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HORMONAL CONTROL OF TRANSMITTER PLASTICITY IN INSECT PEPTIDERGIC NEURONS

I. STEROID REGULATION OF THE DECLINE IN CARDIOACCELERATORY PEPTIDE 2 (CAP_2) EXPRESSION

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Summary

Transmitter plasticity, the ability to alter transmitter expression, has been documented in several different preparations both *in vivo* and *in vitro*. One of these is the tobacco hawkmoth, *Manduca sexta*, whose central nervous system contains four individually identified lateral neurosecretory cells (LNCs) that undergo a postembryonic transmitter switch *in vivo*. In larvae, the LNCs express high levels of a myoregulatory peptide, cardioacceleratory peptide 2 (CAP_2). In contrast, the predominant LNC transmitter in adult moths is bursicon, a classic insect peptide hormone responsible for cuticular tanning. Here we show that the CAP_2-to-bursicon conversion by the LNCs is a multi-step process beginning with a decline in CAP_2 levels midway through the final larval stage. We provide several lines of evidence that this CAP_2 drop is regulated by the insect steroid hormone 20-hydroxyecdysone (20-HE). The LNCs exhibit a fall in CAP_2 levels at the beginning of metamorphosis, immediately after the commitment pulse of 20-HE when steroid levels are elevated. LNCs not exposed to this 20-HE rise do not exhibit a decline in CAP_2 level. The transmitter switch can also be prevented by using an analog of juvenile hormone to create a larval hormonal environment during the commitment pulse of 20-HE. The CAP_2 decline in the LNCs could be directly induced by exogenous steroid application, but only under conditions where the LNCs remained connected to the brain. Thus, the first step in the transmitter switch by the LNCs, the decline in CAP_2 levels, is triggered by the commitment pulse of 20-HE, which may act indirectly through a set of steroid-sensitive cells in the brain.

Introduction

Although the transmitter phenotype of most fully mature neurons remains invariant throughout their lifespan, a few have the ability to adjust their transmitter profile postembryonically. Transmitter plasticity in post-mitotic neurons has been best explored

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in sympathetic neurons, which are usually noradrenergic but, under certain conditions, can be induced to express a cholinergic phenotype (Patterson and Chun, 1977). For example, rat sympathetic neurons innervating peripheral sweat glands possess intense catecholamine histofluorescence and immunoreactivity for catecholamine synthetic enzymes shortly after they arrive at the developing sweat glands. These properties, diagnostic for catecholamines, slowly disappear as the gland matures and are replaced by increases in choline acetyltransferase activity and cholinergic transmission (Landis and Keefe, 1983; LeBlanc and Landis, 1986; Stevens and Landis, 1987; Schotzinger and Landis, 1988). Other mammalian neurons, including ciliary ganglion cells (Landis *et al.* 1987), superior cervical cells (Potter *et al.* 1986) and sympathoadrenal derivatives (Anderson *et al.* 1991), also exhibit transmitter plasticity postembryonically. Such plasticity is not limited to the mammalian nervous system: transmitter variations have also been reported in several invertebrate phyla, including coelenterates (Koizumi and Bode, 1986, 1991), molluscs (Gesser and Larson, 1985) and arthropods (Tublitz and Sylwester, 1990). Thus, there are several well-documented instances of transmitter-switching neurons.

Although phenotypic changes in transmitter expression have been well described, much less is known about the regulatory mechanisms that control this type of neuroplasticity. The most detailed work to date has been performed on the adrenergic-to-cholinergic switch by sympathetic neurons that innervate sweat glands. Cultured sympathetic neurons treated with sweat gland extracts undergo many of the changes seen *in vivo*, including the induction of choline acetyltransferase activity and a reduction of catecholaminergic properties (Rao and Landis, 1990). Recent biochemical analyses of sweat gland homogenates have isolated a heat-labile, trypsin-sensitive diffusible factor that triggers cholinergic differentiation in cultured sympathetic cells (Rohrer, 1992). Other soluble factors, including cholinergic differentiation factor (CDF; Yamamori *et al.* 1990), membrane-associated neurotrophic substance (MANS; Wong and Kessler, 1987) and ciliary neurotrophic factor (CNTF; Lin *et al.* 1989), have also been shown to induce phenotypic changes in transmitter expression in sympathetic neurons.

The purpose of this study was to elucidate the mechanisms controlling transmitter expression in one set of transmitter-switching neurons. We have previously shown that the central nervous system (CNS) of the tobacco hawkmoth, *Manduca sexta* (L.), contains a set of four identified lateral neurosecretory neurons (LNCs) that alter their transmitter profile *in vivo* during postembryonic development (Tublitz and Sylwester, 1990). The primary transmitter of the LNCs during embryonic and larval stages is cardioacceleratory peptide 2 (CAP_2), a group of at least three related myoregulatory neuropeptides that modulate gut activity (Broadie *et al.* 1990; Tublitz *et al.* 1991, 1992*a*; Cheung *et al.* 1992). During metamorphosis, these cells switch their transmitter phenotype: CAP_2 content decreases markedly to less than 2% of its original level and the LNCs begin to produce a second neuropeptide, bursicon, which is responsible for tanning the insect cuticle after each molt (Reynolds, 1983). By the final molt from pupa to adult, the LNCs express high levels of bursicon with no detectible CAP_2 bioactivity. In this report, we focus on the regulatory mechanisms underlying the first part of the transmitter

switch by the LNCs, the CAP_2 decline, and show that the decrease in CAP_2 is under the influence of the insect steroid hormone 20-hydroxyecdysone (20-HE). This work has previously been reported in abstract form (Tublitz and Loi, 1990; Loi and Tublitz, 1991).

Materials and methods

Animals

Tobacco hornworms were raised in the *Manduca sexta* rearing facility at the University of Oregon and were staged as previously described (Tublitz, 1989). Briefly, *Manduca sexta* larvae were raised on an artificial diet modified only slightly from that of Bell and Joachim (1976). Shortly after hatching, larvae were placed in individual plastic cups, where they remained until wandering, the period during the fifth instar that marks the beginning of metamorphosis. At the onset of wandering, larvae were transferred to individual chambers in wooden blocks for the remainder of the fifth instar. Animals were removed from the blocks 2 days after the larval–pupal molt and placed in marked plastic bags for the duration of adult development. Larvae and pupae were housed in a controlled environmental chamber using a 17 h:7 h L:D regime with a superimposed thermal period (27°C during the light period, 25°C during the dark period) to improve developmental synchrony. Prior to emergence, these adults (called pharate adults) were placed in a large enclosed chamber (50% relative humidity) for breeding purposes.

Staging of larvae and pupae was based on previously described *Manduca sexta* staging series (Truman and Riddiford, 1974; Weeks *et al.* 1992), which used developmental events as markers. These include ecdysis from the fourth to the fifth instar (day 0, D_0), the onset of wandering behavior (wandering day 0, W_0) and larval–pupal ecdysis (pupal day 0, P_0). To stage animals more precisely throughout the fifth instar (8 days) and the first 10 days of adult development, animals were staged daily. Unless noted otherwise, we only used gate I animals, those that wandered during the fourth night of the fifth instar (Dominick and Truman, 1984).

