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Neuroendocrine regulation of corpus allatum activity in *Manduca sexta*: Sequential neurohormonal and nervous inhibition in the last-instar larva

(allatohibin/medial neurosecretory cells/brain/neural inactivation/corpora allata)

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ABSTRACT **The inactivation of corpora allata (CA) in the last-instar larva of the tobacco hornworm *Manduca sexta* was studied using *in vivo* assay methods that test the capacity of the glands to induce an extra larval molt. Results indicate that the CA are inactivated by a two-step process. In the first step, a neurohormonal inhibition occurs by day 1 after ecdysis, resulting in a decrease in the ability of the glands to respond to allatotropin. The inhibitory hormone appears to be secreted by the medial neurosecretory cells in the brain and remains in the hemolymph only for a short period of <2 days. The second step of inactivation occurs by day 3; it is neurally mediated and essentially terminates juvenile hormone secretion. Neither the neurohormonal nor the neural inhibition is reversible under the conditions used in our larval assays. The timing of the two stages of inhibition coincides with the two periods of reduction in hemolymph juvenile hormone titer; this suggests that the regulation of corpus allatum activity plays an important role in reducing the juvenile hormone titer during the last larval instar—a prerequisite for the transformation of the larva into the pupa.**

Metamorphosis in insects is controlled by the level of juvenile hormone (JH) in the hemolymph at the time of initiation of a molt by ecdysone. Thus in holometabolous insects, a high JH titer leads to a larval molt and a low titer leads to a pupal molt; in the absence of JH the insect molts into an adult (1–3). Although it has been known for a long time that the corpora allata (CA) secrete JH, very little is known about the mechanism of regulation of CA activity in the immature stages. A number of earlier studies have indicated that the CA become inactive in the final larval instar (4–7) and that this inactivation is not autonomous but is regulated by the brain in response to changes in external and internal milieu (1, 2). Evidence suggests that the neurosecretory centers in the brain (and perhaps in other ganglia) influence the activity of CA through hormones either transported directly to the CA by axons (allatal nerves) or released into the blood. These hormones may be stimulatory (8, 9) or inhibitory (10, 11). Direct neural control has been demonstrated in some insects and is mainly inhibitory in nature (12, 13). Thus, the mechanism of regulation of CA appears to differ between various species of insects.

It has been postulated for the tobacco hornworm *Manduca sexta L.* that when last-instar larvae attain a critical weight, an unidentified abdominal factor triggers the brain to stop allatotropin secretion and instead to release through the allatal nerves a CA-inhibiting factor, allatohibin (14). Shortly thereafter the brain secretes prothoracicotropic hormone, which stimulates the prothoracic glands to secrete ecdysone. Thus, a molt is initiated in the virtual absence of JH and larvae undergo pupation.

Because experimental evidence for the preceding hypothesis is fragmentary, the purpose of this study was to determine whether the change in CA activity is due to absence of allatropin or to inactivation by neurohormonal or neural stimuli. The results reported here demonstrate that in the last-instar *M. sexta* larvae, the CA are inactivated by the brain in a two-step mechanism—an early neurohormonal and a later neural inhibition.

MATERIALS AND METHODS

Rearing and Staging of Larvae. Larvae were reared on an artificial diet at 27°C and 60% relative humidity on a 16:8 light:dark 24-hr cycle (lights-off at midnight). Larvae of identical age were collected and staged as described (9, 15). Under our laboratory conditions, a majority of fourth-instar larvae show head-capsule slippage (a sign of the impending larval molt) on day 3 and ecdyse to fifth-instar larvae on day 4. Fifth-instar larvae fed on day 0 exhibit frosted frass (a chalky, white material in the feces) by early day 3; stop feeding and enter the wandering stage on day 4; transform into prepupae by day 6; and pupate between days 9–10.

Surgical Techniques. Allatectomy, nerve severance, cautery of neurosecretory cells, and implantation techniques have been described (9).

Assay with Starved Fifth-Instar Larvae for CA Responsiveness to Allatotropin. This assay is based on our observations (9) that allatectomy prevents the starvation-induced supernumerary molt of fifth-instar larvae and that implantation of day 0, fifth-instar CA restores the supernumerary molt. Therefore, the test CA (one pair per host) were implanted into allatectomized, day 0, fifth-instar larvae (unfed, <24 hr old), which were then starved for 3 days and subsequently fed a regular diet. The nature of the succeeding molt shows whether or not the implanted CA were comparable to day 0 CA in activity. This assay actually measures the response of CA to allatotropin secreted by the host brain (9, 15). Hence, the failure of implanted CA to induce supernumerary molting in the assay animals would indicate that the CA were unable to increase JH production to a level that is required for promoting a larval molt.

