rats exposed to constant light for 7 days and then placed in darkness for up to 3 hr was no different from that measured in the pineal glands of rats kept in constant light. However, if the norepinephrine re-uptake inhibitor desmethylimipramine (DMI) was injected into rats 1 hr before placing them in the dark, then exposure to darkness caused a rapid, sustained increase in pineal gland concentrations of cyclic AMP. While bilateral adrenal demedullation did not prevent the increase in pineal cyclic AMP in rats treated with DMI and exposed to darkness, bilateral superior cervical ganglionectomy abolished completely the darkness-induced rise in cyclic AMP in DMI-treated rats. Furthermore, prior treatment of rats with the β-adrenergic antagonist (±)-propranolol prevented the darkness-induced increase in cyclic AMP in rats treated with the inhibitor of norepinephrine re-uptake. It appears that increases in sympathetic activity to the pineal gland, induced by darkness, can raise the concentration of cyclic AMP in the gland but only when norepinephrine re-uptake is prevented.

Whereas pharmacological stimulation of β-adrenergic receptors increases adenosine 3′, 5′-monophosphate (cyclic AMP) concentrations in many different organs [1, see also Ref. 2], very few instances have been reported where increased noradrenergic neuronal activity raised cyclic AMP concentrations in the innervated organ. The reason for this apparent paradox is not well understood. We have been unable to find any reports of sympathetic nerve stimulation increasing cyclic AMP concentrations in organs receiving such innervation. Siggins *et al.* [3] reported that stimulation of the locus coeruleus increases cyclic AMP concentrations in cerebellar Purkinje cells, as measured by an immunocytochemical procedure. Even here, though, the involvement of cyclic AMP in mediating the effects of norepinephrine (NE) on cerebellar Purkinje cells is somewhat controversial [4], and there is some uncertainty as to how quantitative the immunocytochemical procedure for cyclic AMP is (see Ref. 5).

The mammalian pineal gland is an ideal organ to use for further study of this issue. Its sole innervation is that of post-ganglionic sympathetic fibers originating at the superior cervical ganglion [6], and it contains a high density of β-adrenergic receptors coupled to adenylate cyclase [7, 8], activation of which leads to increased concentrations of cyclic AMP in the gland [9, 10]. The rise in cyclic AMP caused by norepinephrine is thought to mediate some of the effects of norepinephrine on the pineal gland [11, 12]. Most interestingly, the pineal gland responds to changes in environmental lighting via a complex multisynaptic pathway originating at the retina and terminating with the sympathetic innervation to the gland [13–17]. Darkness increases the activity of the noradrenergic fibers to the pineal, as evidenced by darkness increasing the turnover of NE in the gland [18], as well as increasing the electrical activity of the gland [19].

To investigate whether NE, released endogenously from sympathetic nerves, can raise cyclic AMP concentrations in the organ innervated, we have studied whether exposure of rats to darkness would elevate cyclic AMP concentrations in the pineal gland. In addition, as re-uptake into the sympathetic neuron is the primary mechanism for terminating the activity of NE in the synapse (see Ref. 20), the effect of desmethylimipramine, an inhibitor of NE uptake [21], was examined in this system as well.

METHODS

Male Sprague–Dawley rats (200–250 g) were purchased from Zivic–Miller Laboratories, Allison Park, PA. All surgical and sham procedures were performed by the supplier. Surgical procedures were performed on the rats 2 weeks prior to their being used in experiments [22]. All animals had access to food and water *ad lib*. Rats were given intraperitoneal injections of either desmethylimipramine (DMI, 10 mg/kg) or 0.9% NaCl. In addition, some rats received (±)-propranolol (30 mg/kg, i.p.) 15 min before a subsequent injection of either DMI or saline.

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Animals were housed in an environmental room (Hotpack Corp., Philadelphia, PA) which was maintained at 22°. For most experiments, this room was kept in constant light and the rats acclimated to it for 7 days prior to the experimental procedure so as to reduce diurnal fluctuations in beta-adrenergic receptor density [23]. For another experiment, animals were housed under conditions of alternating light and dark periods (lights on from 7:00 a.m. to 7:00 p.m.) and were killed at the end of the light cycle.

