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Circadian Pineal Modulation of Pituitary Effect on Murine Corticosterone *In Vitro*[1]

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SÁNCHEZ DE LA PEÑA, S., F. HALBERG, F. UNGAR, E. HAUS, D. LAKATUA, L. E. SCHEVING, E. SÁNCHEZ AND P. VECSEI. *Circadian pineal modulation of pituitary effect on murine corticosterone* in vitro. BRAIN RES BULL **10**(4) 559–565, 1983.—An old controversy is resolved as a novel effect: In a rhythmic fashion, aqueous pineal homogenate (APH) enhances, attenuates or leaves unaffected the production of corticosterone by mouse adrenals incubated with pituitary media. All glands stem from the same circadian stage in these (isophasic) studies on 72 female CD2F$_1$ mice, standardized for two weeks in L 0600–1800 and D 1800–0600. Every 4 hours during a 24-hour span, 12 mice were killed. Pineals were removed for the preparation of APH and stored at 4°C. Hypothalami, pituitaries and adrenals were removed, bisected and placed in wells containing 1 ml Krebs-Ringer buffer (K), at 4°C, until incubation. At each circadian stage, bisected adrenals were incubated with 95% O_2 and 5% CO_2 at 37±1°C for 5 hours, with K only or with the addition of 0.05 IU ACTH 1–17 or APH or with isophasic pituitary or hypothalamic preincubation media with and without APH or muscle. Media were stored at −20°C until corticosterone RIA. A circadian rhythm ($p<0.05$) characterized corticosterone production after stimulation by the pituitary alone or with APH. The overall modulatory effect of APH is an increased circadian amplitude of adrenal corticosterone production, in response to the isophasic pituitary.

Pineal Pituitary Adrenal Circadian Corticosterone Modulation Mouse
Heterophasic sequential incubation Isophasic sequential incubation

BY an approach based upon the inspection of recordings of activity and rest, so-called behavior-day charts, phase responses and characteristics of free-running activity rhythms in the golden hamster have been reported to be independent of the pineal gland [2]; however, more generally, with respect to hormones, it has been suggested that a coherent picture of the temporal organization of vertebrates must take into account the way in which the neuroendocrine systems interact in generating intermodulating circadian rhythmicity [28]. Herein, such interactions between the murine pituitary, pineal and adrenal are studied by *in vitro* incubation [22] against the background of earlier work demonstrating a circadian adrenal cycle *in vivo* [7–10, 12, 13] and *in vitro* [8, 29, 30]. We explore adrenal cortical functions in the absence of the many extraglandular agents, known and unknown, impinging upon the glands *in vivo*, except for those present at the time of tissue harvest. In our hands [23–25], the incubation assay used [22] reproduces the *in vivo* state faithfully, to an extent similar to that of isolated cells [26]. A comparison of these methods with other useful approaches, e.g., by superfusion [3] or organ culture [1], remains beyond our scope. We here follow up on recent studies [24] suggesting that, as a function of the stage of rhythms with several frequencies in the pineal, pituitary and adrenal, aqueous pineal homogenates (APH) *in vitro* modulate (enhance or attenuate) corticosterone production of bisected mouse adrenals in response to pituitary preincubation media.

METHOD

Seventy-two female CD2F$_1$ mice, 8 weeks of age, were standardized for over 1 week, 2 per cage, in L 0600–1800 D 1800–0600, with food (Purina Laboratory Chow) and water freely available at a room temperature of 24±1°C and ~50% humidity. Twelve mice were killed by decapitation with scissors every 4 hours during a 24-hour span (at each of 6 circadian stages 02, 06, 10, 14, 18 and 22 *h*ours *a*fter *l*ight *o*nset, HALO, on March 8–9, 1982). Pituitaries, hypothalami and adrenals were cleaned and bisected and two pituitaries, one hypothalamus or 4 halved adrenals each were placed in

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(separate) plastic wells containing 1 ml of Krebs-Ringer buffer (pH 7.35) with 0.01 M glucose and albumin (1%) added (KRG) in an atmosphere of 95% O_2 + 5% CO_2 at 37±1°C. The pineals of all 12 mice were cleaned, pooled and homogenized with 1.2 ml of 0.9% NaCl solution (APH).

