

NYCTOHEMERAL RHYTHM IN MELATONIN RESPONSE
TO ISOPROTERENOL *IN VITRO*: COMPARISON OF
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Abstract—1. The *in vitro* response of melatonin synthesis was assessed by incubation of individual whole pineal glands for 4 hr without or with different concentrations of isoproterenol in the medium.
2. Pineals were taken either at the end of the 14-hr light phase (day), or at 6 hr into the 10-hr dark phase (night) after a 30-min exposure of the animals to light just before sacrifice at night.
3. The response was greater in pineals taken at night than in those taken at the end of the light phase in rats, but was absent at the end of the light phase in Syrian hamsters.
4. Hamster pineals taken at night responded, though higher isoproterenol concentrations were required than in the rats.
5. Unresponsiveness of hamster pineals during the day may explain previous failure of isoproterenol administration to stimulate pineal MEL content in this species.

INTRODUCTION

Pineal melatonin (MEL) synthesis in rats rises in response to beta-adrenergic agonists applied *in vitro* or administered *in vivo* during the day (Klein *et al.*, 1971; Deguchi and Axelrod, 1972; Brownstein *et al.*, 1973; Axelrod, 1974; Parfitt and Klein, 1976). In these experiments, isoproterenol (ISO) was commonly used as the beta-agonist because its action is not influenced by the nerve ending uptake mechanism (Parfitt and Klein, 1976). In contrast to the studies in rats, injection of ISO into Syrian hamsters (with intact or denervated pineals) during the day did not increase pineal melatonin content (Lipton *et al.*, 1982). These results seem incongruent with the previously shown ability of injections of the beta-antagonist propranolol to block the normal *in vivo* nocturnal surge of melatonin content in the Syrian hamster pineal (Lipton *et al.*, 1981). However, the results in the hamster could be explained by a day/night difference of pineal end-organ sensitivity to the beta-adrenergic action of the endogenous sympathetic neurotransmitter sensitivity to a beta-agonist could be demonstrated at night when the sympathetic nerves to the pineal ordinarily mediate the nocturnal surge of melatonin synthesis (Bowers and Zigmond, 1980; Vaughan and Reiter, 1986). The purpose of the present study was to determine whether the pineal melatonin response to ISO *in vitro* differs between the end of the light phase and 6 hr into the dark phase.

MATERIALS AND METHODS

Adult male Sprague Dawley rats (*Rattus norvegicus*) and Syrian hamsters (*Mesocricetus auratus*) were adapted for

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more than 2 weeks to a light cycle with darkness between 2000 and 0600 hr. Animals were sacrificed by guillotine at the end of the light phase (2000 hr) or at 0230 hr and the pineals were taken for incubation. For the sacrifice at 0230 hr, the animals were brought into the light 30 min prior to sacrifice in order to lower *in vivo* pineal melatonin production acutely to near-daytime levels (Rollag *et al.*, 1980). Pineals were incubated individually as described (Vaughan *et al.*, 1986) in 1 ml Minimal Essential Medium containing 10% fetal calf serum at pH 7.4 and 37°C under an atmosphere of 95% O₂ and 5% CO₂, without or with isoproterenol (ISO) at one of several tenfold different concentrations ranging from 10⁻⁸ to 10⁻⁵ M for pineals taken at 2000 hr or from 10⁻⁹ to 10⁻⁶ M for those taken at 0230 hr. There were 5-6 pineals in each dose group. At the end of the 4-hr incubation, melatonin (MEL) was determined by radioimmunoassay (Vaughan *et al.*, 1985) in the medium, and in the pineal glands after sonication. The least detectable MEL was 500 pg/ml medium (rat), 50 pg/ml medium (hamster), and 10 pg/pineal. MEL was undetectable in media incubated without a pineal and with ISO at the highest concentration. For graphic purposes, best fit curves were drawn from a three-parameter exponential regression of the MEL data against ISO concentration, which allows a sigmoid plot with respect to log concentration, and data were analyzed by *t*-tests with the Bonferroni correction for multiplicity of comparisons, analyses of covariance, and in one case a two-way analysis of variance (Dixon, 1983).

