STEROID-REGULATED MORPHOLOGICAL PLASTICITY IN A SET OF IDENTIFIED PEPTIDERGIC NEURONS IN THE MOTH *MANDUCA SEXTA*

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Summary

The lateral neurosecretory cells (LNCs) in the tobacco hornworm *Manduca sexta* undergo a switch in neurotransmitter phenotype during pupation. Concurrent with this change in function, the LNCs undergo a major morphological reorganization. This study characterizes the morphological change and its underlying cause. In the larva, the LNC has a very compact dendritic arborization in a small volume of neuropil ipsilateral to the cell soma. In the adult moth, the LNC arborization extends through a much larger volume of neuropil, including the contralateral side of the ganglion. Using both *in vivo* manipulations and a single-cell culture system, we show that this change in morphology is probably triggered by two pulses of the steroid hormone 20-hydroxyecdysone (20-HE): a small commitment peak and a larger prepupal pulse. These are the same two pulses of 20-HE as those previously shown jointly to cause the change in transmitter phenotype. This work, in conjunction with a previous study on the transmitter switch, documents the orchestration of major morphological and biochemical changes in a set of identified neurons by a single hormone.

Key words: steroid, hormone, 20-hydroxyecdysone, neuronal plasticity, insect, neurosecretion, *Manduca sexta,* neuropeptide, bursicon, insect cardioacceleratory peptide.

Introduction

Neural plasticity is essential for the proper functioning of any animal because it is the mechanism through which such processes as learning and memory occur. Among the possible types of plasticity found in the central nervous system (CNS) are synaptic plasticity, in which neurons either create and break connections with other neurons or change the strength of their existing connections (Bottjer and Johnson, 1997), physiological plasticity, in which neurons change their electrophysiological characteristics (Manseau *et al.* 1998), and transmitter plasticity, in which neurons change the type of neurotransmitter that they express (Landry *et al.* 1997).

Significant morphological changes also occur in neurons. Some neurons rebuild large areas of their dendritic fields by reducing their existing projections and growing new ones. One example of this is the significant seasonally mediated changes in the volume of a song control nucleus, the robustus archistriatalis, in songbirds (Hill and DeVoogd, 1991). Such major changes in neural architecture are particularly important in holometabolous insects which, because they undergo complete metamorphosis, have very different larval and adult forms with vastly different sensory systems and behavioral repertoires. During pupation in these animals, much of the CNS must be completely reorganized; many larval functions are no longer needed and, therefore, many neurons controlling these functions are programmed to die (Taylor and Truman, 1974; Truman *et al.* 1992; Levine and Weeks, 1996). Numerous new neurons arise *de novo* from imaginal cells which remained undifferentiated in the larval stage to produce the new behavior patterns required in the adult (White and Kankel, 1978; Booker and Truman, 1987). Other neurons, co-opted from now-defunct larval roles (Levine and Truman, 1985), must be respecified to perform their new roles, with all the structural, physiological and biochemical changes that this may imply.

The purpose of this study is to assess the extent of and to explore the mechanisms underlying the changes in morphology that occur in a set of identified neurons, the lateral neurosecretory cells (LNCs) in the abdominal nerve cord of the tobacco hawkmoth *Manduca sexta*. These peptide-containing cells are of particular interest because they undergo a well-described switch in neurotransmitter phenotype during metamorphosis (Tublitz and Sylwester, 1990). In the larva, these cells produce a set of insect cardioacceleratory peptides collectively known as the CAP_2s, which are involved in gut-emptying at the wandering stage (Tublitz *et al.* 1992). Production of the CAP_2s is down-regulated at the end of the last (fifth) larval instar and, during pupal development, the LNCs begin to produce a different neurotransmitter, bursicon,

an insect neurohormone responsible for cuticular tanning (Reynolds, 1983). These two events, the down-regulation of the CAP$_2$ group and the up-regulation of bursicon, are independently triggered by two separate pulses of the insect steroid hormone 20-hydroxyecdysone (20-HE) that together initiate the events surrounding the pupal molt (Loi and Tublitz, 1993; Tublitz and Loi, 1993; Fig. 1). The first peak of 20-HE, called the commitment pulse (CP), commits the larva to a pupal molt. This commitment peak is followed by the much larger prepupal pulse (PP) of 20-HE, which formally triggers the physiological events underlying the pupal molt (Bollenbacher et al. 1981).

Loi and Tublitz (1993) have shown that the switch from the secretion of the CAP$_2$ group to the secretion of bursicon is a multi-step process beginning with a sharp drop in CAP$_2$ levels in the LNCs midway through the last (fifth) larval instar. This is accompanied by an increase in the levels of CAP$_2$ in the hemolymph, indicating that the CAP$_2$s from the LNCs are released directly into the blood (Tublitz et al. 1992). The drop in the levels of CAP$_2$ is triggered by the commitment pulse of 20-HE and is also dependent on an intact connection to the brain (Loi and Tublitz, 1993). Additional studies have shown that the subsequent rise in bursicon levels in the LNCs is directly triggered by the prepupal pulse of 20-HE (Fig. 1) and, unlike the initial drop in the levels of CAP$_2$, the increase in bursicon level is independent of a connection to the brain (Tublitz and Loi, 1993).

In conjunction with the change in neurotransmitter phenotype, the LNCs undergo a major alteration in morphology in preparation for their new functions in the adult. This paper presents a quantification of this morphological change and provides evidence that it is evoked by the same two pulses of 20-HE that cause the transmitter switch.

Materials and methods
Animals

Manduca sexta were individually raised in the Manduca rearing facility at the University of Oregon using procedures and rearing conditions described in detail previously (Loi and Tublitz, 1993). In brief, newly hatched larvae were placed in individual plastic cups containing a small cube of artificial diet (Bell and Joachim, 1978). Larvae were maintained in a long-day photoperiod (17 h:7 h L:D) superimposed on a 27 °C:25 °C thermal cycle. Animals from three age groups were used in these experiments unless otherwise stated: (i) on day 2 of the fifth (final) instar (approximately 6 days before pupation), (ii) in the first hour after pupation (pupal day 0) or (iii) in the final few hours before adult emergence.

Visualization of lateral neurosecretory cells in vivo

Each unfused abdominal hemiganglion contains 4–5 LNC somata, usually situated in a cluster or row at the point along the lateral edge of the dorsal side where the ventral nerve exits from the ganglion. These studies generally focused on the most posterior pair of LNCs, which are biochemically, immunologically and morphologically indistinguishable from each other. A silver-intensified cobalt staining method was used to visualize the morphology of larval and adult LNCs. For the larval morphology studies, larvae were used at the same stage as those used for culture (day 2 of the fifth instar). For investigations of adult LNC morphology, pharate adults were