The following tissues were "preincubated" for 30 minutes: pituitaries (2) with 1 ml of KRG, hypothalamus (1) + 1 ml of KRG, pituitaries (2) with APH (0.1 ml) and 0.9 ml of KRG, hypothalamus (1) with APH (0.1 ml) and 0.9 ml KRG. The preincubation fluid was then removed and stored at 4°C.

The adrenal pools (4 halves) were first incubated for 1 hour in 0.5 ml of KRG. The first-hour incubation fluid was then removed and frozen at −20°C. The fluid was replaced immediately by 0.3 ml of KRG containing 0.2 ml of the preincubation fluid of pituitaries (2 wells), pituitaries and an ~20-mg fragment of masseter muscle (1 well), pituitaries + APH (2 wells), hypothalamus (1 well), and hypothalamus + APH (1 well), or were replaced by 0.5 ml KRG containing 0.025 ml APH (2 wells) or 0.05 IU of ACTH 1–17, kindly provided by Hoechst Italy and tested by us earlier [7] (1 well) or by KRG alone (2 wells). Every hour for the next 4 hours, the incubation fluid was removed, frozen and replaced by the same treatment. Within 10 minutes after completion of each incubation, the medium was stored at −20°C for corticosterone RIA. This procedure was repeated at each of the six timepoints of the study. The data were analyzed by the fit of a 24-hr cosine curve (Fig. 1), yielding the parameters shown in Fig. 2 [11].

RESULTS

A statistically significant circadian rhythm ($p<0.05$) was found to characterize corticosterone production after stimulation by pituitary alone (2nd to 5th hr of incubation) and by pituitary and APH (2nd, 4th and 5th hr of incubation). The circadian rhythm parameters as obtained by single cosinor [11] are shown in Table 1. It can be seen that the percent rhythm, i.e., the percentage of the overall variability accounted for by the fit of a single 24-hour cosine curve, varied among series. When extensive data were available, as in the case of incubation with Krebs-Ringer only, documented by 48 data points, the (percentage) rhythm (of 22) was significant below the 1% level. In the case of series documented more sparsely, the percentage rhythm was at least 12 or 22%; yet in the majority of series, sparse documentation notwithstanding, it was ≥50%. Accordingly, the p-values are significant below the 5% level in 9 of the 11 series.

On this basis, it seems reasonable to fit cosine curves to each series separately; these are presented in Fig. 3 for the series involving the incubation of adrenals with pituitary preincubation medium only or with both pituitary preincubation medium and APH. On the left and in the middle of Fig. 3, results are separated for each hour of incubation. Thus, one can see that the large differences occur during the fourth hour of incubation when the pineal response at 10 HALO reaches a rather high value. The actual data underlying the fit in the 4th hour of incubation are shown in Fig. 4. The scale in this figure should be noted, to see the difference at 10 HALO at its face value in conjunction with the finding of no-difference at 2 HALO and a difference of opposite sign at 18 HALO as compared to the effect at 10 HALO.

Figure 3 (right) compares results pooled for all treatments of a given kind. This figure visualizes, as does Fig. 4, that a pineal effect upon adrenal responsiveness varies with time, from intensification at certain times to the point of attenuation

