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HORMONAL CONTROL OF TRANSMITTER PLASTICITY IN INSECT PEPTIDERGIC NEURONS

II. STEROID CONTROL OF THE UP-REGULATION OF BURSICON EXPRESSION

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Summary

Each abdominal ganglion of the central nervous system of the tobacco hawkmoth, *Manduca sexta*, contains four individually identified lateral neurosecretory cells (LNCs) that undergo a postembryonic transmitter switch *in vivo*. In the embryonic and caterpillar stages, the primary LNC transmitter is cardioacceleratory peptide 2 (CAP_2), a myoregulatory peptide. During metamorphosis, these cells stop expressing CAP_2 and instead produce bursicon, a classic insect peptide hormone responsible for cuticular tanning. We have previously reported that this transmitter plasticity is under the control of the insect steroid hormone 20-hydroxyecdysone (20-HE), which surges twice during the last larval instar. In that report we showed that the CAP_2 decline is indirectly regulated by the first 20-HE rise, the commitment pulse (CP). Here we provide evidence that the rise in bursicon levels in the LNCs is directly triggered by the second 20-HE surge, the prepupal peak (PP). We performed several experimental manipulations that exposed LNCs to the PP without the CP; cells treated in this manner exhibited a significant rise in bursicon content. In contrast, bursicon levels remained unchanged in those LNCs exposed only to the CP. Exposure to the PP triggered a precocious increase in bursicon expression in LNCs from the penultimate larval stage. Increased bursicon levels in the LNCs were also induced by direct infusion of 20-HE. Taken together, the results of these experiments suggest that the rise in bursicon in the LNCs during metamorphosis is due to the direct action of the PP on the LNCs. Thus, the two 20-HE surges combine to regulate the CAP_2-to-bursicon switch in the LNCs, the first acting indirectly to cause a decline in CAP_2 levels and the second triggering a rise in bursicon expression, possibly by a direct action on the LNCs.

Introduction

The adult central nervous system (CNS) is the ultimate example of biological plasticity, enabling most multicellular organisms to behave appropriately in a complex

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world of ever-changing conditions. Underlying this phenomenal behavioral flexibility by the adult CNS is the plasticity exhibited at the level of a single neuron. Adult neurons are dynamic elements capable of undergoing significant morphological and physiological changes long after the completion of embryogenesis and differentiation. For example, motoneurons in the postembryonic CNS can undergo extensive morphological remodelling of central and peripheral processes with important physiological ramifications (e.g. Levine and Truman, 1985; Lichtman *et al.* 1987). Biochemical changes in postembryonic neurons can also have profound physiological consequences, as in the case of neurons that alter their transmitter phenotype. Qualitative changes in transmitter expression have been demonstrated across a very broad range of metazoans, ranging from mammalian sympathetic cells (Landis *et al.* 1987) to neurons in the coelenterate *Hydra* (Koizumi and Bode, 1986, 1991). The occurrence of transmitter plasticity in phylogenetically diverse organisms suggests that this is a prominent evolutionary strategy to increase neuronal flexibility that emerged relatively early in phylogeny.

One preparation where transmitter plasticity has been studied in some detail is the tobacco hawkmoth *Manduca sexta* (L.) (Tublitz *et al.* 1991), whose nerve cord is subdivided into discrete ganglia, roughly one for each thoracic and abdominal segment. Each abdominal ganglion contains four pairs of individually identifiable neurons that change transmitter phenotype during metamorphosis (Tublitz and Sylwester, 1990). In the caterpillar stage, these cells, known as the lateral neurosecretory cells (LNCs; see Fig. 1 inset), synthesize and secrete cardioacceleratory peptide 2 (CAP_2), one of a family of recently sequenced myoregulatory neuropeptides (Loi *et al.* 1992). During metamorphosis, the LNCs cease expression of CAP_2 and instead produce a second neuropeptide, bursicon, a classical insect neurohormone responsible for cuticular sclerotization (Reynolds, 1983). The change from CAP_2 to bursicon expression occurs during the initial transition from larva to pupa (Loi and Tublitz, 1993). Midway through the last (fifth) larval stage (instar), CAP_2 content in the LNCs drops by 50%. The CAP_2 decline is followed shortly thereafter by an increase in bursicon level in the same neurons. The CAP_2 drop and the bursicon increase are independent of each other (Loi and Tublitz, 1993), suggesting autonomous regulation. We have recently demonstrated that the drop in CAP_2 is indirectly controlled by the insect steroid hormones, the ecdysteroids (Loi and Tublitz, 1993). This paper addresses the mechanisms controlling the second and final step of the transmitter switch, the up-regulation of bursicon.

Metamorphosis in *Manduca sexta*, as in other insects, is under the control of the two ecdysteroids, ecdysone and 20-hydroxyecdysone (20-HE). Of the two ecdysteroids, 20-HE is the predominant bioactive form. In contrast to earlier larval–larval molts, the molt from fifth-instar larva to pupa requires two distinct surges in the titers of 20-HE in the hemolymph during that last larval stage (Fig. 1). The first, known as the commitment pulse (CP), obliges epidermal cells to produce a pupal phenotype at the next molt. The CP marks the first instance of an increase in 20-HE in the absence of juvenile hormone, a sesquiterpenoid that, among its other roles, determines the identity of cuticular proteins secreted by the epidermis at each molt (Riddiford, 1985; Fig. 1). A second, larger 20-HE pulse, the prepupal peak of 20-HE (PP), initiates the actual construction of a pupal

epidermis and the rest of the events associated with molting. The PP is secreted during a period of elevated juvenile hormone titers (Riddiford, 1985). Experimental manipulation of ecdysteroid titers during the fifth instar has established that the CP triggers the CAP_2 decline in the LNCs, probably by activating a set of steroid-sensitive neurons in the brain (Loi and Tublitz, 1993). Here we show that the increase in bursicon in the LNCs is also due to the action of the ecdysteroids, but in this case it is the prepupal peak and not the CP that is the causal factor. Moreover, we provide evidence that the PP-induced rise in bursicon is due to a direct action of the ecdysteroids on the ganglion containing the LNCs. 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 staged as previously described (Loi and Tublitz, 1993). Briefly, *Manduca sexta* larvae were individually housed in plastic cups and fed with an artificial diet modified only slightly from that of Bell and Joachim (1976). Larvae were transferred to individual chambers in wooden blocks at the onset of wandering (Dominick and Truman, 1986), which occurs midway through the last (fifth) larval instar. A few days after the larval–pupal molt, pupae were removed from the blocks and placed in marked plastic bags for the remainder of adult development. Adult development in our *Manduca sexta* colony takes place over a period of 18 days. On the last day of adult development, animals were transferred to a large enclosed chamber for adult emergence and breeding. All stages were raised in a controlled environmental chamber using a 17h:7h L:D regime with a superimposed thermal period (27˚C during the light period, 25˚C during the dark period) to improve developmental synchrony.

