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THE LARVAL ECLOSION HORMONE NEURONES IN *MANDUCA SEXTA*: IDENTIFICATION OF THE BRAIN–PROCTODEAL NEUROSECRETORY SYSTEM

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Summary

Larval and pupal ecdyses of the moth *Manduca sexta* are triggered by eclosion hormone (EH) released from the ventral nervous system. The major store of EH activity in the latter resides in the proctodeal nerves that extend along the larval hindgut. At pupal ecdysis, the proctodeal nerves show a 90 % depletion of stored activity, suggesting that they are the major release site for the circulating EH that causes ecdysis. Surgical experiments involving the transection of the nerve cord or removal of parts of the brain showed that the proctodeal nerve activity originates from the brain. Retrograde and anterograde cobalt fills and immunocytochemistry using antibodies against EH revealed two pairs of neurons that reside in the ventromedial region of the brain and whose axons travel ipsilaterally along the length of the central nervous system (CNS) and project into the proctodeal nerve, where they show varicose release sites. These neurons constitute a novel neuroendocrine pathway in insects which appears to be dedicated solely to the release of EH.

Introduction

Studies on a variety of insects over the past 20 years have shown that ecdysis behavior is triggered by a neurohormone, the eclosion hormone (EH; Truman & Riddiford, 1970; Truman *et al.* 1981; Fugo *et al.* 1984). In the moth *Manduca sexta*, EH is a 62 amino acid peptide (Marti *et al.* 1987; Kataoka *et al.* 1987), which is secreted at the culmination of each moult through the life history of the insect (Truman *et al.* 1981). An intriguing aspect of these secretion events, however, is that the release site varies according to developmental stage. For larval and pupal ecdyses, release comes from the ventral nervous system, whereas for adult eclosion, the corpora cardiaca/corpora allata (CC–CA) complex serves as the

Key words: eclosion hormone, *Manduca*, neurosecretion, ecdysis.

major site of secretion. The various stages also differ in the factors that regulate EH release. In the larval and pupal stages, release appears to depend solely on the appearance and then withdrawal of ecdysteroids. By contrast, secretion at adult ecdysis requires an appropriate input from a circadian clock in addition to the steroid withdrawal (Truman *et al.* 1983).

By using a variety of techniques, Copenhaver & Truman (1986) identified the location of a set of neurons that released EH at adult eclosion. These are five paired cells that are located in both of the lateral neurosecretory cell groups of the brain and that send their axons to the ipsilateral CC–CA complex. The present paper identifies a second, distinct set of EH neurons, whose products are secreted from the ventral nervous system. We report that the release site of this second set is the proctodeal nerve, but that the neurosecretory somata reside in the brain. These neurons define a new neurosecretory pathway in insects, the brain–proctodeal pathway.

Materials and methods

Experimental animals

Larvae of *Manduca sexta* were reared at 26·5 °C on an artificial diet as previously described by Bell & Joachim (1976). During the moult to the fifth stadium, larvae were staged according to Copenhaver & Truman (1982). The timing of events during the larval–pupal transition was as described in Truman *et al.* (1980). The stages most relevant to the present study were the 'anterior shrinkage' (AS) stage, which occurs about 4 h before pupal ecdysis and is characterized by the formation of a circumferential fold in the larval cuticle of segment A1, due to endocuticle breakdown and molting fluid resorption, and the 'posterior shrinkage' stage (PS, about 2·5 h before ecdysis), characterized by the formation of folds in the larval cuticle over the posterior abdomen.

Assays for eclosion hormone activity

Freshly dissected tissues were immediately transferred to ice-cold, acidified methanol (90:9:1 methanol:water:acetic acid). The samples were then homogenized and the particulate matter pelleted by centrifugation. The supernatant was blown to dryness under a stream of nitrogen and the residue dissolved in 10 % acetonitrile (HPLC/SpectroGrade; Alltech. Assoc.).

The amount of EH in the samples was determined using the *Manduca* pupal ecdysis assay (Truman *et al.* 1980). Prepupae were selected at the AS stage (about 4 h before ecdysis) and test samples were injected into the posterior abdomen. The presence of EH activity was indicated by the onset of ecdysis behavior within 140 min of injection. The response latency was inversely related to the dosage of EH injected and was used to estimate the amount of hormone present in each sample. An ecdysis score was calculated as (1/latency)$\times 10^4$. Negative assays, which showed a latency greater than 140 min, were given a standard score of 51.

