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A comparison of four techniques for mapping the distribution of serotonin and serotonin-containing neurons in fixed and living ganglia of the snail, *Lymnaea*

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Summary

The distribution of serotonin and serotonin-containing neurons was studied in the ganglia of the CNS of the snail *Lymnaea stagnalis*. Results of the application of three different labelling techniques on wholemount preparations were compared with each other and with the serotonin content of the ganglia, measured by high-performance liquid chromatography. Serotonin immunocytochemistry resulted in the highest number of labelled neurons, but the more recently developed *in vivo* method of 5,6- or 5,7-dihydroxytryptamine-induced pigmentation also proved to be a reliable technique for the visualization of serotonin-containing cell bodies. In comparison with these two techniques, the glyoxylic acid fluorescence method appeared to be less sensitive. The distribution and number of serotonin-containing neurons and biochemically measured serotonin in specific ganglia showed a close correlation. By combining the results of the three labelling techniques, a detailed map of serotonin-containing neurons was constructed, and this was compared with maps of identified neurons prepared from earlier electrophysiological studies. Previously described serotonergic neurons were consistently found, as well as several new serotonin-containing cell types in the cerebral, visceral and parietal ganglia. A network of serotonin-containing inter- and intraganglionic axon tracts, and thin serotonergic fibres in the perineurium were also demonstrated. This *in vivo* and *in vitro* identification of serotonin-containing neurons will facilitate further neurophysiological analysis of serotonergic neural mechanisms in *Lymnaea*.

Introduction

Serotonergic transmission and neuromodulation play an important role in the regulation of a variety of behaviours in gastropod molluscs (S.-Rózsa, 1984; Walker, 1986). As a first step in understanding the more detailed role of serotonin in defined neural circuits, it is necessary to map the serotonin-containing neurons and devise methods which allow the identification of these neurons for electrophysiological study. A number of techniques are available for identifying serotonin in neural tissue, and it is important to use more than one method to confirm the identity of serotonin as the specific substance and to determine different features of its distribution. The use of immunocytochemistry and histofluorescence techniques such as glyoxylic acid allow the whole CNS to be screened; when used in wholemounts, the location of the cell bodies on the ganglionic surface can be mapped accurately. Both techniques also allow serotonin fibres to be traced within the CNS and peripheral nerves. However, once the techniques have been applied, labelled cells cannot be used for subsequent electrophysiological analysis. A new technique, involving the injection of the serotonin analogues 5,6- or 5,7-dihydroxytryptamine (5, 6-DHT or 5,7-DHT) into live snails, has recently been developed in *Helix*. This induced the formation of brown pigment in serotonin-containing neurons while keeping the cells alive for electrophysiology (Balaban *et al.*, 1985; S.-Rózsa *et al.*, 1986; Kemenes & S.-Rózsa, 1987; Jahan-Parwar *et al.*, 1987; Vehovszky *et al.*, 1988; Hernádi *et al.*, 1989). The identification of serotonin-containing cells is obviously facilitated if a detailed map of the location of different types of cells is already available. This is the case with the CNS of the pond snail *Lymnaea stagnalis* (Benjamin *et al.*, 1980, 1985; Elliott & Benjamin, 1985), the preparation used for the present study. This snail has been the subject of a number of major neurobiological studies, several of

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which examined the role of serotonin-containing neurons (e.g. McCrohan & Benjamin, 1980; Kemenes *et al.*, 1988).

Cellular localization of serotonin in the CNS of *Lymnaea* has so far mainly been analysed using the Falck–Hillarp method (Sakharov & Zs.-Nagy, 1968; Cottrell *et al.*, 1979) or glyoxylic acid fluorescence histochemical method (Audesirk, 1985; Casey & Winlow, 1985).These studies concentrated on the localization of giant neurons or clusters of neurons in specific ganglia, and were limited in their scope. The only immunocytochemical study has been performed on the cerebral giant cells (CGCs) which were shown to contain serotonin as well as two other putative neurotransmitters, dopamine and VIP (Boer *et al.*, 1984). Microchemical analysis confirmed the presence of serotonin in the CGCs (McCaman *et al.*, 1984).

The main objective of the present work was to evaluate the usefulness of four different techniques for studying serotonin-containing cell bodies and fibres in the CNS of the pond snail, *Lymnaea stagnalis*. Two of the methods, the 5,6- or 5,7-DHT pigment-induction technique and the immunocytochemical mapping of wholemounts, had not been applied to the *Lymnaea* CNS before. The results of these techniques were compared with those obtained after glyoxylic acid application. The fourth technique involved the neurochemical analysis of serotonin in specific ganglia and nerves, using high-performance liquid chromatography (HPLC).

A further important objective of the study was to identify labelled neurons within our previously published maps of neurons obtained from electrophysiology (Benjamin, 1983; Benjamin *et al.*, 1985). This confirmed the presence of some of the previously identified serotonin-containing neurons but also discovered many new ones. This will allow further analysis of the role of serotonin in the feeding (Benjamin, 1983; Benjamin & Elliott, 1989) and other circuits (Benjamin *et al.*, 1985) of the *Lymnaea* CNS. A final major objective was achieved when it was shown that 5,6- or 5,7-DHT induced pigmentation in the same identifiable neurons revealed by the histochemical techniques. This showed the specificity of the method and confirmed its value in identifying serotonergic neurons for electrophysiology (S.-Rózsa *et al.*, 1986; Kemenes & S.-Rózsa, 1987).

Methods

Pond snails, *Lymnaea stagnalis*, were bought from animal suppliers in the UK or collected locally on the Tihany peninsula in Hungary. No difference in the neuroanatomical organization of specimens of these two geographically distant populations could be detected. The snails were maintained under standard laboratory conditions (12:12 h light–dark cycle; 20 ± 2° C) in aerated standard snail water (SSW2; Thomas *et al.*, 1975) and fed on a standard lettuce diet.

Pigment labelling technique induced by 5, 6-DHT and 5, 7-DHT

The pigment labelling technique previously developed for *Helix* (S.-Rózsa *et al.*, 1986) was adapted for use on *Lymnaea*, the only differences being in the composition of *Helix* and *Lymnaea* saline and the drug concentrations used. Preliminary experiments revealed that higher concentrations of the neurotoxins were necessary for reliable pigment induction in *Lymnaea* compared to *Helix*. The tryptamine derivatives 5,6- or 5,7-dihydroxytryptamine creatinine sulphate (SIGMA) were dissolved in HEPES-buffered saline (Benjamin & Winlow, 1981) containing 0.5 mg ml^{-1} ascorbic acid as an antioxidant. Fifty snails of body weight 1.5–3 g were injected with 25–50 µl solutions of either drug, equivalent to a 200 mg per kg body weight dose of the neurotoxins. A group of 50 control snails was injected with the carrier solution alone. The 5,7-DHT-induced red and the 5,6-DHT-induced brown pigment were used as markers to localize serotonin-containing neurons.

Every 3 days after injection, three snails were taken from both experimental and control groups. These snails were dissected in HEPES-buffered saline (Benjamin & Winlow, 1981) and the CNS pinned out in a Sylgard-lined dish. The CNS was desheathed and the fresh preparations were examined under a stereomiscroscope. Preparations containing pigmented cells were then dehydrated in graded alcohols, cleared in methyl salicylate and mounted on concave slides in DPX. During this process the ganglia lost all their natural orange pigmentation and only the drug-induced dark pigment remained visible. The locations of the pigmented cells were mapped with the aid of a drawing apparatus attached to a stereomicroscope and by taking photographs of the cleared wholemounts. Drawings showing the locations of the labelled neurons in standardized brain diagrams were prepared. Cell diameters were measured by an ocular micrometer, and counts of cell numbers made for each ganglion of the CNS.

Wholemount glyoxylic acid procedure

The histochemical glyoxylic acid procedure (Axelsson *et al.*, 1973), modified for wholemounts by Audesirk (1985), was used to detect yellow serotonin fluorescence in wholemounts of *Lymnaea* CNS. After the glyoxylic acid treatment the brains were viewed under a Zeiss epifluorescence microscope and photographed on colour and black and white film. This allowed the locations, numbers and cell body diameters of yellow fluorescing neurons to be obtained.

