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MORPHOLOGY OF IDENTIFIED NEURONES IN THE BUCCAL GANGLIA OF *LYMNAEA STAGNALIS*

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SUMMARY

The morphology of seven types of identified neurones in buccal ganglia of *Lymnaea* was investigated by intracellular injection of Procion Yellow and retrograde injection of cobaltous chloride into the nerve roots of the buccal ganglia. The results provided anatomical support for the electrophysiological findings that some cells are motoneurones for muscles of the buccal mass (type 4-group cells, types 6 and 8 cells). Others project to nerves innervating oesophageal tissue (types 2, 3 and 5 cells) and to pro-oesophageal tissue (types 3 and 5 cells). The type-1 cells project to the salivary gland ducts and are similar to the salivary gland motoneurones found in other molluscan species.

INTRODUCTION

The neural geometry and axonal projections of gastropod neurones have been investigated by a number of techniques in the last few years in attempts to correlate morphology, at the light-microscope level, with the large amount of electrophysiology recently carried out on mulluscan neurones (for review, see Dorsett, 1973). A Golgi study revealed the general features of molluscan neuronal morphology (Benjamin & Ings, 1972) but techniques which allow the morphology of identified neurone types to be reconstructed have also been used. These include injection of Procion Yellow (Kater & Kaneko, 1972; Benjamin, 1976; Benjamin, Swindale & Slade, 1976; Blackshaw, 1976), cobalt chloride (Winlow & Kandel, 1976), radioactive tracers (Pentreath & Cottrell, 1974) and staining of specific neurosecretory cell types using Alcian Blue–Alcian Yellow (Swindale & Benjamin, 1976).

In the present study the neural geometry of *Lymnaea* buccal neurones, identified in the previous study of Benjamin & Rose (1979), were investigated by intracellular iontophoretic injection of Procion Yellow and by retrograde injection of cobalt chloride into the nerve roots of the buccal ganglia. The main object of this study was to discover the peripheral nerve projections of motoneurones in the feeding system of *Lymnaea*, and to correlate these findings with a parallel electrophysiological study of muscular activity during feeding (Rose & Benjamin, 1979).

We have also carried out dissections to confirm the innervation of the buccal mass by nerves of the buccal ganglia, as described previously by Carriker (1946) and Goldschmeding & de Vlieger (1975).

MATERIALS AND METHODS

Iontophoresis of Procion Yellow

Procion Yellow (M-4RS) filled microelectrodes were used to penetrate the cell bodies of buccal neurones which were identified on the basis of size and position or by their electrical properties (see Benjamin & Rose, 1979), in an isolated preparation which is shown diagrammatically in Fig. 1. One or two cells were injected in each

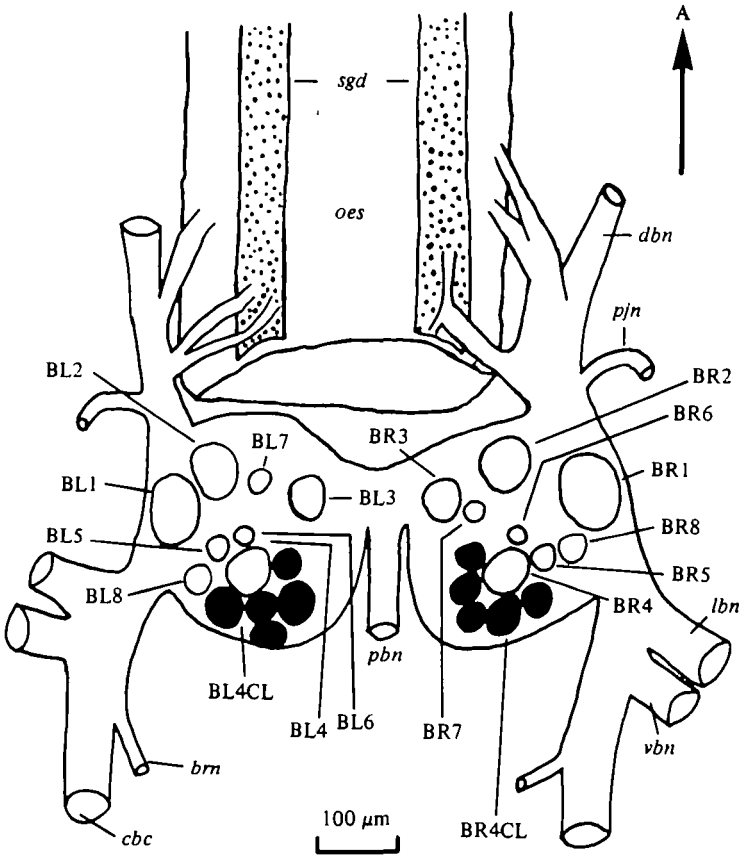

Fig. 1. Diagram of the dorsal surface of the buccal ganglia of *Lymnaea* showing the locations of identified neurone types BL1–BL8 and BR1–BR8. The dorsobuccal nerves (*dbn*) innervate the oesophagus (*oes*) and the salivary gland ducts (*sgd*). All the nerve roots of the buccal ganglia are paired except the postbuccal nerve (*pbn*). Other abbreviations are: A, anterior; *brn*, buccal retractor nerve; *cbc*, cerebrobuccal connective; *lbn*, laterobuccal nerve; *vbn*, ventrobuccal nerve; *pjn*, posterior jugalis nerve.

preparation which was then kept in snail saline overnight at 4 °C. The material was then fixed in Stieve's fluid, which is used routinely in this laboratory for the histology of the snail brain. We have not noticed that the brightness of injected cells under ultraviolet light is any less with this fixative compared with the usual ones used for Procion dye work (such as buffered glutaraldehyde). Fixed material was embedded in wax after dehydration and serially sectioned at 10 μm. Neurones were reconstructed using a *camera lucida* attached to a Leitz 'orthoplan' microscope. This was a complex

process because many of the larger cells (e.g. the 3 cell of Fig. 5*a*) needed to be traced through more than 100 sections. Large-scale drawings of cells were prepared by fitting together drawings of parts of the cell projected in sequence on to the same sheet of tracing paper. Finally, a continuous outline of the cell was drawn into a reconstructed ganglion outline also obtained from serial sections. It was found that the most accurate two-dimensional drawing of buccal cells could be obtained if the preparation was sectioned 'horizontally', that is in the plane of the drawing shown in Fig. 1. All the cells shown in this paper were reconstructed from horizontal sections, and their orientation can easily be compared with the arrangement of nerves and location of cell bodies shown in this figure. Photographs of Procion-filled neurones were taken with Gaf 500 colour film or Kodak 2475 black and white film. Some examples of photographed material are shown in Fig. 6 (*a–e*), but the best impression of filled cells can only be obtained from drawings, which are used in all the other figures in this paper.