These experiments utilized fourth- and fifth-instar larvae as well as pupae. Accurate staging of these animals was based on the appearance of stage-specific developmental and/or behavioral events (Truman and Riddiford, 1974). Staging markers included day 3 fourth-instar larvae, ecdysis from fourth to fifth instar (day 0, D_0), onset of wandering behavior during the fifth instar (wandering day 0, W_0) and larval–pupal ecdysis (pupal day 0, P_0; see Fig. 1 for developmental time line). To ensure precision, animals were staged daily throughout the fifth larval instar and the first 10 days of adult development. Only gate I animals, those that wandered during the fourth night of the fifth instar (Dominick and Truman, 1984), were used in these experiments.

Isolation of the lateral neurosecretory cells (LNCs)

There is a cluster of four LNCs situated on each side of each abdominal ganglion (Fig. 1, inset). For these experiments, LNCs were taken only from the fourth abdominal ganglion (A4). The techniques for isolating the LNCs have been published in detail elsewhere (Tublitz and Sylwester, 1990; Loi and Tublitz, 1993). In brief, each four-cell cluster was dissected from surrounding tissue with glass needles and transferred into a small volume (2–5 μl) of distilled water 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. One sample was tested on the isolated *Manduca sexta* heart for CAP_2 activity (Loi and Tublitz, 1993) and the other was assayed for bursicon.

Bursicon bioassay

Bursicon levels in each LNC cluster were quantitatively determined using the adult wing bioassay. The details of the bursicon bioassay have been published previously (Taghert and Truman, 1982*a*,*b*; Tublitz and Sylwester, 1990; Loi and Tublitz, 1993). To summarize, samples were hydrated in 10 μl of physiological *Manduca sexta* saline (Tublitz, 1989) and then injected directly into the lumen of the costal vein of one of the mesothoracic wings removed from a pre-emergent adult. The contralateral wing received a saline-only injection as a control. Following a 3h incubation at room temperature, each wing was descaled to visualize tanning level of the wing veins. Tanning is proportional to bursicon concentration (Tublitz and Sylwester, 1990) and bursicon activity was

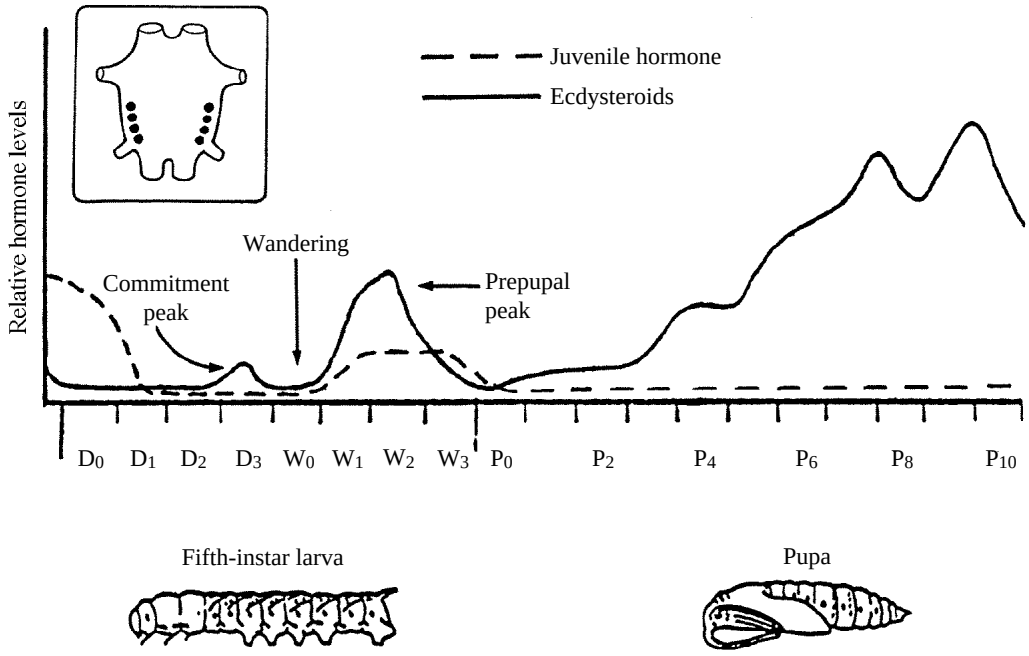

Fig. 1. Summary of the major hormonal changes during metamorphosis in the tobacco hawkmoth, *Manduca sexta*. 'Relative hormone levels' refers to relative blood titers of both ecdysteroids and juvenile hormone. The curves depict the relative timing and magnitude of hormonal changes, not absolute titers, since the minimal levels shown are undetectable. Hormonal titers are taken from Bollenbacher *et al.* (1981) and Riddiford (1985). The solid line represents hemolymph levels of both ecdysone and 20-hydroxyecdysone. The dashed line represents the juvenile hormone titer. Time is in days. D_0–D_3, days during the feeding period in fifth (final) instar larva; W_0–W_3, days during and immediately after wandering behavior; P_0–P_{10}, first 10 days after pupation. Inset: cell body location of lateral neurosecretory cells in the fourth abdominal ganglion.

determined by 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*; Loi and Tublitz, 1993). Bursicon levels are expressed in terms of bursicon units; 1 unit is the quantity of bursicon bioactivity in the ventral nerve cord of a pharate adult.