The experimental procedure consisted of either keeping the rats exposed to light or placing them in the dark for periods of time ranging from 1 to 180 min. Rats were exposed to darkness between 10:00 and 11:00 a.m. To place the rats in darkness, the animals were removed first from the environmental room into an adjacent lighted area. The animals were maintained in this area, in individual cages, for several hours. They were then returned, one at a time, to the environmental room and lights in this room were turned off for the appropriate periods of time. All rats exposed to darkness, i.e. those receiving saline or desmethylimipramine, were handled in this manner. Initially, rats killed in constant light were handled in this way also. However, preliminary experiments showed that there was no difference in the results if this were done or if the rats were just kept in the environmental room and killed in the light. Since this latter procedure was simpler to perform, most of the rats killed in constant light were handled in this way.

Darkness was defined as the absence of all light except that from a red 25 W Sylvania light bulb protected by a red acetate filter. Previous studies have shown that the activities of pineal enzymes sensitive to white light are not altered by this intensity of red light [24]. Rats killed in the dark had their pineal glands removed in the dark.

Preliminary studies, that were run for another, related investigation, showed plasma and pineal concentrations of DMI to be maximal 30–60 min after its intraperitoneal administration [25]. Therefore, 1 hr after the DMI or saline injection, the rats were decapitated and the pineal glands removed and frozen on dry ice within 30 sec to control for a post-decapitation rise in cyclic AMP [26]. With this experimental design, then, rats exposed to darkness for longer than 60 min received their injection of DMI or saline in the dark.

Cyclic AMP was extracted by sonicating the pineal glands for 15 sec in 1 ml of ice-cold 2.5% perchloric acid. The sonicate was centrifuged at 49,000 *g* for 15 min at 4°.

The supernatant fluid was decanted, neutralized with excess $CaCO_3$ [27], and centrifuged at 12,000 *g* for 10 min at 4°. The cyclic AMP concentration of the neutralized extract was measured by radioimmunoassay using [^{125}I]antigen and antiserum purchased from New England Nuclear, Boston, MA. All unknown samples were assayed in triplicate. Standard solutions of cyclic AMP were prepared in 2.5% perchloric acid that had been neutralized with $CaCO_3$.

Protein determinations were performed according to the method of Lowry *et al.* [28].

Desmethylimipramine was donated by USV Pharmaceutical Corp. NY. (±)-Propranolol was purchased from the Sigma Chemical Co., St. Louis, MO.

RESULTS

Because the level of activity of the noradrenergic innervation to the pineal gland shows circadian vari-

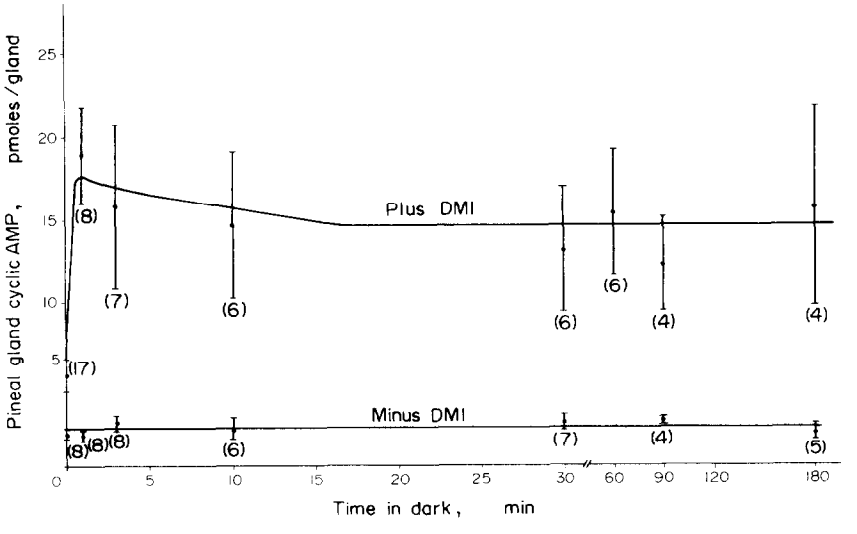

Fig. 1. Effect of desmethylimipramine on pineal gland cyclic AMP concentrations in rats placed in darkness. Rats received intraperitoneal injections of either saline (lower trace, labeled "minus DMI") or DMI (upper trace; 10 mg/kg). 1 hr prior to being killed, the rats were placed in the dark for the times indicated. Each point represents the mean value for the number of experiments indicated in the parentheses. Each bracket indicates the S.E.M. In rats receiving DMI, each mean value of cyclic AMP in the pineal glands of rats placed in darkness for different periods of time was significantly higher than the mean value measured in rats receiving DMI and not placed in the dark ($P < 0.025$).

