Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 2960–2964, April 1994
Physiology

Nervous control of juvenile hormone biosynthesis in *Locusta migratoria*

(insect brain/pars lateralis/corpora allata/semiintact preparation)

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Communicated by Howard A. Bern, December 6, 1993

ABSTRACT **In *Locusta migratoria migratorioides* R. and F., two types of brain neurons innervate the juvenile hormone (JH)-producing corpora allata (CA). Thirteen cells in each pars lateralis (PL) innervate the ipsilateral CA, while four cells (two in each PL) innervate both glands. We investigated possible influences of these two neuronal types on JH production by a newly developed method. A radiochemical assay was used to measure hourly JH production by a CA with intact nerve connections to the brain. Then, changes in hormone production due to selective nerve stimulation or transection were assessed. In control preparations JH production per h remained approximately constant for at least 9 h. Simultaneous electrical stimulation of all neurons innervating one CA (i.e., 13 ipsilateral plus 4 bilaterally innervating cells) always inhibited JH production, while their transection led to a rapid progressive increase in JH biosynthesis in CA from females with oocytes longer than 4.5 mm. Thus, there is strong neurally mediated inhibition of the CA at certain phases of the vitellogenic cycle. The dramatic effects of nerve transection show that *in vitro* rates of JH production are an unreliable indicator of *in vivo* levels. Selective stimulation of the four neurons innervating both CA suggests that they do modulate JH biosynthesis but the effect varies qualitatively depending on the phase of the vitellogenic cycle.**

Juvenile hormone (JH) is a key insect hormone. Its controlled production is crucial for postembryonic development, metamorphosis, sexual maturation, and reproductive physiology (1, 2). It is produced exclusively by a pair of endocrine glands, the corpora allata (CA), which together with the corpora cardiaca (CC) and associated nerves form the retrocerebral complex. In locusts, two neurosecretory centers in each brain hemisphere, the pars intercerebralis and pars lateralis (PL), project to the CC via the nervi corporis cardiaci 1 and 2 (NCC1, NCC2), respectively. Each CA is connected to the CC and subesophageal ganglion via the nervi corporis allati 1 and 2 (NCA1, NCA2), respectively. Although modulation of JH production via a hormonal route may also occur, there is strong evidence that an important control mechanism involves brain neurosecretory cells that project to each CA and exert a paracrine influence on gland cells (3). Candidate neuropeptides, termed allatotropins and allatostatins, which stimulate and inhibit JH production, respectively, have recently been isolated from the insect brain (4–6).

Studies of regulation of JH production by means of short-term *in vitro* radiochemical assay (RCA) (7, 8) indicate that nerve inputs from the subesophageal ganglion are not involved and that the type of CA regulation via brain neurons varies between species (3, 9). Allatostatic control exists in the cockroach *Diploptera punctata* (10), the beetle *Leptinotarsa decemlineata* (11), and probably in the cricket *Gryllus bimaculatus* (12), whereas nerve-mediated allatotropic effects have so far been observed only in locusts (13–18). However, nerve-mediated inhibitory control of JH production has been reported in some locust strains (19). Possibly, excitatory and inhibitory mechanisms of neural control of CA activity exist side by side, with one or the other mechanism being dominant at particular stages of larval development and oocyte maturation.

A recent study of the CA-innervation pattern in locusts indicates a possible neuroanatomical substrate for antagonistic control mechanisms of the CA, since two morphologically different types of brain neurons with somata in the PL innervate the CA (20). Two cells in each PL extend axons bilaterally and innervate both CA, while 13 cells in each PL project to the ipsilateral gland only (Fig. 1*A*). No connections from the brain to the CA via NCC1 were found.

Several alternative mechanisms of CA regulation can be hypothesized on the basis of this innervation pattern. Assuming that each morphological type is physiologically homogeneous, then the 4 bilaterally innervating cells could mediate allatotropic effects while the 13 ipsilaterally projecting cells might have an allatostatic influence, or vice versa. Alternatively, the latter group could consist of two subpopulations, one with a stimulatory and the other with an inhibitory influence on CA activity. The cells with bilateral projections resemble morphologically the prothoracicotropes of moths (21–24). Thus, they may be a source of prothoracicotropic hormone (PTTH), with the CA acting as the release site for this hormone rather than its target. However, PTTH itself or other peptides produced by the prothoracicotropes might also modulate JH production (23, 25, 26).