Isolation of the lateral neurosecretory cells (LNCs)

LNCs were isolated using a modification of a method described elsewhere (Tublitz and Sylwester, 1990). Briefly, the fourth abdominal ganglion (A4) was removed from animals at various stages, pinned in a small Sylgard-containing Petri dish and immersed in paraffin oil. Each cluster of four LNCs (see inset in Fig. 1) was removed from the ganglion with glass needles using dark-field optics to visualize the cells (Rowell, 1976). Previous work has shown that all four LNCs are identical in terms of peptide content (Tublitz and Sylwester, 1990). Isolated LNC clusters were individually transferred into a small volume of distilled water (2–5 μl) to promote cell lysis (Berlind and Maddrell, 1979). The LNC-containing water droplet was then divided into two equal samples, both of which were frozen at −20°C until needed, usually a period of not more than 7 days. Each cluster was assayed for both bursicon and CAP_2 bioactivities unless otherwise noted. This paper focuses on the CAP_2 results.

CAP_2 bioassay

CAP_2 levels were quantitatively assayed on an isolated pharate adult *Manduca sexta* heart according to a previously published protocol (Tublitz and Truman, 1985*a*). Briefly, a piece of the abdominal heart was attached to a force transducer and superfused with standard physiological saline. Previous studies have determined that heart rate is directly proportional to CAP_2 concentration (Tublitz and Truman, 1985*a–d*). Each frozen sample (half of a cluster) was thawed and resuspended in 95 μl of physiological saline immediately prior to assaying for cardioacceleratory activity. Data are expressed in terms of CAP units, where 1 unit is equal to the total amount of CAP bioactivity (CAP_1 and CAP_2 combined) measured in the ventral nerve cord of a single pharate adult moth. The limit of resolution of this bioassay is 0.02 CAP units.

Bursicon bioassay

The remaining sample from each cluster was assayed for bursicon bioactivity using the adult wing bioassay (Taghert and Truman, 1982*a*,*b*; Tublitz and Sylwester, 1990). After thawing, enough saline was added to each sample to bring the final volume up to 10 μl, which was then injected directly into the lumen of the costal vein of the mesothoracic wing that had been removed from a pre-emergent pharate adult. The contralateral wing from the same individual received a control injection of 10 μl of saline. Wing pairs were incubated for 3h at room temperature in a moist environment to prevent dehydration. Following the incubation period, each wing was descaled with paraffin wax and 70 % ethanol to visualize the wing veins. Tanning of the injected wing veins is proportional to bursicon concentration and bursicon activity was determined by visually comparing the tanning level of the control and test injections using an arbitrary scale of 1–5 as previously described (Taghert and Truman, 1982*a*,*b*; Tublitz and Sylwester, 1990). Bursicon levels are expressed in terms of bursicon units, where 1 unit is equal to the quantity of bursicon bioactivity in the ventral nerve cord of a pharate adult. The wing bioassay can detect as little as 0.005 bursicon units.

Isolation of LNCs from the steroid-secreting glands by ligation

The source of the ecdysteroids in *Manduca sexta* is the prothoracic glands, located in the first thoracic segment. To prevent the ecdysone-secreting prothoracic glands from affecting the LNCs in the abdominal CNS, animals were ligated with suture thread at the thoracic–abdominal juncture following a 10–30min anesthetization period in iced water. In those cases where suture thread proved unsuitable, animals were also clamped with small hemostats. After ligation, the anterior end of the animal was removed and discarded. Ligated animals were returned to the colony rearing chamber until further manipulation. Ligated animals died over a period, with approximately 95% remaining alive and healthy after 1 week and about 95% of these still living 14 days after ligation. The health of the animals was determined by measuring cuticle coloration and hydration state; those deemed unhealthy were discarded. Ligations were performed early in the day, prior to any changes in LNC peptide levels.

Ganglion and CNS implantation

For the single-ganglion implantation experiments, fourth abdominal ganglia (A4) were removed from day 2 fifth-instar caterpillars (D_2 is the second full day after ecdysis from the fourth larval instar) and prepared for implantation by rinsing in sterile physiological saline. Prior to implantation, host animals were anesthetized in ice and/or CO_2 and the tip of the dorsal 'horn' was cut off. Every host received a single rinsed ganglion, which was injected with a 1ml sterile syringe through polyethylene tubing (PE 160) into the body hemocoel *via* the opening in the dorsal horn. Following implantation, the opening in the horn was ligated with 6-0 surgical suture thread, and after post-operative recovery the animal was returned to the colony rearing facility. Hosts were allowed to develop until the equivalent of pupal day 8 ('P_8'), at which time both implanted and host A4 ganglia were removed. Identification and proper orientation of implanted ganglia on 'P_8' were facilitated by tying 6-0 sutures of differing lengths around both anterior and posterior connectives prior to implantation. LNCs from hosts and implanted ganglia were separately dissected, processed and assayed for CAP bioactivity as described above.

The CNS implantation experiments were carried out using the same protocol as the ganglion implantation except that the entire CNS from a day 2 fifth-instar caterpillar was isolated and injected through the dorsal horn into another D_2 larva. Some hosts received a brainless CNS, i.e. a CNS with all ventral ganglia except a supraesophageal ganglion. Hosts were killed on wandering day 1 (W_1) and the A4 ganglion was removed from both the host CNS and the implanted CNS. Dissection of LNCs and bioassay for CAP activity were identical to those for the ganglion implantation experiments.

Application of a juvenile hormone mimic

As a means of blocking the metamorphic effects of the commitment pulse (CP) of 20-HE that occurs in fifth-instar larvae (see Fig. 1A), animals were treated with methoprene, a juvenile hormone agonist (Levine *et al.* 1986). Methoprene (7S isomer no. 312008, a gift of Dr D. Cerf, Sandoz Crop Protection, Palo Alto, CA) was dissolved in cyclohexane ($1\,mg\,ml^{-1}$) and $25\,\mu l$ was topically applied twice onto the cuticle of feeding larvae, once on day 2 at 17:00h and again on day 3 at 09:00h. A control population of identically staged animals was treated with cyclohexane only. After treatment, control and experimental animals were returned to our animal rearing facility where they remained until the controls reached pupal day 8 (P_8). When controls had developed to P_8, each cluster of four LNCs from ganglion A4 was removed from controls and experimental animals and assayed for bursicon and CAP_2 levels using the wing and heart bioassays, respectively.