ation, being higher during darkness than light [18, 19], we examined whether exposure to the dark would elevate cyclic AMP concentrations in the pineal gland. Figure 1 shows the pineal gland concentrations of cyclic AMP in animals kept in constant light for 7 days and then given a single injection of either DMI or saline before being exposed to darkness for the times indicated. In those animals receiving only saline, at no time up to 180 min did exposure to darkness raise the levels of pineal cyclic AMP over that measured in pineal glands of rats not placed in the dark. In contrast, pineal cyclic AMP increased rapidly, within 1 min of darkness, in animals which received a single injection of DMI; concentrations of cyclic AMP remained elevated for up to 3 hr of exposure to darkness in rats injected with DMI. Because the elevation of pineal gland cyclic AMP was maximal after 1 min of exposure to darkness, this time was chosen for all subsequent experiments involving rats killed in the dark.

The results in this paper are expressed as picomoles of cyclic AMP per pineal gland rather than in terms of pineal protein content, as no significant change was noted in pineal protein during the relatively short time period the rats were placed in the dark. Thus, the results would be essentially the same as those presented if the data were expressed in terms of pineal protein. For example, in rats given DMI and kept in constant light, the pineal concentration of cyclic AMP was significantly lower (65 ± 13 pmoles/mg protein) than that measured in rats given DMI and placed in the dark for 1 min (167 ± 33 pmoles/mg protein; $P < 0.005$).

Injection of DMI is effective in raising cyclic AMP levels in rats kept in constant light as well as in rats placed in the dark. This was studied by giving rats an injection of saline or DMI and killing them, in

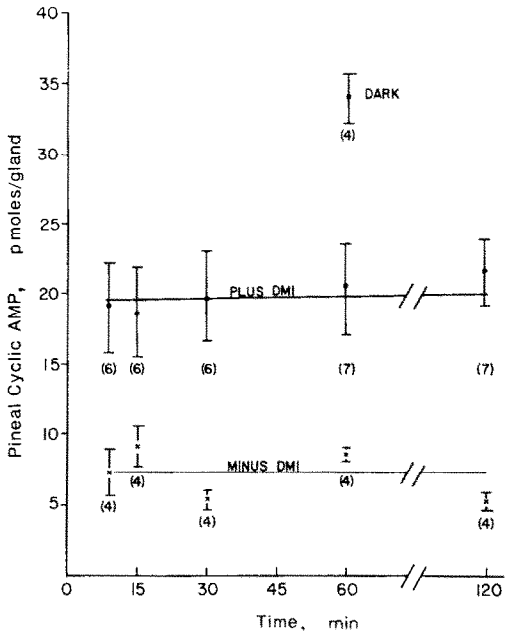

Fig. 2. Effect of desmethylimipramine on pineal cyclic AMP in rats kept in constant light. Rats were injected with either 0.9% NaCl or DMI (10 mg/kg) and were killed either 7.5, 15, 30, 60 or 120 min later. Each point represents the mean value for the number of experiments indicated in the parentheses. Each bracket indicates the S.E.M. At all time points, the concentrations of cyclic AMP in the pineal glands of rats given DMI were higher than those found in control animals ($P < 0.05$, Student's t-test). The concentrations of pineal cyclic AMP in rats placed in the dark for 1 min after being treated with DMI is indicated as "dark". This value is significantly higher than that measured in any of the groups of rats given DMI and kept in the light ($P < 0.025$).

Table 1. Effect of DMI treatment on pineal gland concentrations of cyclic AMP

Drug treatment	Housing conditions	Exposure to darkness for 1 min	Pineal cyclic AMP (pmoles/gland)	P*
0.9% NaCl	Constant light	−	5.0 ± 1.1† (5)‡	
0.9% NaCl	Constant light	+	4.8 ± 1.0 (5)	> 0.5
0.9% NaCl	Light–dark	−	6.9 ± 0.9 (8)	
0.9% NaCl	Light–dark	+	5.6 ± 0.8 (8)	> 0.2
DMI§	Constant light	−	11.5 ± 1.1‖ (14)	
DMI	Constant light	+	22.6 ± 1.5 (14)	< 0.001
DMI	Light–dark	−	11.7 ± 1.6‖ (7)	
DMI	Light–dark	+	22.7 ± 1.9 (7)	< 0.001

