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A SUBOESOPHAGEAL GANGLION CELL INNERVATES HEART AND RETROCEREBRAL GLANDULAR COMPLEX IN THE LOCUST

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Summary

The suboesophageal ganglion of the migratory locust *Locusta migratoria* contains a pair of large neurosecretory cells located posteriorly, close to the sagittal plane. By means of double labelling, it is shown that the cells are immunoreactive to bovine pancreatic polypeptide. Using a combination of electrophysiological, neuroanatomical and immunocytochemical methods, it is shown that the neurones project into the corpora cardiaca with ascending anterior axons and into the lateral cardiac nerve cords with posterior axons that descend into the thoracic and abdominal nerve cord.

Introduction

It has recently been shown that, in the locust, the nervus corporis cardiaci III (NCC III), one of the nerves supplying the retrocerebral endocrine complex, consists mainly of axons derived from neurones located within the suboesophageal ganglion (Bräunig, 1990). The cell body of the largest of these neurones is located ventrally and posteriorly, close to the sagittal plane of the ganglion. For this reason, the cell has been called the 'ventral posterior median' neurone (VPM). The cell sends a small-diameter anterior axon towards the retrocerebral complex *via* the circumoesophageal connective and the NCC III. A larger posterior axon proceeds into the neck connective and descends into the thoracic nerve cord.

Both in the size of the cell bodies of the two VPM neurones of both sides and in their location within the suboesophageal ganglion these cells closely resemble the large cell bodies immunolabelled with antisera against bovine pancreatic polypeptide (BPP; Myers and Evans, 1985*a*,*b*). One aim of the present study, therefore, was to find out whether the two cell pairs are identical. The second aim was to disclose the peripheral target(s) of both the anterior and the posterior axons of the VPM neurones.

As will be shown here, the neurones project into the corpora cardiaca with their

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anterior axons. The posterior axons descend through the thoracic nerve cord and through almost the entire length of the abdominal nerve cord to project into the lateral cardiac nerve cords with collaterals passing through the dorsal segmental nerves of the abdominal ganglia. Thus, the present study establishes the astonishing fact that suboesophageal ganglion cells participate in the innervation of the heart in insects. It also shows that in certain cases the structure of a neurone can only be elucidated by a combination of neuroanatomical, neurophysiological and immunocytochemical techniques.

Materials and methods

Insects

Adult male and female locusts, *Locusta migratoria migratorioides* (R. & F.), were taken from the laboratory culture maintained at our institute. Insects were selected 2–4 days after the last moult, since at that stage fatty tissue around nerves is not yet fully developed, which very much aids both dissection and the penetration of immunocytochemical reagents in whole mounts. For the same reason, whole-mount immunocytochemistry was also performed using the smaller ventral nerve cords of fourth and fifth instar hoppers.

Recording and staining

Conventional hook or suction electrodes were used to stimulate or record from peripheral nerves. Electrodes for intracellular recording were filled with 2 mol l^{-1} potassium acetate, or, when staining was required, 0.1 mol l^{-1} hexamminecobaltic chloride (Brogan and Pitman, 1981). In the first case they had resistances between 30 and 40 MΩ, in the latter between 60 and 80 MΩ. Cobalt ions were ionophoretically injected into the cells with 5–10 nA depolarizing current pulses for 30–50 min (pulse duration 200 ms; frequency 2.5 Hz). Ganglia were left overnight in the refrigerator. After precipitation of Co^{2+} with ammonium sulphide (1 drop of an approximately 20 % solution per millilitre of saline) and subsequent thorough washing, ganglia were fixed in alcoholic Bouin's solution, washed in 70 % isopropanol, and rehydrated for subsequent silver intensification of stains (Bacon and Altman, 1977).

To study the morphology of the neurones in the suboesophageal ganglion, isolated ganglia or parts of the ventral nerve cord were treated as described previously (Bräunig, 1990). To stain the peripheral ramifications in the retrocerebral glandular complex, the following procedures were used. After immobilization by chilling, the locust was decapitated and the head was opened with a parasagittal cut with a razor blade that removed the mouthparts and parts of the tentorium on one side, but left the hypopharynx, pharynx, oesophagus and central nervous system (CNS) intact (except for the optic lobe on the operated side). The head was pinned open side up into a Sylgard-lined dish and covered with locust saline.

The suboesophageal ganglion was exposed and its peripheral nerves were cut as far away from the ganglion as possible. The ganglion was then rigidly fixed ventral

side up on a small supporting Sylgard block using its peripheral nerves and the cervical connectives as 'guy ropes'. After staining, the retrocerebral complex was partially exposed, freed from tracheae, air sacs and fatty tissue, and oriented such that its supplying nerves, the small branches of NCC III in particular, were in an optimal position for the final dissection after fixation.

After precipitation and fixation, the retrocerebral complex and surrounding tissues (hypocerebral ganglion, aorta and some of the pharyngeal dilator muscles innervated by NCC III; Bräunig, 1990) were carefully dissected, rehydrated and intensified with silver. These rather complicated measures had to be taken because it is almost impossible to identify particular nerve branches in this very complex system (Bräunig, 1990) if they are not fixed in their natural position prior to all subsequent histological procedures.

Whole-mount immunocytochemistry

Single ganglia, entire ventral nerve cords and the dorsal part of the abdomen containing the heart were fixed overnight in aqueous Bouin's fluid at 4°C. They were thoroughly washed at room temperature in 10–20 changes of phosphate-buffered saline containing 0.1 % Triton X 100 (PBS–TX) until the washing fluid no longer showed any yellow coloration from dissolved picric acid. The tissues were then transferred into PBS–TX containing the primary antibody directed against bovine pancreatic polypeptide (BPP, code-no. 162 II) in a 1:500 dilution and incubated for 3–4 days at 4°C. (The antibody was a kind gift of Dr C. J. P. Grimmelikhuijzen, University of Hamburg.) After thorough washing (1 day at room temperature), tissues were immersed overnight in PBS–TX containing biotinylated secondary antibody (Vector laboratories, Burlingame, California; dilution according to the Vectastain ABC kit protocol) at 4°C. This was followed by washing in phosphate-buffered saline (PBS) without Triton X 100 (1 day at room temperature) and incubation (overnight in the cold) with avidin–horseradish peroxidase complex (dilution according to Vectastain protocol). After washing in PBS (2–4 h at room temperature), tissues were preincubated with a solution of 25 mg of 3,3′diaminobenzidine (DAB; Sigma) in 100 ml of PBS for 1 h in the dark. For development, they were transferred into fresh DAB solution containing 0.01 % H_2O_2. Finally, tissues were briefly washed in PBS, dehydrated in a graded series of isopropanol, cleared in methylsalicylate and mounted in natural Canada Balsam (solid resin dissolved in methylsalicylate).