Back injection of cobalt chloride

Cobalt chloride was passively introduced into the axons of buccal neurones by diffusion into the cut ends of buccal nerves dipped into cobaltous chloride and left for 2–3 days in a refrigerator. After treatment with ammonium sulphide the brains were fixed in Carnoy's fluid, dehydrated and cleared in methyl salicylate in a manner which was similar to the method used by Winlow & Kandel (1976). Neurones filled by this method were easily observed in whole mounts, and were drawn or photographed using a Wild M7 binocular microscope. It was difficult to prevent the cobalt chloride diffusing into the connective tissue surrounding the nerves and thence into the surface tissue of the ganglia itself, and this spoilt many preparations and made good photography difficult. In general, as with the Procion reconstructions, drawings were more useful than photographs for showing the morphology of injected cells, but an example of photographed material is shown in Fig. 6 (*f*).

RESULTS

Innervation of the buccal mass by nerves of the buccal ganglion

The innervation of the buccal mass of *Lymnaea* has been described by Carriker (1946) and Goldschmeding & de Vlieger (1975). As an introduction to our account of the motoneurone control of muscles, their nerve supply is summarized in semi-diagrammatic form in Fig. 2, based largely on these earlier descriptions. We have chosen this form of representation so that the reader can quickly verify that a given identified neurone and its axon branch could supply a given muscle based on anatomical evidence. To avoid the complication of showing a nerve supplying both superficial and deep muscles, the buccal mass has been cut along the dorsal and ventral line and the two halves splayed out (Fig. 2). The anterior jugalis has been removed from the right side and only deeper nerve branches are shown on that side, while the more superficial muscles and nerve branches are shown on the intact left side.

The main nerve trunks leaving the buccal ganglia are (using the terminology of Carriker, 1946) the dorsobuccal, laterobuccal, ventrobuccal and postbuccal nerves, while the ganglia themselves are connected to the cerebral ganglia by the paired cerebrobuccal connectives (Fig. 2). The buccal nerves are all paired with the exception

of the postbuccal nerve, which arises between the buccal ganglia and passes ventrally to the radular sac (Fig. 2). Although this nerve innervates secondary structures such as the collostylar hood and the epithelium of the pro-oesophagus, the main point is that it supplies the suspensor muscles and the radular tensor muscles (Fig. 2).

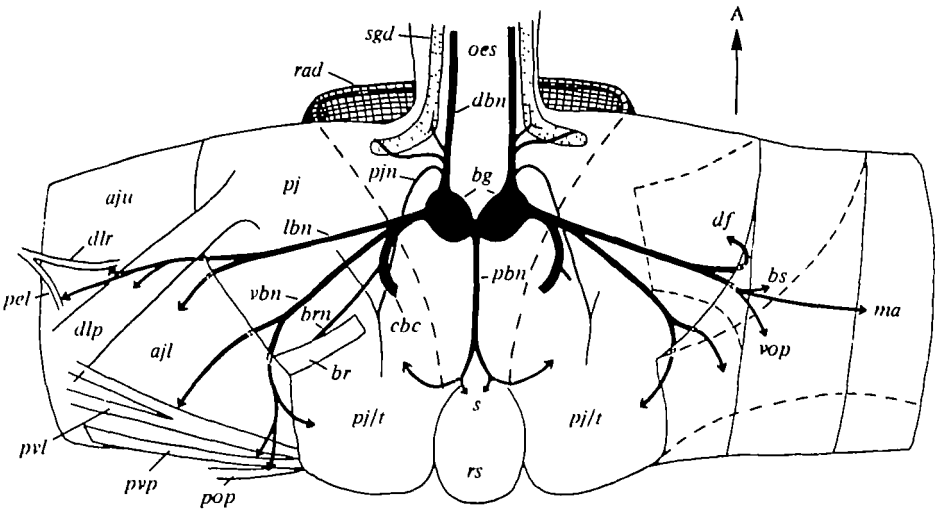

Fig. 2. Innervation of the muscles of the buccal mass. The buccal mass has been cut along the dorsal and ventral mid-line and the two halves spread out to show the superficial innervation (left) and deep innervation (right). To show the deep innervation the anterior jugalis muscle has been removed on the right side. The radula (*rad*) is mostly hidden from view, but has been pulled forward slightly to show its position. There are five pairs of nerves leaving the paired buccal ganglia (*bg*): *dbn*, dorsobuccal nerve; *pjn*, posterior jugalis nerve; *lbn*, laterobuccal nerve; *vbn*, ventrobuccal nerve; *pbn*, postbuccal nerve. In addition the buccal ganglia are joined to the cerebral ganglia by the paired cerebrobuccal connectives (*cbc*), both of which has a small nerve branch to the buccal retractor muscle (*brn*, buccal retractor nerve). Muscle abbreviations: *pj*, posterior jugalis; aj_u, upper anterior jugalis; aj_l, lower anterior jugalis; *dlp*, dorsolateral protractor; *dlr*, dorsolateral retractor; *pel*, preventral levator; *pvl*, postventral levator; *pvp*, preventral protractor; *pop*, postventral protractor; *br*, buccal retractor; *df*, dorsal odontophoral flexor; *bs*, buccal sphincter; *vop*, ventral odontophoral protractor; *ma*, mandibular approximator; *s*, suspensor muscles. Other abbreviations: *rs*, radula sac; *oes*, oesophagus; *sgd*, salivary gland duct. The innervation pattern is based on dissections and the results of Carriker (1946) and Goldschmeding and de Vlieger (1975) and is semi-diagrammatic. The laterobuccal nerve (*lbn*) supplies the largest number of muscles, including *dlp*, *dlr*, *pel*, aj_u, aj_l (see left) and *df*, *bs*, *ma* and *vop* (see right). The posterobuccal nerve supplies *pj/t* and *s*. The posterior jugalis nerve goes to the *pj* muscles, while the dorsobuccal nerve supplies *sgd* and *oes*.

The paired laterobuccal and ventrobuccal nerves pass over the sides of the buccal mass up to the posterior edge of the anterior jugalis muscle. From here the laterobuccal nerve continues forward to supply the anterior jugalis muscle itself, the superficially placed dorsolateral protractor, and the small dorsolateral retractor and preventral levator (Fig. 2, left). The same nerve supplies all the deeper muscles lying underneath the anterior jugalis (Fig. 2, right), these being the mandibular approximator, ventral odontophoral protractor, dorsal odontophoral flexor and buccal sphincter.

The ventrobuccal nerves supply two important sets of muscles – firstly the group of strand-like muscles running anteriorly, which comprise the postventral levator and postventral protractor, and secondly the large tensor muscles (Fig. 2). The tensor muscles thus receive a dual supply, from this nerve and from the postbuccal nerve. The

ventrobuccal nerve also innervates the ventral odontophoral protractor (Fig. 2, right).

The dorsobuccal nerves run along the length of the oesophagus and innervate it at various points (Fig. 1), but close to the buccal ganglion send small branches to the salivary gland ducts and posterior jugalis muscle (Figs. 1, 2). We refer to the last nerve as the posterior jugalis nerve. The posterior jugalis nerves sometimes originate from the anterior surface of the buccal ganglion rather than the root of the dorsobuccal nerve.

The last nerves to be mentioned are the buccal retractor nerves which originate from the cerebrobuccal commissure (Fig. 2, left) and innervate the buccal retractor muscle.

