

Neural and hormonal regulation of growth of corpora allata in the cockroach, *Diploptera punctata*

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Abstract

DNA synthesis and mitosis in the corpora allata (CA) of adult *Diploptera punctata* males were investigated with total cell count after 5'-bromo-2'-deoxyuridine immunodetection and colchicine arrestment both in vivo and in vitro. The CA exhibited a single wave of DNA synthesis followed by cell division during the first 4 days after the imaginal ecdysis. A second mitotic wave was experimentally induced after the nervous connections between the CA and the brain were severed on day 4. Spontaneous mitosis was abolished in cockroaches treated with a juvenile hormone (JH) analog. This inhibitory regulation in vivo appeared to act through brain neurosecretory cells since in the denervated CA mitotic activity was unaffected by JH treatment. An in vitro system supporting growth of the corpus allatum was established to study direct hormonal effects. By using continuous bromodeoxyuridine labeling in vitro for 6 days, we showed that DNA synthesis of corpus allatum cells was unaffected by direct contact with JH. In contrast, 20-hydroxyecdysone exerted direct mitogenic action on allatal cells. These and previous results suggest that CA cells alternate between JH synthesis and a proliferative state in which they divide in a self-renewing fashion to yield differentiated progeny. We propose that in newly enclosed adult *Diploptera punctata* males, low JH titer and high ecdysteroid titer promote mitosis in CA cells. As the ecdysteroid titer declines, JH produced by the CA acts on brain neurosecretory cells which dispatch inhibitory signals through nerves to prevent continuous proliferation of CA cells.

Keywords: Corpora allata; Juvenile hormone; 20-Hydroxyecdysone; DNA synthesis; Cockroach

1. Introduction

Insect corpora allata (CA) produce juvenile hormone (JH) and are considered the analog of the anterior pituitary of vertebrates (Scharrer et al., 1987). In both the brain-cardiacum-allatum axis and the hypothalamic-pituitary system, the brain dispatches peptide hormones that control specific physiological activities by either directly addressing target sites or by instructing intervening endocrine glands, CA or anterior pituitary respectively. It is well known that pituitary development is also regulated by hypothalamic-releasing factors; for example somatotroph proliferation is stimulated by growth hormone-releasing factor and inhibited by somatostatin (Voss and Rosenfeld, 1992;

Montminy, 1993). In insects, several brain neuropeptides have been isolated and shown to stimulate (allatotropins) or inhibit (allatostatins) JH synthesis by the CA (Kataoka et al., 1989; Woodhead et al., 1989). However, only scant information is available on how these and other extracellular signals influence the ontogeny of allatal cells.

Postembryonic growth of insect CA is achieved by either increases in cell number, as in most exopterygotes, or in cell polyploidization, as in some endopterygotes (Cassier, 1990). In cockroaches, the number of CA cells increases late in each intermolt period and continuously during the entire last stage coincident with high ecdysteroids and low JH in the hemolymph (Kikukawa and Tobe, 1986; Chiang et al., 1991b, 1993). In adult females of the viviparous cockroach *Diploptera punctata*, CA cells increase in number immediately after the imaginal molt (Szibbo and Tobe,

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1981; Chiang et al., 1993) before the prothoracic gland (source of ecdysteroids) completely degenerates and before JH synthesis increases (Stay et al. 1984). The imaginal proliferation of CA cells, however, does not occur in the two oviparous cockroach species, *Blattella germanica* and *Supella longipalpa* (Chiang et al. 1991b, 1994). During the ovarian cycle, the CA of all three cockroach species exhibit cycles of JH synthesis and undergo volumetric changes due to rapid proliferation of organelles followed by their synchronous autophagy (Chiang et al., 1989 1991a,c; Johnson et al., 1989, 1993; Piulachs et al., 1989; Chiang and Schal, 1991, 1994; Cheng and Chiang, 1995). To study mechanisms involved in regulation of allatal development, we used adult males as a model system since their CA contain a sufficient number of cells for most in vivo and in vitro tests and, after an initial activation of CA activity, JH synthesis remains relatively constant; regulation of the male CA thus appears to be less complex than the female CA due to lack of ovarian factors.

