J. exp. Biol. (1982), **98**, 385–401
With 11 *figures*
Printed in Great Britain

IDENTIFICATION OF THE BURSICON-CONTAINING NEURONES IN ABDOMINAL GANGLIA OF THE TOBACCO HORNWORM, *MANDUCA SEXTA*

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(*Received* 28 *September* 1981)

SUMMARY

The abdominal ganglion neurones responsible for the secretion of the peptide hormone bursicon from the transverse nerves of the tobacco hornworm *Manduca sexta* have been localized. Each unfused abdominal ganglion produced bursicon during adult development. The hormone was then transported to the next posterior transverse nerve for subsequent release following adult emergence. Of the two efferent pathways that connect ganglia to posterior transverse nerves, only one had ultrastructural features consistent with a neurosecretory function. All axons in this pathway contained numerous, elementary, dense-cored granules with diameters ranging from 100 to 300 nm.

In pharate adults, cobalt back-filling of this neurosecretory pathway revealed two groups of cell bodies in abdominal ganglia: three dorso-laterally and eight at the mid-line. Hormonal activity was present in lateral regions of the ganglion but not in mid-line regions. In the dorso-lateral region, four blueish cell bodies could be seen in the living tissue. The two combined clusters of four cells from both sides of the ganglion contained more than 60% of the bursicon activity present in the entire ganglion. More careful dissection of the cluster detected the presence of activity in cell pairs and individual members.

Individual dye-fills of these blue cells demonstrated that three of these were the three lateral neurones that were back-filled from the transverse nerve. The fourth blue cell may be identical to a neurone whose axon travels in the descending, contralateral connective and exits the nervous system via the next posterior ganglion. On the basis of dendritic geometry, each of the neurones in the bursicon cell cluster could be identified as a unique, individual neurone.

INTRODUCTION

The insect neuroendocrine system has been studied with respect to the hormones that it produces and to the cytological and ultrastructural features of individual neurosecretory cells (Maddrell, 1974). However, studies of its physiology have been

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hindered because, in general, a particular hormone product has not been unequivocally ascribed to a particular identified neurone. Knowledge of the specific cells that secrete these factors could provide the basis for more detailed study of the physiological controls over neurosecretory processes. To date, there are two examples of specific neuropeptides being localized to individual neurones in the insect central nervous system. Agui *et al.* (1979) have described a single neurone on either side of the brain of the tobacco hornworm, *Manduca sexta*, that contains the prothoracicotropic hormone; this brain hormone stimulates the release of the steroid moulting hormone, ecdysone. More recently, O'Shea & Adams (1981) have identified a single neurone in the unfused abdominal ganglia of cockroaches that contains a myotropic substance that is chemically indistinguishable from the pentapeptide proctolin (Brown, 1975).

In many respects, the neurosecretory cells of the insect ventral nerve cord present a more attractive system for cellular analysis than do those of the brain because the nerve cord is a 'simpler system'. The number of cells in a single ganglion is generally two or more orders of magnitude less than in the brain (Bullock & Horridge, 1965). Release of neurosecretory products from the ventral nerve cord is thought to occur primarily from the neurohaemal perivisceral organs (PVO) located on the segmentally repeated transverse nerves (Raabe *et al.* 1974). However, until now the identities of cells that secrete particular PVO hormones were unknown.

Bursicon is a protein hormone that is stored in and released from the transverse nerves of the abdomen as a late part of the eclosion motor programmes that are initiated by another peptide – the eclosion hormone (Truman, 1973, 1980). Bursicon release is effected in a highly predictable and pulsatile fashion (Reynolds, Taghert & Truman, 1979). In *Manduca sexta* the hormone functions to trigger the sclerotization of those portions of newly synthesized cuticle that undergo post-ecdysis inflation. Bursicon is present in the nervous system of *Manduca* as one or more peptides with homogeneous size and charge (Taghert & Truman, 1982). The present report describes experiments to localize and identify the peptidergic neurones responsible for the release of bursicon from the transverse nerves. A preliminary report of some aspects of this work has been published elsewhere (Taghert & Truman, 1979).

MATERIALS AND METHODS

Animals

The rearing and selection of animals are described by Taghert & Truman (1982). Male *Manduca sexta* were used throughout these experiments.

Biological assay

For the measurement of bursicon activity, tissues were dissected out of abdomens and homogenized in ice-cold saline (Ephrussi & Beadle, 1936). Homogenates were centrifuged at 1000 *g* for 5 min without prior heating, then frozen at −20 °C until used. Isolated wing assays were performed as previously described (Taghert & Truman, 1982).

In situ staining

In situ staining of the entire abdominal nervous system was done on isolated abdomens that had been opened mid-dorsally and pinned out in a wax-bottomed chamber. Preparations were flooded with simple saline containing either Methylene Blue (a 1:100 dilution of a saturated solution) or Neutral Red (MC/B Corp.; 0·01 mg/ml, filtered just before use (Evans & O'Shea, 1978)) and incubated overnight.

Surgery

Single abdominal ganglia were removed from male pupae 12–24 h after pupal ecdysis. Following anaesthesia under CO_2, a rectangular piece of cuticle was removed from the ventral surface of the fifth abdominal segment and the target ganglion excised by cutting all tracheal and neural connexions and removing it. A few crystals of phenylthiourea were placed in the exposed region to inhibit tyrosinase activity and the patch of cuticle replaced and sealed with molten paraffin.

Cobalt chloride back-filling

Portions of the abdominal nervous system were dissected out of the animal in a modified Lepidopteran saline (Weevers, 1966). The cut stumps of the transverse nerve were immersed in distilled water for 30 sec then placed in pools of cobaltous chloride (0·1 M) to allow the retrograde diffusion of the stain (Iles & Mulloney, 1971; Pitman, Tweedle & Cohen, 1972). The abdominal ganglia were immersed in a pool of saline and the two pools were separated by a barrier of paraffin oil. Small pieces of fat body were included with the ganglia during the diffusion period. Ions were allowed to diffuse for a period of 1–4 days at 4 °C. Cobalt was precipitated in a solution of approximately 0·1% $(NH_4)_2S$ in saline for ½–1 min. Tissues were subsequently examined either as sectioned material or as whole mounts. For sectioning, ganglia were fixed in alcoholic Bouin's for 12–24 h, dehydrated in an ethanol series and then embedded in paraffin. Ten-micron sections were intensified according to the methods of Tyrer & Bell (1974). For whole mounts, tissues were fixed in Carnoy's for 1 h at 4 °C then dehydrated starting with 70% ethanol. Intensification was performed according to the procedure of Bacon & Tyrer (1977).