Immunocytochemistry

For wholemount anti-serotonin immunostaining, the brains were rapidly removed from the snails in cold HEPES-buffered saline (Benjamin & Winlow, 1981), pinned out on Sylgard, and fixed for 6 h at 4° C with 4% paraformaldehyde diluted in 0.1 mol l^{-1} phosphate buffer (PB, pH 7.4). After fixation the brains were washed for 36 h at 4° C in several changes of 0.1 mol l^{-1} PB. During the wash the outer sheath

surrounding the CNS ganglia was removed. Before incubating with the primary antiserum, the brains were treated with 0.3–2% H_2O_2 in 0.1 mol l^{-1} PB for 30 min and then with 5 or 10% normal goat serum for a further 30-min period. Both solutions were diluted in phosphate-buffered saline (PBS) containing 0.25% Triton X (PBS–TX). The brains were incubated for 20 h at 4° C with anti-serotonin antiserum (Immunonuclear Corp., Stillwater, USA) diluted to 1:1000, 1:3000 or 1:5000 in PBS–TX containing 0.25% bovine serum albumin. The ganglia were then processed according to the peroxidase–antiperoxidase (PAP) method (Sternberger, 1979). All steps of antiserum treatment were followed by washes in PBS–TX. For the development of the peroxidase histochemical reaction product 0.05% diaminobenzidine in 0.05 mol l^{-1} Tris HCl, to which 0.01% H_2O_2 was added, was applied. Time of development varied between 3 and 7 min and was controlled under a stereomiscroscope. The specificity of this immunonuclear antiserotonin antiserum has been well demonstrated in invertebrates (Klemm, 1983; Nässel, 1987) including gastropods (Ono & McCaman, 1984; Kistler *et al.*, 1985). In our controls the primary antiserum was replaced with the preimmune normal rabbit serum in the same dilution. For light microscopy, immunostained wholemounts were dehydrated in graded ethanol, cleared in methylbenzoate and xylene, and mounted on slides in Canada balsam. Mapping of serotonin-immunoreactive neurons and fibres used the same procedures described for the pigment-labelling method. A table comparing the localization, size and number of the labelled cells after the application of each of these three methods was constructed (see Table 1).

Biochemical measurement of serotonin levels in ganglia of Lymnaea *CNS*

Serotonin levels in selected ganglia from 10 untreated *Lymnaea* brains were measured using previously described HPLC methods (Saller & Salama, 1984). Tissue samples were homogenized in 40 volumes (vol/wt) of mobile phase and centrifuged at 30 000 *g* for 20 min at 4° C. Aliquots of the clear supernatant were subjected to HPLC on a reverse phase C18 Nucleosil column (150 × 4.6 mm, 5 μm) using a mobile phase of 0.1 M sodium phosphate, 1 mM EDTA, 1 mM sodium octane-sulphonic acid, 10% acetonitrile, adjusted to pH 4.5 with citric acid, at a flow rate of 1 ml min^{-1}. The elution of serotonin was monitored by electrochemical detection at 0.7 V. Quantitative determinations were made by comparisons with appropriate standards. The following pieces of tissue were analysed separately: buccal ganglia, cerebro-buccal connectives together with the latero- and ventrobuccal nerves, cerebral ganglia, pedal ganglia and the viscero-parietal complex. The serotonin levels were expressed in pmol per ganglia, for these values could be most directly related to the number of serotonin-containing neurons in a particular ganglion.

Results

ORGANIZATION OF THE CENTRAL GANGLIA IN *LYMNAEA*

The CNS of *Lymnaea* is formed of 11 ganglia, nine of which form the main ganglionic ring, with two more distally located buccal ganglia connected to the rest of the brain via the cerebrobuccal connectives. All the ganglia are paired except for the visceral ganglion. The ganglia on each side are generally similar in size and shape apart from the right parietal ganglion which is much larger than the left. For a summary of the distribution of cells found in the present study and the arrangement of the ganglia and nerve roots leaving the CNS, see Fig. 7: it can be used as a reference figure for understanding the relationships of individual ganglia to the whole CNS in the rest of the results.

COMPARISON OF PIGMENT-LABELLED, SEROTONIN-IMMUNOREACTIVE AND YELLOW SEROTONIN-FLUORESCENT NEURONS IN THE *LYMNAEA* CNS

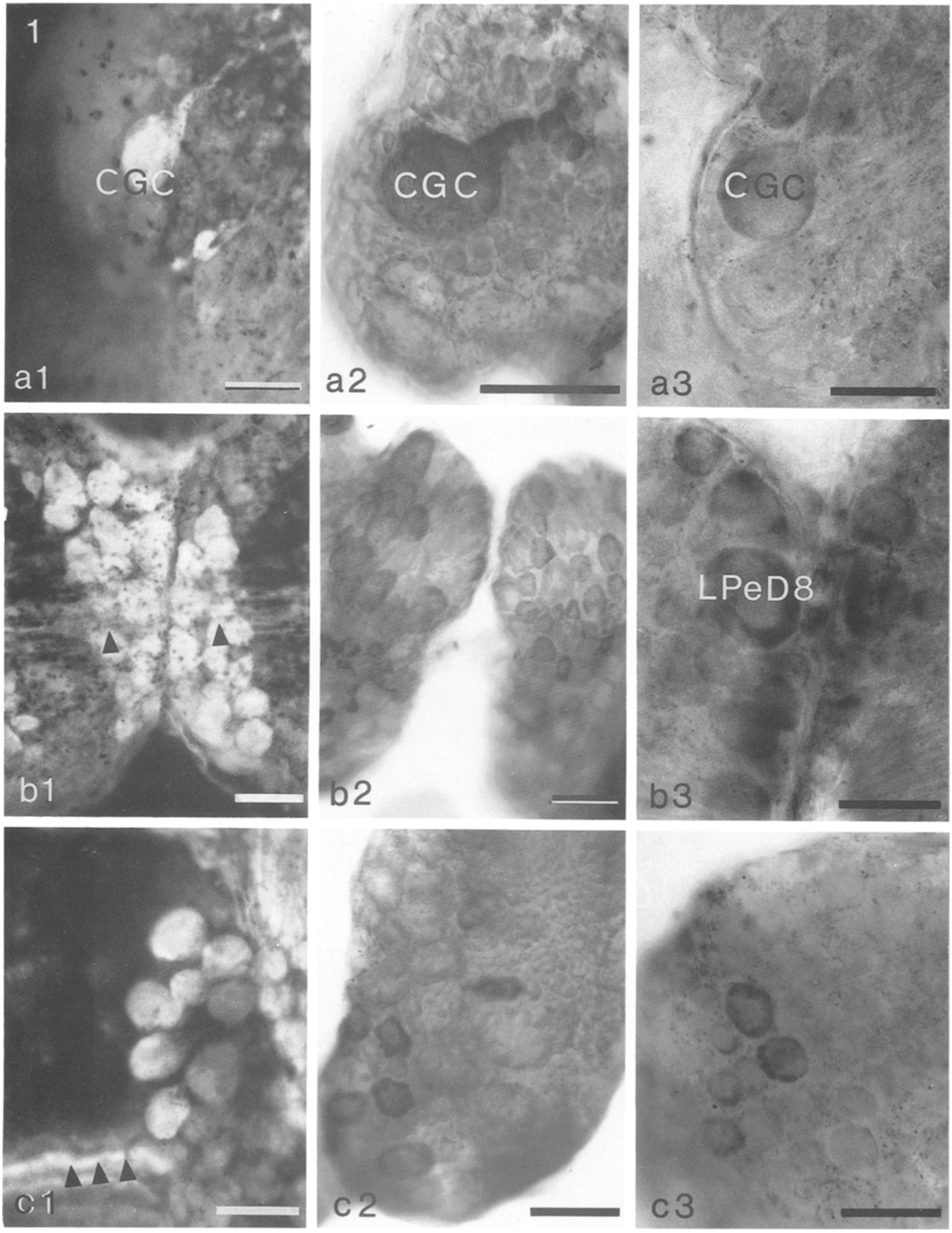
Fig. 1 shows that many of the most prominent giant neurons (Fig. 1a) and clusters of neurons (Fig. 1b, c) were visualized by all three histological methods. However, the numbers of cells and even the basic identification of some large cells consistently varied with the method used; details of this variation will become clear when each ganglion is considered in the following sections. No difference was found on the effectiveness of 5,6-DHT versus 5,7-DHT in identifying serotonin-containing cells, although 5,6-DHT produced a darker brown pigmentation, making it easier to see the cells.

