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The insect neuropeptide prothoracicotropic hormone is released with a daily rhythm: Re-evaluation of its role in development

(*Rhodnius prolixus*/neurohormones/ecdysteroid/circadian rhythm/molting)

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ABSTRACT **Prothoracicotropic hormone (PTTH) is the central cerebral neurohormone in insect development. Its release has been believed for decades to be confined to one (or two) critical moments early in each developmental stage at which time it triggers prolonged activation of the prothoracic glands to synthesize and release the steroid molting hormones (ecdysteroids), which elicit developmental responses in target tissues. We used an *in vitro* assay for PTTH released from excised brains of the bug *Rhodnius prolixus* and report that release of PTTH does occur at the expected time on day 6, but that this release is merely the first in a daily rhythm of release that continues throughout most of the 21 days of larval–adult development. This finding, together with reports of circadian control of ecdysteroid synthesis and titer throughout this time, raises significant challenges to several features of the current understanding of the hormonal control of insect development. New questions are raised concerning the function(s) of PTTH, its relationship with the prothoracic glands, and the significance of circadian rhythmicity throughout this endocrine axis. The significance of the reported observations derives from the set of entirely new questions they raise concerning the regulation of insect development.**

The insect prothoracicotropic hormone (PTTH) is one of the oldest known neurohormones. In 1922 it was reported (1) that the brain was necessary for the inception of metamorphosis in a caterpillar. This finding was extended using the hemimetabolous blood-feeding bug, *Rhodnius prolixus*, in which a "brain hormone" was found to be essential for larval-adult development (2) by activating the prothoracic glands (PGs) to secrete the steroid molting hormones (3), now known as ecdysteroids. Release of PTTH from the insect brain is thought to occur at only one or two discrete moments early in each larval instar and to induce sustained activation of steroidogenesis in the PGs (4–6). These ecdysteroids elicit the developmental changes in target tissues by inducing temporally precise sequences of gene expression. Although much has been learned recently of the molecular mechanism of gene activation by ecdysteroids (7), the manner by which these responses are orchestrated into temporal patterns during development, either within a cell or among groups of cells, is essentially unknown.

The present work uses an *in vitro* assay for PTTH (8) released from isolated brain-retrocerebral complexes of *Rhodnius* throughout larval–adult development. We report that PTTH is released with a daily rhythm that is maintained for at least 11 consecutive days, indicting that the function of PTTH extends beyond an initial activation of the PGs. This finding, taken together with reports that the synthesis and titer of ecdysteroids are regulated by a circadian clock in the PGs (9–11), raises previously uncontemplated questions concerning the functions and regulation of these two hormones and, consequently, the endocrine regulation of development. The discovery of pronounced circadian rhythmicity throughout this endocrine axis raises further new questions concerning its function and possible relationship with the temporal patterns seen in target tissues during development.

MATERIALS AND METHODS

Animals. Male fifth (last) instar larvae of *Rhodnius prolixus* were reared at 28 ± 0.5°C in 12-hr light: 12-hr dark regime. Larval–adult development was initiated with a large blood meal. The day of feeding is designated day 0, and ecdysis to the adult occurs around day 21. Brain complexes were extirpated at 6-hr intervals (at 1, 7, 13, and 19 hr after lights off, unless otherwise stated) on the days specified. The time of feeding on day 0 had no influence on the results.