Histology

Manduca abdomens were isolated, opened mid-dorsally, and pinned to a wax-bottomed chamber. The nervous system was fixed *in situ* for 1 h at room temperature in a 2% paraformaldehyde/0·5% glutaraldehyde fixative in Millonig's phosphate buffer (pH 7·4). Tissues of interest were then dissected out of the abdomens and fixed for an additional 2 h. Following a 5 min rinse in buffer, they were post-fixed in 2% OsO_4 in 1·25% bicarbonate buffer (pH 7·4) for 1 h. After embedding in Epon 812, tissues were sectioned at 1 μm thicknesses and stained with Richardson's stain (Richardson, Jarret & Finke, 1960). Selected blocks were further sectioned at 600 Å, stained with uranyl acetate and lead citrate (Reynolds, 1963) and examined in a Phillips 300 electron microscope.

Cell body assays

Developing male adults were staged with respect to cuticular markers (M. M. Nijhout, unpublished results). Animals at days 7–11 of development were chilled on ice prior to dissection of the three unfused abdominal ganglia. Each ganglion was pinned in a drop of modified Lepidopteran saline (Weever's, 1966) that was completely immersed in paraffin oil. Cell clumps or individual cells were teased out of the ganglia with pulled glass needles and transferred through the oil into small (1–4 μl) drops of distilled water, according to the methods of Berlind & Maddrell (1977). Cell lysis occurred within seconds of transferral. An equivalent volume of twice concentrated saline that contained 0·02% bacitracin was added to the drops in preparation for the biological assay. The drops were frozen while still immersed in oil for 12–24 h, thawed, then assayed for the presence of bursicon using the isolated wing assay.

Intracellular cobalt dye filling

Animals were chilled on ice for $\frac{1}{4}$–$\frac{1}{2}$ h before dissection. Abdomens were isolated from days 8–10 developing adults and the ventral portion was pinned to a wax-bottomed chamber. A physiological lepidopteran saline (Truman & Sokolove, 1972) filled the semi-dissected abdomen while the external surface remained dry (Bate, 1973). Abdomens from pharate adults (day 18, whose cuticle is less brittle) were opened with a mid-dorsal incision, then pinned over a hole that had been cut in a Sylgard (Dow Corning) chamber. Air was supplied to the spiracles through this hole and was driven by a peristaltic pump. High-vacuum grease (Dow Corning) was applied to the edges of the abdomen to ensure a water-proof seal of the external surface. All other procedures (except as noted) were similar for the two developmental stages.

The gut, reproductive tract and some loose fat body were removed and the abdomen flushed with saline. The fourth or fifth abdominal ganglia was pinned through surrounding connective tissue on to a Sylgard-coated platform. All tracheal trunks and all nerves, except the connectives anterior to the ganglion, were left intact. Recordings were made from relevant neuronal somata using glass microelectrodes that were filled with 10% cobalt nitrate and had resistances in the range of 50–90 MΩ. Developing adult neurones were impaled under visual guidance. Pharate adult neurones, once impaled, were identified according to information previously accumulated (Taylor & Truman, 1974; this report) on the axonal pathways and soma locations of certain cells. To test particular pathways, peripheral nerve trunks were stimulated with glass suction electrodes. Antidromic (versus synaptic) activation was judged by the lack of a discernible post-synaptic potential when hyperpolarizing current sufficient to block the action potential was injected. Axon pathways were subsequently confirmed by tracing the dye-filled axons towards or to the point of stimulation.

Current was passed into cells via a bridge circuit that was built into the high-impedance pre-amplifier. Cobalt ions were injected for 20–30 min with 1 s depolarizing pulses. The tissue was then processed as described above for wholemount cobalt chloride back-fills.

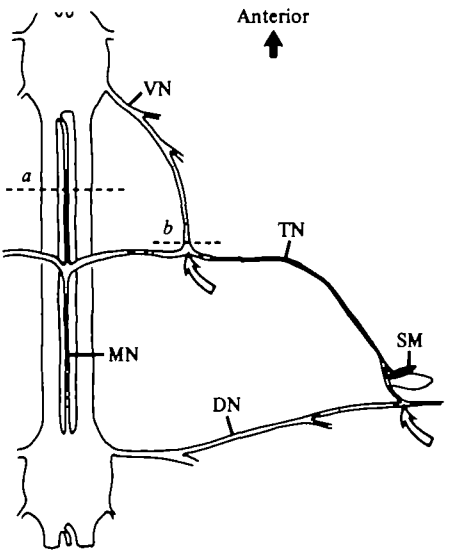


Fig. 1. The organization of nerves on one side of two abdominal segments in a pharate adult *Manduca*. The pattern is similar in segments 3–5. Each abdominal ganglion (AG) – one per segment – has two segmental nerves: a dorsal (DN) and a ventral (VN) nerve. The median nerve (MN) runs between the two interganglionic connectives and divides to form the two branches of the transverse nerve (TN). The TN innervates the closer muscle of the spiracle (SM) and is connected peripherally with the DN and the VN by separate anastomoses (open arrows). Dashed lines *a* and *b* refer to planes of cross-sectioning in Fig. 4A and B, respectively. Proportions in the drawing are not to scale.

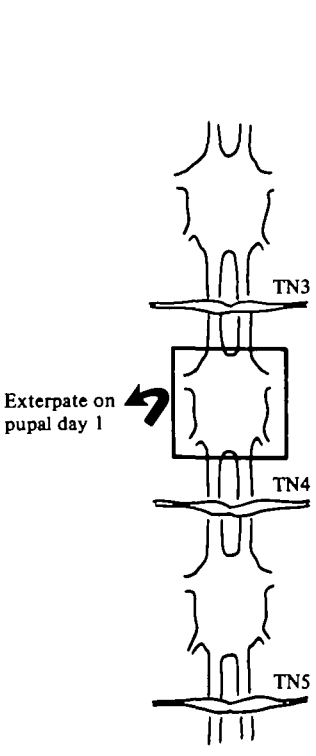
RESULTS

The gross anatomy of the transverse nerve

Fig. 1 schematizes the arrangement of ganglia and nerves in two abdominal segments of a pharate adult *Manduca sexta*. Each ganglion has two paired, segmental nerves: the dorsal (DN) and ventral (VN) nerves. In addition, an unpaired median nerve (MN) runs between the two connectives from one ganglion to the next; this nerve gives off the two branches of the transverse nerve (TN) at a point approximately mid-way between the two ganglia. Perivisceral organs are apparent as slight swellings along the proximal lengths of the TN. Peripheral anastomoses are present between the TN and the VN of the next anterior ganglion and the DN of the next posterior ganglion. The transverse nerve innervates the closer muscle of the spiracle. Methylene Blue and Neutral Red staining gave no evidence for the presence of peripheral nerve cell bodies along the length of the transverse nerve.