Fig. 1. Temporal sequence of steroid-induced transmitter plasticity in the lateral neurosecretory cells (LNCs) during metamorphosis in the tobacco hawkmoth Manduca sexta. Upper panels: relative levels of CAP$_2$s 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 commitment pulse (CP). This small pulse of steroid activates a groups 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 decline in CAP$_2$ levels is followed by a gradual rise in bursicon levels in the LNCs induced by the prepupal pulse (PP). The PP presumably acts directly on the LNCs to stimulate transcription of the bursicon gene. D$_0$–D$_3$, days 0–3 of the fifth larval instar; W$_0$–W$_3$, days 0–3 of the wandering stage of the fifth larval instar; P$_0$–P$_{10}$, days 0–10 of the pupal stage. Inset: cell body location of the lateral neurosecretory cells in larval abdominal ganglion A4 (from Tublitz and Loi, 1993).
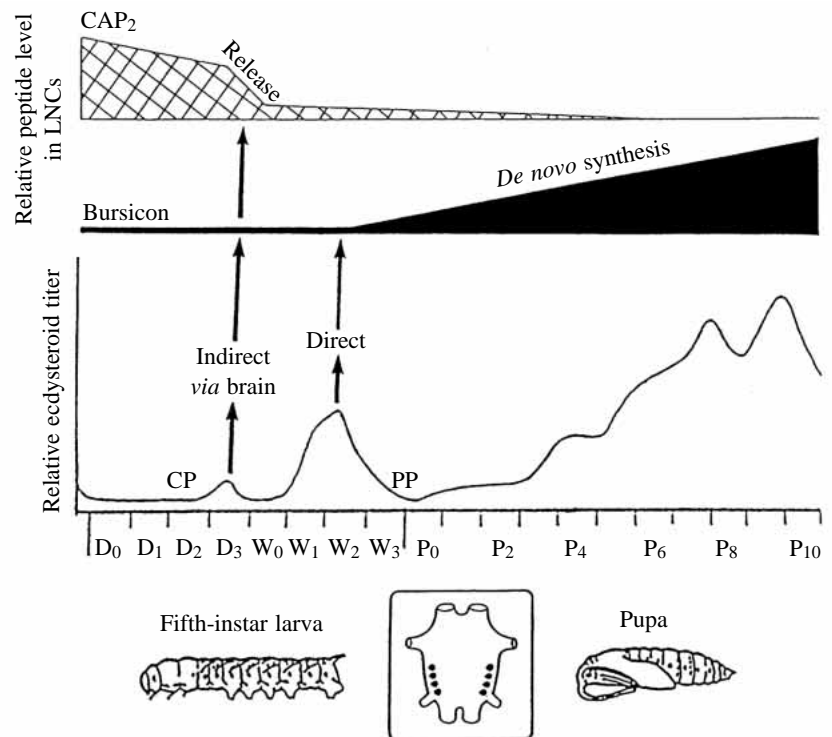
used several hours before adult emergence from the pupal cuticle. Following anesthetization on ice, the larval abdominal nerve cord from abdominal ganglion 3 (A3) to A8 was dissected out and pinned to a Sylgard-coated Petri dish containing the saline of Miyazaki (1980) (140 mmol l^{-1} NaCl, 4.8 mmol l^{-1} KCl, 28 mmol l^{-1} D-(+)-glucose, 5 mmol l^{-1} Hepes, 5 mmol l^{-1} CaCl$_2$·2H$_2$O; pH adjusted to 7.0 with 1 mol l^{-1} NaOH). The nerve cord was pinned out dorsal side up, and the dorsal perineurium was removed using two pairs of specially sharpened forceps. The same procedure was used for the nerve cord of the pharate adult, with the addition of a step removing the dorsal pad over each ganglion using a pair of fine iridectomy scissors.

The prepared abdominal nerve cord was then placed under dark-field illumination in a Faraday cage and one LNC per abdominal ganglion was impaled with a glass microelectrode containing 100 mmol l^{-1} hexamminic CoCl$_2$ in 1.5 mol l^{-1} KCl. The resistance of the electrodes was approximately 25 MΩ. Cells were dye-filled ionophoretically for 10–15 min using 1 s depolarizing steps of 0.7 nA at a frequency of 0.5 Hz. After filling, the electrode was removed and the ganglia were allowed to stand for at least 30 min to allow dispersal of the cobalt through the cell. Ganglia were then suspended in Miyazaki saline saturated with H$_2$S for 5 min to precipitate the Co^{2+} to CoS, rinsed in fresh saline for 20 min and then fixed in Carnoy's fixative (six parts 100 % ethanol, three parts chloroform, one part glacial acetic acid) for at least 1 h. Following fixation, the ganglia were washed in 70 % ethanol for 15 min and stored in fresh 70 % ethanol overnight at 4 °C.

The CoS precipitate in the LNCs was intensified using the silver intensification method of Mesce *et al.* (1993). Ganglia were rehydrated in a descending alcohol series (10 min each in 70 %, 50 % and 30 % ethanol, then 10 min in distilled water), transferred to a solution of 2 % (w/v) sodium tungstate for 15 min, and then into a preincubation solution of an 8:1 solution of (2.25 g l^{-1} sodium acetate·3H$_2$O, 7.5 % (v/v) acetic acid, 0.75 g l^{-1} silver nitrate):5 % sodium tungstate. After preincubation for 15 min, ganglia were transferred to a developing solution consisting of nine parts of fresh preincubation solution plus one part of 0.25 % (w/v) L-ascorbic acid for 6 min. Although this protocol is nominally light-insensitive, development was carried out in the dark as an added precaution. The intensification reaction was stopped by transferring the ganglia to a dish of distilled water and rinsing for 10 min. The ganglia were then dehydrated in an increasing alcohol series (10 min each in 30 %, 50 %, 70 %, 80 %, 90 % and 100 % ethanol), cleared in methyl salicylate, and mounted between two coverslips with DPX mounting medium.

Analysis of the extent of arborization *in vivo*

Individual dye-filled LNCs were viewed on a standard compound microscope and traced through a drawing tube. The *camera lucida* drawing was then photocopied onto a grid and taken back to the microscope, where each grid square was scored for the presence or absence of in-focus LNC processes at a single plane of focus. Each grid square measured 25 μm×25 μm at 250× magnification. This was repeated at 5 μm vertical intervals through the whole ganglion, yielding a number of grid cubes containing processes. This number was divided by the total number of cubes in the ganglion, yielding a percentage of the ganglion volume containing LNC processes.

A similar analysis was performed on individual subtypes of processes: primary, secondary and tertiary processes. The primary process was defined as that arising directly from the soma and traced out to the longest branch. The secondary processes were defined as any process branching directly from the primary process. All other branches were defined as tertiary processes.

In vivo *hormone manipulations*

Day 2 fifth-instar larvae that had not yet been exposed to either the commitment pulse or the prepupal pulse of 20-HE were ligated with 6 lb nylon fishing line at the second abdominal segment, and the anterior portion of the animal was removed. Since 20-HE is secreted into the hemolymph from glands in the prothorax, ligation at this time effectively deprived the developing nerve cord of these two 20-HE pulses. Newly molted pupae (pupal day 0) that had been exposed to both the native commitment pulse and the prepupal pulse, but not yet to the adult development peak, of 20-HE were also ligated at the second abdominal segment, and the anterior portion of the animal was removed. Both sets of ligated animals were kept in normal rearing conditions for 10 days followed by dissection of the nerve cords, and visualization and quantification of LNC projections as described above. The success rate for the ligation experiments was greater than 80 %.

Isolation and culture of individual LNCs

Posterior LNC somata were individually identified using size, ganglionic location and opalescence as visual markers on the basis of a prior classification through immunostaining (Tublitz and Sylwester, 1990). Following a 30 min anesthetization on ice, abdominal ganglia A1–A6 were dissected from the day 2 fifth-instar larvae and stored in Miyazaki (1980) saline. Individual ganglia were pinned dorsal side up in a Sylgard-coated dissection dish and illuminated using dark-field optics. The dorsal perineurium was removed using specially sharpened forceps, and the ganglion was incubated in a 2 mg ml^{-1} collagenase/dispase (Sigma) solution for 10 min to loosen connective tissue surrounding the cells. After the enzyme treatment, the ganglion was rinsed with Hanks' balanced salt solution (5 mmol l^{-1} KCl, 0.3 mmol l^{-1} KH$_2$PO$_4$, 4 mmol l^{-1} NaCl, 0.3 mmol l^{-1} NaHCO$_3$, 0.3 mmol l^{-1} Na$_2$HPO$_4$, 5.6 mmol l^{-1} D-(+)-glucose, pH 7.0, 350 mosmol l^{-1}), and individual posterior LNCs were dissected from the ganglion using fine glass needles. Each isolated cell was transferred using a glass micropipette to a tissue culture dish with a glass-bottomed miniwell made by sealing a glass coverslip over an 8 mm hole punched in the bottom of a 30 mm plastic Petri dish (Hayashi and Hildebrand, 1990). The

coverslips had previously been coated with a solution of $400\,\mu g\,ml^{-1}$ Concanavalin A and $4\,\mu g\,ml^{-1}$ laminin to provide a substratum for cell adhesion.