Endocrine manipulations

The same techniques and methods were employed for all of the ganglion transplantation experiments and these are reported in detail in Loi and Tublitz (1993). An overview of this procedure is provided here. The fourth abdominal ganglion (A4) was removed, rinsed in sterile *Manduca sexta* saline and injected into a host animal through a hole made in the posterior dorsal 'horn'. Hosts, upon reaching pupal day 8 (P_8), were killed and LNC clusters were dissected from both the implant and the host A4 ganglion. 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. After dissection, each four-cell cluster was independently assayed for bursicon bioactivity using the isolated wing bioassay.

The protocol for the 20-HE infusions was essentially identical to those performed by Weeks *et al.* (1992). The only source of the ecdysteroids in larvae is the prothoracic glands located in the first thoracic segment. To remove the endogenous source, wandering day 0 (W_0) caterpillars were anesthetized and ligated at the thoracic–abdominal juncture (Loi and Tublitz, 1993). After ligation, the tip of the dorsal horn was removed and small-bore polyethylene tubing (PE 10) was inserted through the opening. 20-HE, a gift of Dr Janis Weeks, was dissolved in E & B saline (Ephrussi and Beadle, 1936), adjusted to a concentration of $1 mg ml^{-1}$ using spectrophotometric analysis (Meltzer, 1971), divided into 50 μl samples, and stored at −20°C until needed. 20-HE was infused through the PE 10 tubing into animals using a syringe pump at a constant rate of $3.0 \mu l h^{-1}$ ($3.0 \mu g h^{-1}$) for 12h. This 20-HE infusion regimen was sufficient to induce pupal development in these ligated abdomens, including synthesis of pupal cuticle, larval–pupal ecdysis and pupal maturation (Weeks *et al.* 1992). Following the infusion period, the polyethylene tubing was clamped or melted closed and left attached to the horn. Infused animals remained in our animal colony until the equivalent of pupal day 8 (P_8), at which time each LNC cluster was removed from ganglion A4 and assayed separately for bursicon bioactivity using the adult wing bioassay. Two other groups of animals, an untreated group and a ligated but not infused group, were run in parallel.

Results

Hormonal manipulations

Ligation

As a first attempt to understand the relationship between 20-HE titers and bursicon expression by the LNCs, the endogenous ecdysteroid titer was experimentally perturbed by ligating animals at the thoracic–abdominal juncture. This procedure effectively isolated the abdominal LNCs from the prothoracic glands, the source of the ecdysteroids.

Fifth-instar larvae were ligated on day 2 (D_2), on the day of wandering (W_0) or shortly after larval–pupal ecdysis on pupal day 0 (P_0; see Fig. 2A). These developmental times were chosen to subject the LNCs to various portions of the changing 20-HE titer during the last larval instar. For example, LNCs from abdomens ligated on D_2 were not exposed to either 20-HE peak, whereas W_0 or P_0 ligations exposed LNCs to either the CP alone or to both CP and PP, respectively. Ligated abdomens were maintained until the equivalent of P_8, at which time the LNCs were removed and assayed for bursicon content. LNCs

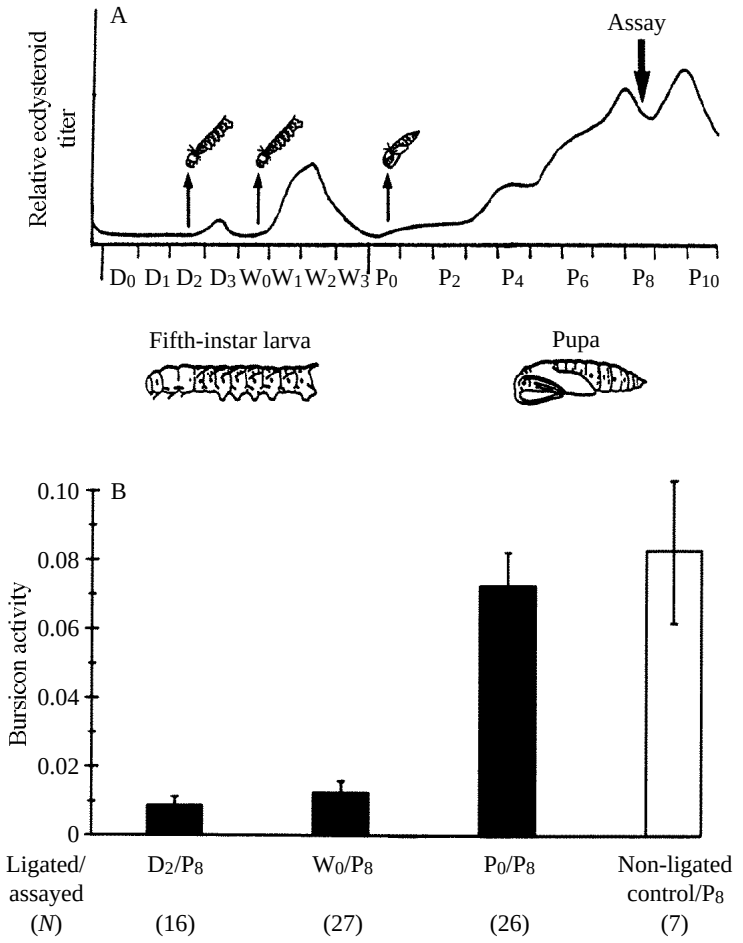

Fig. 2. Effects of ligation on bursicon expression in the LNCs during metamorphosis in *Manduca sexta*. (A) Schematic representation of the experimental procedure. Animals were ligated on D_2, W_0 or P_0 and allowed to develop until the equivalent of P_8. On 'P_8', A4 ganglia from all animals were removed and the LNCs were dissected, processed and assayed as described in Materials and methods. (B) Bursicon 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 bursicon 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 the larval–pupal molt; P_8, the equivalent of the eighth full day after the larval–pupal molt. Bursicon activity is measured in units (see Materials and methods).

taken from the D_2- or the W_0-ligated abdomens on P_8 contained very low levels of bursicon compared with the high bursicon content of the untreated controls (Fig. 2B). In contrast, elevated bursicon activity was detected in LNCs from abdomens ligated on P_0. The bursicon increase in the P_0-ligated group was equivalent to the bursicon accumulation seen in untreated controls. Since the normal rise in bursicon (Loi and Tublitz, 1993) occurred only in LNCs exposed to both 20-HE peaks, these results suggested that the increase in bursicon levels in the LNCs may be mediated by the combination of CP and PP or by the PP alone.