Ganglionic origin of the activity in the transverse nerves

Although bursicon activity is highly concentrated in the abdominal portion of the *Manduca* central nervous system (> 70% of the total), its presence can also be detected in tissue homogenates of the brain, subesophageal ganglion and thoracic ganglia as well (Taghert & Truman, 1982). Therefore, we could not presume that an abdominal ganglion contained bursicon cell bodies without first demonstrating



		Hormone units		
		Pupal day 1	Pupal day 18 (pharate adult)	
		Unoperated controls (n = 5)	Unoperated controls (n = 5)	Operated controls (n = 8)	Experimentals (n = 10)
TN3	Assay →	(< 0·01)	(0·18) ± 0·02	0·14 ± 0·02	0·18 ± 0·03
TN4	Assay →			0·14 ± 0·02	< 0·01
TN5	Assay →			0·18 ± 0·03	0·14 ± 0·03

Fig. 2. Results of extirpating a single abdominal ganglion on the bursicon content of three separate transverse nerves. For unoperated controls of both pupal days 1 and 18, TN's from segments 3–5 were pooled from five animals each. Values from operated controls and experimentals represent the means ± S.D. that were combined from two separate experiments. All values represent those dilutions of nerve homogenates yielding 50% positive responses in the biological assay. 1 unit = the amount of hormonal activity present in 1 pharate adult abdominal nerve cord (Taghert & Truman, 1982).

its ability to produce the hormone. This ability was assessed by surgically removing single abdominal ganglia and subsequently determining the effect of that ablation on the bursicon titre of the transverse nerves that are points of hormone storage.

During adult development, there is a greater-than-tenfold increase in the amount of activity that can be extracted from a transverse nerve (Fig. 2 – compare values between unoperated controls on day 1 and on day 18). Ganglion A_4 was extirpated from a series of animals on the first day of the pupal stage and bursicon titres in the three transverse nerves that were situated both anteriorly and posteriorly to that ganglion were then measured individually just before adult eclosion. Essentially normal bursicon titres were found in TN's anterior (TN 3) and two segments posterior (TN 5) to the lost ganglion in both experimental and sham-operated animals. By contrast, TN 4, immediately posterior to the lost ganglion, had a level that was comparable to that found at the time of the operation (both less than 0·01 units). The fact that bursicon accumulation was essentially normal on either side of the surgical interruption (TN 3 and 5) suggests that bursicon is probably not transported over long distances in the abdomen (i.e. through two or more ganglia). Rather, ea

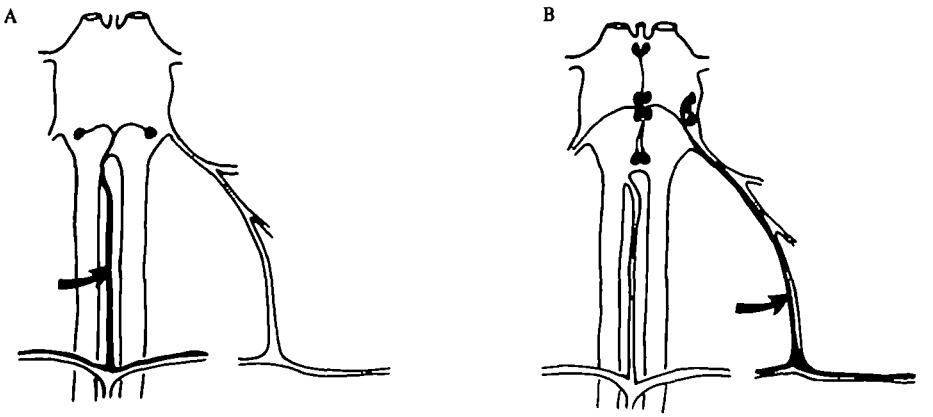


Fig. 3. Cell body maps of neurones that were back-filled with cobaltous chloride from the two different axon routes that connect a transverse nerve with the next anterior ganglion. (A) Route 1, via the anterior median nerve, and (B) Route 2, via the ventral nerve anastomosis.

abdominal ganglion appears capable of producing bursicon and transporting it to the next posterior transverse nerve.

A map of cell bodies with axons in the transverse nerve

Cobalt back-fills of the TN revealed two possible axon routes from a segmental abdominal ganglion to the next posterior transverse nerve (Fig. 3). Route 1 was via the anterior median nerve: in the pharate adult stage (n = 22) this route contained two axons whose cell bodies were located symmetrically along the ventral surface of the ganglion (Fig. 3A). These axons branched once at the origin of the TN to supply both sides of the segment. Route 2 axons excited the ganglion by way of the ventral segmental nerve and they reached the TN through a peripheral anastomosis (Fig. 3B). In the pharate adult, 11 neurones were back-filled from this pathway (n = 13). Three cell bodies were situated dorso-laterally and ipsilateral to the side of the segment in which their axons projected. The remaining eight cells were present at the mid-line of the ganglion as four distinct pairs and they had anterior, posterior, dorsal and ventral positions respectively. Each mid-line cell had axons supplying both sides of the segment. All 11 Route 2 neurones could be back-filled from either the central or peripheral cut end of the TN (relative to the anastomosis with the VN). This indicates that the 11 neurones, once they had reached the TN, branched to travel both peripherally towards the spiracle and also centrally back towards the CNS.

The ultrastructure of nerve routes 1 and 2

The two axon pathways were examined in the electron microscope to determine if either possessed profiles with ultrastructural features characteristic of neurosecretory axons. Animals mid-way through the adult development were chosen for this experiment in the hope that bursicon transport to the periphery might be at near maximal rate. The axons of Route 1 cells were clearly identifiable as the only [redacted] axons in the anterior median nerve (Fig. 4A – also see Fig. 1). They were also

found to be the largest profiles along the entire length of the TN. They were never seen to branch in the nerve, nor did they ever lose their complex glial wrapping. A single, dense-cored granule was seen in a Route-1 axon on a single occasion, but in all other cross-sections examined at many points along their length, these two axons appeared devoid of neuroendocrine specializations.