Cultured LNCs were maintained in an enriched serum-free Leibovitz's L15 cell culture medium containing the following additives per liter of L15 medium: 370 mg of α-ketoglutarate, 364 mg of D-(−)-fructose, 700 mg of D-(+)-glucose, 536 mg of D,L-malic acid, 2 g of lactalbumin hydrosylate, 2 g of TC yeastolate, 20 μg of nicotinic acid, 60 mg of imidazole, 60 mg of succinic acid, 15 mg of aspartic acid, 15 mg of cystine, 5 mg of β-alanine, 0.02 mg of biotin, 2 mg of vitamin B12, 10 mg of inositol, 10 mg of choline chloride, 0.5 mg of lipoic acid, 5 mg of p-aminobenzoic acid, 25 mg of fumaric acid, 0.4 mg of Coenzyme A and 15 mg of glutamic acid. The pH of this enriched L15 medium was adjusted to 7.0 using HCl, and the osmolarity was adjusted to $350\,mosmol\,l^{-1}$ using mannitol. Serum was not added to the culture medium because it was found to interfere with the adhesion of the LNCs to the substratum coverslip and because the cells were able to survive and grow in its absence. The hormone 20-HE and antibiotics [100 μg of gentamicin and 1 ml of penicillin/streptomycin solution containing 5000 units of penicillin and 5000 μg of streptomycin (Gibco BRL)] were added to 100 ml volumes of medium just prior to use.

Following plating, LNCs were incubated for 24 h in 100 μl of enriched L15 medium to allow cells to adhere to the substratum. The volume of medium was then increased to 4 ml per dish, and the dishes were sealed with Parafilm to prevent evaporation of the medium and to protect the dish from contamination. LNCs were incubated in low-density culture (generally 1–2 cells per dish) for approximately 2 weeks in medium containing varying levels of 20-HE. The medium was changed every 5 days. Levels of hormone exposure were designed to mimic the two endogenous 20-HE pulses that take place during the last larval instar: the initial small commitment pulse (CP) and the second larger prepupal pulse (PP). Cells were exposed to a constant level of 0, $0.1\,\mu g\,ml^{-1}$ (=CP) or $1.5\,\mu g\,ml^{-1}$ (=PP) 20-HE for the entire duration of the incubation. An additional set of LNCs was exposed to $0.1\,\mu g\,ml^{-1}$ 20-HE for the first 5 days of growth and to $1.5\,\mu g\,ml^{-1}$ 20-HE for the last 5 days as a first approximation of the endogenous fluctuations in hormone levels. The growth of processes was analyzed daily as described in the next section. The success rate for the culture experiments varied greatly between replicates and there were no signficant differences in LNC survival among the different 20-HE treatment conditions: 50–60 % of LNCs cultured in each condition survived for the 10 day monitoring period if culture dishes remained uncontaminated.

As a control, individual cells from a set of unidentified neurons found along the anteriodorsal midline just anterior to the D-IV motoneurons of the abdominal ganglion were dissected and cultured in the same manner as the LNCs. These midline cells were exposed to either 0.1 or $1.5\,\mu g\,ml^{-1}$ 20-HE for 2 weeks in culture. Data from these cells were collected for the first, fifth and tenth day of process growth, following the same procedures as that for the measurement of the growth of LNC processes.

Analysis of LNC growth in culture

Changes in LNC cell morphology were measured by monitoring the outgrowth of cell processes daily. The dissection procedure removed all processes that the cell had grown *in vivo*; thus, the cells began incubation in culture as only a soma. Cells that were placed into culture with intact processes tended to fare badly and were not included in the final data set. Healthy LNCs began to grow processes 1–4 days after being placed in culture, and this first day of growth was designated as day 1 for each cell. The health of the cells in culture was determined by the presence of processes and by the integrity of the cell membrane; LNCs that showed little or no neurite growth or had large vacuoles in the soma were considered to be unhealthy. Unhealthy cells did not extend processes and tended not to survive after the first 5 days in culture. The data in this paper were obtained only from cells that survived and grew for at least 10 days and were deemed healthy using the above criteria.

Neurite outgrowth was monitored using a Zeiss Axiovert inverted microscope with phase contrast illumination. The extent of process growth was quantified using an ocular micrometer grid inserted into one eyepiece of the microscope. At 400× magnification, each unit on the grid was $50\,\mu m \times 50\,\mu m$. Data on process growth were gathered for 10 days starting from the first day that neurite growth became visible (day 1). As with the *in vivo* data collection, *in vitro* processes were categorized into three types for the purposes of measurement and analysis. Neurites extending directly from the soma were counted as primary processes, branches from a primary process were counted as secondary processes and further branches were counted as tertiary processes.

Statistical analyses

Statistical analyses were performed on the total arborization volumes of *in vivo* LNCs as well as on the individual process subgroups. A one-tailed Mann–Whitney U-test was used to determine the statistical significance of differences between larval and adult morphologies and between ligated larval and pupal morphologies. The same test was used to determine whether there were any significant differences in the extent of LNC arborization between normal and ligated larvae, and between normal adults and ligated pupae. Statistical significance was set at $P=0.05$.

For the *in vitro* experiments, statistical analyses were also performed on the day 10 growth data for each 20-HE condition and for all neurite types within each hormone condition. A one-way analysis of variance (ANOVA) was used to determine the statistical significance of differences between average growth at day 10 for each condition. Fisher's least-significant-difference (LSD) test was used to determine significant differences between the extent of individual neurite types in different culture conditions on day 10. Statistical significance was set at $P=0.05$.

Results

In vivo *morphology*

LNC morphology changes dramatically during the metamorphic transition from larva to adult (Fig. 2A). In the larva, the neuritic processes are limited to a fairly small volume of the neuropil (3.31±0.44 % of total ganglion volume; mean ± S.E.M., *N*=5), and all processes are limited to the side of the ganglion ipsilateral to the soma. In the adult, the extent of arborization increases significantly (*P*<0.005 using a one-tailed Mann–Whitney *U*-test): processes spread through a much larger volume of the ganglion (8.74±1.38 %; mean ± S.E.M., *N*=5; Fig. 2B) and extend to the contralateral side of the ganglion.

The branching patterns of the larval and adult forms are also qualitatively different. The larval LNCs are composed of a single primary process with many short and densely packed secondary and tertiary branches, whereas the adult LNCs have a much longer primary process, several long secondary processes reaching to the contralateral side of the ganglion and many diffuse tertiary branches. The relative proportions of the primary, secondary and tertiary processes of both the larval and adult LNCs are compared in Fig. 3. The mean length of the primary process, defined as the longest process arising directly from the soma, doubles between the larval and the adult form

Fig. 3. Extent of primary, secondary and tertiary processes of the larval and adult forms of the lateral neurosecretory cells (LNCs) *in vivo* expressed as a percentage of the total ganglion volume containing each type of process. Values are mean ± S.E.M. (*N*=5 for each set). *The mean extent of all three process types was significantly greater in the adult than in the larva, using a one-tailed Mann–Whitney *U*-test, *P*<0.005.

(from 0.65±0.052 % of ganglion volume in the larva to 1.34±0.17 % in the adult; means ± S.E.M., *N*=5). The extent of secondary processes, defined as those branching directly from the primary process, and of tertiary processes, defined as all other processes, almost triples. The mean percentage of ganglion volume containing secondary processes increases from 1.44±0.14 % in the larva to 3.81±0.52 % in the adult (means ± S.E.M., *N*=5), and the mean ganglion volume containing tertiary processes increases from 2.93±0.36 % to 8.29±1.28 % (means ± S.E.M., *N*=5). In all cases, the change from larval to adult form was significant (*P*<0.005 using a one-tailed Mann–Whitney *U*-test). Thus, these cells undergo a major morphological alteration during the metamorphic period between larva and adult.