Procion Yellow-injected neurones

In the previous paper eight different neurone types were identified in the buccal ganglia of *Lymnaea*, and their cell body locations are shown in Fig. 1. We have made reconstructions of 1, 2, 3, 4 group, 5 and 8 cells, but not the 6 and 7 cells. The buccal cells could be divided into two main classes based on their nerve projections. The first class included those cells which projected to the dorsobuccal nerve and subsequently innervated the pro-oesophagus (2 and 5 cells) or salivary gland ducts (1 cells) while the second class included those cells which projected to nerves innervating the buccal musculature (4 group and 8 cells). One cell type, the 3 cell, showed features common to both of these classes in that it had axonal projections to both the dorsobuccal nerves (and pro-oesophagus) and to the laterobuccal nerves.

Cells projecting to the salivary gland ducts (1 cells)

The cell bodies of the 1 cells are the largest in the buccal ganglia, easy to find, and consequently frequently filled with Procion Yellow. We have obtained eight good fills of these cells (3 of BR1 and 5 of BL1) which we considered complete enough for reconstruction, including one preparation in which both BL1 and BR1 were filled (Fig. 3*a*).

Every reconstructed 1 cell shows an axonal projection to both the left and right dorsobuccal nerve. The ipsilateral nerve branch of 1 cells divides and sends one or two branches to the salivary gland duct (Fig. 3*a*, *b*) and a finer branch along the part of the dorsobuccal nerve which innervates the gut (Fig. 3*a*, *b*). However, we have been unable to trace this latter branch to the pro-oesophagus, and it may be that it terminates in gut tissue more distal to that retained in our preparations. The contralateral 1 cell axon could also be traced into salivary and oesophagal branches (Fig. 3*a*) of the dorsobuccal nerve in some preparations. The ipsilateral 1 cell axon to the salivary gland duct may well continue to the salivary gland tissue proper (see Kater, Murphy & Rued, 1978), but this could not be ascertained in the present experiments.

We have carried out several double injections of 1 cells, and the most successful is shown in Fig. 3*a*. At first we thought it might be difficult to trace the axonal pathways of two similarly projecting neurones injected with the same dye. In practise, it was possible because the amount of dye injected and thus the brightness of processes of the cells was sufficiently different to separate even the more distal axons of the two cells. Another source of confusion in double injections could arise from Procion Yellow travelling across electrotonic junctions. It is known from electrophysiological experi-

ments (Benjamin & Rose, 1979) that I cells are coupled by junctions of this type, and indeed the axons of BLI and BRI are closely apposed at various points, particularly in the buccal commissure and in the dorsobuccal nerves (Fig. 3*a*). However, in none of our single fills did we see any discontinuities of brightness of fluorescence or sudden changes in axonal diameters without branching, which would suggest that dye movement between electrotonically coupled neurones is occurring in insufficient quantity to confuse our reconstructions of I cells.

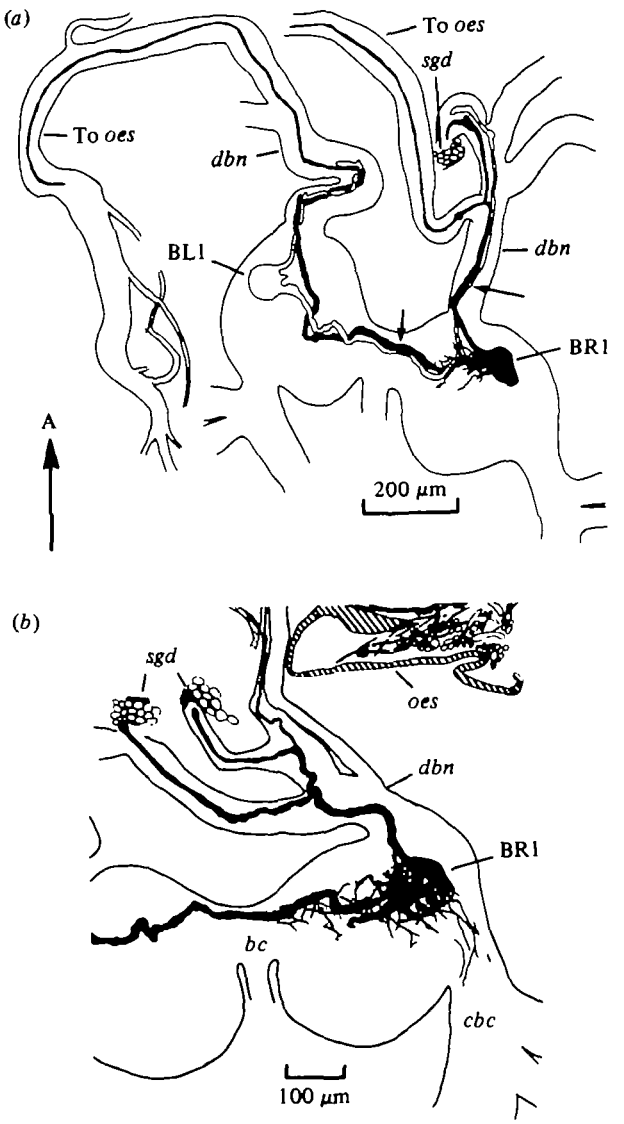

Fig. 3. Drawings of Procion Yellow injected I cells reconstructed from serial sections. (*a*) BLI and BRI injected in the same preparation with closely apposed axonal projections (arrows) in the buccal commissure and dorsobuccal nerves (*dbn*). Both cells have ipsilateral and contralateral axonal projections to the dorsobuccal nerves and axons can be traced to the salivary gland duct (*sgd*) on the right side (see also (*b*)). Another finer axon continues along the oesophageal branches (to *oes*) of the dorsobuccal nerves. (*b*) shows the complicated dendritic processes of I cells which are extremely dense close to the cell body. Several of these fibres project to the cerebrobuccal connective (*cbc*).

We were impressed by the symmetry in the axonal projections of the 1 cells in Fig. 3(*a*), the arrangement of processes in one cell forming a mirror image of the processes in the other.

A dense mass of short fine dendritic branches occur on the proximal axon of 1 cells (Fig. 3*b*). These dendrites often branch once or twice and then terminate in the neuropile of the ipsilateral buccal ganglion. Occasionally fine processes, originating from the proximal axon, projected as far as the root of the cerebrobuccal connective (Fig. 3*b*). No fine branching was seen on 1 cell axons once they left the ipsilateral ganglion neuropile (Fig. 3*a*, *b*).