20-Hydroxyecdysone (20-HE) infusions

Infusion of 20-HE was carried out using a minor variation of the protocol of Weeks *et al.* (1992). Following 1h of chilling on ice to anesthetize the animal, day 2 fifth-instar larvae were ligated and implanted with an intact day 2 CNS including the brain. These animals were allowed to recover from the implantation and infused 24h later with 20-HE, which was delivered by syringe pump into the hemolymph *via* small-bore polyethylene

tubing (PE 10) inserted into the dorsal horn (Weeks, 1987). 20-HE, a gift of Dr Janis Weeks, was hydrated in a physiological saline containing no carbohydrates (Ephrussi and Beadle, 1936) at a concentration of $1 mg ml^{-1}$. After spectrophotometric analysis to confirm the concentration (Meltzer, 1971), the 20-HE stock solution was frozen in 50 μl samples and stored at −20°C until needed. 20-HE was infused into animals at a rate of $3.0 \mu l h^{-1}$ ($3.0 \mu g h^{-1}$) for 6h, a concentration designed to mimic the CP (Dominick and Truman, 1985). Following infusion, the polyethylene tubing was clamped or melted closed and left attached to the horn. Infused animals remained in our animal colony for an additional 2 days, after which the LNCs from ganglion A4 were removed from the host and from the implanted CNS and assayed separately for CAP_2 bioactivity using the isolated heart bioassay.

Results

Time course of the CAP_2-to-bursicon switch in the LNCs

Previous work has demonstrated that the LNCs undergo a transmitter switch between the last larval instar and midway through adult development (Tublitz and Sylwester, 1990), but the time course of this change was unknown. To identify more precisely when the conversion from CAP_2 to bursicon occurred, single LNC clusters were removed from ganglion A4 using standard techniques (Tublitz and Sylwester, 1990). Every A4 ganglion contains two identical sets of four LNCs whose cell bodies are bilaterally situated on the posterolateral margin of the ganglion and both sets were used for all the experiments described here (Fig. 1, inset). Individual clusters are easily identifiable *in situ* under a dissecting microscope by the position and opalescent reflectiveness of the LNC somata (Taghert and Truman, 1982*a*,*b*; Tublitz and Sylwester, 1990). After dissection, the contents of each LNC cluster were divided into two equal samples, one of which was assayed for CAP bioactivity on an *in vitro* pharate adult heart (Tublitz and Truman, 1985*a*–*c*), while the remaining sample was analyzed for bursicon activity on an isolated adult wing bioassay (Taghert and Truman, 1982*a*,*b*; Reynolds, 1983; Tublitz and Sylwester, 1990). Both bioassays are very specific and highly sensitive; the myogenic heart responds in a dose-dependent fashion to CAP_2 application by increasing beat frequency, while samples containing bursicon induce a dose-dependent premature tanning of wing veins when injected into wings isolated from moths just prior to the pupal–adult molt.

Our results, depicted in Fig. 1B, indicate that the level of CAP_2 remains relatively unchanged during days 0–3 (D_0–D_3) of fifth (final) instar larvae, followed by a precipitous drop on wandering day 0 (W_0). W_0 is the day when the caterpillar stops feeding and commences wandering behavior, a period of sustained locomotory activity in preparation for pupation (Dominick and Truman, 1986). The present data show that the LNCs lose approximately 50% of their total CAP_2 content during wandering (Fig. 1B). The drop in CAP_2 levels at W_0 is followed by a second, slower CAP_2 decline during the remainder of larval life, so that, by the second day following pupation (pupal day 2, P_2), CAP_2 activity is below the limit of detection (Fig. 1B). In contrast, bursicon levels in the LNCs are minimal throughout most of the final larval instar, and it is not until the second

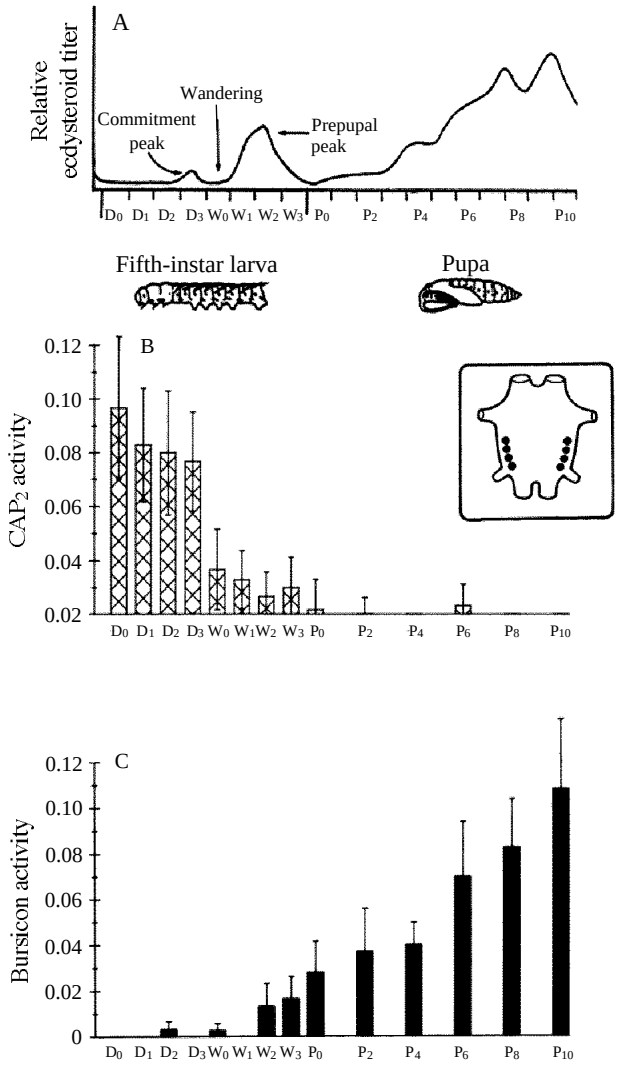


Fig. 1. Time course of peptide changes in the lateral neurosecretory cells (LNCs) of the moth *Manduca sexta.* (A) Ecdysteroid blood titers during development. 'Ecdysteroid titers' refers to blood levels of both ecdysone and 20-HE ecdysone. Time is in days. The curve shows the relative timing and magnitude of ecdysteroid events, not absolute titers, since the minimal levels shown are undetectable. Ecdysteroid titer is taken from Bollenbacher *et al.* (1981). D_0–D_3, days during the feeding period in fifth (final) instar larvae; W_0–W_3, days during and immediately after wandering behavior; P_0–P_{10}, first 10 days after pupation. (B,C) Time course of transmitter changes in the LNCs during metamorphosis: (B) CAP_2 disappearance; (C) bursicon accumulation. Each group of four LNC cell bodies was dissected and assayed for both peptides. Left and right LNC clusters were used interchangeably in these experiments. CAP_2 and bursicon activities are each expressed in units of biological activity, where 1 unit is equivalent to the amount of each peptide found in the ventral nerve cord of a pharate adult, the stage just prior to adult emergence. Minimal detection levels are 0.02 units for CAP_2 and 0.005 units for bursicon. Each bar represents the mean ± S.E.M. of CAP_2 or bursicon activity in at least nine LNC clusters. Inset: cell body location of lateral neurosecretory cells in the fourth abdominal ganglion.

day of wandering (W_2) that bursicon activity first becomes measurable in physiologically significant amounts (Fig. 1C). From W_2 until P_{10}, bursicon levels in the LNCs continue to increase so that, by P_{10}, bursicon activity in these cells is over 50-fold higher than in larvae.