It is difficult to test the above hypotheses by using the conventional *in vitro* assay for JH production. Isolating the CA destroys both types of nerve connection simultaneously, and recent evidence that allatostatins are quick acting (27) suggests that the effect of removing a CA might mask the effect of a previous treatment. Therefore, we developed a preparation in which the hourly JH production of a CA, with intact and functional nerve connections to the brain, could be measured by RCA. Using this preparation, the time course of changes in JH production in one gland due to selective nerve stimulation or nerve transection was monitored.

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Abbreviations: CA, corpora allata; CC, corpora cardiaca; JH, juvenile hormone; NCA1, nervus corporis allati 1; NCC2, nervus corporis cardiaci 2; PL, pars lateralis; PTTH, prothoracicotropic hormone; RCA, radiochemical assay.

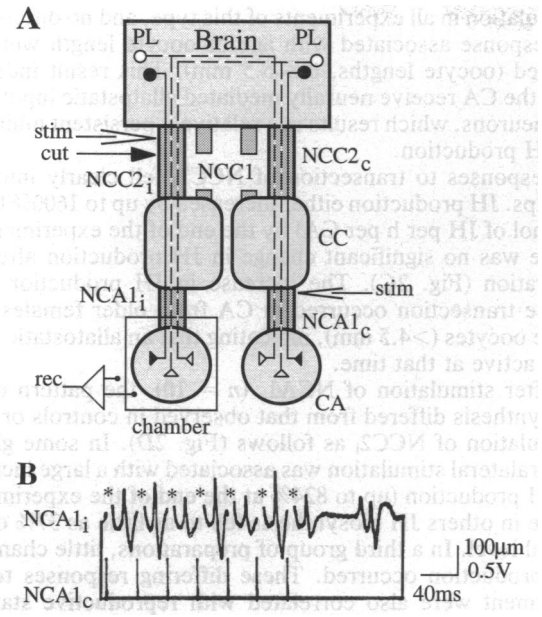

FIG. 1. (*A*) Diagram of preparation used to study effects of PL cells on JH production in *L. migratoria*. Thirteen cells project to the ipsilateral gland only (●), while two cells in each PL innervate both glands via paired axons (○). These connections from brain to CA via NCA1 and NCC2 remained intact bilaterally; all other nerves were cut and the CC was split along the midline. One CA was positioned in a Teflon chamber in which the RCA was carried out. Suction electrodes (stim) were positioned on the $NCA1_c$ and $NCC2_i$ and activity in $NCA1_i$ was measured differentially between the inside and outside of the incubation chamber via a pair of stainless steel wires (rec). Arrow indicates site of nerve transection. (*B*) Typical responses, recorded from $NCA1_i$, to stimulation of $NCA1_c$ with a train of brief stimuli (typical parameters: train duration, 200 ms; train repetition rate, 1 per s; pulse frequency, 30–40 Hz; pulse duration, 0.05–0.1 ms; mean spike frequency, 8 per s). Each spike is a compound action potential produced by simultaneous activation of four PL neurons with bilateral projections via antidromic stimulation of their contralateral branches. *, Stimulus artifacts.

MATERIALS AND METHODS

Animals. Experiments were performed on adult female *Locusta migratoria migratorioides* R. and F. taken from a crowded laboratory culture. They were reared under a 12:12 h (light/dark) photoperiod and fed on wheat seedlings and bran. The temperature in the cages varied from 30°C to 36°C during the day and from 20°C to 25°C at night. Females laid their first egg pod 2–3 weeks after adult emergence.