Since the antiserum was only used as a means of selectively staining a particular neurone in order to study its morphology, apart from controls in which the primary antiserum was omitted, no further specificity tests were carried out. Data concerning the specificity of the primary antiserum are listed in Verhaert *et al.* (1985) and Nässel *et al.* (1988).

Double labelling

Cells were penetrated with cobalt-filled microelectrodes and identified using electrophysiological criteria. They were injected only briefly (10–15 min, 5 nA).

Ganglia were fixed immediately afterwards in aqueous Bouin's fluid. After washing briefly in PBS, they were dehydrated and embedded in paraplast. Alternate 10 μm transverse sections were placed on different slides. One slide was used for BPP immunohistochemistry. The procedure was identical to the one described above for whole mounts, but incubation times were shorter (primary antiserum overnight, all other steps completed in 1 day according to the Vectastain protocol). The profiles of the cobalt-injected neurone in the second set of sections were intensified with silver (Tyrer and Bell, 1974).

Results

Structure of the neurone as revealed by intracellular staining

Intracellular staining of the VPM neurone in isolated nerve chains showed that the cell has dendritic ramifications only within the suboesophageal ganglion (Fig. 1; SOG). Details concerning the branching pattern of the neurone (e.g. the course of major dendritic arborizations in relation to the fibre tracts of the suboesophageal ganglion) have been described previously (Bräunig, 1990). The anterior axon ascends through the circumoesophageal connective and exits through NCC III without any ramifications in the brain (Fig. 1). Similarly, the posterior axon passes through the pro- and mesothoracic ganglia into the metathoracic ganglion (where staining always faded) without forming any ramifications within their neuropiles (Fig. 1; T1, T2).

Tracing the posterior axon with electrophysiological methods

Both the anterior and the posterior axons of the VPM neurone run within the dorsal lateral longitudinal fibre tracts of the CNS (Bräunig, 1990). Because of this, the axons in the connectives come to lie dorso-laterally and just below the surface. This allows excitation of the cell antidromically, using focal stimulation of connectives with a small suction electrode placed dorso-laterally on the outer surface. It was found that, upon stimulation of the cervical connective with 4–10 V, phase-locked spikes (marked with dots in Fig. 2) could be recorded from the anterior axon of the cell running in the ipsilateral NCC III (Fig. 2; stimulus site *a*). When the connective between pro- and mesothoracic ganglia was stimulated, the spikes in NCC III appeared with slightly longer latency (Fig. 2; stimulus site *b*). These experiments confirm the results obtained with intracellular staining (Fig. 1).

In this fashion the stimulating electrode was moved step by step down the thoracic and abdominal nerve cord. Spikes in NCC III could still be elicited by stimulation of the connective between abdominal ganglia 4 and 5 (Fig. 2; stimulus site *e*), but not by stimulation of that connecting ganglia 5 and 6 (Fig. 2; site *f*). The latency of the spikes increases considerably in the recordings shown in Fig. 2*a–e*. As will be shown below, this increase is entirely due to the increasing distance between the stimulating and recording electrodes. There is no contribution from polysynaptic pathways.