Hormone manipulation *in vivo*

Because the morphological changes in the LNCs occurred during metamorphosis, it was hypothesized that the CP and PP of 20-HE were the triggering events for these morphological alterations. An alternative hypothesis was that these changes were caused by the large adult developmental peak of 20-HE during pupal development (Bollenbacher *et al.* 1981). To test both these hypotheses, animals were ligated at the second abdominal segment as described in the Materials and methods section. Ten days after ligation, the LNCs were dye-filled and the extent of their processes was quantified.

LNCs from ligated day 2 fifth-instar larvae, which had not been exposed to any of the endogenous pulses of 20-HE, remained in a larval form, spreading through a mean of only 3.88±0.44 % of the ganglion volume (mean ± S.E.M., *N*=5) (Fig. 4). Although slightly higher than the mean value for unmanipulated larval LNCs (3.31±0.44 %), the two values are not significantly different (*P*=0.1). LNCs from ligated pupae,
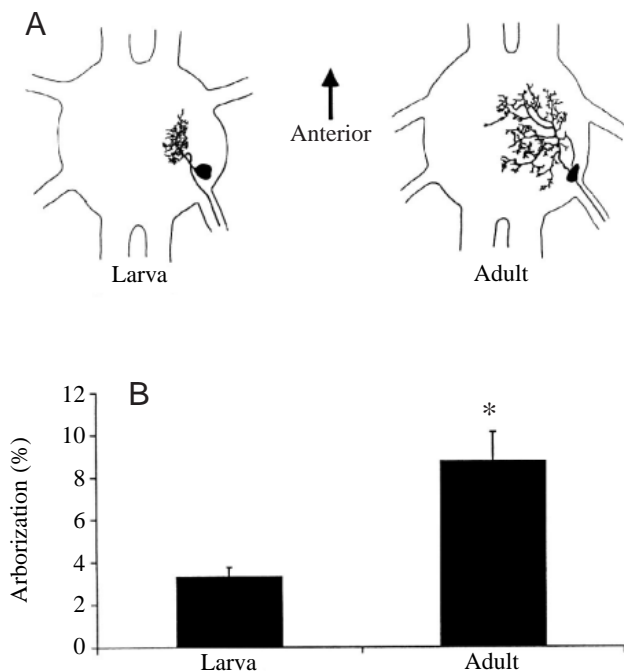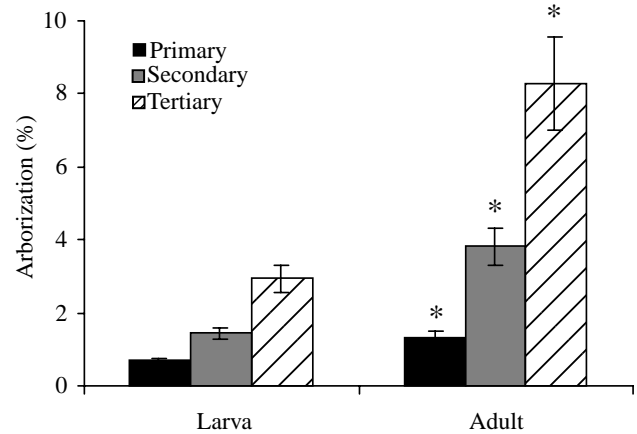
Fig. 2. A comparison of the total extent of arborization in larval and adult lateral neurosecretory cells (LNCs). (A) *Camera lucida* drawing of the central processes of an individual LNC taken from a larva (left) and an adult (right). (B) Extent of arborization of the larval and adult forms of the LNCs expressed as a percentage of the total ganglion volume containing processes. Values are mean + S.E.M. (*N*=5 for each set). *The mean extent of arborization in the adult was significantly greater than that in the larva, using a one-tailed Mann–Whitney *U*-test, *P*<0.005.

which had been exposed to the CP and the PP but not to the adult development peak, were similar to adult LNCs in morphology, with a long primary process and diffuse secondary and tertiary branches that spread into the contralateral side of the ganglion. These processes covered 8.19±1.04 % of the ganglion volume (mean ± S.E.M., N=5) (Fig. 4). The values for the total amount of process growth in ligated pupal LNCs that had not been not exposed to the adult developmental peak but had been exposed to the CP and PP are not significantly different (P=0.28 using a one-tailed Mann–Whitney U-test) from that for untreated adult LNCs that had been exposed to all three 20-HE peaks. It therefore seems likely that it is the CP and PP that determine the change in morphology of these cells, independently of the adult developmental peak of 20-HE.

A similar comparison can be made by examining the occurrence of processes subdivided into individual subtypes. There is a significant increase in the quantity of primary,
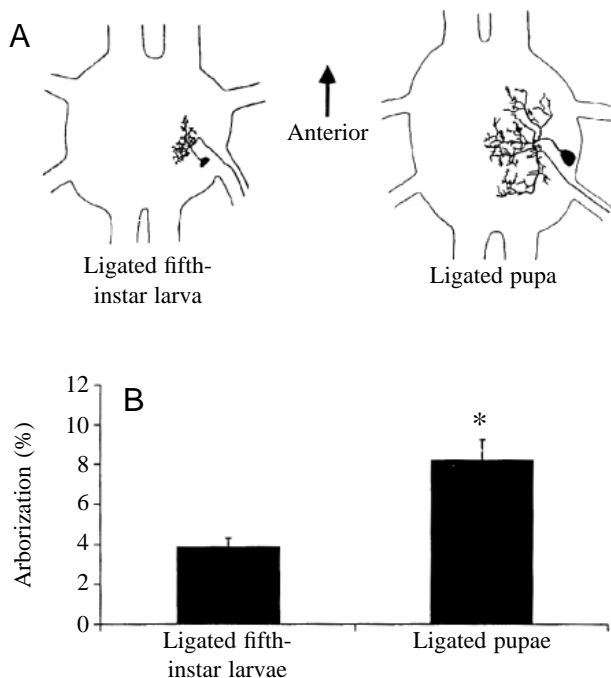

Fig. 4. A comparison of the total extent of arborization of the lateral neurosecretory cells (LNCs) in *Manduca sexta* ligated as larvae and early pupae. (A) *Camera lucida* drawing of LNCs from animals ligated on the second day of the fifth instar (left) or just after pupation (right). Ligation on day 2 of the fifth instar deprives the LNCs of all 20-hydroxyecdysone (20-HE) peaks. Ligation after pupation allows exposure of the LNCs to the endogenous commitment pulse and the prepupal pulse, but prevents exposure to the adult development peak. (B) Extent of arborization of the LNCs in animals ligated on day 2 of the fifth instar or just after pupation expressed as a percentage of total ganglion volume containing processes. Values are mean + S.E.M. (N=5 for each set). *The mean extent of arborization in the ligated pupae was significantly greater than in the ligated larvae, using a one-tailed Mann–Whitney U-test, P<0.005.
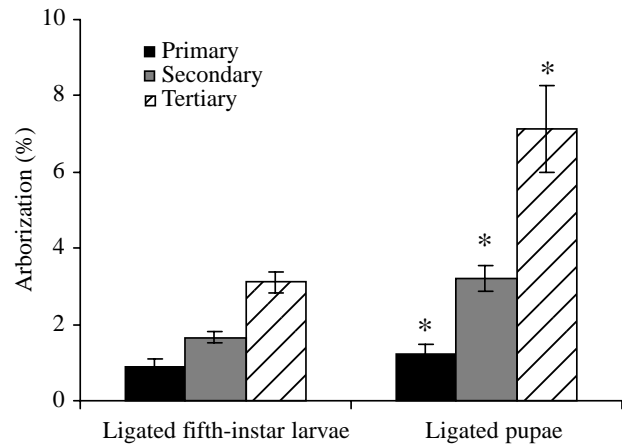

