Abundant distribution of locustatachykinin-like peptide in the nervous system and intestine of the cockroach *Leucophaea maderae*

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SUMMARY

An antiserum raised to the locust neuropeptide locustatachykinin I (LomTK I) was used for analysis of the distribution of tachykinin-related peptide in the cockroach *Leucophaea maderae*. Extracts of dissected brains, suboesophageal ganglia, thoracic ganglia and midguts were separated by high performance liquid chromatography and the fractions analysed in enzyme-linked immunosorbent assay with use of the LomTK antiserum. Each of the tissues was found to contain LomTK-like immunoreactive (LomTK-LI) components with retention times corresponding approximately to synthetic LomTK I and II and callitachykinins I and II. The LomTK antiserum was also used for immunocytochemical mapping of peptide in the nervous system and intestine of *L. maderae*. A large number of LomTK-LI interneurons were detected in the proto-, deuto- and tritocerebrum of the brain and in the suboesophaegeal ganglion. The immunoreactive neurons supply processes to most parts of the brain: the central body, protocerebral bridge, mushroom body calyces, antennal lobes, optic lobe and most regions of the non-glomerular neuropil. A few protocerebral neurons send LomTK-LI processes to the glandular lobe of the corpora cardiaca. In each of the thoracic ganglia there are six LomTK-LI interneurons and in each of the unfused abdominal ones there are two interneurons. The fused terminal ganglion contains some additional cell bodies in the posterior neuromers. LomTK-LI cell bodies were detected in the frontal ganglion and fibres were seen in this ganglion as well as in the hypocerebral ganglion. The frontal ganglion supplies LomTK-LI processes to the muscle layer of the pharynx. The muscle layer of the midgut is innervated by LomTK-LI fibres from the stomatogastric system (oesophageal nerve and associated ganglia). Additionally the midgut contains numerous LomTK-LI endocrine cells. A number of the pharyngeal dilator muscles were also found to be innervated by LomTK-LI fibres, probably derived from cell bodies in the suboesophageal ganglion. All the LomTK-LI neurons of the central nervous system appear to be interneurons, suggesting a neuromodulatory role of the endogenous tachykinins. The tachykinin-like peptides from peripheral ganglia may be involved in the control of foregut and midgut contractility and possibly the peptide of the endocrine cells in the midgut has additional actions related to intestinal function.

1. INTRODUCTION

Although many insect neuropeptides have been isolated by means of their myotropic actions on visceral muscle in convenient and sensitive assays, it is quite clear that their physiological functions may be much more diverse (Holman *et al.* 1991; Goldsworthy *et al.* 1992; Schoofs *et al.* 1993; Nässel 1994). The fascinating possibility that given insect neuropeptides, and their isoforms, have multiple functions became apparent largely owing to immunocytochemical mapping: specific peptides are often distributed in a variety of neuron types as well as in neurosecretory and endocrine cells (see: Hökfelt 1991; Nässel 1993*a*, 1994; Homberg 1994). Reverse endocrinology, the search for functions of isolated substances (Lafont 1991), has to some extent confirmed the functional multiplicity for some of the myotropic peptides (Goldsworthy *et al.* 1992; Schoofs *et al.* 1993). We have initiated studies of the distribution of myotropic neuropeptides in the cockroach nervous system (Nässel *et al.* 1992; Muren *et al.* 1993), with the aim of revealing potential sites of peptide action accessible for functional analysis.

A number of myotropic neuropeptides have been isolated from whole heads of the cockroach *Leucophaea maderae* with the aid of a bioassay that monitors changes in contractions of the cockroach hindgut (Holman *et al.* 1990*a*, *b*, 1991). The twelve cockroach peptides thus isolated have been grouped into sulphakinins, myosupressins, pyrokinins and leucokinins (Holman *et al.* 1990*a*, *b*, 1991). With the same bioassay related peptides were also isolated from brains and corpora cardiaca of the locust, *Locusta migratoria* (Schoofs *et al.* 1993). Among some additional peptides found in the locust were five peptides related to vertebrate tachykinins, the locustatachykinins I–V (LomTK I–V) (Schoofs *et al.* 1991*a*, *b*, 1993). Representatives of this peptide family have also been isolated from the blowfly *Calliphora vomitoria* (Lundquist *et al.* 1994*a*) and the mosquito *Culex salinarius* (Clottens

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et al. 1993; Holman *et al.* 1995), but so far not from *L. maderae*. These insect peptides are characterized by a sequence FX_1GX_2Ramide in their carboxy terminus (X_1 is F, H, M, T, V or Y and X_2 is M or V) and have been referred to as Arg-tachykinins by Holman *et al.* (1995), in contrast to the common vertebrate tachykinins which end with methionine-amide and which hence are termed Met-tachykinins. Peptides of the Met-tachykinin type have also been isolated from invertebrates, eledoisin from a cephalopod (Erspamer & Anastasi 1962) and sialokinins I and II from a mosquito (Champagne & Ribeiro 1994).

The physiological function of insect tachykinins is not known, but *in vitro* studies on the locust have shown that LomTKs stimulate contractions of foregut and oviduct muscle and stimulate the hindleg extensor tibia muscle (Schoofs *et al.* 1991). Immunocytochemical analysis of LomTK distribution in the brain of *Locusta migratoria* with an antiserum to LomTK I has indicated that LomTKs may also function as neurotransmitters or neuromodulators in interneurons (Nässel 1993*b*).

The LomTK I antiserum was subsequently used for mapping of locustatachykinin-like immunoreactive (LomTK-LI) neurons in the nervous system and intestine of the blowfly *Calliphora vomitoria* (Lundquist *et al.* 1994*b*) and for monitoring LomTK-LI fractions during purification of two blowfly peptides, callitachykinin I and II (CavTK I and II), with sequence similarities to the LomTKs (Lundquist *et al.* 1994*a*). The antiserum which recognizes a preserved region of the locust and blowfly peptides (Lundquist *et al.* 1994*b*) may thus be a good probe for LomTK-like peptides in other species, including the cockroach *L. maderae*. Since previous studies of the distribution of LomTK immunoreactivity were on dissected brains and corpora cardiaca of the locust (Nässel 1993*b*) and on dissected brains, thoracic–abdominal ganglia and intestines of the blowfly (Lundquist *et al.* 1994*b*), we have no information about possible LomTK-LI neurons in other issues. Thus, in the present study we decided to make a more extensive analysis of the distribution of LomTK-LI material in the cockroach *L. maderae*. The antiserum against LomTK I was employed for immunocytochemical mapping of neurons in the central and peripheral nervous system and intestine of *L. maderae*. Furthermore we used enzyme-linked immunosorbent assay (ELISA) to measure the amount of LomTK-LI material in extracts of dissected brains, ganglia and intestines separated through reversed phase high performance liquid chromatography (HPLC). The HPLC retention times of the immunoreactive material in the different tissues were compared with that of synthetic insect Arg-tachykinins. Each of the investigated ganglia and the midgut contains several immunoreactive components with different retention times. In the central nervous system LomTK-LI material was, with a few notable exceptions, found exclusively in interneurons, whereas in the peripheral nervous system (stomatogastric system) efferent neurons innervating visceral muscle were also resolved. LomTK-LI endocrine cells were seen in the midgut.

2. METHODS

(*a*) *Animals*

Cockroaches of the species *Leucophaea maderae* were from a crowded laboratory colony kept at 25 °C with a 16:8 light–darkness cycle. The animals were reared on dog chow and water (and occasionally fed apples). For most experiments adult cockroaches were used; nymphs were used for some of the wholemount immunocytochemistry and whole head sections. For the latter recently moulted animals were also used to facilitate cryostat sectioning.

(*b*) *Tissue extraction and preparation*

Tissue extraction was as described earlier (Muren *et al.* 1993). In short, the tissues were rapidly dissected in phosphate buffer (0.1 M, pH 7.4) and transferred to 50 ml polypropylene tubes kept on dry ice. Brains, suboesophageal, thoracic and abdominal ganglia and midguts were collected, typically 40 tissue samples in each tube (fresh mass between 0.2 and 1 g). To each tube 10 ml of boiling water was added. The tubes were boiled for 20 min (while vortexed every 5 min), cooled on ice and centrifuged (2900 *g* at 4 °C for 10 min). The supernatant was removed and the pellet was resuspended in 10 ml of acetic acid (1.0 M, 70 °C) following the same procedure. The two supernatants from each sample were pooled, centrifuged (12000 *g* at 4 °C for 10 min) and freeze dried.

(*c*) *High performance liquid chromatography* (HPLC)

Before separation on reversed phase HPLC the samples were prepurified by using SepPak C_{18} cartridges (Millipore), activated with 10 ml of acetonitrile (CH_3CN) followed by 30 ml of water (H_2O). The cartridges were then equilibrated with 10 ml of H_2O with 0.1 % trifluoroacetic acid (TFA) followed by 10 ml of 10 % CH_3CN + 0.1 % TFA. The freeze dried samples from the extraction step were dissolved in 15 ml of 10 % CH_3CN + 0.1 % TFA and applied to the cartridge. They were eluted with 6 ml of 50 % CH_3CN + 0.1 % TFA. The eluates were further purified by ultrafiltration (at 2900 *g*) with use of Ultrafree-CL filters (Millipore) to separate proteins larger than 30 kDa from the samples.

Further separation was achieved by using a Waters/Millipore HPLC system with a 600E controller and a 486 ultraviolet (UV) detector set at 214 nm. For reversed phase separation a Waters Delta Pak column (C_{18} bonded phase, 100 Å pore diameter, 5 μm particle size, dimensions 3.9 mm × 150 mm) was used. The mobile phase was 10 % CH_3CN + 0.1 % TFA (solvent A) and 50 % CH_3CN + 0.1 % TFA (solvent B). Separation was done at room temperature under the following operating conditions: 100 % solvent A for 5 min after loading the sample, then a linear gradient to 100 % solvent B for 40 min. The flow rate was set at 1 ml min^{-1} and 1 min fractions were collected. Fractions were lyophilized in a Savant Speed-Vac before being assayed in ELISA (see below). The UV

absorption and retention times of synthetic LomTK I and II and CavTK I and II were determined by running 1 nmol of each of the peptides, separately as well as mixed together. The synthetic LomTK I and II were from Peninsula (Belmont, California) and CavTK I and II were synthesized by Dr R. J. Nachman, College Station, Texas (see Lundquist *et al.* 1994*a*).

Chromatographic separation was also done on whole heads, which before extraction had been ground to powder in liquid nitrogen with a ceramic mortar. Extraction was as described above. Prepurification was done on a SepPak cartridge which after activation was equilibrated with 10 ml of 20% CH_3CN + 0.1% TFA. The sample (40 heads in 8 ml of 20% CH_3CN + 0.1% TFA) was applied and eluted with 5 ml of 80% CH_3CN-0.1% TFA. The ultrafiltration step was omitted. The eluate was diluted four times in H_2O + 0.1% TFA before being pumped directly onto a Delta Pak column (Waters C_{18}, 100 Å, 15 μm, 3.9 mm × 300 mm). After loading the sample, the pump was run in an isocratic mode for 5 min with 100% solvent A (20% CH_3CN + 0.1% TFA) and then a linear gradient to 100% of solvent B (80% CH_3CN + 0.1% TFA) for 50 min. The HPLC system was used with the detector set at 214 nm and the pump operating at 1.5 ml min^{-1} at room temperature. One minute fractions were collected and freeze dried. After running the head extract the retention time of synthetic LomTK I was determined.

The HPLC fractions collected from the midguts were split into two portions: one half was used for ELISA determinations; the other half of the fractions that were found immunoreactive in the ELISA were assayed in the cockroach hindgut contraction bioassay (Holman *et al.* 1991). This assay was kindly done by Dr G. M. Holman (College Station, Texas).

(*d*) *Enzyme-linked immunosorbent assay* (ELISA)

The fractions were assayed for LomTK-like immunoreactivity in an ELISA, with the same protocol as described earlier (Lundquist *et al.* 1994*a*, *b*). The HPLC fractions and synthetic LomTK-I peptide were dissolved in 80% CH_3CN + 0.1% TFA and freeze dried to the wells of a MaxiSorp immunoplate (Nunc). To each well was added 150 μl of 10 mM phosphate buffered saline (PBS buffer) with 0.1 M NaCl (pH 7.2). The samples were incubated overnight at 4 °C. Non-specific binding of the antibody was blocked with 300 μl of wash buffer (20 mM PBS, 0.15 M NaCl, 0.05% Tween-20, pH 7.2) with 0.05% gelatin for 1 h at 37 °C. After blocking, the plate was incubated for 2 h at 37 °C with 150 μl of LomTK-I antiserum (as described below), diluted to 1:2000. This was followed by washing and incubation with 150 μl of secondary antiserum (alkaline phosphatase-labelled goat anti-rabbit antiserum, dilution 1:1000, Pierce) for 1 h at 37 °C. The plate was washed with wash buffer and DEA buffer (10 mM diethanolamine, 0.5 mM $MgCl_2$, pH 9.5) and the reaction was visualized with 100 μl of p-nitrophenyl phosphate (Pierce, 1 mg ml^{-1} in DEA buffer). The reaction was stopped after 20 min with 100 μl of 3 M sodium hydroxide and monitored at 405 nm in a Labsystems Multiskan Plus ELISA plate reader. ELISAs were done both in a competitive and in a non-competitive fashion (see Lundquist *et al.* 1994*b*). In competitive ELISAs all wells were coated with 5 pmol of LomTK-I peptide overnight at 4 °C. The antibody was preincubated (1 h, room temperature) in Eppendorf tubes with known concentrations of peptide and the fractions from the HPLC before they were added and incubated as above.

(*e*) *Immunocytochemistry* (ICC)

Tissue was fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 4–16 h at 4 °C (commonly 16 h was used). Brains and ganglia of the ventral cord as well as frontal ganglia and corpora cardiaca were fixed *in situ* after removal of cuticle and muscles. After thorough washes in phosphate buffer the tissues were dissected and used either for whole-mount immunocytochemistry (ICC) or for cryostat sectioning followed by ICC as described below. Several heads were fixed after opening of the cuticle at the back, leaving most of the muscles intact. For analysis of the intestine the abdomen was opened dorsally and the midgut was taken out, cut sagittally and pinned out in fixative to make a spread preparation.

The antiserum production was described by Nässel (1993*b*). In brief the antiserum (9207-7) was raised in rabbit against LomTK I conjugated to human serum albumin (HSA) with carbodiimide. The specificity and cross reactivity of this antiserum has been extensively tested in ELISA, dot–blot and preabsorption ICC experiments (Nässel 1993*b*; Lundquist *et al.* 1994*b*). This antiserum was used for all the ICC described in this paper (as well as in the ELISAs described above). Another antiserum raised against LomTK II (Agricola & Weiss 1993; Agricola & Bräunig 1994) was tested for comparison. Since it produced the same labelling pattern as the LomTK I antiserum it was not further employed.

For wholemounts the dissected tissues were incubated for four to five days at 4 °C in anti-LomTK I diluted to 1:1000 in a dilution buffer (DiB) consisting of 0.01 M phosphate buffered saline (PBS) with 0.25% Triton X-100 and 0.5% bovine serum albumin (BSA). After thorough washes in PBS with Triton the tissues were incubated in unlabelled swine anti-rabbit IgG (DAKO, Copenhagen), diluted to 1:50 in DiB, for 24 h and thereafter in rabbit peroxidase anti-peroxidase (PAP, DAKO), at 1:100 in DiB, for 24 h. The peroxidase reaction was done in 0.03% diaminobenzidine (Sigma) and 0.01% hydrogen peroxide for 30 min. The specimens were dehydrated and embedded between coverslips in Durcupan (Fluka).

Cryostat sections were incubated with primary antiserum (1:1000) for two days at 4 °C, and then with secondary and tertiary antisera as above, but for 1 h each. The enzyme reaction was performed as above, but for 10–15 min. The slides were dehydrated and mounted under coverslips with Permount. Some sets of cryostat sections and some wholemounts were used for

immunofluorescence. The primary antiserum was applied as above, but as secondary antiserum we used either fluorescein isothiocyanate (FITC) or tetramethyl rhodamine (TRITC) tagged swine anti-rabbit IgG (DAKO) both diluted to 1:30 in DiB (see also Nässel *et al.* 1992).

As controls the following ICC tests were made on tissue sections by the peroxidase anti-peroxidase technique: application of preimmune antiserum (9207-0, diluted 1:1000) from the same rabbit; application of antiserum (diluted 1:1000) that had been preabsorbed with synthetic LomTK I or II (Peninsula) or LomTK I–HSA conjugate, at 20 or 50 nmol ml^{-1} diluted antiserum, overnight at 4 °C.

3. RESULTS

(*a*) ELISA *and* HPLC

The amount of antiserum and peptide needed in the ELISA was determined in a dilution series of peptide and antiserum (chequer board ELISA). The peptide was added to the plate in a range of 1 fmol to 10 pmol per well. The antiserum was applied at concentrations from 1:500 to 1:20000. Optimal response was obtained at a dilution of 1:2000 with the peptide added to the plate at a concentration of 5 pmol per well. The cross reactivity of the LomTK antiserum has been characterized in ELISA by Lundquist *et al.* (1994*b*). Immunoreactive material in crude extracts from whole heads of *L. maderae* was found to dilute in parallel to a dilution series of synthetic LomTK I (figure 1). The amount of immunoreactive material in crude extracts was estimated to 0.15 pmol per head and 0.5 pmol per midgut.

Extracts of heads, brains, suboesophageal ganglia, thoracic ganglia (T1–3) and midguts were separated on HPLC. Fractions collected every minute were assayed for LomTK-like immunoreactivity. For comparison

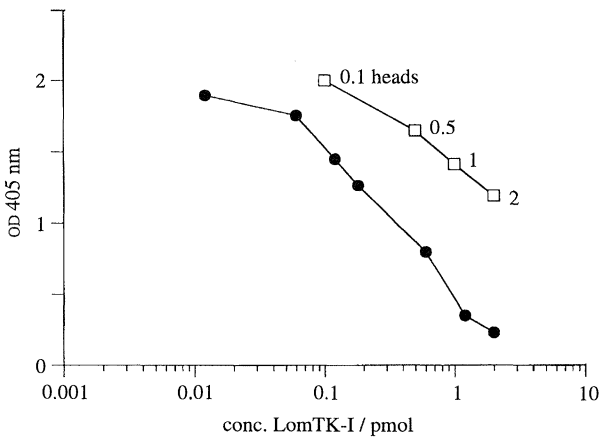

Figure 1. Competitive ELISA showing the binding of locustatachykinin antiserum to crude extract from *Leucophaea* heads (open squares) compared with the binding of the antiserum to synthetic locustatachykinin I (LomTK-I) (filled circles). The *y*-axis represents the optical density (OD, filter 405 nm) of the colour reaction and the *x*-axis represents the amount of locustatachykinin preincubated with the antiserum before incubation in the wells. The amount of head extract preincubated with the antibody is indicated in the figure.