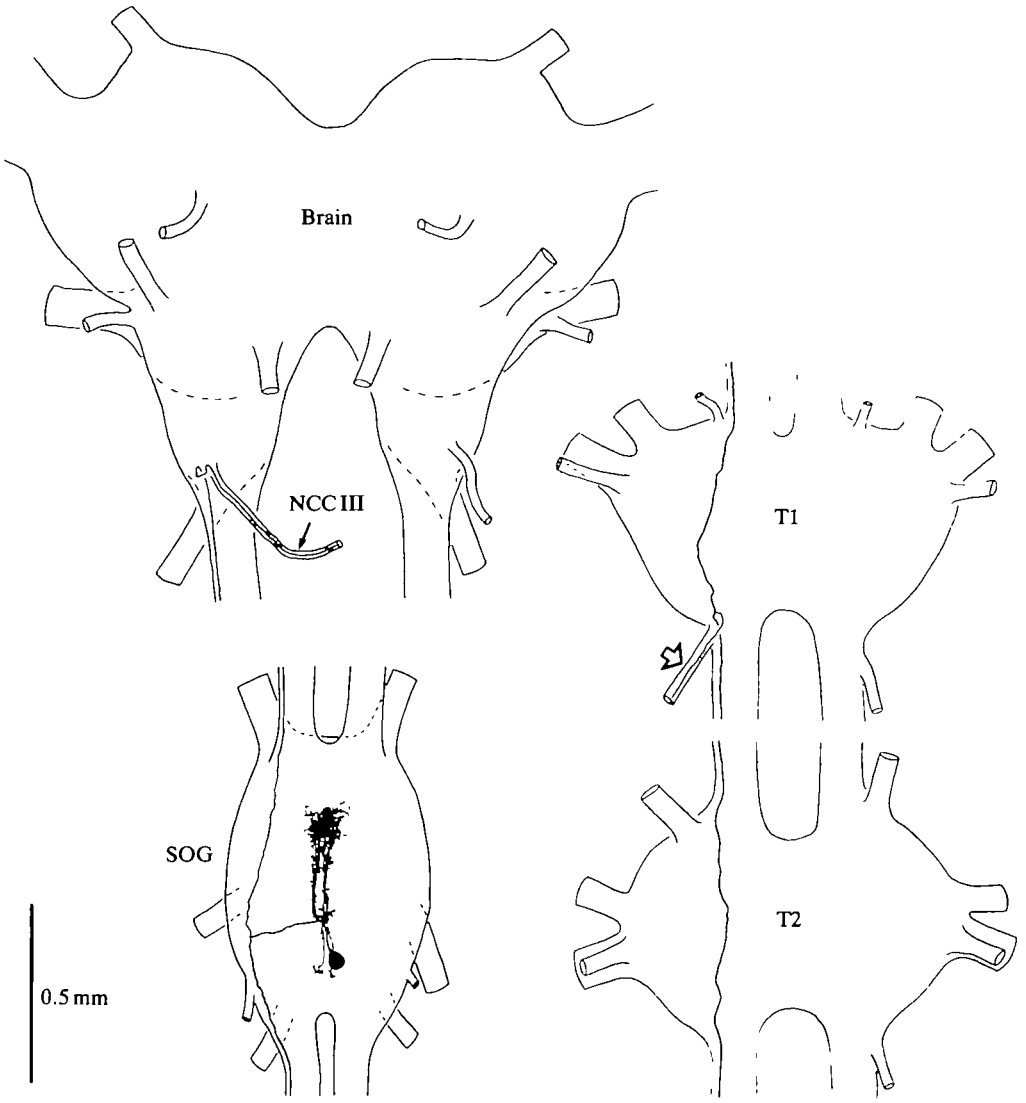

Fig. 1. Morphology of the ventral posterior median (VPM) neurone as revealed by intracellular staining. *Camera lucida* drawings of the supraoesophageal (Brain), suboesophageal (SOG), pro- (T1) and mesothoracic (T2) ganglia of an adult locust (dorsal views, anterior to the top). NCC III, nervus corporis cardiaci III.

Constant-latency spikes in NCC III could also be elicited by stimulation of the dorsal segmental nerves of the abdominal ganglia (Fig. 2; sites *g–j*). Moving the stimulating electrode along the dorsal segmental nerve and its peripheral branches, it was found that NCC III responds as long as the electrode is placed on the main trunk of this nerve, but not when one of its numerous side branches is stimulated. In this way it was established that the axon in question runs through

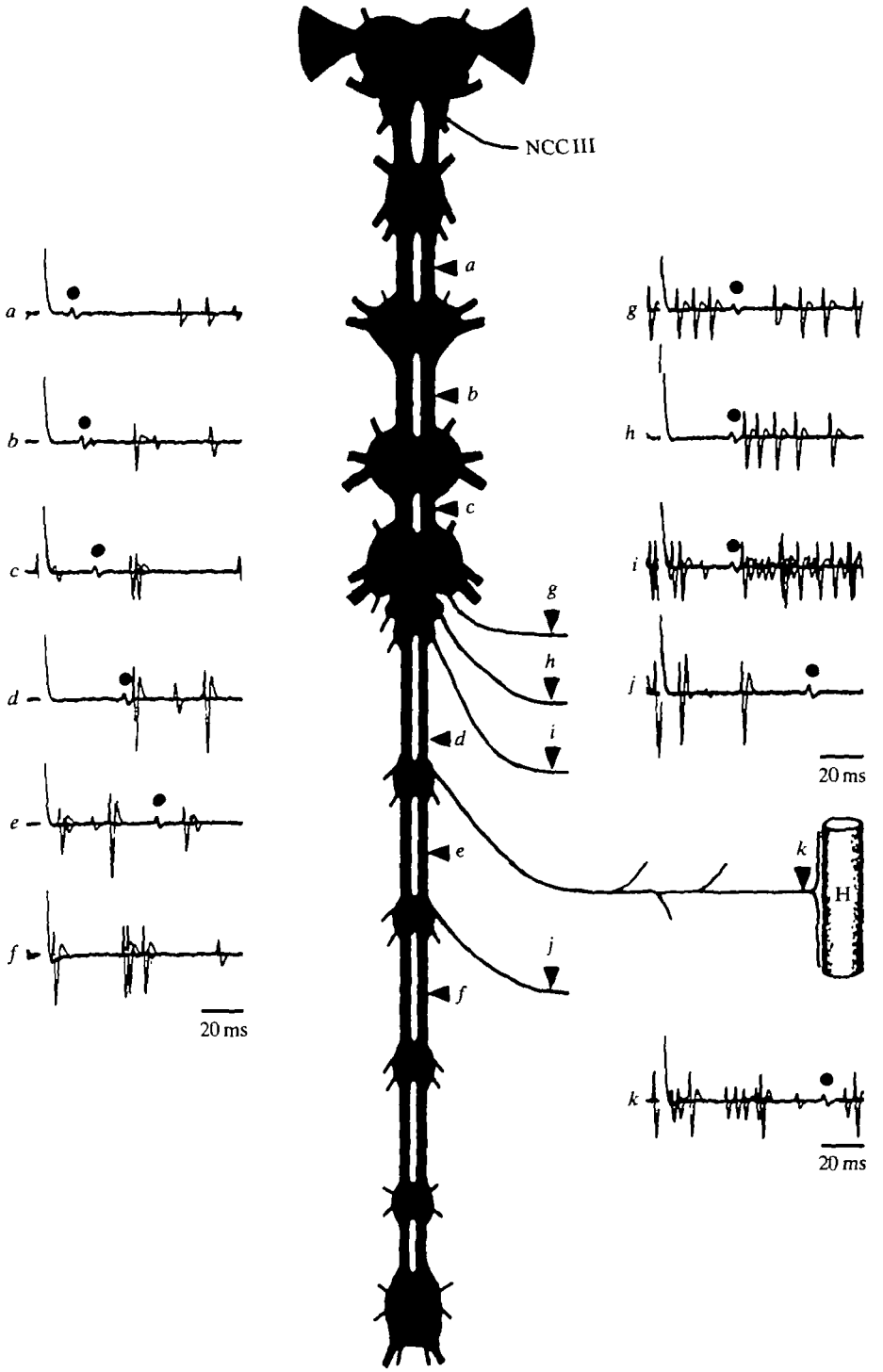

Fig. 2

Fig. 2. Electrophysiological tracing of the posterior axon. The connectives of the ventral nerve cord were stimulated at sites *a–f* (arrowheads). On the left are shown recordings from the nervus corporis cardiaci III (NCC III) ipsilateral to the stimulated connective. Note that constant-latency spikes (marked with dots) appear in NCC III upon stimulation at sites *a–e*, but not *f*. The recordings on the right (*g–j*) show that stimulation of the dorsal segmental nerves of abdominal ganglia 1–3 and 5 also causes phase-locked spikes in NCC III. In abdominal segment 4, the dorsal segmental nerve was exposed and stimulated where its distalmost end merges with the lateral cardiac nerve cord (*k*). 7–10 sweeps of the oscilloscope, pre-triggered by the stimulus, are superimposed in traces *a–k*. H, heart. Connectives and peripheral nerves are considerably foreshortened in this schematic representation of the nervous system.