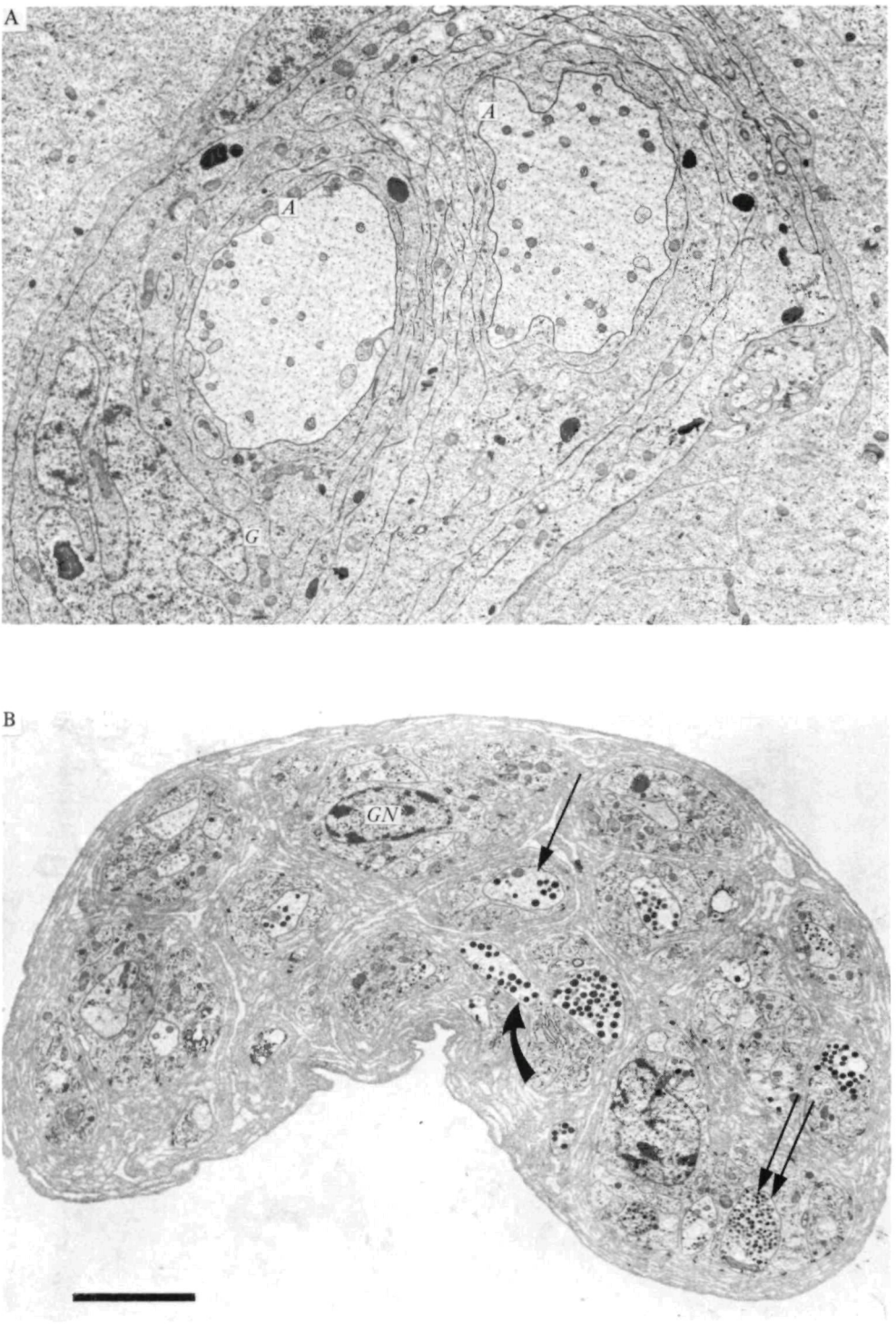
The axons of Route 2 were studied at a point immediately before their anastomosis with the TN (see Fig. 1). In cross-section, many elementary, dense-cored granules (diameter range = 100–300 nm) were visible in all axon profiles (Fig. 4B). There were at least two populations of granules and they were present in distinct axons. In addition, a number of Route 2 axons were observed to branch and separate from their glial wrapping; these desheathed axonal extensions travelled through the extracellular matrix of the nerve in a manner comparable to that of neurosecretory axons in the transverse nerves of other insect orders (Brady & Maddrell, 1967). On ultrastructural grounds, Route 1 cells appeared exclusively motor and indeed are probably homologous to the spiracular closer motoneurones that have been described in locusts (Burrows, 1978). This route was therefore excluded from further consideration as a potential one for bursicon-containing neurones. We then concentrated on the eleven Route 2 cells, which had the ultrastructural features consistent with a neurosecretory function, as the prime candidates for the bursicon-containing neurones.

Biological assays of ganglion regions and isolated cell bodies

Within an abdominal ganglion, Route 2 cells comprised two groups with eight neuronal somata located at the mid-line and three situated dorso-laterally on either side. Groups of cell bodies and individual cells were dissected from abdominal ganglia, and extracts prepared and tested for the presence of bursicon using the isolated wing assay. For these experiments, animals on days 7–11 of adult development were chosen because individual cells could be most easily visualized at this stage. Initially, the ganglion was divided into regions, one of which contained the entire mid-line cell group and another the entire lateral cell group (Fig. 5). The mid-line region was consistently devoid of activity, even in assays that employed mid-line regions from as many as three ganglia. In contrast, single lateral cell regions consistently gave positive responses.

In order to localize this bursicon activity to specific neurones, cells and cell clusters were dissected from the lateral region. Under epi-illumination, most of the nerve cell bodies in this area appeared grey and opaque, but there were four neurones that displayed a distinct Tyndall effect and appeared blue to yellow. They lay in a dorso-lateral position, between the two segmental nerves (Fig. 6A). Clusters of

Fig. 4. Ultrastructure of Routes 1 and 2. (A) Cross-section of Route 1 (cf. Fig. 1 – dashed line *a*). Note absence of neurosecretory specializations in the two axons. *A*, Axon; *G*, glia. (B) Cross-section through Route 2 axons (cf. Fig. 1 – dashed line *b*). Most axon profiles are wrapped within glial bundles. Note that some profiles are branching out of bundles and into the extracellular matrix (curved arrow). Nearly all profiles in this section contain many electron dense granules; two apparent populations are visible: a large (single arrow) and a small (double arrow). *GN*; Glial nucleus. Bar = 4 μm (A) and 5 μm (B).