Buccal ganglia

Fig. 2 shows 5, 6- or 5, 7-DHT-induced pigmentation in three small (15–20 μm) neurons on the dorsal side of the buccal ganglia (arrows). Two of the cells occurred in similar locations in left and right ganglia and thus appeared to be a bilaterally symmetrical pair. The third occurred on its own in the right buccal ganglion in a more anterior position to the other cell. In cleared preparations it was possible to identify other unmarked larger cells in the buccal ganglia, particularly the feeding motor neurons. This allowed us to locate accurately the paired pigmented cells on the ganglion surface. Thus, in most preparations the paired cells could be seen to occur just posterior (Fig. 2, arrowhead; Fig. 7a) to the motor neuron cell type known as the three cells (Benjamin & Rose, 1979). None of the three pigmented cells appeared to correspond to any of the previously mapped interneuron or motor neuron types (summarized in Benjamin & Elliott, 1988). Neither of the other two labelling techniques showed neurons in the buccal ganglia (Table 1).

Cerebral ganglia

Joosse (1964) described three lobes in the cerebral ganglia of *Lymnaea*: the dorsal, ventral and anterior lobes. The anterior and ventral lobes were larger on the right side (Fig. 7). This division of the cerebral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ganglia provided a useful framework for describing the location of serotonin cells, with bilaterally symmetrical groups of cells occurring in all three lobes.

The best known serotonin-containing cells in *Lymnaea* were the paired CGCs (Sakharov & Zs.-Nagy, 1968; McCaman *et al.*, 1984). These occurred on the more ventral edge of the anterior lobes and were consistently labelled by all three techniques (Figs 1a, 3b–e). Associated with each CGC was a group of three or four medium-sized cells (30–50 μm in diameter) which formed a crescent-like arrangement (Fig. 3a). These cells also stained with all three methods, as did another group of small to medium-size neurons (15–40 μm in diameter) which lie in the anterior lobe close to the cerebral commissure (Fig. 3c); this group numbered either four or five neurons, depending on the staining method used (Table 1).

In the dorsal lobes, a single identifiable giant cell (60–70 μm) could be observed on the lateral surface of each side associated with a cluster of three smaller cells (20–30 μm diameter). This giant cell was seen after applying both 5,6- or 5,7-DHT and immunocytochemistry (Fig. 3d, e), but not with the glyoxylic acid method (Table 1). Another cluster of 9 to 13 cells (20–30 μm diameter) occurred more deeply within the dorsal lobe on each side, located between the origin of cerebropedal and cerebropleural connections (Fig. 3b).

An interesting group of small (15–40 μm diameter) cells occurred in a characteristic location in the ventral lobes at the root of the cerebro-buccal connectives on both sides. These appear to correspond to neurons of the cerebral feeding system called CV5 (cerebral ventral 5), CV6, CV7 and CV8 cells, some of which, e.g. CV7, are motor neurons for the lips (McCrohan, 1984). These cells were detected by the immunostaining method but not the other labelling techniques (Table 1).

Pedal ganglia

The serotonin-containing neurons of the pedal ganglia have been extensively mapped by Audesirk (1985) and Casey & Winlow (1985) using the glyoxylic acid technique. Neurons corresponding to identifiable single cells, or clusters of neurons described in the mapping studies of Slade *et al.* (1981) were identified (Fig. 7). The present study showed that similar cells could be located by immunostaining and 5,6- or 5,7-DHT pigment induction as well as glyoxylic acid treatment (Fig. 1b), confirming the presence of serotonin in these neurons. Thus the giant neurons left and right PeD4 (pedal dorsal 4), PeD7 and PeD8 (Fig. 1b3) stained as well as PeV1 (pedal ventral 1), PeV3 (Fig. 7). Only left and right PeV1 cells (Fig. 7b) appeared not to have been stained in the previous glyoxylic acid fluorescence study of Casey & Winlow (1985). The only giant cell not stained by all three methods was LPeD1 (L, left) which was not detected by the pigment induction technique (Table 1). Other neurons arranged as clusters (Table 1) corresponded to the cells described by Casey & Winlow (1985), apart from cells of the D or H group which appear not to have been stained in their study.

Pleural ganglia

No labelled cells were found in the pleural ganglia with any of the three labelling techniques (Fig. 7a, b, Table 1).

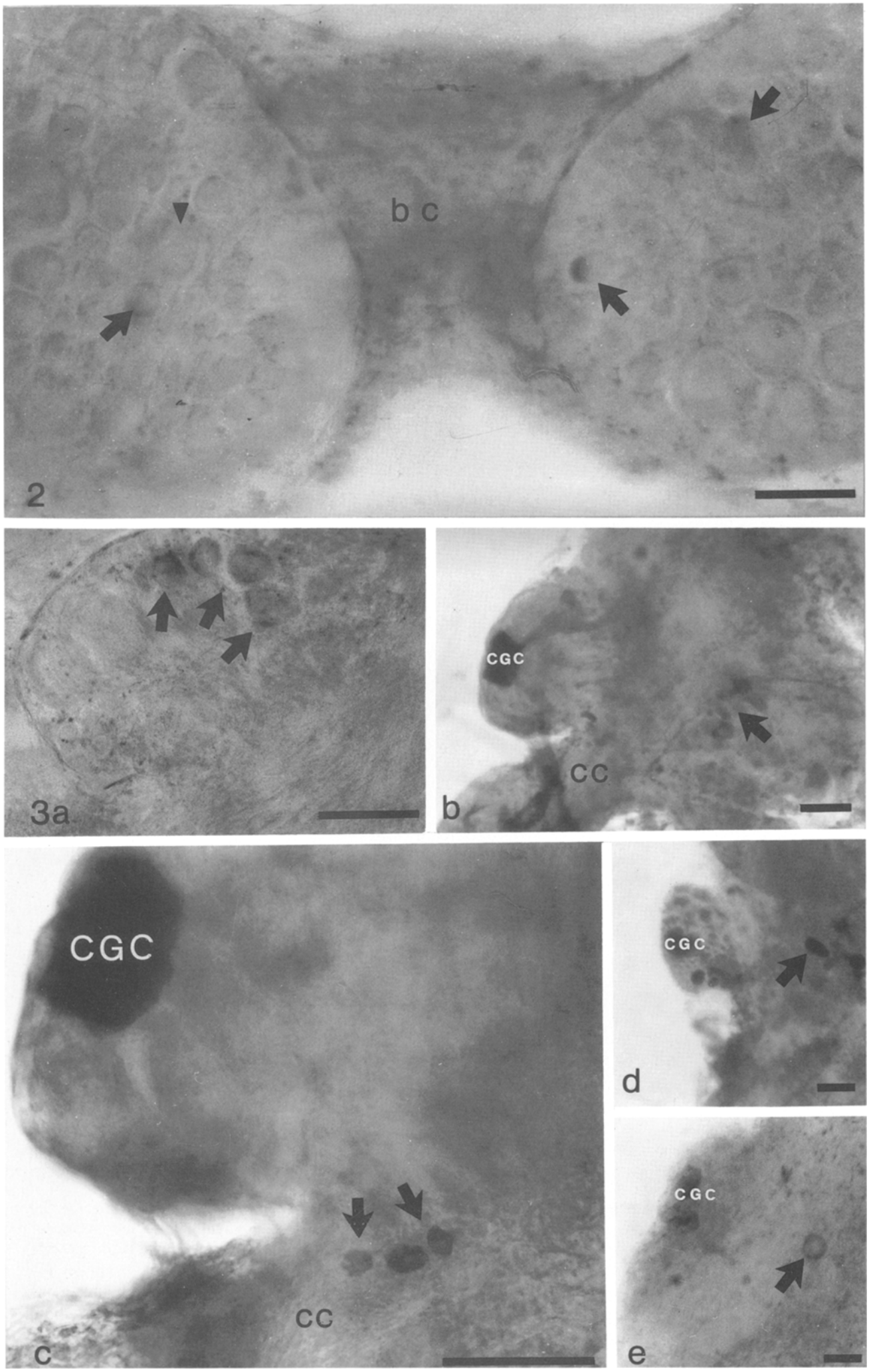
Left parietal ganglion

Between four and six medium to large cell bodies (40–70 μm diameter) on the ventromedial surface of the left parietal ganglion (Figs 4a, 5a), were shown to be serotonin-immunoreactive, but were not detected by the glyoxylic acid fluorescence or 5, 6- or 5, 7-DHT pigment induction methods. These cells did not appear to correspond to any of the other previously mapped cells (Benjamin *et al.*, 1985), such as giant cells LP1 (left parietal 1), LP2 or LP3, or the D group (Fig. 7a, b).