Fig. 5. Extent of primary, secondary and tertiary branches of the lateral neurosecretory cells (LNCs) in animals ligated on day 2 of the fifth instar or ligated just after pupation expressed as a percentage of the total ganglion volume containing each type of process. Values are mean ± S.E.M. (N=5 for each set). *The mean extent of all three process types was significantly greater in the adult than in the larva, using a one-tailed Mann–Whitney U-test, P<0.03 (primary) and P<0.005 (secondary and tertiary processes).

secondary and tertiary processes in the ligated pupal LNCs compared with the ligated larval LNCs (Fig. 5). The extent of primary processes increases from a mean of 0.90±0.19 % of the ganglion volume in the ligated larval nerve cords to 1.24±0.22 % in the ligated pupal nerve cords. Similarly, the extent of secondary processes increased from a mean of 1.66±0.14 % to 3.21±0.35 %, and the extent of tertiary processes increased from 3.10±0.27 % to 7.12±1.14 %. The change in primary process extent is moderately significant (P<0.03), and the change in mean secondary and tertiary process extent are both highly significant (P<0.005). These arborization values are very similar to those obtained from untreated larval and adult LNCs (Fig. 3), further supporting the hypothesis that the CP and PP are primarily responsible for the change in morphology, independently of the peak level of 20-HE that occurs during the adult developmental peak.

In vitro *studies*

The *in vivo* data collected for the LNCs indicate that these cells increase the size and complexity of their dendritic arborization during the transformation from the larval to the adult form and that this increase is mediated by the CP and PP of 20-HE. The controlled environment provided by low-density cell culture enabled the dynamics of this increase in arborization to be studied in detail. We therefore cultured single LNCs from fifth-instar day 2 larvae in various levels of 20-HE for 10 days as described in detail in the Materials and methods section. Our results indicate that the increase in neurite outgrowth is strongly correlated to varying levels of 20-HE (Fig. 6). By carefully scoring the daily growth of individually cultured LNCs, it was possible to assess both the total growth and the growth of individual neurite subclasses.
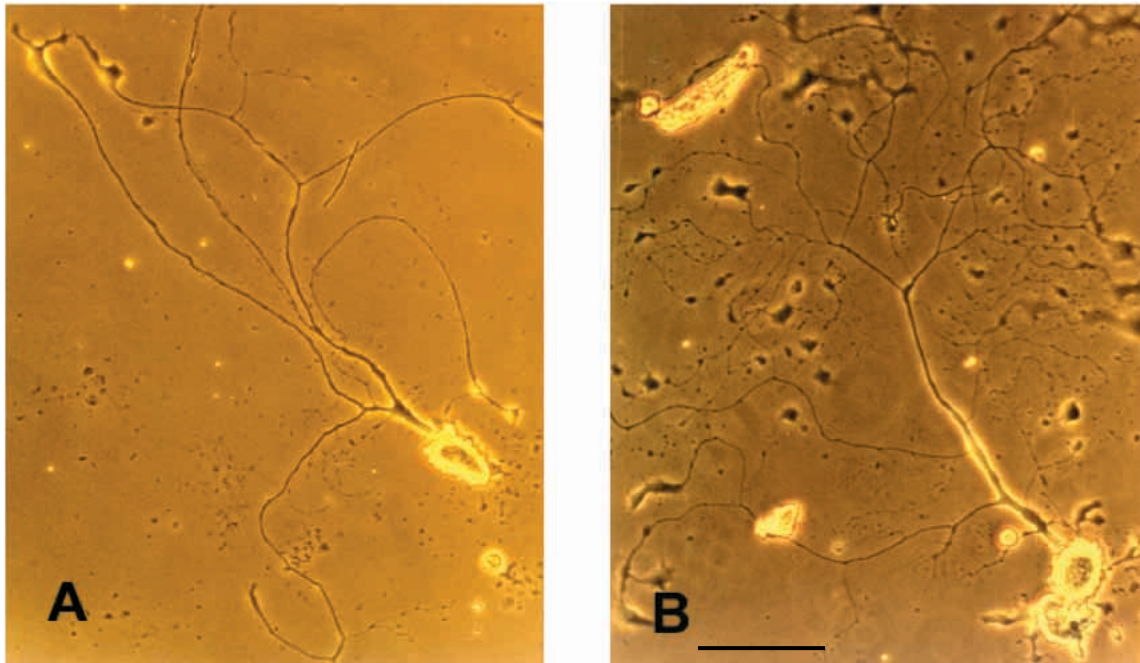
Fig. 6. Photomicrographs of single lateral neurosecretory cells (LNCs) *in vitro*. (A) A single LNC in culture exposed to $0.1\,\mu g\,ml^{-1}$ 20-hydroxyecdysone (20-HE) for the entire culture period. The cell is shown on day 10 of growth. (B) A single LNC grown in a regimen of $0.1\,\mu g\,ml^{-1}$ 20-HE for days 1–5, mimicking the native level of the commitment pulse, and $1.5\,\mu g\,ml^{-1}$ for days 6–10, mimicking the native level of the prepupal pulse. The cell is shown on day 10. Scale bar, $40\,\mu m$.

The sum of all cell process types (primary, secondary and tertiary) was used to determine a general pattern of neurite outgrowth in response to the differing levels of 20-HE (Fig. 7). These data show an increase in the total growth of processes for cells in the presence of 20-HE compared with cells in the absence of 20-HE ($P<0.005$ using a one-way ANOVA). By day 10, there was a clear dose-dependent relationship among the groups of cells exposed to single concentrations of 20-HE for the entire culture period, with the greatest amount of growth in cells exposed to the higher levels of 20-HE and the least growth in LNCs cultured in the absence of 20-HE. Importantly, the greatest level of total growth was observed in

LNCs exposed to the CP dose ($0.1\,\mu g\,ml^{-1}$) of 20-HE for the first 5 days of growth followed by 5 days at the PP level ($1.5\,\mu g\,ml^{-1}$) of 20-HE.

Primary, secondary and tertiary processes were also measured and analyzed separately to determine whether the pattern of growth seen in the sum of all types of process is the same for each individual type. Primary process growth did not seem to be affected by the presence or absence of 20-HE (Fig. 8). Although by day 10 there was a slight enhancement of the growth of primary processes in the presence of the highest 20-HE concentration, this difference was not significant compared with untreated controls. Furthermore,