the entire length of this nerve as far as its very distal end (the 'segmental heart nerve'; Alexandrowicz, 1926) which, makes a T-branch and merges with the lateral cardiac nerve cord (Fig. 2; site *k*).

Stimulation of the ventral segmental nerves of the abdominal ganglia or of the lateral nerves of the thoracic ganglia did not cause spikes in NCC III. Occasionally, stimulation of the dorsal nerve of a particular abdominal ganglion failed to elicit spikes in NCC III. This indicates that in single segments peripheral axon collaterals of the neurone may be missing, a result that was confirmed by the immunohistochemical studies (see below; Fig. 5, arrow). Another result, confirmed by both intracellular staining (Fig. 1, open arrow) and immunocytochemistry (Fig. 5, open arrows), is that the cell occasionally sends axon collaterals into the intersegmental nerves between thoracic ganglia. Accordingly, stimulation of these nerves in a few preparations also caused phase-locked spikes in NCC III.

The results described above suggested that the VPM neurone projects into both the NCC III of the retrocerebral complex and into the lateral cardiac nerve cord with segmental collaterals in the dorsal segmental nerves of abdominal ganglia. This was confirmed by penetrating the cell body of the neurone in the suboesophageal ganglion with a microelectrode while simultaneously recording the activity from both types of nerves (Fig. 3). As described previously (Bräunig, 1990), the cell produces large-amplitude soma spikes with a pronounced undershoot. Each soma spike is followed one-to-one by axon spikes in both NCC III and the distalmost branch of the dorsal segmental nerve (the segmental heart nerve) in an abdominal segment (Fig. 3A,B). Stimulation of the dorsal segmental nerve of another abdominal segment causes phase-locked spikes in all three recordings (Fig. 3C). In this case the soma spikes look different, probably because the undershoot is cancelled by an attenuated spike invading the soma. The dorsal segmental nerves of abdominal ganglia 1–3 are very long (because of the anterior position of their ganglia resulting from their fusion with the third thoracic ganglion), and the segmental collaterals have a smaller diameter (and lower conduction velocity) than the main axon in the ventral nerve cord. This means that, as in the example shown in Fig. 3C, the antidromic spikes take longer to arrive in the segmental heart nerve than in the soma and the NCC III.

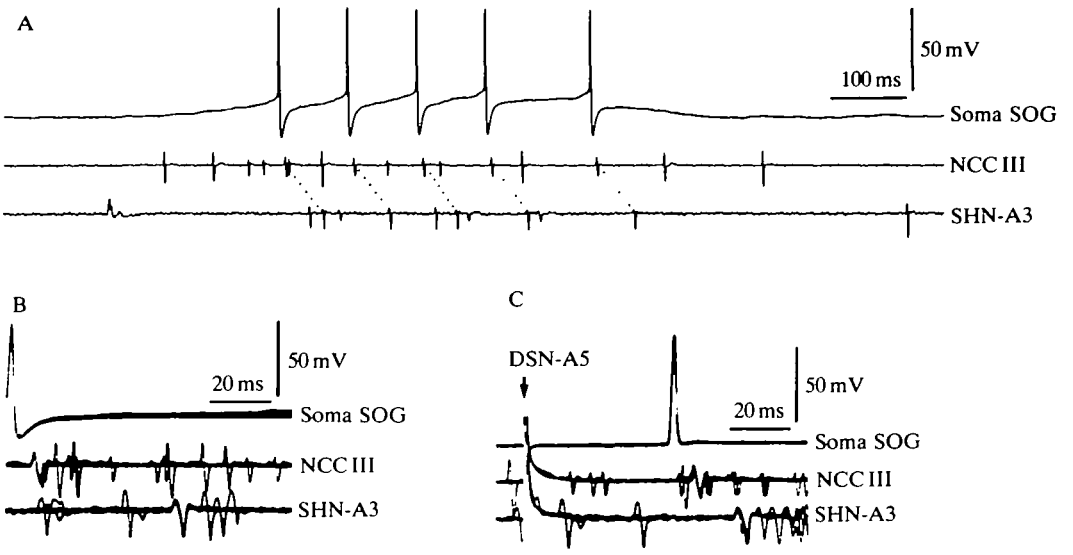

Fig. 3. (A) Simultaneous recording from the VPM soma (Soma SOG), the nervus corporis cardiaci III (NCC III), and the segmental heart nerve of abdominal ganglion 3 (SHN-A3). The VPM spikes are connected by dotted lines in the extracellular recordings. (B) Ten sweeps of the oscilloscope triggered by a VPM soma spike superimposed to illustrate the temporal relationships more clearly (traces as in A). (C) Stimulation of the dorsal segmental nerve of abdominal ganglion 5 (DSN-A5) causes phase-locked spikes in all three traces (10 sweeps superimposed). These occur with the latencies to be expected given the geometry of the neurone.

Immunocytochemistry

Double labelling of electrophysiologically identified neurones with both cobalt and antibodies directed against bovine pancreatic polypeptide (BPP) showed that the cells are indeed identical with previously described BPP-immunoreactive cells (Fig. 4). During these experiments, it turned out that the antiserum stained the VPM neurones particularly well, while other neurones were labelled comparatively weakly. Thus, it was possible to trace the axons in the central and in the peripheral nervous system, and to show their continuity. If a sketch had been drawn of the morphology of the neurone based on the results of the electrophysiological experiments, it would not have differed much from Fig. 5, which shows the results obtained with whole-mount immunocytochemistry. Thus, the results obtained with both methods match rather nicely.