RESULTS

Figure 1 (upper panels) shows that after 4-hr incubation of rat pineals with ISO, MEL content in the medium (though responding to ISO in both day and night experiments) was more elevated for pineals taken at 0230 hr (night) than for those taken at 2000 hr (day). No difference between day and night was seen for MEL remaining in the incubated pineals. A two-way analysis of variance (not shown) compared medium MEL between unstimulated (zero ISO) and stimulated (pool of ISO 10⁻⁷ and 10⁻⁶ M) pineals, each with respect to the two times of col-

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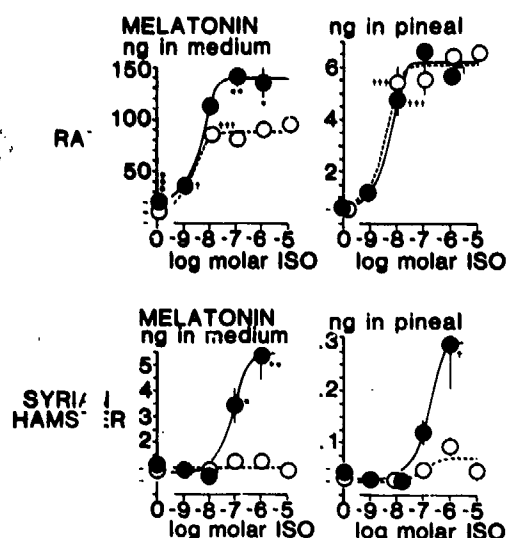


Fig. 1. Melatonin ($\pm$ SE) in the medium or pineal after a 4-hr incubation of individual pineals in 1 ml medium without (zero abscissa point) or with various concentrations of isoproterenol (ISO). Pineals were taken at the end of the light phase (2000 hr, open circles) or 6½ hr after the beginning of the dark phase (0230 hr) just after 30 min exposure of the animals to light (closed circles). * $P < 0.05$, ** $P < 0.01$ vs 2000 hr group at the same ISO concentration; + $P < 0.05$, +++ $P < 0.001$ vs respective group with zero ISO.

lection of the pineals. As expected, the main effects of ISO and time of collection were significant. In addition, the significant interaction ($P < 0.01$) indicated that for rat pineals the stimulation of MEL in the medium in the presence of ISO was different (greater) at night from that at the end of the day.

Figure 1 (bottom panels) shows that hamster pineals taken at 2000 hr did not respond to ISO, in that none of the medium or pineal MEL mean values for groups with ISO significantly differed from those of respective controls without ISO. However, medium and pineal MEL for hamster pineals taken at 0230 hr rose with ISO exposure. Even at night, hamster pineals were less sensitive to ISO than were rat pineals, in that the minimal dose producing elevation of medium MEL in hamsters and rats differed by two orders of magnitude. In addition, all mean MEL values for rats were more than tenfold greater than the respective ones for hamsters.

Figure 2 shows that for increasing amounts of MEL in the pineal at the end of the incubation, the proportion of MEL in the medium decreased (common slope $P < 0.001$ for either species). Analysis of covariance showed that this relationship was different between night and the end of the day, in that in either species, for a given amount in the pineal, a greater proportion of the total MEL was in the medium (and consequently a lesser proportion in the pineal) at night. For hamsters, the day/night difference in distribution between the medium and pineal was not evident at the lower amounts in the pineal but this difference was found over the whole range of observed post-incubation pineal MEL in the rats.

DISCUSSION

After incubation, the quantities of MEL remaining in the glands represented only 3–7% of the total amount present after incubation with or without ISO. Furthermore, in ISO-exposed groups with medium MEL content elevated above the level seen in the comparison group without ISO, the amount of MEL remaining in the ISO-exposed glands was not less than that seen in glands incubated without ISO in either species. Thus, the elevation of medium MEL in groups incubated with ISO represents a response of MEL synthesis, not a net loss of intrapineal MEL. It is evident also from another perspective that the amount of MEL in the medium (in ng quantities) represents MEL synthesized during incubation, because even in the groups incubated without ISO, the mean amounts remaining in the pineal were the same as (hamsters) or in excess of (rats) the usual daytime pineal MEL content in glands removed without subsequent incubation in this laboratory (Vaughan *et al.*, 1985).

For rats, the response to ISO, present at the end of the light phase, was even greater at night. For hamsters, a significant response occurred only in the pineals taken during the night, with no response of end-light-phase pineals even at an ISO dose two orders of magnitude higher than the lowest dose producing a response in medium MEL from pineals taken during the night. Whether even higher doses of ISO would stimulate daytime or further stimulate night-time pineals from hamsters was not assessed. Also, since only one incubation time (4 hr) was used, it was not assessed whether a time delay in response to ISO contributed to the observed reduction in response at 2000 hr (vs that at 0230 hr) in either species. There is evidence in the rat that the pineal *N*-acetyltransferase (NAT) responses to injected ISO (Deguchi and Axelrod, 1972) and to electrical stimulation of the cervical sympathetic trunk (Bowers and Zigmond, 1982) are delayed during the day as compared to the night. However, even those daytime responses were evident by 2 hr.