JH and ecdysteroids regulate a multiplicity of functions during development and reproduction (Gilbert et al., 1980; Riddiford, 1980). Injection of 20-hydroxyecdysone has been shown to mimic the inhibitory effect of a mature ovary on JH synthesis in *D. punctata* (Stay et al., 1980) and, in addition, it also induces a decrease in CA cell size in ovariectomized *B. germanica* (Chiang et al., 1991a,c). Topical application of JH analog also modulates rates of JH synthesis (Tobe and Stay 1979) and has been shown to stimulate the release of allatostatins in *D. punctata* (Stay et al., 1994). However, the influence of these hormones on proliferation of CA cells has not been fully investigated. Here we report on DNA synthesis and division of CA cells both in vivo and in vitro and show that proliferation of CA cells is regulated by negative feedback of JH acting indirectly through the brain and by direct mitogenic effects of the molting hormone, 20-hydroxyecdysone.

2. Materials and methods

2.1. Insects

Cockroach colonies were reared at $27 \pm 1^\circ\text{C}$ under a 12-h light/12-h dark photoperiodic regime and supplied with pelleted Purina dog food No. 1780 and water ad libitum. Adult males were collected from the colony within 24 h after ecdysis and housed in groups of two to ten. Age in the text refers to age from the time of adult ecdysis. All males used in the present study were unmated.

2.2. Organ culture

Corpora allata were dissected from *D. punctata* adult males, cleaned from adjacent tissues, and separated in sterile cockroach saline solution containing 9.27 g NaCl, 1.314 g KCl 0.324 g NaHCO_3 , 0.189 g

$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 1.206 g Na_2HPO_4 , and 2.7 g glucose per liter (modified from Kurtti and Brooks, 1976). This solution is iso-osmotic with adult *D. punctata* hemolymph (360 mOsm per kg H_2O) measured with a micro osmometer (Advanced Instruments, MA). Individual CA were incubated in 10 μl L-15B 15B (Munderloh and Kurtti, 1989) supplemented with 10% FBS and 10% cockroach hemolymph derived from day-4 *Periplaneta americana* adult males (Tsai et al., 1995). Hemolymph was collected into a known volume of L-15B medium on ice, and stored at -70°C until needed. To prepare culture media the stored hemolymph solution was diluted with L-15B to the desired concentration. The media were subsequently centrifuged ($9300 \times g$ for 20 min) and then sterilized by filtration. All incubations were carried out in 96-well culture plates sealed with parafilm membrane and maintained in a humidified chamber at $27 \pm 1^\circ\text{C}$. The incubation contains low amount of endogenous 20-hydroxyecdysone (ca. 0.32 ng/ml; Weaver et al., 1984) and JH III (ca. 0.23 ng/ml; Weaver et al. 1990) derived from the 10% cockroach hemolymph.

2.3. Mitotic index and total cell number

The gland was desheathed with collagenase and spread into a whole-mount nuclear monolayer under a coverslip as described earlier (Chiang et al., 1989). The number of metaphase cells per corpus allatum was determined by total count under a Nikon Optiphot microscope 4 h after injection of colchicine in saline (0.02 $\mu\text{g}/\text{mg}$ body mass). Total cell number within a single corpus allatum was determined by hemocytometric sampling as described previously (Chiang et al., 1993); individual glands were desheathed with 0.1% collagenase, stained with 0.1% safranin and dissociated into a nuclear suspension with 0.2% Triton-X 100 and 0.2 M citric acid in cockroach saline solution.