Average scores were converted into units of EH activity using the dose–response curve in Truman *et al.* (1980).

Surgical techniques

Larvae that were to be hosts for central nervous system (CNS) implants were anesthetized by submersion in water for 20–30 min. Each was then blotted dry, the tip of a proleg removed, and the donor tissue inserted through the wound into the body cavity. A small crystal of phenylthiourea (PTU; Williams, 1959) was then placed in the wound and the distal end of the proleg tied off with a silk thread ligature.

Pupae were anesthetized using CO_2. A square of cuticle and the underlying epidermis were removed from the region of interest. In the case of the pupal brain, it was readily visible after the square of head cuticle had been removed. It was bisected and the right brain lobe removed. For the ventral CNS, gentle probing in the underlying fat body was required for snagging the nerve cord. A ganglion was then removed with as much anterior and posterior connectives as possible. The wounds were treated with a few crystals of PTU, covered with the excised piece of cuticle, and sealed with melted wax.

Immunocytochemistry

Immunostaining utilized a serum antibody raised against *Manduca* EH (Copenhaver & Truman, 1986). Tissues were fixed in either Bouin's fixative or 4 % paraformaldehyde in phosphate-buffered saline (PBS; $15\,mmol\,l^{-1}$ phosphate; pH 7·4; $0{\cdot}15\,mol\,l^{-1}$ NaCl) for 2–4 h. The fixed tissue was then repeatedly rinsed in buffer and incubated at room temperature in a 0·05 % solution of collagenase (Sigma, St Louis, MO) for 1–2 h. The enzyme treatment improved the penetration of the antibody into whole-mount preparations. The samples were then run up and down through an alcohol series to further permeabilize the tissues, exposed to 3 % hydrogen peroxide to reduce endogenous peroxidase levels, and preincubated in 10 % normal goat serum (Sigma). Samples were typically exposed to 1:100 dilutions of the primary antiserum in PBS with 0·3 % Triton X-100 (Sigma, PBS–TX) for 36–60 h at 4 °C with constant agitation. The tissues were then subjected to 6–8 rinses with PBS–TX and then incubated with a biotinylated goat anti-rabbit IgG (Vector Labs, Burlingame, CA) at a dilution of 1:200 in PBS–TX for 36 h at 4 °C. After a series of subsequent rinses with PBS–TX, the tissues were incubated at room temperature with a preformed avidin–peroxidase conjugate as provided by a Vectastain ABC kit (Vector Labs). The location of peroxidase activity was visualized using diaminobenzidine (Sigma) as a chromagen ($5\,mg\,ml^{-1}$ in PBS) and hydrogen peroxide generated by the glucose oxidase method (Watson & Burrows, 1981). After the reaction, the tissues were dehydrated, cleared in xylene, and mounted in Canada balsam.

Cobalt backfilling

Nerves and connectives were filled *in situ* by retrograde or anterograde cobalt

diffusion. Typically, animals were opened dorsally, rinsed with saline, most of the viscera removed, and a small cup of Vaseline molded around the stump of a nerve or connective. The cup was filled with a cobalt–lysine complex (Lazar, 1978) and covered over with more Vaseline to prevent evaporation. The animals were then placed in a humidified chamber at 4 °C for up to 4 days. The filled tissues were then dissected, fixed with 2 % paraformaldehyde, and immersed in saline saturated with hydrogen sulphide gas to precipitate the cobalt. The cobalt was then intensified using the whole-mount technique of Bacon & Altman (1977).