***In Vitro* Assay for Released PTTH.** Brains were excised under saline (12) with the retrocerebral complex (corpus cardiacum and corpus allatum) attached. This tissue complex is termed a brain complex. Each complex was washed thoroughly and then incubated in a 25-μl standing drop of saline for 4 hr. The electrophysiological properties of the *Rhodnius* brain complex in this saline are indistinguishable from those in hemolymph (13). Incubations were performed in room lighting when the donor animal was in the photophase and in darkness when the donor was in the scotophase. Incubation media were then heated to 100°C for 2 min and stored at −80°C until further use. The PTTH activity of incubation medium was assayed by its ability to augment synthesis of ecdysteroids by *Rhodnius* PGs *in vitro* (see ref. 8). The specificity of this assay for PTTH has been documented in dose–response protocols (8). PGs for assay of PTTH were from day 7 animals maintained in continuous light for >3 weeks before the blood meal; continuous light eliminates the endogenous rhythmicity of ecdysteroid synthesis by PGs (14), and PGs are maximally responsive to PTTH on day 7 (8). PGs were excised as described earlier (15) under the same saline as that used for brain complexes. This saline has been extensively used for *in vitro* incubation of PGs (15). Test samples were assayed in groups of 60–80 on PGs excised at the same time of day (± 1 hr). One PG from each animal was incubated with test medium for 4 hr (unless noted otherwise), and the contralateral PG was incubated in saline alone as control. Ecdysteroid content of PG incubation medium derives entirely from synthesis (15) and was quantified by RIA (below). The degree of stimulation of synthesis by test medium was evaluated using two complementary methods. (*i*) The difference in amount of synthesis [ng of 20-hydroxyecdysone (20E) equivalents/4 hr] between treated and control members of each PG pair was calculated and expressed as mean difference ± SEM for a group of pairs. (*ii*) These differences in synthesis in a group of pairs were compared using the paired-sample *t* test (two-tailed), and the

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Abbreviations: HCP, head critical period; PG, prothoracic gland; PTTH, prothoracicotropic hormone; 20E, 20-hydroxyecdysone; SI, stimulation index; Ar, activation ratio.

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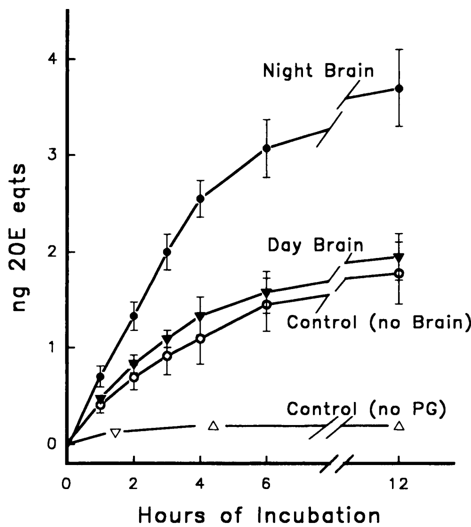

FIG. 1. Time-course of ecdysteroid synthesis by day-7 PGs incubated in saline alone (control, no brain, ○) or in medium containing material released from day-11 brain complexes excised during the scotophase at 7 hr after lights-off (night brain, ●) or the photophase at 7 hr after lights-on (day brain, ▼). Complexes were incubated singly for 4 hr. One member of each PG pair was then incubated in this medium, while the contralateral PG was incubated in saline alone (control). Data from control PGs for day brains and night brains are combined for clarity (○). After each time point, PG medium was replaced with fresh saline; therefore, test PGs were exposed to material released from brain complexes for only the first hour of incubation. One-hour exposure yields maximal stimulation of steroidogenesis (8). Leakage of endogenous ecdysteroids from scotophase brains alone is minimal (control, no PG). Each point is mean ± SEM of n = 6 separate incubations, except control PG, where n = 12. eqts, Equivalents.

resulting t value was plotted as a Stimulation Index (SI) (8). SI reveals the level of significance of the stimulation calculated in the first method. For comparison, Activation Ratios (Ars) were also calculated for one experiment. Ar is the synthesis of stimulated PGs divided by synthesis of control PGs (16).

RIA for Ecdysteroids. Ecdysteroids were quantified by RIA using H3 antiserum, which recognizes both ecdysone and 20E (17), which are the major molecules synthesized by *Rhodnius* PGs *in vitro* (ref. 15 and unpublished work). The labeled ligand was α-[23,24-^{3}H(N)]ecdysone (88.6 Ci/mmol; 1 Ci = 37 GBq) (New England Nuclear). Results are expressed as 20E equivalents, as this steroid was used as the standard (Sigma). The RIA procedure has been described (18).

Proteolysis of PTTH. Twelve brain complexes were excised at each of 1 hr and 7 hr after lights-off on day 11. Complexes were incubated in pairs in 50 μl of saline for 4 hr. The incubation medium was then divided into two equal parts. One-half was stored at −80°C for use as control; the other half was digested with pronase E at 1 mg/ml (Sigma) at 37°C, heated to 100°C for 2 min, and then stored at −80°C until used. The digested and undigested halves of medium were assayed for PTTH activity on the paired PG assay described above.