Ganglion transplantations

The data from the ligation experiments provided supporting evidence for the hypothesis that 20-HE is involved in the LNCs' accumulation of bursicon during metamorphosis. To test this hypothesis, we developed another method of manipulating the hormonal milieu during the last larval instar. This involved surgical transplantation of a single abdominal ganglion into the abdomen of an older host, thereby exposing the LNCs in the implanted ganglion to an abbreviated 20-HE titre. This implantation technique is effective because *Manduca sexta*, like all other insects, has an open circulatory system and implanted ganglia are therefore subject to the same hormonal environment as the host.

To determine which 20-HE pulse during the last larval instar was critical for the bursicon rise in the LNCs, single A4 ganglia from D_2 caterpillars were implanted into hosts at various stages (Fig. 3A). D_2 animals were chosen as donors for this experiment because D_2 LNCs have not yet been exposed to the two major fifth-instar 20-HE pulses. D_2 ganglia were implanted into D_2, W_0 or W_3 hosts, where they remained until the hosts reached P_8. This experimental paradigm exposed the implanted LNCs to the CP and PP ($D_2 \rightarrow D_2$), only the PP ($D_2 \rightarrow W_0$) or neither of the 20-HE pulses ($D_2 \rightarrow W_3$). When the host reached P_8, each cluster of four LNCs from the host and the implanted A4 ganglion was removed individually and separately analyzed for bursicon content using the adult wing bioassay. High bursicon levels were detected in implanted LNCs exposed to both ecdysteroid peaks ($D_2 \rightarrow D_2$) or to the PP alone ($D_2 \rightarrow W_0$; Fig. 3B). Despite the relatively high variability in these data sets, statistical analyses did not identify significant differences in bursicon levels between hosts or implanted LNCs in these experimental conditions.

Bursicon failed to be expressed only in those LNCs implanted into hosts on W_3, the day before pupation, when prepupal ecdysteroid levels are returning to basal. In all groups, LNCs removed from the host animal on P_8 expressed their normal transmitter phenotype for that stage, i.e. high amounts of bursicon (Fig. 3B; Loi and Tublitz, 1993). These data indicate that the PP by itself, without a contribution from the CP, can induce bursicon accumulation in the LNCs.

The preceding results showed that LNCs can express bursicon if exposed to the PP without the CP. Is the converse also true? Is the CP by itself capable of inducing bursicon accumulation in the LNCs? This question was explored empirically in a second set of transplantation experiments in which LNCs were exposed to the CP only. An A4 ganglion from a W_0 caterpillar was implanted into a W_3 host where it remained until the host reached P_8 (Fig. 4A). At that point, both the implanted and the host A4 ganglia were

removed and individual LNC clusters were assayed for bursicon content. Implanted LNCs exposed only to the CP exhibited very low levels of bursicon on P_8, whereas host LNCs that had been exposed to both 20-HE peaks, expressed normal, high amounts of

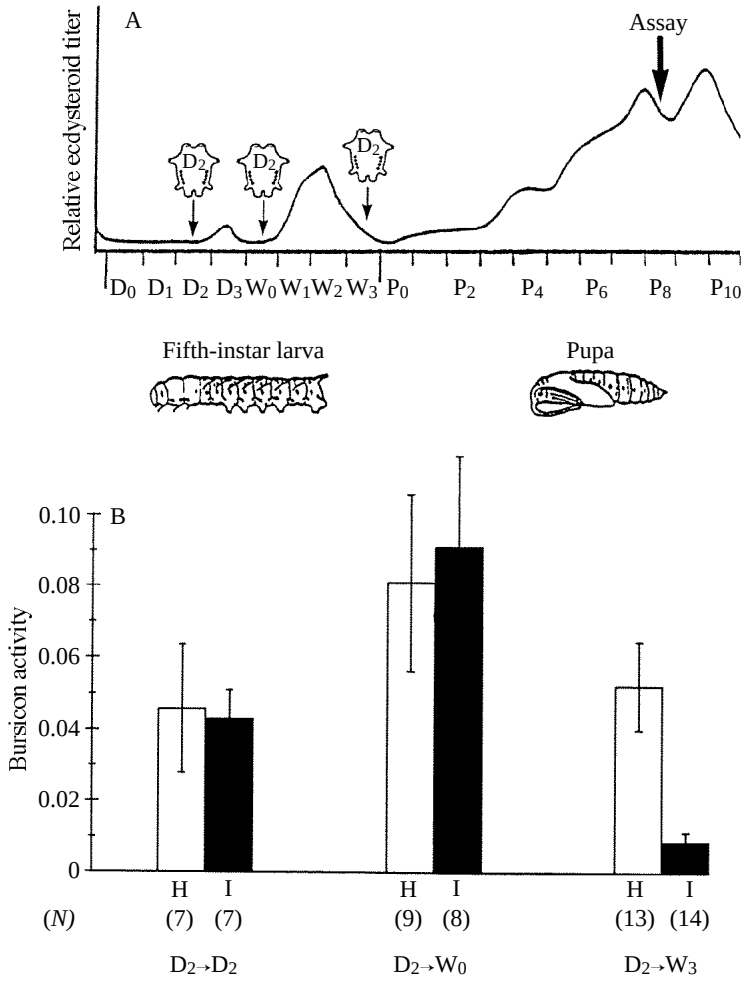

Fig. 3. Effects of ganglion transplantation on bursicon expression in the LNCs during metamorphosis in *Manduca sexta*. (A) Schematic representation of the experimental procedure. A4 ganglia were removed from D_2 fifth-instar larvae and individually implanted into host animals on D_2, W_0 or W_3 to expose 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 for bursicon. (B) Bursicon levels in host (H) and implanted (I) LNCs. Each bar represents the mean ± S.E.M. of bursicon activity from (*N*) separate LNC clusters. Bursicon levels in the $D_2 \rightarrow D_2$ and the $D_2 \rightarrow W_0$ experiments are not significantly different from each other in the host and implanted LNCs. 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 from a D_2 animal that was implanted into a W_0 caterpillar. Abbreviations as in Figs 1 and 2.

bursicon (Fig. 4B). Thus, the CP alone is not sufficient to play a major role in the regulation of bursicon levels in the LNCs.