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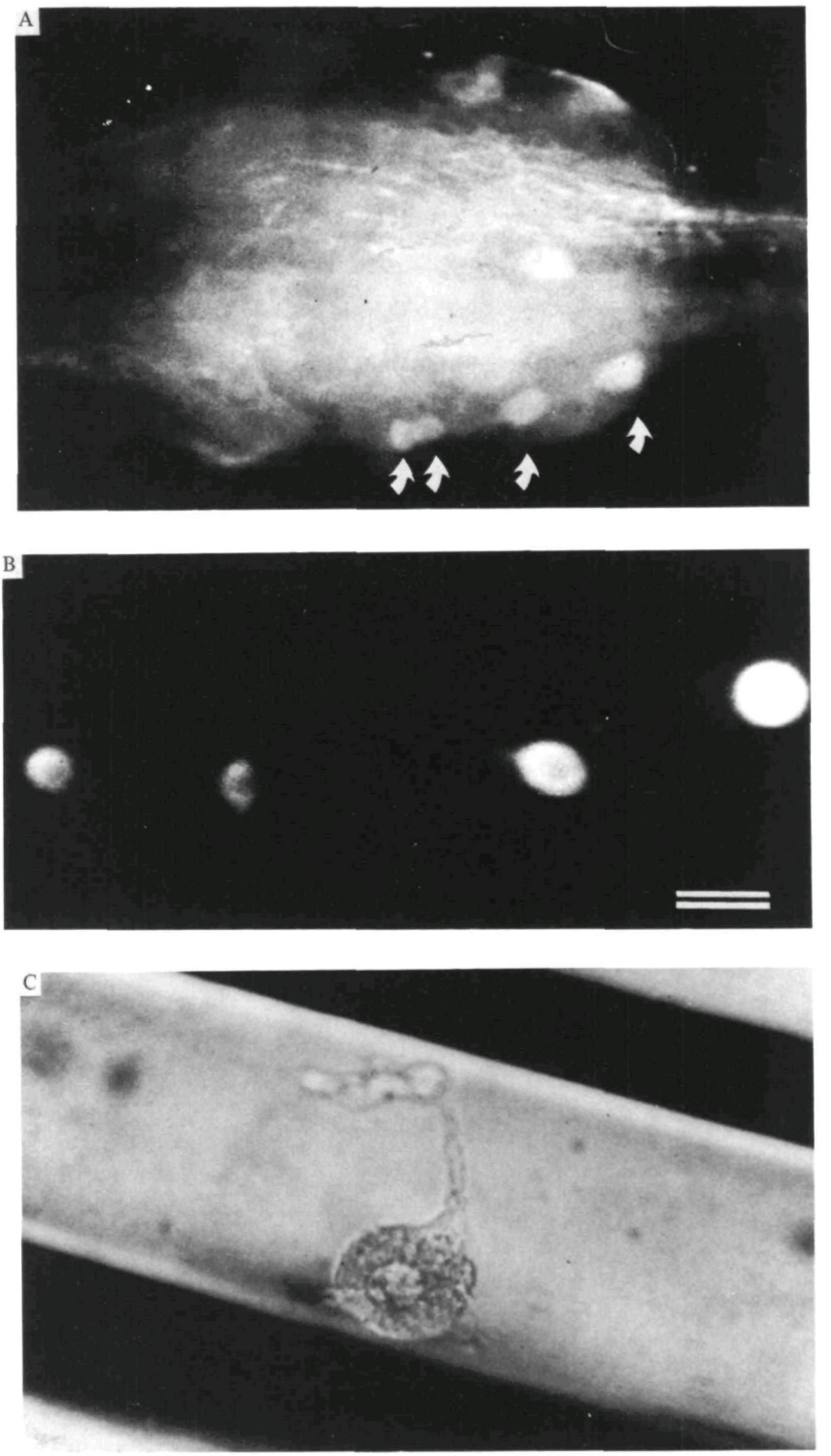
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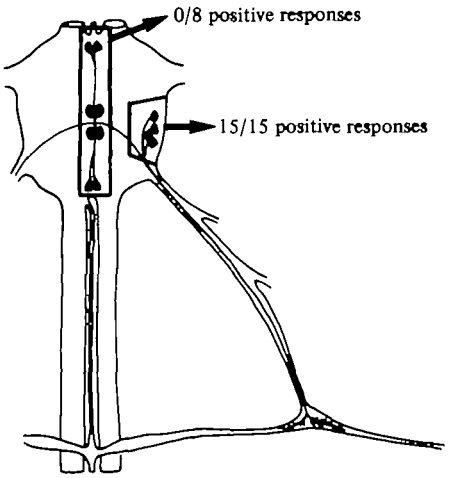

Fig. 5. Bursicon activity present in large subsections of an abdominal ganglion. Rectangular boxes represent the approximate lines of tissue sectioning. Areas were chosen to encompass the cell bodies of Route 2 neurones. Ratios indicate positive scores in the biological assay.

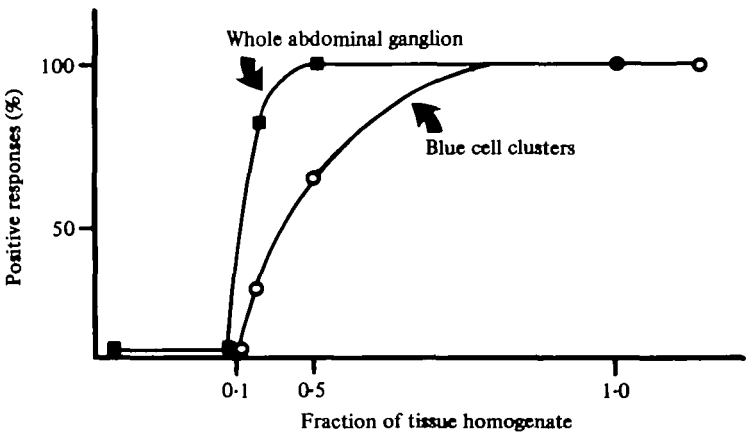

Fig. 7. Bursicon activity in the lateral blue cell group. Dose–response curves compare the amount of activity present in the group with that present in the entire ganglion. For the lateral group, 1 dose unit = 8 cells.

these four cells were teased out of the ganglion, combined and assayed for bursicon. These cell clusters were positive for bursicon and accounted for more than 60% of the bursicon activity in the entire ganglion (Fig. 7). Thus, these four-cell clusters appear to contain the major bursicon-containing neurones in the abdominal ganglia. Bursicon activity was never detected in 'non-Tyndall' cells from this region (n = 12).