* Compared to corresponding value in rats not exposed to darkness.
† $\bar{X}$ ± S.E.M.
‡ Number of observations.
§ Ten mg/kg, intraperitoneally, 1 hr before animals were killed.
‖ $P < 0.025$, compared to saline-treated animals housed under similar lighting conditions.

the light, at different times thereafter. The results of this experiment are shown in Fig. 2. At the earliest time of measurement after administration of DMI, namely 7.5 min, cyclic AMP concentrations rose about 2.5 times as compared to the concentration measured in rats injected with 0.9% NaCl. The rise in cyclic AMP, observed in the rats given DMI, persisted for at least 2 hr. In the same experiment, some rats were placed in the dark for 1 min after they had been given DMI 1 hr previously. As shown before (Fig. 1), exposure to the dark in rats treated with DMI raised pineal cyclic AMP concentrations still further.

The increase in cyclic AMP in rats administered DMI and then exposed to darkness was observed in animals housed under conditions of alternating light and dark periods as well as in constant light. In a separate experiment, rats were housed either in constant light for 7 days or in 12 hr light–12 hr dark periods for the same time. Animals were then injected with either saline or DMI and 1 hr later were exposed to the dark for 1 min. The results of this experiment are shown in Table 1. As before, injection of DMI caused a rise in cyclic AMP in animals killed in the light. However, treatment with DMI raised pineal cyclic AMP significantly higher if the rats were placed in darkness for 1 min prior to death; this occurred regardless of the lighting conditions in which the rats were housed.

To determine if the increase in cyclic AMP

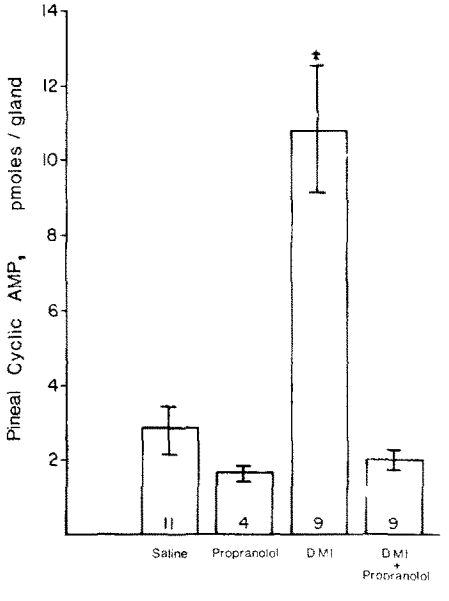

Fig. 3. Effect of propranolol on the darkness-induced rise in pineal cyclic AMP in rats treated with desmethylimipramine. Rats received intraperitoneal injections of either (±)-propranolol (30 mg/kg) or saline 15 min before a subsequent injection of either saline or DMI (10 mg/kg). One hr after the second injection, the animals were exposed to darkness for 1 min prior to decapitation. Each bar represents the mean value of cyclic AMP and the brackets show the S.E.M. The figures in each bar show the number of experiments. The asterisk (*) indicates $P < 0.025$ compared to either the saline group or the group receiving (±)-propranolol and DMI.

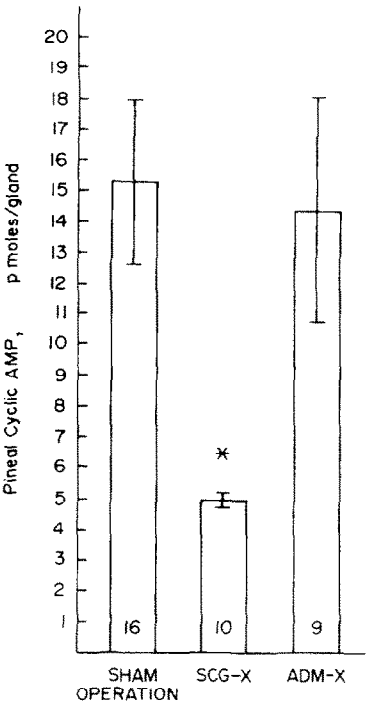

Fig. 4. Effect of bilateral superior cervical ganglionectomy (SCG-X) or bilateral adrenal demedullation (ADM-X) on the darkness-induced rise in pineal cyclic AMP in rats treated with desmethylimipramine. Rats underwent (1) a sham operation, or (2) bilateral superior cervical ganglionectomy (SCG-X), or (3) bilateral adrenal demedullation (ADM-X) 2 weeks prior to the experiment. All animals received DMI (10 mg/kg) 1 hr before being killed. All animals were exposed to darkness for 1 min prior to decapitation. The asterisk (*) indicates $P < 0.01$ compared to the sham operated group.