Endocrine manipulation of CAP_2 levels in the LNCs

The temporal association between the timing of the CAP_2-to-bursicon transmitter switch and the onset of metamorphosis suggested that the LNC transmitter switch might be regulated by one or more of the hormones involved in the control of molting in insects. One likely set of candidates are the ecdysteroids, ecdysone and 20-HE; the titers of these two substances in the blood show fluctuations during the last larval instar that are temporally correlated with changes in transmitter type in the LNCs. Ecdysone is synthesized and secreted from the prothoracic glands located in the first thoracic segment. After its release into the blood, it is converted into the biologically more active 20-HE form. There are two, distinct, physiologically important ecdysteroid pulses in fifth-instar *Manduca sexta* (Bollenbacher *et al.* 1981; Hoffman, 1983; Riddiford, 1985): the first, known as the commitment pulse (CP), occurs early on D_3 and serves to commit larval tissues to pupal differentiation as well as triggering wandering behavior (Fig. 1A). Actual expression of the pupal phenotype occurs only in response to the second larger ecdysteroid rise, the prepupal peak (PP), which commences on the day after wandering, W_1. To ascertain whether either of these ecdysteroid pulses was responsible for the decline in CAP_2 expression, ecdysteroid levels were experimentally manipulated in several ways.

First, we exposed the LNCs to different portions of the fifth-instar ecdysteroid titer. One simple method of accomplishing this *in vivo* was to ligate animals at the thoracic–abdominal juncture. This procedure physically isolated the abdominal LNCs from the ecdysteroid-secreting prothoracic glands, located in the first thoracic segment, since the portion of the animal anterior to the ligation, i.e. the head and thorax, was discarded. About 90% of abdomens ligated in this fashion remained alive and healthy for 2–3 weeks. Ligations were performed on D_2, W_0 or P_0 in order to subject LNCs to no ecdysteroids (D_2 ligations), to the CP alone (W_0 ligations) or to the normal two-peak sequence (P_0 ligations; Fig. 2A). For these experiments, ligated animals were allowed to develop until the equivalent of P_8, the stage in normal development when the LNCs acquire maximal bursicon activity. For example, abdomens isolated on D_2 were held for 14 days, those isolated on W_0 for 12 days and those isolated on P_0 for 8 days in order that they could be dissected on the day that would normally correspond to the eighth day of the pupal stage in intact animals. On 'P_8', each cluster of four LNCs from ligated animals was removed from ganglion A4 and assayed for CAP_2 content using the isolated heart bioassay. We found that the normal decline in CAP_2 was prevented only by the earliest ligation, that is, on D_2 prior to the CP (Fig. 2B). LNCs removed from animals ligated on W_0 or P_0 exhibited the normal CAP_2 decline (Fig. 2B).

To test whether the ecdysteroids directly regulate the drop in CAP_2, individual ganglia were implanted into host caterpillars at different developmental stages and assayed for CAP_2 content when the hosts reached P_8 (Fig. 3A). A4 ganglia were removed from D_2

larvae, the stage prior to the commitment and prepupal peaks. These ganglia were then individually implanted into the abdominal hemocoel of D_2, W_0 or W_3 caterpillars. These stages were chosen because they exposed the implanted ganglion to different ecdysteroid regimens; e.g. implanted ganglia were exposed to both ecdysteroid peaks (D_2 hosts), the PP alone (W_0 hosts) or neither peak (W_3 hosts). When hosts reached pupal day 8 (P_8), both host and implanted A4 ganglia were removed and the LNCs were dissected. Each cluster was subsequently bioassayed for CAP_2 content. LNCs removed from implanted ganglia never showed a decline in CAP_2 levels regardless of the type of ecdysteroid

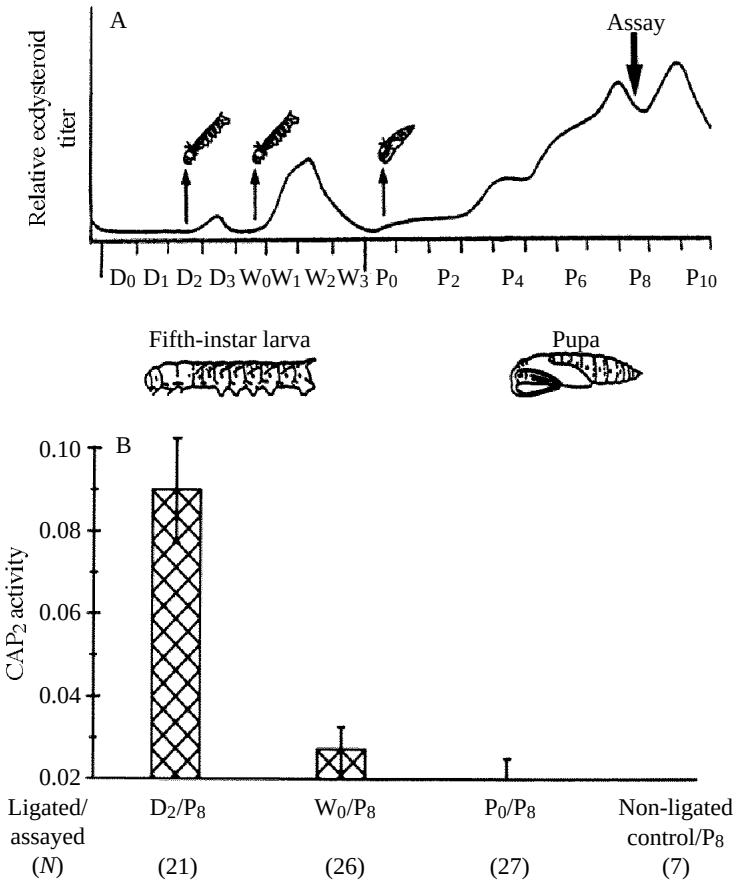


Fig. 2. Effects of ligation on CAP_2 expression in the LNCs during metamorphosis in *Manduca sexta*. (A) Schematic representation of the experimental procedure. Animals were ligated on D_2, W_0 or P_0 and allowed to develop until the equivalent of P_8. On 'P_8', A4 ganglia from all animals were removed and the LNCs were dissected, processed and assayed as described in Materials and methods. (B) CAP_2 levels in LNCs from ligated and control animals. Control LNCs received the same treatment except that control animals were not ligated. Each bar represents the mean ± S.E.M. of CAP_2 activity from (*N*) separate LNC clusters. D_2, day 2 fifth-instar larva; W_0, day of wandering; P_0, pupal day 0, which is the day of larval–pupal molt; P_8, the equivalent of the eighth full day after the larval–pupal molt.

exposure (Fig. 3B). The normal drop in CAP_2 levels was seen, however, in LNCs from host animals whose nervous systems were intact.