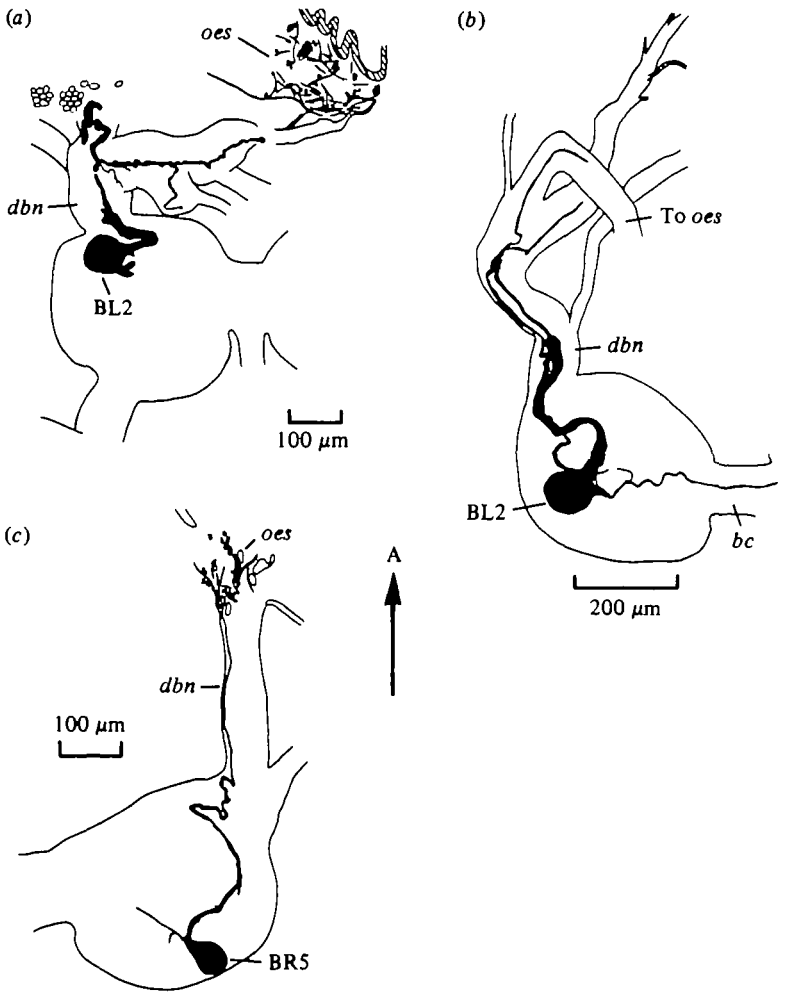

Fig. 4. Drawings of Procion Yellow injected 2 and 5 cells showing their projections to the dorsobuccal nerves (*dbn*). Although both BL2 neurones have axonal branches to the oesophageal branches of the dorsobuccal nerves these branches could not be traced to oesophageal tissue itself, although they were close to it in (*a*), whereas the 5-cell dorsobuccal axon in (*c*) terminated in the pro-oesophagus (*oes*). The 2 cell of (*b*) was unusual in that it had two dorsobuccal nerve branches originating from the cell body. None of the cells shown had prominent axonal branches to the contralateral buccal ganglia although the BL2 of (*b*) had a fine axonal branch to the buccal commissure (*bc*) which could not be traced beyond the edge of the contralateral ganglion neuropile.

Cells projecting to the pro-oesophagus (2 and 5 cells)

The dorsobuccal nerves innervate the oesophagus (Figs. 1, 2), which is the part of the alimentary canal posterior to the buccal mass (Carriker, 1946). Three pairs of neurones projected to the pro-oesophagus, the 2, 3 and 5 cells. The 3 cells have an additional projection to the laterobuccal nerve and will be considered in the next section.

BL2 and BR2 are the second largest neurones in the buccal ganglia (Fig. 1) and can easily be recognized on the basis of their size and location. BL5 and BR5 are much smaller cells (Fig. 1), and can only be identified after electrode penetration by their receipt of double inhibitory inputs (Benjamin & Rose, 1979).

Five 2 cells were reconstructed, and all had a large axonal projection to the ipsilateral dorsobuccal nerve (Fig. 4*a*). No equivalent contralateral projection is present (Fig. 4*a*, *b*). In one example, two axons arose from separate but adjacent points on the cell body and projected to the dorsobuccal nerve (Fig. 4*b*). Whether there were one or two branches of a 2 cell to the dorsobuccal nerve, their axons always projected to oesophagal branches of the dorsobuccal nerve rather than to its salivary gland roots (Fig. 4*a*, *b*). We were not able to trace the 2 cell axons into gut tissue itself, although some axons could be traced very close to it (Fig. 4*a*).

In some 2 cells a second finer axonal branch could be traced to the buccal commissure (Fig. 4*b*), but this branch never extended beyond the edge of the buccal commissure close to the contralateral ganglion neuropile. It certainly never reached the area of the contralateral ganglion where the 2 cell of the opposite ganglion is usually positioned.

The 5 cells were extremely similar to the 2 cells in that the two reconstructed neurones had a single nerve projection to the ipsilateral dorsobuccal nerve, and this could be traced to its terminal in pro-oesophageal tissue (Fig. 4*c*). The 5 cell of Fig. 4(*c*) had a second finer axon which divided and terminated in the ipsilateral ganglion neuropile.

Unlike other buccal neurones the 2 and 5 cells had no prominent axonal projections to the opposite buccal ganglion and this lends support to the electrophysiological experiments (Benjamin & Rose, 1979), which failed to find connexions between pairs of 2 cells or pairs of 5 cells. At no point do either of these cell types' axons come close enough for such connexions to be possible.

Cells projecting to the pro-oesophagus and buccal mass (3 cells)

3 cells have quite large cell bodies which occur in characteristic locations in the buccal ganglia (Fig. 1). Like the 1 cells they have axonal projections to nerves of the left and right buccal ganglia but they are more complicated than 1 cells in that they project to two nerve roots on each side. Each of the four reconstructed 3 cells had axonal projections to the left and right laterobuccal and dorsobuccal nerves (Fig. 5). The juxtaposition of BL3 and BR3 in the same figure (Fig. 5) emphasizes the mirror image axonal symmetry of these cells. Some of the axonal projections of another 3 cell were photographed and are shown in Fig. 6 (*b–e*). These photographs show that BR3 has a thick axonal projection to the contralateral buccal ganglion (Fig. 6*b*)) with two finer axonal branches to the right laterobuccal and dorsobuccal nerves (Fig. 6*d*). A small

part of the dorsobuccal axon branch is shown in the vicinity of oesophageal tissue (Fig. 6*e*). An overall impression of this neurone can be gained from the reconstruction shown in Fig. 6(*a*) with the cell body locations confirmed by examination of the