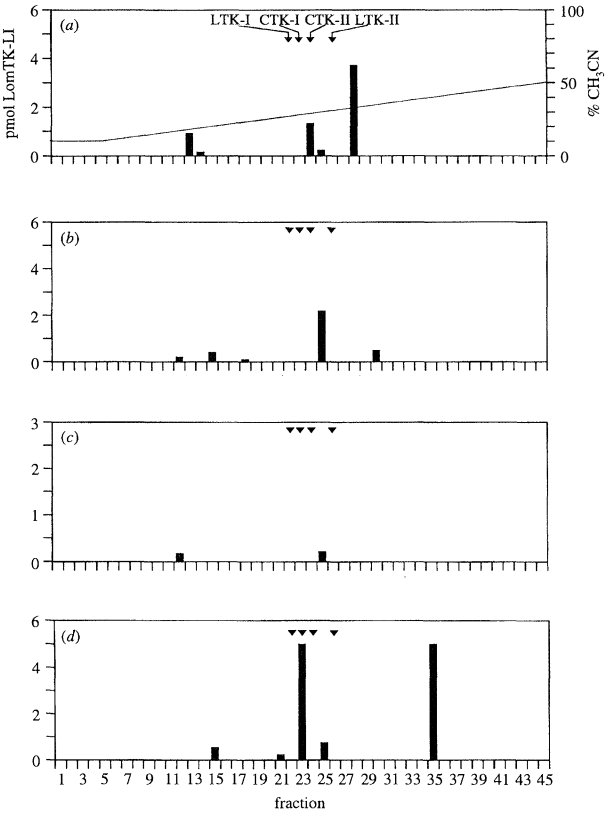

Figure 2. Locustatachykinin immunoreactivity in fractions separated on reversed phase HPLC (Waters C_{18} column) from different tissues measured in non-competitive ELISAs. Fractions were split in two and assayed as duplicates from (*a*) brains ($n = 20$), (*b*) suboesophageal ganglia ($n = 60$), (*c*) thoracic ganglia (T1–T3) ($n = 40$), and (*d*) midguts ($n = 10$). After 5 min of isocratic condition a linear gradient from 10 to 50% acetonitrile with 0.1% trifluoroacetic acid was run over 40 min as shown in (*a*). The retention times of the peptides locustatachykinin I and II (LTK-I, LTK-II) and callitachykinin I and II (CTK-I and CTK-II) are indicated (arrows, arrowheads). The midgut fractions 22–25 and 35 were split in two and half of each was used in the *L. maderae* hindgut contraction assay (Holman *et al.* 1991). Fractions 22–24 were found bioactive, whereas 25 and 35 were not.

1 nmol of each of the synthetic peptides CavTK I and II and LomTK I and II were run for determination of their retention times (figure 2). Each tissue assayed contained several immunoreactive components, some of which were in the range of the synthetic peptides (figure 2). One of these components was detected in all tissues assayed and had a retention time close to CavTK II and LomTK II. Another LomTK-LI component was found in the brain and the ventral ganglia, but not the intestine (fraction 12–13). The brain and suboesophageal ganglia contained an additional later component (fractions 28 and 30 respectively). The intestine contained yet another late component in fraction 35 not seen in the other tissues. In a slightly different HPLC system the bulk of the immunoreactive material of the crude head extract coeluted with the synthetic LomTK I (data not shown).

The fractions collected from the midguts were divided in two parts; one was assayed in ELISA and the other in the *L. maderae* hindgut contraction assay. The immunoreactive fraction 23 was found to be myotropic

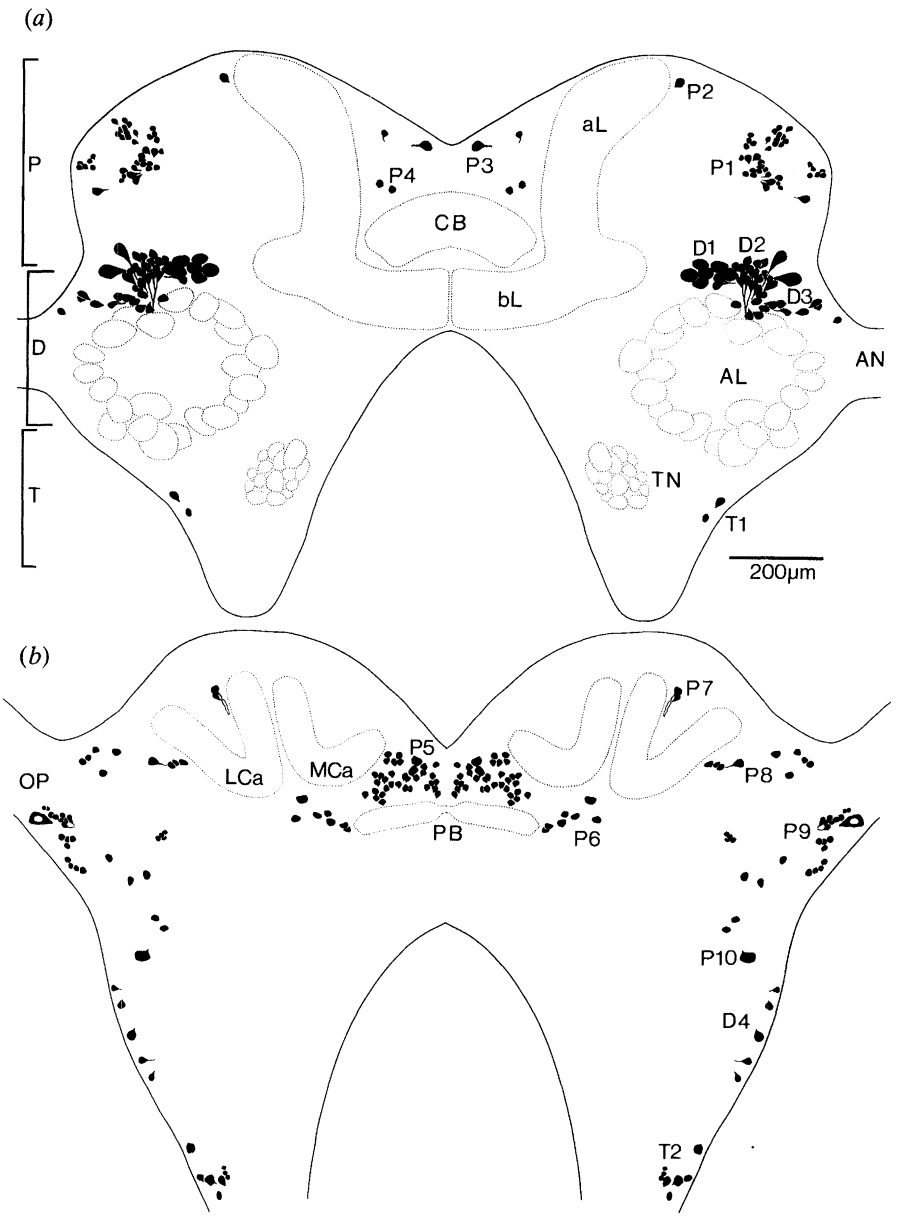

Figure 3. Distribution of LomTK-LI cell bodies in the brain of *L. maderae* seen in frontal view. The anterior portion of the brain is shown in (*a*) and the posterior part is displayed in (*b*). Cell bodies are distributed in a bilateral symmetric fashion. (*a*) Anteriorly in the protocerebrum (P) there are a few clusters of cell bodies or single identifiable cell bodies: a large lateral cluster P1, the single dorsal cell bodies P2 and P3 and the pair P4 near the central body (CB). In the deutocerebrum (D) there are three anterior clusters of cell bodies (D1–D3) dorsal to the chemosensory antennal lobes (AL). The D1 neurons have large cell bodies, D2 and D3 smaller ones. In the tritocerebrum only two LomTK-LI cell bodies (T1) can be identified in each hemisphere. Other structures in the anterior brains are the mushroom bodies with α- and β-lobes (aL and bL), the central body (CB), antennal nerve (AN) and a glomerular tritocerebral neuropil (TN). (*b*) In the posterior protocerebrum there is a larger number of immunoreactive cell bodies. The largest cluster (P5) is located dorsal to the protocerebral bridge (PB) and smaller ones (P6–P8) are seen around the calyces (Ca) of the mushroom bodies. A cluster P9 is associated with the optic peduncle (OP) at the base of the optic lobe. About five cell bodies (D4) can be found in the posterior deutocerebrum and about eight in the tritocerebrum (T2).

in the hindgut assay (fractions 22 and 24 were also found myotropic, but not immunoreactive). The immunoreactive material in the midguts that eluted later (fraction 35), however, showed no bioactivity.

(*b*) *Specificity of antiserum on tissue sections*

The LomTK I antiserum (9207-7) was tested for immunocytochemistry on cockroach tissue after preabsorption with LomTK I and II and LomTK-I–HSA conjugate (20 and 50 nmol ml^{-1} antiserum, overnight). The preabsorbed antiserum and the pre-immune serum of the rabbit 9207 did not produce any immunolabelling in *L. maderae*. A further support for the specificity of our antiserum was that an antiserum raised to the almost identical peptide, LomTK II, by a different protocol (Agricola & Weiss 1993; Agricola & Bräunig 1994) gave identical immunolabelling.

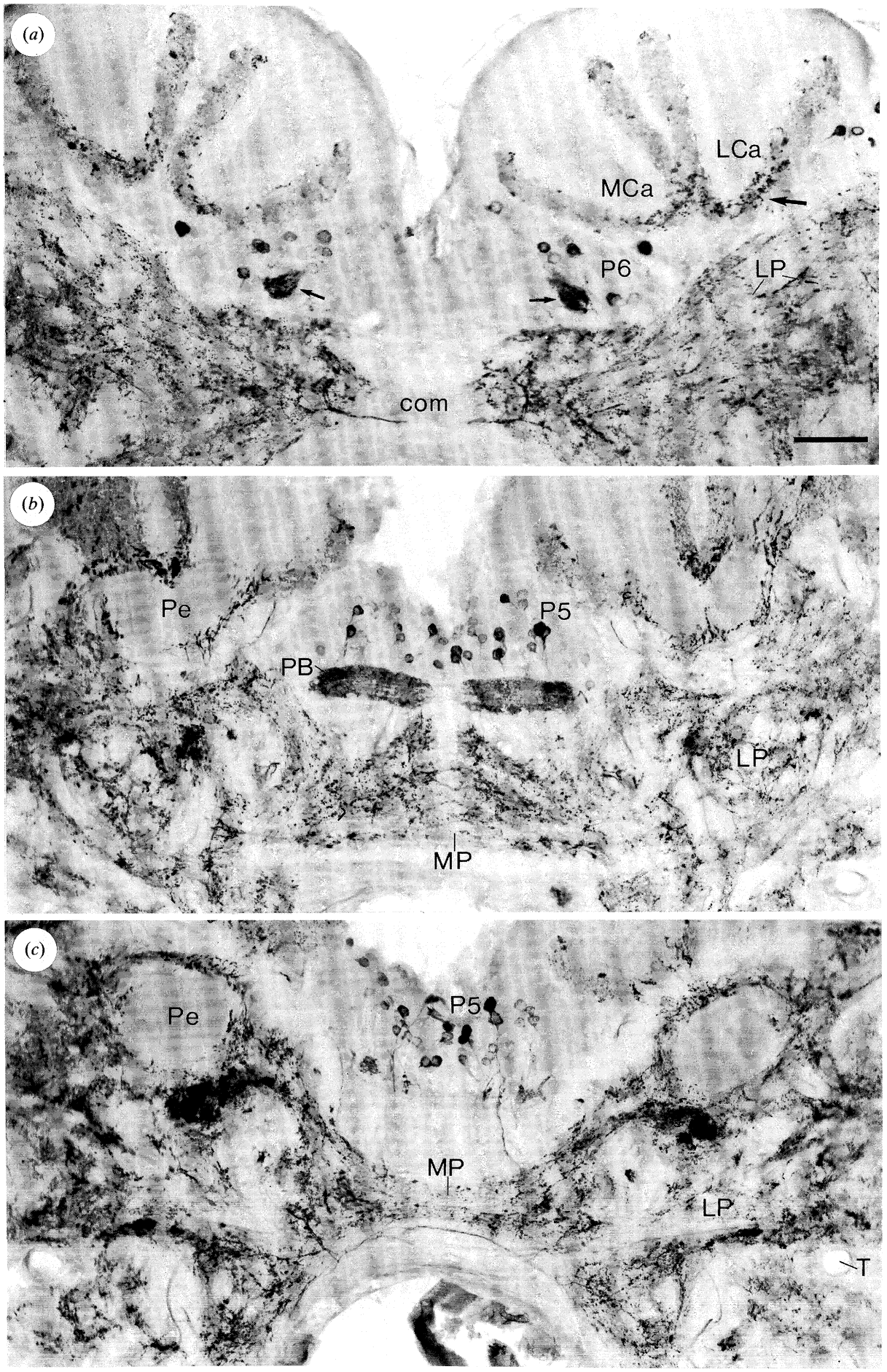

Figure 4. For description see opposite.

(*c*) *Distribution of LomTK immunoreactive cell bodies and neural processes in the brain*

Locustatachykinin-like immunoreactive (LomTK-LI) neurons were found throughout the central nervous system of *L. maderae*. The largest number of LomTK-LI cell bodies was seen in the brain. In the suboesophageal ganglion and the thoracic and abdominal ganglia only a smaller number of cell bodies could be detected in each neuromer. Most immunoreactive processes branch extensively and often form long projections. Thus it was not easy to resolve the morphology of entire single cells (or even populations of cells). In this account we therefore made no attempts to trace arborizations of neurons in detail. We wish to emphasize that, with a few possible exceptions, the LomTK-LI neurons of the CNS are interneurons.

About 360 LomTK-LI cell bodies are located in the two hemispheres of the brain (only intensely labelled cell bodies being counted). Of these about 240 are in the protocerebrum, 100 in the deutocerebrum and 20 in the tritocerebrum. An additional 50, or more, small cell bodies were seen in each of the optic lobes. The cell bodies are distributed as bilateral pairs or in bilateral clusters so that the two hemispheres appear roughly mirror symmetric in terms of number of LomTK-LI cell bodies. The largest clusters of cell bodies are seen at the protocerebral bridge (about 32 in each hemisphere) and at the antennal lobes of the deutocerebrum (about 40 cell bodies in each antennal lobe).

In the protocerebrum the 240 cell bodies are distributed in a few main regions as shown in figure 3. Large clusters of immunoreactive cell bodies (P1) are located laterally in the anterior protocerebrum. Other large clusters of cell bodies are found posteriorly above the protocerebral bridge (P5) and lateral to it (P6), as well as at the base of the optic peduncle (P9). The remaining protocerebral LomTK-LI cell bodies are found scattered as bilateral pairs or smaller bilateral clusters as seen in figure 3. In the protocerebrum LomTK immunoreactive neurons innervate a number of regions (figures 4–6). Of the well delineated neuropils (glomerular neuropils) the following receive immunoreactive fibres: the lower portion of the mushroom body calyces (figures 4*a*, *b*, 6*f*, *g*), the protocerebral bridge (figures 4*b*, 6*a*), the fan-shaped body (figures 5*a*, 6*b*, *c*), the nodules (figure 6*b*) and the lateral accessory lobes of the central complex (figure 6*d*), and the medulla and lobula neuropils in the optic lobes (figure 5*c*, *d*). On the other hand some glomerular neuropils are devoid of immunoreactive fibres: the peduncles and α- and β-lobes of the mushroom bodies (figures 5*a*, *b*) and the optic lobe lamina. It is apparent that LomTK-LI fibres also supply numerous extensive regions of less defined synaptic neuropil (non-glomerular neuropil). In the figures 5 and 6*a*, *b* densely packed immunoreactive fibres are seen in lateral and median protocerebrum. The neuropil surrounding the mushroom body peduncles (figures 4*c*, 5*a*) and the α- and β-lobes (figure 5*b*) is especially densely innervated. A substantial number of LomTK-LI fibres can be seen connecting the two brain hemispheres in tracts and median neuropils indicating that quite a number of the LomTK-LI neurons supply processes bilaterally to both sides of the brain.

The central complex is innervated by the small cell bodies (P5) seen in figures 4*b* and 6*a*. From these cell bodies fibres invade the protocerebral bridge (figure 6*a*) where they arborize before entering the upper division of the central body (fan-shaped body) in which they form further arborizations (figure 6*b*). LomTK-LI fibres of undetermined origin also invade the lower division of the central body (figure 6*c*) and portions of the noduli (figure 6*b*). Tracts of immunoreactive fibres run from the central body to the lateral accessory lobes of the central complex (figure 6*d*). As seen in the sagittal section of the central body in figure 6*e*, the distribution of immunoreactive fibres forms complex arborizations in the neuropil.

It is not clear which LomTK-LI cell bodies supply fibres to the mushroom body calyces (owing to superposition of immunoreactive fibres in critical regions). Both the median and the lateral calyces appear to be innervated by the same systems of fine varicose fibres which also supply branches to the dorsolateral protocerebrum. The most dense fibre supply is seen in the basal portion of the calyces (figures 4*a*, 6*f*, *g*).

In the optic lobes LomTK-LI fibres can be resolved in the medulla, especially a basal layer (figure 5*c*), and in the distal portion of the lobula (figures 5*c*, *d*). These fibres are in part derived from cell bodies located adjacent to the medulla and lobula (figure 5*c*); others may be derived from cell bodies in the optic peduncle (figure 3*b*).

In the deutocerebrum most of the LomTK-LI cell

Figure 4. Series of micrographs displaying LomTK-LI structures in the cockroach brain (from the same series of 25 μm frontal cryostat sections, PAP method). Only the midregion of the brain is shown (compare with figure 3). (*a*) Most caudal section with the median (MCa) and lateral (LCa) calyces of the mushroom bodies. Dense distribution of LomTK-LI fibres is seen in the most basal portion of the calyx neuropil. A cluster of cell bodies (P6) is located in this region. Note the dense innervation of the posterior neuropil of the lateral protocerebrum (LP). Arrows point at posterior portions of the protocerebral bridge. (*b*) More anterior section including the protocerebral bridge (PB) and mushroom body calyces at the point where they form the peduncle (Pe). The cell bodies of the P5 cluster innervate the protocerebral bridge. Immunoreactive fibres are seen in the calyces, in the protocerebral neuropil ventral to the calyces and in diffuse neuropil in the median protocerebrum (MP). Commissural fibres (com) connect the two hemispheres. (*c*) Adjacent more anterior section with anteriormost portion of calyces and with peduncles (Pe) of mushroom bodies. Note the dense distribution of LomTK-LI fibres around the peduncles and diffuse neuropil in lateral protocerebrum (LP). Commissural fibres can be seen in median protocerebrum (MP). The anterior cell bodies of cluster P5 are located dorsally. Scales: (*a*)–(*c*) 100 μm.

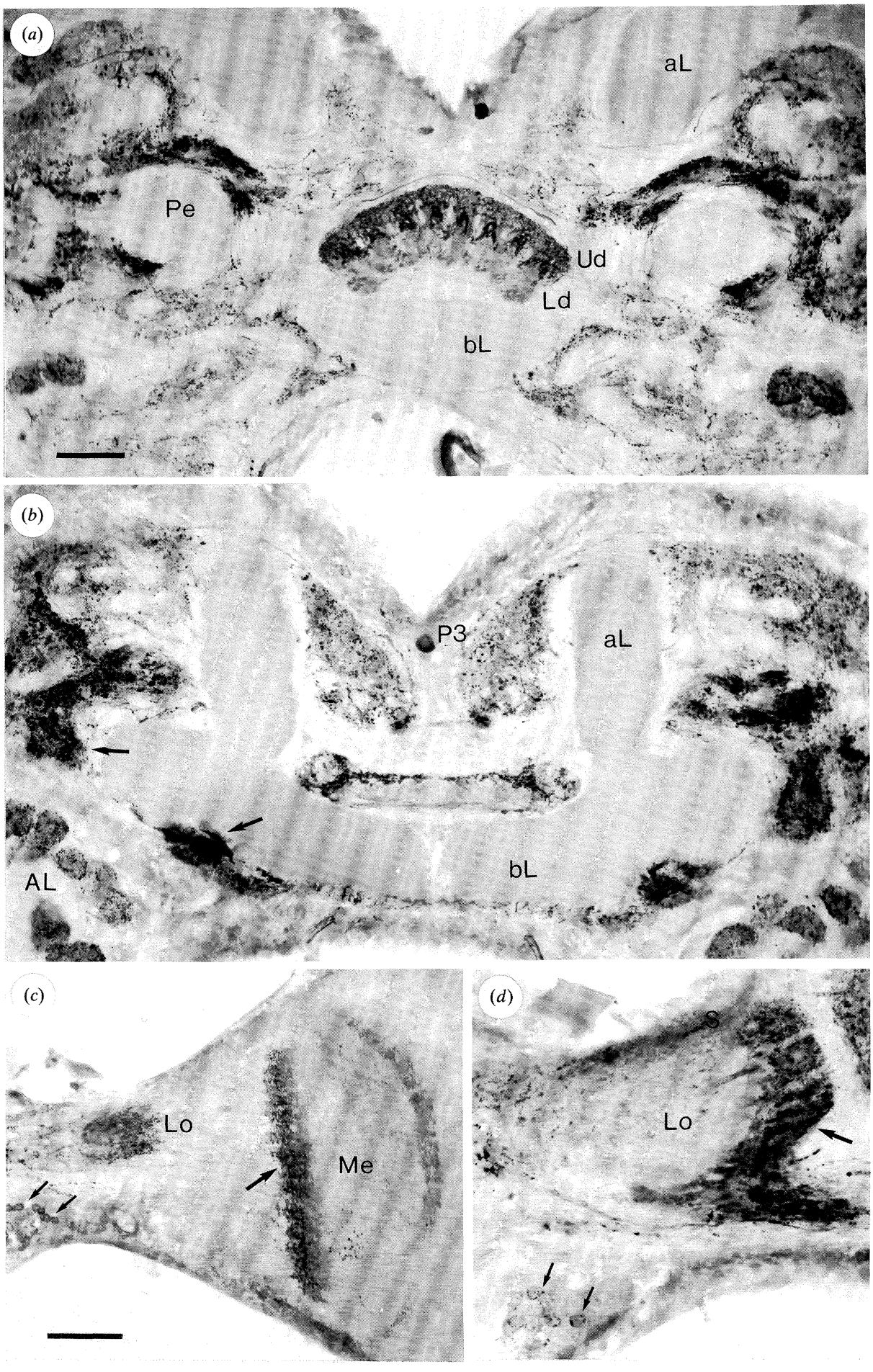

Figure 5. For description see opposite.

bodies are clustered anteriorly in a region dorsal to the antennal lobes (figure 3*a*). Here we found three types of neurons (D1–3), judged by cell body diameters and axonal projections (figures 3*a*, 7*a*, *b*): one group consists of large cell bodies (D1), the other two (D2–3) have smaller cell bodies and the neurites of each group fasciculates separately. The neurons of groups D1 and D2 innervate the glomeruli of the antennal lobe on the ipsilateral side (figure 7*a*, *b*), whereas it is not clear what neurite trajectories the D3 neurons have. All antennal glomeruli appear to be supplied by varicose LomTK-LI fibres (figures 7*a*, *b*). No immunoreactive fibres could be seen in the antennal nerves (figure 7*a*). In the posterior deutocerebrum a small number of LomTK-LI cell bodies (D4) are located laterally in each hemisphere (figure 3*b*); immunoreactive fibres could also be resolved (not shown).