Another approach to studying 20-HE effects is to manipulate the titers of another hormone, juvenile hormone (JH). JH, a sesquiterpenoid, is an essential hormonal factor in the larval-to-pupal transformation in *Manduca sexta* because its absence during the

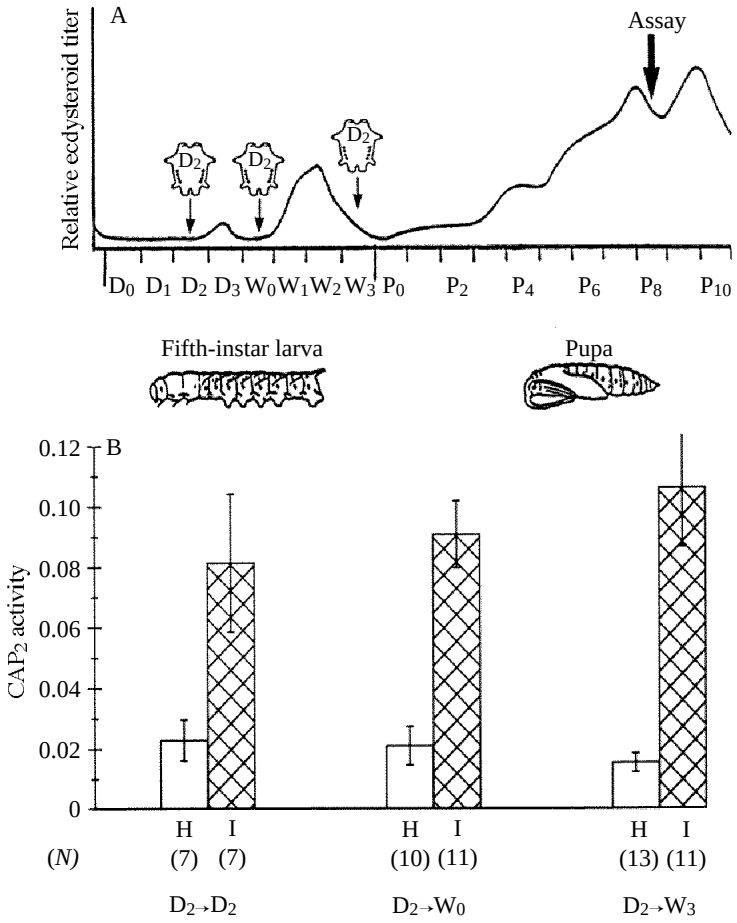


Fig. 3. Effects of ganglion implantation on CAP_2 expression in the LNCs during metamorphosis in *Manduca sexta*. (A) Schematic representation of the experimental procedure. A4 ganglia were removed from D_2 fifth-instar larvae and individually implanted into host animals on D_2, W_0 or W_3 to expose LNCs to different ecdysteroid regimens. In all cases, hosts were allowed to develop until P_8, at which time both implanted and host ganglia (A4) were removed. LNCs from hosts and implanted ganglia were separately dissected, processed and assayed as described in Materials and methods. (B) CAP_2 levels in host (H) and implanted (I) LNCs. Each bar represents the mean ± S.E.M. of CAP_2 activity from (*N*) separate LNC clusters. D_2, day 2 fifth-instar larva; W_0, day of wandering; W_3, the third full day following wandering. The label under each pair of histograms describes the stage of the implanted ganglion and the stage of the host, e.g. $D_2 \rightarrow W_0$ refers to an A4 ganglion taken from a D_2 animal implanted into a W_0 caterpillar.

CP is the crucial signal that triggers the onset of metamorphosis. If JH levels remain elevated during the CP, fifth-instar caterpillars will molt into supernumerary sixth-instar larvae, not into pupae (Riddiford, 1985). To explore further the relationship between the CP and transmitter expression in the LNCs, animals were treated with 7S-methoprene, a pharmacological juvenile hormone agonist (Levine *et al.* 1986). As detailed in the Materials and methods section, experimental animals were exposed to 7S-methoprene dissolved in cyclohexane late in the afternoon of D_2 and again in the morning of D_3 to ensure elevated hemolymph levels of this agonist throughout the

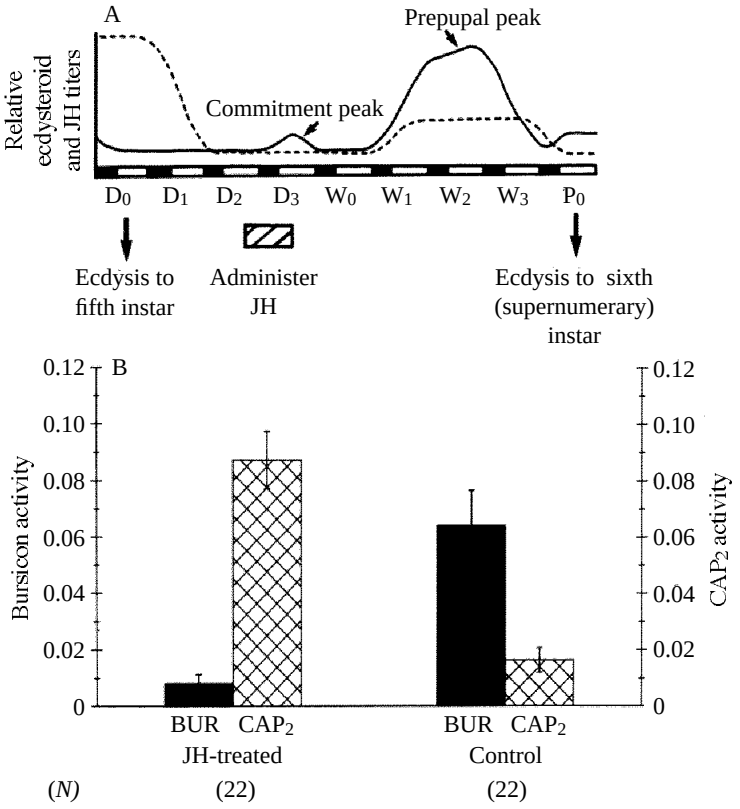


Fig. 4. The effect of 7s-methoprene on transmitter phenotype in the LNCs. The experimental design is schematically illustrated in A. Relative hormone titers of 20-hydroxyecdysone (solid line) and juvenile hormone (dotted line) during the last (fifth) larval instar are shown. Day and night are marked by open and filled bars, respectively. Methoprene, a juvenile hormone agonist, was dissolved in cyclohexane and administered in the afternoon of D_2 and again in the morning of D_3 as described in Materials and methods. Controls received cyclohexane only. LNCs were dissected from both groups 2 weeks later, when the controls had reached P_8. LNCs were assayed for CAP_2 and bursicon content using the *in vitro* heart and pharate adult wing bioassays, respectively. (B) The results of methoprene application on transmitter choice in the LNCs. Each bar represents the mean ± S.E.M. of CAP_2 activity (hatched bars) and bursicon activity (filled columns) from (*N*) separate LNC clusters. CAP_2, cardioacceleratory peptide 2; BUR, bursicon; other abbreviations are as described in Fig. 1.