We performed a third set of ganglion transplantation experiments to determine whether developmental age was a determining factor in regulating the transmitter phenotype of these cells. In these experiments, an A4 ganglion was removed from a fourth-instar caterpillar (day 3, the day prior to molting from fourth to fifth instar) and surgically implanted into a D_2 fifth-instar larva. LNCs transplanted in this fashion thus bypassed the last day of the fourth instar and the first 2 days of the fifth instar (Fig. 5A), a period of minor fluctuations of the ecdysteroid titer (Riddiford, 1985). As with all previous transplantation experiments, the implanted and host LNCs were removed when the host

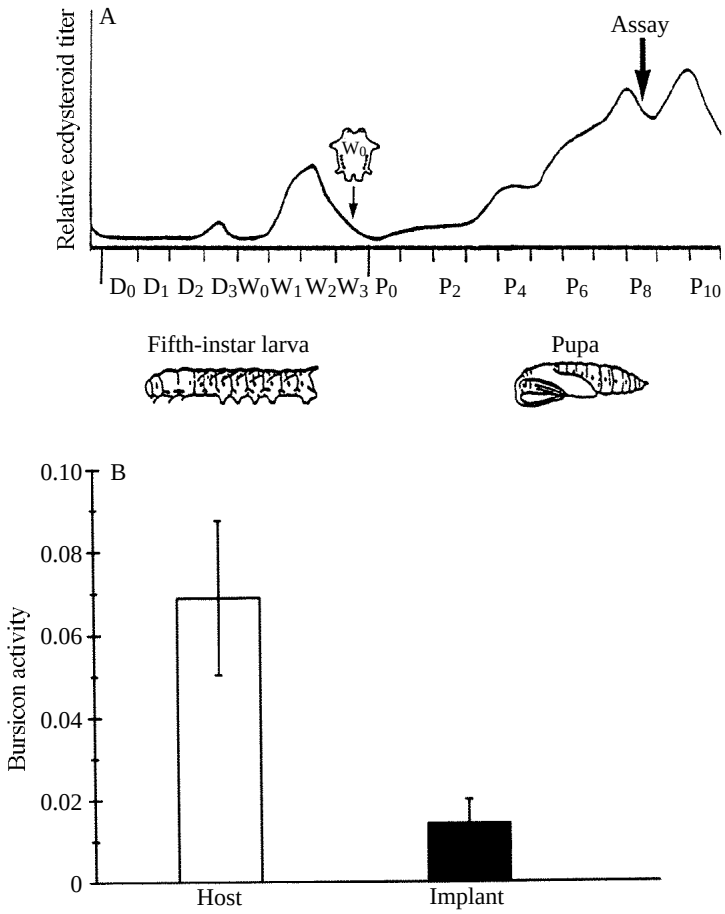

Fig. 4. The effects of the commitment pulse on bursicon expression in the LNCs. (A) Schematic representation of the experimental procedure. A4 ganglia were removed from W_0 fifth-instar larvae and individually implanted into W_3 hosts. This procedure exposed LNCs to the CP only. After transplantation, hosts were allowed to develop until P_8, at which time both implanted and host ganglia (A4) were removed and assayed for bursicon. (B) Bursicon levels in host and implanted LNCs. Each bar represents the mean ± S.E.M. of bursicon activity from eight separate LNC clusters. Abbreviations as in Figs 1–3.

reached P_8. After removal, each LNC cluster was assayed on the adult wing to determine bursicon content. High levels of bursicon were found in both host and implanted LNCs (Fig. 5B). This result shows that exposure to the normal 20-HE titer can induce precocious bursicon expression in the LNCs and suggests that acquisition of 20-HE sensitivity occurs many days prior to any steroid-induced transmitter changes.

Endocrine manipulations: 20-HE infusion

The results from the ligation and ganglion transplantation experiments show that the PP and the rise in LNC bursicon levels are temporally correlated but do not prove causality. To address this issue, we carried out a set of experiments in which the

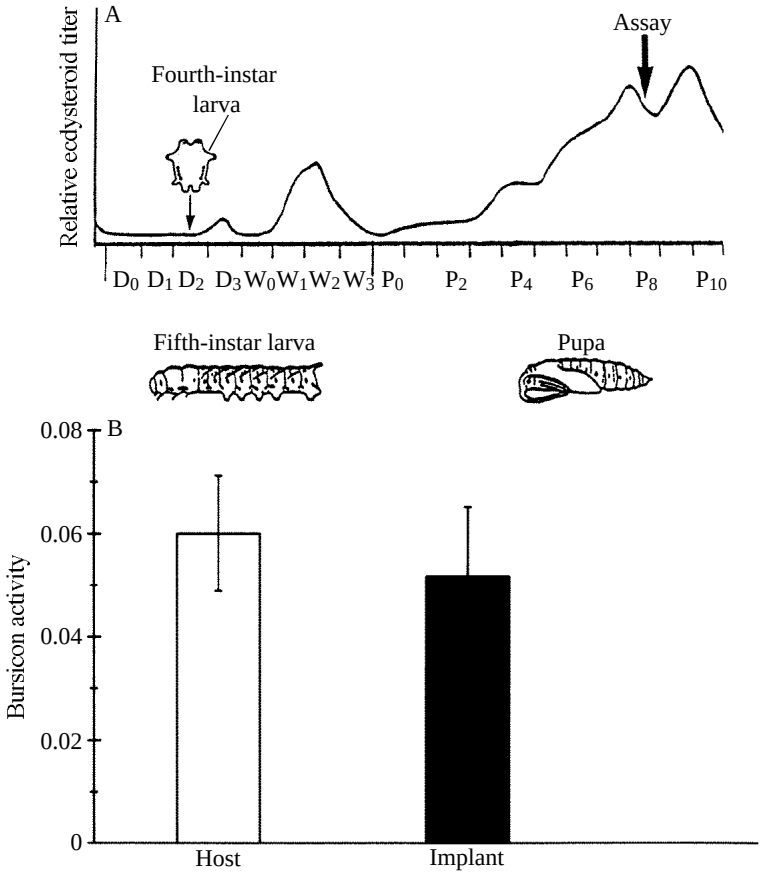

Fig. 5. The prepupal ecdysteroid peak induces precocious bursicon expression in fourth-instar LNCs. (A) Schematic representation of the experimental procedure. A4 ganglia were removed from fourth-instar larvae and individually implanted into D_2 fifth-instar caterpillars. This procedure exposed the implanted LNCs to both the CP and PP in the proper sequence. After transplantation, hosts were allowed to develop until P_8, at which time both implanted and host ganglia (A4) were removed and assayed for bursicon. (B) Bursicon levels in host and implanted LNCs. Each bar represents the mean ± S.E.M. of bursicon activity from eight separate LNC clusters. Abbreviations as in Figs 1–3.