Right parietal ganglion

The right parietal ganglion is much larger than the left, and electrophysiological mapping studies showed that it contains many more cell types (Benjamin *et al.*, 1985). One conspicuous group of neurons on the dorsal surface was the A group. Cells from this group were labelled by all three methods (Figs 1c, 4a, c). The location of the labelled neurons close to the right parietal-visceral connective (Fig. 4a), and the relatively large size of the cell bodies (40–80 μm diameter) suggested that the cells were the so-called A group e.p.s.p. cells rather than the A group b.p.s.p. or K cells (Benjamin & Allison, 1985) which are smaller, but form part of the same cluster (Fig. 7a). A more scattered group of serotonin-containing neurons, more variable in location, occurred among the B group (Fig. 4a). All three methods detected these neurons,

Fig. 1. Some of the most characteristic serotonin-containing neurons or clusters of neurons identified by all three labelling techniques. (a1, b1, c1) Wholemount glyoxylic acid staining. (a2, b2, c2) Wholemount serotonin imnmunostaining. (a3, b3, c3) *In vivo* 5,6- or 5,7-DHT pigment labelling. (a) Left CGC (Sakharov & Zs.-Nagy, 1968). (b) A cluster of neurons on the dorsal surface (Slade *et al.*, 1981) of the pedal ganglia. The individually identifiable neuron RPeD8 can be seen in b3. (c) A-group e.p.s.p. neurons on the dorsal surface of the right parietal ganglion (Benjamin & Winlow, 1981). Arrowheads indicate stained fibres running in the pedal commissure (b1) and the right parieto-visceral connective (c1). Scale bars: 100 μm.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but the 5,6- or 5,7-DHT method produced more labelled cells than the other two (Table 1). The small size of the cell bodies (15–40 μm) and their variable location suggested that they were not the main B group cells identified by Benjamin & Winlow (1981) and shown in Fig. 7a. This indicates that cells in this part of the right parietal ganglion are more heterogenous than previously thought. Finally, two small neurons which occurred at the base of the internal right parietal nerve (Fig. 4a) showed serotonin immunoreactivity but were not labelled by the other two techniques (Table 1).

Visceral ganglion

The most striking serotonin-containing neurons were two giant cells lying on the anterior surface of the ganglion (Fig. 4b). Their size (100–110 μm diameter) and location suggested that they were the cells VV1 (visceral ventral 1) and VV2 first described by Winlow & Benjamin (1976). Cells VV1 and VV2 showed both 5,6- or 5,7-DHT-induced pigmentation and serotonin immunoreactivity, but yellow fluorescence was not observed after the glyoxylic acid treatment. All three methods revealed a second large cluster of small to medium-size neurons (15–70 μm) lying on the dorsal surface of the visceral ganglion (Fig. 4a, c). The number of cells and their maximum size varied with the method used (Table 1), but their location was consistent, suggesting that at least some of the same cells were stained by all three methods. They occurred between the H, I, J, K cells and the G group cells (Fig. 7a), but lie much deeper in the ganglion. They probably represent a new type of cell, lying too deep in the ganglion tissue to have been observed in previous mapping experiments which relied on electrophysiological recording of cell bodies located superficially on the ganglion surface (Benjamin & Winlow, 1981). The last type of cells found in the visceral ganglion showed only serotonin immunoreactivity. These were a few small neurons scattered across the central region of the dorsal surface of visceral ganglion (Fig. 4a), but also on the left side in the F-group region (Fig. 5a). Immunoreactive cells also occurred on the ventral surface where the F-group were also found (Fig. 7b). Cells on either surface were unlikely to be the main type of F-group cell identified in electrophysiological studies (Benjamin & Winlow, 1981) because of their small size and scattered distribution. One of the immunoreactive cells on the dorsal surface (Fig. 4a) could correspond to the cell known as the visceral white interneuron (Benjamin, 1984), but its small size makes it difficult to identify with certainty by the methods employed here.

AXON TRACTS AND FIBRES

Of the three serotonin labelling techniques, only the immunocytochemistry and the glyoxylic acid histofluorescence demonstrated serotonin-containing fibres. The 5,6- or 5,7-DHT method only labelled neuronal somata. In all parts of the CNS, interganglionic immunostained or serotonin-fluorescent fibres could be traced through the ganglia, commissures and connectives (Figs 1b1, c1, 4a, 5a, 6a, c–e). All the nerves leaving the CNS also contained serotonin-containing axons. Examples of these are shown in Figs 4a, 5c, 6a–c. A network of thin serotonin-immunoreactive fibres, often possessing varicosities, was also found in the perineurium of the CNS (Fig. 5b).

Specific pathways could only be mapped out when a uniquely identifiable giant neuron was present. A good example of this was the CGCs. This pair of giant cells send their thick axons to the buccal ganglia via the cerebro-buccal connectives (McCrohan & Benjamin, 1980). The CGC axon was easy to detect by both immunostaining (Fig. 6d, e) and glyoxylic acid histofluorescence (Fig. 6c). The projections of the main CGC axon into the latero-, ventro-, dorso- and postbuccal nerves, and the buccal ganglion neuropile (ipsilateral and contralateral to the CGC) could also be followed by both techniques (Fig. 6a–c). Fine branching of the serotonin-immunostained CGC fibres could be traced within the nerves (Fig. 6b). These corresponded closely to the CGC projections revealed by intracellular staining of the CGCs (McCrohan & Benjamin, 1980).

COMPARISON BETWEEN BIOCHEMICALLY ASSAYED SEROTONIN AND NUMBERS OF SEROTONIN-CONTAINING NEURONS IN THE *LYMNAEA* CNS

In Table 2 the mean number of cells occurring in different parts of the CNS was compared with the mean amount of serotonin measured by HPLC (expressed in pmol per ganglion). The percentage of cells and serotonin occurring in each part of the CNS

Fig. 2. Arrows show 5,6-DHT-induced pigment labelled neurons on the dorsal surface of the paired buccal ganglia. Arrowhead indicates unlabelled identified feeding motor neuron type 3 (Benjamin & Rose, 1979). The more anterior pigment-labelled neuron (top of figure) in the right buccal ganglion is slightly out of focus because it lies deeper than the other two stained cells. bc, Buccal commissure. Scale bar: 50 μm.

Fig. 3. New types of serotonin-containing neurons (arrows) in the cerebral ganglia of *Lymnaea*. (a) 'Crescent' cells in the anterior lobe (5,6-DHT pigment labelling). (b) Cluster of 'deep' cells between cerebro-pleural and cerebro-pedal connectives (immunostaining). (c) Anterior cerebral commissure cluster (immunostaining). (d, e) Lateral giant cell in the dorsal lobe shown by immunostaining (d) or pigment labelling. (e) cc, Cerebral commissure. Scale bars: 100 μm.

Table 1. Maximum and mean (±S.D.) number and size (maximum cell body diameter) of serotonin-containing neurons in the *Lymnaea* CNS, demonstrated by three different labelling techniques. For identified cells and clusters, nomenclatures of Benjamin *et al.* (1985) were used. Completely new serotonergic cell types are indicated by an asterisk.