duration of the endogenous CP (Fig. 4A). Controls were treated with a equivalent volume of cyclohexane. After the final treatment, both experimental and control animals were returned to our animal colony where they remained for 12 days, at which

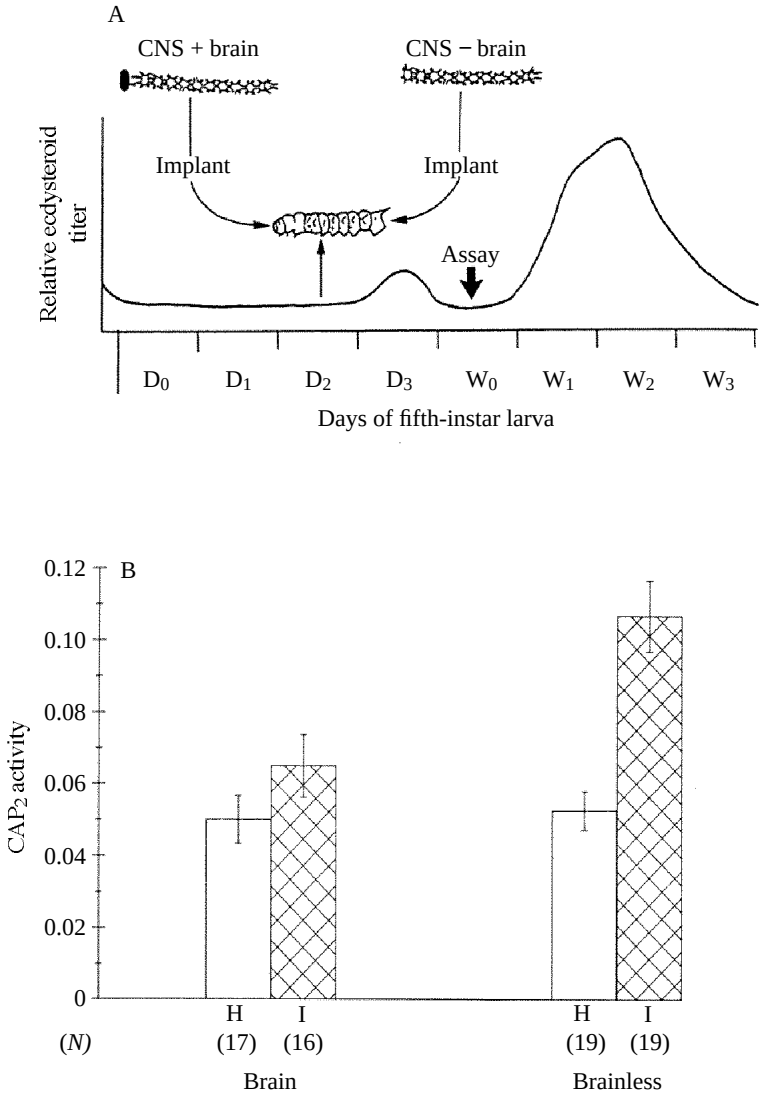
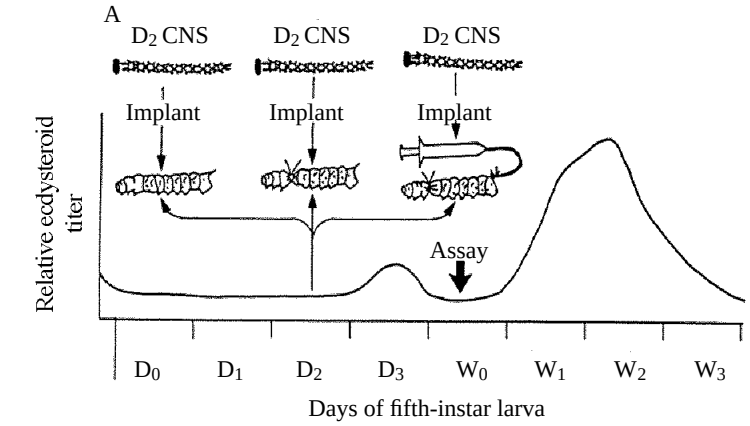


Fig. 5. CAP_2 levels in LNCs from intact and brainless CNSs implanted into a pre-wandering caterpillar. (A) Schematic diagram of the experimental design. The CNS from a D_2 animal was removed and implanted into another D_2 caterpillar. D_2 hosts were implanted with either a complete CNS or one without a brain. LNCs were removed from the host and the implanted CNS on W_0 and assayed separately for CAP_2 content. (B) CAP_2 levels in host and implanted LNCs taken from complete or brainless CNSs. Each histogram represents the mean ± S.E.M. of CAP_2 activity from (*N*) separate LNC clusters. H, host CNS; I, implanted CNS; other abbreviations are as stated in Fig. 1.



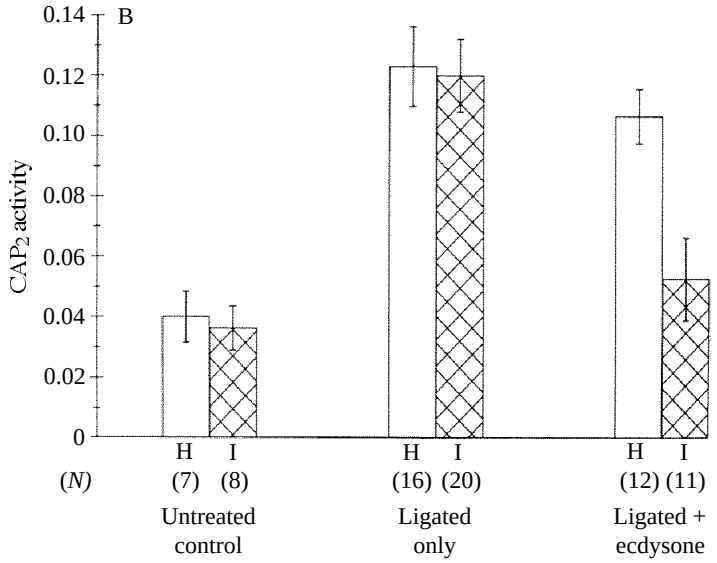


Fig. 6. The effect of 20-hydroxyecdysone (20-HE) on CAP_2 levels in the LNCs. (A) Schematic representation of the experimental procedure. Caterpillars were ligated on D_2 and then implanted with a complete CNS taken from another D_2 larva. Some of these ligated, implanted preparations were then infused with sufficient 20-HE to mimic the commitment pulse (see Fig. 1 and text for details). One set of controls was ligated and implanted but not infused, while a second control group received CNS implants without ligation or infusion. Host and implanted LNC clusters from all three groups were separately removed on W_0 and assayed individually for CAP_2 content. (B) CAP_2 levels in LNCs from control and hormonally manipulated animals. Each bar represents the mean ± S.E.M. of CAP_2 activity from (*N*) separate LNCs. H, host CNS; I, implanted CNS; other abbreviations are as stated in Fig. 1.

time the controls had reached P_8 and the experimentals were 3–5 days into a supernumerary sixth-instar larva. Analysis of CAP_2 and bursicon levels in LNC clusters from the methoprene-treated animals revealed that these cells were predominantly CAP-containing, whereas the same cells removed from the controls primarily expressed bursicon with CAP_2 levels being below the limit of detection (Fig. 4B). Thus, treatment with methoprene blocked the normal transmitter changes from occurring in the LNCs.