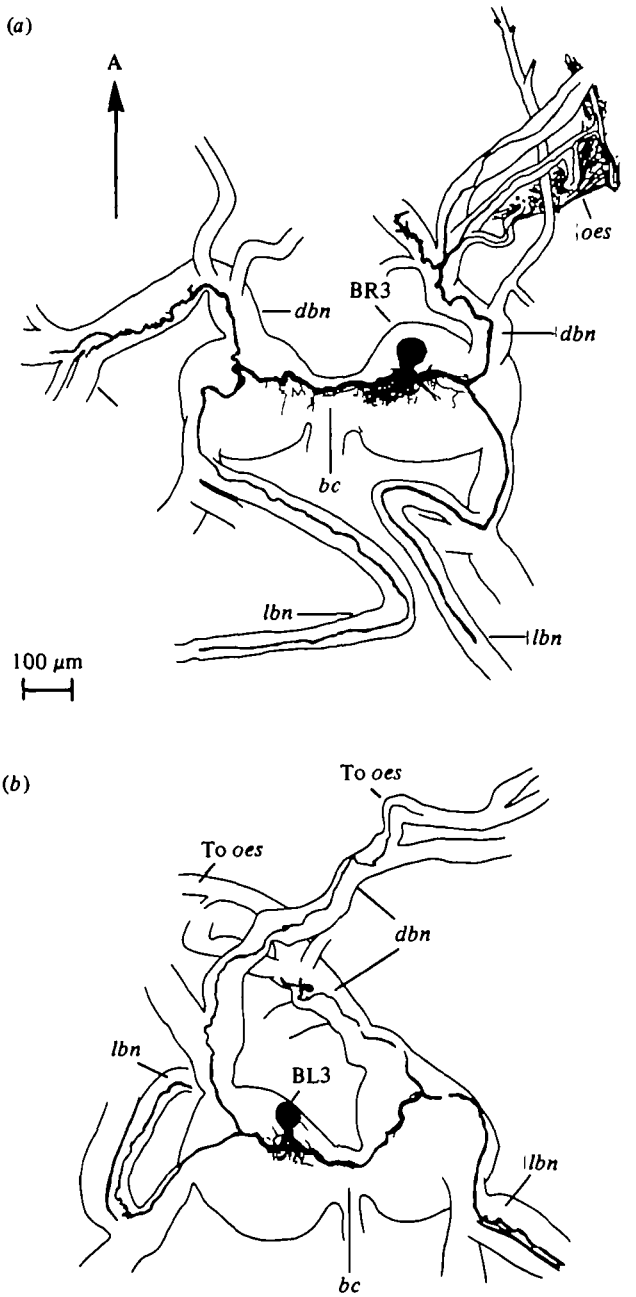

Fig. 5. Reconstructions of Procion Yellow injected BR3 (*a*) and BL3 (*b*) showing their complex axonal projections. Both 3 cells have axonal projections to the left and right laterobuccal (*lbn*) and dorsobuccal (*dbn*) nerves. Axonal projections could be traced to the pro-oesophagus (*oes*) or oesophageal branches (to *oes*) but not to salivary gland branches of the dorsobuccal nerves. Note the complex dendritic branching of 3 cells close to the cell bodies.

adjacent photograph of Fig. 6(*c*). Examination of Fig. 6(*a*) shows that a second cell (BL3) was injected in this preparation. Enough of the cell was revealed to indicate that the proximal axons of BL3 and BR3 are sufficiently close in the buccal commissure for this to be a likely site for the electrotonic junction connecting these cells (Benjamin & Rose, 1979).

It was not possible to follow the projections of the 3 cells in the laterobuccal nerves to a peripheral tissue, and the large number of muscles which this nerve innervates (Fig. 2) does not allow us to identify a muscle which a 3 cell might innervate. It is known that 3 cells fire during the first phase of retraction of the buccal mass (Rose & Benjamin, 1979) and this is consistent with the fact that a number of muscles innervated by the laterobuccal nerve fire during this phase. However, in electrophysiological experiments we were unable to find any muscle whose electrical activity was influenced by 3 cell firing. It is interesting to note that the laterobuccal nerve splits into three main branches, the most dorsal one of which branches profusely over the buccal glands of the buccal mass (Carriker, 1946). It is therefore quite possible that the 3 cells control the secretory activity of these glands, which would account for our failure to record 1:1 firing in any of the muscles.

3 cell axons in the dorsobuccal nerve divide and project along several nerve branches to the pro-oesophagus (Fig. 5*a*). In one case the ipsilateral dorsobuccal nerve axons could be traced into extremely fine nerve terminals ending in the pro-oesophagus (Fig. 5*a*). In the contralateral dorsobuccal nerve the 3 cell axons could be traced for long distances (Fig. 5*a*, *b*), but never to terminals in gut tissue. This was also the case for many ipsilateral dorsobuccal 3 cell axons (Fig. 5*a*, *b*), which suggests that some branches of 3 cells may be innervating gut tissue at some distance (at least more than a few mm) away from the buccal ganglion.

One characteristic feature of all 3 cells was the dense dendritic branching which occurred close to the cell body (Figs. 5*a*, *b*, 6*b*). The BR3 of Fig. 5(*a*) had a particularly complex system of proximal branching which was as complicated as the dendritic branching of large neurones outside the buccal ganglia, revealed by Golgi techniques (Benjamin & Ings, 1972). These fibres terminate within 200 μm of the cell body in the ipsilateral ganglion neuropile. A few fine fibres originate from 3 cell axons in the buccal commissure and contralateral ganglion neuropile (Fig. 5*a*, *b*). These also terminate locally in the neuropile adjacent to the axon.

Projections to nerves innervating the buccal musculature (4 *group and* 8 *cells*)

4-group and 8 cells have axons which project to the laterobuccal and ventrobuccal nerves (Fig. 7). These nerves innervate a number of muscles in the buccal mass (Fig. 2).

4 cells (BL4 and BR4) are large identifiable neurones which are surrounded by a cluster of smaller cells (BL4 cluster and BR4 cluster) with similar electrical properties, but which are not individually identifiable. The arrangement of these cells is shown in Fig. 1 and their electrical properties described in Benjamin & Rose (1979). Many 4 cells were injected and three reconstructed. A smaller number of 4 cluster neurones were injected and two of them reconstructed. However, the results from Procion Yellow injections should be compared with the cobalt chloride back fills which are described in the next section and give a better idea of the overall projections of the 4 cluster cells.