PTTH Extraction from Hemolymph. Hemolymph was collected through an amputated leg at 4°C, diluted with an equal volume of saline, and promptly subjected two times to ultrafiltration (Microcon 3YM membrane, 3-kDa cut-off, Amicon) at 4°C to separate free ecdysteroids. The retentate was reconstituted to initial volume and assayed for its stimulatory action on PGs as above. The residual ecdysteroid content of the retentate was determined by RIA (≈10% of the initial amount in the hemolymph) and subtracted from that in the PG incubation medium at the end of incubation.

PTTH Extraction from Brain Complexes. Individual complexes were homogenized in saline, heat-treated at 100°C for 2 min, and centrifuged at 3000 × g for 10 min. The supernatant was assayed for PTTH activity with the paired PG assay above. Thus, each test PG was incubated with the extract of one brain complex.

RESULTS

Larvae of *Rhodnius prolixus* remain in a state of arrested development resembling diapause (2), in which ecdysteroids are absent from the hemolymph (18), until given a blood meal. Development into the next stage is initiated by a single blood meal and the underlying physiological changes unfold with remarkable temporal precision (2). A head critical period (HCP) (2) occurs on day 6 in male fifth-instar larvae (19), after which removal of the head fails to prevent the formation of adult cuticle beneath that of the fifth instar. Therefore, PTTH release from the brain is not essential for continued cuticle secretion after day 6. A comparable HCP occurs in all insects

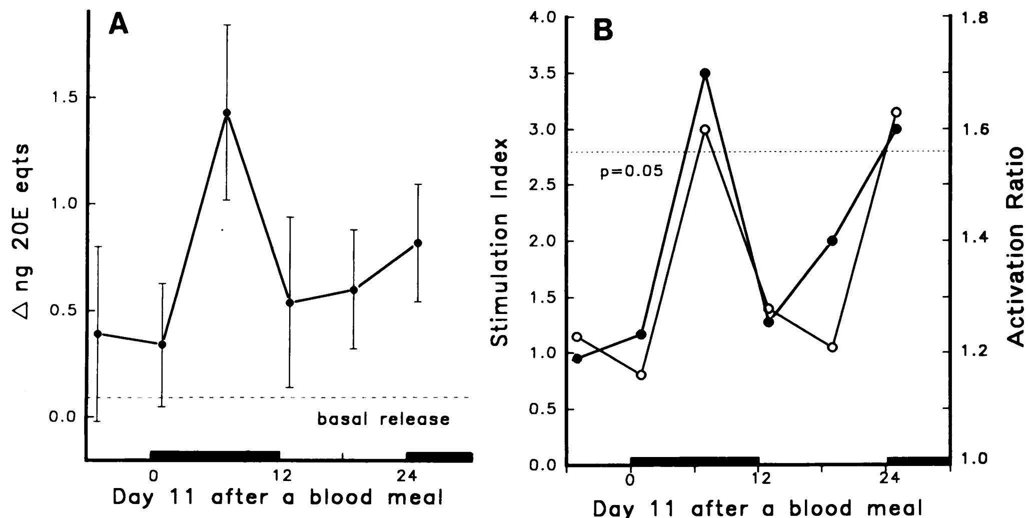

FIG. 2. Stimulation of ecdysteroid synthesis by PTTH released from complexes of day-11 larvae removed at 6-hr intervals. (*A*) Stimulation is expressed as the numerical difference in synthesis between stimulated and unstimulated (contralateral) members of each PG pair plotted as mean difference ± SEM of n = 6 separate determinations. (*B*) SI values derived from the statistical analysis of the data in *A* (●); data points above the line at P = 0.05 represent significant stimulation; activation ratios (Ars) are derived from data in *A* (○); the significance line does not apply to Ars. Dark bars indicate scotophases. eqts, Equivalents.