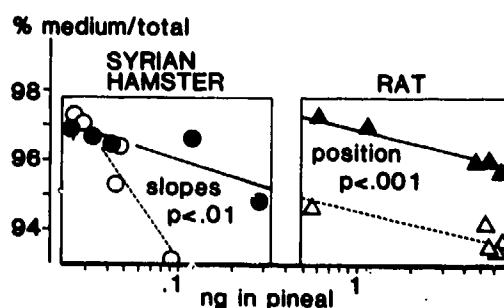


Fig. 2. Mean proportion of melatonin in the medium (from the data in Fig. 1) expressed as a function of the amount of melatonin in the pineal after incubation. The total refers to the combined amount of melatonin in the medium and pineal. Open symbols represent day pineals (2000 hr), and closed symbols night pineals (0230 hr). The difference between day and night slopes is indicated for the hamsters. While the day and night slopes were not significantly different for the rats, the ordinate position of the night values was higher.

The reduced sensitivity of hamster pineals (higher doses of ISO required for a response than in rat pineals) and the lower overall MEL values for hamster incubations might have resulted from an effect of light exposure of the animals (including the 30-min exposure at night) prior to obtaining the pineals. Though it has not yet been adequately tested, we must consider the hypothesis that light exposure itself may have produced a signal in hamsters resulting in less ability of their pineals to respond subsequently, during incubation, to the residual endogenous neurotransmitter (without ISO) and exogenously added ISO. Such an effect in the hamsters (absent or less marked in the rats) might be suspected, because in the conditions of the present study, the highest *in vitro* intrapineal MEL content achieved at night for rats (about 6 ng) was higher than the normal *in vivo* peak nocturnal pineal MEL content (about 2 ng); whereas, for hamsters, the highest observed *in vitro* value (0.3 ng) was lower than the usual mean *in vivo* nocturnal peak (about 1 ng in this species). The *in vivo* values (Vaughan *et al.*, 1985) were obtained without nocturnal exposure of the animals to light. Other recent experiments (Vaughan *et al.*, 1986) have also demonstrated nocturnal responsiveness and daytime unresponsiveness to norepinephrine in Syrian hamster pineals; the somewhat smaller pineal weight in hamsters (25% of that in rats) did not fully account for the much greater difference of *in vitro* norepinephrine-stimulated MEL production between species after light exposure.

The altered distribution of MEL between gland and medium after incubation (propensity for a greater proportion to be in the medium for a given amount in the pineal at night than at the end of the day) in both species is not yet understood. However, one explanation might be that there is a mechanism to enhance transport of MEL out of the pineal gland and that this mechanism is more active at night. Whether these *in vitro* observations reflect a more active *in vivo* MEL secretory process at night is not yet known.

The end of the light phase was chosen for measuring daytime sensitivity, because in rats this was the time of greatest sensitivity of pineal NAT stimulation by ISO during the light phase (Romero and Axelrod, 1974). In the present studies, the *in vitro* pineal response of MEL synthesis to beta-adrenergic stimulation for 4 hr was greater $6\frac{1}{2}$ hr into the dark phase than at the end of the light phase in rats, and it was absent at the end of the light phase in hamsters under the conditions of the present study. Hamster pinea's taken at night responded (though with less sensitivity than those of rats). Whether this represents a relative receptor or post-receptor defect for beta-adrenergic stimulation during the day is not yet known. However, a contributory role for a nyctohemeral difference in receptors is likely in that pineal beta-receptor density was lower during the day than at night in the rat (Esquifino *et al.*, 1985). Whatever the mechanism, the relatively profound unresponsiveness of hamster pineals during the day may explain the lack of *in vivo* daytime pineal MEL response to injected ISO in this species during or near the end of the light phase (Lipton *et al.*, 1982; Reiter, personal communication). In conformity with

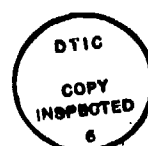
the present demonstration of *in vitro* responsiveness at night, *in vivo* elevation of pineal MEL content 1-3 hr after ISO injections in Syrian hamsters acutely exposed to light at night has recently been achieved (Reiter, personal communication; Vaughan, unpublished observations).

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