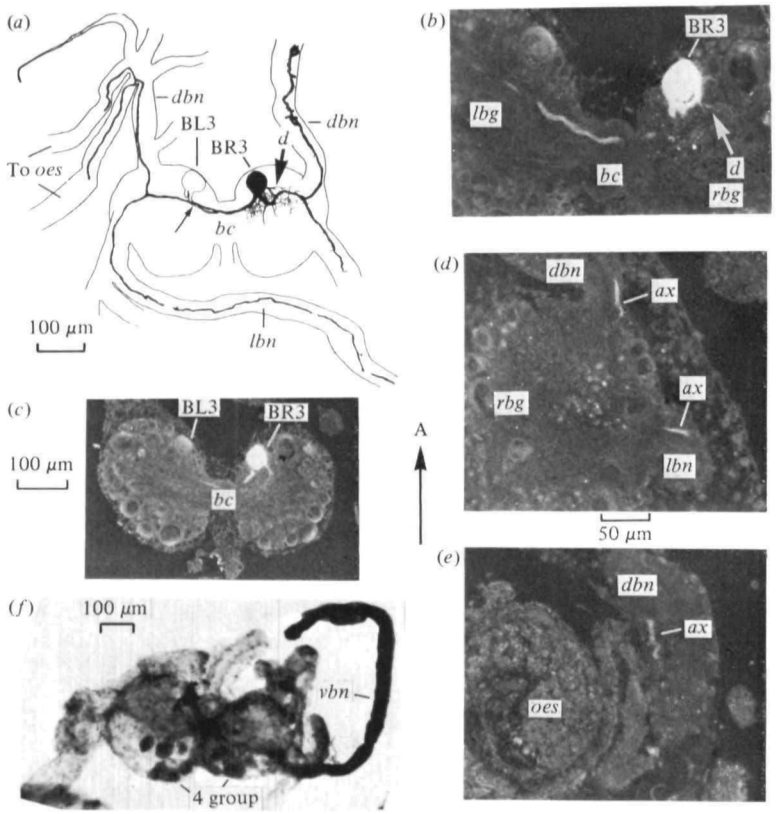

Fig. 6. (*a*) Drawing of reconstructed Procion Yellow injected BR_3 and BL_3 with photographs of BR_3 from the same preparation shown in (*b–e*). The location of cell bodies in (*a*) confirmed by the photograph in (*c*). Axonal projections of BR_3 to left buccal ganglion (*lbg*) shown in (*b*), to the left dorsobuccal nerve (*dbn*) and laterobuccal nerve (*lbn*) in (*d*). (*e*) shows the dorsobuccal nerve projection close to oesophageal tissue (*oes*). Dendritic branch (*d*) originating from the cell body of BR_3 shown by arrow in drawing of (*a*) and photograph in (*b*). (*f*) Cobalt chloride back injection of the right ventrobuccal nerve (*vbn*) filled 4-group neurones in both the right and left buccal ganglia (drawing of this fill shown in Fig. 8*d*). Other abbreviations are: *bc*, buccal commissure; *rbg*, right buccal ganglion.

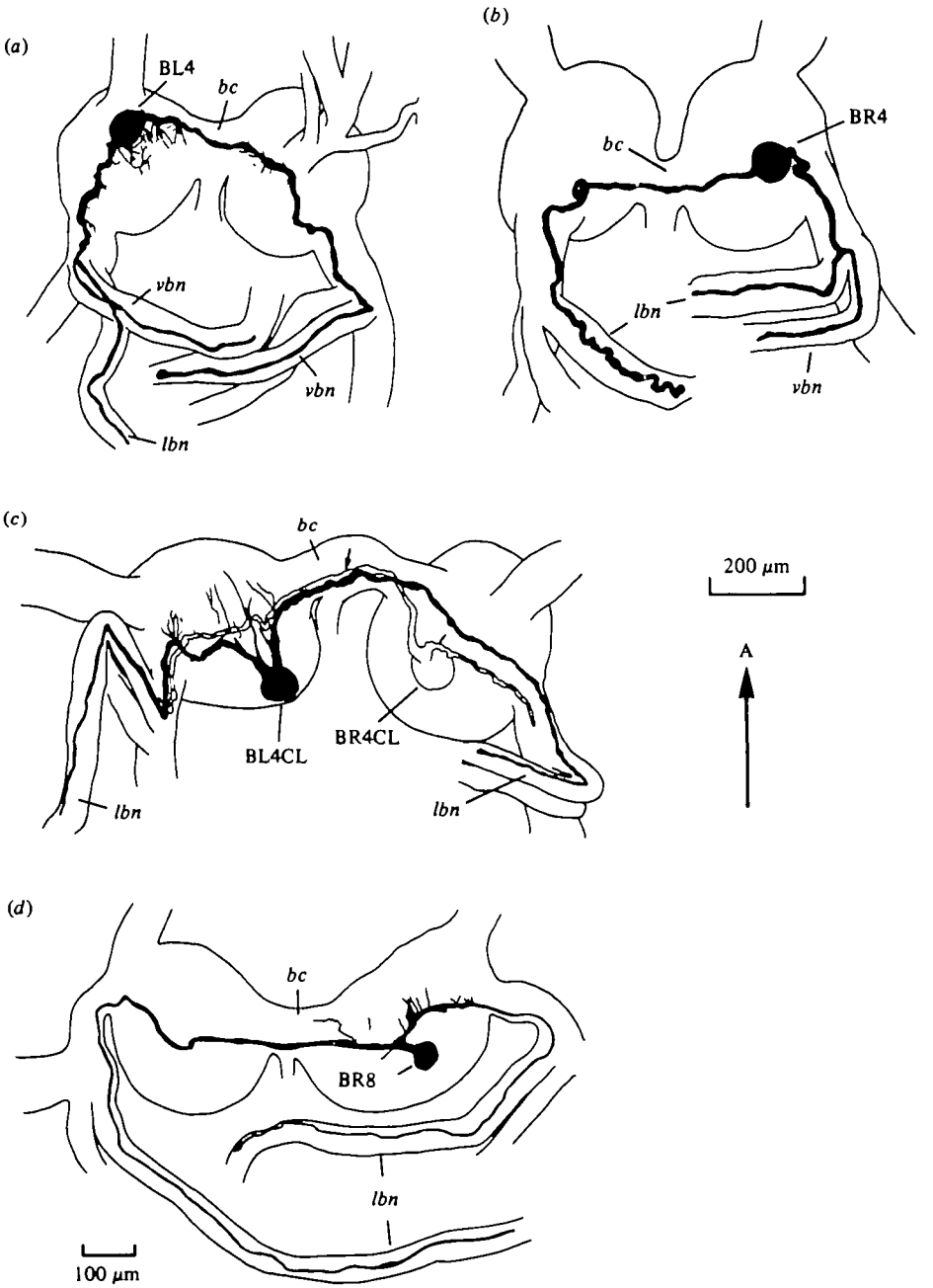

Fig. 7. Drawings of Procion Yellow-filled 4 group and 8 cells projecting to the laterobuccal (*lbn*) and ventrobuccal (*vbn*) nerves. (*a*) and (*b*) are 4 cells which project ipsilaterally to the ventrobuccal and laterobuccal nerves but contralaterally to the ventrobuccal nerve (*a*) or to the laterobuccal nerve (*b*). Note the tufted, serially arranged dendritic processes originating from BL_4 in (*a*). (*c*) BL_4 cluster cell (BL_4CL) and BR_4 cluster cell (BR_4CL) injected in the same preparation. Arrow marks point of close contact between these cells in the buccal commissure (*bc*). Axonal branches of the best filled cell (BL_4CL) were to the left and right laterobuccal nerves. (*d*) BR8 neurone with axonal projections to the right and left laterobuccal nerves.

There appears to be some variation in the peripheral projections of the 4 cells. BL4 and BR4 neurones shown in Fig. 7 (*a*, *b*) both have ipsilateral projections to the laterobuccal and ventrobuccal nerves, but BL4 projects contralaterally to the ventrobuccal nerve (Fig. 7*a*) whilst BR4 projects contralaterally to the laterobuccal nerve (Fig. 7*b*). In a third 4 cell reconstruction (a BR4) only one ipsilateral 4 cell projection occurred (to the ventrobuccal nerve) together with a single contralateral projection to the ventrobuccal nerve. This variability in the axonal projections of 4 cells seems to be genuine, and is not related to problems of tracing fibres in serial sections. All three cells we have described as having variable nerve projections, had plenty of Procion Yellow in them and were easy to follow.