Table 1. Effect of enzymic proteolysis on released PTTH activity

Hours after lights off	Pronase treatment	Ecdysteroid synthesis, ng of 20E equivalents/PG	
		+PTTH	−PTTH
7	−	2.35 ± 0.22	1.35 ± 0.17**
7	+	1.25 ± 0.25	1.30 ± 0.30*
1	−	1.48 ± 0.27	1.43 ± 0.34*
1	+	1.25 ± 0.25	1.30 ± 0.30*

The two numbers on each line are the mean ecdysteroid synthesis by treated (+PTTH) or untreated contralateral control (−PTTH) members of PG pairs. One member of each PG pair was incubated with PTTH released from day-11 brain complexes at the times shown, whereas the other member was incubated without PTTH. Values are mean ± SEM of $n = 6$ PGs. *$P > 0.10$; **$P < 0.01$, for comparison between control and PTTH-treated PGs using paired sample t test.

examined (see ref. 5), and it has been assumed throughout the literature that a brief release of PTTH at the HCP activates the PGs and that further release of PTTH does not occur.

Nocturnal Release of PTTH *in Vitro*. Brain complexes were removed from day 11 larvae during scotophase or photophase and incubated individually *in vitro* for 4 hr. Media were assayed for stimulation of steroidogenesis by PGs *in vitro*. Media from scotophase complexes were highly stimulatory, whereas media from photophase complexes had no activity (Fig. 1). Because PGs do not store ecdysteroids, all ecdysteroids in the medium result from synthesis (15). Synthesis is roughly linear for at least 4 hr. The increase in ecdysteroid content of the medium is not due to leakage of hemolymph ecdysteroids from within the brain complexes, because scotophase complexes incubated in the absence of PGs leaked only minute quantities of ecdysteroid (Fig. 1). Other scotophase nervous ganglia of comparable size had no stimulatory activity (data not shown). Thus, PTTH activity is released from brain complexes in scotophase, but not in photophase.

The daily pattern of release of PTTH activity was examined with more frequent samples using the paired PG assay and statistical analysis in Fig. 2. A large numerical increase in synthesis above that of contralateral (control) PGs is induced by scotophase brain complexes (Fig. 2*A*). This increase is statistically significant, whereas the modest increase induced by photophase brain complexes is not (Fig. 2*B*). These data expressed as Ar values show the same trend as SI values (Fig. 2*B*), but significance cannot be inferred (8); hence Ar was not used in later experiments. It was essential to determine whether the observed release of PTTH activity was artifactual—e.g., induced by trauma to the brain complex during dissection or inappropriate *in vitro* conditions. These possibilities were examined using brain complexes of unfed larvae, which do not release PTTH *in vivo* (2, 20) but contain large quantities of biologically active PTTH (8). Any release from such complexes would therefore be artifactual. These complexes released a nominal, constant amount of PTTH activity (yielding a stimulation above control PGs of 0.094 ± 0.10 ng of 20E equivalents), which showed no variation within a day. This amount is designated "basal release" (Fig. 2*A*). Physiologically relevant release must exceed this basal amount. The known PTTHs are peptides (5, 21). To examine the possibility that some PTTH activity could be due to release of nonpeptides, media from incubation of day-11 brains were treated with pronase (Table 1). Brain complexes excised at 7 hr after lights off released abundant stimulatory material that induced significant stimulation of steroidogenesis above that of contralateral (untreated) PGs (line 1). All this activity was destroyed by pronase (line 2). Conversely, media from complexes excised at 1 hr after lights off were unaffected by Pronase (line 3) because these media contained no PTTH activity before treatment (line 4). Therefore, all released stimulatory activity is destroyed by pronase, and the enzyme treatment did not affect the behavior of the PGs used in the assay. We conclude that PTTH activity is due to peptide(s) released only from brain complexes and only during scotophase.

PTTH Rhythms *in Vivo*. Complete substantiation that the rhythmic release of PTTH observed *in vitro* occurs also *in vivo* requires detection of related rhythmicity *in vivo*. (*i*) The existence of a rhythm in PTTH in the hemolymph was examined. Hemolymph was collected at 6-hr intervals