2.4. DNA synthesis

For in vivo labeling of S-phase cells, insects were injected with 1 μl cockroach saline containing 3 μg 5'-bromo-2'-deoxyuridine (BrdU), a thymidine analog. For in vitro labeling, individual CA were cultured in 10 μl of supplemented L-15B medium containing 3 ng BrdU. To prepare a single corpus allatum into a monolayer of nuclei without using a coverslip, the BrdU-labeled CA was digested with 0.1% collagenase in a hypotonic solution composed of equal parts of cockroach saline and distilled water. After 10 min at 27°C the CA was transferred into a 2 μl drop of 0.1 M citric acid solution on a gelatin subbed slide. Fifteen min later, as CA cells became swollen and loosely attached to each other, the CA was spread into a monolayer of nuclei by slowly removing the incubation solution under a dissecting microscope. The specimen was fixed and dried quickly by passing it through a flame three

times. After washing the specimen three times in PBS BrdU-labeled cells were visualized by immunodetection according to the RPN 20 protocol (Amersham Corp., Arlington Heights, IL). Briefly, cells were covered with 10 μ l of anti-BrdU working solution (diluted anti-BrdU 1:100 in Tris-buffer containing 1% BSA and nuclease) for 1 h at 27°C. After washing the specimen three times in PBS, cells were incubated in 10 μ l peroxidase anti-mouse-IgG2a working solution (diluted antiserum 1:60 with PBS) for 1 h at 27°C. It was washed three times in PBS. The immunoreactive cells were visualized by incubating in a solution of 3,3'-diaminobenzidine in PBS containing hydrogen peroxide and nickel chloride. The number of BrdU-labeled nuclei per corpus allatum, indicated by black 3,3'-diaminobenzidine precipitate, was counted directly under the microscope.

2.5. CA denervation and transplantation

Unilateral transection of nervus corporis allati-I was performed as described by Pipa (1986). The right corpus allatum was always denervated and the innervated left corpus allatum was used as an internal control. In some cases, pairs of corpora cardiaca-allata from day-4 adult males were transplanted into day-0 adult males near the host's CA.

2.6. Hormone treatments

Some cockroaches were treated with JH analog by continuous tarsal contact in a round petri dish, the bottom of which was coated with 2.8 μ g/cm² fenoxycarb (HLR Sciences) applied in 1 ml ethanol. To examine the effects of ecdysteroids cockroaches were reared normally except that the supplied distilled water contained 0.02 ng/ml 20-hydroxyecdysone (Sigma). To determine the direct effects of JH, CA from day-0 adult males were separated and while one gland was incubated in 10 μ l L-15B medium supplemented with 3.75 μ M JH III (1 μ g/ml; Sigma), 0.05% ethanol and 0.05% glycerol, the contralateral gland acted as a control; it was incubated in the same medium without JH. To determine the in vitro effects of 20-hydroxyecdysone, CA from day-4 adult males were separated and while one gland was incubated in 10 μ l L-15B medium supplemented with 1 μ M 20-hydroxyecdysone (480.6 ng/ml) and ethanol (0.01%), the contralateral gland was incubated in the same medium but without 20-hydroxyecdysone. After incubation for 6 days, BrdU-labeled cells were visualized by immunodetection.

3. Results

5'-Bromo-2'-deoxyuridine (BrdU) labeling to visualize S-phase cells revealed that the CA of newly ecdysed adult males exhibited a synchronous cycle of DNA synthesis that peaked on day 2 (Fig. 1). It was followed closely by a peak of cell division on day 3. This single

wave of cell proliferation resulted in a 20% increase in the average cell number per corpus allatum from 5606 \pm 220 cells (mean \pm SEM, $n = 19$) on day 0 to 6739 \pm 397 cells (mean \pm SEM, $n = 9$) on day 4. In contrast to the mitotic wave seen in untreated adult males, most CA cells became quiescent following treatment with JH analog fenoxycarb, resulting in 24.7, 99.5, 99.9 and 83.8% inhibition of cell proliferation for days 1, 2, 3 and 4 respectively. Transplantation of an extra pair of CA, as a source of natural JH, from day-4 males into newly-ecdysed males resulted in a similar inhibition of cell proliferation in the innervated CA (Fig. 1). Conversely, treatment with 20-hydroxyecdysone accelerated the normal increase in mitotic activity of CA cells (Fig. 1). Adult males that drank distilled water containing 0.02 ng/ml 20-hydroxyecdysone for 2 days had significantly more mitotic CA cells on day 2 (118 \pm 25 metaphase cells per CA, $n = 14$) than males drinking distilled water only (63 \pm 15 metaphase cells per CA $n = 13$; t -test, $P < 0.05$).