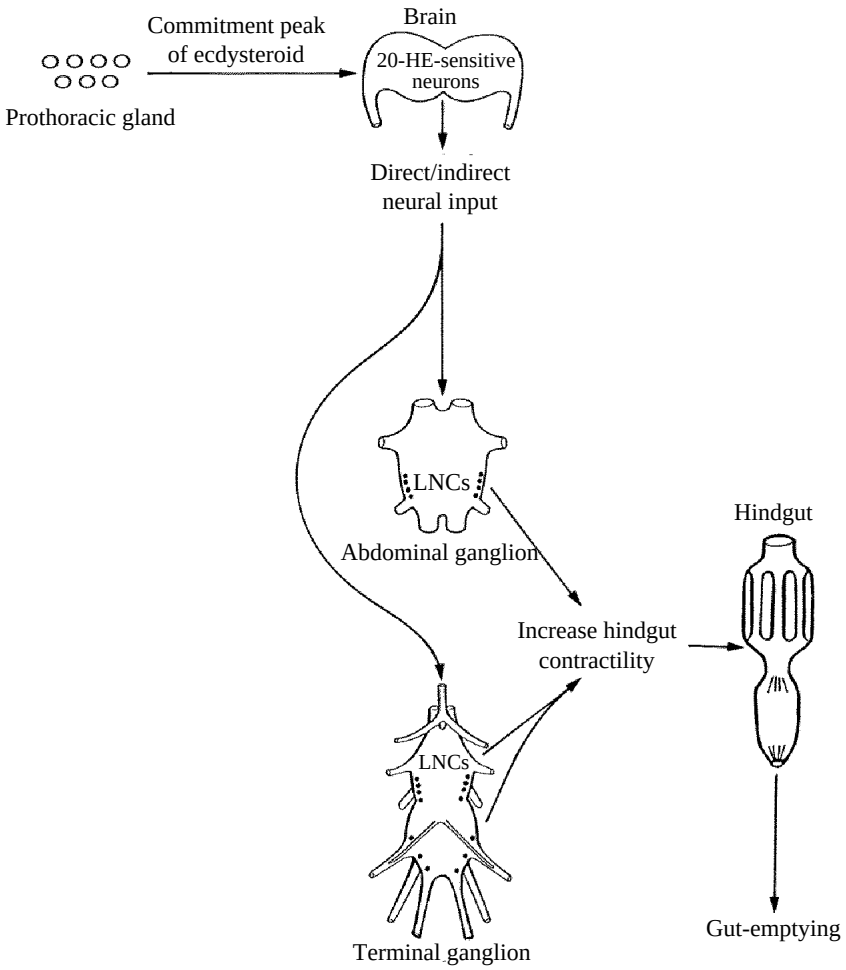


Fig. 7. Working model of the cellular pathway mediating the effects of the commitment pulse of 20-HE on the CAP_2 decline in the LNCs. The commitment pulse of the ecdysteroids, released by the prothoracic glands, stimulates a population of 20-HE-sensitive neurons located in the brain. The 20-HE-sensitive brain neurons in turn activate a set of descending interneurons that directly or indirectly excite the LNCs in all the abdominal ganglia, including the terminal ganglion. LNC stimulation results in CAP_2 release, the primary role of which at this stage is to increase gut contractility during gut-emptying behavior.

CNS implantation

The extent of the nervous system required for the drop in CAP_2 levels in the LNCs was tested by removing the complete CNS, i.e. brain+ventral nerve cord, from a D_2 fifth-instar larva and implanting it in its entirety into another D_2 caterpillar (Fig. 5A). The procedure for this experiment was the same as described above for the ganglion implantation experiments. In this case, the host larvae contained two complete central nervous systems each with its own set of LNCs. A parallel set of D_2 animals received D_2 CNSs without brains, i.e. containing all neural components between the subesophageal and terminal abdominal ganglia. Following surgery and recovery, host animals were maintained in standard rearing conditions until the day after wandering, W_1, the stage in untreated controls when CAP levels have already undergone a major decline (Fig. 1). Individual LNC clusters were removed from the A4 ganglion of the host and the implanted CNS and separately assayed for CAP content. Animals that received an intact CNS exhibited a normal CAP_2 decline in both implanted and host LNCs (Fig. 5B). In contrast, a drop in CAP_2 was not seen in the implanted LNCs dissected from a brainless CNS, although the LNCs of their hosts did undergo the normal CAP_2 decrease. These data indicate that the decline in CAP_2 in the LNCs at wandering occurs only if the LNCs are able to receive neural information from the brain.

20-Hydroxyecdysone infusions

To examine more directly the role of the CP in down-regulating LNC CAP_2 levels, we devised an experimental protocol that enabled us to control exogenous 20-HE levels. The experimental design is depicted in Fig. 6A. D_2 caterpillars were ligated and the isolated abdomens were implanted with a complete D_2 CNS, including the brain, using the procedures described in Materials and methods. Each of these preparations consisted of an isolated abdomen containing the host's abdominal CNS (A1–A8) plus a complete CNS from the donor. Implantation of an intact CNS was necessary because the ganglion and CNS implantation results showed that intact neural connections between the LNCs and the brain were required for the CAP_2 decline. The day following implantation, some of the ligated and implanted abdomens received a 6h infusion of 20-HE at a concentration designed to approximate closely the CP (refer to Materials and methods for infusion and dosage details). Infused and uninfused abdomens were maintained until the equivalent of W_0, at which time ganglion A4 LNCs were isolated from both the implanted and the host CNS and were assayed separately for CAP_2 content. 20-HE infusion induced a CAP_2 decline in implanted LNCs that was equivalent to the normal CAP_2 drop measured in untreated controls (Fig. 6B). In contrast, CAP_2 levels in LNCs from the host CNS, when exposed to an identical 20-HE treatment, were unaffected. CAP_2 levels also remained unaltered, i.e. high, in those preparations that were ligated but not infused with 20-HE (Fig. 6B). Hence, exogenous 20-HE induced a major reduction in CAP_2 levels in the LNCs, but only in preparations containing a complete CNS.