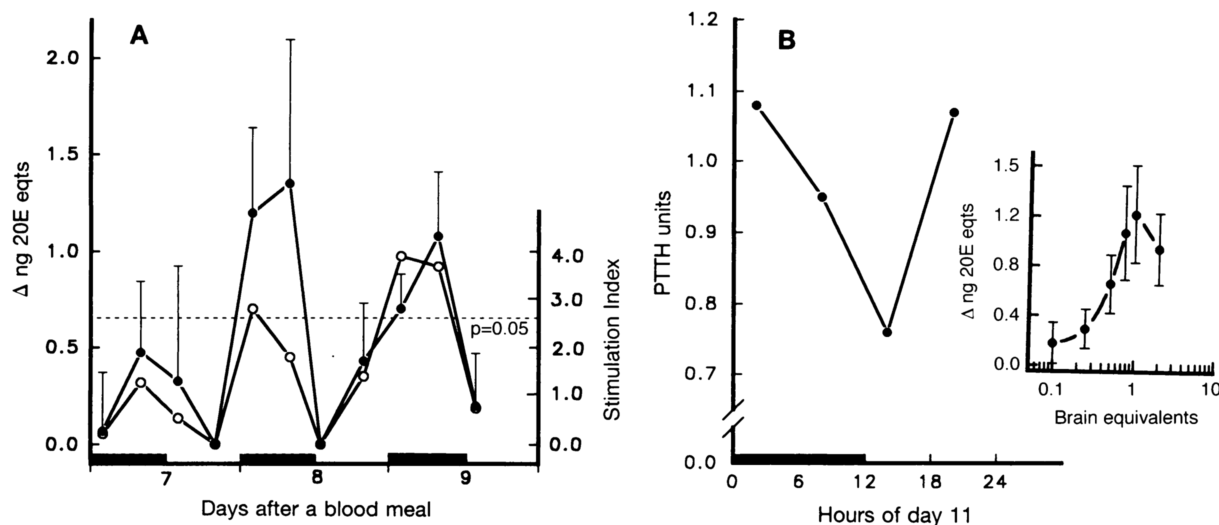

FIG. 3. PTTH rhythms *in vivo*. (*A*) Stimulation of ecdysteroid synthesis by PTTH activity present in larval hemolymph. Day-7 to -9 larvae were used because they contain naturally low amounts of ecdysteroids in their hemolymph (15). Any ecdysteroid remaining after ultrafiltration (see *Materials and Methods*) was determined by RIA and subtracted from the total amount in the medium. Hemolymph was diluted 1:1 with saline before PG incubation. Solid points show increase in ecdysteroid synthesis above that of contralateral (control) PGs, plotted as mean difference ± SEM of $n = 6$ separate pairs of PG incubations. Open circles are SI values calculated from the numerical differences and reveal times when stimulation is significant. Points above the horizontal dashed line at $P = 0.05$ represent significant stimulation. (*B*) PTTH activity extracted from whole brain-retrocerebral complexes. PTTH content is expressed as PTTH units, which were derived from a dose–response curve (*Inset*) constructed from the mean increase in ecdysteroid synthesis by stimulated PGs over contralateral (control) PGs (± SEM of $n = 6$ separate determinations) elicited by various increments of brain-complex extracts from day-11 larvae rendered arrhythmic by maintenance in continuous light. One PTTH unit is therefore the amount present in one day-11 brain complex of an animal in continuous light.

throughout days 7–9, processed to eliminate endogenous ecdysteroids and other small molecules, and assayed for PTTH activity. Large increases in ecdysteroid synthesis above that of contralateral (control) PGs are induced by scotophase hemolymph, and little or no increase is induced by photophase hemolymph on each day (Fig. 3*A*). Significant stimulation is obtained only with scotophase hemolymph. Therefore a rhythm of PTTH activity is seen in hemolymph, which has the same temporal pattern as does PTTH release *in vitro*. (*ii*) We examined the PTTH content of the brain complex for rhythmic changes. We were encouraged in this approach by recent evidence that PTTH content varies systematically during the 21 days of development (8). Fig. 3*B* shows that the PTTH content varies dramatically within a day. Highest values are seen in early scotophase, when no release was detected *in vitro*; PTTH content declines throughout the remainder of the scotophase—i.e., at the time when release was observed from complexes *in vitro*. The content of PTTH is restored, presumably by PTTH production, during the photophase. Therefore, the daily changes in brain complex content of PTTH reflect the observed time of release from complexes *in vitro*. The existence of rhythms of PTTH in both hemolymph and brain complexes *in vivo* strongly argues that the rhythm of PTTH release seen *in vitro* occurs also in the intact animal.