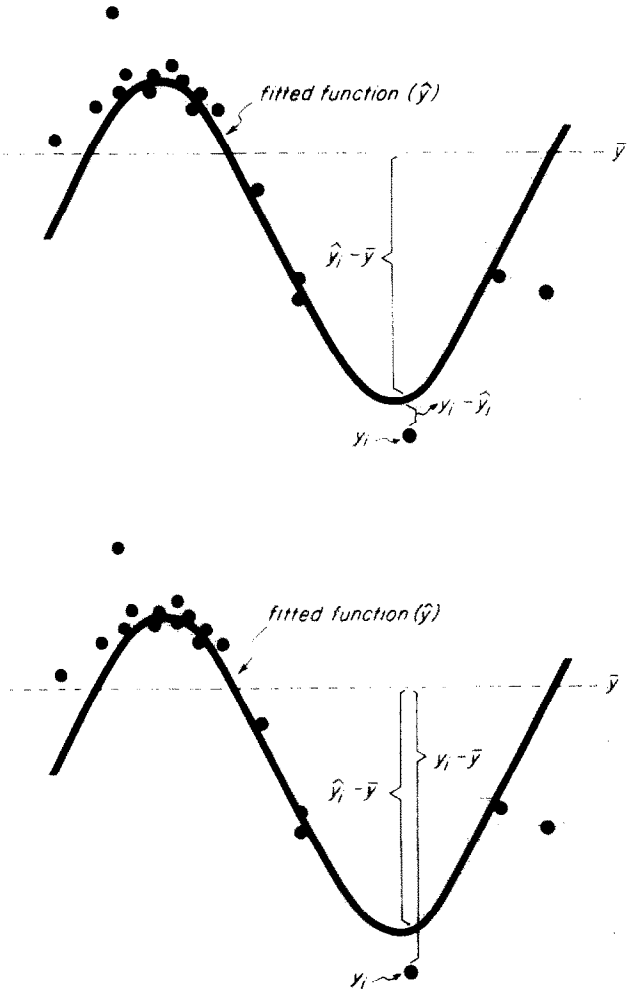

FIG. 1. Rhythm description by zero-amplitude test yields a p-value (top) and a percentage rhythm (bottom).

Indices of rhythm's statistical significance, p-value obtained by computing:

$$F = \frac{\Sigma_i(\hat{y}_i - \bar{y})^2/2}{\Sigma_i(y_i - \hat{y}_i)^2/(N-3)}$$

where y_i = datum, $\bar{y}$ = arithmetic mean, $\hat{y}_i$ = value of fitted function and N = number of data in series.

If $F \geq F_{.95}(2, N-3)$, rhythm is considered statistically significant.

Percent rhythm, PR, percentage of overall variability of data (y_i) about arithmetic mean ($\bar{y}$) attributable to rhythm defined by fitted function ($\hat{y}$):

$$PR = 100 \times \Sigma_i (\hat{y}_i - \bar{y})^2 / \Sigma_i (y_i - \bar{y})^2$$

$$\text{i.e., } 100 \times \left(\frac{\text{sum of squared deviations, from mean, of values derived from fitted function at each sampling time}}{\text{sum of squared deviations of data from mean}} \right)$$

= 100 × variability ratio. Note that PR and P-value are related by expression:

$$P = \left(1 - \frac{PR}{100} \right)^{\frac{N-3}{2}}$$

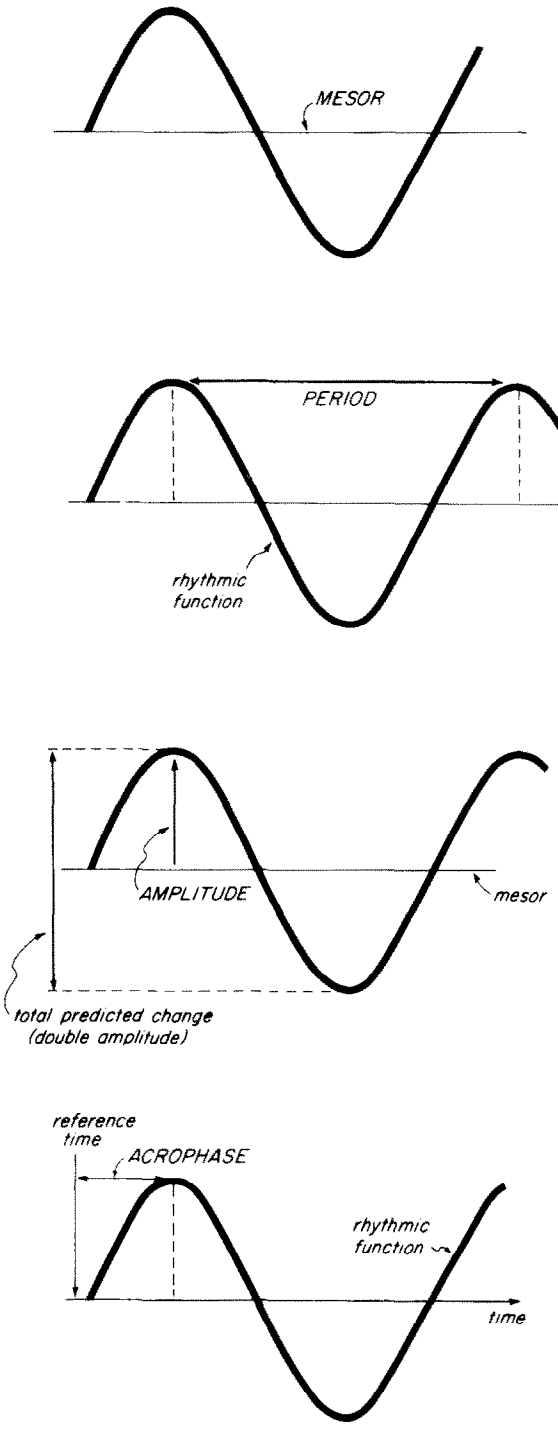

FIG. 2. Rhythm characteristics, e.g., obtained by equating the period to 24 hours for a so-called linear least-squares analysis. The mesor, amplitude and acrophase are each obtained with confidence limits, given in tables (not shown in this figure). Definition of rhythm parameters: Mesor, M, rhythm-adjusted mean (midline-estimating statistic of rhythm), defined as average value of rhythmic function (e.g., cosine curve) fitted to data; expressed in same units as original data. Note that mesor will differ from arithmetic mean if data unequidistant (e.g., concentrated near crest of rhythm) and/or cover non-integral number of cycles. Period, τ, duration of one complete cycle in rhythmic function; expressed in time units, such as seconds, hours, days or years, or in physiologic units such as complete cardiac, respiratory or menstrual cycle; equated to 360° for angular expression of acrophase. Amplitude, A, half of total predictable change in rhythm, defined by rhythmic function fitted to data; expressed in original or "relative" units, e.g., as percentage of series mean or mesor. Acrophase, Ø lag from reference time of rhythm's crest-time, defined by rhythmic function fitted to data; usually expressed in (negative) degrees, with 360°=period, 0°=reference time; customary time units (e.g., clock-hours and minutes, days, weeks, months or years) or physiologic units (e.g., number of heart beats, respiratory or menstrual cycles) also appropriate for rhythm synchronized with corresponding period.

at other times. The major overall effect of the pineal during the 4 hours of incubation is an increase in the circadian amplitude of corticosterone production, perhaps accompanied by a lesser increase in mesor.

Table 2 shows this rhythmic pineal interaction with the equally rhythmic effect of the pituitary upon the adrenal. In a parameter test, the difference is of borderline statistical significance, the p-values being 0.088 and 0.054. The data are not normally distributed, however; the fit of a sinusoidal curve can serve only as a first approximation (Fig. 3). A two-way analysis of variance on the log-transformed data pool for all 4 incubation hours in the lower section of Table 2 reveals the statistically highly significant effect of both treatment (presence or absence of APH addition to the pituitary incubation) and circadian stage. Table 2 shows further that the treatment-time interaction during these 4 hours of incubation is also statistically highly significant. Against this background, the difference shown in Fig. 3 (right) can be regarded as established, the deviation from sinusoidality notwithstanding.