Transection of nervous connections (nervus corporis allati-I) between the brain and only one corpus allatum of a CA pair in newly ecdysed males clearly showed that the inhibitory action of JH on CA cell division was mediated by the brain. In males treated with fenoxycarb a mitotic wave occurred in the denervated CA, but it was totally eliminated in the contralateral innervated CA (Fig. 2a). Unilaterally denervated CA of control males that were not exposed to JH analog experienced a mitotic wave of similar magnitude to that in the denervated CA of fenoxycarb-treated males (Fig. 2a,b). Likewise cell division was also partially inhibited in the

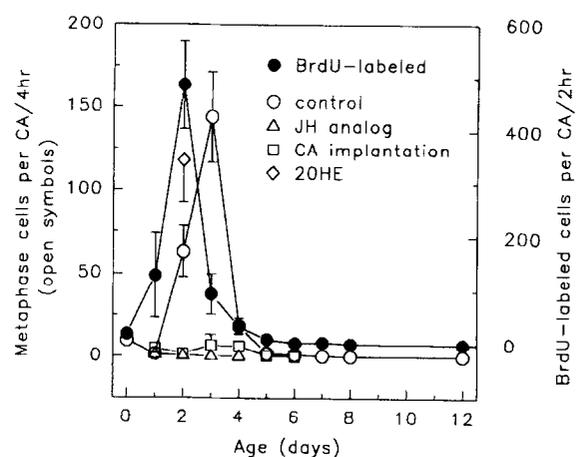


Fig. 1. Hormonal effects on allatal growth in vivo. In control insects, a surge of DNA synthesis (solid circles) is followed by a mitotic wave (open circles). Some cockroach were supplied distilled drinking water containing 0.02 ng/ml 20-hydroxyecdysone (20HE). The mitotic wave was not seen in insects exposed to the JH analog fenoxycarb (triangles) or implanted an extra pair of day-4 CA (squares). Pairs of corpora cardiaca-allata from day-4 adult males were transplanted into day-0 adult males near the host's CA. Each point represents the mean \pm SEM from 6–14 measurements.

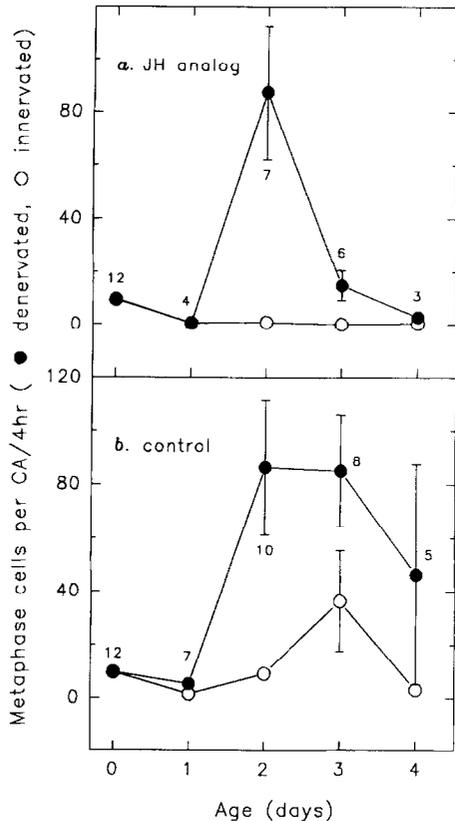


Fig. 2. Brain mediation of JH inhibition of CA cell division. Unilateral transection of the nervus corporis allati-I was performed on newly ecdysed adult males. The right corpus allatum was always denervated, and the innervated left corpus allatum was used as an internal control. Numbers of metaphase cells per corpus allatum were determined by total count 4 h after colchicine injection. (a) Exposure to the JH analog fenoxycarb abolished the mitotic wave in the innervated CA but not in the denervated CA. (b) In control insects, a clear mitotic wave in the denervated CA was significantly inhibited in the contralateral innervated CA, probably due to inhibition by JH released from the companion denervated CA. Data for the two members of each CA pair were collected from the same insects. Each point represents the mean \pm SEM of the number of insects indicated.