Preparation. Experiments were performed on isolated head preparations. Locusts were decapitated, the mouthparts were removed, the head was mounted dorsal side up in a small dish, and the retrocerebral complex was exposed by dorsal dissection. The brain and subesophageal ganglion together with their tracheal supply remained intact, along with the ocelli, compound eyes, and antennae. The viability of the preparation was maintained by ventilating the interconnected trachea of the head via a polythene cannula connected to an air supply. Under these conditions, spontaneous antennal movements, visual and tactile antennal reflexes, and spontaneous activity in the nerves of the retrocerebral complex remained for the 10-h duration of the experiments. The NCC1 and NCA2 were cut bilaterally together with the recurrent nerve, and the CC were split along the midline. Thus, only the connections from the brain to CA via NCC2, CC, and NCA1 on each side remained intact (Fig. 1*A*). One CA was isolated in a small Teflon chamber (≈5 µl) in which the RCA of JH production was later carried out. The intact NCA1 passed through a narrow slot in one wall of the chamber. The slot was sealed with petroleum jelly and the chamber was filled with insect Ringer's solution. Suction electrodes for nerve stimulation and recording were applied to $NCC2_i$ (ipsilateral) and $NCA1_c$ (contralateral) (the terms ipsilateral and contralateral are relative to the side of the CA in the chamber). Spontaneous and evoked activity in $NCA1_i$ was recorded differentially between the inside and outside of the chamber via two stainless steel wire electrodes (Fig. 1).

Assay of JH Production. JH3 is the only JH produced in insects other than Lepidopterans (3). JH3 production was measured at room temperature using a RCA (7, 8) adapted slightly for the *in vivo* conditions.

Incubation Medium. L-[*methyl*-^{3}H]Methionine (TRK583) was obtained from Amersham at a specific activity of 83.4 Ci/mmol (1 Ci = 37 GBq). Basic tissue culture medium was made from 90% (vol/vol) TC199 (Serva) [without methionine but with Hanks' salts, bicarbonate, glutamine, 50 mM Hepes (pH 6.95), and 20 µg of Ficoll per ml] and 10% (vol/vol) fetal calf serum with low molecular weight components removed by Sephadex G-25 separation. This basic medium was mixed 1:1 with insect Ringer's solution (140 mM NaCl/10 mM KCl/7 mM $CaCl_2$/1 mM $MgCl_2$/4 mM $NaHCO_3$/4 mM Trehalose/5 mM Tes buffer, pH 7.3) and substituted with nonradioactive methionine and L-[^{3}H]methionine to reach a final total concentration of 55 µM methionine with a final specific activity of 7.1 Ci/mmol.

Initially, the Ringer's solution in the chamber was replaced with this medium for a 30-min preincubation. Subsequently, the medium was exchanged at 1-h intervals for, in most cases, 9 h. To measure the amount of labeled JH produced, each sample was mixed with 100 µl of ethanol containing 20 µg of nonradioactive JH3 and applied directly to analytical silica plates (Merck; silica gel 60 F245; 0.2 mm). Lipophilic substances were concentrated to a line 3 cm above the application area by using ether, the gel of the application area was removed, and the plate was developed in *n*-hexane/ether (2:1, vol/vol). JH3 spots were located under UV light and the corresponding silica gel was scraped off for liquid scintillation counting (minivials; Emulsifier Safe, Beckman LC6000C, set to dpm mode). Control experiments in which calibrated amounts (50,000 dpm) of ^{3}H-JH3 (NEN/DuPont), dissolved in 5 µl of nonradioactive medium, were pipetted into the incubation chamber and, 1 h later, processed in the same way revealed >95% recovery of ^{3}H-JH3 by this method.

Experimental Protocols. Four types of experiment were performed. (*i*) In control experiments, the level and variability of JH production over 9 h were measured. (*ii*) Strong stimulation of $NCC2_i$. This should excite the axons of all 17 brain neurons terminating in the gland under study (13 ipsilateral cells and 4 cells that innervate both CA). (*iii*) Transection of $NCC2_i$. This removes the effects of all currently active nervous inputs from the brain. (*iv*) Stimulation of $NCA1_c$. This selectively excites only four neurons projecting to the CA under investigation—i.e., those with bilateral projections—by antidromic stimulation of their contralateral projection.