Discussion

LNC transmitter plasticity and function

The LNCs of the tobacco hawkmoth, *Manduca sexta*, because of their location, size and accessibility, have been a model system for investigations into the neurobiology of peptide-containing neurons (Taghert and Truman, 1982*a*,*b*; Tublitz and Sylwester, 1988, 1990). One outcome of these studies has been the discovery of the unexpected transmitter plasticity exhibited by these neurons. The initial work on these cells by Taghert and Truman (1982*a*,*b*) unequivocally showed that the LNCs in late larvae and adults contained bursicon, the classic insect tanning hormone (Reynolds, 1983). That these cells in larvae also expressed CAP_2 was first demonstrated immunocytochemically (Tublitz and Sylwester, 1988) and later confirmed biochemically (Tublitz and Sylwester, 1990). This latter report also showed that bursicon levels were low in LNCs removed from larvae and high in LNCs dissected from adults. It was concluded from these experiments that the LNCs underwent a qualitative alteration in transmitter expression from CAP_2 to bursicon during metamorphosis.

The current study shows that the transmitter switch by the LNCs from CAP_2 to bursicon is a multi-step process commencing with a decline in CAP_2 at the onset of metamorphosis (Fig. 1B). CAP_2 levels in the LNCs drop precipitously on W_0, with the LNCs losing more than 50% of their accumulated CAP_2. The timing of this transmitter depletion is temporally correlated with wandering behavior, a complex series of motor activities, including increased locomotion, which in nature enables the caterpillar to crawl off its host plant and burrow underground. One of the activities associated with wandering is ‘gut-emptying’ behavior, when the alimentary canal is completely cleared of undigested materials in preparation for metamorphosis. Recent work has demonstrated that ‘gut-emptying’ is accomplished in part by a CAP_2-mediated increase in gut activity (Edwards *et al.* 1990; Tublitz *et al.* 1992*a*,*b*). Because the LNCs in larvae are strongly CAP-immunopositive (Tublitz *et al.* 1991, 1992*a*), it has recently been proposed that the LNCs are the source of the CAP_2 released at wandering. Our measurements of the time course of the drop in the LNC content of CAP_2 (Fig. 1B) provide important additional support for the hypothesis that the LNCs are the major source of CAP_2 at wandering.

Does 20-hydroxyecdysone trigger the CAP_2 decline in the LNCs at wandering?

The primary goal of this study was to identify the mechanisms responsible for the reduction in CAP_2 expression in the LNCs. The data presented in this paper implicate the CP of 20-HE as the causal factor in this process. The results of the ligation experiments (Fig. 2) and the endocrine manipulations using a juvenile hormone agonist (Fig. 4) demonstrate the existence of a temporal relationship between the CP and the CAP_2 decline in the LNCs. Although this evidence by itself is not conclusive, it does indicate that manipulation of the endocrine milieu, specifically the CP, is sufficient to alter significantly the normal progression of transmitter expression in the LNCs. A more definitive test of the hypothesis that the CP triggers the CAP_2 drop is furnished by the 20-HE infusion experiments (Fig. 6). The results of these experiments demonstrate that 20-HE, infused at levels equivalent to that of the endogenous CP, is capable of reducing

CAP_2 levels in the LNCs. These data, taken together, provide strong evidence that the CP is the proximate cause of the CAP_2 release by the LNCs at wandering.

The CAP_2 decline in the LNCs requires an intact brain

The nature of the role played by the CP was also explored. In particular, we were interested in determining whether 20-HE acted directly on the LNCs. On the basis of the work presented here, it is unlikely that all the effects of the CP on the LNCs can be accounted for by a direct steroid action on the LNCs. This conclusion is based in part on the results of the D_2-to-D_2 ganglion implantation experiments (Fig. 3). A decline in CAP_2 levels was detected only in the host LNCs and not in the LNCs removed from the implanted CNS, although both host and implanted LNCs were exposed to the endogenous CP (Fig. 3B). The observation that the LNCs from the host CNS exhibited a normal decline in CAP_2 suggested that intact neural connections were required in order for the CP to be effective, and this hypothesis was tested by the CNS implantation experiments. The results from those experiments unequivocally demonstrated that the normal decline in CAP_2 in the LNCs occurred only if the LNCs were in direct neural contact with the brain (Fig. 5).

On the basis of these data, we propose the following model illustrated in Fig. 7: the larval brain in *Manduca sexta* contains a set of steroid-activated neurons that drive a population of interganglionic descending interneurons. These descending interneurons project posteriorly down the entire length of the nerve cord and synapse directly onto the LNCs in each ganglion. Activation of the brain neurons by the CP stimulates the descending interneurons which, in turn, excite the LNCs and cause them to release CAP_2 at wandering. The proposed model is attractive because of its parsimony, because all its neuronal elements are known to be present in *Manduca sexta* and because it is testable. The existence of CP-sensitive neurons in the insect brain affecting neuronal activity in the ventral nerve cord was first postulated by Dominick and Truman (1986) during their investigations into the hormonal control of wandering behavior in *Manduca sexta* larvae. They proposed a model in which the CP initiates wandering behavior by suppressing the activity of a set of pre-motor brain neurons, which, in the absence of 20-HE, normally inhibit the central neurons that generate the wandering motor program. These neurons described by Dominick and Truman are potential presynaptic elements in the LNC circuit, not only because of their steroid sensitivity but also because they are activated at the same time as the LNCs. Our model also postulates the existence of a second class of neurons, a set of descending interganglionic neurons with processes in the *Manduca sexta* ventral nerve cord. The presence of such neurons in *Manduca sexta* has recently been documented using a combination of intracellular dye-filling and confocal microscopy (Mesce and Klukas, 1991). Although the connectivity between individual descending interganglionic interneurons and the LNCs is not yet known, it is plausible that some of these descending neurons are presynaptic to the LNCs, since stimulation of the interganglionic connectives in adult moths elicits a barrage of excitatory postsynaptic potentials in the LNCs and other CAP-containing neurons (N. J. Tublitz, unpublished results). Although we favor this model for the reasons stated above, it should be noted that other hypothetical models can be constructed to fit the existing data. In one alternative model, 20-HE acts directly on the LNCs in conjunction with the descending input from the brain. Studies are currently under way to distinguish between these two models.

In this paper, we have demonstrated that the initial step in the CAP_2-to-bursicon transmitter switch by the LNCs, the decline in CAP_2 levels, is hormonally regulated by the CP of the insect steroid hormone 20-HE. The developmental decline in CAP activity in these cells appears to be a two-step process: a release of CAP_2 followed by suppression of its further synthesis. Our data show the steroid dependence of the initial peptide release step but provide no evidence about the relationship between 20-HE and the down-regulation of CAP synthesis. It would not be surprising, however, if CAP synthesis were suppressed by 20-HE since steroid-dependence of peptide expression has been documented in several neuroendocrine systems (Alexander and Miller, 1982; Gross *et al.* 1987). Steroid-induced effects on transmitter phenotype have also been described in embryonic neurons during embryogenesis in invertebrates (Witten and Truman, 1991*a*,*b*) and in vertebrates (Anderson, 1989; Anderson and Axel, 1986). This study provides evidence that steroids control transmitter expression in mature, functional neurons. Whether the molecular mechanisms underlying steroid-dependent transmitter plasticity are the same for developing and mature neurons must await future studies.

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