RESULTS

The Change in Responsiveness of the Fifth-Instar CA to Allatotropin. To determine whether fifth-instar CA undergo a change in activity similar to that observed in Cecropia (5) and *Bombyx* (4), CA from fifth-instar larvae of various age groups

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Abbreviations: JH, juvenile hormone; CA, corpora allata; NSC, neurosecretory cells; M-NSC, medial neurosecretory cells, L-NSC, lateral neurosecretory cells.

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were assayed by using the starved-fifth-instar assay. Donors were selected on the basis of chronological age (beginning from the time of feeding on day 0) and corresponding morphological changes, which establish the time-frame relative to the impending pupal ecdysis (7, 9). Our selection of donors was also influenced by the JH titer curve (16), which shows two discrete periods of marked decrease in hemolymph JH concentration. A drastic decrease occurs by day 1, and the titer is maintained at this low level during the next 2 or 3 days, after which JH levels become undetectable.

CA were excised from donors on days 0, 1, 3, 4, 6, and 8 and implanted into allatectomized hosts prior to starvation. Table 1 shows that in contrast to day 0 CA, CA of day 1 and older donors were unable to promote supernumerary molting of hosts. This demonstrates that by day 1, the CA had undergone a physiological change that decreased their responsiveness to allatotropin.

Evidence for Allatohibin. The data in Table 1 imply that, contrary to the previous assumptions, the CA may actually be turned off by day 1. To test whether the inhibitory signal is neural or neurohormonal, the nervi corporis allati were severed on day 0, thereby detaching the CA from the brain–corpora cardiaca complexes. On day 1 the CA were removed and assayed. Of the 22 hosts, 20 survived and all molted into pupae, as did the recipients of CA from sham-operated larvae (Table 2). The inhibition of CA by day 1 is, therefore, not mediated by neural stimuli that require intact allatal nerves.

We then examined whether the brain is the source of an inhibitory hormone by selective destruction of various groups of neurosecretory cells (9). The anteromedial region containing group II, but possibly also some of the group I neurosecretory cells (NSC), was cauterized in one group and the lateral region containing groups III and IV NSC (L-NSC) in another group of day 0 larvae. On day 1, CA from each group of larvae were assayed. Only cautery of the medial neurosecretory cells (M-NSC) prevented inhibition of CA (Table 2), which suggests that the inhibitory neurohormone allatohibin originates from the M-NSC.

Is allatohibin present in the hemolymph continuously until pupation? An extensive study was carried out to test this. Day 0, fifth-instar CA were implanted into fifth-instar larvae of different ages (interim host). One day later the implanted CA were recovered from the hosts, and their activity was tested by the starved-fifth-instar assay. In every case the intact CA of the interim hosts were also assayed in the same manner, and these served as controls.

Table 1. The change in activity of CA in the fifth-instar larvae of *Manduca*

Age of donor	Hosts	Survivors No.	Survivors %	Survivors molted to Sixth instar larvae, %	Survivors molted to Pupae, %
Day 0 (newly ecdysed)	40	29	73	93	7
Day 1	29	21	72	0	100
Day 3 (frosted frass)	25	19	76	0	100
Day 4 (exposure of dorsal vessel)	25	20	80	5	95
Day 6 (prepupae)	30	22	73	0	100
Day 8	10	10		0	100

CA were excised from normally fed donor larvae and implanted into allatectomized, day 0, fifth-instar hosts. The latter were then starved for 3 days and refed a normal diet. Supernumerary molting of host larvae indicates that allatotropin released by the host brain (9, 15) stimulated the implanted CA to secrete high amounts of JH, causing a larval molt. Pupation of the hosts indicates that biosynthesis of JH was not stimulated and, hence, the CA were refractory to allatotropin. In all experiments only a single pair of CA was implanted.

Table 2. Effects of denervation of CA or of cautery of neurosecretory cells in newly ecdysed fifth-instar larvae on activity of CA

Treatment of day 0 donor	Hosts	Survivors No.	Survivors %	Survivors molted to Sixth-instar larvae, %	Survivors molted to Pupae, %
Severance of nervi corporis allati	22	20	91	0	100
Cautery of M-NSC	16	13	81	100	0
Cautery of L-NSC	16	14	88	0	100

In all cases the CA were excised on day 1 and assayed in the same manner as described for Table 1. The M-NSC contain group II neurosecretory cells and perhaps some cells of group I; L-NSC contain groups III and IV neurosecretory cells.