innervated CA of control males (Fig. 2b). We reasoned that, after experiencing a single mitotic wave on days 2–3, CA cells of adult males remained quiescent in part because of inhibitory signals from the brain. A key observation in support of this hypothesis was that unilateral denervation of the CA in 4-day-old adult males induced a second wave of DNA synthesis and cell division in the denervated CA but not in the contralateral innervated gland (Fig. 3). However, the peak level of the induced second wave of cell division in the denervated CA was only about half of that in the denervated CA during the first 4 days (Fig. 2b). We tested the hypothesis that the humoral environment of older males was less supportive of CA cell proliferation by transplanting CA from day-4 into day-0 adult males. These denervated older CA in the humoral environment of young males exhibited a similar peak level of

cell division as CA that were denervated and remained in the older male (Fig. 4). In both cases, cell proliferation eventually ceased even without an intact nerve connection to the brain (Figs. 3 and 4).

To investigate direct hormonal effects on allatal growth, we established a culture system in which adult male CA exhibit a complete cycle of DNA synthesis and mitosis in vitro (Tsai et al., 1995). A fast method using BrdU immunodetection together with nuclear-monolayer preparation was developed to count the total number of S-phase cells in single corpus allatum during the incubation period (Fig. 5). Under these organ culture conditions, direct contact with JH III ($3.75 \mu\text{M}$) showed no significant inhibitory effect on DNA synthesis in CA cells. After continuous BrdU labeling in vitro for 6 days, the mean number of BrdU-labeled cells did not differ significantly between CA incubated in medium supplemented with JH (610 ± 65 BrdU-labeled cells/CA) and without JH (592 ± 104 BrdU-labeled cells/CA) ($n = 4$, paired t -test, $P > 0.05$).

In contrast, 20-hydroxyecdysone ($1 \mu\text{M}$) exerts a direct stimulatory action on DNA synthesis in CA cells in vitro. CA treated with 20-hydroxyecdysone for 6 days showed significantly more BrdU-labeled cells (743

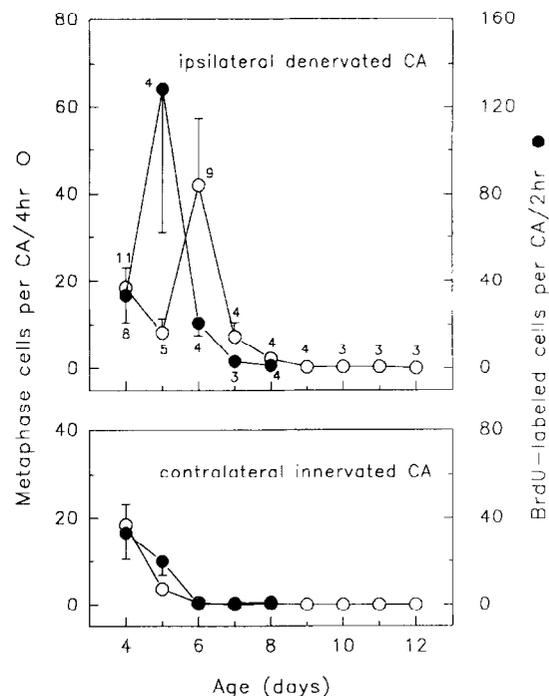


Fig. 3. Neural inhibition on DNA synthesis and mitosis. Unilateral transection of the nervus corporis allati-I was performed on day-4 adult males. The right corpus allatum was always denervated and the innervated left corpus allatum was used as an internal control. The number of BrdU-labeled cells per corpus allatum was determined by total count 2 h after injection of BrdU, while number of metaphase cells was counted 4 h after colchicine injection. Each point represents the mean \pm SEM of the number of insects indicated. Data for the two members of a CA pair were collected from the same insects.