In addition, whole-mount immunocytochemistry (Fig. 6) showed that the axon collaterals in each abdominal segment (Fig. 5, A1–A5) pass through the entire length of the dorsal segmental nerves without branching (Fig. 6C) and establish varicose terminal branches along the lateral cardiac nerve cords (Figs 6D, 7).

The target of the anterior axon

In contrast to the posterior axon, the anterior axon could not be traced with

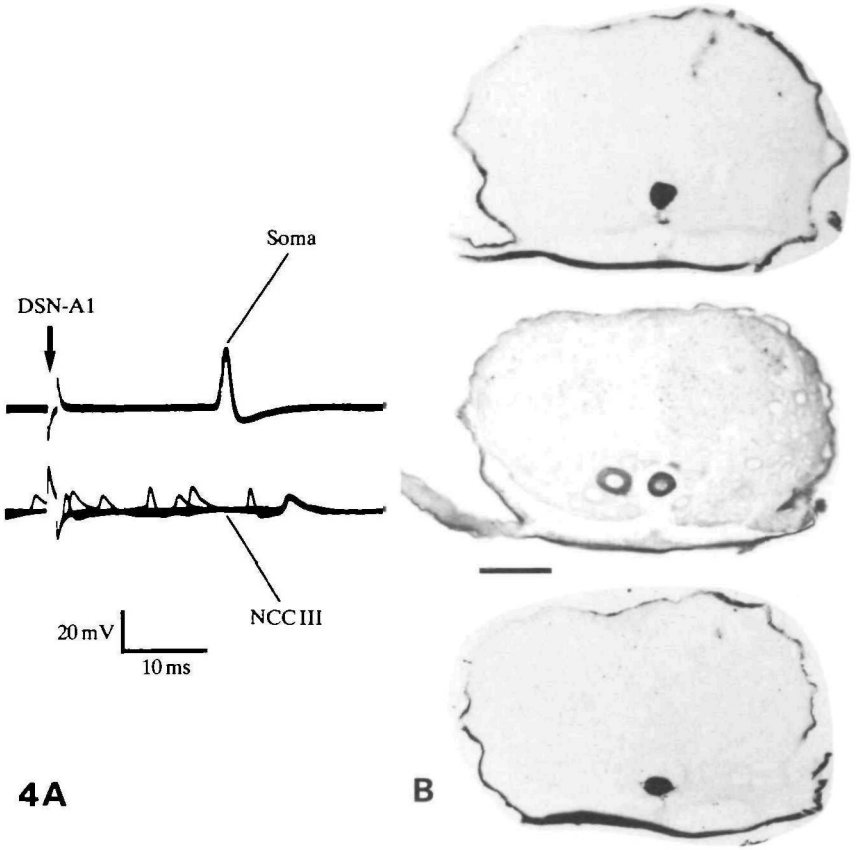

Fig. 4. Double labelling of the VPM neurone with cobalt and BPP antiserum. (A) Electrophysiological identification of the cell by stimulation of the dorsal segmental nerve of abdominal ganglion 1 (DSN-A1) and simultaneously recording from the VPM soma in the suboesophageal ganglion (Soma), and the nervus corporis cardiaci III (NCC III). (B) Three consecutive 10 μm paraplast transverse sections of the suboesophageal ganglion (dorsal to the top). In the top and bottom sections, cobalt staining was intensified with silver. The middle section was treated with the BPP antiserum. Scale bar, 100 μm.

immunocytochemical methods. As Fig. 8A shows, NCC III anastomoses with the NCA I/NCA II system, a direct nervous connection between the retrocerebral complex and the suboesophageal ganglion. This system contains many fibres immunoreactive to BPP that derive from populations of neurones of both the brain and the suboesophageal ganglion (Böhme and Bräunig, 1990; P. Bräunig, unpublished results) and obscure the single fibre entering the system *via* NCC III.

Of 20 attempts to stain the peripheral branches of the anterior axon by injecting cobalt into the cell body in the suboesophageal ganglion, only two were partially successful. Both showed that the axon branches into two collaterals in the