Rhythmic PTTH Release During Development. PTTH release from brain complexes was studied every 6 hr throughout days 10–16. The pattern seen on day 11 is repeated every day, with high numerical differences between stimulated and contralateral (control) PGs in each scotophase, and minimal values (close to basal release) in each photophase (Fig. 4*A*). The PTTH released during the scotophase always elicits significant stimulation, whereas release from photophase complexes is never significant (Fig. 4*B*). Decapitation of larvae at the end of day 6 does not prevent the deposition of adult cuticle, and hence this rhythm is involved with mechanisms other than the continuation of development (see *Discussion*). To determine whether the HCP on day 6 is associated with a change in the pattern of PTTH release, brain complexes of days 2–7 were studied. Throughout days 2–5, release is close to basal release (Fig. 4*C*) and is never significant (Fig. 4*D*). The first significant release is seen during the scotophase of day 6—i.e., during the HCP. PTTH release on day 7 resembles that on day 6 and days 10–16. Therefore, the rhythm of PTTH release commences at the HCP and continues for most, if not all, of the remaining 15 days before emergence of the adult insect.

DISCUSSION

The daily rhythm of release of PTTH *in vitro* reported here is accompanied by corresponding rhythms in both the brain

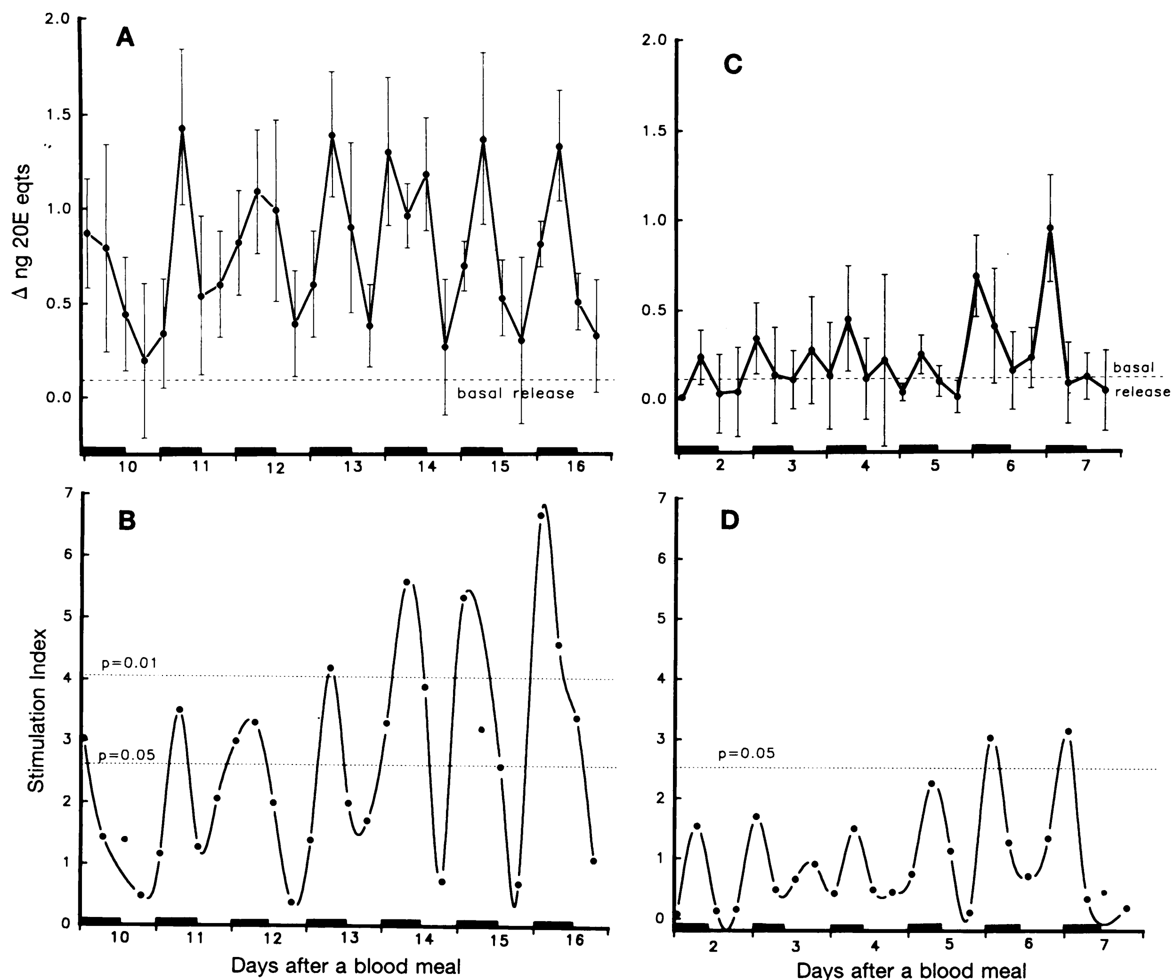

FIG. 4. Daily rhythm of PTTH release from brain complexes of day-10 to -16 larvae, expressed as mean increase (± SEM of $n = 6$ separate determinations) in ecdysteroid synthesis of test PGs above contralateral (control) PGs (*A*), and as statistical significance of stimulation (SI) (*B*). Note that numerical stimulation during the photophase is always close to the basal release (*A*) and yields daily minima in SI values (*B*). Significant stimulation is obtained only from scotophase brain complexes (*B*). PTTH release from complexes on days 2–5 is close to basal release (*C*) and is never significant (*D*). At the HCP (day 6) an increase in numerical stimulation occurs (*C*) to statistical significance (*D*) during the scotophase, marking the onset of rhythmicity.

content and hemolymph levels of PTTH seen *in vivo.* We infer that the rhythm of release also occurs *in vivo.* The rhythm occurs throughout most of the 21 days of larval–adult development. It persists in aperiodic conditions and is under circadian control by the brain (22). Previous studies have shown that both ecdysteroid synthesis by the PGs and the hemolymph titer of ecdysteroids are also under circadian control during this time (9) and that a second circadian clock is located within the PGs themselves (10, 11). Together, these observations raise significant challenges to several features of the current understanding of the hormonal control of insect development. The observations raise new questions concerning (*i*) the function(s) of PTTH and, by implication, its functional relationship with the PGs and (*ii*) the significance of pronounced circadian rhythmicity throughout this endocrine axis.