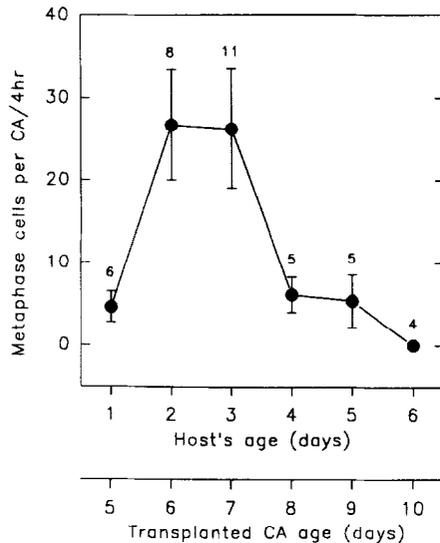


Fig. 4. Effects of changing humoral environment on mitosis in denervated CA. Pairs of corpora cardiaca-allata from day-4 adult males were transplanted into day-0 adult males near the host's CA. The number of metaphase cells per corpus allatum was determined by total count 4 h after injection of colchicine. Each point represents the mean \pm SEM with the number of measurements indicated.

± 107 cells/CA) than the untreated contralateral glands (424 ± 76 BrdU labeled cells/CA) (paired *t*-test, $n = 6$, $P < 0.05$) (Figs. 5 and 6).

4. Discussion

We have demonstrated that the CA of *D. punctata* adult males exhibited a single wave of DNA synthesis followed by cell division that resulted in a 20% increase in cell number during the first 4 days after the imaginal ecdysis (Fig. 1). This surge of cell division clearly did not require intact nervous connection with the brain since it occurred in denervated CA both in vivo (Fig. 2b) and in vitro (Fig. 5; Tsai et al., 1995). The relation between changes in CA cell number and JH synthesis under various conditions has attracted much attention but with little consensus among researchers, mainly because of differences in methodologies and among animals (Chiang and Schal, 1994). Here, using BrdU immunodetection and metaphase arrest techniques, together with nuclear monolayer separation we were able to count the total number of CA cells in DNA replication and cell division independently of the history of CA growth, which affects the total cell number. Furthermore long-term organ culture in the presence of BrdU provided a cumulative count of cells undergoing DNA synthesis. This very sensitive method will be of great utility in the search for factors that control cell proliferation in the CA.

We reasoned that the early mitotic wave in adult male CA can be facilitated by three possible mechanisms: (1) removal of neural inhibition or exertion of

neural stimulation; (2) appropriate humoral conditions after ecdysis (e.g., low JH, high ecdysteroids, or other factors); or (3) an intrinsic mitotic program that is expressed only shortly after the molt. In *D. punctata* adult females, the number of CA cells does not increase after ecdysis until females are mated or their brain lateral neurosecretory cells are cauterized (Szybbo and Tobe 1981; Tobe et al., 1984). Using the metaphase arrest technique we confirmed that the mitotic wave occurs only in CA of mated females and in brain-denervated CA of virgin females (unpublished data from the authors). These observations argue that while the humoral environment can support CA cell proliferation, signals from the brain inhibit CA growth.

In adult males, it is not clear which mechanism facilitates the increase in DNA synthesis and mitosis in the CA. The timing of the spontaneous increase in DNA synthesis and mitosis appears to coincide with high ecdysteroid titers and low JH titers in the hemolymph of adult males during the first 3 days after emergence (Tobe et al., 1979; Stay et al., 1984). Our results show that low or no JH in the hemolymph is essential for CA cells to proliferate (Figs. 1 and 2). Cell proliferation in the CA was inhibited when JH (or JH analog) levels in the hemolymph were elevated by implantation of an extra pair of day-4 CA (Fig. 1) or denervation of the contralateral gland (Fig. 2b), or exposure to fenoxycarb (Fig. 1). JH did not act directly on CA cells as indicated by the in vitro test. Rather, it acts through the brain, which exerts neural inhibition on the proliferation of CA cells, since severing the nerves between the CA and the brain removed this inhibitory action of the JH analog (Fig. 2). Consistent with this idea is a recent report showing strong JH-induced inhibition of JH synthesis when the nerves between the brain and CA are intact, but less so when CA nerves are severed (Stay et al., 1994). Treatment of virgin *D. punctata* females with the JH analog hydroxyprogesterone also induces a dose-dependent decrease in CA cell number (Kikukawa et al. 1988).