In nerve stimulation and transection experiments, the first 2 h of incubation were used to estimate JH production prior to the treatment. Then, $NCC2_i$ was cut and JH measurements were made for 4 or 7 h. Alternatively, the selected nerve ($NCA1_c$ or $NCC2_i$) was stimulated for 3 h with trains of pulses and JH production was monitored for a further 4 h poststimulation. Stimulus intensity (0.6–10 V) was chosen to induce the maximum amplitude of compound action potentials (i.e., recruitment of all units). Spontaneous activity of $NCA1_i$ was also monitored, and spikes were counted automatically by using a window discriminator linked to a computer.

RESULTS

Production of JH by untreated glands varied between preparations from 0.06 to 1 pmol of JH3 per h per CA. The variability is partly attributable to variation in the age and reproductive state of the females used. However, *in vitro* JH biosynthesis rate varies considerably even between individuals of the same age (28, 29), and great asymmetry between left and right CA activity of an individual locust occurs (30, 31). Therefore, to facilitate comparison among preparations treated in the same way, and to emphasize changes in JH biosynthesis during experiments irrespective of the absolute amounts produced, JH production in each incubation period was expressed as a percentage of mean production during the first 2 h (i.e., before any treatment).

In control trials (females with oocytes 0.4–4.3 mm long), JH production of individual CA varied somewhat in the course of 9 h, but in most cases no progressive increase or decrease of JH production was observed. Thus, mean percentage JH production remained approximately constant (Fig. 2*A*). The SD of JH production in control experiments served as a criterion for assigning a stimulatory or inhibitory effect to experimental treatments. A progressive increase in JH3 biosynthesis rate to final values exceeding 152% (control JH production in last hour + 1SD) was declared a stimulatory effect of the treatment while a progressive decline to <65% (control JH production in last hour − 1SD) was declared inhibitory.

Stimulation of $NCC2_i$ resulted in a progressive decline of JH production to levels as low as 20% of pretreatment values (Fig. 2*B*). Production of JH was lower during and after stimulation in all experiments of this type, and no differences in response associated with age or oocyte length were observed (oocyte lengths, 0.8–5.5 mm). This result indicates that the CA receive neurally mediated allatostatic input from PL neurons, which results in a relatively persistent inhibition of JH production.

Responses to transection of $NCC2_i$ fell clearly into two groups. JH production either increased by up to 1600% (up to 6 pmol of JH per h per CA) by the end of the experiment, or there was no significant change in JH production after the operation (Fig. 2*C*). The increase in JH production after nerve transection occurred in CA from older females with large oocytes (>4.5 mm), indicating that an allatostatic input was active at that time.

After stimulation of $NCA1_c$ (n = 10), the pattern of JH biosynthesis differed from that observed in controls or after stimulation of $NCC2_i$ as follows (Fig. 2*D*). In some glands contralateral stimulation was associated with a large increase in JH production (up to 824% at the end of the experiment), while in others JH biosynthesis fell to as little as 37% of the initial level. In a third group of preparations, little change in JH production occurred. These differing responses to the treatment were also correlated with reproductive state as measured by oocyte length (32).

The relationship between the phase of the vitellogenic cycle and the effect of the treatments is shown in Fig. 3. Stimulation of $NCC2_i$ resulted in an inhibition of JH biosynthesis at all tested stages of the oocyte maturation cycle. The increase in JH production after $NCC2_i$ transection occurred in preparations from females in the later stages of the oocyte development cycle—i.e., late vitellogenesis and choriogen-