In the prevailing understanding of the role of PTTH in insect development, a brief release of PTTH early in development triggers the PGs to undergo a prolonged period (15 days in *Rhodnius*) of ecdysteroid synthesis in the presumed absence of further PTTH release; ecdysteroid synthesis gradually increases during the first half of the instar and progressively declines during the second half (4–6, 15). This view derives from the existence early in each instar of a HCP, which is the period of time (usually ≈24 hr) during which the continuation of development (as scored by subsequent cuticle secretion) acquires an independence from the presence of the head (as revealed by neck ligations). The HCP is understood to represent the time at which PTTH is released (5). Our findings confirm that PTTH is released at the HCP in *Rhodnius* (day 6) but reveal this release is only the first of many daily releases. What occurs at the HCP that enables development to continue in the absence of the head? Rhythmicity in ecdysteroid synthesis commences at the HCP (9); could it be that the first daily release of PTTH sets in motion the rhythm of steroidogenesis that can subsequently be sustained by the PG oscillator? The numerous subsequent daily releases of PTTH have no precedent in the prevailing understanding of the control of development. What is their function? The obvious temptation is to suppose that the PTTH rhythm drives the rhythm of steroidogenesis (as in mammals, see ref. 23)—i.e., that the PGs are merely slave oscillators to a brain pacemaker (controlling PTTH release), whose coupling to the external light cycle assures entrainment of the two-oscillator system to the outside world. This possibility is seriously challenged by the fact that the PGs contain their own photosensitive clock (10, 11); it is not clear why the PGs would require a second, potentially redundant, entraining signal (PTTH) from the brain. The discovery of circadian rhythmicity in these two hormones adds a new dimension of complexity to understanding their functional relationships.

What is the significance of this periodicity? Insect cells *in vitro* respond to pulses (≈ 2 hr) of ecdysteroids but seldom to their continued presence; pulses of different concentrations of hormone lead to synthesis of different proteins (24) and induction of different transcripts (e.g., ref. 25). In *Rhodnius*, the circadian "pulses" of ecdysteroids in the hemolymph are superimposed upon mean daily titers, which themselves change progressively during development (15); therefore, the pulses occur within a different range of hormone concentration on each day of development. Does this pattern provide the basis for a temporal program for reading the genome during development? The significance of the observations reported here derives from the set of entirely new questions they raise.

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