Ecdysteroids have been shown to have growth-promoting effects on many insect cells in vitro and ecdysteroid receptors have been identified from insect CA (Riddiford, 1980; Bidmon and Koolman, 1989). Our in vitro study indicates that 20-hydroxyecdysone causes an acceleration of DNA synthesis and that it acts directly on CA cells of *D. punctata* adult males (Figs. 5 and 6). This is supported by the finding that during nymphal development, the proliferation of CA cells is coincident with the timing of hemolymph ecdysteroid peaks (Kikukawa and Tobe, 1986; Chiang et al., 1993).

20-Hydroxyecdysone appears to exert different and almost opposite effects on CA cells: in addition to acting as a mitogen, it also induces a regression in size of CA cells and a decline in JH synthesis (Stay et al., 1980; Chiang et al. 1991c). Furthermore, injection of

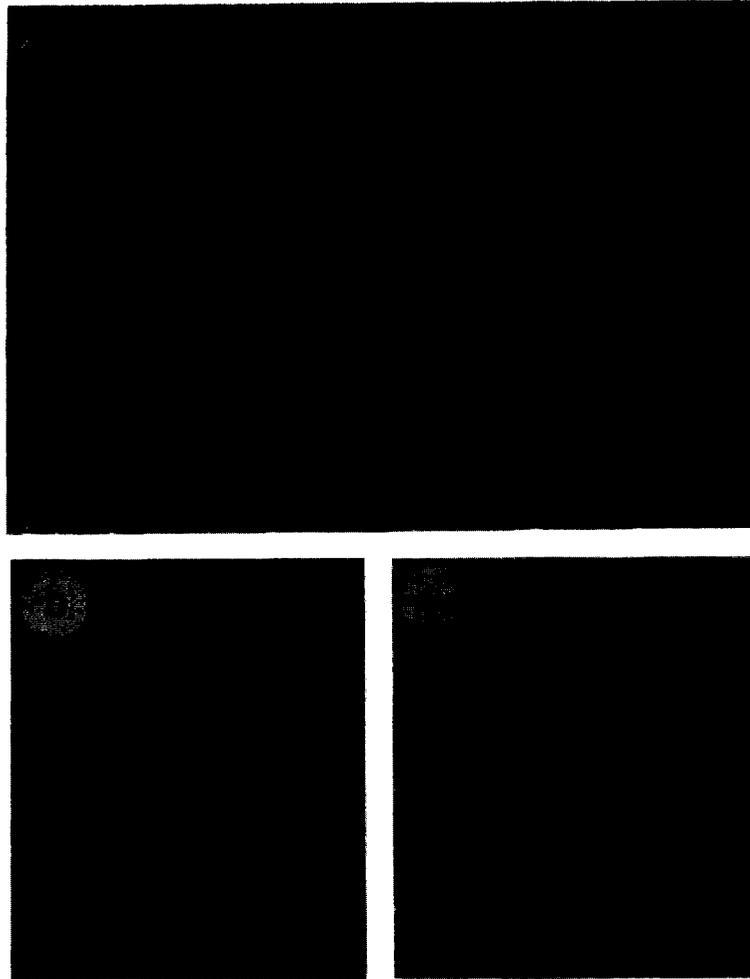


Fig. 5. BrdU immunodetection in CA nuclear monolayers. To label CA cells during DNA synthesis, BrdU was incorporated into media for CA organ culture. After various periods of time, each individual corpus allatum was prepared into a nuclear monolayer and S-phase cells were visualized with BrdU immunodetection. (a) Nomarski DIC photomicrograph showing a portion of (c) at higher magnification. DNA replicating nuclei labeled with BrdU are identified by intensified black 3,3'-diaminobenzidine precipitation. Most of the nuclei are not labeled. (b,c) Photomicrographs at the same low magnification showing whole-mounts of nuclear monolayers from two members of a CA pair prepared after 6 days of incubation in medium containing BrdU with (c) or without 20-hydroxyecdysone (b). At this low magnification only diaminobenzidine-positive nuclei were visible. Note that the CA in (b) is smaller and shows fewer BrdU-labeled nuclei than in (c).