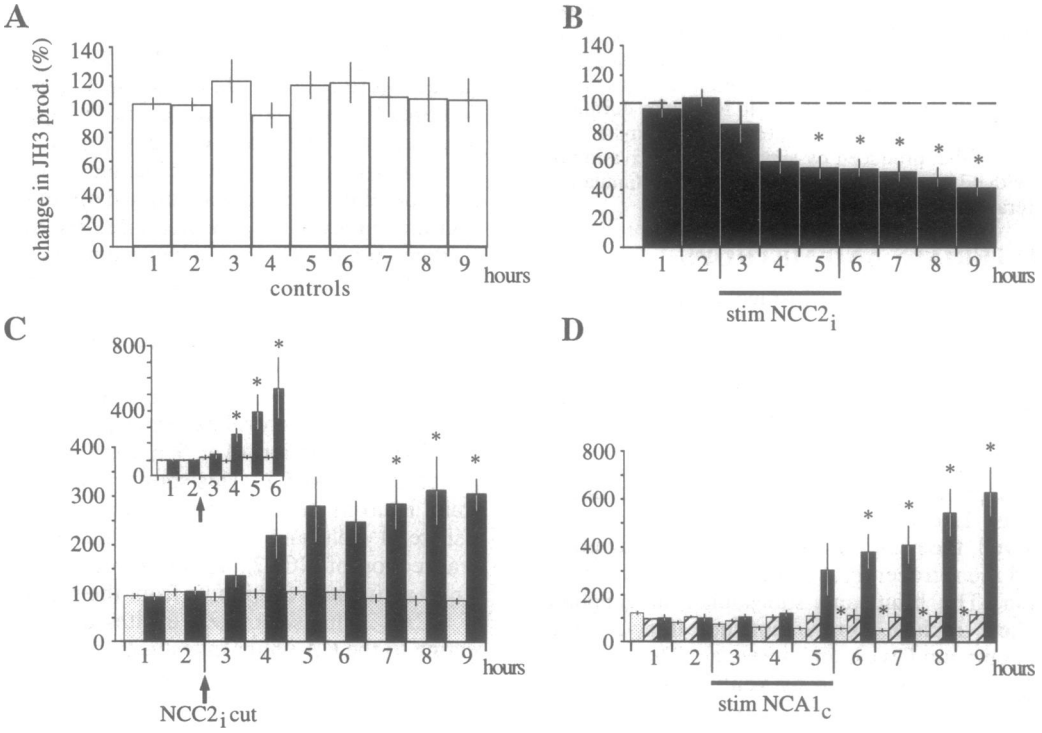

FIG. 2. Influence of PL cells on JH production in *L. migratoria*. Histograms show mean changes in JH production per h (±SEM) over 9 h as a percentage of mean production in the first 2 h. *, JH production rates differing significantly from that measured in the first 2 h—i.e., prior to treatment (Wald–Wolfowitz runs test; $0.05 < P < 0.1$). (*A*) JH production in control experiments (n = 7). (*B*) Stimulation of $NCC2_i$ leads to a decrease in JH production, which persists after the stimulation period (n = 6). (*C*) Effects of transecting $NCC2_i$. Two types of response were obtained. No effect of the treatment was found in CA from females with oocytes < 4.6 mm long (shaded columns) (n = 3–5). In CA from females with oocytes > 4.6 mm long, JH production increased after transection (solid columns) (n = 3–4). (*Inset*) Comparison between JH production in control experiments (open columns) and from denervated CA (solid columns) including additional data from several short-term (6-h duration) experiments (n = 7). (*D*) Effect of stimulating $NCA1_c$. Responses are divided into three groups according to oocyte length. In CA from females with oocytes < 1 mm long, JH production decreased (shaded columns; n = 4). No effect of the treatment was seen for oocytes 1–3.4 mm long (hatched columns; n = 3), and there was a large increase in hormone production when oocytes were > 3.4 mm long (solid columns; n = 3).

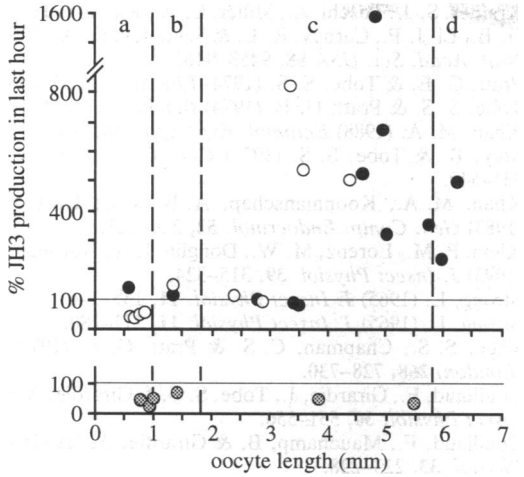

FIG. 3. Relationship between phase in reproductive cycle, as indicated by length of terminal oocytes (32), and effects on JH production of $NCC2_i$ transection (solid circles), $NCA1_c$ stimulation (open circles), and stimulation of $NCC2_i$ (shaded circles; separate axes). Dashed lines demarcate ovarian maturation stages: a, previtellogenesis; b, first phase of vitellogenesis; c, second phase of vitellogenesis; d, choriogenesis.