observed in rats treated with DMI and placed in the dark was due to activation of β-adrenergic receptors, propranolol was administered to rats 15 min before administration of DMI and 75 min before placing the rats in darkness for 1 min. The results of this experiment are shown in Fig. 3. As before, the pineal cyclic AMP concentration of rats receiving DMI 1 hr before being placed in darkness was significantly higher than that measured in the glands of rats not receiving the tricyclic drug. Pretreatment with (±)-propranolol, however, completely eliminated the darkness-induced rise in pineal gland cyclic AMP in rats given DMI. This indicates that the DMI-induced increase in pineal cyclic AMP in animals placed in darkness is mediated by activation of pineal β-adrenergic receptors.

The source of the catecholamines that were responsible for the rise in pineal cyclic AMP in rats given DMI and placed in the dark was determined by treating three different groups of rats with DMI 60 min prior to placing them in the dark for 1 min: (1) sham-operated rats; (2) rats receiving bilateral superior cervical ganglionectomy (SCG-X); and (3) rats receiving bilateral adrenal demedullation (ADM-X). Ablation of both superior cervical ganglia

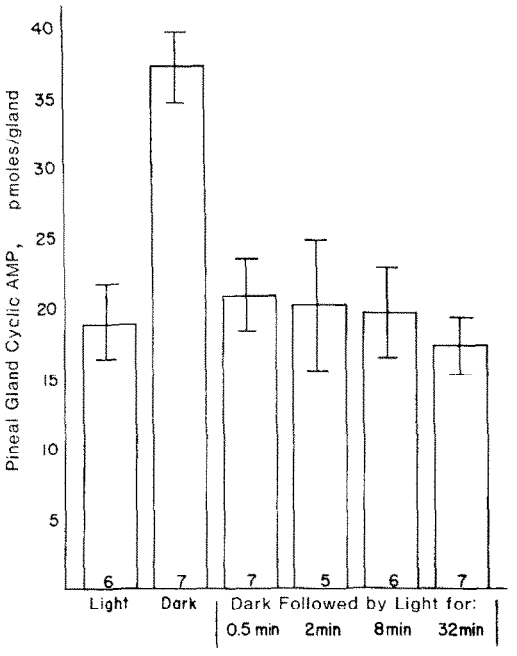

Fig. 5. Effect of re-exposure to light on pineal gland cyclic AMP in rats given desmethylimipramine and placed into darkness. All rats received injections of DMI (10 mg/kg). One hr later, the animals were (1) killed without being placed in the dark ("light") or (2) were placed in the dark for 1 min ("dark") or (3) were placed in the dark for 1 min and re-exposed to light for the times indicated. Each bar represents the mean value of cyclic AMP ± S.E.M. The number in each bar shows the number of experiments. The value of cyclic AMP in the pineal glands of rats killed in the dark is significantly higher than that measured in any of the other groups of animals ($P < 0.005$, Student's t-test).

will result in complete denervation of the pineal, as this is the exclusive innervation of this gland [6], whereas bilateral adrenal demedullation removes the primary source of catecholamines found in the circulation. The results of this experiment are shown in Fig. 4. Adrenal demedullation had no effect on the rise in cyclic AMP seen in DMI-treated rats placed in the dark. In contrast, bilateral superior cervical ganglionectomy abolished the rise in pineal gland concentration of cyclic AMP. These results indicate that DMI is potentiating the effects of NE released in the dark from the post-ganglionic sympathetic fibers innervating the pineal gland.

It was of interest to determine whether re-exposure to light would cause a fall in pineal cyclic AMP concentrations in rats given DMI and placed in the dark. To see if this would happen, rats were injected with DMI (10 mg/kg) and 1 hr later placed in the dark for 1 min. The lights were then turned on and the animals killed either 0.5, 2, 8 or 32 min later. The results of this experiment are presented in Fig. 5. As expected, the concentration of cyclic AMP in the pineal glands of rats given DMI and placed in the dark for 1 min was significantly higher than that measured in the glands of rats not exposed to darkness. Within 30 sec of re-exposure to light, though, the concentration of cyclic AMP fell to the value found in rats not exposed to the dark.