Fig. 7. The effects of 20-hydroxyecdysone (20-HE) on the total outgrowth of processes from lateral neurosecretory cells (LNCs) *in vitro*. A unit on the growth index scale is equivalent to $50\,\mu m^2$. Each data point represents the mean ± S.E.M. of total growth. $0\,\mu g\,ml^{-1}$ 20-HE, $N=10$ cells; $0.1\,\mu g\,ml^{-1}$ 20-HE, $N=23$ cells; $1.5\,\mu g\,ml^{-1}$ 20-HE, $N=14$ cells; $0.1/1.5\,\mu g\,ml^{-1}$ 20-HE, $N=16$ cells. Day 1 refers to the first day of growth after plating. *The mean values at day 10 for every 20-HE condition were found to be significantly different from untreated controls using a one-way ANOVA, $P<0.005$. ‡Significant differences in the extent of process outgrowth on day 10 were found between the $0.1/1.5\,\mu g\,ml^{-1}$ and $0.1\,\mu g\,ml^{-1}$ 20-HE conditions using Fisher's LSD test, $P<0.002$.
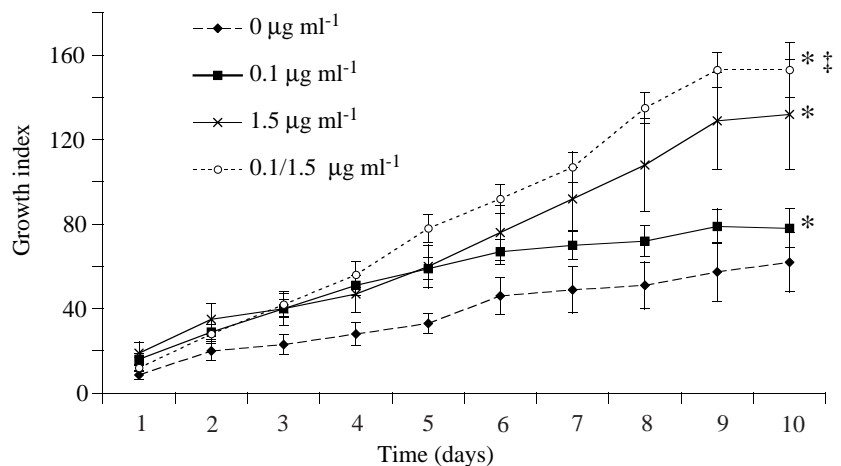
Fig. 8. The effects of 20-hydroxyecdysone (20-HE) on the outgrowth of primary processes from lateral neurosecretory cells (LNCs) *in vitro*. A unit on the growth index scale is equivalent to $50 \, \mu m^2$. Each data point represents the mean ± s.e.m. of total growth. $0 \, \mu g \, ml^{-1}$ 20-HE, $N=10$ cells; $0.1 \, \mu g \, ml^{-1}$ 20-HE, $N=23$ cells; $1.5 \, \mu g \, ml^{-1}$ 20-HE, $N=14$ cells; $0.1/1.5 \, \mu g \, ml^{-1}$ 20-HE, $N=16$ cells. Day 1 refers to the first day of growth after plating.
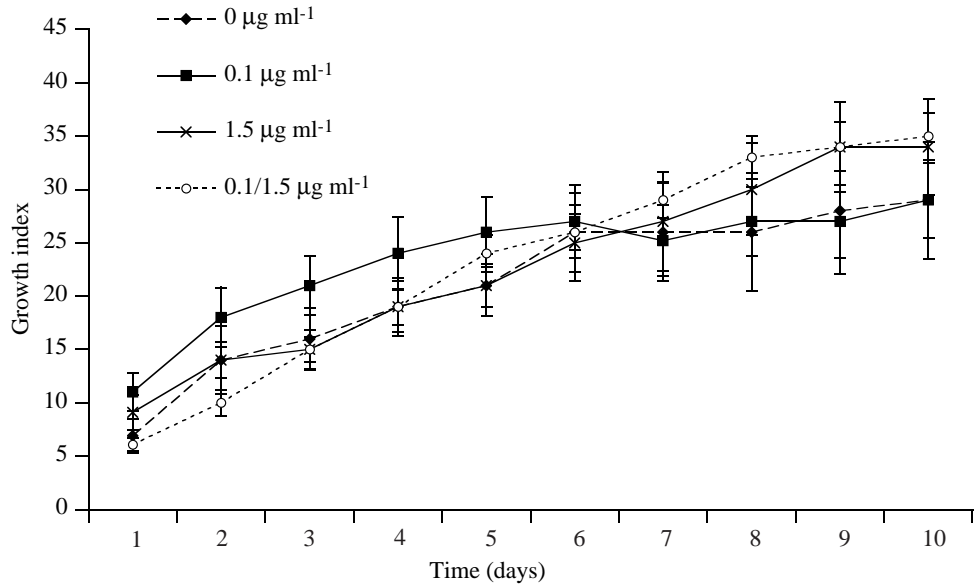
cells not exposed to any 20-HE extended primary processes to the same extent as cells exposed to the lower levels of 20-HE.

In contrast to the pattern of growth of the primary processes, the secondary and tertiary processes did show a marked increase in process growth in cells exposed to 20-HE. Differences between cells exposed to any level of 20-HE and cells cultured in the absence of 20-HE were observed as early as day 1 in culture (Figs 9, 10). For the whole culture period, there was a significant effect of 20-HE on the outgrowth of secondary and tertiary processes ($P<0.002$ for secondary processes and $P<0.003$ for tertiary processes using a one-way ANOVA). By day 10, there was a clear hierarchy of growth among the cells treated with a constant 20-HE concentration, with the least growth in the absence of 20-HE, more growth in

the presence of $0.1 \, \mu g \, ml^{-1}$ 20-HE, and the most growth in the presence of $1.5 \, \mu g \, ml^{-1}$ 20-HE. Cells exposed to $0.1/1.5 \, \mu g \, ml^{-1}$ 20-HE showed a significantly greater amount of growth of secondary ($P<0.005$) and tertiary ($P<0.005$) processes than cells exposed to any of the other culture conditions (Fig. 11), suggesting that sequential exposure to the two hormone peaks is important for proper neurite growth.

To determine the specificity of 20-HE on LNC neurite outgrowth, a separate group of cells, the upper midline cells found just anterior to the D-IV motoneurons, were exposed to a low ($0.1 \, \mu g \, ml^{-1}$) or a high ($1.5 \, \mu g \, ml^{-1}$) level of 20-HE for 10 days. Data were evaluated on days 1, 5 and 10 in culture, and the neurite outgrowth of these cells was analyzed in the same manner as for the LNCs (Fig. 12). These cells did not