Tables 3 and 4 present the results of control incubations. When preincubation media of muscle were added to the pituitary preincubation medium and the adrenal response was studied, both the mesor and the amplitude were rather similar to the values found with only the addition of pituitary preincubation media (Table 3).

Table 4 presents results of incubation, during 4 consecutive hours, of bisected adrenals with media from preincubations with hypothalamus alone. These media have no obvious effect with the design here employed. The rhythm characteristics are similar to those with Krebs-Ringer alone, shown in Table 1.

DISCUSSION

In a follow-up study, performed in April 1982 under the conditions described herein on 8-week-old female B6D2F$_1$/J (rather than CD2F$_1$) mice, a rhythmic circadian pineal modulation of the pituitary effect upon the adrenal corticosterone produced *in vitro* was reproduced. The principal statistically significant change exerted by the pineal again involved the circadian amplitude. In the absence of any effect upon the mesor, this effect means a rhythmic change involving attenuation, no effect and amplification by the pineal of the pituitary effect upon the adrenal. During the third hour of incubation, this rhythmic effect was most prominent. In terms of ng of corticosterone/ml/hr, the effect of the pituitary alone was associated with an amplitude of 66 as compared with an amplitude of 221 for the effect of the pineal interaction with the pituitary.

In these studies of a pituitary-pineal interaction with re-

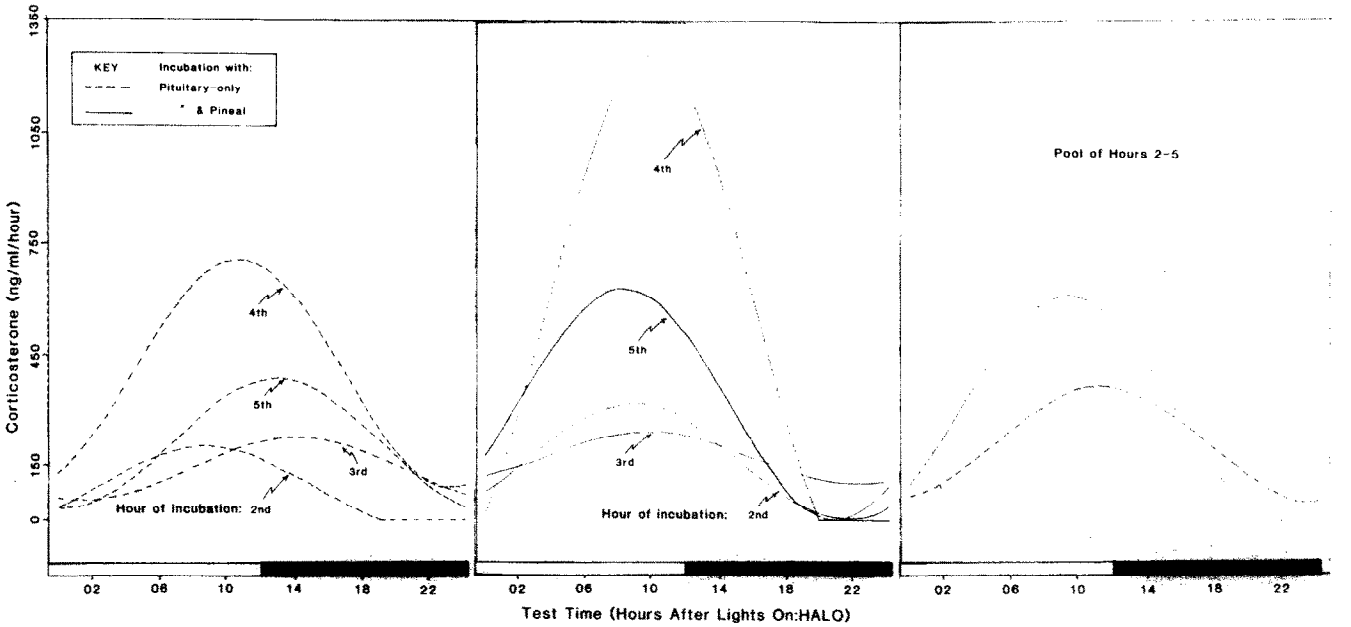

FIG. 3. Direct pineal modulative effect upon adrenal cortex and its marked time dependence upon circadian stage and hour of incubation. Original data illustrated in Fig. 4. Tables 1 and 2, summarize statistical significance of results displayed in Figs. 3 and 4, while Tables 3 and 4 show analysis of control data. Reconstructed curves of circadian pituitary-pineal interaction on murine adrenal corticosterone production *in vitro*. Adrenals removed from female $CD2F_1$ mice at each of 6 circadian test times incubated with tissues from same mice at same test time.

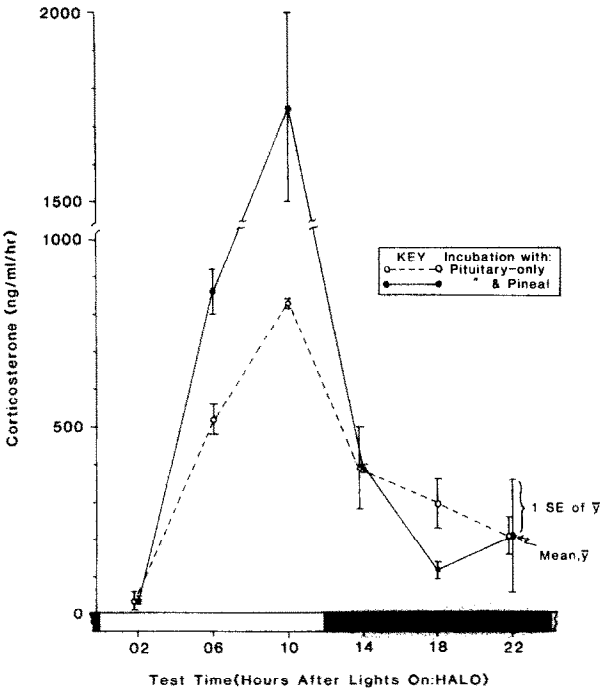