Ganglion	*Cluster or cell*	*5,6- or 5,7-DHT*		*Glyoxylic acid*		*Immunocytochemistry*	
		Size (μm)	*Max. no. (L + R)*	*Size (μm)*	*Max. no. (L + R)*	*Size (μm)*	*Max. no. (L + R)*
Buccal (see Fig. 2)	*Dorsal symm. pair near buccal commissure	15–20	2	—	—	—	—
	*Single right dorsal cell	15–20	1	—	—	—	—
	Σ		3		0		0
Cerebral (see Figs 1, 3, 6)	Ventrol lobe group at base of cerebro-buccal connective (CV5-8 cells)	—	—	—	—	10–20	8
	*'Crescent' cells near CGCs	30–50	8	30–50	6	30–50	8
	CGCs	70–100	2	80–100	2	90–120	2
	*'Deep' cells between pleural and pedal connectives	20–30	18	20–30	26	20–30	20
	*Anterior cerebral commissure cluster	15–40	8	20–40	10	20–40	10
	*Lateral symm. pair of giant cells in dorsal lobe	60–70	2	—	—	60–80	2
	*Small cells near lateral symm. giant cells	20–30	6	—	—	20–30	6
	Σ		44		44		56
Pedal (see Fig. 1)	L + RPeD7	60–70	2	60–70	2	60–80	2
	L + RPeD8	60–70	2	50–60	2	60–70	2
	L + RPeD4	50–60	2	50–60	2	50–60	2
	L PeD1	—	—	60–90	1	60–90	1
	L + RPeV1	60–80	2	60–80	2	60–80	2
	L + RPeV3	70–80	2	60–70	2	60–80	2
	L + RPeV2	60–70	2	60–70	2	60–80	2
	L + R A, B, C, E, F, I and H or D group	30–70	90	30–80	100	15–70	120
	Σ		102		113		133
Pleural		—	—	—	—	—	—
Left parietal (see Figs 4, 5)	*Ventro-medial group	—	—	—	—	40–70	6
	Σ		0		0		6
Right parietal (see Figs 1, 4)	*Anterior cluster of small cells (among B group)	15–25	15	10–20	10	25–40	8
	A group e.p.s.p.	25–50	9	30–70	8	40–80	12
	*Small cells near base of int. right par. nerve	—	—	—	—	10–20	2
	Σ		24		18		22
Visceral (see Figs 4, 5)	VV1, VV2	100–110	2	—	—	100–110	2
	*'Deep' visc. cluster	15–30	18	15–40	10	25–70	10
	*'Scattered' visceral cells	—	—	—	—	20–40	4
	Σ		20		10		16
Max. no. cells labelled by staining method			193		185		233
Mean no. cells			178 ± 17		175 ± 7		210 ± 13
Max. no. cells					236		

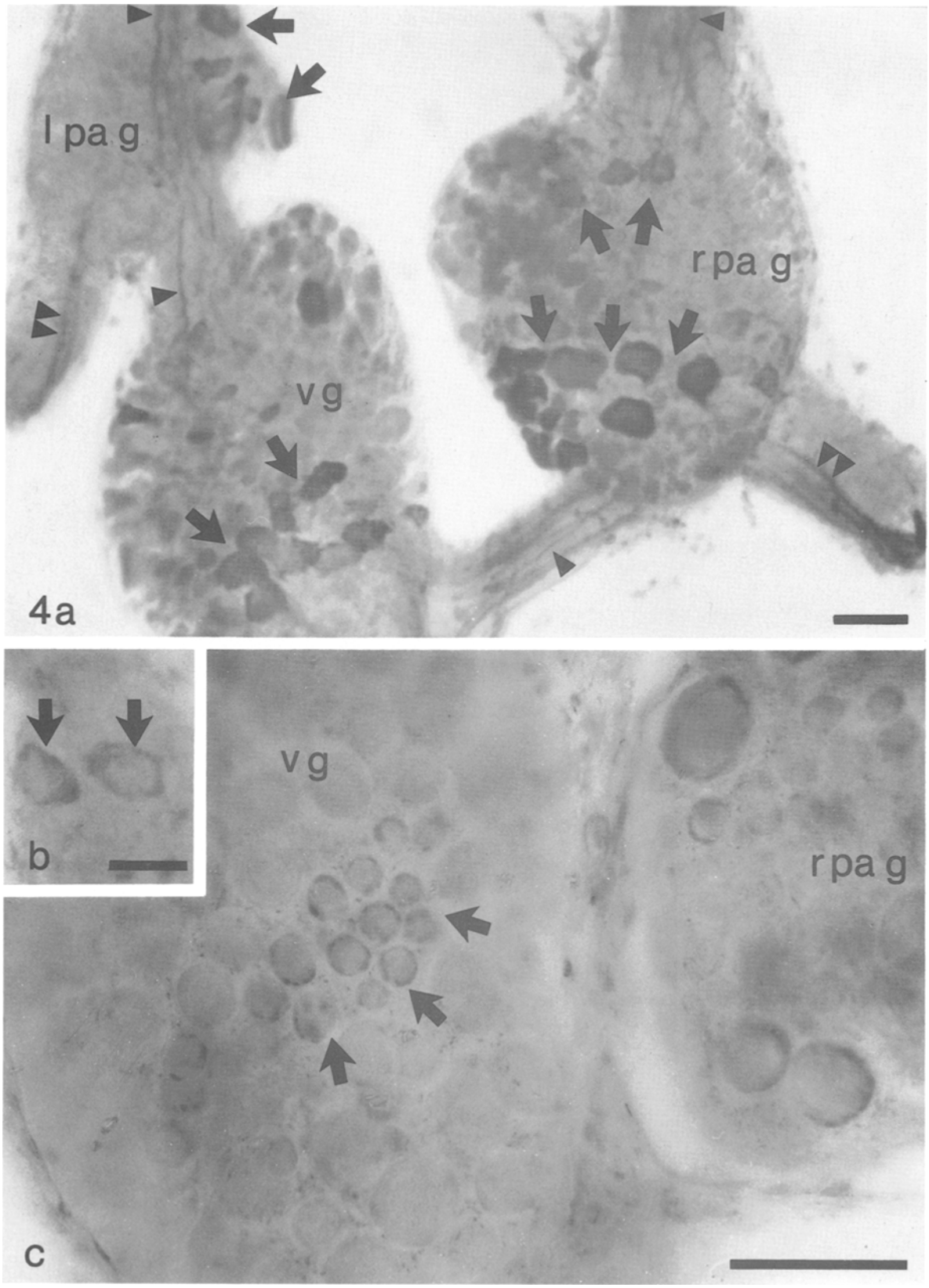

Fig. 4. Immunostained (a) and 5, 6-DHT pigment-labelled (b, c) serotonin-containing neurons (arrows) in the left and right parietal ganglia and visceral ganglion. (a) Ventromedial group in the left parietal ganglion (l pa g), 'deep' visceral ganglion cluster (vg), anterior cluster of small cells and A-group e.p.s.p. cells (Benjamin & Winlow, 1981) in the right parietal ganglion (r pa g). Arrowheads show serotonin-immunoreactive axons in the left parietal and internal right parietal nerves. (b) VV1 and VV2 neurons (Winlow & Benjamin, 1976) on the anterior surface of the visceral ganglion. (c) 'Deep' visceral ganglion cells (arrows). Scale bars: 100 μm.