endogenous source of the steroids, the prothoracic glands, was removed and replaced by exogenous application of 20-HE. The procedure for this set of experiments involved ligating caterpillars on W_0, after the completion of the CP but prior to the onset of the PP (Fig. 6A). As with the ligation experiments described previously, a ligature was tied at the thoracic–abdominal juncture and the anterior end of the animal, including the prothoracic glands, was discarded. A calibrated syringe pump was used to infuse 20-HE directly into ligated abdomens *via* a small plastic catheter inserted into the dorsal horn. 20-HE infusion rate and concentration were designed to mimic the endogenous PP as closely as possible (Weeks *et al.* 1992). Following infusion, abdomens were maintained

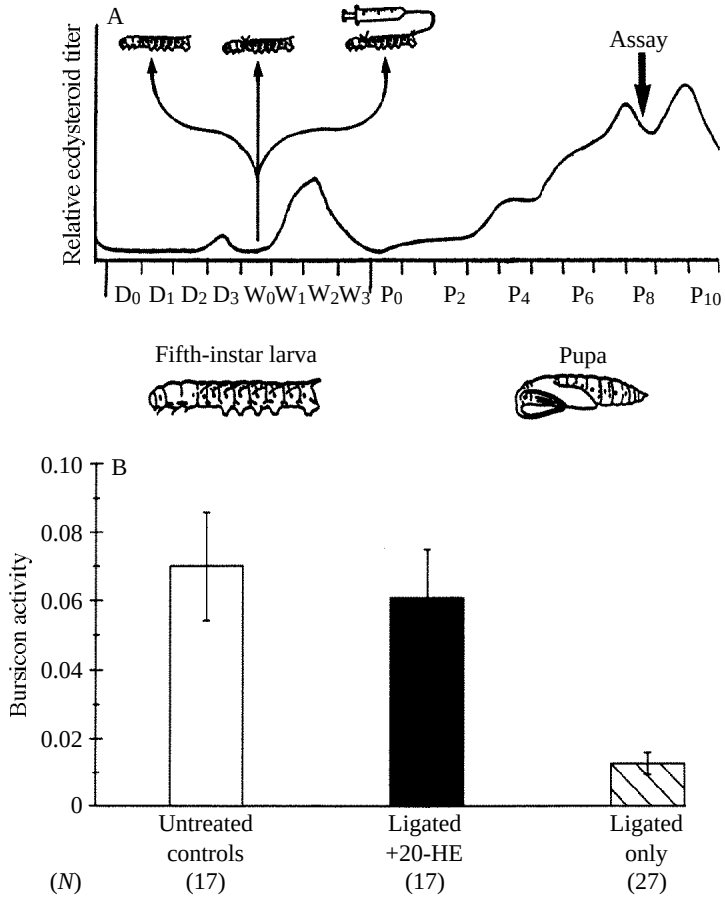

Fig. 6. The effect of 20-hydroxyecdysone (20-HE) infusions on bursicon levels in the LNCs. (A) Schematic representation of the experimental procedure. Caterpillars were ligated on W_0, then infused with sufficient 20-HE to mimic the prepupal peak (see Fig. 1 and text for details). One set of controls was ligated but not infused, while a second control group was untreated. LNC clusters from the fourth abdominal ganglion of all three groups were separately removed on P_8 and assayed individually for bursicon content. (B) Bursicon levels in LNCs from control and hormonally manipulated animals. Each bar represents the mean ± S.E.M. of bursicon activity from (*N*) separate LNC clusters. Abbreviations as stated in Figs 1–3.