Results

Site of EH release from the ventral CNS at pupal ecdysis

Both larval and pupal ecdyses in *Manduca* are triggered by the secretion of EH from the ventral nervous system, but the precise hormonal release site was unknown. To address this issue, ventral nervous systems were removed from *Manduca* 4–12 h before pupal ecdysis. As indicated in Table 1, each was divided into the CNS (ganglia plus connectives) and the various sets of peripheral nerves, and assayed for EH activity. For the CNS, the chain of unfused segmental ganglia (T1–A6) contained moderate levels of EH activity, whereas levels in the terminal ganglion were below detectability when assayed at our standard dosage of 0·5 animal equivalents (i.e. one half-ganglion) per assay. Of the peripheral nerves, extracts from the lateral segmental nerves (dorsal, ventral and transverse) from ganglia T1–A6 gave consistently negative scores (levels <0·02 units). The combined collection of the most posterior nerves, dorsal A8, ventral A8 and the terminal nerves (minus their proctodeal branch), showed a trace of activity, whereas the proctodeal branch contained more activity than the remainder of the

Table 1. *Distribution of eclosion hormone activity in the ventral nervous system of prepupal* Manduca sexta

Tissue*	*N*	Assay score† ($\bar{x} \pm$ S.E.)	EH activity† (units/insect)
Ganglia T1–A6 and connectives	8	90 ± 10	0·08
Terminal ganglion (A7,A8)	9	51 ± 0	<0·02
Terminal nerve (minus proctodeal nerve)	9	55 ± 5	0·03
Proctodeal nerve	9	133 ± 7	0·22
Dorsal nerve‡	9	51 ± 0	<0·02
Ventral nerve‡	10	51 ± 0	<0·02
Transverse nerve‡	10	51 ± 0	<0·02

*Tissue was pooled from five prepupae 4–10 h before ecdysis.

† Average scores were obtained after assaying the various tissues at the level of 0·5 insect equivalents. The assay score was converted into units of EH activity using the dose–response curve in Truman *et al.* (1980) and the value multiplied by 2 to yield the estimate per insect.

‡ Pooled nerves from T1–A6.

ventral nervous system combined. A similar set of extractions carried out on pharate fifth-instar larvae likewise showed a major concentration of EH in the proctodeal nerves (data not shown).

The significance of the high levels of EH activity in the proctodeal nerves was revealed by examining changes in its EH content at the time of pupal ecdysis (Fig. 1). The proctodeal nerve extends anteriorly from the rectum to the midgut–hindgut border (Reinecke *et al.* 1973). Along its length it sends off numerous branches to innervate the hindgut musculature. In removing the proctodeal nerve, we included as long a segment as possible. Tissues were removed at the PS stage (about 2·5 h before ecdysis) and from animals in the act of ecdysis. As seen in Fig. 1A, the EH activity in the proctodeal nerve dropped by almost 90 % during the 2·5 h between the PS stage and pupal ecdysis. By contrast, the EH titers in the remainder of the ventral nervous system (T1–A8 plus associated lateral nerves) remained relatively stable over this period (Fig. 1B). These data confirm that the proctodeal nerve is the major site of EH storage in the prepupa. The dramatic depletion seen in the levels of stored EH activity in the proctodeal nerves suggests that they constitute the major release site for the circulating EH that triggers pupal ecdysis.

The brain is the source of the EH in the proctodeal nerves

The source of the EH found in the proctodeal nerves was determined by surgically removing parts of the nerve cord of day 1 pupae and then measuring the levels of EH activity rostral and caudal to the lesion after the completion of adult

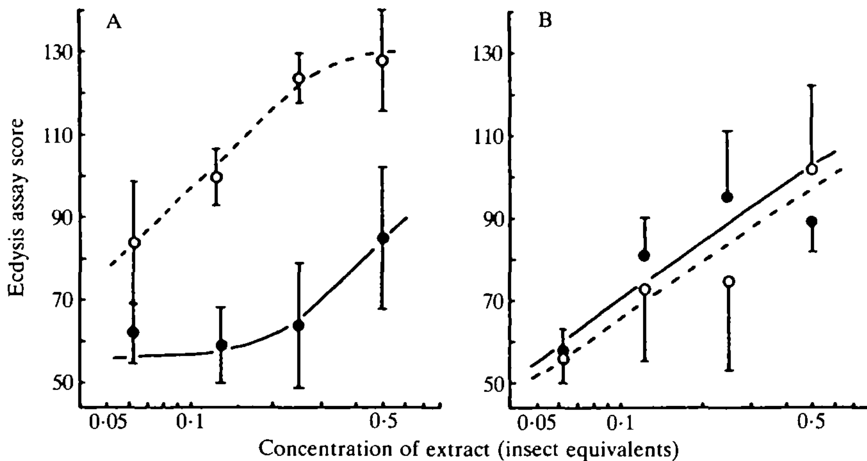

Fig. 1. Changes in the eclosion hormone content in various regions of the pupal ventral nervous system during the 2·5 h between the PS stage (open circles) and ecdysis (filled circles). (A) The proctodeal branch from the terminal nerve; (B) the remainder of the ventral CNS from T1 to A8 including all peripheral nerves except the terminal nerve and dorsal and ventral nerves for A8. The symbols give the mean (±S.E.) assay score for various dilutions of extracts prepared from the respective tissues. Five assays were performed at each dilution.