In the tritocerebrum only a smaller number of LomTK-LI cell bodies were found anteriorly and posteriorly (figure 3). Immunoreactive fibres are scattered in the tritocerebral neuropil and axon tracts. The most prominent neuropil structure in the tritocerebrum is a glomerular neuropil anteriorly in each hemisphere, approximately at the site of the common root of the labral nerve and frontal connective (figures 3*a*, 7*c*, *d*). This neuropil is densely supplied by fine LomTK-LI fibres in a fashion rendering each glomerulus-like structure almost uniformly labelled (figures 7*c*, *d*). The cellular source of this immunolabelling could not be determined. In the tritocerebral connectives, which connect the brain to the suboesophageal ganglion, numerous immunoreactive fibres could be resolved indicating the presence of ascending and possibly descending LomTK-LI neurons (see below).

(*d*) *LomTK-LI neurons in the suboesophageal, thoracic and abdominal ganglia*

The ventral cord of the cockroach consists of a suboesophageal ganglion (a fusion of three ganglia), three thoracic ganglia, five unfused abdominal ganglia and a fused terminal abdominal ganglion. The last thoracic ganglion, the metathoracic ganglion, is fused with the anteriormost abdominal ganglion (A1).

In the suboesophageal ganglion about 25 LomTK-LI cell bodies were detected (figure 8*a*). It should be noted that for analysis of the cell body distribution in the suboesophageal ganglion we mainly used wholemounts of nymphal ganglia (antibody penetration was a problem in adult ganglia). These LomTK-LI cell bodies are distributed as 12 pairs and one dorsal unpaired median (DUM) cell. The cell body of the immunoreactive DUM neuron is located anteriorly in the mandibular neuromer of the ganglion, but we could not resolve its processes. As seen in figure 9 the neuropil of the three neuromers of the suboesophageal ganglion is densely innervated by LomTK-LI fibres, both in ventral and in dorsal neuropils. A substantial number of LomTK-LI fibres traverse the ganglion midline in commissures and median neuropil regions (figures 9*b–d*), indicating the presence of neurons with bilateral processes. Immunoreactive axons can be seen in the tritocerebral connectives and the posterior connectives joining the prothoracic ganglion. We could, however, not resolve immunoreactive efferent or afferent fibres in the suboesophageal nerve roots.

In each of the three thoracic ganglia three pairs of LomTK-LI cell bodies were found ventrally (figures 8*b–d*). These neurons all seem to be interneurons with processes restricted to the central nervous system, possibly with ascending axons (see abdominal ganglia), since no axons were seen in peripheral nerve roots. The morphology of the central neuropil branches could not be determined from the wholemount preparations.

In the first abdominal ganglion (fused with the metathoracic ganglion) and in each of the five unfused abdominal ganglia (A2–A6) we could label one pair of cell bodies (figures 8*d*, *e*, 10). Owing to the small size of the abdominal ganglia and the thin connectives (especially in subadult specimens) we could resolve the detailed anatomy of the abdominal LomTK-LI neurons (figure 10*a*). Each neuron has neuropil processes in the ipsilateral side of the native neuromer, both laterally near the cell body and medially in the neuropil. From the neurite an axonal process ascends anteriorly into the ipsilateral connective. This axonal process traverses at least the two next anterior ganglia, where it gives off some collateral branches posteriorly in each ganglion. It appears that the axon terminates in the posterior portion of the third ganglion from the native ganglion (anterior to it). It cannot be excluded that the axons project further anteriorly, but are too weakly immunolabelled to be detected (figure 10*a*). No immunoreactive fibres were detected in the peripheral nerve roots of the abdominal ganglia.

In the terminal abdominal ganglion there are eight pairs of LomTK-LI cell bodies, all of which appear to be interneurons (figure 8*f*). In the two anterior

Figure 5. LomTK-LI structures in frontal cryostat sections of brain (continuation of section series from figure 4). (*a*) Section at level of central body and α-lobes of mushroom body. In the central body immunoreactive fibres are most dense in the upper division (Ud) and less dense in the lower division (Ld). No immunoreactivity is seen in the α-lobes (aL), β-lobes (bL) and peduncles (Pe) of the mushroom bodies, whereas protocerebral neuropil surrounding the peduncles is densely innervated by LomTK-LI fibres. (*b*) More anterior section with α- and β-lobes (aL, bL) of mushroom bodies (in which no immunoreactivity could be revealed). Immunoreactive fibres are, however, distributed in surrounding protocerebral neupils and in one of the P3 neurons. Part of the posterior portion of the antennal lobes (AL), with immunoreactive fibres, is seen here. (*c*) Overview of immunoreactivity in the right optic lobe. Fibres (large arrow) were resolved in the medulla (Me) and lobula (Lo) neuropils. Cell bodies (small arrows) are seen ventral to the lobula. (*d*) Detail of the immunoreactive fibres distally in the lobula neuropil (Lo). Scales: (*a*), (*b*) 100 μm; (*c*), (*d*) 50 μm.

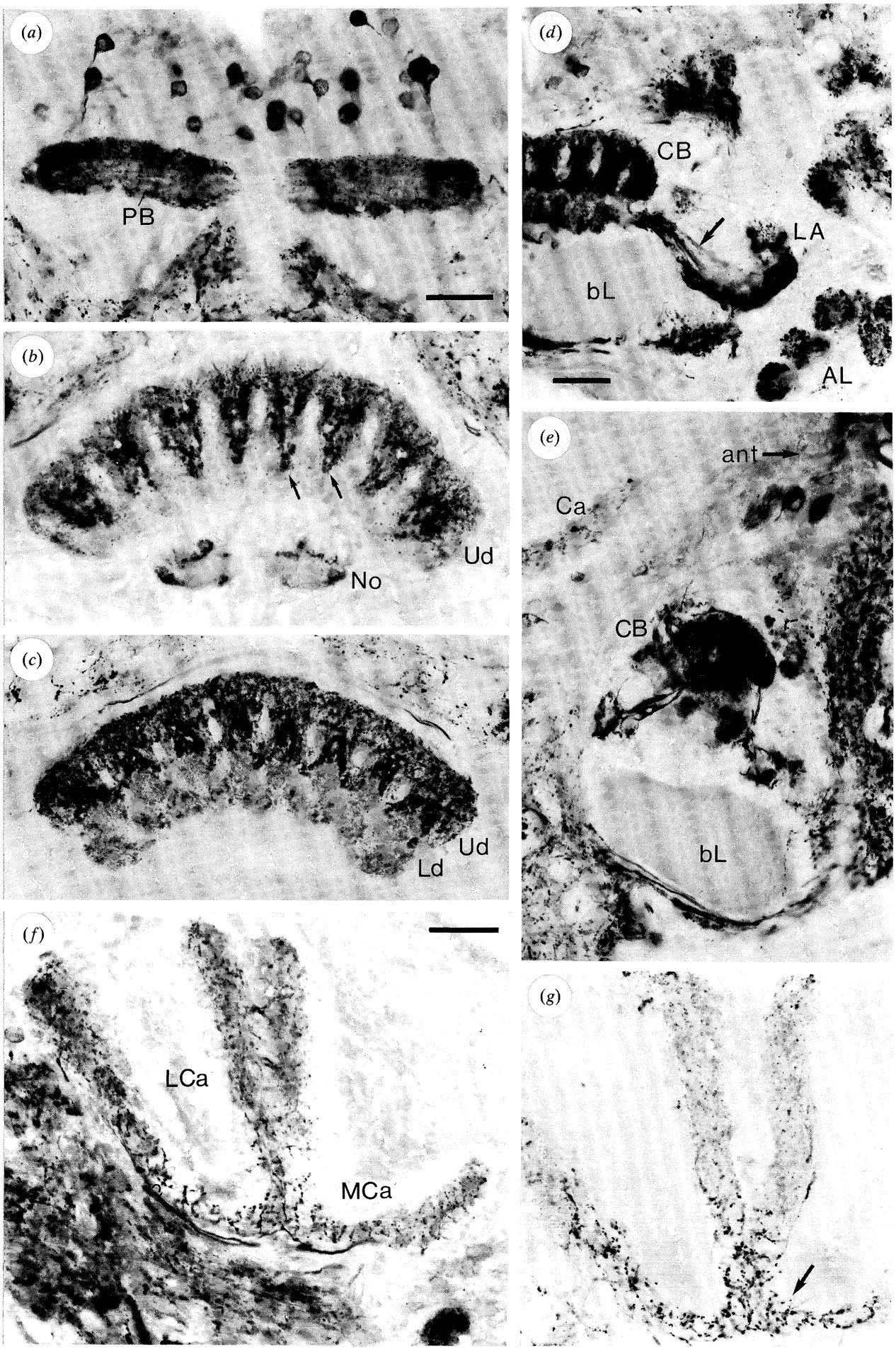

Figure 6. For description see opposite.

neuromers ascending neurons, of the type described above in the unfused ganglia, can be seen. The organization of the more posterior interneurons is not clear. No peripheral fibres could be labelled with the LomTK antiserum.

(*e*) *Retrocerebral complex and peripheral nervous system associated with the pharynx*

The cephalic neurosecretory system consists of neurosecretory cells in the protocerebrum, tritocerebrum and suboesophageal ganglion supplying axons to the neurohaemal organs corpora cardiaca and corpora allata which are attached to the cephalic aorta (see: Wiley 1961; Gundel & Penzlin 1978; Koontz & Edwards 1980). This neurosecretory complex, the retrocerebral complex, is connected by nerves from the brain (NCC I–III) and suboesophageal ganglion (NCA II) and to the stomatogastric system (NCS). The stomatogastric system consists of (1) the frontal ganglion connected to the brain by the two frontal connectives and the unpaired *nervus connectivus* and (2) the hypocerebral ganglion which is connected to the frontal ganglion by the recurrent nerve and to the non-cephalic stomatogastric system with a posterior nerve termed the oesophageal nerve. The frontal ganglion and the recurrent nerve supply nerve branches to pharyngeal muscles.

A sparse innervation of the glandular lobe of the corpora cardiaca with LomTK-LI processes could be seen (figure 11*a*, *d*). The fibres are interspersed between the glandular cells. Very few immunoreactive fibres were detected in the storage lobes of the corpora cardiaca and no fibres in the corpora allata. The cell bodies supplying fibres to the corpora cardiaca could not be unambiguously identified owing to the massive immunolabelling of the brain; they appear to be cells of the median neurosecretory cell group (MNC), since a few cell bodies in this region were labelled and some LomTK-LI fibres were seen in the corpora cardiaca nerve NCC I which is supplied by the MNCs. Further supply from the NCC II and III cannot be excluded.

In the frontal ganglion one large and two smaller LomTK-LI cell bodies were seen in the rind (figure 11*b*, *c*). These neurons form processes in the neuropil of the frontal ganglion (figure 11*b*, *c*). Immunoreactive axonal processes can be detected both in the frontal connectives that connect the frontal ganglion to the tritocerebrum and in the recurrent nerve that joins the hypocerebral ganglion (figure 11*a*, *c*). LomTK-LI fibres but no cell bodies were detected in the hypocerebral ganglion (figure 11*a*) and the fibres continue into the oesophageal nerve. Some of the nerve branches originating from the frontal connectives and recurrent nerve of the frontal ganglion contain LomTK-LI fibres that supply the muscle layer of the pharynx (figure 11*e*).

LomTK-LI fibre terminations were seen on the pharyngeal dilator muscles (figure 11*d*). The dorsoventral muscles seen in figure 11*d* are pharyngeal dilator muscles that probably correspond to muscles 37 (pharyngeal dilator muscles) in the locust (Hughes 1980; Bräunig 1990). Other pharyngeal dilator muscles, anterior and ventral ones, are also supplied by LomTK-LI fibres (Figure 11*d*). We were not able to trace the origin of the LomTK-LI fibres to these muscles. In the locust cobalt filling has revealed innervation of pharyngeal dilator muscles derived from neurons in the suboesophageal ganglion whose axons run via the tritocerebrum and NCC III to their peripheral targets (Bräunig 1990).

No immunoreactive fibres were detected in the muscle of the antennal pulsatile organ (antennal heart; Pass *et al.* 1988). We specifically investigated this muscle since it is known to be a target of innervation by e.g. allatostatin immunoreactive and FMRF-amide immunoreactive fibres (Woodhead *et al.* 1992; D. R. Nässel, unpublished).

(*f*) *Immunoreactive fibres and endocrine cells in the intestine*

The muscle layer of the pharynx is innervated by LomTK-LI fibres derived from the frontal ganglion and recurrent nerve (figure 11*d*). In the midgut LomTK-LI endocrine cells were seen unevenly distributed (figure 12*a–f*). These cells are elongated and do not appear to make contact with the intestinal lumen (Figure 12*a–d*). A fairly dense supply of varicose LomTK-LI fibres could be seen in the wall of the midgut (figure 12*f*, *g*). Most of these fibres are longitudinally oriented. In several regions the fibres form arborizing varicose terminals (figure 12*g*, *h*). No fibres were seen at the border between midgut and hindgut. The origin of the fibres appears to be the stomatogastric system via the oesophageal nerve.

Figure 6. Details of LomTK-LI neurons in the protocerebrum. All micrographs, except (*e*), show frontal sections of the cockroach brain (PAP method). (*a*) Immunoreactive fibres in the protocerebral bridge derived from P5 cell bodies. (*b*), (*c*) The central body. In the more caudal section in (*b*) the eight palisades of immunoreactive fibres in the upper division (Ud) are prominent. Some fibres are seen in the noduli (No). In the next anterior section (*c*) fibres can also be seen in the lower division (Ld) of the central body. (*d*) In a nymphal cockroach the immunoreactive fibres are more densely packed. The fibre connection between the central body (CB) and lateral accessory lobe (LA) can be seen (arrowed). A portion of the antennal lobe (AL) is visible. (*e*) A sagittal section through the midbrain (of nymph) with the central body (CB) and β-lobe (bL). This section is not through the midline, but somewhat lateral, at the level of the median calyx (Ca). Anterior is to the right with dense neuropil innervation of LomTK-LI fibres. (*f*) LomTK-LI fibres in the calyces (LCa and MCa) of a nymphal cockroach. In the more compact brain of nymphs the innervation of the calyces appears denser (compare with (*g*)). (*g*) The calyces of an adult cockroach. Here the innervation by immunoreactive fibres appears dense only in the basal portion. Scales: (*a*)–(*c*) 50 μm; (*d*), (*e*) 50 μm; (*f*), (*g*) 50 μm.

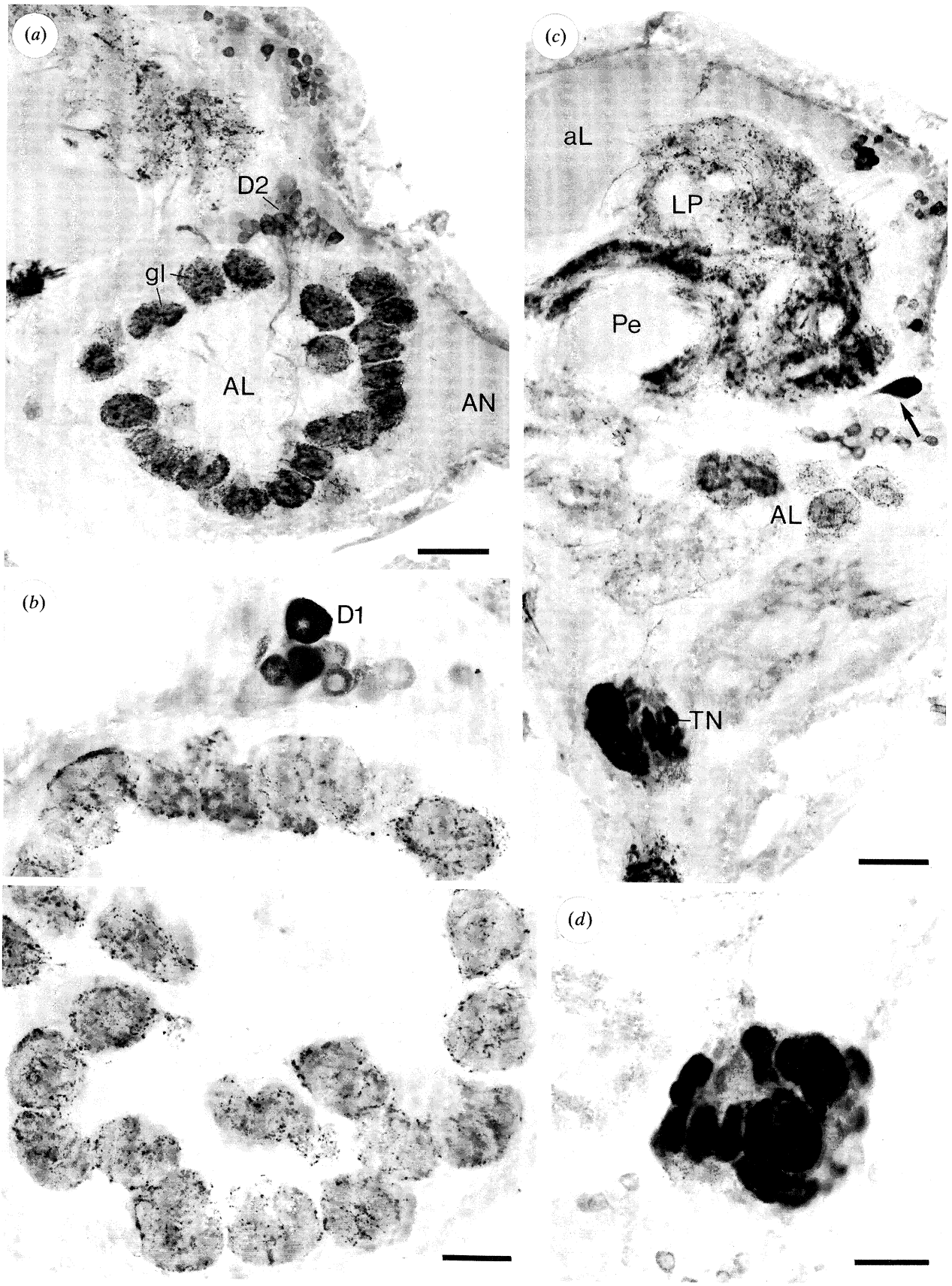

Figure 7. LomTK-LI neurons in the deutocerebrum and tritocerebrum of the cockroach brain (frontal sections, PAP method). (*a*) Overview of right antennal lobe (AL) with the antennal nerve (AN). Two of the antennal glomeruli are indicated (gl). In this micrograph the D2 neurons can be seen innervating the AL. (*b*) Detail of the antennal lobe in higher magnification (more posterior section). Here the D1 neurons are visible. Note the dense innervation of each of the glomeruli. (*c*) Overview of the right brain hemisphere with LomTK-LI fibres innervating neuropil in the lateral protocerebrum (LP), the most posterior glomeruli of the antennal lobe (AL) and a dense glomerular neuropil (TN) of tritocerebrum. The large protocerebral neuron P10 can be seen at arrow. Pe, peduncle. (*d*) Higher magnification