Fig. 9. The effects of 20-hydroxyecdysone (20-HE) on the outgrowth of secondary processes from lateral neurosecretory cells (LNCs) *in vitro*. A unit on the growth index scale is equivalent to $50 \, \mu m^2$. Each data point represents the mean ± s.e.m. of total growth. $0 \, \mu g \, ml^{-1}$ 20-HE, $N=10$ cells; $0.1 \, \mu g \, ml^{-1}$ 20-HE, $N=23$ cells; $1.5 \, \mu g \, ml^{-1}$ 20-HE, $N=14$ cells; $0.1/1.5 \, \mu g \, ml^{-1}$ 20-HE, $N=16$ cells. Day 1 refers to the first day of growth after plating. *Significant differences in the extent of process outgrowth on day 10 were found between all the 20-HE conditions and the $0 \, \mu g \, ml^{-1}$ control condition using a one-way ANOVA, $P<0.005$. ‡Significant differences in the extent of process outgrowth on day 10 were found between the $0.1/1.5 \, \mu g \, ml^{-1}$ and $0.1 \, \mu g \, ml^{-1}$ 20-HE conditions using Fisher's LSD test, $P<0.005$.
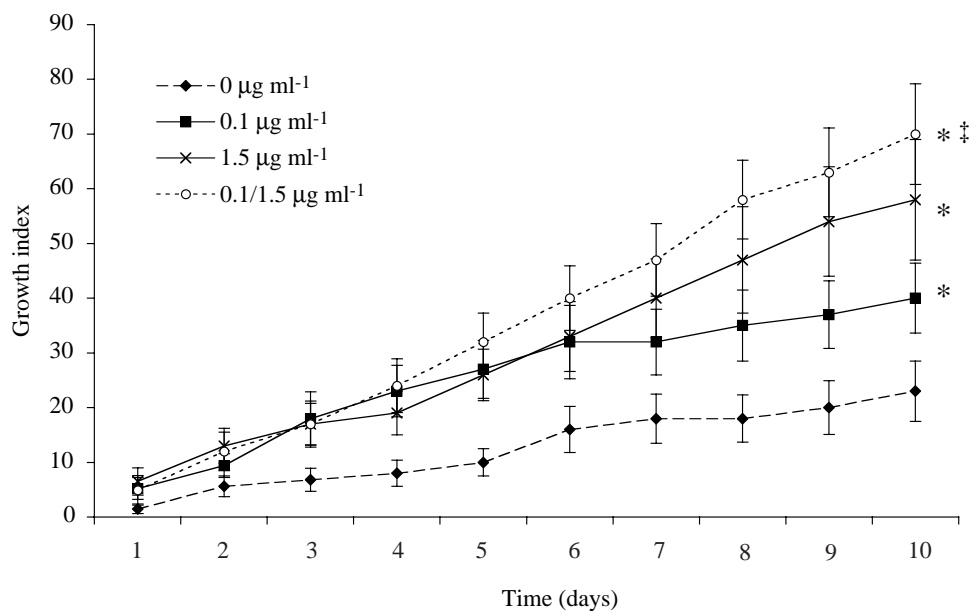
Fig. 10. The effects of 20-hydroxyecdysone (20-HE) on the outgrowth of tertiary processes from lateral neurosecretory cells (LNCs) *in vitro*. A unit on the growth index scale is equivalent to $50\,\mu m^2$. Each data point represents the mean ± S.E.M. of total growth. $0\,\mu g\,ml^{-1}$ 20-HE, $N=10$ cells; $0.1\,\mu g\,ml^{-1}$ 20-HE, $N=23$ cells; $1.5\,\mu g\,ml^{-1}$ 20-HE, $N=14$ cells; $0.1/1.5\,\mu g\,ml^{-1}$ 20-HE, $N=16$ cells. Day 1 refers to the first day of growth after plating. *Significant differences in the extent of process outgrowth on day 10 were found between all of the 20-HE conditions and the $0\,\mu g\,ml^{-1}$ control condition using a one-way ANOVA, $P<0.0003$. ‡Significant differences in the extent of process outgrowth on day 10 were found between the $0.1/1.5\,\mu g\,ml^{-1}$ and $1.5\,\mu g\,ml^{-1}$ 20-HE conditions using Fisher's LSD test, $P<0.045$. §Significant differences in the extent of process outgrowth on day 10 were found between the $1.5\,\mu g\,ml^{-1}$ and $0.1\,\mu g\,ml^{-1}$ 20-HE conditions using Fisher's LSD test, $P<0.045$.
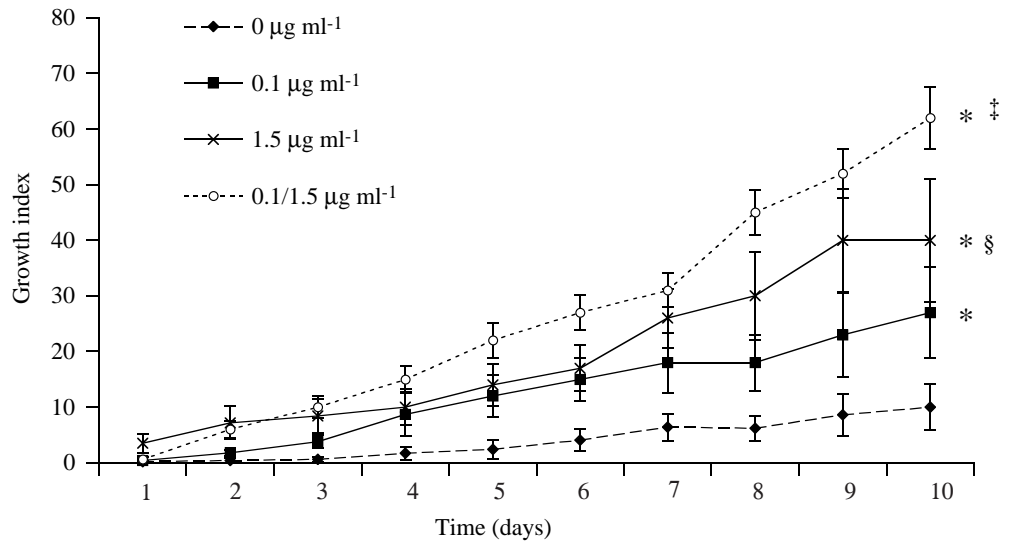
show any hormone-dependent increase in levels of process growth, although they did extend processes and grow in culture.

Discussion

The LNCs undergo a major enhancement of their central arborization during metamorphosis

The data presented here indicate that transmitter phenotype is not the only property altered in the LNCs during metamorphosis (Tublitz and Sylwester, 1990). *In vivo* dye-fills of larval and adult LNCs reveal a large increase in the complexity and extent of arborization, with the greatest increase observed at the level of the tertiary processes (Fig. 3). In larvae, the processes of LNCs are limited to one side of the ganglion, with many connections in a fairly small volume of neuropil. The adult LNCs, however, extend across the midline and throughout a much larger volume of the neuropil, suggesting that they receive input from both sides of the ganglion. During metamorphosis, therefore, the neurons must be directed to start new growth of neurites to reach their new targets. These results demonstrate that the LNCs undergo a major alteration in their central processes during metamorphosis. The extent of the morphological alterations described here for the LNCs is similar in magnitude to that seen in other neurons from *Manduca sexta*, most notably in motoneurons (Levine and Truman, 1985; Weeks and Truman, 1986). The dendritic remodeling in those cases is associated with a functional respecification of these neurons resulting from the metamorphic transition from larva to adult.

Functional significance of LNC outgrowth during metamorphosis

As is the case for *M. sexta* motoneurons, the morphological changes in the LNCs correlate well with the very different functions performed by these cells in the larval and adult stages. Larval LNCs release the CAP_2s into the hemolymph prior to the wandering stage of the last larval instar to facilitate gut purging (Tublitz *et al.* 1992). In contrast, the LNCs in the newly emerged adult are responsible for releasing bursicon into the hemolymph to ensure that the cuticle is properly tanned and that the wings harden after they have been inflated (Taghert
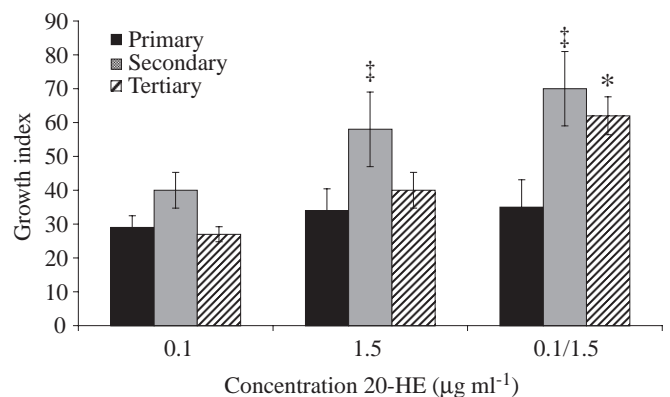
Fig. 11. Mean outgrowth of primary, secondary and tertiary processes on day 10 for $0.1\,\mu g\,ml^{-1}$, $1.5\,\mu g\,ml^{-1}$ and $0.1/1.5\,\mu g\,ml^{-1}$ 20-hydroxyecdysone (20-HE) conditions. A unit on the growth index scale is equivalent to $50\,\mu m^2$. Each data point represents the mean ± S.E.M. of total growth. $0.1\,\mu g\,ml^{-1}$ 20-HE, $N=23$ cells; $1.5\,\mu g\,ml^{-1}$ 20-HE, $N=14$ cells; $0.1/1.5\,\mu g\,ml^{-1}$ 20-HE, $N=16$ cells. *There are significant differences in the extent of tertiary processes between the $0.1/1.5\,\mu g\,ml^{-1}$ 20-HE condition and both the $1.5\,\mu g\,ml^{-1}$ 20-HE ($P<0.044$) and the $0.1\,\mu g\,ml^{-1}$ 20-HE ($P<0.005$) conditions using Fisher's LSD test. ‡There are significant differences in the extent of secondary processes between both the $1.5\,\mu g\,ml^{-1}$ and the $0.1/1.5\,\mu g\,ml^{-1}$ 20-HE conditions and the $0.1\,\mu g\,ml^{-1}$ 20-HE condition using Fisher's LSD test; $P<0.05$ for $1.5\,\mu g\,ml^{-1}$ 20-HE and $P<0.0015$ for $0.1/1.5\,\mu g\,ml^{-1}$ 20-HE.
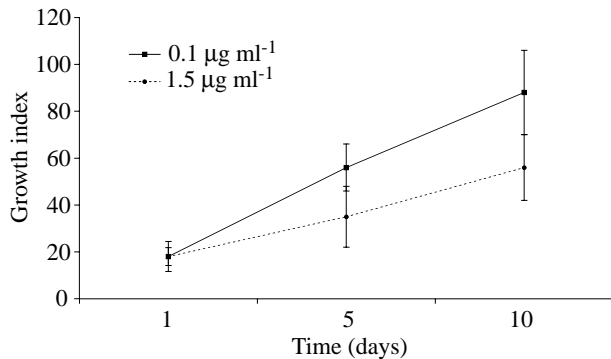
Fig. 12. Mean growth of upper midline cells *in vitro*. Cells were treated with either $0.1\,\mu g\,ml^{-1}$ or $1.5\,\mu g\,ml^{-1}$ 20-hydroxyecdysone (20-HE) for the entire culture period. A unit on the growth index scale is equivalent to $50\,\mu m^2$. Each data point represents the mean ± S.E.M. of total growth. $0.1\,\mu g\,ml^{-1}$ 20-HE, $N=7$ cells; $1.5\,\mu g\,ml^{-1}$ 20-HE, $N=6$ cells. Day 1 refers to the first day of growth after plating.