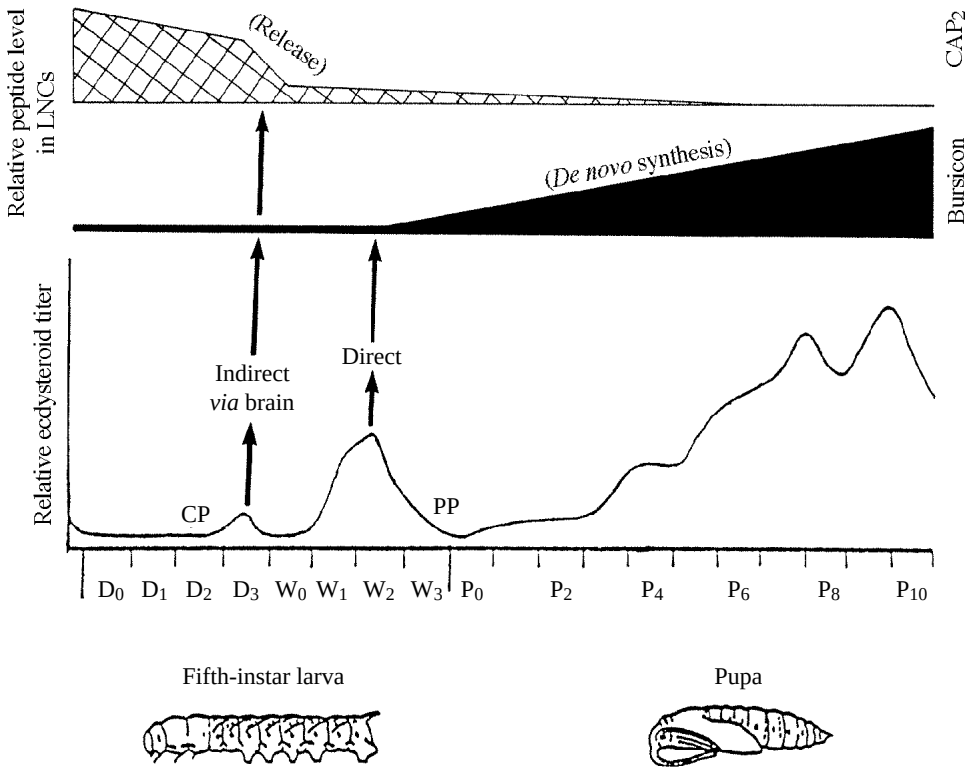

Fig. 7. Temporal sequence of steroid-induced transmitter plasticity in LNCs during metamorphosis in the tobacco hawkmoth, *Manduca sexta*. Upper panels: relative levels of CAP_2 and bursicon in the LNCs during the last larval instar and the first half of adult development. Bottom panel: relative ecdysteroid titer during the same period. The switch from CAP_2 to bursicon in the LNCs begins on D_3 with the CP. This small steroid rise activates a group of 20-HE-sensitive neurons in the brain that, by way of a set of descending interneurons, stimulate the LNCs into releasing CAP_2 on W_0 (Loi and Tublitz, 1993; Tublitz *et al.* 1992). The CAP_2 decline is followed by a gradual rise in bursicon levels in the LNCs induced by the PP. The PP presumably acts directly on the LNCs to stimulate transcriptional activity of the bursicon gene. CP, commitment pulse; PP, prepupal peak: other abbreviations are as stated in Fig. 1.

until P_8 at which time the LNCs from ganglion A4 were removed and quantitatively assayed for bursicon levels. Our results (Fig. 6B) demonstrate that 20-HE can trigger bursicon expression since LNCs from ligated abdomens infused with 20-HE expressed high amounts of bursicon. Bursicon levels in these infused preparations were equivalent to those of a control group of animals which had been left untreated and had therefore been subject to the normal endogenous 20-HE titer (Fig. 6B). LNCs from another control group that had been ligated but not infused contained minimal levels of bursicon. Thus, exogenous 20-HE produces an increase in bursicon levels in the LNCs that is qualitatively and quantitatively similar to that occurring during normal development.

Discussion

Up-regulation of bursicon expression in the LNCs by the prepupal peak of 20-HE

The primary goal of the present study was to determine the mechanism(s) responsible for the increase in bursicon in the LNCs during metamorphosis in *Manduca sexta*, and the data presented here strongly implicate the PP as the proximate factor in this process. The results of the ligation experiments (Fig. 2) demonstrated a temporal correlation between changes in the 20-HE blood titer in fifth-instar larvae and bursicon accumulation in the LNCs. The ganglion transplantation data (Figs 3–5) established that bursicon levels in the LNCs were influenced primarily by the PP, the second and larger of the two 20-HE pulses. For example, LNCs exposed to only the PP ($D_2 \rightarrow W_0$ implants, Fig. 3B) expressed high levels of bursicon, equivalent to that of untreated controls (data from host LNCs, Fig. 3B). The converse manipulation, testing the role of the CP in the absence of the PP, produced LNCs containing low levels of bursicon (Fig. 4). Direct confirmation of the causal relationship between the PP and bursicon levels in the LNCs was provided by the results of the 20-HE infusion experiments, in which LNCs from animals lacking an endogenous 20-HE source were able to produce normal amounts of bursicon if, and only if, they were treated with exogenous 20-HE (Fig. 6). Those LNCs not exposed to 20-HE, whether from endogenous or exogenous sources, failed to develop measurable levels of bursicon. Thus, the up-regulation of bursicon in the LNCs during metamorphosis appears to be triggered by the PP.

Are the LNCs direct targets of the prepupal peak of 20-hydroxyecdysone?

Does the PP act directly or indirectly on the LNCs? Although this question was not the primary focus of the current study, the experiments discussed here shed some light on this issue. As a first approximation, the site of ecdysteroid action must be localized to the ganglion containing the LNCs since the 20-HE-induced bursicon accumulation occurs in single, transplanted ganglia (Figs 3 and 4). Although it is possible that ecdysteroid action on the LNCs may be mediated indirectly by another target within the same ganglion, the most parsimonious view is that the LNCs are direct targets for the PP, which causes an increase in LNC bursicon levels *via de novo* synthesis (Fig. 7). This explanation is consistent with our transplantation data and compatible with much of the literature on steroid-dependent changes in the CNS. Direct steroid action is, of course, well established in a myriad of neuronal and non-neuronal systems (Riddiford, 1985; McEwen *et al.* 1990; McEwen, 1991). It is unlikely that 20-HE exerts its effects on the LNCs indirectly through other, neighboring cells in the same ganglion since there is little evidence for this particular type of indirect regulation in other preparations. The second aspect of our proposal, that 20-HE directly increases the transcription rate of the bursicon gene, is also well documented in other systems. Steroid-induced transcriptional regulation has been described in detail for numerous mammalian CNS genes, including the oxytocin receptor (DeKloet *et al.* 1985; Schumacher *et al.* 1990) and ribosomal RNA (Jones *et al.* 1986). In *Manduca sexta*, many physiological and/or developmental events involving the CNS are directly controlled by 20-HE and those that have been studied, such as expansion of sensory arborizations (Levine *et al.* 1991), peptide-induced effects (Morton and Truman,

1988) and neuronal cell death (Montemayor *et al.* 1990; Schwartz *et al.* 1990), appear to require gene activation. Thus, our work meshes nicely with the existing literature and argues in favor of a direct genomic effect of 20-HE on the LNCs. One way to test this model more rigorously would be to culture individual LNCs *in vitro* in the presence and absence of 20-HE; experiments along these lines are currently in progress.