The results summarized in Table 3 show that CA implanted into interim hosts on day 0 and removed on day 1 were unable to cause a supernumerary molt in the assay hosts, whereas CA implanted into older interim hosts and taken out one day later were able to promote supernumerary molting on implantation into assay hosts. As expected, the intact CA of day 1 or older interim hosts were inactive. We conclude from these observations that allatohibin was not present in the hemolymph of day 2 and older larvae.

Evidence for Neural Inhibition. The preceding data do not support the conclusion of Nijhout and Williams (17) that in fifth-instar *Manduca* larvae, the CA are turned off approximately 24 hr before larvae enter the wandering stage. The hemolymph JH titer curve (17) does show a sharp decrease 1 day prior to the wandering stage, which may be due in part to the onset of a rapid increase in hemolymph JH esterase levels (18). However, it also is conceivable that after exposure to allatohibin on day 1, the CA gradually decrease in activity and become virtually inactive within 2–3 days. The plateau in the JH titer curve between days 1 and 3 and the sharp decrease on day 3 argue against such a conclusion. Further, Fukuda (4), using allatectomized, young, fourth-instar hosts as test animals, has observed a sharp decline in CA activity midway through the fifth (last larval) instar of the silk moth *Bombyx mori*. In Cecropia (5) also, inactivation of CA seemed to occur at the end of the feeding period in the last larval instar. If a second level of inactivation does occur in *Manduca* larvae, it is obvious that the assay with starved, fifth-instar larvae is not sensitive enough to allow discrimination between less active and inactive CA. In an attempt to distinguish between these two levels of activity, we transplanted fifth-instar CA into allatectomized, young, fourth-instar larvae. Because the volume of hemolymph in fourth-instar larvae is smaller than in fifth-instar larvae, dilution of the small quantities of JH secreted by the less active CA would presumably be minimized and the hosts might molt into fifth-instar larvae.

First, we determined the length of time during the fourth instar when the CA are required to cause the molt to the fifth instar. Groups of fourth-instar larvae were allatectomized on days 0, 1, 2, and 3. In the control groups, the CA were removed and reimplanted. All of the surviving 20 or more larvae allatectomized on day 0 or day 1 pupated. Only 14 of the surviving 42 larvae allatectomized on day 2 pupated; the remaining 28 larvae molted into black, fifth-instar larvae (JH deficiency

Table 3. Test for the presence of the inhibitor neurohormone allatohibin in the hemolymph of fifth-instar larvae of *Manduca*

Interim host age at				Survivors		Survivors molted to	
CA* implantation	CA excision	CA source	Hosts†	No.	%	Sixth instar larvae, %	Pupae, %
Day 0	Day 1	Implant	20	14	70	0	100
		Host	10	7	70	0	100
Day 2	Day 3	Implant	20	15	75	100	0
		Host	10	8	80	0	100
Day 3	Day 4	Implant	20	17	85	100	0
		Host	10	9	90	0	100
Day 6	Day 7	Implant	20	14	70	100	0
		Host	10	10	100	0	100

* Donors of Ca were day 0, fifth-instar larvae.
† For assay procedure refer to Table 1.

syndrome, see ref. 16) and many of them had pupal mouth parts. All 10 allatectomized day 3 larvae molted into structurally normal but black, fifth-instar larvae. Among the controls, all of the surviving larvae in every group molted into normal, fifth-instar larvae. We conclude that the CA are required until day 2 for a larval molt, thus confirming the observations of Kiguchi and Riddiford (19).

In the fourth-instar larvae, JH titer begins to decline from day 1 (16), although no JH esterase is detectable in the hemolymph (20). This suggests that the decrease in JH levels is due to reduced activity of CA, and yet the CA are required at this time for the larval–larval molt (see earlier results). Consequently, we assumed that even less active fifth-instar CA would effectively substitute for day 1, fourth-instar CA and, therefore, chose day 1, allatectomized larvae as hosts for testing CA activity. CA from 0-, 1-, 3-, 4-, 6-, and 8-day-old fifth-instar larvae were tested. Table 4, shows that day 0 and day 1 CA induced allatectomized, fourth-instar hosts to molt into fifth-instar larvae, whereas CA from day 3 and older animals were unable to do so. These results indicate that CA do undergo a further decrease in activity between day 1 and day 3, although both behaved identically in the starved-fifth-instar assay. Day 1 CA thus appear to retain a low level of activity that is abolished by day 3. (Only those exhibiting frosted frass were used in this experiment, because preliminary findings showed that CA from larvae that had not excreted frosted frass by day 3 were as active as day 1 CA). The CA remain in this inactive state until at least day 8 (i.e., 1–2 days before pupation).