development approximately 3 weeks later. The developing adult was selected for these experiments because its ventral CNS shows at least a fivefold accumulation of EH activity between pupal ecdysis and adult eclosion (Truman *et al.* 1981). Also, once adult development is initiated, any or all of the CNS can be removed without disrupting the remainder of adult differentiation.

As seen in Fig. 2A, the EH activity in the abdominal CNS of the pharate adult was distributed relatively equally among the three unfused segmental ganglia and the terminal ganglion with its associated nerves. In the former, the majority of the activity was found in the connectives (data not shown). Substantial levels of EH activity were also present in the hindgut. This hindgut activity disappeared when the terminal ganglia (A7, A8) were removed early in development (Fig. 2B), indicating that the hindgut EH arises from central neurons that normally project to the hindgut *via* the proctodeal nerve. By contrast, ganglion A6, situated just rostral to the lesion, showed over 0·2 units of activity, which was especially impressive since a single ganglion (without anterior or posterior connectives) normally does not have sufficient EH activity to evoke a positive assay response.

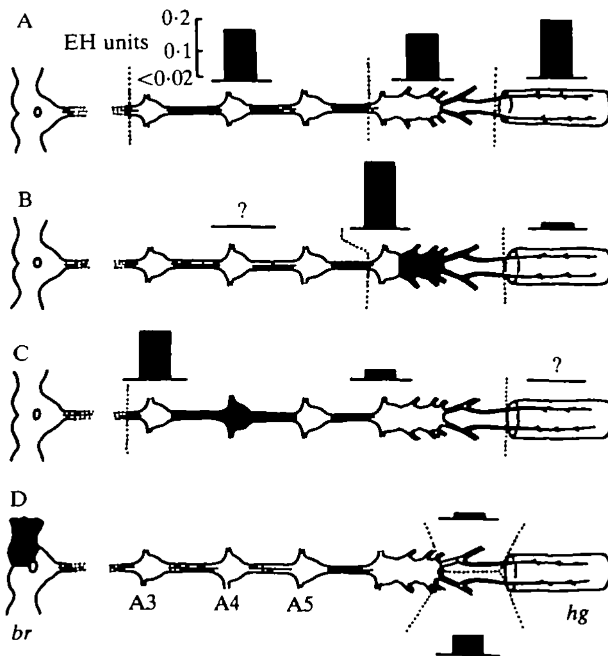

Fig. 2. Distribution of eclosion hormone (EH) activity in pharate adult *Manduca sexta* that had been surgically manipulated at the onset of adult development. Blackened areas on the drawings indicate the areas of the CNS that were removed. Histograms show the mean level of EH activity contained in the regions enclosed by the dotted lines. EH values were based on the average score obtained from 8–10 assays at the level of 0·5 animal equivalents of tissue per assay. (A) Intact controls; (B) removal of ganglia A7,A8; (C) removal of ganglion A4; (D) removal of right brain lobe. *br*, brain; A*n*, *n*th abdominal ganglion; *hg*, hindgut; ?, not assayed.

Removal of ganglion A4 early in adult development similarly resulted in a redistribution of EH activity (Fig. 2C); again, substantial levels of activity were found in the ganglion immediately rostral to the lesion with only trace amounts in more caudal ganglia. This pattern of redistribution of EH activity after ganglion removal suggested that EH is transported to the proctodeal nerves from a synthetic site in the anterior end of the insect.

Evidence that this anterior source is the brain was provided by experiments in which the right brain lobe was removed from 2-day-old pupae. Later, at the completion of adult development, the right and left terminal nerves were removed and assayed for EH activity. As seen in Fig. 2D, the left terminal nerve contained almost 0·07 units of EH whereas the right nerve was essentially devoid of activity (only 1 weak positive assay out of 11). These data suggest that neurons originating in the brain project ipsilaterally down the length of the ventral CNS to the proctodeal nerve, where they store EH for release at ecdysis.