The most complete 4 cluster cell we have reconstructed (the dark-shaded cell of Fig. 7*c*) had an axonal branch in both the left and right laterobuccal nerves. Double injections of left and right 4 cluster cells (Fig. 7*c*) showed that the axons of such cells come close together at various points, particularly in the buccal commissure. These points of contact could be the sites of the electrotonic junctions found in electrophysiological experiments (Benjamin & Rose, 1979).

The dendritic branching of 4-group cells was not as dense on the proximal axons as 1 or 3 cells and consisted of single fine fibres, which could themselves have side branches, or tufts of fine fibres arising from a small segment of axon (Fig. 7*a*, *c*). These fibres spread serially along the two main branches of the 4-group cells but do not usually occur beyond the ipsilateral ganglion neuropile. Single, short undividing dendrites were seen on more distal axon branches in the contralateral neuropile (Fig. 7*a*).

The significance of the 4-group projections to buccal nerves will be considered in the cobalt chloride section which follows this one.

We have obtained only one good fill of an 8 cell which is shown in Fig. 7(*d*). Axonal projections to the left and right laterobuccal nerves were found (Fig. 7*d*), which is consistent with electrophysiological findings which showed that the 8 cells are motoneurones for the anterior jugalis muscle (Rose & Benjamin, 1979). Fig. 2 shows the laterobuccal nerve is alone in innervating this muscle.

Cobalt chloride back injections

We needed to correlate neurones filled with cobalt chloride, by retrograde nerve injection, with our map of identified neurones in the buccal ganglia. This was possible in the case of the 4-group cells which form an easily recognized cluster on the posterodorsal surface of each buccal ganglion (Fig. 1). In addition, we have obtained some useful information by back injection of the posterior jugalis nerve, about the identification of probable motoneurones of the posterior jugalis muscle.

4-group cells

Back injection of the laterobuccal nerves and ventrobuccal nerves filled 4-group cells (Figs. 6*f*, 8*a–d*). Left or right nerves were filled with cobalt chloride and cell bodies from both ipsilateral and contralateral 4-group clusters were marked irrespective of which nerve was injected. However, more cells seemed to be injected from backfills of ventrobuccal nerves compared with laterobuccal nerves (compare Fig. 8*c*, *d* with

Fig. 8*a*, *b*). The largest cell bodies which were stained with cobalt sulphide were probably BL4 or BR4 and these were most commonly seen after back injection of the ipsilateral ventrobuccal nerve (e.g. Fig. 8*c*), although a contralateral 4 cell appeared to be filled in Fig. 8 (*d*) (photographed in Fig. 6*f*). Overall, it appeared that a number of 4-group cells could be injected from any of the four nerves concerned, and this applied to cells located on the same or opposite side to the injected nerve.

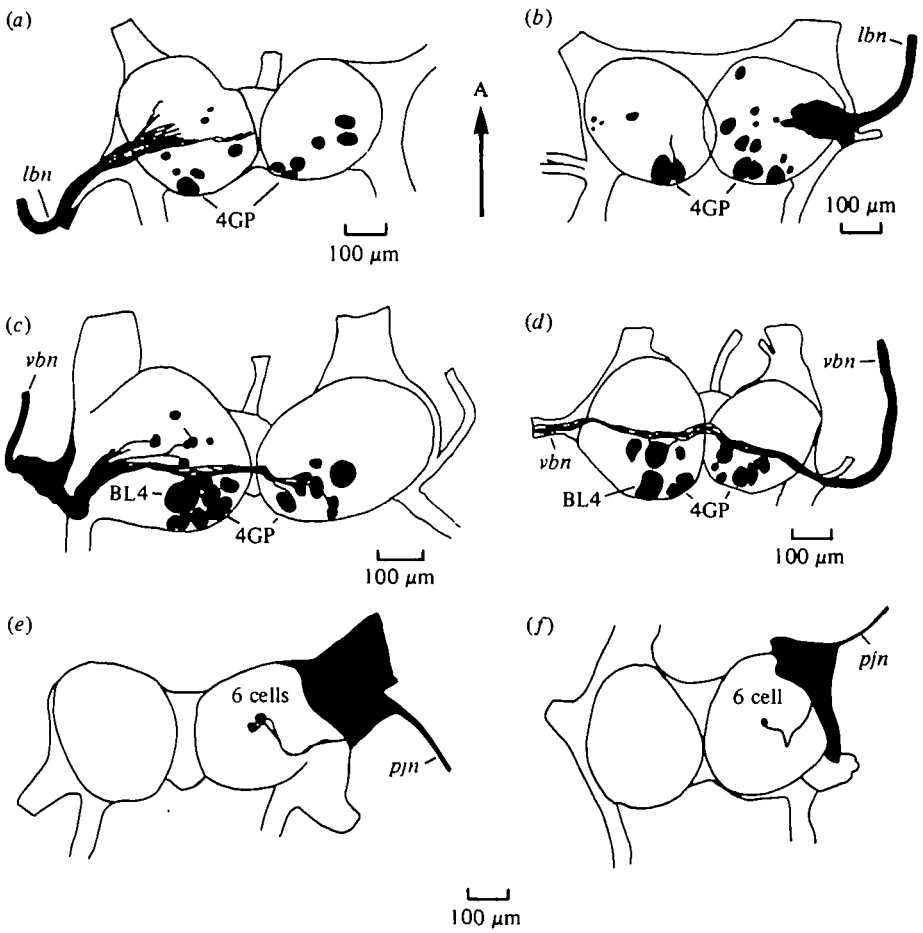

Fig. 8. Drawings of 4-group and 6 cells filled by retrograde injection of cobalt into the nerve roots of the buccal ganglia. (*a*–*d*) Back-fills of 4-group cells and other smaller neurones from laterobuccal (*lbn*) and ventrobuccal nerves (*vbn*). Injection of any of these nerves filled cells in both left and right buccal ganglia. More cells were filled from the ventrobuccal nerves compared with laterobuccal nerves. (*e*, *f*) Back fills of probable 6 cells from the posterior jugalis nerves. The back-fill of (*d*) has been photographed and shown in Fig. 6(*f*) (note that the distal part of the right ventrobuccal nerve has not been drawn in (*d*)).

The back-filling results using cobalt chloride agreed with those obtained by Procion Yellow injections of particular cell bodies and showed in addition that the 4 cluster cells could project along the ventrobuccal nerves as well as the laterobuccal nerves.