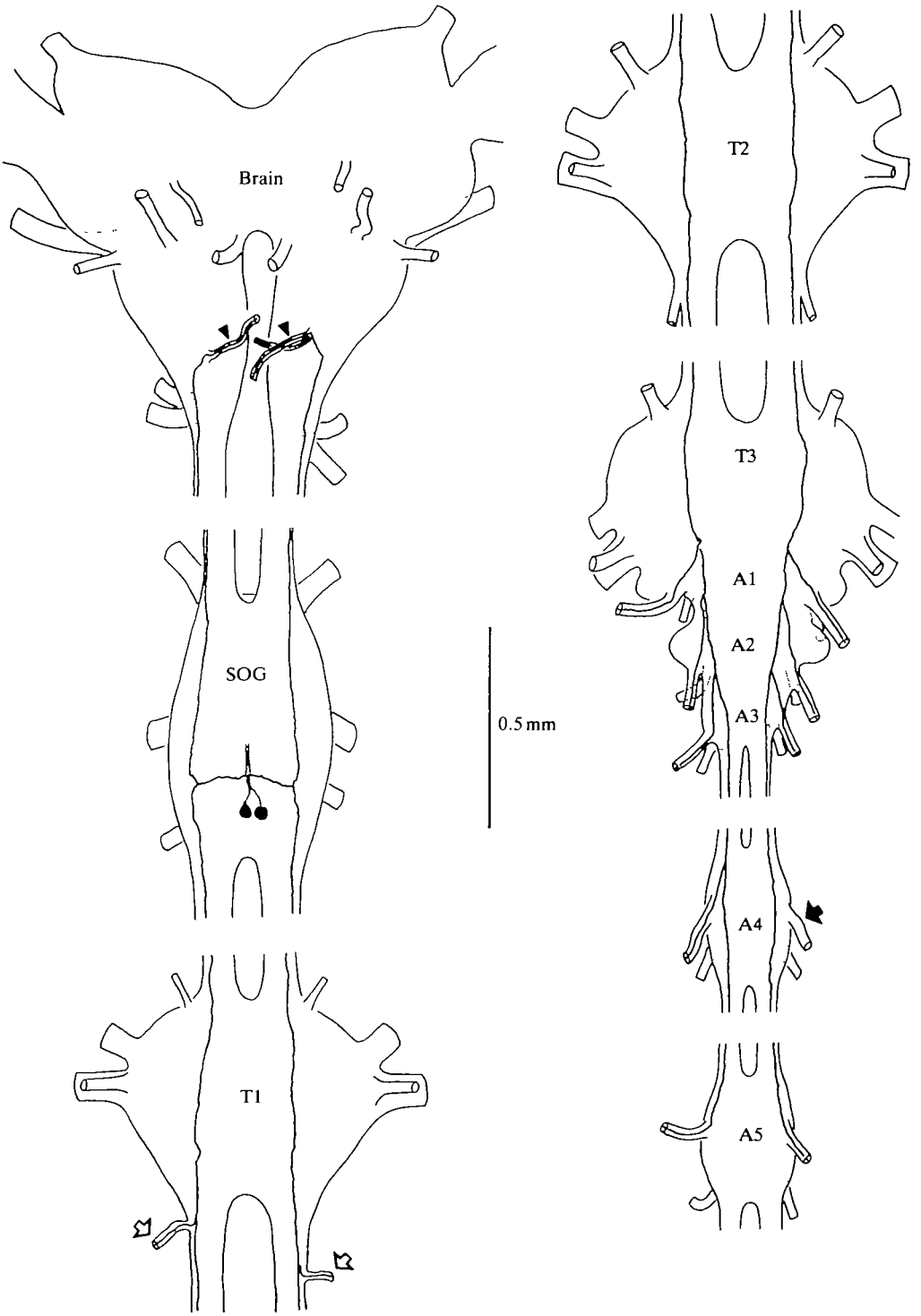

Fig. 5

Fig. 5. Morphology of the VPM neurones as revealed by wholemount immunocytochemistry. *Camera lucida* drawings (dorsal views) of the ganglia of a fourth-instar hopper (connectives omitted) stained with BPP antiserum. From the two cell bodies in the suboesophageal ganglion (SOG) anterior axons run towards the brain and exit through the nervi corpororum cardiacorum III (Brain, arrowheads). The posterior axons run through the thoracic (T1–T3) and abdominal ganglia (A1–A5). Axon collaterals exit through the dorsal segmental nerves of all abdominal ganglia, except for the right nerve of ganglion 4 (A4, filled arrow). Additional axon collaterals exit through the intersegmental nerves of the prothoracic ganglion (T1, open arrows).

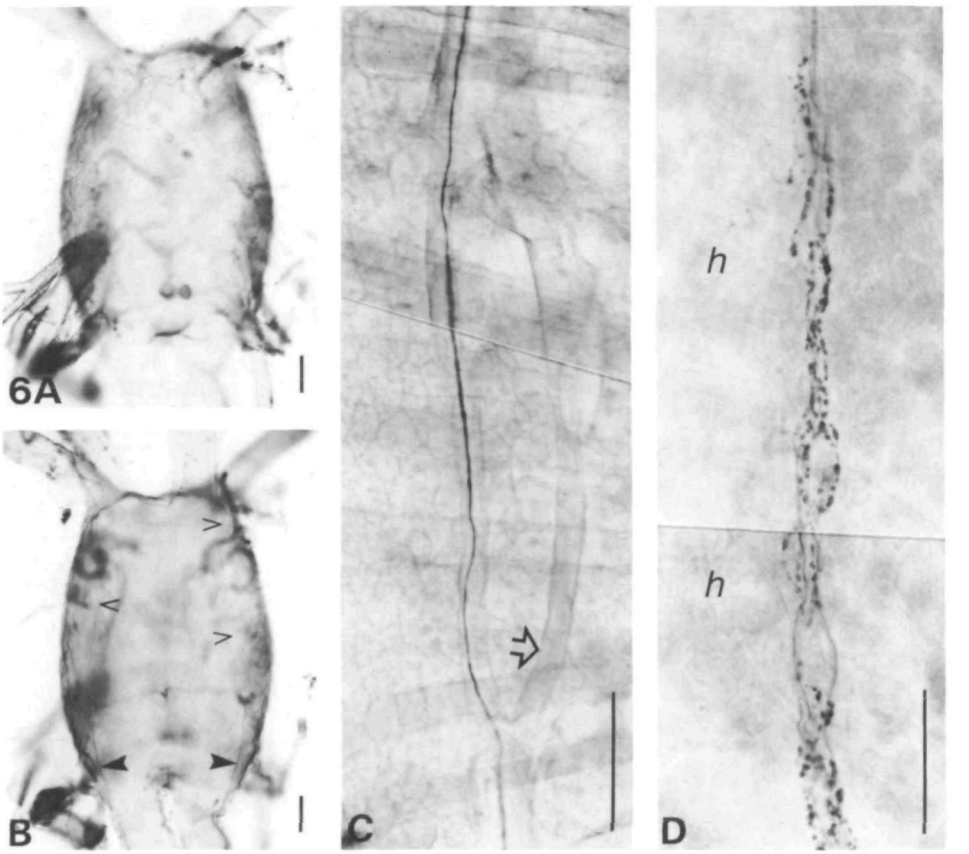

Fig. 6. Immunocytochemistry of VPM neurones. (A) Ventral view of the suboesophageal ganglion (anterior to the top) showing the two VPM somata posteriorly in the ganglion. (B) Dorsal view of the same ganglion showing the anterior (open arrowheads) and posterior axons (filled arrowheads). (C) The single BPP immunoreactive axon in the dorsal segmental nerve of an abdominal segment (dorsal to the top). Note that the axon follows the main nerve and does not branch into a side branch (arrow). (D) Varicose terminal ramifications along the lateral cardiac nerve cord established by the axon collateral shown in C (*h*, heart; compare Fig. 7). Scale bars, 100 μm.