Although the above experiments were performed on developing adults, the larval brain also possesses these posterior-projecting EH cells. During the larval instars, EH accumulation occurs during the feeding period of each instar (Truman *et al.* 1981). Nerve cord transection at this time severely interferes with behavior and growth, so we adopted a different strategy to examine EH transport from the larval brain. Newly ecdysed fifth-stage larvae received implants of either an attached brain–subesophageal ganglion (SEG) or the subesophageal–T1 ganglia from larvae of the same age. Five days later, after the hosts had begun wandering, the implants were recovered and the SEG portion of each was extracted and assayed for EH activity. As seen in Table 2, the SEG portion of the implant contained substantial levels of EH only in the case when it was implanted with intact connections to a brain.

Identification of the brain cells by cobalt backfilling

Candidates for the posterior-projecting EH cells were identified by anteriorly directed cobalt fills of the ventral connectives from below ganglion A3. Four large brain neurons routinely filled from this location: one with its cell body contralateral and three with their cell bodies ipsilateral to the connective that was filled. Of the latter, two were situated frontally in the ventromedial region (Fig. 3A) and

Table 2. *Levels of eclosion hormone activity recovered from the subesophageal portion of CNS implants implanted into day 0 fifth-stage larvae and recovered after the host began wandering*

Implant	Number of assays	Number positive	Score ($\bar{x} \pm$ S.E.)	EH activity (units)
Brain + subesophageal ganglion*	8	8	106 ± 7	0·055
Subesophageal ganglion + T1*	8	1	56 ± 6	0·016

* Only the subesophageal portion of the implant was assayed; assayed at the level of 0·9 ganglion equivalents.

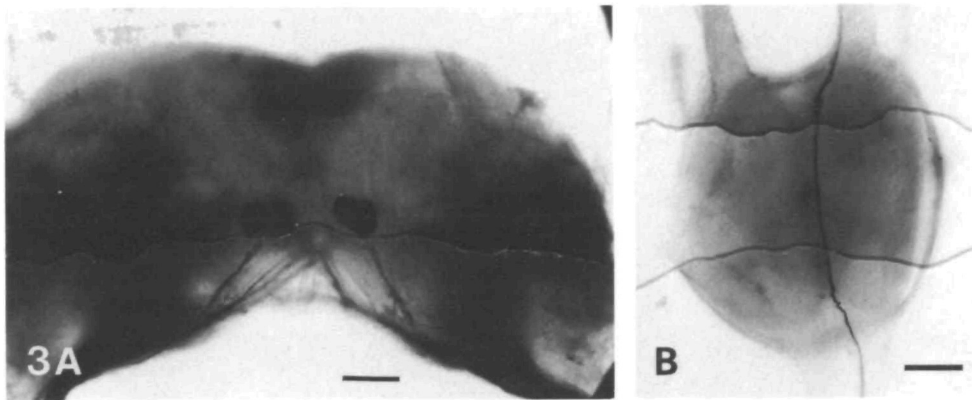

Fig. 3. Photomicrographs of cobalt fills showing various aspects of ventromedial cell morphology. (A) Brain of a day 2 fourth-instar larva whose connectives had been filled anteriorly from below ganglion A3. The two pairs of large, ventromedial cells are evident on both sides. (B) Dorsal view of ganglion T2 from a day 3 fourth-stage larva showing anterior-projecting axons stained by unilateral filling of the terminal nerve. Scale bar, 50 μm.

one was medial on the back side of the brain. When the CNS was filled from the terminal nerve, we found that most of the efferent axons in that nerve arose from neurons whose cell bodies were in the terminal ganglion (A7,A8) and a few from neurons situated in ganglia A5 and A6. In addition, a pair of fasciculated axons were routinely seen projecting anteriorly through the ganglia beyond A5. In one favorable preparation, these axons were traced through the length of the ventral CNS to the pair of ipsilateral ventromedial cells in the brain. As seen in Fig. 3B they take a medial trajectory through the segmental ganglia and show little, if any, branching during their journey from the brain. The cobalt fills revealed a relatively sparse ventral arbor for these cells within the brain (Fig. 4).