esis (32). This suggests that the CA are under strong inhibitory influence from PL cells during this time. All animals in which stimulation of the four cells with bilateral projections caused a decrease of JH production were at the beginning of the first oocyte maturation cycle (previtellogenesis). Increase in JH production as a result of stimulation of $NCA1_c$ was observed in females with oocytes >3 mm long.

Low spontaneous activity in $NCA1_i$ measured during the first 2 h was associated with high JH production in preparations of females with oocytes >4.5 mm long, suggesting that an allatostatic influence via the nerve predominates at this stage. This agrees with the results from $NCC2_i$ transection experiments, which also suggest that inhibitory inputs are active at this time. However, there was no consistent relationship between $NCA1_i$ activity and hormone production at other stages of the reproductive cycle, possibly due to simultaneous activity in both allatotropic and allatostatic units or in units with no influence on CA activity.

DISCUSSION

The rates of JH production in the current study are at the low end of the wide range of values found in locust CA in *in vitro* studies (29, 30). While the results show that low rates are partly due to inhibitory influences from the intact nerves, the different and nonoptimal experimental conditions of the *in vivo* assay, compared to most *in vitro* studies, are probably also a contributing factor (i.e., smaller volume of medium, lower temperature, no shaking). The main purpose of the study, however, was not to measure absolute values of hormone produced but to investigate the time course of changes in hormone production in a single CA after treatment.

Stimulation of $NCC2_i$ decreased JH production by CA from females of all stages tested of the vitellogenic cycle, indicating that this pathway mediates a powerful allatostatic influence. This is almost certainly mediated via paracrine release of neurosecretory products from PL neurons that terminate within the CA. Neurohormones produced in other PL cells and released from the CC by $NCC2_i$ stimulation cannot reach the CA under our experimental conditions. In *L. migratoria*, there is also no evidence for the existence of intrinsic CC cells with processes extending to the CA, which could mediate an indirect action of brain neurons on JH production (20). Furthermore, since the four neurons innervating both CA were not spontaneously active under the experimental conditions, the inhibition removed by nerve transection originated from neurons in the ipsilateral PL.

Nerve transection experiments indicate that an inhibitory mechanism, already present at the beginning of adult life, is particularly effective during the later stages of the oocyte maturation cycle. This might be a result of increased activity in allatostatic units in the nerve but it could also involve increased physiological sensitivity of the CA to allatostatin during the final stages of oocyte maturation, as shown in *D. punctata* (27).

In vitro assays of CA activity have been used to estimate JH production *in vivo* on the assumption that no significant changes in JH biosynthesis take place during the 3 h after denervation (3). However, the present study demonstrates that during later stages of the oocyte maturation cycle this assumption does not hold. Increases of JH production up to 16-fold within 4 h of nerve transection occur. These results confirm predictions, based on rapid action of allatostatin *in vitro*, that the *in vitro* assay may at certain times overestimate *in vivo* production (27).

Most previous studies of locusts have shown evidence for stimulatory control of the CA from the PL (13–18, 33), whereas the current findings resemble more the situation in beetles, crickets, and cockroaches, where the PL is a source of allatostatin (34–36). The PL may, therefore, be the source of both allatotropins and allatostatins. If so, then the experimental design may determine whether allatotropic or allatostatic effects are detected. Previous studies on locusts, involving sectioning of NCA1 immediately after the imaginal molt and followed by measurements of JH production using *in vitro* RCA on subsequent days, may have revealed the absence of a stimulatory factor(s), but not of inhibitory ones, since JH production was already low due to the absence of allatotropin(s) (16, 17). In other studies, nerve transection was carried out at a stage of the reproductive cycle when JH biosynthesis was known to be very high—i.e., during the first part of vitellogenesis (15, 18). At that time, it is very likely that allatotropic control is dominant and inhibitory control is at its weakest.