FIG. 4. Modulation by the pineal of adrenal function: at one time (1000 HALO), the pineal homogenate enhances; at another time (1800 HALO), it attenuates the ACTH effect upon corticosterone production by adrenal cortex *in vitro*, while there is no such effect at still another time (0200 HALO). Circadian pituitary-pineal interaction on murine adrenal corticosterone production during 4th hour of incubation *in vitro*. Adrenals removed from female $CD2F_1$ mice at each of 6 circadian test times incubated with tissues removed from same mice at same test time. Each plotted value represents 2 determinations.

spect to adrenal corticosterone production, any continuing effect of the hypothalamus beyond that present at the time of tissue harvest has been removed. Hence, *in vitro* studies of CRF release from the hypothalamus [4–6, 15, 16] provide an interesting background but are not directly pertinent to the effect here studied. A direct inhibitory effect of bovine pineal extract on the adrenal cortex is quite pertinent. The effect(s) studied by these authors and others, however, were not explored with respect to any systematic role of changes in response as a function of different circadian stages [14].

Feed-backs in neuroendocrinology are usually viewed without an explicit qualification in time. Here, we document a rhythmically changing effect of the pineal upon the circadian adrenal cycle previously studied in the context of pituitary-hypothalamic interactions [8]. We here report a temporally-qualified, non-hierarchical so-called feed-sideward by the pineal upon the pituitary in the absence of the hypothalamus *in vitro*.

In this feed-sideward, namely a rhythmic pineal interaction with the equally rhythmic response of the adrenal to the pituitary, multiple factors are likely to play a role, and *a priori* the response may not be specific. The extent of specificity was checked herein by the results of preincubations with (1) masseter muscle and (2) hypothalamus and pineal, but it will have to be checked further on a larger scale with many more tissues and eventually with pure agents (even if a circadian rhythm was not demonstrated herein for any interaction by muscle preincubation medium with the adrenal response to the pituitary, Table 3, or by preincubation of hypothalamus alone or with the pineal, Table 4).