Fig. 6. (A) Photomicrograph of a living ganglion from a day 9 developing adult. Note a number of visible somata along the lateral and mid-line regions that display a Tyndall effect. Anterior is to the left. The ganglion was slightly damaged on both sides during the removal of trachea that obscured visiblity. (B) The four blue cells (marked by arrows in (A)) photographed following their individual dissection from the ganglion. Note that the two cells on the left are slightly smaller than the two on the right. (C) A single blue cell (from the right-hand pair of (B)) suitably dissected for assay and drawn up into pulled capillary glass. Bar = 68 μm (A), 50 μm (B) and 27 μm (C).

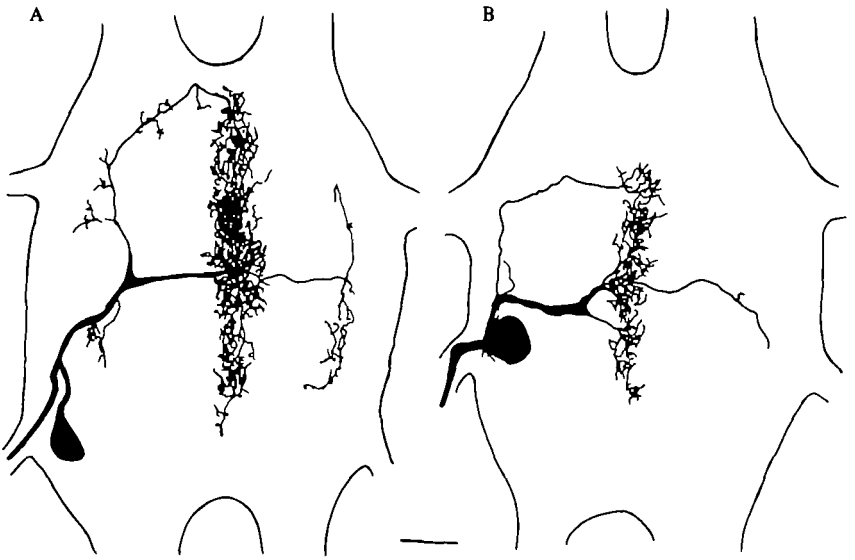

Fig. 8. Comparison of the dendritic and axonal morphologies of a lateral blue cell with those of a lateral Route-2 cell. (A) A back-filled Route 2 neurone from a pharate adult. (B) Blue cell from a day-10 developing adult that was intra-somatically filled. The outline of the ganglion is drawn in both cases and both are viewed from the dorsal aspect. Note similarity in details of dendritic geometry. Bar = 50 μm.

The cell bodies in each cluster could be resolved as two distinct pairs of cells. Two of these neurones had round cell bodies with diameters of approximately 25–30 μm and appeared blue; the other two cells had more oblong somata that were slightly larger than the first pair (Fig. 6B). Within the second pair, one consistently displayed a darker colour than the other. Cells of each type were dissected free of adhering tissue (Fig. 6C) and the large and small pairs assayed separately. Both pairs contained significant bursicon activity: large pair – 11/20 positive assays; small pair – 12/16 positive assays. Using the colour difference to discriminate between members of the larger pair, cells of both light and dark appearance were collected and tested (2–6 cells per assay). Both members of the larger pair contained activity: light – 5/11 positive, dark – 4/8 positive. There were no available criteria by which the smaller two cells could be discriminated from each other and assayed separately. However, their similar appearance and staining response to the neurosecretory stain, paraldehyde fuchsin (Taghert, 1981), suggest that both cells of the smaller pair contain the hormone. Thus all four cells in the cluster appear to be bursicon-containing cells.

Relationship of the bursicon-containing cells to the Route 2 neurones

The fact that the bursicon-containing cells and the lateral Route 2 neurones are both situated in the same dorso-lateral region of the ganglion suggests that they are identical but does not prove it. Therefore we attempted to penetrate and stain lateral, Tyndall-positive cells in ganglia from animals mid-way through adult development. The fragility of the nervous system at this stage made this experiment difficu

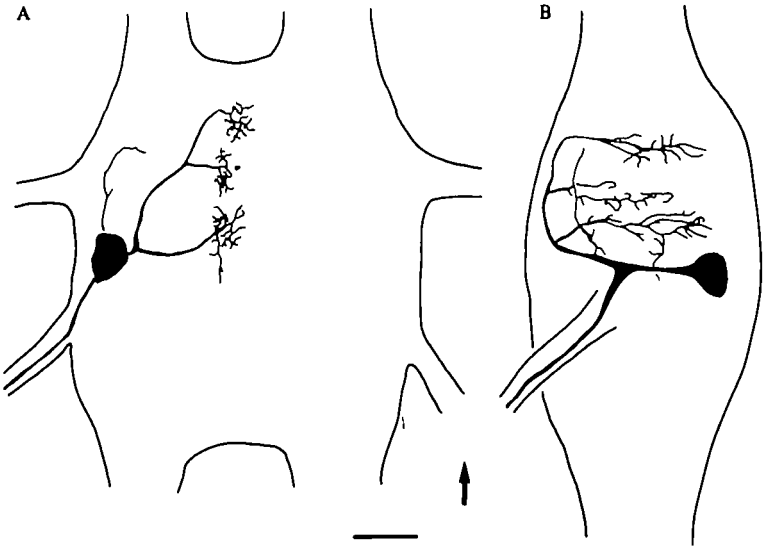

Fig. 9. Two perspectives of a cobalt-filled bursicon-containing neurone from pharate adult. (A) From the dorsal aspect. (B) From a lateral aspect with dorsal to the right. The axon exits the ganglion via the ventral segmental nerve. Large processes cross to the mid-line along the most ventral surface of the neuropile. Most of the fine branching is confined to a dorso-ventral plane at the mid-line. Arrow points anteriorly.

but a single, blue cell was dye-filled. It had an axon that exited the ganglion via the ipsilateral ventral segmental nerve and proceeded unbranched to the posterior transverse nerve. Moreover, the unusual configuration of dendrites in the neuropile corresponded exactly to that seen in all three back-filled lateral Route 2 neurones: fine branching was mostly in a dorso-ventral plane at the mid-line of the ganglion; major dendrites connecting the cell body to that plane proceeded along the most superficial ventral surface of the neuropile (Fig. 8). The extent of branching was somewhat reduced but this was reasonable because of the earlier stage of development of the nervous system. This demonstration directly identifies one of the blue, 'Tyndall-effect' cells as a lateral Route 2 neurone and strongly suggests that all lateral Route 2 cells are bursicon-containing neurones.