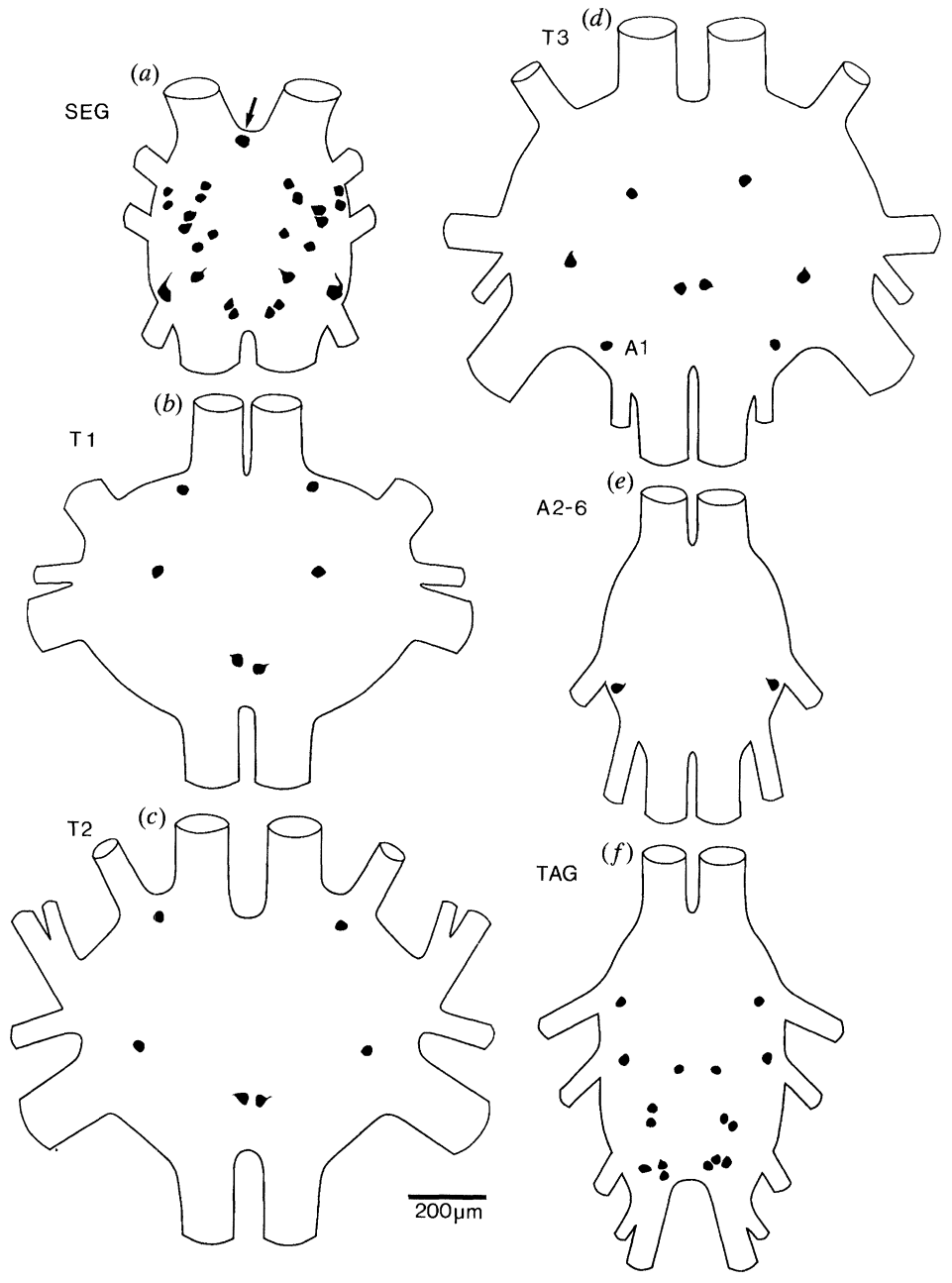

Figure 8. LomTK-LI cell bodies in the ganglia of the ventral cord. All cell bodies, except a few in the suboesophageal ganglion, are ventrally located. (*a*) In the suboesophageal ganglion the LomTK antiserum labels at least 12 pairs of cell bodies and one dorsal unpaired median neuron (arrow). (*b*), (*c*) Pro- and mesothoracic ganglion respectively, each with three pairs of immunoreative neurons. (*d*) The metathoracic ganglion also contains three pairs of immunoreactive neurons. The first abdominal ganglion has fused with the metathoracic ganglion posteriorly. In this abdominal neuromer one pair of LomTK-LI neurons can be seen. (*e*) The unfused abdominal ganglia A2–6 contain one pair of LomTK-LI neurons each. (*f*) The terminal abdominal ganglion is a fusion of at least three ganglia. At least eight pairs of immunoreactive neurons were detected.

4. DISCUSSION

(*a*) *The immunoreactive material detected in the cockroach is related to Arg-tachykinins*

Peptides of the tachykinin family have been extensively studied in mammals. We know that these peptides have an abundant distribution in the central and peripheral nervous system as well as in the intestinal tract and that they have multiple roles as excitatory neurotransmitters and as regulators of numerous physiological functions (Maggio 1988; Otsuka & Yoshioka 1993). Peptides with certain structural similarities to vertebrate tachykinins have been identified in several invertebrates such as insects

of immunoreactivity in the glomerular tritocerebral neuropil in other specimen. Even at this magnification it is hard to resolve individual fibres or terminals. Scales: (*a*), (*c*) 100 μm; (*b*), (*d*) 50 μm.

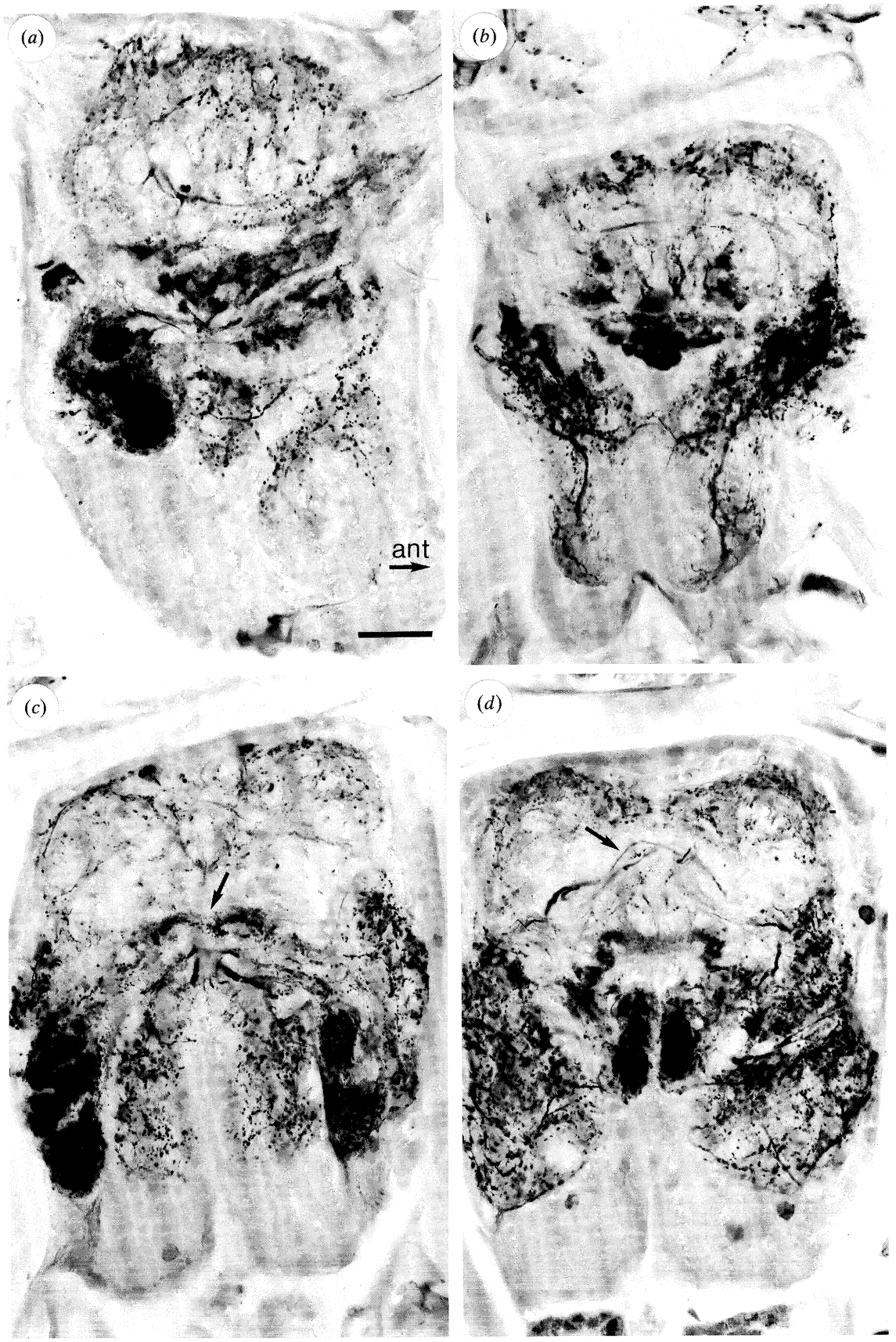

Figure 9. LomTK-LI neurons in the suboesophageal ganglion. (*a*) Sagittal section slightly off the midline. Immunoreactive fibres are seen in all three neuromers. (*b*) Anterior portion of the ganglion. (*c*) Midportion of the ganglion. (*d*) Slightly more posterior position. Scales: (*a*)–(*d*) 50 μm.

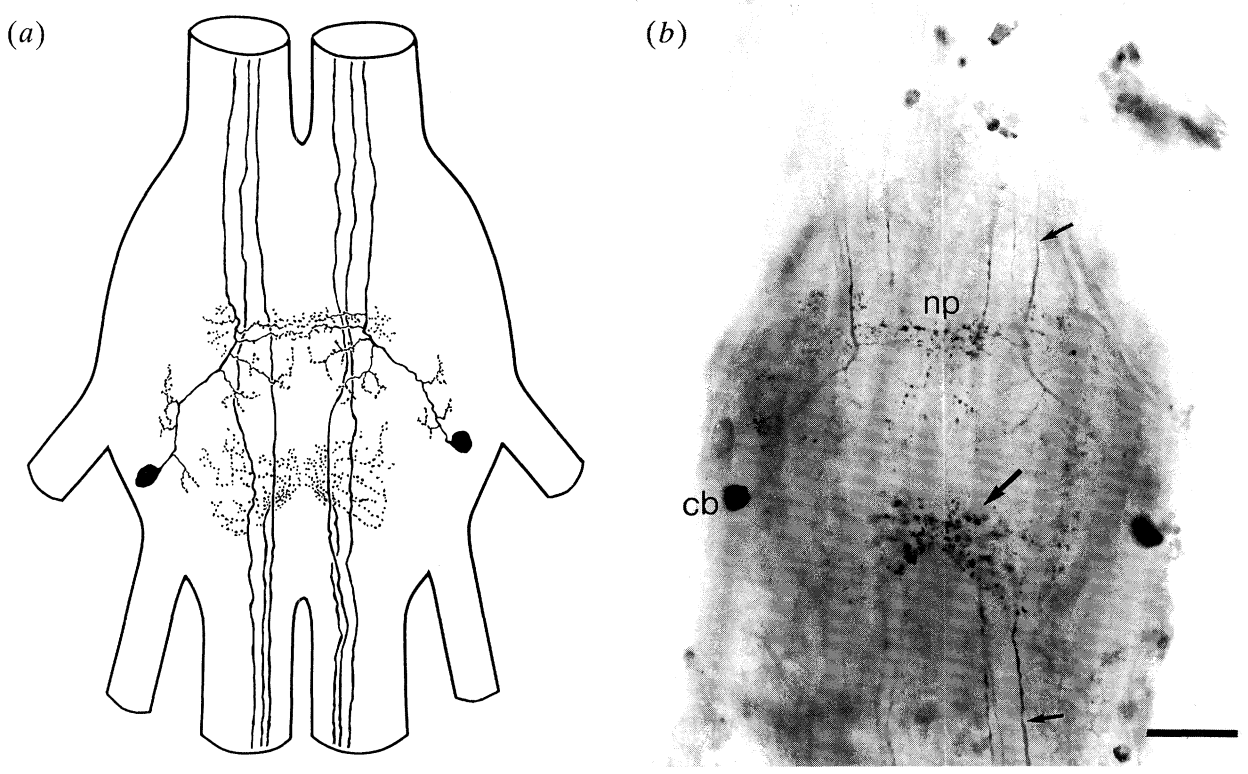

Figure 10. LomTK-LI neurons in an unfused abdominal ganglion. (*a*) Tracing of the pair of neurons located in the third unfused abdominal ganglion. The axons of this pair of neurons project anteriorly and appear to terminate three ganglia further up the cord. Thus three axons are seen in the connectives between the abdominal ganglia. In the posterior connective one axon (from three ganglia down the cord) terminates posteriorly in the ganglion neuropil, another is derived from two ganglia down the cord and the final from the previous posterior ganglion (the last two continue past the ganglion). (*b*) Micrograph of an unfused abdominal ganglion with the LomTK-LI cell bodies (cb) and axons in connective (arrows) and neuropil arborizations (np). Scale: 50 μm.

(Schoofs *et al.* 1990*a*, *b*, 1993; Clottens *et al.* 1993; Champagne & Ribeiro 1994; Holman *et al.* 1994; Lundquist *et al.* 1994*a*), echiuroid worms (Ikeda *et al.* 1993) and molluscs (Erspamer & Anastasi 1962; Fujisawa *et al.* 1995). These are of two major types: Arg-tachykinins with an FX_1GX_2Ramide C-terminus and Met-tachykinins with an FX_1GX_2Mamide C-terminus (see Holman *et al.* 1994). At present there are no reports of the isolation of both Arg- and Met-tachykinins from the same species. In fact, in invertebrates the Met-tachykinins have so far only been isolated from salivary glands (Erspamer & Anastasi 1962; Champagne & Ribeiro 1994), whereas the Arg-tachykinins appear to be neuronal peptides (Schoofs *et al.* 1993; Nässel 1993*a*, 1995; Lundquist *et al.* 1994*a*, *b*). Immunocytochemistry has, however, indicated that in both insect and mollusc species there are peptides recognized by antisera to LomTK and others recognized by antisera to Met-tachykinins, such as substance P and neurokinin A (Nässel *et al.* 1992*b*; Nässel 1993*a*; Elekes *et al.* 1994; Elekes & Nässel 1995). In this account we shall concentrate on the distribution of cockroach Arg-tachykinins, or more specifically peptides recognized by an antiserum to LomTK I.

With a well characterized antiserum to LomTK I we have mapped the distribution of immunoreactive material in the nervous system and intestine of the cockroach *Leucophaea maderae*. Partly purified extracts of nervous tissue and midgut from this cockroach were shown to contain components that are immunoreactive to the LomTK antiserum in ELISA. The bulk of this material eluted in HPLC with retention times roughly similar to the Arg-tachykinins LomTK I–II of locusts and CavTK I–II of blowflies. Previous analysis of the specificity of the antiserum by ELISA revealed that a preserved portion of the LomTKs and CavTKs is recognized: the sequence FYGVRamide shared by LomTK I–III and V and CavTK I (Lundquist *et al.* 1994*a*, *b*). The antiserum does however also recognize peptides with FHGVRamide and FVGVRamide, Like LomTK IV and CavTK II (Lundquist *et al.* 1994*a*, *b*). This makes it likely that the material demonstrated in the cockroach tissues is closely related to the insect Arg-tachykinins.

(*b*) *Is there more than one Arg-tachykinin in L. maderae?*

The HPLC analysis of extracts from brains, suboesophageal ganglia, thoracic ganglia and midguts revealed several components that reacted with the LomTK antiserum in ELISA. One of these components was present in all tissues assayed and had a retention time close to CavTK II and LomTK II. Some other LomTK-LI components appeared to be present in the brain and the ventral ganglia, but not the intestine, whereas the intestine contained another, later component not seen in the other tissues. It has not been determined whether the material eluting earlier and later than the synthetic Arg-tachykinins contains

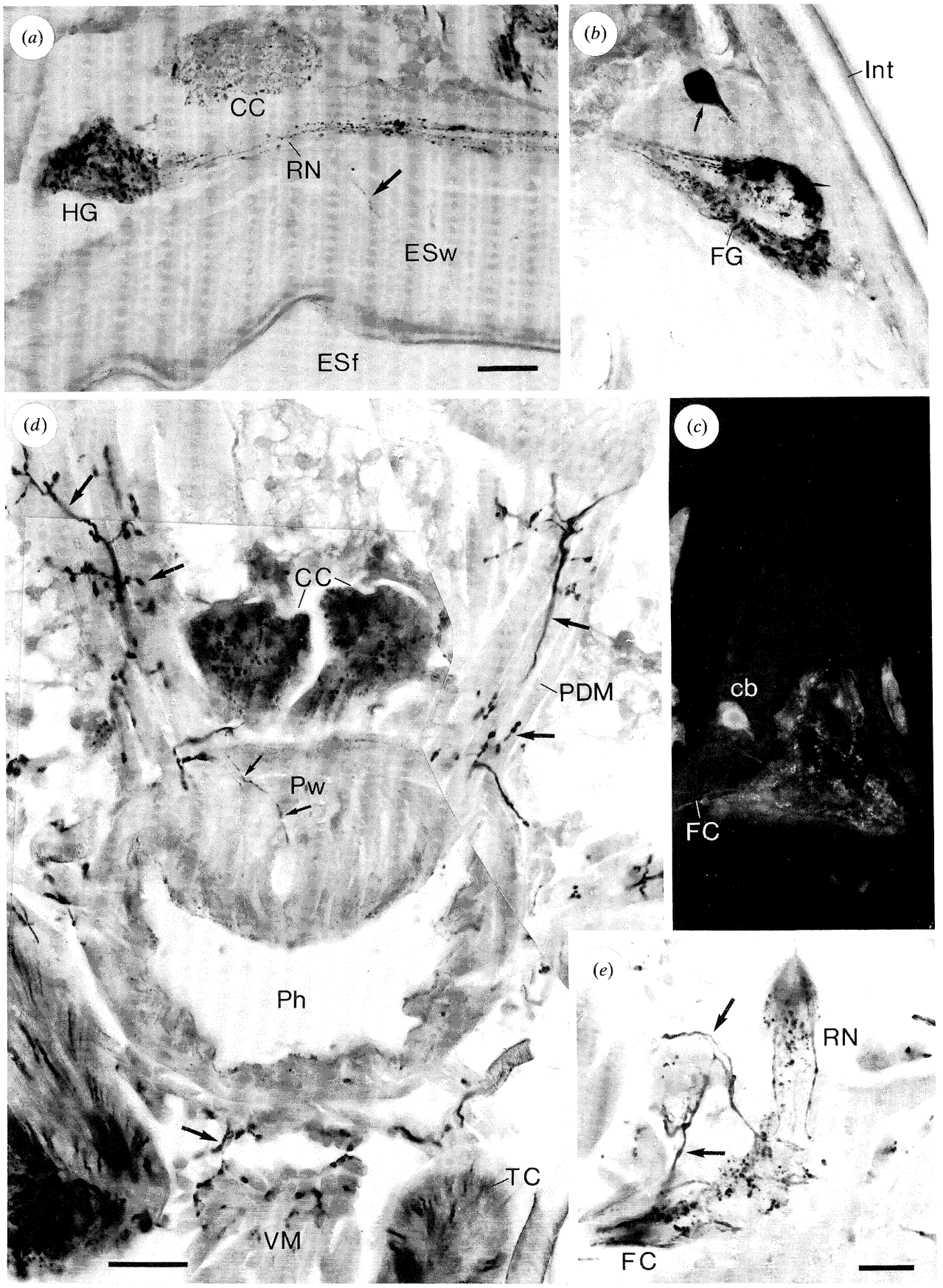

Figure 11. Lom TK-LI neurons in the peripheral nervous system. (*a*) Sagittal section through cockroach head. LomTK-LI fibres supply the hypocerebral ganglion (HG) from the recurrent nerve (RN). A portion of the corpora cardiaca (CC) is seen with immunoreactive fibres. The muscle layer of the oesophagus (ESw) is also supplied with LomTK-LI fibres (arrow) from the stomatogastric system. ESf, oesophageal lumen. (*b*) The frontal ganglion (FG) with immunoreactive fibres in sagittal section. The large cell body (arrowed) is seen in the ganglion. Int, integument.