At this point, it seemed desirable to use a fourth-instar assay to discriminate between day 0 and day 1 CA not only to confirm the results of the starved-fifth-instar assay (Table 1) but also to provide a more rapid *in vivo* assay. Because JH titer is very high in day 0, fourth-instar larvae (16), we assumed that only highly active or responsive CA would be able to maintain the JH concentration at that level in allatectomized, day 0, fourth instar larvae and cause a larval molt. Table 4 clearly demonstrates that only day 0, fifth-instar CA had that capacity. CA from all other groups were ineffective, and the hosts underwent precocious pupation. These results are identical to results obtained with starved-fifth-instar assay. The most important fact to emerge from these experiments is that the two fourth-instar assays clearly identify three distinct levels of activity of fifth-instar CA: high activity on day 0, less activity on day 1, and undetectable activity on day 3 and beyond.

The marked loss of activity of day 3, fifth-instar CA indicates that the second inhibitory event probably occurs during the scotophase preceding day 3, when larvae first excrete frosted frass. It is interesting to note that in our synchronized cultures, most of the larvae attain weights of 5 g or more prior to this period. The second inhibition is likely to be neurally but not neurohormonally mediated, because active (day 0) CA implanted into fifth-instar larvae on either day 2 or 3, removed on day 3 or 4, respectively, and assayed in starved, fifth-instar larvae remained active (Table 3). Therefore, the following experiments were done to establish the timing and nature of the second inhibition: (*i*) the nerves to the CA were severed on day 2, and the CA were recovered on day 4 (wandering larvae); (*ii*) the nerves were severed on day 3 (after appearance of the frosted frass), and the CA were taken out on day 4; and (*iii*) the sham operation was done on day 2, and the CA were taken out on day 4. In all cases the CA were then assayed in allatectomized, day 1, fourth-instar larvae. Table 5 shows that when de-

Table 4. Evidence for a two-step inactivation of CA of the last-instar *Manduca* larvae*

Age of CA donor	Hosts	Survivors		Survivors molted to	
		No.	%	Pupae at end of fourth instar, %	Fifth instar, %
		Day 1 fourth-instar assay			
Day 0	20	19	95	0	100
Day 1	25	24	96	4	96
Day 3 (frosted frass)	30	26	87	100	0
Day 4 (exposed dorsal vessel)	10	10	100	100	0
Day 6 (prepupae)	10	8	80	100	0
Day 8	10	8	80	100	0
		Day 0 fourth-instar assay			
Day 0	40	32	80	0	100
Day 1	47	42	89	100	0
Day 3	14	11	79	100	0
Days 6 & 8	20	17	85	100	0

* CA from fifth-instar larvae (donors) were implanted into day 1 or day 0, allatectomized, fourth-instar larvae (hosts). The biosynthetic capacity of the implanted CA is indicated by the nature of the host's molt.

Table 5. Effect of denervation on the second inhibition of CA

Procedure	Hosts*	Survivors No.	Survivors %	Survivors molted to: Pupae at end of fourth instar, %	Survivors molted to: Fifth instar, %
Allatal nerves severed on day 2†	30	25	83	0	100
Allatal nerves severed on day 3† (frosted-frass stage)	10	9	90	100	0
Sham operation on day 2†	20	16	80	100	0

* Assays were done in day 1, allatectomized, fourth-instar larval hosts as described for Table 4.

† In all cases CA were excised and assayed after the larvae exposed the dorsal vessel on day 4.

nervation of CA was done on day 2, the CA retained the same level of activity as that of day 1 CA (Table 4). Denervation of CA in larvae excreting frosted frass had no effect, because the CA recovered a day later from wandering larvae remained inactive. CA from sham-operated larvae were also inactive. Evidently the second inhibition, which occurs by day 3 before larvae excrete frosted frass, required the presence of intact allatal nerves. Therefore, denervation effectively prevents this inactivation.

DISCUSSION

The data presented here describe a two-step mechanism of inactivation of CA in the last larval instar of a holometabolous insect. The first stage of inactivation takes place within 1 day after ecdysis to the fifth instar and is mediated by a neurohormone secreted by the M-NSC in the brain. The second inactivation step occurs by day 3 and requires the presence of intact allatal nerves.