Anatomical characteristics of the individual bursicon-containing neurones

The constant features of size, shape and colour in the four cells of the bursicon-containing cluster suggested that each peptidergic cell may have unique and identifiable properties. To test this hypothesis, individual neurones were dye-filled with cobalt ions and their axonal and dendritic morphologies compared. Ganglia from pharate adult moths (fully differentiated) were used. Bursicon-containing neurone cell bodies were not plainly visible at this stage and so they were identified by the antidromic stimulation of their axons in the transverse nerve. It cannot therefore be stated with absolute certainty that all neurones within the cluster were impaled and dye-filled. However, the nature of the results and their correspondence with

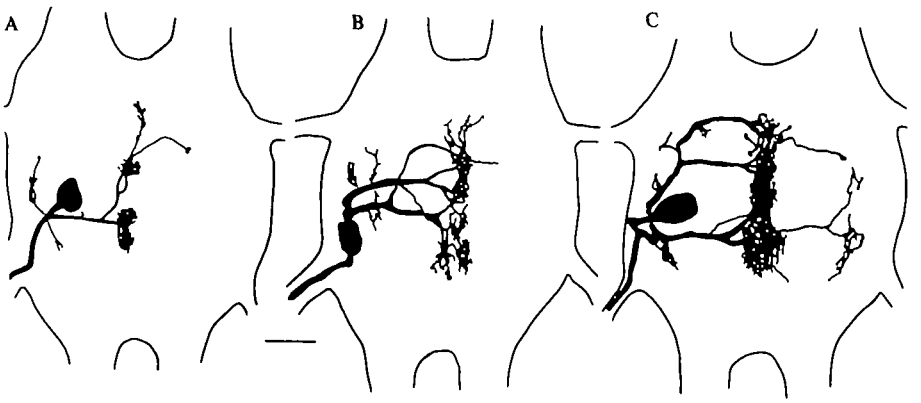

Fig. 10. Individual morphologies of the three Route 2 bursicon-containing cells: (A) no. 24, (B) no. 25 and (C) no. 26. All primary branches extend along the ventral-most surface of the neuropile except for the branch marked with the open arrow in cell no. 25 (B), which is on the dorsal-most surface.

back-filling data gave confidence that representatives of all four neurones were examined.

From the best examples of back-filling, the following characteristics of the three Route 2 bursicon-containing neurones were clearly seen: one cell was slightly larger than the other two; fine branching was present in a dorso-ventral plane at the mid-line; a single dorsal, and at least two ventral, dendrites extended from cell bodies to the mid-line. Examined individually, these neurones were found to share many morphological features in common. A single dye-filled neurone is shown from two perspectives in Fig. 9 and it highlights many of these features. The neurones were monopolar; a ventrally projecting neurite branched once to form the axon and the dendritic tree. The tree was formed by large dendrites that travelled to the mid-line along the ventral-most and/or dorsal-most surfaces of the neuropile. At the mid-line, fine branching took place along as many as four dorso-ventral tracts that extended through the entire depth of the neuropile. Additional fine branching took place along both extreme lateral edges of the neuropile in a dorso-ventral orientation.

On the basis of the shape of the cell body, the degree of fine branching and the presence of contra-lateral branching, it was possible to distinguish three unique Route 2 bursicon-containing neurones. They were designated cells nos. 24, 25 and 26 following the convention of Taylor & Truman (1974). In the course of examining this area of the ganglion, one other cell (no. 27) was found which had morphological and physiological properties that were comparable to those of cells nos. 24–26. We describe these properties below and tentatively conclude it to be the fourth bursicon-containing cell. In the following descriptions, the major dendritic branches that connect mid-line to lateral areas are referred to as primary branches; fine dendrites in the dorso-ventral planes at the mid-line and lateral edges are referred to as secondary branches.

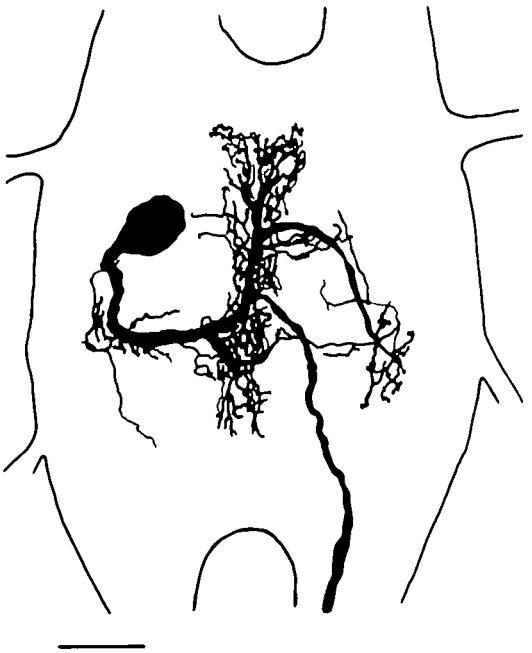

Fig. 11. Individual morphology of a neurone (no. 27), which may be identical to the fourth bursicon-containing blue cell. All primary branches extend along the ventral surface of the neuropile. Note similarities to the form of cell no. 26 (Fig. 10C). The axon exits the central nervous system in the dorsal segmental nerve of the next posterior ganglion.

Cell no. 24

Fig. 10A shows an example of cell no. 24. It had a small, round or slightly triangular soma (mean diameter ± S.E. of eight examples = 35 ± 9 μm) and an axon in the ventral nerve that proceeded unbranched to the next posterior transverse nerve. The primary branches were along the ventral-most surface of the neuropile and the amount of secondary branching was typically slight. Cell no. 24 showed few, if any, primary branches that extended past the mid-line to the contralateral side of the ganglion. When present, these were extremely thin and were also restricted to the ventral surface.