The limitations of the studies here reported stem from the facts that (1) they focus only upon females; (2) the investigation bears only upon a single frequency, the circadian one; and (3) the interactions found describe glands all removed at

TABLE 1

INTERACTION FROM PINEAL HOMOGENATE WITH EFFECT OF PITUITARY INCUBATION FLUID UPON BISECTED ADRENALS *IN VITRO**

Incubation with (duration)	No. Data	Percent rhythm	*p*	Mesor ± SE¶	Amplitude ± SE¶	Acrophase ± SE (degrees)†
Krebs-Ringer (4 hrs)	48	22	0.004	29.4 ± 2.3	11.7 ± 3.3	−103° ± 16
Pineal homogenate (4 hrs)	47	12	0.064	25.3 ± 2.4	8.3 ± 3.4	−77° ± 24
ACTH 1–17 0.05 IU (4 hrs)	24	49	0.001	333.7 ± 39.7	251.4 ± 56.1	−176° ± 13
Pituitary 2nd (1 hr)	12	64	0.010	92.2 ± 19.2	109.1 ± 27.2	−123° ± 14
Pituitary 3rd (1 hr)	12	54	0.030	137.2 ± 18.9	86.8 ± 26.7	−201° ± 18
Pituitary 4th (1 hr)	12	70	0.004	395.8 ± 47.4	309.3 ± 67.1	−152° ± 12
Pituitary 5th (1 hr)	10	68	0.019	205.4 ± 32.4	177.7 ± 47.7	−185° ± 14
Pit + Pineal 2nd (1 hr)	12	68	0.006	165.0 ± 25.4	157.2 ± 35.9	−127° ± 13
Pit + Pineal 3rd (1 hr)	12	22	0.331	169.5 ± 30.7	68.8 ± 43.4	−138° ± 36
Pit + Pineal 4th (1 hr)	12	67	0.007	560.7 ± 115.9	705.0 ± 163.9	−141° ± 13
Pit + Pineal 5th (1 hr)	12	51	0.041	314.0 ± 73.4	316.1 ± 103.8	−118° ± 19

*In parameter comparison, a difference in amplitude during the 4th hour of incubation with and without the pineal homogenate added to the pituitary preincubation fluid is significant below the 5% level.

†360°=24 hr; 0°=light onset; LD12:12.

¶Expressed in ng/ml/hr.

one given circadian stage, whereas one should test at each adrenal incubation time, material from all possible stages of rhythms characterizing the interactions.

The isophasic work here reported involves sampling (at each timepoint) of different variables from animals all killed at the same stage of the lighting regimen, the synchronizer of rhythms under the conditions of this study. In the case of concomitant and/or sequential incubations or of extracts, e.g., of glands, however, the original starting material need not be obtained at each time point from tissues all harvested

TABLE 2

COMPARISON OF RHYTHM PARAMETERS FROM POOLED 4 HOURLY-INCUBATIONS OF BISECTED ADRENALS WITH PITUITARY PREINCUBATION MEDIA, ALONE OR WITH PINEAL HOMOGENATE (APH)

Index studied	Pituitary Alone	Pituitary With APH	*df*	F	*p*
N of points	46	48			
Data limits (ng/ml/hr):					
Low	7	10			
High	840	2000			
Percent rhythm	29	31			
p (zero-amplitude)	0.001	0.001			
			Parameter Comparison*		
Mesor ±SE (ng/ml/hr)	208 ± 26	302 ± 48	1,69	2.998	0.088
Amplitude ±SE (ng/ml/hr)	156 ± 38	307 ± 68	1,70	3.833	0.054
Acrophase ±SE (95% CL) (360°≡24 hr)	−162° ± 13 (−136, −188)	−133° ± 13 (−108, −158)	1,88	2.272	0.135
Amplitude, acrophase*			2,88	2.853	0.063
Mesor, amplitude, acrophase*			3,88	2.808	0.044
		Analysis of Variance*			
Source of variation					
R_x (pituitary without vs. with APH)			1	7.918	0.006
Circadian stage (C)			5	9.777	0.001
$R_x \times C$			5	3.604	0.005

*Homogeneity of variance rejected for original data here analyzed ($p<0.001$). Homogeneous variance assumed for multiple parameter (but not for single parameter) test results. Analysis of variance on log-transformed data.