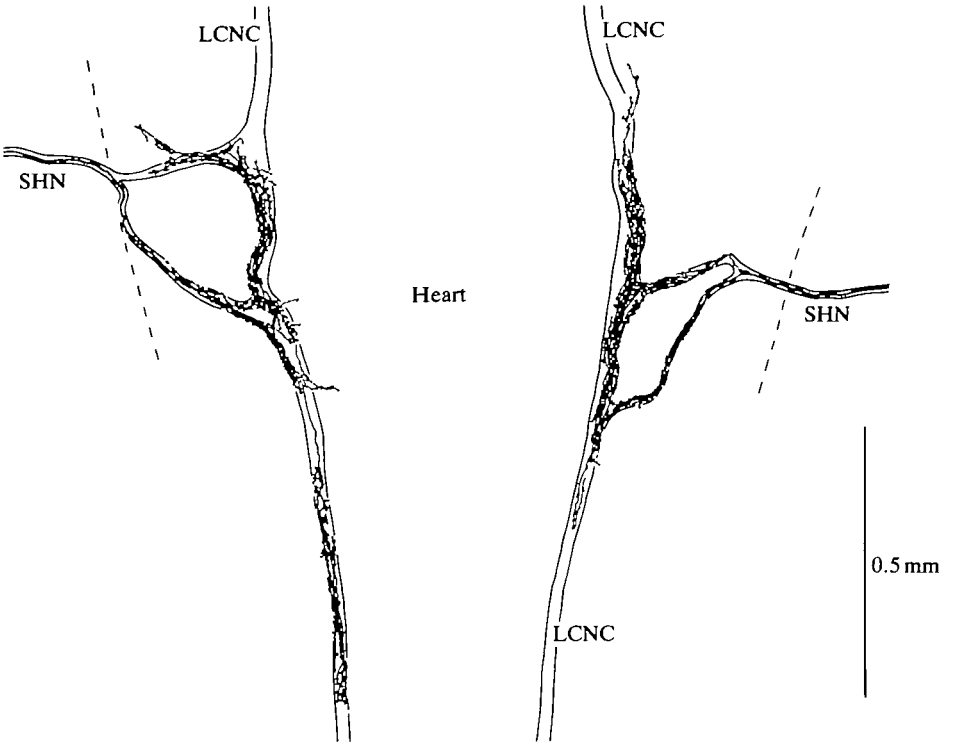

Fig. 7. *Camera lucida* drawing (ventral view, anterior to the top) of BPP immunoreactive axons in the segmental heart nerves (SHN) and varicose profiles along the lateral cardiac nerve cords (LCNC) in an abdominal segment. The broken lines indicate the dorsal rim of the dorsalmost intersegmental muscles.

periphery. One collateral proceeds into the corpora cardiaca *via* NCC IIIa, where it forms diffuse terminal ramifications (Fig. 8B). In both preparations, the majority of ramifications occurred in the storage lobe of the corpora cardiaca, but a few also extended into the glandular lobes. In both preparations, a second collateral entered NCC IIIb. This nerve innervates pharyngeal dilator muscles 43–45 (Bräunig, 1990), but its main trunk is also a neurohaemal organ (see Discussion). In one preparation, the anterior VPM axon was traced almost as far as muscle 45 (the most posterior of the three muscles). There, staining faded. In this preparation, it did not branch into the side branches of NCC IIIb that innervate muscles 43 and 44.

Discussion

Innervation of the insect heart by identified neurones

Following the pioneering work of Zawarzin (1911), it has been shown that, in the more primitive insect orders, the lateral cardiac nerve cords receive innervation from the abdominal ganglia *via* the distalmost extensions of the dorsal