The results from both sorts of anatomical technique are consistent with electrophysiological evidence of 4-group function reported in detail in the following paper of

Rose & Benjamin (1979). Electrophysiological evidence suggests that the 4 cells (BR4 and BL4) are motoneurones for both the anterior jugalis and tensor muscles. In order to innervate these muscles, the 4 cells need to project down the laterobuccal nerve to innervate the anterior jugalis muscle, and the ventrobuccal nerve to innervate the tensor muscle (Fig. 2). The anatomical evidence presented here is consistent with 4 cells being motoneurones for these muscles because, at least in their ipsilateral projections, the 4 cells project along these appropriate nerves. The same argument applies to the 4 cluster neurones. Again the electrophysiological evidence shows that these cells are motoneurones for the anterior jugalis and tensor muscles and again the 4 cluster cells project down the appropriate laterobuccal and ventrobuccal nerves.

Possible back injections of 6 cells

Fig. 8(*e*, *f*) shows that one or two small cells are filled with cobalt chloride after back injection of the posterior jugalis nerve. These cells are in the correct location for them to be the 6 cells identified in electrophysiological experiments (Benjamin & Rose, 1979). These results are not unambiguous because we were unable to prevent spread of cobalt chloride over the root of the dorsobuccal nerve (Fig. 8*e*, *f*) which makes it impossible to be sure that only the posterior jugalis nerve has been filled. However, a similar result was obtained by Goldschmeding, Bruins & Everts (1977) in their retrograde filling of the same nerve in *Lymnaea*. If these cells are 6 cells then it is consistent with the electrophysiological results of Rose & Benjamin (1979), which show that the 6 cells are motoneurones for the posterior jugalis muscle, whose only innervation is by the posterior jugalis nerve (Fig. 2).

DISCUSSION

The results in this paper can be compared with those from the electrophysiological experiments of Rose & Benjamin (1979), which were concerned with the motoneurone innervation of muscles of the *Lymnaea* buccal mass. Simultaneous recordings of buccal neurones and muscles by Rose & Benjamin (1979) showed 1:1 neurone and muscle action potentials for several neurones identified in the initial study of Benjamin & Rose (1979). Thus 4-group neurones appear to be motoneurones for the anterior jugalis and tensor muscles, 8 cells motoneurones for the anterior jugalis (upper) muscle and 6 cells the motoneurones for the posterior jugalis muscle. What the present study showed was that these neurones had axonal projections along the nerves which were known to innervate these muscles, thus providing further evidence for them being motoneurones. Thus the 4-group neurones projected along the laterobuccal and ventrobuccal nerves, the 8 cells the laterobuccal nerves and the 6 cells probably to the posterior jugalis nerves. Rose & Benjamin (1979) failed to record responses in buccal mass muscles from the activation of 1, 2 and 5 cells and this is also consistent with the anatomical results from the present experiments, because the 1 cell projected to the salivary gland duct and the 2 and 5 cells to the branches of the dorsobuccal nerves innervating the pro-oesophagus. None of these three cell types had projections along the nerves appropriate for innervating muscles of the buccal mass.

The work of Goldschmeding *et al.* (1977) provides confirmation of some of the data

discussed above and additional information about the 7 cells whose anatomy was not investigated in the present study. Cobalt chloride injections of the cell bodies of neurones which probably correspond to our 2, 3 and 4 cells resulted in axons being filled which were identical to those reported in the present paper. Back injection of the dorsobuccal nerve by Goldschmeding *et al.* (1977) filled a cell which is the right size and occurs in the same position as one of the 7 cells shown in Fig. 1. If this is true then it would suggest that all the double input cells (except the 8 cells) of Benjamin & Rose (1979) have projections to gut tissue. We have no proof that the 2, 5, 7 cells are motoneurones for the gut but this seems likely in view of the electrophysiological evidence (Benjamin & Rose, 1979) that the spike activity of these cells is related to the activity of known motoneurones in the isolated buccal ganglion (also there is direct evidence that spike activity in 2 cells results in contraction of the gut, Cunliffe, personal communication). It is likely that the 2 and 5 cells innervate different parts of the gut because the 5 cells terminate in pro-oesophagus close to the buccal ganglion whereas 2 cells apparently innervate more distal tissue. The 3 cells had interesting double projections both to the laterobuccal nerve and the dorsobuccal nerve (and thence to the pro-oesophagus) which would be sensible if these branches innervated glandular tissue in both the oesophagus and the buccal mass (see Results section). However, we have no direct evidence for this at present. Overall we can say that there is anatomical evidence that four cell types innervate the oesophagus (the 2, 3, 5 and 7 cells) with direct electrophysiological evidence that the 2 cells are motoneurones (from unpublished results), and a suggestion that the 3 cells may innervate the glandular tissue of the buccal mass and pro-oesophagus. The 1 cells project to the salivary gland duct and may well continue to the salivary gland itself which would make them similar to the 4 cells of *Helisoma* (Kater *et al.* 1978) and the autoactive salivary gland motoneurone of *Limax* (Prior & Gelperin, 1977).

Another feature of *Lymnaea* neural morphology can also help to explain some physiological findings of Benjamin & Rose (1979). These authors found that bilaterally symmetrical buccal neurones of the same type were electrotonically coupled (the 1, 3 and 4 group cells) whereas others were not (2, 5 and 7 cells). In the case of electrotonically coupled neurones, morphological studies in the present paper showed contralateral axonal projections from both members of a bilaterally symmetrical pair, which brought the two cells together at various points, particularly in the buccal commissure. These points of contact between cells of the same type need to be investigated in the electron microscope for evidence of gap junctions, but they seem the likely site for the electrotonic synapses revealed by electrophysiology. On the other hand, cells with no electrotonic connexions had only ipsilateral axonal projections (5 cells) or only fine contralateral projections (2 cells) which did not project far enough for synaptic contacts to be possible between cells in the opposite ganglia.

Motoneurones which project to the same nerve on both sides of the buccal ganglia were found in *Lymnaea* and seem common in other molluscs (Siegler, Mpitsos & Davis, 1974; Kater *et al.* 1978). Goldschmeding (1977) made the interesting observation in *Lymnaea* that a neurone of this type could innervate the same muscle type on both sides of the buccal mass. This raises the possibility of double innervation of muscles having the same function and could presumably be a mechanism for synchronizing the activity of muscles acting together in buccal mass movements, although

this would seem unnecessary because central mechanisms exist (Benjamin & Rose, 1979) for synchronizing the electrical activity of motoneurones of the same type in opposite ganglia.

Some general observations can be made about the neural geometry of buccal neurones in relation to other studies of molluscan morphology. Each type of identified neurone in the buccal ganglion of *Lymnaea* has a distinct set of morphological features (e.g. axonal projections and dendritic branching) which distinguishes it from its fellows. The same could be said for different types of neurosecretory cells in *Lymnaea* (Benjamin *et al.* 1976; Swindale & Benjamin, 1976). The complexity of dendritic branching of the 1 and 3 cells in particular is as great as revealed by Golgi impregnation (Benjamin & Ings, 1972) or by cobalt chloride injection (Winlow & Kandel, 1976), which suggests that Procion Yellow can penetrate fine axonal branches of molluscan neurones. The variability of axonal projections seen in 4-group cells has been reported for other molluscan neurones (Benjamin, 1976; Winlow & Kandel, 1976). Its functional significance is not always clear but it may occur when several neurones of one type project to the same end organ or where there are several alternative routes to the periphery.

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