DISCUSSION

The mammalian pineal gland, formerly considered nothing more than a vestigial organ, has become a widely studied structure within the past 20 years. It has been shown, for example, that the pineal gland responds to changes in environmental lighting via a complex multisynaptic pathway originating at the retina and terminating with the sympathetic innervation to the gland from the superior cervical ganglion [13–17]. The onset of darkness increases the turnover of norepinephrine (NE) in the sympathetic nerves innervating the pineal gland [18]. Furthermore, darkness increases the electrical activity of the pineal gland [19], and NE causes hyperpolarization of the pinealocyte cell membrane by activation of β-adrenergic receptors on the gland [29].

In view of this, it is somewhat surprising that the onset of darkness has not been reported to cause rapid increases in the concentration of cyclic AMP in the pineal gland. While rats killed after 12 hr of exposure to darkness have higher basal pineal gland concentrations of cyclic AMP than rats killed after being in the light for the same amount of time [23, 30], exposure of rats to darkness for periods up to 6 hr has not been shown to stimulate a rise in pineal gland cyclic AMP concentrations [23].

In agreement with these results [23], we have not been able to measure any significant change in pineal cyclic AMP in saline-treated rats kept in constant light and then exposed to darkness. However, if rats were given a single injection of DMI 1 hr prior to exposure to darkness, then a rapid rise in cyclic AMP concentrations occurred when the rats were placed in the dark. This effect persisted after bilateral adrenal demedullation, but either bilateral superior cervical ganglionectomy or prior administration of propranolol prevented the increase in cyclic AMP from occurring. This indicates that the increases in pineal cyclic AMP seen in DMI-treated rats are due to the action of NE released from the sympathetic nerve terminals innervating the pineal. As prior work has demonstrated no change in the binding of [^{3}H]dihydroalprenolol to pineal gland homogenates of rats given a single injection of DMI [25], the elevated cyclic AMP concentrations seen in rats given DMI and placed in the dark is not caused by DMI increasing the density of pineal gland β-adrenergic receptors. The most likely explanation for the darkness-induced rise in cyclic AMP in rats given a single injection of DMI is that the tricyclic drug, by blocking NE re-uptake into sympathetic terminals [21], causes a persistent elevation in synaptic concentrations of NE so as to stimulate a measurable rise in pineal cyclic AMP. This work establishes that exposure of rats to darkness can rapidly raise pineal cyclic AMP levels.

It should be emphasized, though, that injection of rats with DMI raised pineal gland cyclic AMP levels even in animals kept in constant light. Some release of NE from the sympathetic nerve innervating the pineal probably occurs in the light, and its effect on pineal β-adrenergic receptors would be enhanced in the presence of DMI. However, exposure to darkness raised still further the concentration of cyclic AMP. The darkness-induced rise in cyclic

AMP in rats treated with desmethylimipramine was reversed very rapidly upon re-exposure to light (Fig. 5). The rapid fall in pineal cyclic AMP observed upon re-exposure to light is similar to the quick decrease in activity of serotonin *N*-acetyltransferase [31] observed in rats exposed to light after being placed in the dark. Indeed, the fall in cyclic AMP appears to happen even faster than the decrease in activity of *N*-acetyltransferase, and this would be consistent with the idea [32] that the fall in serotonin *N*-acetyltransferase is a consequence of the drop in cyclic AMP levels.

The present work provides further evidence for sympathetic control of pineal gland activity. When the norepinephrine reuptake inhibitor DMI is present, the onset of darkness via activation of β-adrenergic receptors rapidly raises cyclic AMP concentrations in the pineal. Why the uptake inhibitor must be present to demonstrate the effects of sympathetic nerve activity on levels of pineal cyclic AMP, but not activity of the pineal enzymes serotonin *N*-acetyltransferase [33, 34] or hydroxyindole-*O*-methyltransferase [35], is not readily apparent. It may be that increased sympathetic nerve activity affects the 'turnover' of the cyclic nucleotide so as to cause increases in the activity of these enzymes. Such changes in cyclic AMP turnover may occur without measurable alterations in the concentration of the cyclic nucleotide.

In conclusion, it appears that blockade of NE reuptake may be a necessary condition to demonstrate effects of increased sympathetic nerve activity on end organ concentrations of cyclic AMP.

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