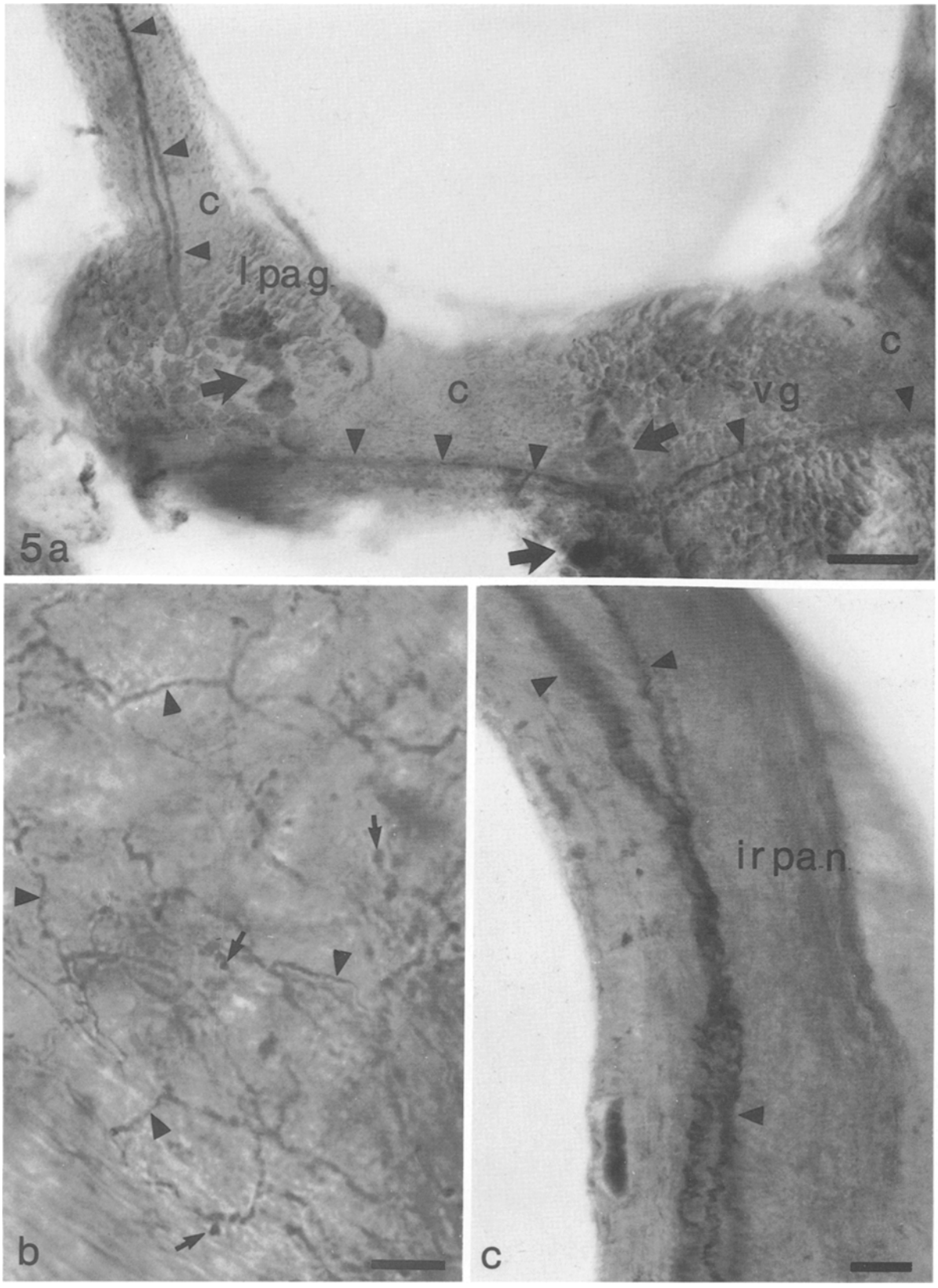

Fig. 5. Serotonin-immunoreactive axon tracts and fibres in the *Lymnaea* CNS. (a) Interganglionic axons (arrowheads) in the left pleuro-parietal, left parieto-visceral and viscero-right parietal connectives. Arrows show the left parietal ventro-medial group and the 'scattered' group of visceral neurons. l pa g, Left parietal ganglion; vg, visceral ganglion; c, connective. (b) Network of fine serotonin-immunoreactive fibres (arrowheads) with varicosities (small arrows) in the perineurium of the cerebral ganglion. (c) Serotonin-immunoreactive axons (arrowheads) in the internal right parietal nerve (i r pa n). Scale bars: (a) 100 μm; (b) 10 μm; (c) 20 μm.

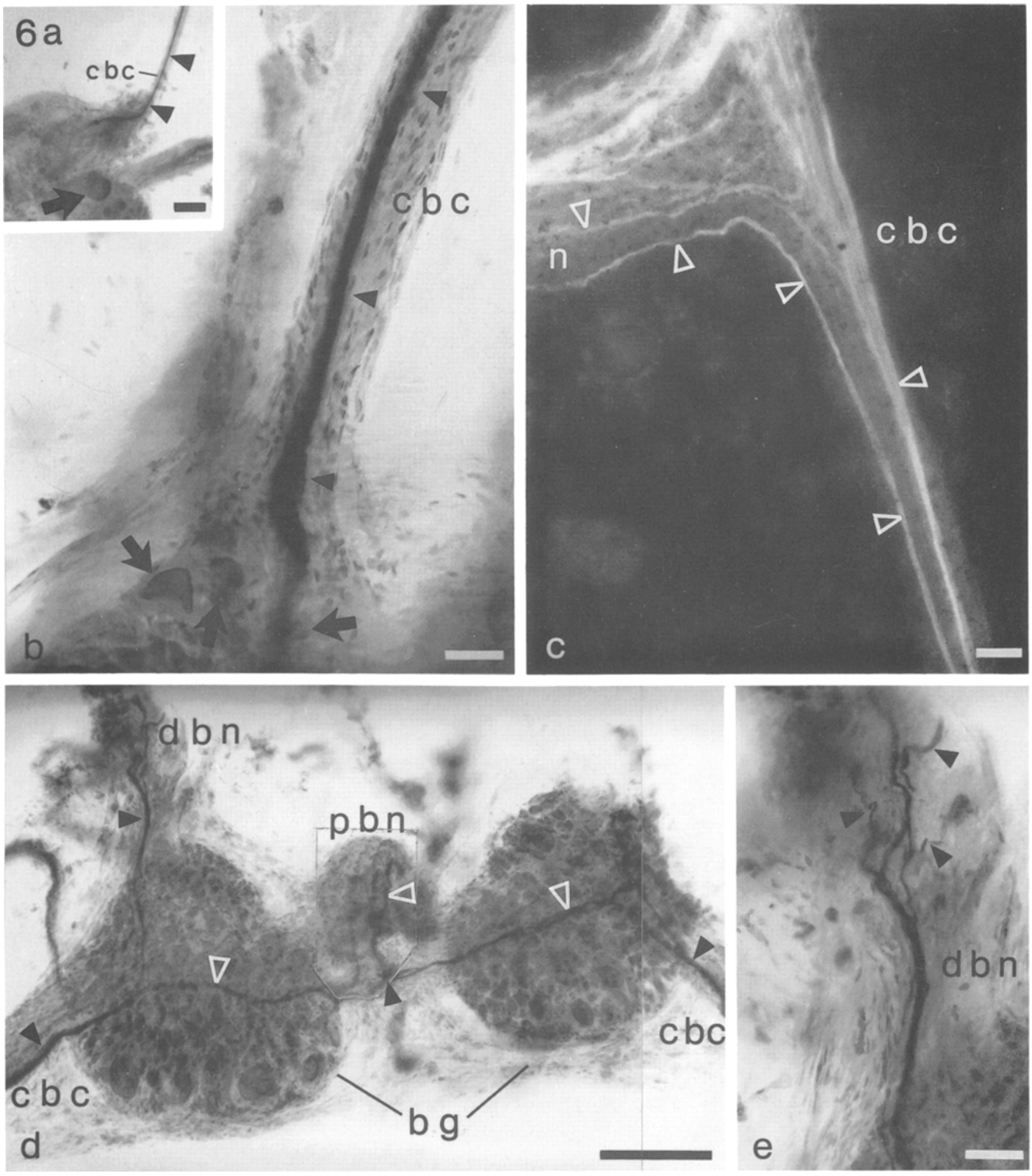

Fig. 6. Immunostained (a, b, d, e) or glyoxylic acid fluorescent (c) axonal projections of the CGCs. (a) Low magnification picture of the right CGC soma (arrow) and main axon (arrowheads) projecting into the cerebro-buccal connective (cbc). (b) Higher magnification photograph of the main CGC axon (arrowheads) leaving the cerebral ganglion and entering the cerebro-buccal connective. Arrows show ventral lobe neurons (CV5–8 cells?; McCrohan, 1984) at the base of the cbc. (c) More anterior segment of the left cbc with serotonin-fluorescent fibres (arrowheads) of the main CGC axon, projecting into the left latero- and ventrobuccal nerves (n). (d) Serotonin-immunoreactive CGC fibres (arrowheads) entering the buccal ganglia and projecting into the dorso-buccal (dbn) and post-buccal (pbn) nerves as well as the contralateral buccal ganglion. (e) High magnification picture of the fine branching (arrowheads) of the CGC axon collateral projecting into the dorso-buccal nerve. Scale bars: (a, d) 100 μm; (b, c, e) 20 μm.