Hormonal control of the transmitter plasticity in the LNCs during metamorphosis

It is now clear that some fully differentiated, mature neurons change their transmitter phenotype *in vivo* (e.g. Adler and Black, 1986; Landis and Keefe, 1983; LeBlanc and Landis, 1986; Schotzinger and Landis, 1988), yet little is known about the underlying regulatory mechanisms. Fortunately, cellular and molecular studies are beginning to furnish a glimpse into the complex nature of this process, and the LNCs, because of their large somata size, characteristic soma location within each ganglion and their physical accessibility, provide a good model system for such explorations. The studies reported here and in the preceding paper (Loi and Tublitz, 1993) provide an empirical foundation on which to build a model that explains the changes in transmitter expression in the LNCs during metamorphosis, and such a model is illustrated in Fig. 7. The conversion from CAP_2 to bursicon commences with the CP on the evening of D_3. This small, but crucial, steroid pulse indirectly triggers a major decline in LNC CAP_2 levels (Loi and Tublitz, 1993). Several different types of endocrine and CNS implantation manipulations unequivocally show that the effects of the CP on the LNCs require intact neural connections between the LNCs and CP-sensitive neurons located in the brain. For example, the CP-induced CAP_2 decline does not occur if the brain is not intact and attached to the rest of the CNS (Loi and Tublitz, 1993). CAP_2 levels continue to fall following the CP until midway through adult development, by which time the LNCs are devoid of measurable cardioexcitatory bioactivity (Tublitz and Sylwester, 1990; Loi and Tublitz, 1993). Meanwhile, bursicon levels in the same cells begin to rise gradually as a result of a direct effect of the second larger 20-HE surge on W_1–W_3. By P_8, the transmitter switch is complete, with the LNCs exhibiting a bursicon phenotype (Fig. 7). Transmitter plasticity in the LNCs is thus a multi-step process, beginning with the CAP_2 decline on W_0 and ending 12 days later with elevated bursicon levels on P_8.

The two major components of the switch, the CAP_2 decline and the subsequent bursicon accumulation, appear to be independently regulated. This conclusion is based on the fact that we were experimentally able to generate LNCs expressing all possible CAP_2/bursicon phenotypes. LNCs expressing high CAP_2 levels and minimal amounts of bursicon were created by the ligation experiments (Fig. 2 from Loi and Tublitz, 1993 and Fig. 2 this paper), whereas the converse phenotype, bursicon-containing cells without measurable CAP_2, were produced by normal exposure to the two-peak 20-HE sequence (Fig. 1 from Loi and Tublitz, 1993). We were also able to generate LNCs that expressed high levels of both peptides (the $D_2 \rightarrow W_0$ ganglion implantation data, Fig. 3 here and Fig. 3 from Loi and Tublitz, 1993) or that expressed neither (combined data from the $D_2 \rightarrow W_3$ ganglion implantation experiments illustrated in the same two figures). The independence of the two steroid-induced events lends additional support to the model that the CP and the PP act *via* completely separate pathways.

The two steroid pulses can have very different effects on the same group of neurons because of the contrasting nature of the two endocrine events. The CP is about fivefold smaller in magnitude and has a shorter duration than the PP. Moreover, the CP occurs in the absence of juvenile hormone (Fig. 1), whereas there is a substantial and physiologically significant juvenile hormone titer during the PP (Riddiford, 1985). The two ecdysteroid surges also produce very distinct physiological and behavioral effects. Differences in the characteristics of the two 20-HE pulses may explain how different responses are generated in the same cells from a single hormonal effector.

One intriguing result emerging from these studies is that the increase in the ecdysteroid titer during adult development beginning on P_3 did not seem to affect bursicon expression in LNCs not exposed to the PP (refer to the $D_2 \rightarrow W_3$ and $W_0 \rightarrow W_3$ ganglion implantation results in Figs 3 and 4, respectively). This result is a bit perplexing since the magnitude of the post-pupation 20-HE surge suggests that it could have substituted for and been interpreted as the PP by implanted LNCs that had skipped the host's PP. A trivial, yet likely, explanation is that the large 20-HE surge in pupa was indeed sufficient to activate bursicon expression in these circumstances, but bursicon levels were not detected because of the short interval between the 20-HE rise and the P_8 assay point. Another possibility is that qualitative and quantitative differences between the PP and the pupal 20-HE peak preclude the latter substituting for the former. The pupal 20-HE peak is at least twice the magnitude and four times the duration of the PP and exerts its metamorphic effects in the absence of juvenile hormone (Fig. 1). Moreover, the PP also has a distinct falling phase that has been shown to be physiologically important for some of the metamorphic changes in the nervous system (Levine *et al.* 1991; Weeks *et al.* 1992). These differences may explain why the pupal 20-HE peak fails to induce bursicon expression in LNCs not exposed to the PP. Experiments are currently under way to distinguish between these two possibilities.

Functional significance of the transmitter switch in the LNCs

Since the primary secretory product of the LNCs varies with developmental stage, it is not surprising that the function of these cells is also stage-specific. The primary role of the LNCs during embryogenesis and in larvae is to modulate hindgut activity. During the later stages of embryonic development, CAP_2 release from the LNCs triggers the first contractions of the hindgut to aid digestion of extra-embryonic yolk (Broadie *et al.* 1990). CAP_2 stimulation of hindgut activity occurs again in fifth-instar larvae at wandering to facilitate purging of the alimentary canal, a type of behavior euphemistically named 'gut-emptying' (Tublitz *et al.* 1992). We have recently shown that the CAP_2 decline in the LNCs at wandering is due to CAP_2 secretion (Tublitz *et al.* 1992). Interestingly, this release is both local and hormonal. Although the neurohormonal LNCs in each abdominal ganglion are all activated during gut-emptying, the primary effectors of the CAP_2-triggered increase in gut activity are the LNC homologues in the terminal ganglion, the most posterior ganglion of the CNS. Dye-fills of these terminal ganglion LNCs have ascertained that they have peripheral axons that project to and terminate on hindgut musculature (Tublitz *et al.* 1992). Thus, the primary role of the LNCs prior to metamorphosis is to modulate visceral muscle.

In contrast to their hindgut-modulating function in embryos and larvae, the LNCs perform a very different role in adults after the switch from CAP_2 to bursicon. The elegant studies by Reynolds and his collaborators on adult LNCs showed that these cells are activated within seconds after the adult moth emerges from the pupal skin (reviewed in Reynolds, 1983). They further showed that LNC activation at adult emergence releases bursicon into the hemolymph, where it acts to facilitate tanning of the adult cuticle. The transformation of LNC function at metamorphosis can therefore be accounted for at the molecular level by the phenotypic switch from CAP_2 to bursicon. Finally, it is interesting to note that, while CAP_2 levels are declining in the LNCs, there is a set of newly arising neurons in the same ganglion that express the CAPs for the first time (Tublitz and Truman, 1985*d*). The appearance of these newly arising CAP-containing cells ensures the availability of the CAPs in the adult, where they act as cardioregulatory neurohormones (Tublitz and Truman, 1985*a–c*; Tublitz, 1989). Thus, the ability of the CNS to use the CAPs at all stages is preserved by temporal and spatial alterations in the number of CAP-containing neurons.

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