The posterior projections of these cells were examined by caudally directed fills of connectives from anterior to A4. Most of the axons filling from this site projected to the terminal ganglion but one pair on each side exited *via* the terminal nerve and turned into the proctodeal branch. They traveled along the length of the proctodeal nerve but did not extend down the short side branches to the hindgut musculature. The axons were confined to the outer area of the sheath, rather than running in the central core of the nerve, and showed numerous swellings along their length. Thus, these two pairs of ventromedial brain cells have a unique anatomy that coincides with the distribution and transport characteristics described above for EH.

Immunocytochemistry

Immunohistochemistry using an anti-EH antiserum confirmed the anatomy of the ventromedial cells and showed that they contain EH-like immunoreactivity. A polyclonal antiserum raised against partially purified EH stains a subset of the

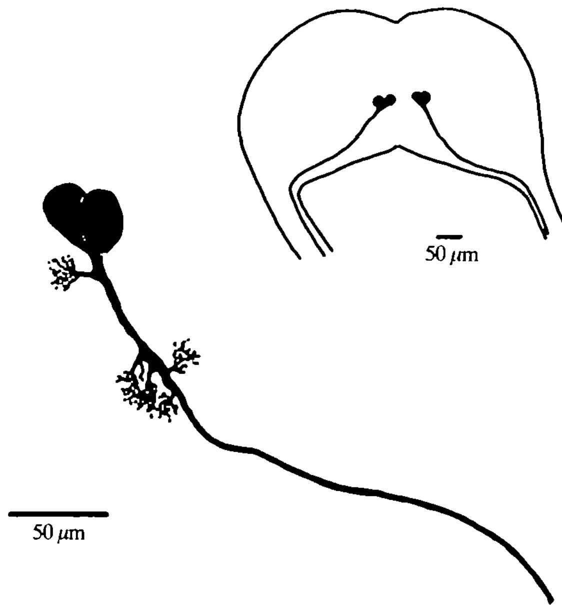

Fig. 4. *Camera lucida* drawings of a cobalt fill of the ventromedial brain cells. The axons of the two cells are fasciculated and cannot be distinguished in this drawing. The inset shows the position of the cells in the larval brain.

lateral neurosecretory cells in the adult protocerebrum and this staining is blocked by preabsorption of the antibody with EH (Copenhaver & Truman, 1986). This antiserum immunoprecipitates EH activity from extracts of pharate adult CC–CA complexes (Copenhaver & Truman, 1986) and also from extracts prepared from prepupal proctodeal nerves (J. W. Truman, unpublished observations). We used this antiserum in the present study to track the distribution of EH activity in the larval nervous system. As seen in Fig. 5A, the ventromedial cells in the larval brain stained strongly with this antiserum. The staining was specific and not observed when preimmune serum was substituted for the anti-EH antiserum. Also, preincubation of the antiserum with extracts from prepupal proctodeal nerves or from CC–CA complexes from pharate adults (both containing major stores of EH) completely blocked staining. The immunostained axons of the ventromedial cells could be followed through the length of the nerve cord and out along the proctodeal nerve. The trajectory of the stained axons through the segmental ganglia (Fig. 5B) was the same as that described for the cobalt-filled fibers described above. The immunostained axons extended the length of the proctodeal nerve and formed numerous varicosities in the sheath (Fig. 5C). Typically, the axons did not send collaterals down the side branches of the proctodeal nerve, but they did bifurcate when the proctodeal nerve split to supply the dorsal and ventral regions of the midgut–hindgut junction. In their pattern of immunostaining, these cells exactly matched the anatomy of the cobalt-filled cells described above.