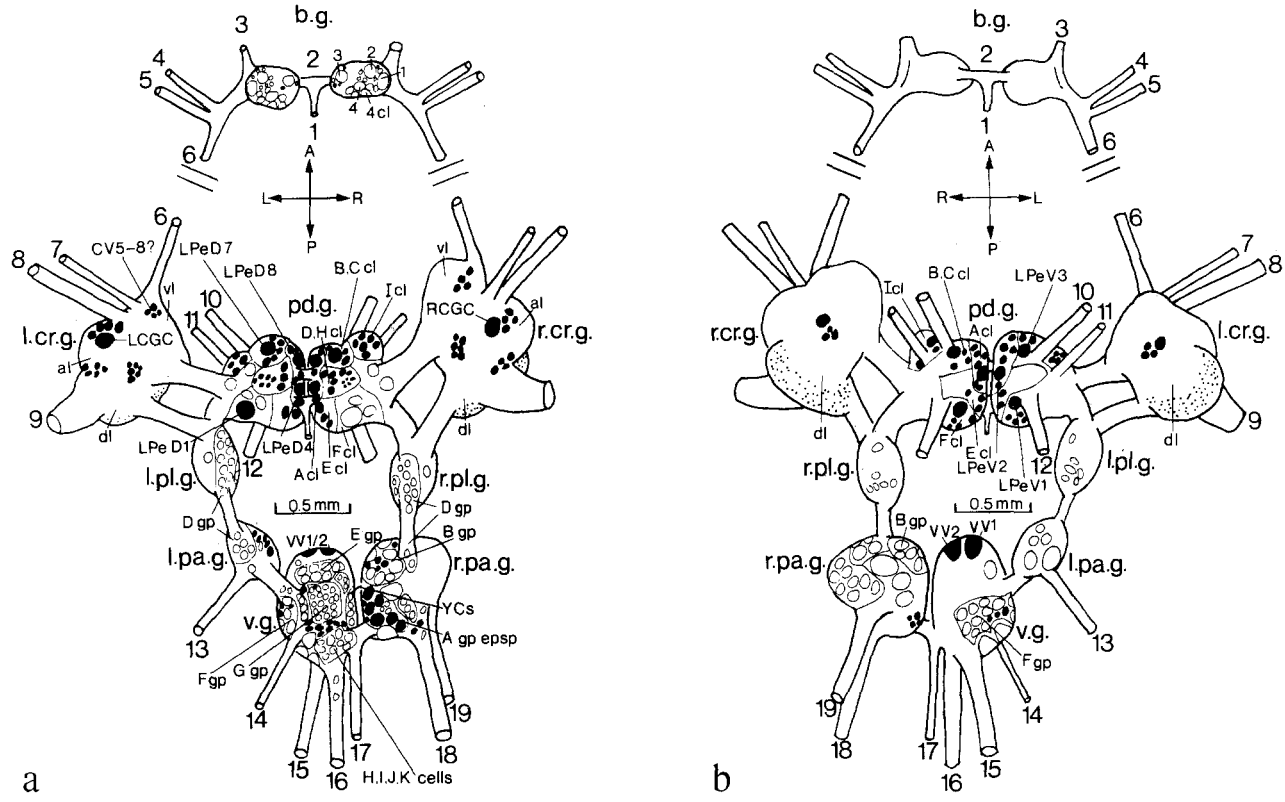

Fig. 7. Maps of neurons on the dorsal (a) and ventral (b) surfaces of the *Lymnaea* CNS, showing the location of serotonin- (shaded) and non-serotonin- (unshaded) containing cells. This summarizes the data from immunostaining, 5,6- or 5,7-DHT pigment labelling, and glyoxylic acid staining. The cerebral ganglia are shown with the cerebral commissure cut and the left and right ganglion folded out to give a flattened, two-dimensional view of the CNS. (a) Dorsal view of the CNS except for the cerebral ganglia which are shown mainly in medial view. (b) Ventral view of the CNS with the cerebral ganglia shown in mainly lateral view. Ganglia: buccal (b.g); cerebral (cr.g.); pedal (pd.g.); pleural (pl.g.); parietal (pa.g.); and visceral (v.g.). L, R and l, r indicates left and right; A, P indicates anterior and posterior. Lobes of the cerebral ganglia (after Joosse, 1964): anterior (al); ventral (vl); and dorsal (dl). Major nerves, connectives and commissures: postbuccal nerve (1); buccal commissure (2); dorsobuccal nerve (3); laterobuccal nerve (4); ventrobuccal nerve (5); cerebro-buccal connective (cut, 6); superior lip nerve (7); median lip nerve (8); cerebral commissure (cut in midline, 9); superior pedal nerve (10); median pedal nerve (11); inferior pedal nerve (12); left parietal nerve (13); cutaneous pallial nerve (14); anal nerve (15); intestinal nerve (16); genital nerve (17); internal right parietal nerve (18); and external right parietal nerve (19). Identified cells are labelled in the right pedal, identified clusters labelled in the left pedal ganglion. Both have symmetrical equivalents in the contralateral ganglion. The nomenclature for pedal neurons and clusters (cl) is after Slade *et al.* (1981) for visceral and right parietal neurons and groups (gp) after Winlow & Benjamin (1976) and Benjamin & Winlow (1981).

was also given and this allowed a simple comparison to be made between the two sets of data. The highest number of neurons labelled by all three serotonin detection methods was found in the pedal ganglia. These cells gave 54.8% of the labelled neurons in the whole CNS. The serotonin content of the pedal ganglia gave 54.6% of the mean total serotonin content of the CNS. A similar correlation between cell number and serotonin level could be observed in the cerebral ganglia (19.2% of mean total serotonin content and 21.6% of mean number of serotonin-containing neurons). The viscero-parietal complex contributed 21.6% to the mean total number of labelled cells and 14.1% to the serotonin content of the whole brain. Much more (6.1% of total) serotonin was found in the buccal ganglia than would have been expected from the low number of labelled cells (1.5% of total), and a surprisingly high amount of serotonin (6% of total) was found in the cerebro-buccal connectives and latero- and ventrobuccal nerves. This high level of serotonin in the buccal ganglia and associated nerves was presumably due to the large diameter and extensive branching of the CGC fibres shown by immunocytochemistry and glyoxylic acid staining (Fig. 6).

Both cell numbers and serotonin content increased in the same order: buccal ganglia viscero-parietal complex and cerebral ganglia pedal ganglia (Table 2).

Table 2. Biochemical analysis (HPLC) of serotonin content of ganglia and nerves of the *Lymnaea* CNS compared with the number of serotonin-containing neurons from histological studies. The data are expressed as the mean (±S.D.) or as a percentage (brackets) of the mean total CNS value for each piece of tissue.

	Buccal ganglia	*Cerebral gangalia*	*Pedal ganglia*	*Viscero-parietal complex*	*Cerebro-buccal connectives plus latero- and ventrobuccal nerves*	*Whole CNS*
HPLC						
Serotonin content (pmol per ganglion) ($n = 10$)	51.6 ± 11	163.2 ± 12.8	463.6 ± 73.5	119.6 ± 5.2	50.8 ± 4	848.7 ± 88.2
Percentage of mean total CNS serotonin content	(6.1)	(19.2)	(54.6)	(14.1)	(6.0)	(100)
Mapping methods						
Number of serotonin-containing neurons ($n = 6$)	3 ± 0	42.8 ± 5.3	108.5 ± 17.1	42.7 ± 4	0	198 ± 22.8
Percentage of mean total number of serotonin-containing cells in CNS	(1.5)	(21.6)	(54.8)	(21.6)	(0)	(100)

Overall, there was a remarkably good correlation between the two sets of data. The biochemical data supported the hypothesis that the anatomical labelling techniques were detecting most of the serotonin-containing neurons.