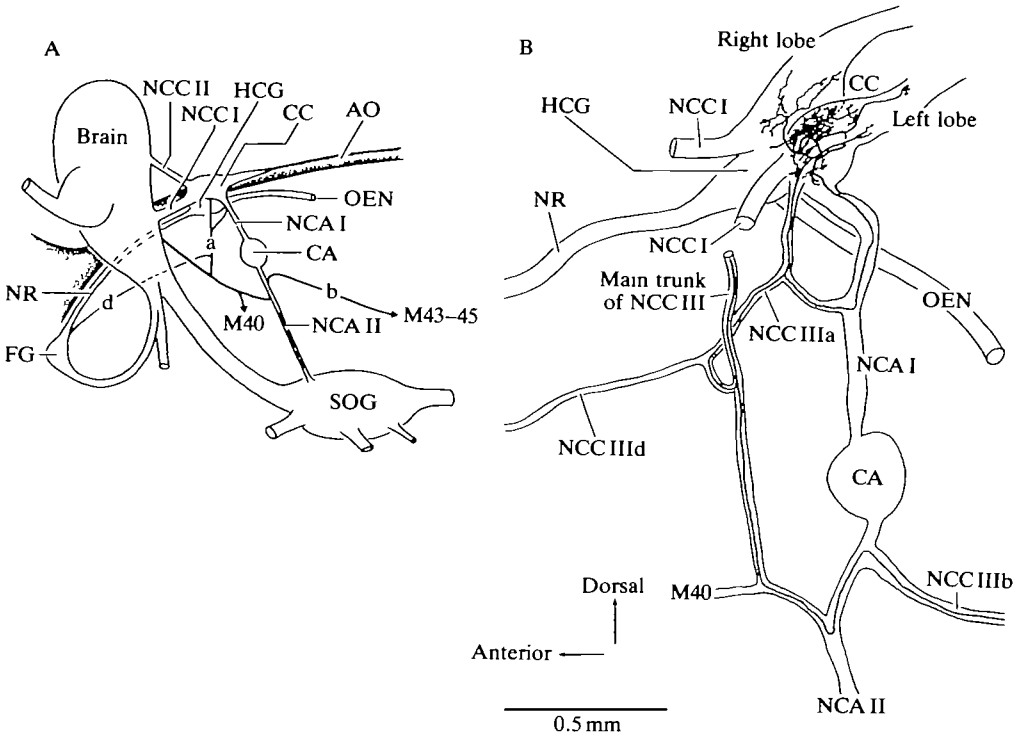

Fig. 8. Targets of the anterior axons of VPM neurones. (A) Semi-schematic lateral view of the retrocerebral complex and its innervation. The major branches of NCC III are labelled a, b and d (not all major branches are included). (B) *Camera lucida* drawing of an anterior VPM axon in nerves NCC IIIa,b, and the corpora cardiaca (CC) as revealed by intracellular staining. Both NCC II have been omitted. AO, aorta; CA, corpus allatum; CC, corpus cardiacum; FG, frontal ganglion; HCG, hypocerebral ganglion; M40, M43–45, pharyngeal dilator muscles; NCA I, II, nervus corpori allati I and II; NCC I, II, III, nervus corporis cardiaci I–III; NR, nervus recurrens; OEN, oesophageal nerve; SOG, suboesophageal ganglion.

segmental nerves, the 'segmental heart nerves' (Alexandrowicz, 1926). In grasshoppers, the segmental heart nerves consist of at least 11 axons (Tyrer, 1971). Although the insect circulatory system has received much attention from physiologists (reviewed in Jones, 1977; Miller, 1985*a*,*b*), it is very surprising that only now, 80 years after Zawarzin's study, have central neurones forming these segmental heart nerves been individually identified. It has been claimed that the 'lateral white neurones' of cricket and cockroach abdominal ganglia innervate the heart (O'Shea and Adams, 1981, 1986), but, to the best of my knowledge, the evidence for this has not yet been presented. Only recently, dorsal unpaired median (DUM) neurones and other bilaterally projecting neurones of locust abdominal ganglia have been shown to send axon collaterals into both the segmental heart nerves and the lateral cardiac nerve cords (Ferber and Pflüger, 1990). The main result of the present study is, therefore, to show that these nerves

also receive axon collaterals from the VPM cells located within the suboesophageal ganglion.

In addition, the VPM neurones provide one of the few examples of intersegmentally projecting neurosecretory cells within the insect CNS. There are only two previous examples of such cells. The first being two large cells located ventrally in the suboesophageal ganglion of locusts (Rémy and Girardie, 1980; Evans and Cournil, 1990), which produce a diuretic peptide hormone (Proux *et al.* 1987, 1988). These cells project into all ganglia of the CNS and, at least in *Locusta migratoria*, establish neurohaemal release sites close to the origins of peripheral nerves (Rémy and Girardie, 1980; Girardie and Rémy, 1980). The second are the eclosion-hormone cells in larval Lepidoptera, which project from the brain to the proctodeal nerve (Truman and Copenhaver, 1989).

Several features of the VPM neurones identify them as neurosecretory cells. The anterior axons project into the principal neurohaemal organ, the corpora cardiaca. We also suspect that the second VPM axon collateral in NCC IIIb (Fig. 8) proceeds into an additional neurohaemal area that is established by the neurones of NCA II in the vicinity of muscle 45 (Böhme and Bräunig, 1990, and in preparation). Whole-mount BPP-immunocytochemistry of this region shows varicose terminals on the outer surface of NCC IIIb but not on the three pharyngeal dilator muscles 43–45 (P. Bräunig, unpublished results). The VPM neurones also establish varicose terminals, typical of neurosecretory cells, along the lateral cardiac nerve cords. These nerves, at least partially, function as neurohaemal organs (Johnson, 1966; Miller and Thomson, 1968; Jones, 1977). Finally, the large soma spikes, typical of insect neurosecretory and neuromodulatory cells, and the obvious peptidergic nature of the VPM neurones further support their identification as neurosecretory cells.

Are the VPM cells 'heart neurones'?

The neurones described in the present study were previously labelled the 'ventral posterior median' (VPM) neurones of the NCC III because of the location of their cell bodies within the suboesophageal ganglion (Bräunig, 1990). Now that their peripheral targets are known, one might call them the 'suboesophageal heart neurones'. However, this should not be taken to imply that the neurones can affect the heart by their activity as no evidence for such effects has been obtained. Stimulation of the neurones affects neither the amplitude nor the frequency of the heartbeat (P. Bräunig, unpublished results).

This result is quite intriguing for two reasons. First, the neurones can be stained with antisera against BPP. This suggests that the cells contain a peptide related to the FMRFamide family. Second, a large number of FMRFamide analogues have been tested in the semi-isolated locust heart preparation (Cuthbert and Evans, 1989) and many of them exerted excitatory, inhibitory or biphasic effects.

There are two possible explanations for these findings. One possibility is that the VPM neurones use the lateral cardiac nerve cords simply as an optimal release site. Agents released in the immediate vicinity of the main circulatory organ are

probably dispersed much faster over the entire haemolymph volume than agents released at other sites. It is interesting to note in this context that at the ultrastructural level the lateral cardiac nerve cords closely resemble neurohaemal organs (Johnson, 1966; Miller and Thomson, 1968). Furthermore, none of the neurones that have so far been shown to innervate the heart, or are suspected of doing so, cause any changes in heartbeat frequency or amplitude when they are stimulated (M. E. Adams, cited in Miller, 1985*b*; Ferber and Pflüger, 1990; and the present study). This is consistent with the observation that it is not possible to 'drive' heartbeat by the stimulation of the segmental heart nerves (Miller, 1985*a*).

The second possibility is that the effects of the neurone on spontaneous heart contractions cannot be observed in intact preparations. The effects of FMRF-amide analogues on the semi-isolated heart (Cuthbert and Evans, 1989) were most pronounced in preparations that showed irregular or bursting activities of the heart. Only small effects were observed when the heart was beating regularly. To test possible effects resulting from stimulation of the VPM neurones, the innervation of the heart had, of course, to be left intact. We never observed irregular or bursting activity of the heart in such preparations. Since the VPM neurones are usually active at low firing frequencies (P. Bräunig, unpublished observations), it could well be that all putative receptors of the heart muscle for the agent produced by the VPM cells are saturated under normal conditions and that stimulation of the neurones does not cause any additional effects. Experiments are currently under way to test this hypothesis.

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