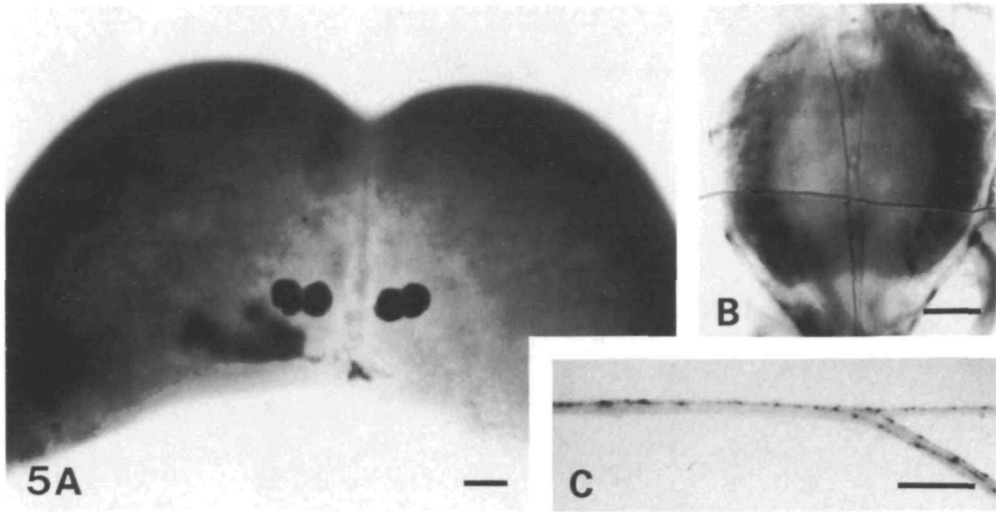

Fig. 5. Whole-mount preparations of tissues from larvae showing the staining of the ventromedial cells using an anti-EH antiserum. (A) Cell body staining in the brain of a day 0 fifth-instar larva; (B) stained axons in an abdominal ganglion from a pharate fifth-instar larva; (C) stained axons in the sheath of the proctodeal nerve of a pharate fifth-instar larva showing varicosities that are the presumed sites of EH release. The axons bifurcate when the nerve splits to form dorsal and ventral branches that supply the hindgut–midgut border. Scale bar, 50 μm.

The immunostaining of ganglia derived from a few animals with CNS lesions was also examined. In the case of insects in which A4 had been removed early in development, a dense ball of processes was formed just caudal to A3, and included tangled, varicose axons that were immunopositive. No staining was observed caudal to the lesion site. In the animals in which one brain lobe had been removed, the lesioned side of the resulting moth lacked immunopositive axons, whereas the intact side had its normal complement of two axons (Fig. 6). These data strongly support the conclusion that the ventromedial cells are the neurons responsible for EH release at larval and pupal ecdysis.

Discussion

Eclosion hormone is used to trigger each ecdysis during the life history of *Manduca* (Truman *et al.* 1981), but the source of that circulating hormone varies depending on the life stage. At adult ecdysis, the hormone is released from the CC–CA complex (Truman, 1973; Copenhaver & Truman, 1986). In the earlier ecdyses, however, EH depletion is seen from stores in the ventral nervous system (Taghert *et al.* 1980; Truman *et al.* 1981) but the exact site of that release was previously unknown. The present study shows that the proctodeal nerves contain a high concentration of EH activity (Table 1) and exhibit a major depletion in EH

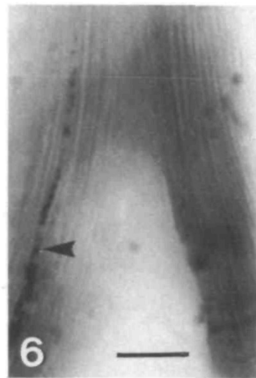

Fig. 6. Whole mount of the basal region of the terminal nerves from a pharate adult' that had had one brain lobe removed at the outset of adult development. Axons that stain with the anti-EH antiserum are present only on the unlesioned side (arrowhead). Scale bar, 50 μm.

levels at the time of pupal ecdysis (Fig. 1). Thus, the proctodeal nerves appear to be the major neurohemal site for EH release at pupal ecdysis.

The surgical lesion experiments in developing adults (Fig. 2) as well as the implants of larval ganglia (Table 2) argue that the source of the proctodeal nerve EH activity is the brain. We have previously shown that the adult brain possesses five pairs of EH-containing neurons, but that these cells send axons only to the CC–CA (Copenhaver & Truman, 1986) and, hence, could not contribute to the EH activity found in the ventral CNS. The ventromedial cells described here represent a second set of brain EH cells, which project the length of the ventral CNS and terminate in the proctodeal nerves (Figs 3, 5). Their anatomy matches exactly the distribution and transport characteristics of EH in the ventral nervous system. Neither the immunostaining nor the cobalt fills revealed any peripheral projections of these cells other than the one to the proctodeal nerves. Thus, the latter appears to be their sole peripheral release site in the larval and pupal stages. Their axons show little or no branching as they course through the ventral CNS but we cannot exclude the possibility that some local release of EH may occur in the CNS coincident with the release in the periphery. Confirmation that the ventromedial cells produce EH has come from recent experiments in which *in situ* hybridization experiments have shown that these cells contain the messenger RNA for the EH precursor (Horodyski *et al.* 1989).