Discussion

Comparison of the labelling methods

Of the three anatomical techniques for serotonin detection used in the present study, the immunocytochemical method produced consistently higher numbers of labelled neurons on average than the other two techniques (Table 1). However, the 5,6- or 5,7-DHT pigment induction technique still showed about 85% of the average result for immunocytochemistry, and the glyoxylic acid method slightly lower. This confirmed the results from *Helix* (Hernádi *et al.*, 1989), showing that serotonin immunocytochemistry and 5,6-DHT give roughly similar results. Although immunocytochemistry appeared to be the most sensitive method, the pigment induction technique had the advantage that a few weeks after injection of 5,6- or 5,7-DHT (both analogues give equivalent results) into intact animals, electrophysiological recordings could be made from the cells in the live brains (S.-Rózsa *et al.*, 1986; Jahan-Parwar *et al.*, 1987; Kemenes & S.-Rózsa, 1987; Kemenes *et al.*, 1988; Vehovszky *et al.*, 1988). The advantage of the glyoxylic acid method was that both serotonergic (fluoresce yellow) and dopaminergic neurons (fluoresce green) could be detected in the same preparations.

Although the overall numbers of cells and their gross distributions were similar, consistent differences were found in the details of the cell types revealed by the three different labelling techniques (Table 1). The only neurons that were detected by the 5,6- or 5,7-DHT labelling technique, but failed to show serotonin-immunoreactivity and glyoxylic acid-induced yellow fluorescence, were the three small cells in the buccal ganglia. The failure of the immunocytochemistry and glyoxylic acid staining methods to detect these cells may have been due to the small size of the cells, low level of serotonin and/or the high background of fluorescence and immunostaining often seen in this part of the CNS.

A number of neurons detected by both the 5,6- or 5,7-DHT and the immunocytochemical labelling methods failed to fluoresce after glyoxylic acid treatment. These were the cerebral lateral symmetrical pair of giant cells and associated cluster of small cells and the giant VV1, VV2 neurons of the visceral ganglion. The glyoxylic acid staining appeared to be the least sensitive of the three methods and may have failed to detect cells with low serotonin concentrations. This is supported by the observation of Audesirk (1985) who only observed yellow serotonin fluoresence in the buccal ganglia after preincubation of *Lymnaea* brains with the serotonin precursor 5-hydroxytryptophan. Both previous glyoxylic acid studies (Audesirk, 1985; Casey & Winlow, 1985) failed to detect a number of cell types shown by other techniques in the present study (e.g. VV1, VV2, CV5–8, LPeV1). This showed the importance of using several different types of techniques to locate serotonin-containing cells.

Cells missed by both the 5,6- or 5,7-DHT labelling method and the glyoxylic acid technique included the cerebral ventral lobe group at the base of the cerebrobuccal connective, the right parietal small cells near the base of the internal right parietal nerve, and the 'scattered' visceral cells (Table 1).

A direct measure of the serotonin content of individual ganglia was determined by HPLC extraction. This confirmed the presence of serotonin in the *Lymnaea* brain and provided detailed evidence for large concentrations of serotonin in the pedal and cerebral ganglia, and smaller but still significant amounts in other ganglia and nerves of the CNS. The mean amount of serotonin present in specific ganglia of the CNS compared well (Table 2) with the mean number of cells revealed by histological detection techniques (both expressed as a percentage of mean total CNS values), despite the fact that there must be considerable variation in their size and the concentration of serotonin present in different neurons.

Serotonergic axon tracts and fibres in the Lymnaea brain

This present work also revealed the existence of extensive serotonin-containing fibre tracts running throughout the *Lymnaea* brain. These were also found in the neuropile of the pleural ganglia where no serotonin-containing cell bodies were found. The function of these interganglionic fibres is not yet known, but they presumably play an important role in the integration of the functions of several ganglia within the CNS. Serotonin-containing fine fibres and varicosities were found in the connective tissue sheath surrounding the central ganglia and nerves of *Lymnaea*. Similar fibres were also reported in *Philine* (Barber, 1982), *Aplysia* (Ono & McCaman, 1984) and *Helix* (Hernádi *et al.*, 1989). Varicosities may be the sites of non-synaptic transmitter release, suggested to occur in *Aplysia* by Schwartz & Shkolnik (1981), Bailey *et al.* (1981) and Hopkins *et al.* (1982).

Identification of serotonin-containing neurons in Lymnaea

Table 1 and Fig. 7 showed that some of the cells stained by the present techniques corresponded to previously mapped cells, but many were new types of cells which require further investigation. Well-known serotonin-containing cells were the CGCs, modulatory cells involved in feeding (Benjamin & Elliott, 1988), and the dorsal pedal neurons like the A cluster which appear to be involved in the control of foot cilia (McKenzie *et al.*, 1987). The left pedal giant cell (LPeD1) has also been shown to contain serotonin in several other studies (e.g. Cottrell *et al.*, 1979), but its function is unknown.

This study has also provided the first evidence for serotonin content in the right parietal A-group e.p.s.p. cells and the visceral giant cells VV1, VV2. Some initial evidence from surface electrical stimulation suggests that at least some of the A-group e.p.s.p. cells might be able to excite the heart (Buckett, 1987). The giant cells VV1 and VV2 were not stained in previous studies; these are particularly interesting for future work because of their large size and ease of identification. Some preliminary evidence suggested that they may play a role in pneumostomal opening (de Vlieger *et al.*, 1976).

Other cells stained in the present study need further work to definitely establish their identity. These include cells which appear to correspond to the lip motor neurons (CV cells) of McCrohan (1984) located in the ventral lobes of the cerebral ganglia (Table 1, Fig. 7), and the crescent of cells occurring close to the CGCs. These and the three small labelled buccal neurons (Fig. 7a) were all likely to be part of the feeding circuitry.

Completely novel groups of cells occurred as clusters between the G-group and H,I,J,K cluster of the visceral ganglia (Fig. 7a), and scattered among the B-group and F-group of the right parietal and visceral ganglia, respectively (Fig. 7a, b). Other small groups of cells of unknown type occurred in the left parietal, visceral and right parietal ganglia (Fig. 7a). Of particular interest were large neurons which occur as a bilaterally symmetrical pair on the lateral surfaces of the cerebral ganglia. It should be possible to find these neurons and some associated smaller cells by electrophysiological studies because of their size and characteristic location. They were not in the lateral lobes of the cerebral ganglia (Joosse, 1964) and so did not correspond to previously identified giant cells such as the canopy cells.

A general serotonergic 'system' for all gastropod molluscs?

Maps of serotonin-containing neurons now exist for a number of gastropod molluscs. The general distribution of cells appear to be very similar for the five species examined in detail (*Aplysia californica*: Tritt *et al.*, 1983; Ono & McCaman, 1984; Jahan-Parwar *et al.*, 1987. *Aplysia depilans*: Salimova *et al.*, 1987. *Helisoma trivolvis*: Land & Crow, 1985. *Helix pomatia*: S.-Rózsa *et al.*, 1986; Hernádi *et al.*, 1989. *Lymnaea stagnalis*: Sakharov & Zs.-Nagy, 1968; Cottrell *et al.*, 1979; Audesirk, 1985; Casey & Winlow, 1985, and present work). In each type of animal the highest number of serotonin-containing neurons were found in the pedal ganglia, and no, or very few, neurons were found in the pleural and buccal ganglia. The presence of the presumably homologous paired giant serotonin-containing cerebral neurons in even distantly related species has already been well documented (Granzow & Rowell, 1981), and we suggest that this homology

may be at least partly true for the whole serotonergic system of gastropods.

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