TABLE 3

POOLING OF RHYTHM PARAMETERS FROM 4 INCUBATIONS (OF 1 HOUR EACH) OF BISECTED ADRENALS WITH MEDIA FROM PITUITARY PREINCUBATION ALONE OR WITH PREINCUBATION MEDIA OF MUSCLES AND PITUITARY

Index studied	Pituitary Alone	Pituitary With muscle			
N of points	46	21	*df*	F	*p*
			Parameter Comparison*		
Mesor (ng/ml/hr)	208	221	1,30	0.0561	0.8144
Amplitude (ng/ml/hr)	156	136	1,32	0.0977	0.7666
Acrophase (360°≡24 hr)	162°	−143°	1,26	0.5294	0.4734
Amplitude, acrophase			2,61	0.4166	0.6611
Mesor, amplitude, acrophase			3,61	0.2709	0.8461

*Variances homogeneous: F-test of homogeneity of variance: $F(43,18)=0.75061$; $p=0.43314$.

TABLE 4

RHYTHM PARAMETERS FROM POOLING OF 4 INCUBATIONS (OF 1 HOUR EACH) OF BISECTED ADRENALS WITH MEDIA FROM PREINCUBATIONS OF HYPOTHALAMUS-ALONE OR WITH PREINCUBATIONS OF PINEAL AND HYPOTHALAMUS

Index studied	Hypothalamus Alone	Hypothalamus With pineal
No. of points	23	22
Data limits (ng/ml/hr):		
Low	1	2
High	99	92
Percent rhythm	13.4	12.6
p (zero-amplitude)	0.236	0.303
Mesor ±SE (ng/ml/hr)	24.69 ± 5.27	31.54 ± 5.4
Amplitude ±SE (ng/ml/hr)	13.41 ± 7.61	12.6 ± 8.0
Acrophase ±SE (360°≡24 hr)	−99° ± 31	−105° ± 33

only in the same HALO stage. HALO-heterophasic studies of pineal pituitary and adrenal interactions [23] are also available, but remain to be extended to the conditions of the study here reported.

Since the modulated responses occur *in vitro*, interactions from neural and humoral factors other than those immediately related to the glands tested are removed. Clearly, intraglandular factors present within the adrenal, pituitary and pineal at the time of gland harvest play a critical role in the responses. Individual contributions of each gland, however, remain confounded in such isophasic work.

A first step toward the isolation of individual contributions by each structure tested is to keep all but one constant and to test variations in the remaining one. In this study, we replaced only the pituitary with a factor that may be partly, if not largely, responsible for the effect upon the adrenal, namely ACTH. For control incubations, an analogue of the natural pituitary hormone, ACTH 1–17, is used in a fixed dose, Table 1.

Interactions of APH, hypothalmi, bisected pituitaries and ACTH 1–17 effects upon B6D2F$_1$ mouse adrenals have been noted elsewhere [24]. Separate tests at 6 circadian stages reveal a circadian stage-dependence of the ACTH 1–17 effect and pineal interaction with it. The pituitary effect can thus be largely duplicated by just one pituitary factor, in at least these particular hybrid mice. The contributions of the pineal may stem from one or several factors. Pineal 5-hydroxytryptamine, melatonin [19–21] and angiotensin I, in our hands (unpublished), among other agents, all undergo circadian rhythms and are candidates for the effects of the pineal homogenate here recorded.

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