and Truman, 1982). This functional plasticity is likely to have a morphological underpinning since each function is presumably activated by a different, and possibly non-overlapping, set of synaptic inputs. If this assumption is true, then two alternatives are possible: either the LNCs are wired for both sets of inputs during embryogenesis or the adult inputs are added *de novo* during metamorphosis. If the former is correct, then we should not have seen any difference in the extent of central processes between larval and adult LNCs. However, such changes were observed, lending support to the argument that the synaptic connections used by the LNCs during adulthood are substantially reconfigured during the transition from caterpillar to moth.

LNC outgrowth is triggered by the CP and PP of 20-HE

The second goal of this study was to elucidate the mechanisms underlying the morphological plasticity seen in the LNCs during metamorphosis. Since the LNC transmitter switch is mediated by both the commitment and prepupal pulses (Loi and Tublitz, 1993; Tublitz and Loi, 1993), our working hypothesis was that one or both of these 20-HE peaks, which occur during the last larval instar, were the proximal triggers for the central remodeling described here. Several different experiments were performed to test this hypothesis. Experiments in which the source of 20-HE, the prothoracic glands, was removed by ligation prior to the CP resulted in LNCs whose central arborizations remained larval in character (Fig. 4). In contrast, ligation after pupation failed to halt the morphological transformation of the LNCs, raising the possibility of the involvement of the CP and/or the PP (Figs 4, 5).

To assess directly the role of 20-HE on LNC growth, we studied the growth patterns of LNCs exposed to different 20-HE regimens. The standard endocrinological tool usually employed in these circumstances is *in vivo* hormonal replacement therapy, a technique in which a fixed amount of exogenous 20-HE is introduced *in vivo* into the blood after the prothoracic glands have been removed either by dissection or by ligation (e.g. Tublitz and Loi, 1993; Levine and Truman, 1985). Although traditionally used in *M. sexta* to identify hormonal effects, this technique has several drawbacks, including an inability to control completely hemolymph 20-HE concentration owing to 20-HE metabolism and tissue absorption (Weeks *et al.* 1992). In addition, hormonal replacement does not address the issue of direct *versus* indirect hormone effects because the nature of the technique – infusion into the hemolymph – ensures that all cells are exposed to the hormone.

To circumvent these issues, we used an *in vitro* approach in which 20-HE was applied to individual LNCs in culture. This technique has the obvious advantages of providing increased control over the concentration and time of application of 20-HE as well as addressing the issue of direct *versus* indirect effects. The results from the studies of single LNCs *in vitro* strongly suggest that the proliferation of neural processes in the LNCs during metamorphosis is produced by the direct action of 20-HE, primarily the two 20-HE peaks that occur during the last larval instar. The PP preferentially causes significant outgrowth of secondary and tertiary branches in culture (Figs 9, 10, 11). The CP appears to facilitate this growth, since cultured LNCs exposed to both the CP and PP levels of 20-HE produce significantly more secondary and tertiary processes than do LNCs exposed to the PP alone. This expansion of central processes is not due to the peak of 20-HE levels during adult development because LNCs in animals ligated on pupal day 0 (animals exposed to the CP and PP but not to the adult development peak) still expressed an adult morphology after a 10 day waiting period. In contrast, LNCs in ligated animals that had been deprived of the two larval 20-HE pulses retained their larval form. Thus, the CP and PP together seem to be both necessary and sufficient to induce the change in the LNCs from larval to adult morphology. One prediction from this conclusion is that similar results would be obtained in hormone replacement studies in which animals were exposed to 20-HE levels equivalent to those in the CP and PP.

Differences between in vitro *and* in vivo *outgrowth in the LNCs*

It is interesting to note that the increases in the extent of primary, secondary and tertiary neurites were not identical *in vivo* and *in vitro*. Cultured LNCs showed far greater primary neurite outgrowth and a selective preference for secondary growth over tertiary growth (Fig. 11). One possible explanation for these differences may lie in the culture methods themselves: cultured LNCs start as axotomized somata and must regrow completely new arborizations, including a fully regenerated primary process. In addition, cultured cells are grown on a two-dimensional substratum that lacks the normal *in vivo* constraints on growth. The different conditions associated with *in vivo* and *in vitro* studies must be taken into account when comparing the two sets of data. Future studies

performed on the LNCs will include hormone replacement therapy.

The roles played by the CP and PP in respecifying the LNCs

The results described in this paper, showing that the CP and PP together play important roles in regulating neurite growth in the LNCs, are surprisingly reminiscent of the results of previous studies on LNC transmitter plasticity (Tublitz and Loi, 1993; Loi and Tublitz, 1993). The switch in transmitter phenotype from CAP$_2$s to bursicon by the LNCs also occurs during adult development and requires both the CP and PP for the shift to be completed properly. As in the case of the morphological studies presented here, the adult developmental peak of 20-HE appears not to be involved in the transmitter switch. Thus, the functional makeover of the LNCs from caterpillar to adult is mediated by a parallel set of biochemical and morphological changes, each of which is triggered by the combined influence of the CP and the PP.

The hormone 20-HE is known to affect the cellular properties of other *M. sexta* neurons. Oland and Hayashi (1993) found that 20-HE increases the growth of olfactory sensory neurons from the antennal lobes and increases the survival of these cells. 20-HE also increases the outgrowth of processes in motoneurons dissociated from ganglia of pupal *M. sexta* in culture (Prugh *et al.* 1992). In contrast with our findings, some motoneurons in the nervous system of *M. sexta* have been found to increase their dendritic arborization in response to the adult developmental peak rather than to the CP or the PP (Kent and Levine, 1993; Levine and Weeks, 1990; Levine and Truman, 1985). Thus, different populations of neuronal cells appear to respond to the timing and concentration of the 20-HE pulses in different ways. The mechanism underlying this selectivity is not yet known, but presents an interesting topic for future study.

However, 20-HE does not universally promote growth of neurons, as demonstrated *in vitro* by our midline control cells, and 20-HE has been shown to trigger programmed cell death in some larval motoneurons (Streichert *et al.* 1997). 20-HE also affects transmitter expression (Witten and Truman, 1996) and physiological properties (Hayashi and Levine, 1992) in other *M. sexta* neurons. The demonstration that 20-HE plays an important modulatory role in insect neurons adds to the ever-growing literature on the effects of steroids in other organisms. For example, steroids regulate cell number and morphology in a song control nucleus in birds (DeVoogd and Nottebohm, 1981). Steroids have also been shown to affect the transmitter/neuropeptide fate of developing adrenal chromaffin/sympathetic neurons in mammalian neural crest (Hofmann *et al.* 1989; Kawata *et al.* 1994). Given their wide-ranging effects, there may be other steroid-triggered changes that occur in the LNCs during metamorphosis. For example, several electrophysiological properties may be altered to enable the LNCs to meet the demands of their new adult function and, if they occur, such changes may also be steroid-dependent. This issue is currently being investigated

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