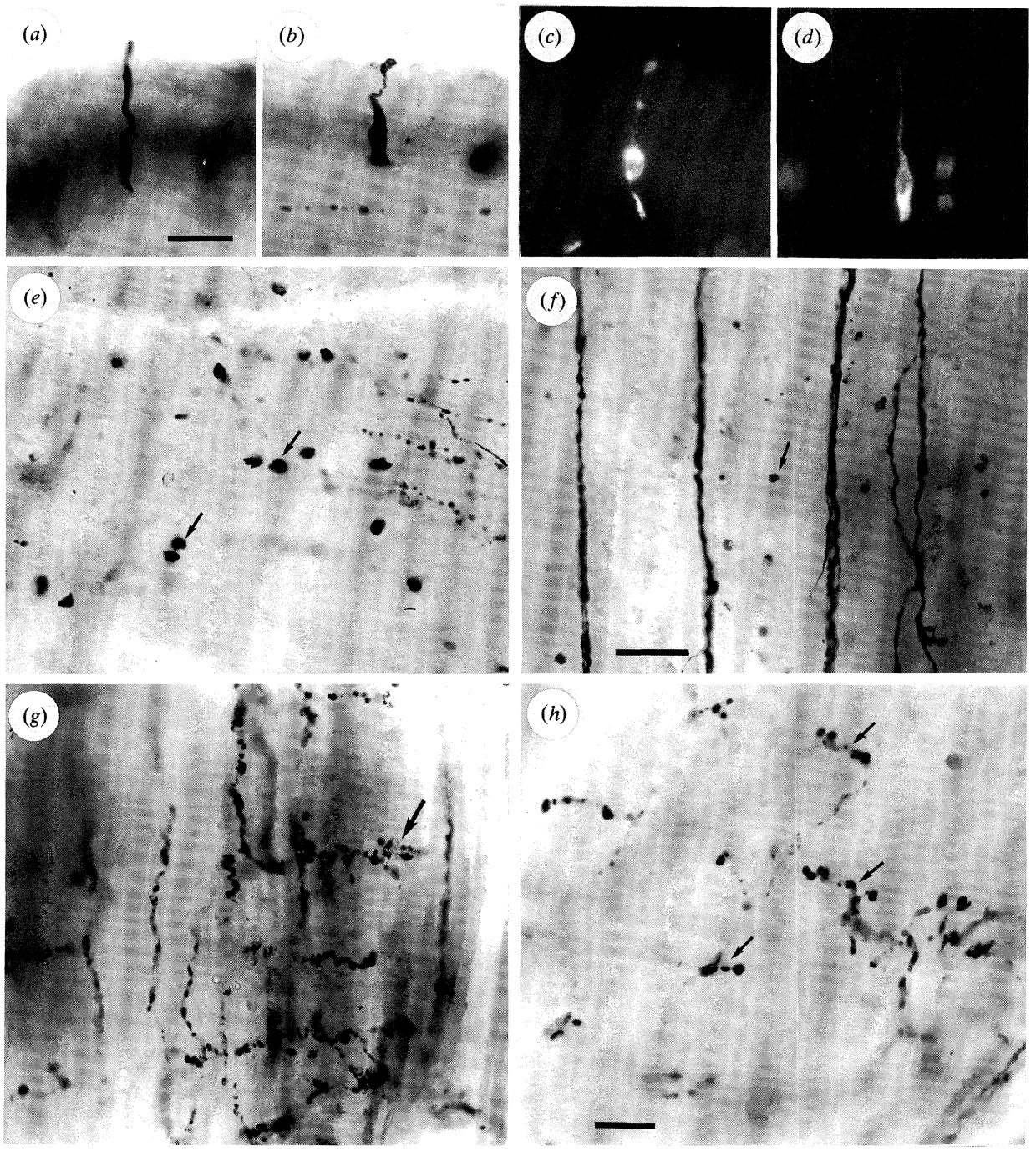

Figure 12. Lom TK-LI immunoreactive cells and fibres in the midgut. (*a*)–(*d*) Immunoreactive endocrine cells in the midgut epithelium (cryostat sections; (*a*), (*b*) PAP, (*c*), (*d*) TRITC). It is not clear whether the morphological variation represents different cell types. (*e*) Endocrine cells (arrowed) seen from the surface of a wholemounted midgut. The cells do not appear to be distributed in a geometric fashion. (*f*) LomTK-LI fibres and endocrine cells (arrowed) in surface view of wholemounted midgut. (*g*) At regular intervals the LomTK-LI fibres arborize and form varicose terminals (arrowed) in the muscle layer of the midgut. (*h*) Detail of varicose terminals (arrowed) in the midgut. Scales: (*a*)–(*e*) 25 μm; (*f*), (*g*) 50 μm; (*h*) 25 μm.

(*c*) Frontal section of the frontal ganglion (TRITC fluorescence) with one of the two smaller cell bodies (cb) and fibres in the ganglion neuropil. FC, frontal connective. (*d*) Frontal section through the pharynx (Ph) and associated structures at a level posterior to the brain. Two of the dorsal pharyngeal dilator muscles (PDM) are innervated by LomTK-LI fibres (large arrows). Some ventral dilator muscles (VM) are also supplied by immunoreactive fibres (large arrows). Also the musculature of the pharynx (Pw) is innervated (small arrows). Portions of the corpora cardiaca (CC) are seen with immunoreactive fibres. TC, tritocerebral connective. (*e*) Oblique frontal section through the frontal ganglion. Immunoreactive fibres in branches of frontal connectives (FC) supply pharyngeal muscles (arrowed). RN, recurrent nerve. Scales: (*a*)–(*c*) 50 μm; (*d*) 50 μm; (*e*) 50 μm.

cockroach tachykinins, degradation products of these tachykinins or some other cross-reacting peptides. The LomTK-LI fractions from the midgut were tested in the *L. maderae* hindgut contraction assay and it was found that the main component eluting with the same retention time as the callitachykinins was bioactive whereas the late fraction (fraction 35) was not, indicating at least that this material either is not related to the Arg-tachykinins or has been degraded in some way. In summary, each of the tissues appears to contain peptide eluting with about the same retention time as synthetic Arg-tachykinins and additional material in varying amounts eluting in other fractions. Although the HPLC column system clearly separates the four synthetic insect tachykinins tested it cannot be concluded whether the brain and suboesophageal ganglia contain more than one cockroach tachykinin. By analogy with locust, blowfly and mosquito (Schoofs *et al.* 1993; Holman *et al.* 1994; Lundquist *et al.* 1994*a*), we, however, assume that several forms are present.

(*c*) *Immunocytochemical mapping in the* CNS *indicates multiple functions of cockroach tachykinins*

In the cockroach we found numerous LomTK-LI neurons and neuronal processes in the central nervous system, stomatogastric system and intestine. As discussed in the previous section the antiserum we employed recognizes the different known isoforms of locust and blowfly Arg-tachykinins and we can therefore not exclude that in the cockroach several isoforms of related tachykinins are also immunolabelled (as indicated in the HPLC analysis). In the brain the abundance of LomTK-LI cell bodies and fibres cannot be rivalled by any other neuropeptide mapped in the cockroach brain so far: proctolin (Bishop & O'Shea 1982), pigment dispersing hormone (Homberg *et al.* 1991), allatostatins (Stay *et al.* 1992), leucokinins (Nässel *et al.* 1992*a*; Chen *et al.* 1994; Meola *et al.* 1994), corazonin (Veenstra & Davis 1993; Predel *et al.* 1994), perisulphakinin (Agricola & Bräunig 1994) and material identified by antisera to different vertebrate neuropeptides (Hansen *et al.* 1982; Verhaert & De Loof 1985; Raabe 1989; Davis & Hildebrand 1992). Almost every major neuropil region, except the peduncle and the α- and β-lobes of the mushroom bodies and the lamina of the optic lobe, is supplied by LomTK-LI processes and the approximately 360 immunoreactive cell bodies are distributed in many regions in each of the three brain divisions, proto-, deuto- and tritocerebrum (and additional ones in the optic lobes).

The morphology of individual brain neurons was not reconstructed owing to the abundance of superimposed processes. As a result we do not know which individual neurons supply processes to many of the neuropils. However, it appears that many neurons arborize quite extensively in large portions of the brain, much of which can be defined as non-glomerular neuropils (neuropils lacking apparent organization such as layers, columns or glomeruli). For a few defined regions we can at least determine which LomTK-LI neurons that supply arborizations and resolve some of the connections between neuropils. These are discussed below.

The different divisions of the central complex are supplied by LomTK-LI neurons with small cell bodies dorsal to the protocerebral bridge; their processes arborize in the protocerebral bridge, the upper division of the fan-shaped body and the lateral accessory lobes. These LomTK-LI neurons appear to be columnar neurons, similar to LomTK-LI neurons resolved by combined intracellular dye injection in the central complex of the meal beetle *Tenebrio molitor* (Wegerhoff 1994; Wegerhoff & Breidbach 1995). It is likely that further types of LomTK-LI neurons send processes to the central complex neuropil of *L. maderae*, since immunoreactive fibres were seen also in the noduli and lower division of the fan-shaped body. In the blowfly and *Drosophila*, where we could resolve individual LomTK-LI neuron types in some detail, there are two sets of neurons, in the proto- and tritocerebrum respectively, which innervate the fan-shaped body (Lundquist *et al.* 1994*b*; Nässel 1994). None of these correspond to the columnar cells seen in the locust, meal beetle or cockroach (Nässel 1993*b*; Wegerhoff 1994). However, a third type of weakly immunolabelled neuron similar to the columnar neurons, but without processes in the protocerebral bridge, appears to be present in the blowfly (Lundquist *et al.* 1994*b*). It is well known that the central complex can be innervated by several different neuronal subsystems using the same transmitter or neuropeptide (Homberg 1991, 1994) and that the different divisions of the central complex are targets of multiple neurotransmitter and neuropeptide systems (Nässel 1993*a*; Homberg 1994). The function of the central complex appears to involve motor control and motor coordination as well as bilateral sensory integration (see Heisenberg 1994; Homberg 1994). The presence of LomTK-LI fibres in the central complex thus indicates a role of insect tachykinins in motor control also suggested for mammalian tachykinins (Maggio 1988; Otsuka & Yoshioka 1993).

The antennal lobes are innervated by three types of LomTK-LI neurons with cell bodies anteriorly in the deutocerebrum. All the glomeruli are supplied by varicose fibres as also found in the locust, the blowfly and *Drosophila* (Nässel 1993*b*, 1994; Lundquist *et al.* 1994*b*). Most of these neurons seem to be local antennal lobe interneurons, but, again owing to the massive superposition of immunolabelled fibres, it cannot be excluded that some neurons have thin axons projecting outside the deutocerebrum e.g. to the calyces of the mushroom bodies (see Homberg *et al.* 1989; Malun *et al.* 1993). To resolve the morphology of the individual LomTK-LI neurons of the antennal lobe (and elsewhere) it would be necessary to apply intracellular dye injection combined with immunocytochemistry or to use the method devised by Wegerhoff & Breidbach (1995) that comprises dye injection into already immunolabelled neurons in fixed tissue. At present we can only assume that LomTK-like peptide is of importance in olfactory processing, not only in the cockroach, but also in all insects studied so far.

Modulation appears important in olfactory processing since the various interneurons innervating the cockroach antennal lobes are 'chemically heterogeneous' and contain several different putative neurotransmitters, monoamines and neuropeptides: GABA, taurine, serotonin, dopamine, histamine, leucokinins, corazonin, allatostatins, perisulphakinin, locustatachykinin-, FMRFamide- and myomodulin-like peptides (Klemm *et al.* 1984; Pirvola *et al.* 1988; Distler 1990; Nässel *et al.* 1992*a*; Pirvola & Panula 1992; Schildberger & Agricola 1992; Nässel 1993*a*; Veenstra & Davis 1993; Agricola & Bräunig 1994; this investigation).

The supply of LomTK-LI fibres to the optic lobes is not as impressive as in the locust (Nässel 1993*b*), but much more prominent than in the blowfly where only some weakly labelled fibres were seen distally in the lobula (Lundquist *et al.* 1994*b*). The role of LomTK-like peptide in visual processing thus seems highly species specific.

The abundance of LomTK-LI fibres in the non-glomerular neuropil surrounding the mushroom bodies and other parts of the protocerebrum was also seen in the locust (Nässel 1993*b*), but was not found to be so prominent in the blowfly and *Drosophila* (Lundquist *et al.* 1994*b*; Nässel 1995). Nothing is known about the function of these neuropil regions, but it is clear that many neurons supplying branches to the glomerular neuropils also have processes in non-glomerular neuropil (see: Strausfeld 1976; Bräunig 1991). It is a common feature of aminergic and peptidergic neurons of the cockroach brain to have profuse arborizations in non-glomerular neuropils (Klemm *et al.* 1984; Pirvola *et al.* 1988; Nässel *et al.* 1992*a*; Davis & Hildebrand 1993; Veenstra & Davis 1993; Predel *et al.* 1994).

In the tritocerebrum the most prominent innervation by LomTK-LI fibres is seen in a glomerular neuropil located anteriorly in each hemisphere. The function of this particular neuropil and the source of the immunoreactive fibres are not known. It is, however, known that the tritocerebrum is involved in control of the foregut and muscle associated with the pharynx and mouthparts and is connected to the stomatogastric nervous system which controls intestinal muscle activity (Willey 1961; Gundel & Penzlin 1978; Bräunig 1990).

As indicated above it seems that all LomTK-LI neurons in the brain are interneurons. The neurons supplying fibres to the corpora cardiaca appear not to be classical neurosecretory cells since the bulk of their terminals is not in the neurohaemal storage lobe. In the locust it is quite clear that the LomTK-LI fibres in the corpora cardiaca contact the adipokinetic hormone (AKH) producing cells of the glandular lobe and may possibly be considered as neurons regulating release of AKH (in preparation). We have not determined the location of the cell bodies of the LomTK-LI neurons innervating the corpora cardiaca in *L. maderae*, but they may be few and located among the median neurosecretory cells. They could also have their cell bodies located in the suboesophageal ganglion and innervate the corpora cardiaca via the brain and NCC III (see Gundel & Penzlin 1978; Bräunig 1990).

The suboesophageal ganglion is associated with the control of mouthparts (gnathal appendages) and can be involved in control of flight, walking, respiration as well as salivary glands, antennal heart and pharyngeal dilator muscles (see Bräunig 1990). We found that the suboesophageal ganglion contains many more LomTK-LI neurons than the other ganglia of the ventral cord. From sectioned material it was also apparent that the suboesophageal neuropil is densely innervated by LomTK-LI fibres, in the form of both local interneurons and neurons passing the ganglion from the brain and thoracic ganglia. We therefore assume that LomTK-like peptide has an important regulatory role in suboesophageal circuits. It may also be that the LomTK-LI fibres innervating the pharyngeal dilator muscles and the corpora cardiaca have their cell bodies among the neurons found laterally in the suboesophageal ganglion, or are derived from the anterior DUM cell, by analogy with findings obtained by cobalt filling in the locust (Bräunig 1990).

We detected very few LomTK-LI cell bodies in thoracic and abdominal ganglia. Also in these ganglia no efferent fibres were detected. The LomTK-LI neurons of the abdominal ganglia could be resolved in detail. These interneurons have a simple organization with intersegmental ascending axons connecting the more anterior abdominal ganglia (and possibly thoracic ones). Their processes appear strictly ipsilateral, in contrast to otherwise similar abdominal interneurons resolved with antisera to serotonin (Bishop & O'Shea 1983) and corazonin (Veenstra & Davis 1993).

(*d*) *Tachykinins may have additional functions in stomatogastric system and intestine*

Three LomTK-LI cell bodies in the frontal ganglion supply fibres to the ganglion neuropil and the hypocerebral ganglion, and are probably the origin of fibres to muscle in the pharynx. Further fibres are seen in the frontal connectives that link the frontal ganglion to the tritocerebrum; these may be derived from cell bodies either in the brain or in the frontal ganglion. The LomTK-LI fibres derived from the frontal ganglion with terminals on visceral muscle of the pharynx furnish one example where Arg-tachykinin-like peptide may be used in an efferent control system. Another example is the extensive LomTK-LI fibre supply to the muscle of the midgut which probably is derived from the oesophageal nerve and/or ganglia of the extracerebral stomatogastric system. In *L. maderae* LomTK-like peptide may thus be involved in the efferent regulation of muscle activity of the pharynx and midgut, a control system that appears to be lacking in the blowfly and *Drosophila* (Lundquist *et al.* 1994*a*; Nässel 1994). This action certainly accords with the locustatachykinins and callitachykinins having stimulatory effects on visceral muscle contraction (Holman *et al.* 1991; Schoofs *et al.* 1993; Lundquist *et al.* 1994*a*). In this investigation we have furthermore indicated that the LomTK-LI material from the *L. maderae* midgut that eluted in the range of insect Arg-tachykinins stimulates contraction of the hindgut of the same species. It seems important to

reveal whether the locust foregut is also supplied by LomTK-LI fibres, since this issue has been quite extensively used in the pharmacological characterization of receptors for proctolin, monoamines, GABA and acetylcholine, and the modelling of their interactions to control muscle contractions (Osborne *et al.* 1990).

In the cockroach midgut we detected LomTK-LI endocrine cells similar to ones observed in the midgut of the blowfly. The function of the peptidergic endocrine cells found in the midgut of several cockroach species is unknown, but it has been suggested that they release peptide locally in a paracrine fashion (Endo *et al.* 1982; Zitnan *et al.* 1993). Since a number of different neuropeptides have been indicated in this type of cells, one may expect that Arg-tachykinins constitute only one of many types of mediators possibly involved in control of motility, enzyme synthesis and release and other intestinal functions.

The prominent supply of LomTK-LI fibres to the pharyngeal dilator muscles is highly interesting. In the locust some of these muscles have an important role in the exuviation process, being involved in the movements generated to split the old cuticle (Hughes 1980; Bräunig 1990). It was found that the locust muscles were innervated by neurons with cell bodies in the suboesophageal ganglion and that these neurons appear to degenerate after the final moult into adults (Bräunig 1990). Owing to technical problems in cutting cryostat sections of whole adult heads we only sectioned cockroach nymphs or just moulted animals (before tanning and sclerotonization of cuticle) in our analysis. It would be of interest to extend the immunocytochemical analysis to adult stages to determine whether the LomTK-LI fibres remain or not. In any case the pharynx dilator muscles may prove to be useful for analysis of peripheral insect tachykinin action both *in vivo* and *in vitro*.