Cell no. 25

Cell no. 25 (Fig. 10B) closely resembled cell no. 24 in the size and shape of its soma, the trajectory of its axon, the paucity of its secondary branches, and the lack of significant contralateral primary branches (n = 5). Cell no. 25 differed from no. 24 in that it possessed an additional, primary branch that reached the mid-line along the dorsal-most surface of the neuropile. In two examples, the ventral primary branches were much reduced in diameter and in another completely lacking. Cells no. 24 and 25 are most likely identical to the smaller pair of blueish cells seen in the living ganglia of developing adults.

Cell no. 26

Cell no. 26 had a larger, more oblong cell body (Fig. 10C) than did the two previous neurones (mean diameter of 8 cells = 47 ± 10 μm). It also had a single, unbranched axon in the ventral nerve that reached the transverse nerve, and had many primary branches, all of which extended along the ventral-most surface of the neuropile. Further characteristics included profuse secondary branching along the mid-line and along both lateral margins of the neuropile.

Cell no. 27

On two occasions, a cell was filled that had a cell body location and dendritic branching pattern comparable to the bursicon-containing cells nos. 24–26 (Fig. 11). In addition, it was the only other cell in this area besides cells nos. 24–26 that we found capable of firing overshooting, somatic action potentials. In contrast to cells nos. 24–26, however, the axon trajectory of cell no. 27 was not out via the ipsilateral ventral nerve but out via the descending contralateral connective. The cell could be antidromically activated from the dorsal nerve of the next posterior segment, indicating that this nerve is its exit point from the CNS. The similarities of both the shape of its cell body and its dendritic morphology to that of cell no. 26 suggest that it may be the other large bursicon-containing neurone.

DISCUSSION

A number of lines of evidence indicate that blood-borne bursicon is derived from the segmentally repeated neurones nos. 24, 25 and 26. The appearance of bursicon in the blood is associated with an 80% depletion in the bursicon activity from the TN (Truman, 1973). Of the neurones whose axons project to the TN, only the lateral Route 2 neurones have the ultrastructure and axonal pathways that are consistent with those required of neurones that secrete bursicon from the TN.

The lateral Route 2 cells share a common position in the ganglion with four Tyndall positive cells whose somata assay positive for bursicon. All bursicon activity that can be extracted from the CNS is due to a single peptide or class of peptides having a net negative charge and an apparent molecular weight of approximately 20 K. This same activity is released from the CNS and is similar to bursicon partially purified from the haemolymph (Taghert & Truman, 1982). Since the four pairs of lateral neurones contain more than 60% of the hormonal activity in the ganglion, the activity in the cells must represent bursicon.

That the lateral Route 2 neurones are identical to three of the four bursicon-containing cells was demonstrated by injecting stain into a cell in the latter group (Fig. 8). As predicted, its axon projected to the posterior transverse nerve via Route 2. In addition, the configuration of its dendrites closely resembled the unusual configuration of back-filled Route 2 neurones. These biochemical, endocrinological and anatomical data support the identification of three of the four lateral blue cells as the neurones responsible for the production of bursicon and its secretion from the transverse nerve.

These neurones include the pair of smaller cells (nos. 24 and 25) and one larg

one (no. 26). The conclusion that cell no. 26 contains bursicon is based on the fact that both of the large cells, when assayed separately for bursicon, showed activity. It was not possible directly to measure the activities of cells nos. 24 and 25 separately because they could not be distinguished from each other in the living tissue nor could the biological assay reliably detect the contents of a single cell body. However, these two cells are histochemically identical to each other (Taghert, 1981). Therefore, we think it is likely that each of the lateral Route 2 neurones contains bursicon and hope to resolve the issue with immunocytochemical techniques employing specific antibodies directed against bursicon.

One unexpected result of this investigation was the presence of a fourth cell that resided in the blue cell cluster, contained bursicon activity, but had a different axon pathway from that of the other three. In probing this region of the ganglion with dye-filled microelectrodes, we found only one cell with the morphological and physiological properties that might be predicted of this fourth cell – no. 27. The final point of termination for this fourth axon, once it has exited the CNS via the next posterior dorsal nerve, is presently unknown. The function of this cell and whether it releases bursicon from an additional neurohaemal site at the same time as the other cells, is likewise unknown.

The cell bodies of these four pairs of neurones contain more than 60% of the bursicon activity present in the ganglion and must therefore represent its major source. A similar situation has been reported in the case of the two prothoracicotropic hormone-containing cells in the brain of *Manduca* (Agui *et al.* 1979): the cell bodies contain 75–80% of the PTTH activity present in the brain. Whether, in either situation, the remainder of the activity resides in the axons and/or dendrites of the identified neurones or whether it resides in other, unidentified cells remains uncertain.

Intracellular cobalt dye-fills of the neurones within the bursicon-containing cell cluster have established the dendritic geometry of these specific neurosecretory cells. These morphological features have provided a basis by which to identify each as a unique neurone. This process of identification made use of cell body size and shape, the location of primary dendritic branches and the extent of secondary dendritic branching. Many of these features demonstrated variability between different examples of the same neurone (Taghert, in prep.); however, the patterns of variability that emerged for sets of features gave confidence that the categorization of unique, individual cells was a valid interpretation (e.g. a single dorsal, primary branch was always associated with a small cell body, profuse secondary branching was always associated with a large cell body, etc.). The significance of morphological uniqueness among neurones that contain a similar hormonal peptide product is at present unclear, but it suggests a certain degree of physiological autonomy for the individual cells.

Despite their individual differences, the dendritic morphologies of these neurones were more similar to each other than to those of other cell types (motorneurones, interneurones) in the ganglion. Together with other neuroendocrine cells in the *Manduca* abdominal ganglia, the central projections of the bursicon-containing cells were restricted to mid-line and extreme lateral margins of the neuropile; the configuration and locations of these areas appears to be specific to neurosecretory neurones (Taghert, 1981; Taghert & Truman, in prep.).

We thank Dr Shirley Reiss for advice in cobalt back-filling procedures, Dr Richard Cloney for assistance in electron microscopy and Dr Richard Levine for advice in intracellular dye-filling. We also thank Drs Lynn Riddiford and John Palka for reading a preliminary draft of this manuscript. Supported by an NSF pre-doctoral fellowship to P.H.T. and by grants from NSF (PCM 77-24878) and NIH (ROI NS13079).

Note added in proof

Since the preparation of this manuscript, J. W. Truman and Shirley Reiss have found that Cell no. 27 projects to the transverse nerve via the dorsal nerve anastomosis. Therefore, all four of the identified bursicon-containing neurons terminate in this neurohaemal organ.

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