Allatohibin is present in the hemolymph during the first 24 hr after ecdysis and is not detectable in day-2 and older larvae. This indicates that the brain secretes the inhibitory hormone only for a short period of time and that the hormone has either a short half-life or is actively removed from the hemolymph. Allatohibin does not totally inactivate the CA; instead, it restricts the responsiveness to allatotropin. This can be deduced from the following observations: (*i*) Unlike day 0, fifth-instar CA, day 1 CA failed to induce a supernumerary molt in the starved-fifth-instar assay. Because starvation appears to stimulate allatotropin production (9, 15), we can infer that day 1 CA were unable to increase JH production in the presence of allatotropin to the level obtained with day 0 CA. (*ii*) We had shown previously that day 0, fourth-instar brain secretes high amounts of allatotropin (15), and yet day 1, fifth-instar CA implanted into allatectomized, day 0, fourth-instar larvae failed to secrete a sufficient amount of JH to promote a larval molt. This again indicates reduced responsiveness to allatotropin.

However, day 1 CA continue to produce JH at a low level; when implanted into allatectomized, day 1, fourth-instar larvae, they promoted a larval molt. This conclusion is also supported by the JH titer determinations (16, 17), which indicate a low but detectable level of JH in the hemolymph between days 1 and 3. Thus, glands exposed to allatohibin show reduced biosynthetic capacity and diminished responsiveness to allatotropin.

The second step in inactivation occurs by day 3, almost 24 hr before larvae enter the wandering stage. This second inhibition is neural because it requires the presence of intact allatal nerves. Furthermore, active glands implanted in day 3 larvae are not inactivated (Table 3). The timing of neural inhibition coincides with the appearance of the frosted frass, which thus appears to be a reliable indicator of the inhibitory event, although the causal relationship, if any, between the two is not clear. It results in virtual inactivation of CA, as seen from the failure of day 3 (and older) CA to induce a larval molt in allatectomized, day 1, fourth-instar larvae. This observation supports the conclusions of Nijhout and Williams (17) that prior to the wandering stage the CA are turned off. We suggest that the inactivation of *Bombyx* CA before termination of the feeding period (4) corresponds to the neural inhibition in *Manduca*.

The two stages of inactivation coincide in time with the two periods of decline in hemolymph JH concentrations (16, 17). Similar hemolymph JH profiles have been reported in other lepidopteran larvae (21–24), which suggests that in these species the CA may be regulated in the same manner as in *Manduca*. Still to be explained is the small peak of JH observed 1 day prior to pupal ecdysis in many lepidopterans including *Manduca* (21–25). It is assumed that the CA resume JH secretion for a brief interval during this time, and yet in none of our experiments did we find any evidence for reactivation of CA. Day 8 (pupal ecdysis occurs on days 9–10) CA were inactive in all tests and, hence, neither denervation nor exposure to allatotropin stimulated detectable activity in these glands. Likewise the last-instar CA of both Cecropia (5) and *Bombyx* (4) did not regain activity on implantation into developing pupae or younger larvae, respectively. Further, Fukuda (4) demonstrated that intact or denervated *Bombyx* CA recovered activity only in pharate adults (that is, after residing for a certain length of time in the metamorphic environment). If the observed increase in hemolymph JH concentration in pharate pupae is due to increased secretory activity of CA, it is necessary to postulate a special, transient neural stimulus for removal of inhibition and stimulation of JH secretion at this time.

In conclusion, based on the findings reported here and elsewhere (9, 15), we propose that the activity of CA in the last-instar *Manduca* larva is regulated by the brain via stimulatory and inhibitory signals. Soon after ecdysis to the fifth instar, the M-NSC stop secreting allatotropin (15). This in itself is sufficient to lower CA activity, because M-NSC cautery, which destroys both allatotropin- and allatohibin-producing cells, prevents supernumerary molting of starved larvae (9). However, within 24 hr after ecdysis, the brain secretes an inhibitory neurohormone, allatohibin, which reduces the biosynthetic capacity of CA. By day 3, the CA become turned off completely by an inhibitory signal from the brain via the allatal nerves. We hasten to add that our findings do not preclude the involvement of other mechanisms, such as release of JH esterase in reducing hemolymph JH levels. In fact, the animal uses all of these modes of regulation of hemolymph JH titer to ensure that in the last larval instar, the molt is initiated when the JH titer decreases to undetectable levels. Collectively, they constitute a very reliable system for assuring a smooth transition from the larval to the pupal stage.

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