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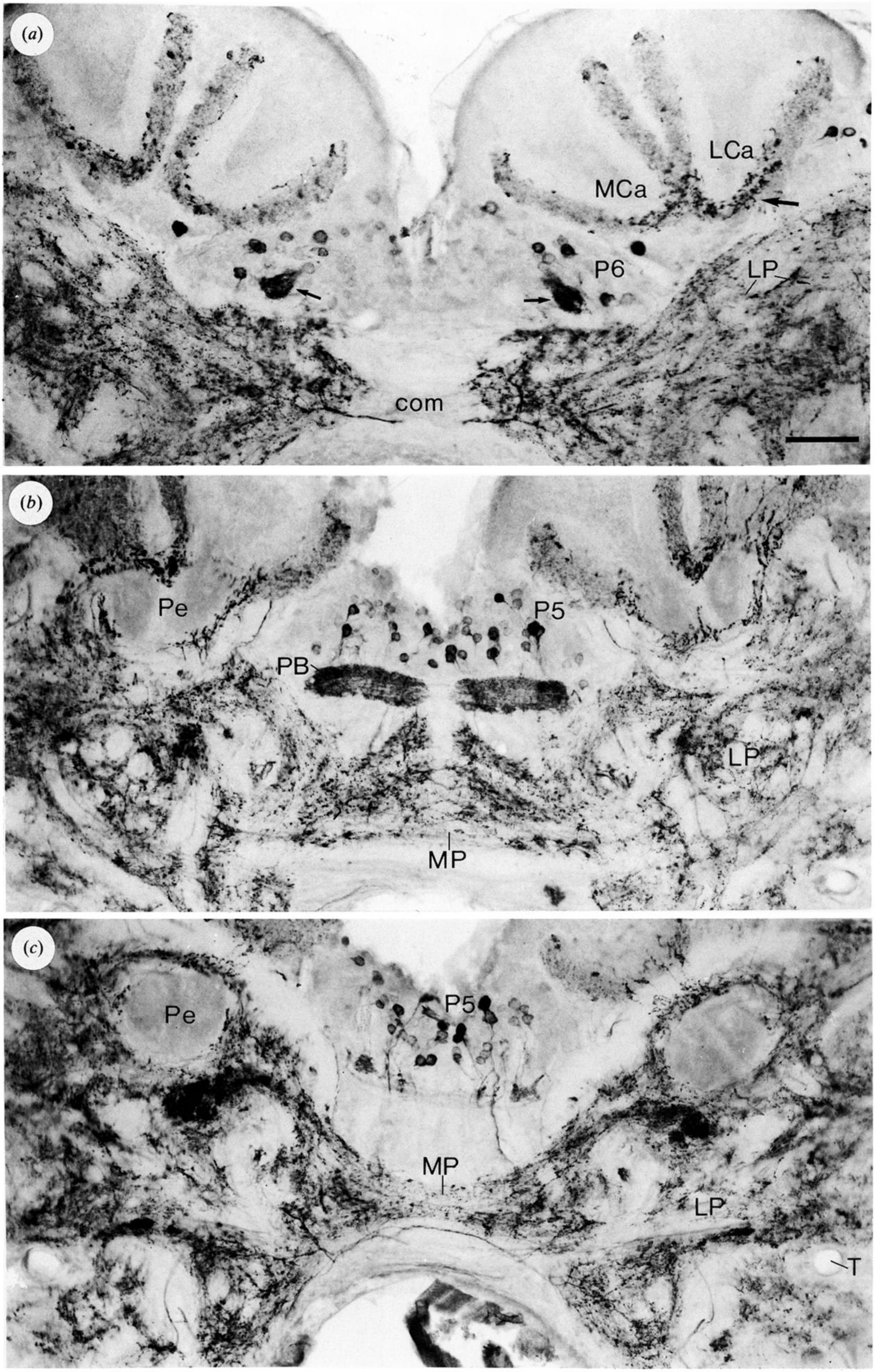

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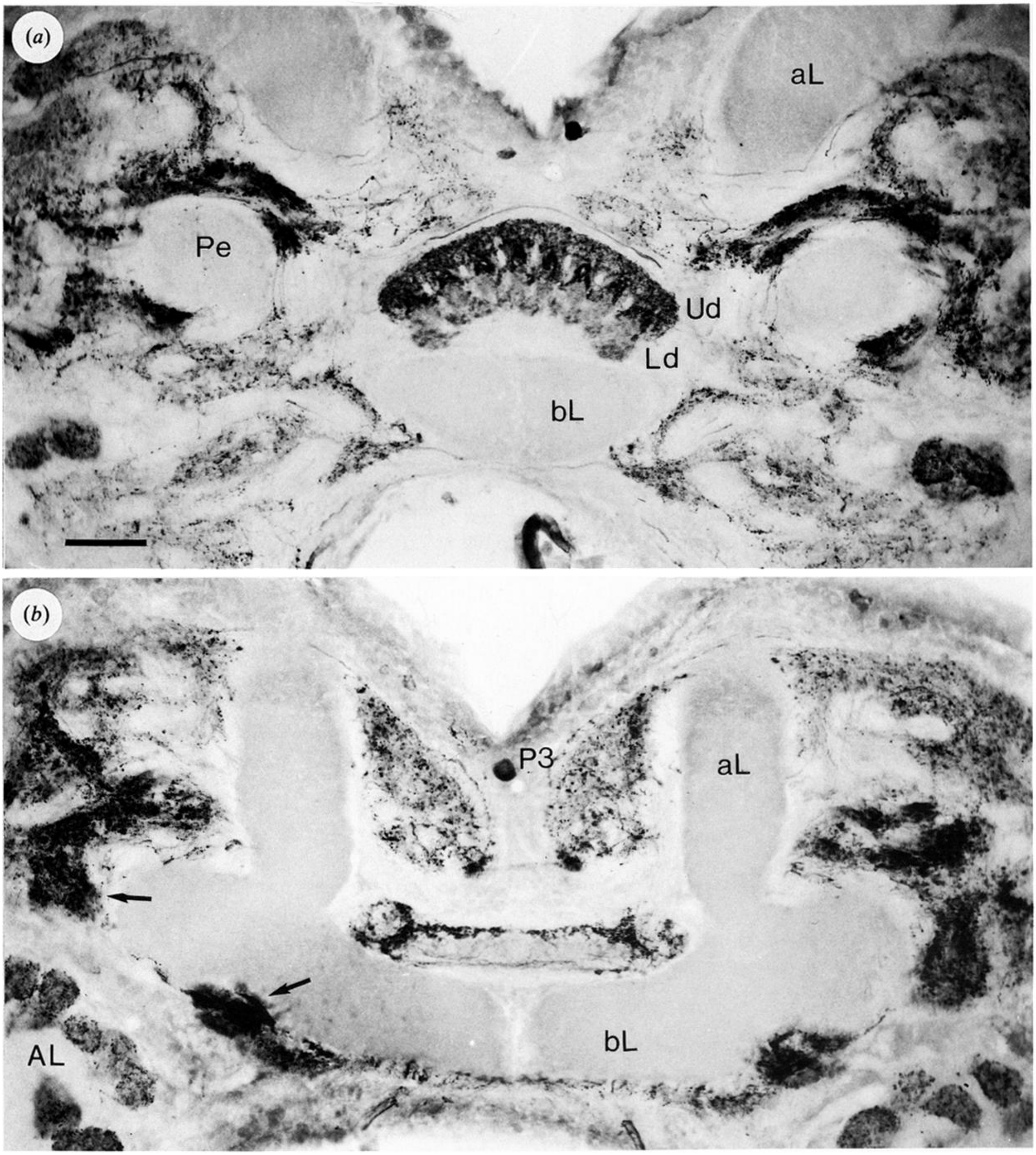

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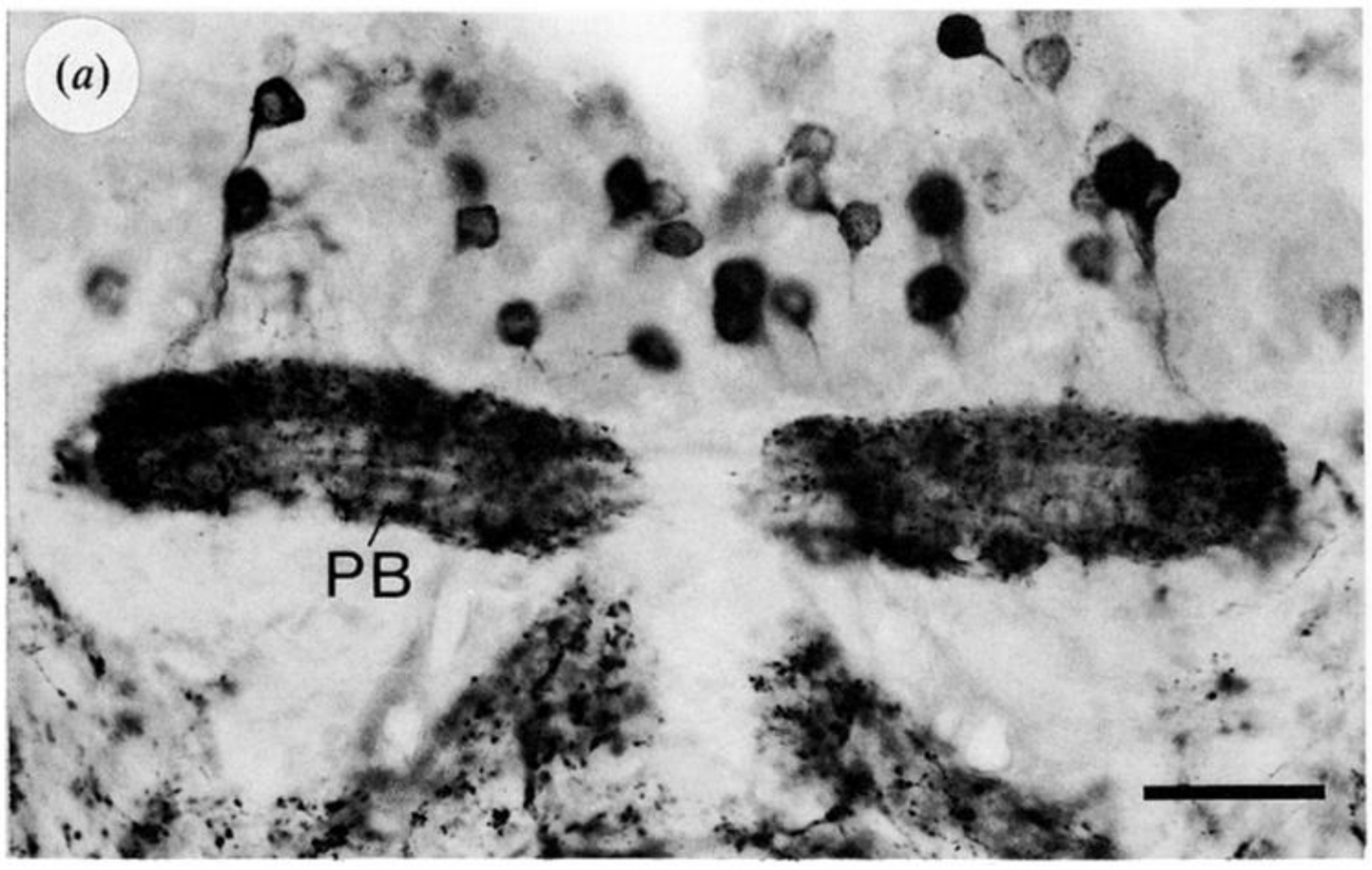