Alternatively, parallel excitatory and inhibitory influences acting via NCA1 may be active on different time scales. If allatostatins, which are known to act on a time scale of minutes (27), have a more rapid and transient effect than allatotropins, then the acute effect of nerve stimulation or transection, as measured in the present study, would be a decrease or increase in JH production, respectively. This effect might be reversed in the longer term (hours to days), as measured by previous *in vitro* studies, due to the effects of the slow-acting allatotropin.

The present study provides some evidence that the population of PL neurons innervating the ipsilateral CA is not homogeneous. For example, the multiunit spontaneous activity in $NCA1_i$ often did not correlate with the amount of JH produced. Also, in several cases, transection of $NCC2_i$ abolished high-frequency spontaneous activity in $NCA1_i$ but had no effect on JH production. Such effects could be the result of qualitative or quantitative differences between neurons in their effects on JH biosynthesis, although the latter is influenced not by nervous input alone but also by the physiological state of the CA (27, 37). In *G. bimaculatus* there is immunocytochemical evidence that some, but not all, PL neurons projecting to the soma ipsilateral NCA1 express allatostatin I-like immunoreactivity (35). The finding that stimulation of $NCC2_i$ always led to inhibition of JH production indicates only that inhibitory effects predominate over allatotropic ones, at least in the short term, and does not imply that all, or even a majority, of the CA-innervating axons in this nerve mediate allatostatic effects.

Role of Cells with Bilateral Projections. These four cells in *L. migratoria* show striking morphological similarities in terms of soma position, axonal pathway, and terminal projection to cells identified as the source of PTTH in moths (21–24). The main morphological difference is the presence of a second axon projecting to the soma ipsilateral CA. Thus, these cells may be locust prothoracicotropes, although this remains to be confirmed by immunocytochemistry and *in situ* hybridization.

In moths, the prothoracicotropes survive the imaginal molt and continue to express the PTTH phenotype (26), possibly stimulating ecdysone production by the ovaries (38). However, it has also been proposed, on the basis of the dense terminal projections ramifying among CA gland cells, that the prothoracicotropes might exert a paracrine influence on CA activity. PTTH itself may play this role (23, 26) or other small peptides produced during the processing of the big PTTH precursor molecule (prepro-PTTH) might be involved (23, 39). In *Manduca sexta* an additional 28-kDa peptide unrelated to big PTTH is also produced by the prothoracicotropes and packaged into neurosecretory vesicles, although its function is as yet unclear (40).

The observed changes in JH production during and after selective stimulation of putative prothoracicotropes lend tentative support to the above hypotheses. The apparent change from an allatostatic to an allatotropic effect, as the vitellogenic cycle proceeds, cannot at present be explained. Conceivably, the expression of specific receptor types on/in CA gland cells in a temporally specific manner may underlie changes in the response of CA gland cells to the substance(s) released from the terminals of these cells, as has been proposed to explain changes in the response of CA gland cells to allatostatins (27, 37).

Alternatively, the response to stimulation of these four cells may be highly dependent on which other CA innervating cells are active at the time, a factor that certainly varies during the reproductive cycle. For example, in some cases, selective stimulation of the putative prothoracicotropes resulted in increased JH production, whereas $NCC2_i$ stimulation, which evoked activity in these cells together with all other CA innervating cells, was always inhibitory. Apparently, the stimulatory effect of these four cells can be swamped by the antagonistic effects of other units.

We thank F. Antoni for making the experimental chambers and A. Blejec for statistical advice. This work was supported by Max Planck Society grants to G.H. and M.V.-D., by a Slovene Research Council grant to M.V.-D., and an A. v. Humboldt award to W.L. G.H. was also partly supported by a Deutsche Forschungsgemeinschaft grant (Ho 463/16-3).

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