The ventromedial EH cells represent a novel neurosecretory system in Lepidoptera, the brain–proctodeal nerve system (Fig. 7). Brain cells have been described in aphids which project down the connectives and exit the CNS to innervate abdominal organs such as the ovary (Johnson, 1962; Steel & Lees, 1977) but this is the first time that similar cells have been described in holometabolous insects. Also, this is the first demonstration that such a system is responsible for

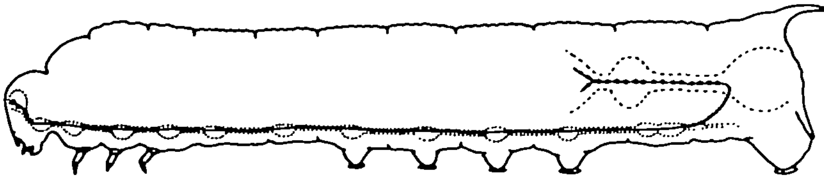

Fig. 7. Drawing of a *Manduca sexta* larva showing the location and projections of the ventromedial EH neurons that make up the brain–proctodeal neurosecretory axis.

the release of circulating hormones. In *Manduca* this system seems to be entirely devoted to EH release, since it apparently consists of only the two pairs of ventromedial cells. Other neurons that project to the proctodeal nerves are confined to the more posterior regions of the ventral nervous system (ganglia A5–A8).

Interestingly, although the ventral medial cells stain with the EH antiserum, they do not stain with the classic neurosecretory cell (NSC) stain, paraldehyde fuchsin (S. R. Reiss & J. W. Truman, unpublished observation), as do the other EH cells that are among the lateral NSC group. However, cell bodies in their location were described by Nijhout (1975) in his analysis of NSCs in the larval *Manduca* brain as being the only ones in the brain to stain with Victoria Blue. The significance of this difference between the two sets of EH cells in their patterns of staining with histochemical dyes is not known.

In our previous studies of the distribution and fluctuations of EH in the ventral nervous system of *Manduca*, we concluded that the synthesis as well as release was due to cells in the ventral CNS but not the brain (Taghert *et al.* 1980; Truman *et al.* 1981). This conclusion was obviously erroneous as indicated by the novel neurons identified in this report. After our lesion experiments (e.g. Fig. 2C), low but detectable levels of hormone activity were often found posterior to the lesion site. This residual activity may reflect low levels of peptide production in neurons we have not yet identified or persistence of some level of activity in the severed axons. The ability of axons to survive for long periods in the absence of their cell bodies is commonly observed within the arthropods (Hoy *et al.* 1967; Clark, 1976; Meyer & Bittner, 1978).

Our interpretations of changes in the distribution of the EH activity in the CNS of *Manduca* through its life history (Truman *et al.* 1981) need to be updated in view of the discovery of the ventromedial cells. The observation that EH is released from the CC–CA at adult eclosion and from a ventral nervous system site during larval and pupal ecdyses remains valid. The identification of the proctodeal nerves as the ventral release site has prompted us to re-examine their role in adult ecdysis. Preliminary results indicate that these nerves also shows substantial EH depletion at adult eclosion (J. W. Truman, unpublished observation). Thus, metamorphosis does not result in the shutdown of the ventral release site as originally thought, but rather in the recruitment of a second release site, the CC–CA complex.

The titers of EH in various regions of the CNS of *Manduca* during its life history showed an anomalous depletion of activity from the head during the embryonic ecdysis and the ecdysis to the second instar (Truman *et al.* 1983). Considering the small size of the animals at those stages, the depletions seen in the head during the few hours between the two samples may be due to the movement of EH out of the brain by fast axonal transport rather than to release from a cephalic site. Indeed, it is not clear how much EH activity in the larval brain, if any, is attributable to the lateral EH cells rather than the ventromedial cells. The identification of the latter cells is an essential step towards the understanding of how EH release is regulated through the life history of the insect.

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