20-hydroxyecdysone has been shown to induce a decrease in CA cell number in ovariectomized *D. punctata* females (Tobe et al., 1984).

In vivo experiments, including denervation of day-4 CA and transplantation of CA from day-4 donors into day-0 males showed that CA cells after day 4 are still able to proliferate at relatively high rates in vivo (Figs. 3 and 4). What then prevents CA cells from continuous proliferation after day 4? A rapid decline of ecdysteroid titer in the hemolymph, due to degeneration of the prothoracic gland after ecdysis, may be partially responsible. In *D. punctata* adult males hemolymph ecdysteroid titers decline to about 9–12 ng/ml 20-hydroxyecdysone equivalents by day-7 (Stay et al., 1984) when DNA synthesis and mitosis of CA cells were undetectable (Fig. 1). However, our results indicate that CA cells can proliferate in a low 20-hydroxyecdysone environment. For instance transection of the nervous

connections between the CA and the brain on day-4 stimulated CA cell proliferation in a low ecdysteroid environment. Moreover, the CA of day-4 adult males which had completed a mitotic wave in situ, exhibited a cycle of de novo DNA synthesis followed by cell division during 6 days of incubation in medium containing only 0.32 ng/ml 20-hydroxyecdysone (calculated from Weaver et al., 1984), which was derived from the 10% hemolymph in the medium.

Inhibitory neural control through intact nervous connections with the brain is most likely directed by feedback from circulating JH upon the brain; rates of JH synthesis double during the first 4 days (Stay et al., 1984), and treatment with JH analog abolishes cell proliferation in intact but not in denervated CA (Figs. 1 and 2). Other, non-neural factors, or an intrinsic mitotic program, may also arrest CA cells at a non-proliferative state, since cell proliferation eventually ceased even in denervated CA (Figs. 2 and 3).

These and previous results suggest that corpus allatum cells alternate between a quiescent state and a proliferative state in which they divide in a self-renewing fashion to yield differentiated progeny. We propose that, in the newly eclosed adult males, low JH titer and high ecdysteroid titer promote mitosis in CA cells. As the ecdysteroid titer declines and JH titer increases, JH produced by the CA acts on brain neurosecretory cells which dispatch inhibitory signals through nerves to prevent continuous proliferation of CA cells. It is not clear if JH induces allatostatins which act on CA cells, as somatostatin inhibits somatotroph proliferation in the pituitary, or whether other brain factors execute this mission. Topical application of JH analog has been shown to modulate rates of JH synthesis and to stimulate the release of allatostatins in *D. punctata* (Tobe and Stay, 1979; Stay et al., 1994). It remains to be determined whether the negative feedback of JH on CA activity and cell proliferation operates on the same intermediate regulatory center.

The model for CA growth in the adult male cockroach, based on ecdysteroid stimulation and JH depression of the mitotic index is consistent not only with CA growth and development in immature stages (above), but also with similar tissue development models in other insects. In *Rhodnius testis* for example, ecdysone clearly stimulates and JH depresses mitosis in vivo in the preadult male (Dumser and Davey, 1974, 1975), and in locust testis ecdysone-induced elevation of the mitotic index has been confirmed in vitro (Dumser, 1980). However, the CA developmental model has a unique characteristic: the CA are the source of JH which in turn modulates its development. Moreover, at each developmental stage the CA can alternate between producing hormone, which requires cell growth and organelle proliferation, and cell proliferation. We have yet to determine whether previous experience of the CA, such as JH synthesis, cell growth, and exposure to extracellular signals plays a role in allatal development.

The complex sequential nature of allatal development and its dependence on humoral and neurosecretory directives explains why early attempts at in vitro CA culture failed to show significant growth (Marks, 1980).

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