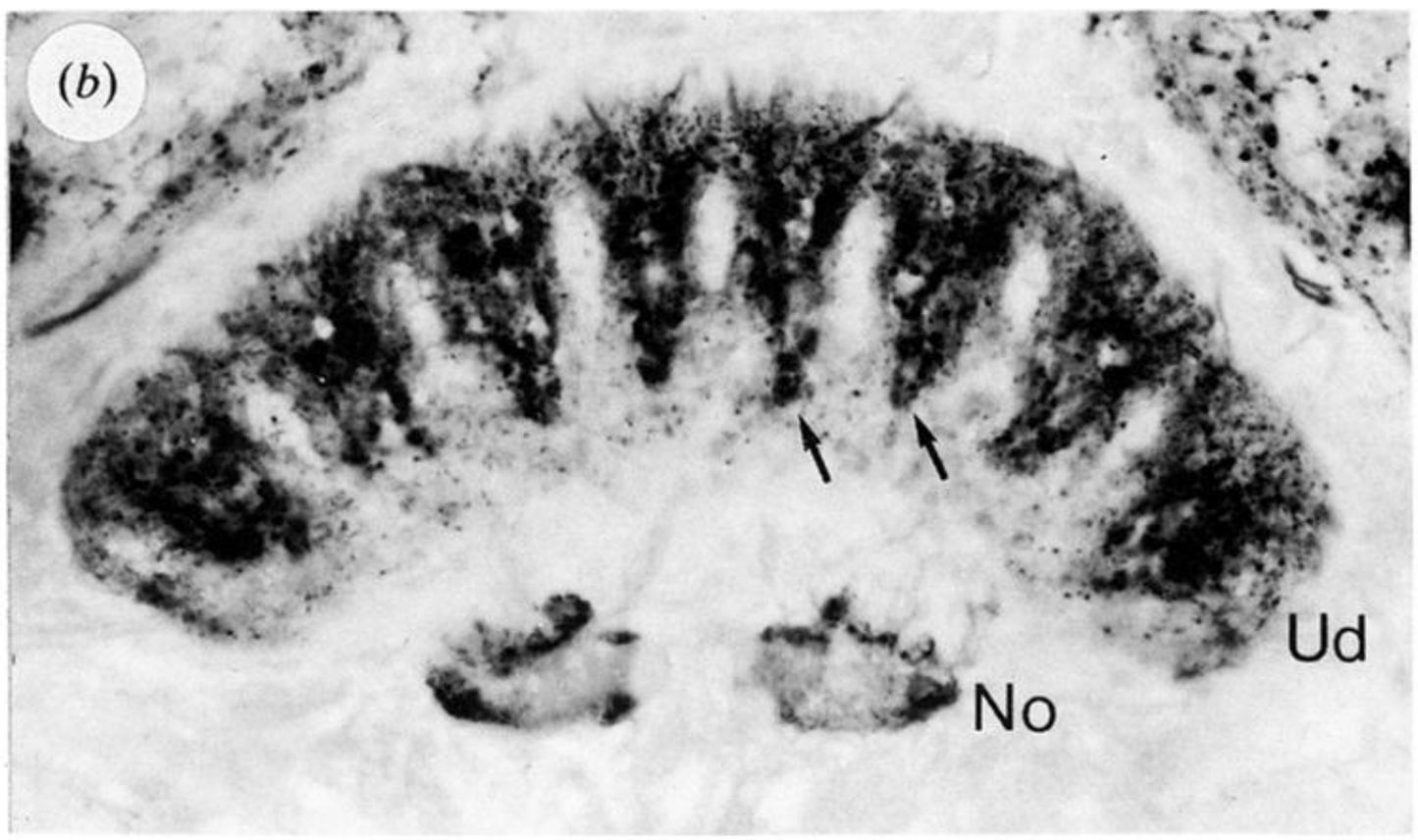

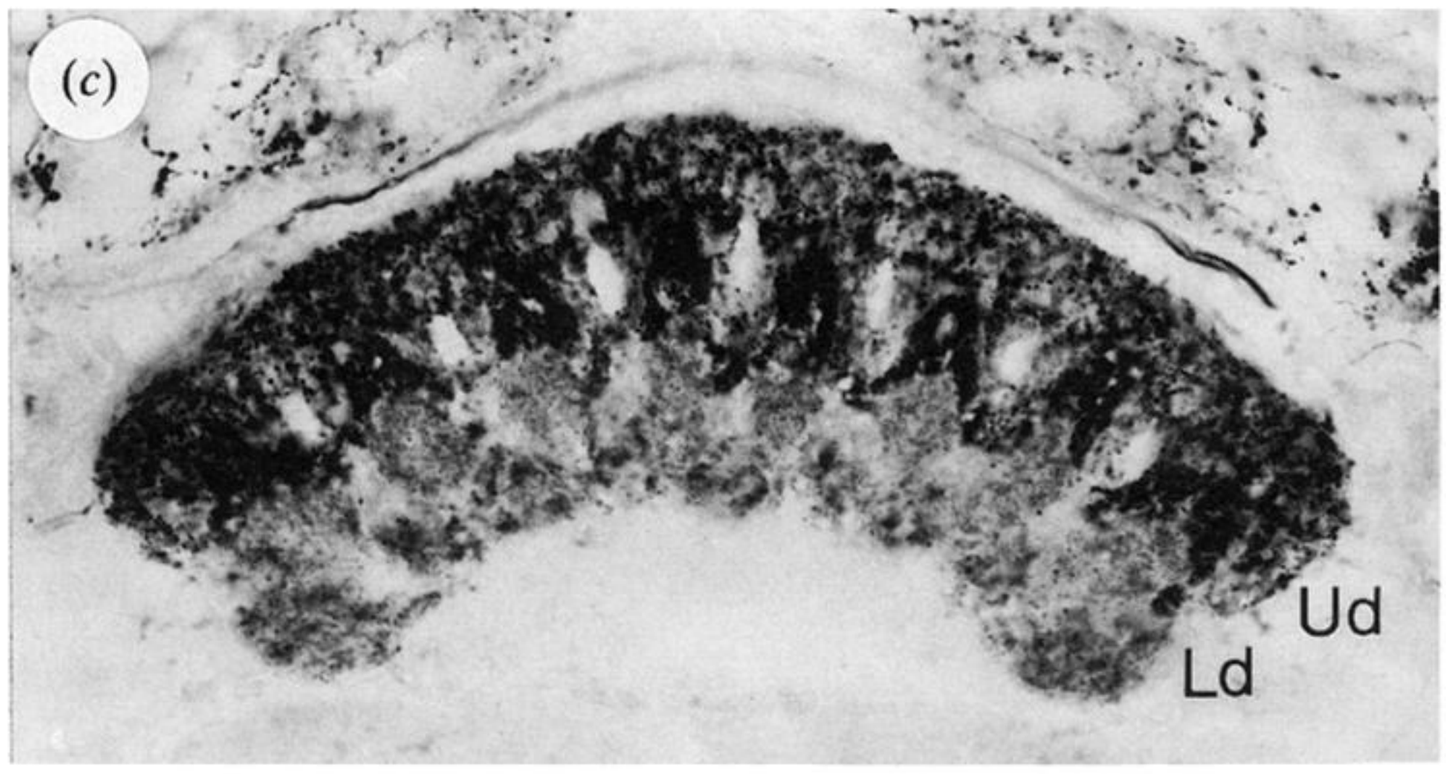

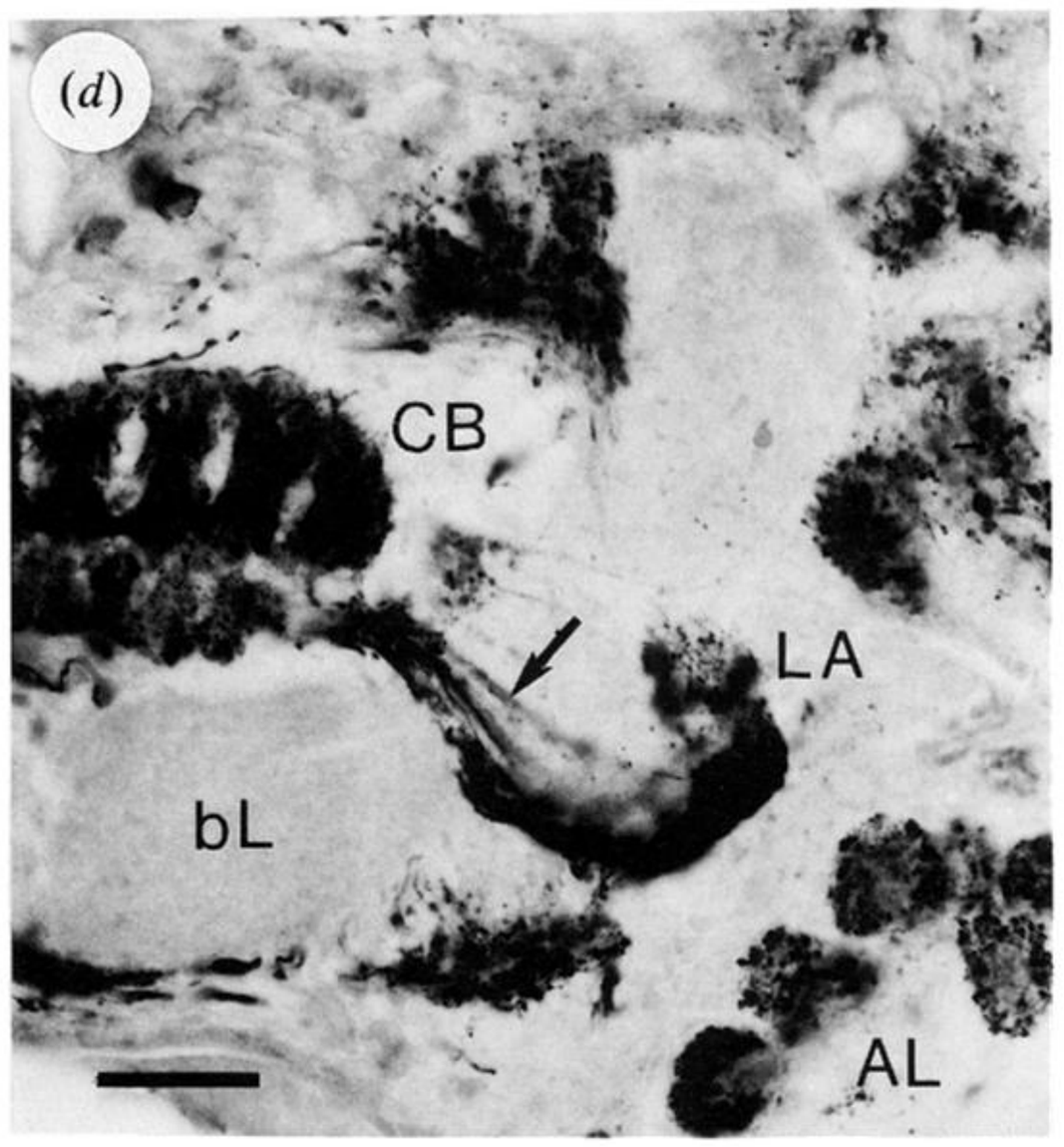

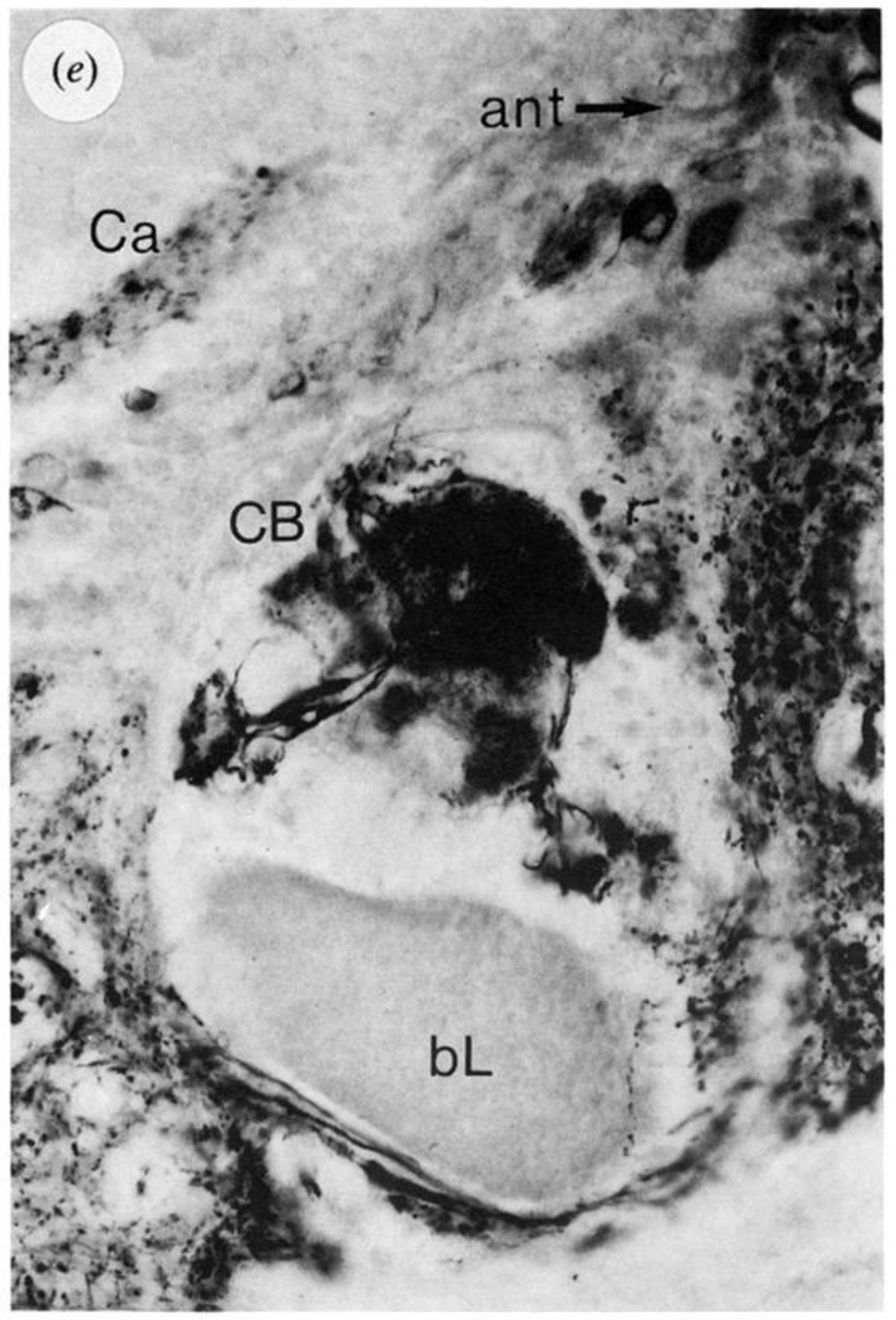

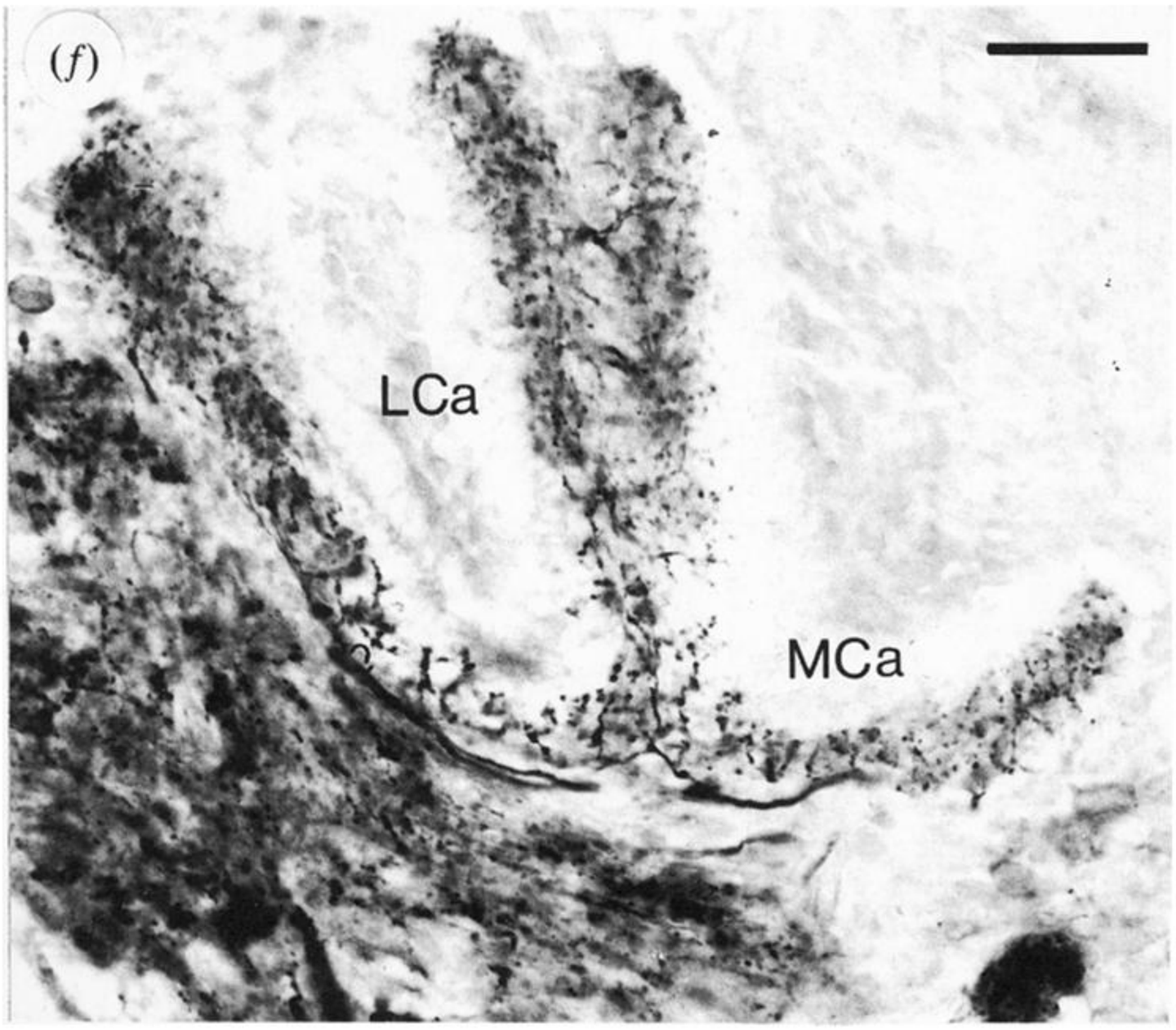

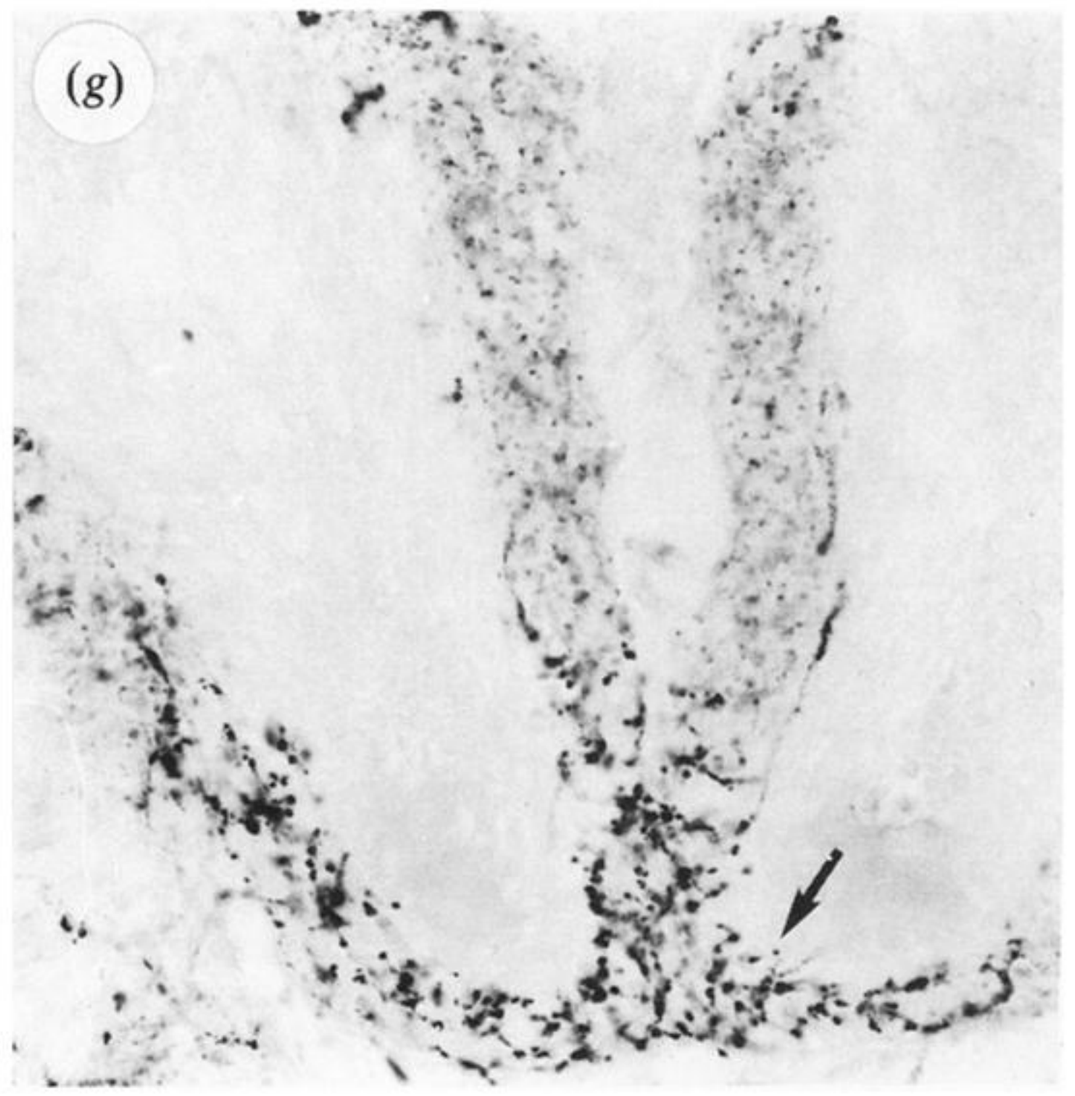

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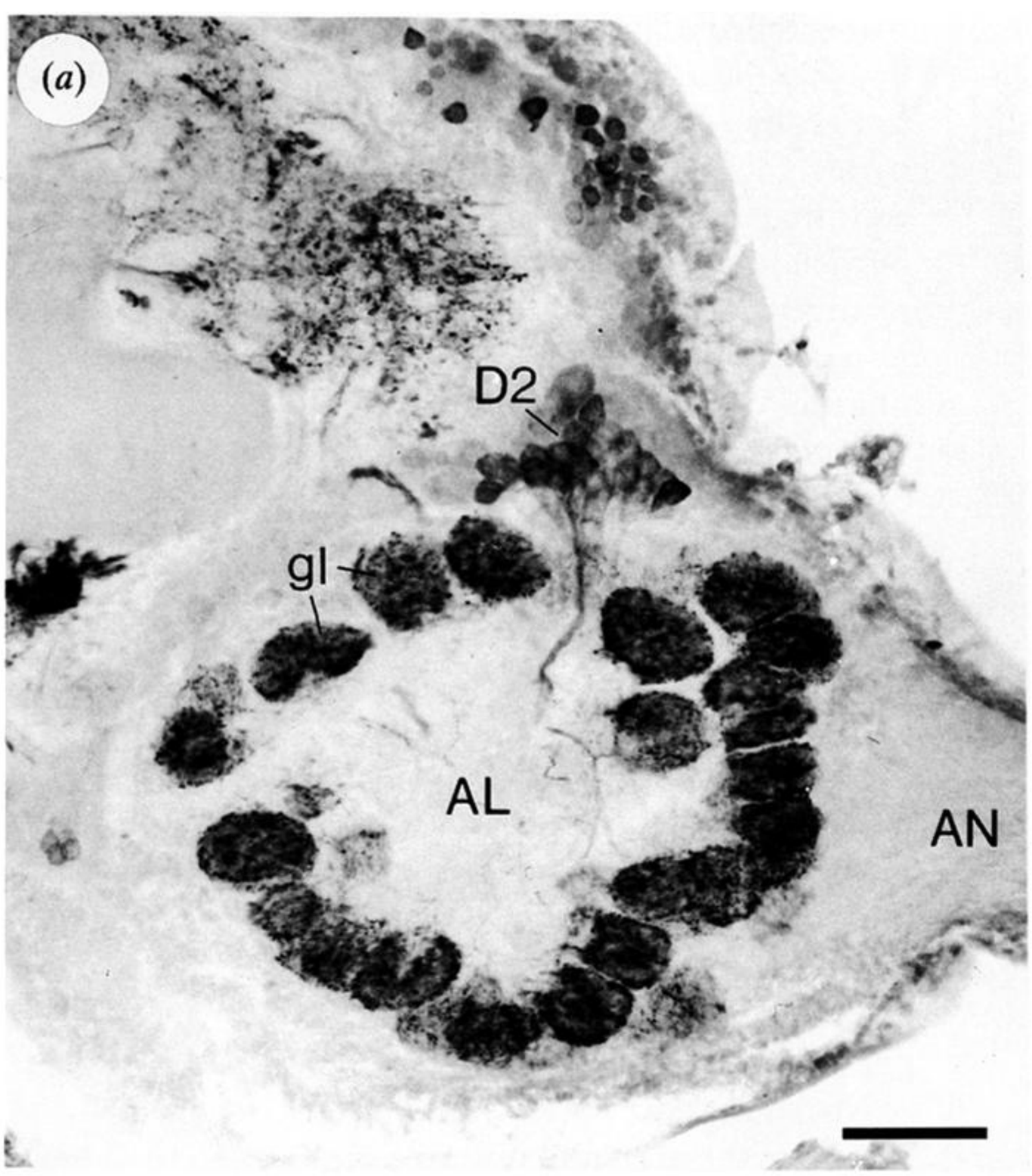
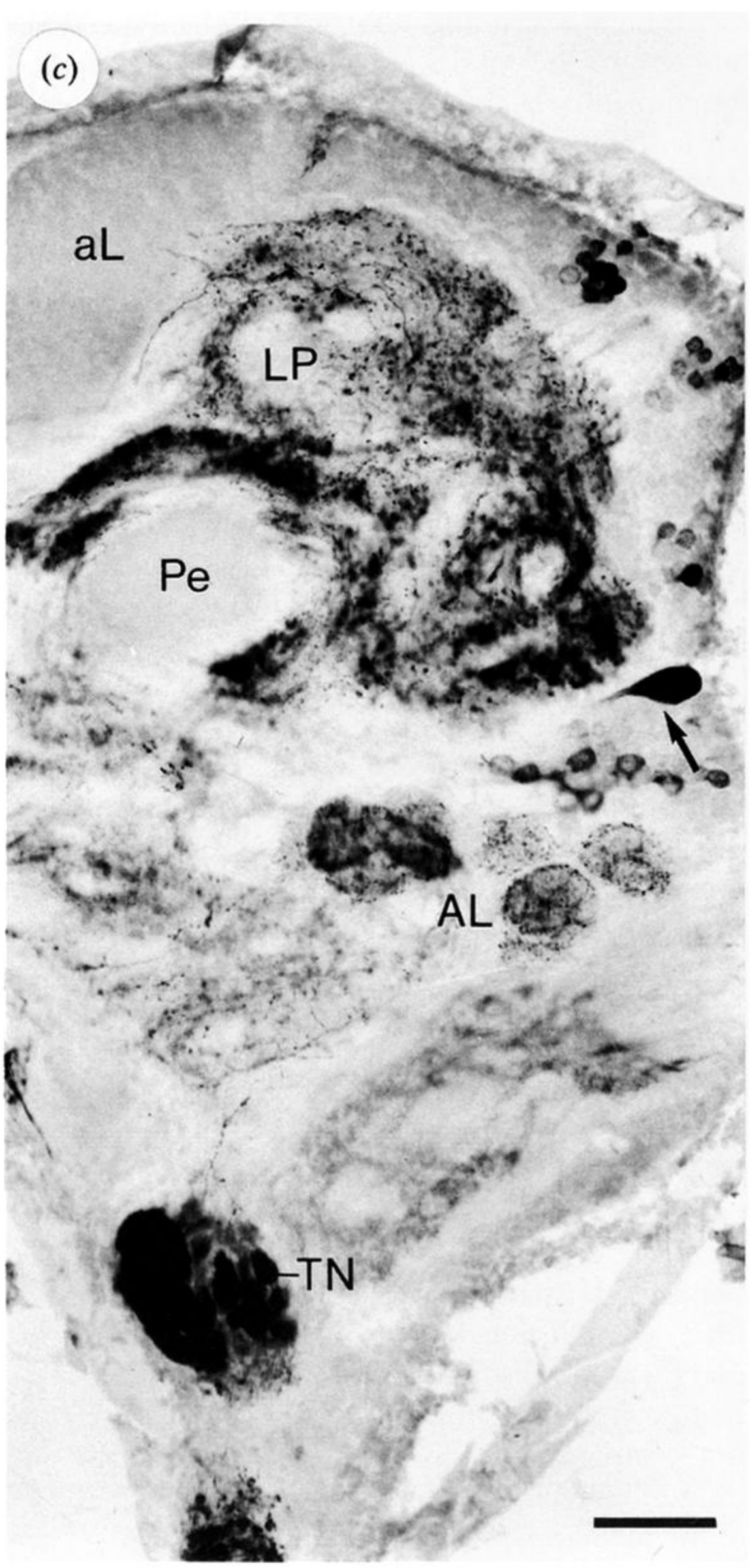
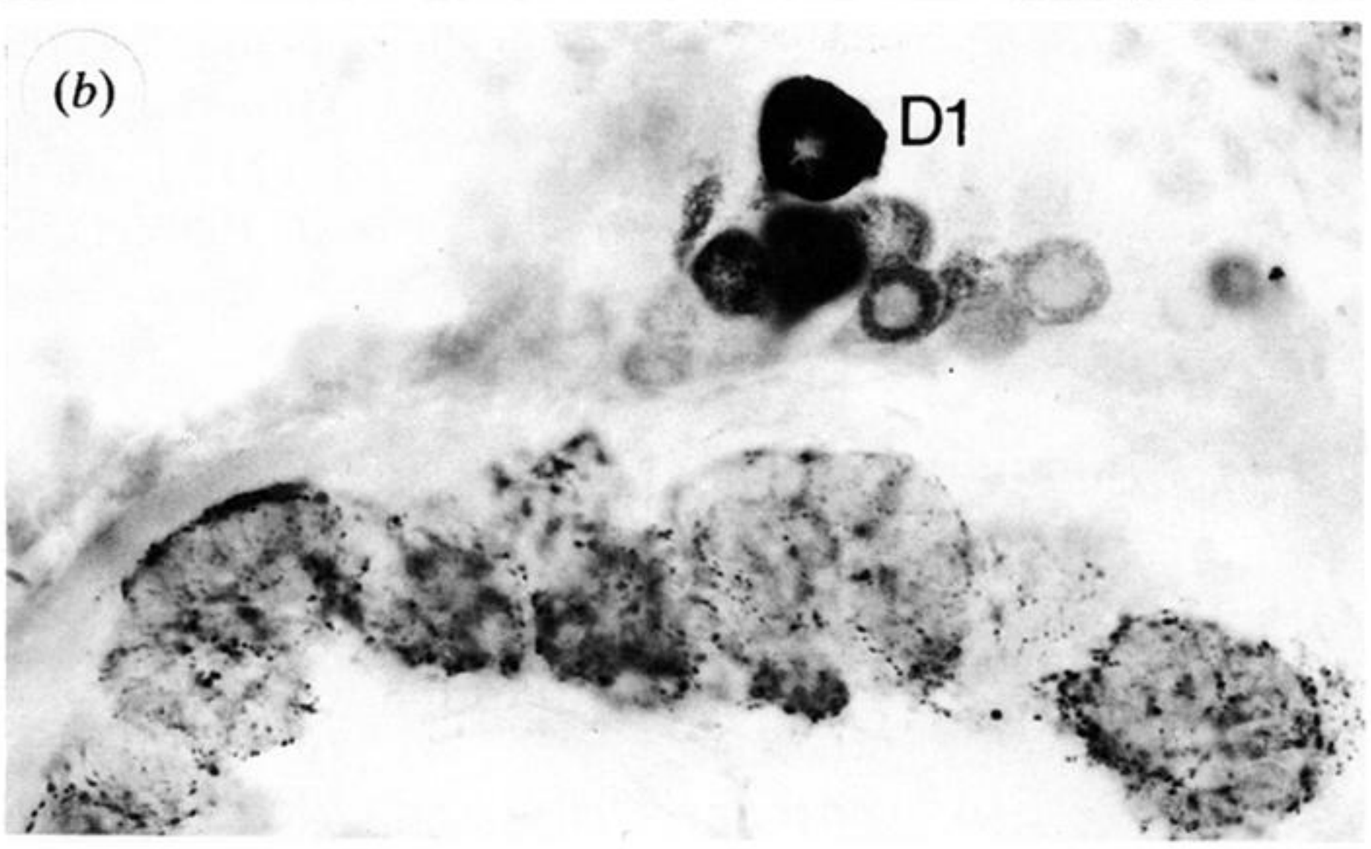
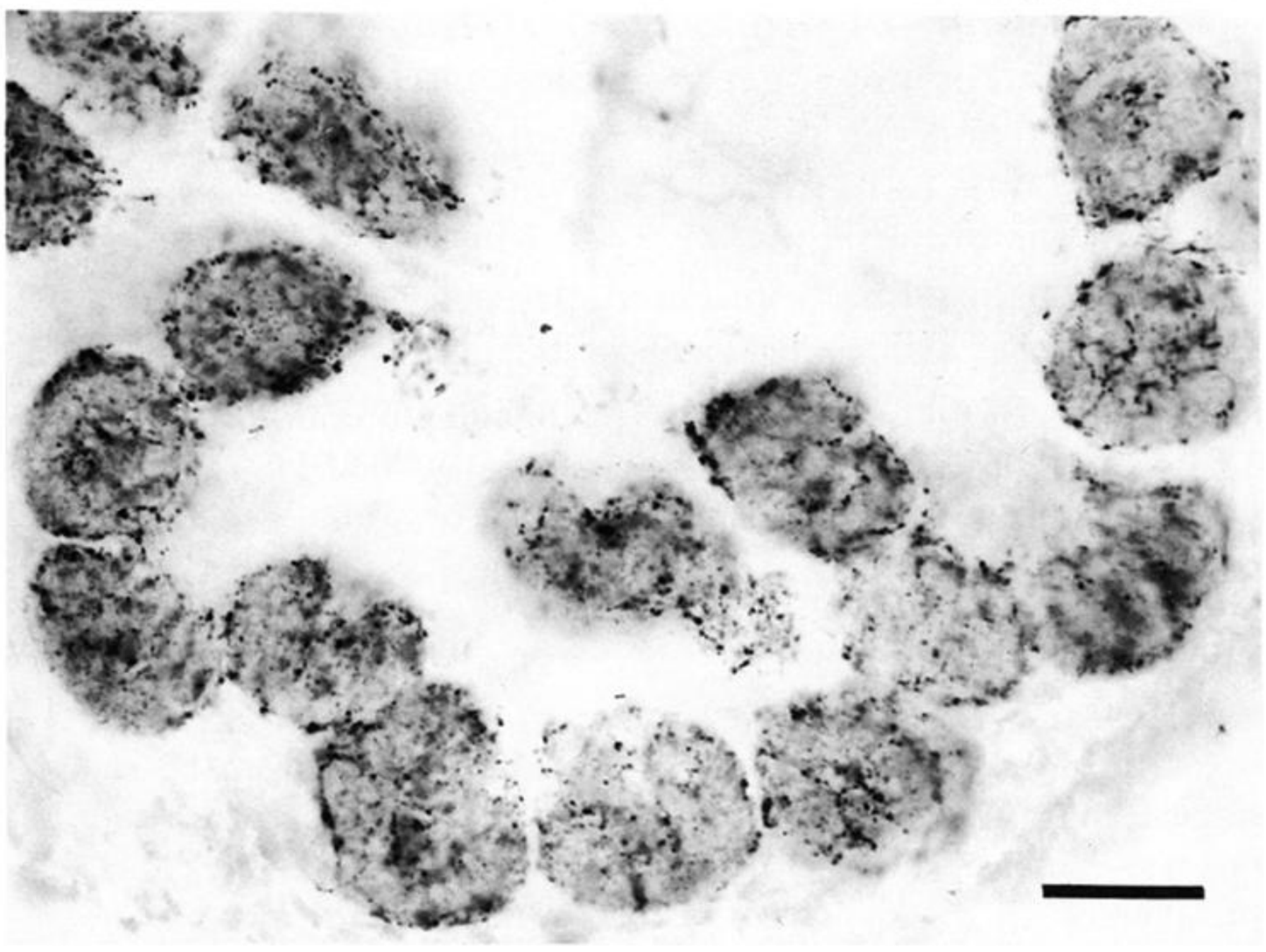








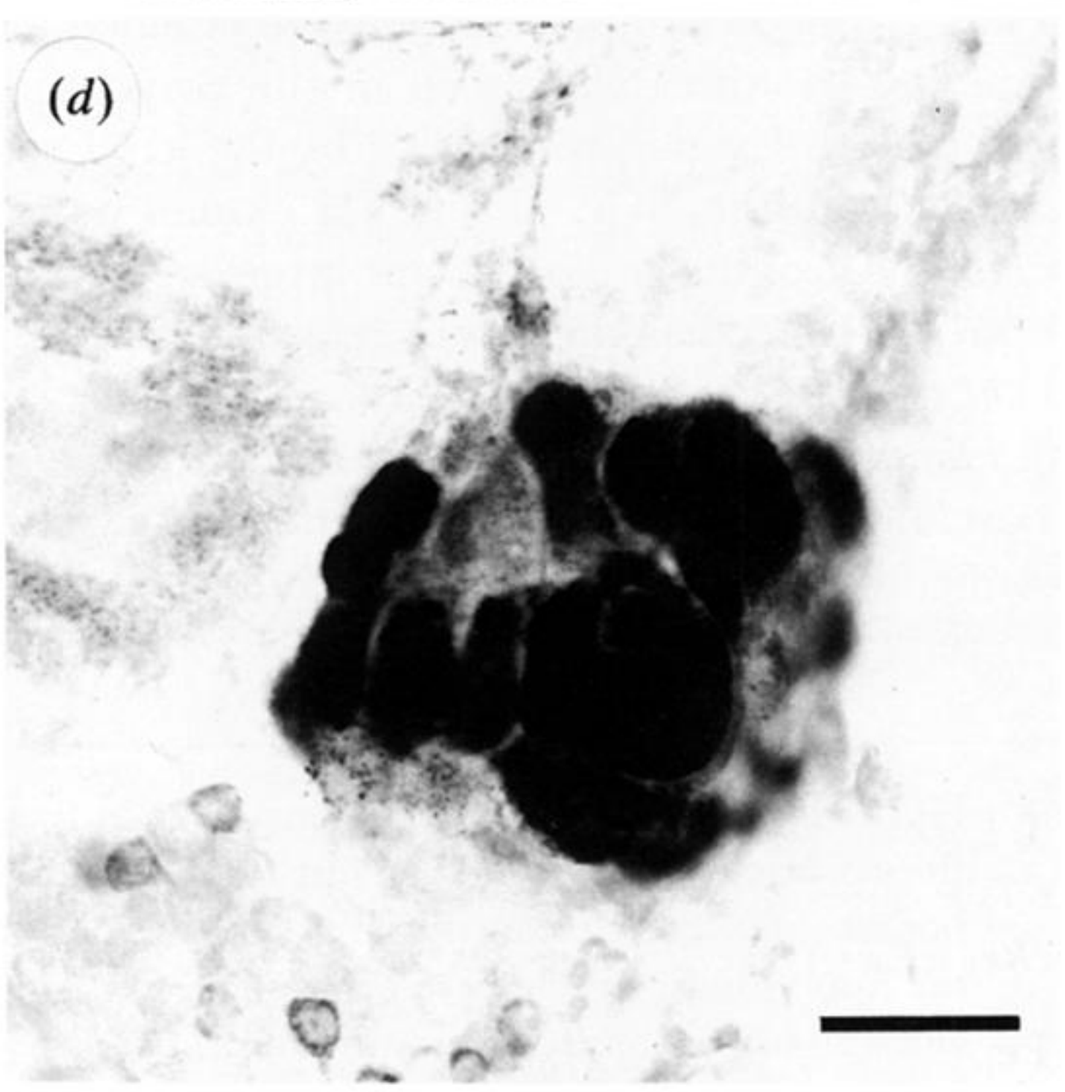
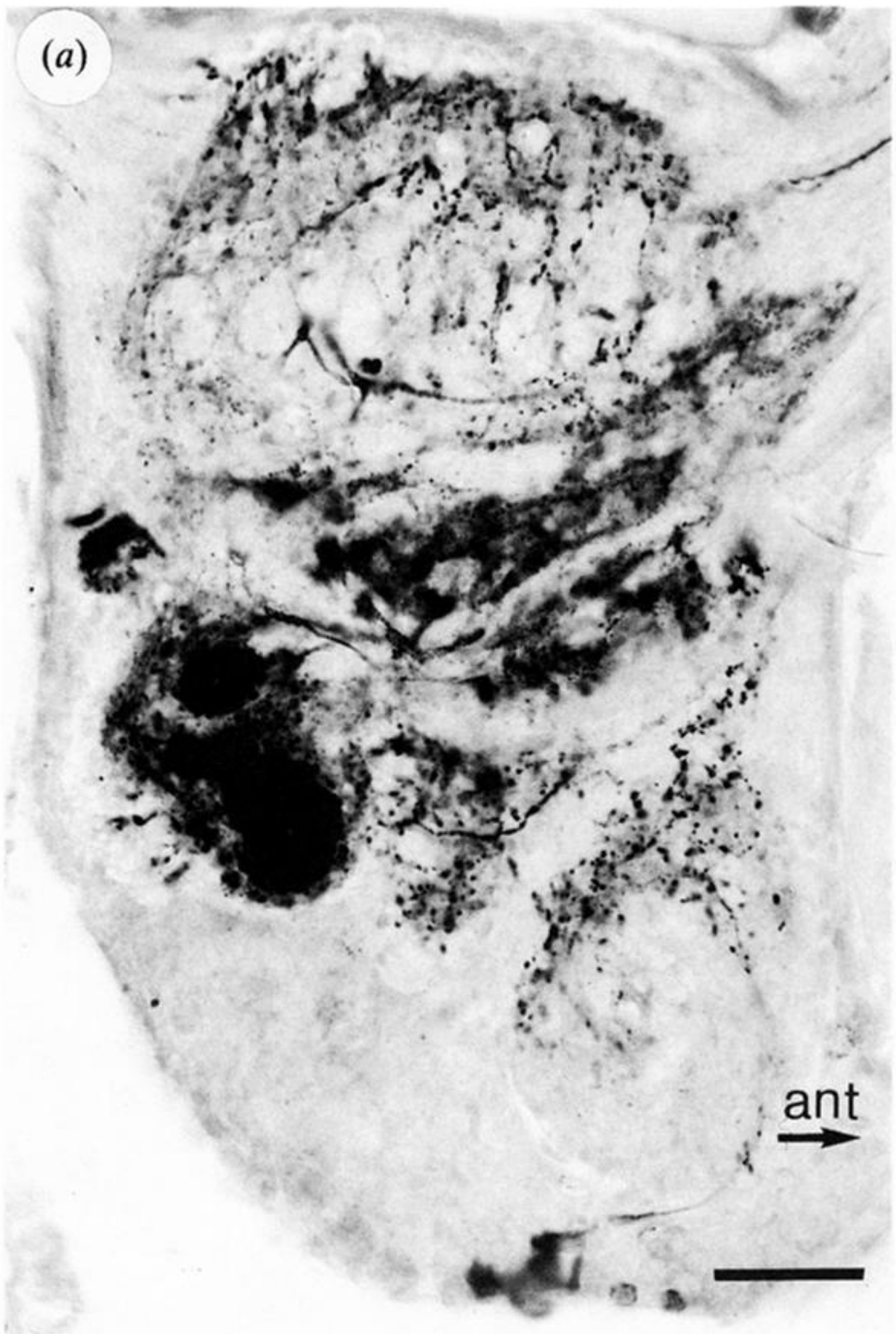
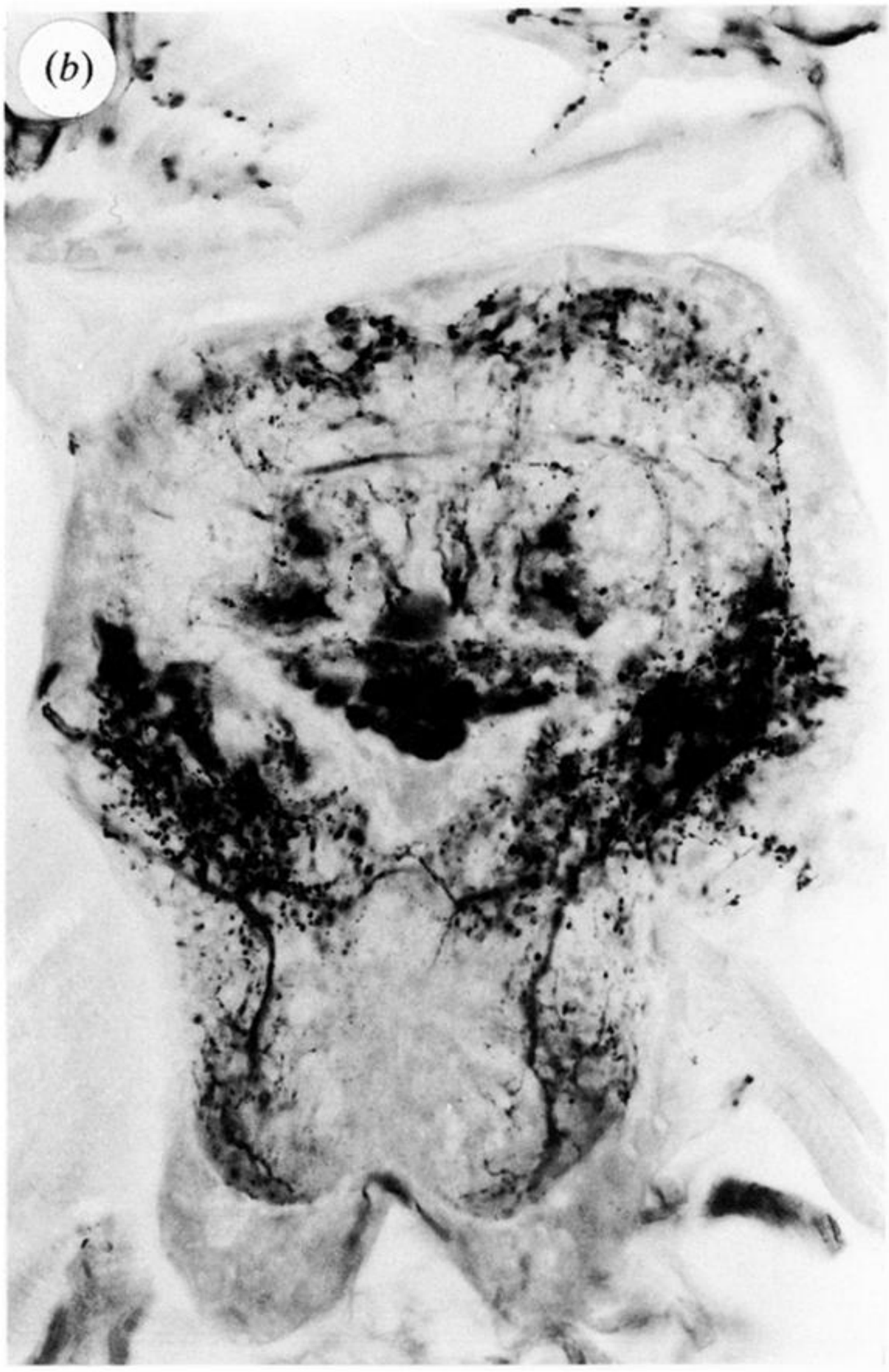


Figure 7. LomTK-LI neurons in the deutocerebrum and tritocerebrum of the cockroach brain (frontal sections, PAP method). (*a*) Overview of right antennal lobe (AL) with the antennal nerve (AN). Two of the antennal glomeruli are indicated (gl). In this micrograph the D2 neurons can be seen innervating the AL. (*b*) Detail of the antennal lobe in higher magnification (more posterior section). Here the D1 neurons are visible. Note the dense innervation of each of the glomeruli. (*c*) Overview of the right brain hemisphere with LomTK-LI fibres innervating neuropil in the lateral protocerebrum (LP), the most posterior glomeruli of the antennal lobe (AL) and a dense glomerular neuropil (TN) of tritocerebrum. The large protocerebral neuron P10 can be seen at arrow. Pe, peduncle. (*d*) Higher magnification of immunoreactivity in the glomerular tritocerebral neuropil in other specimen. Even at this magnification it is hard to resolve individual fibres or terminals. Scales: (*a*), (*c*) 100 μm; (*b*), (*d*) 50 μm.





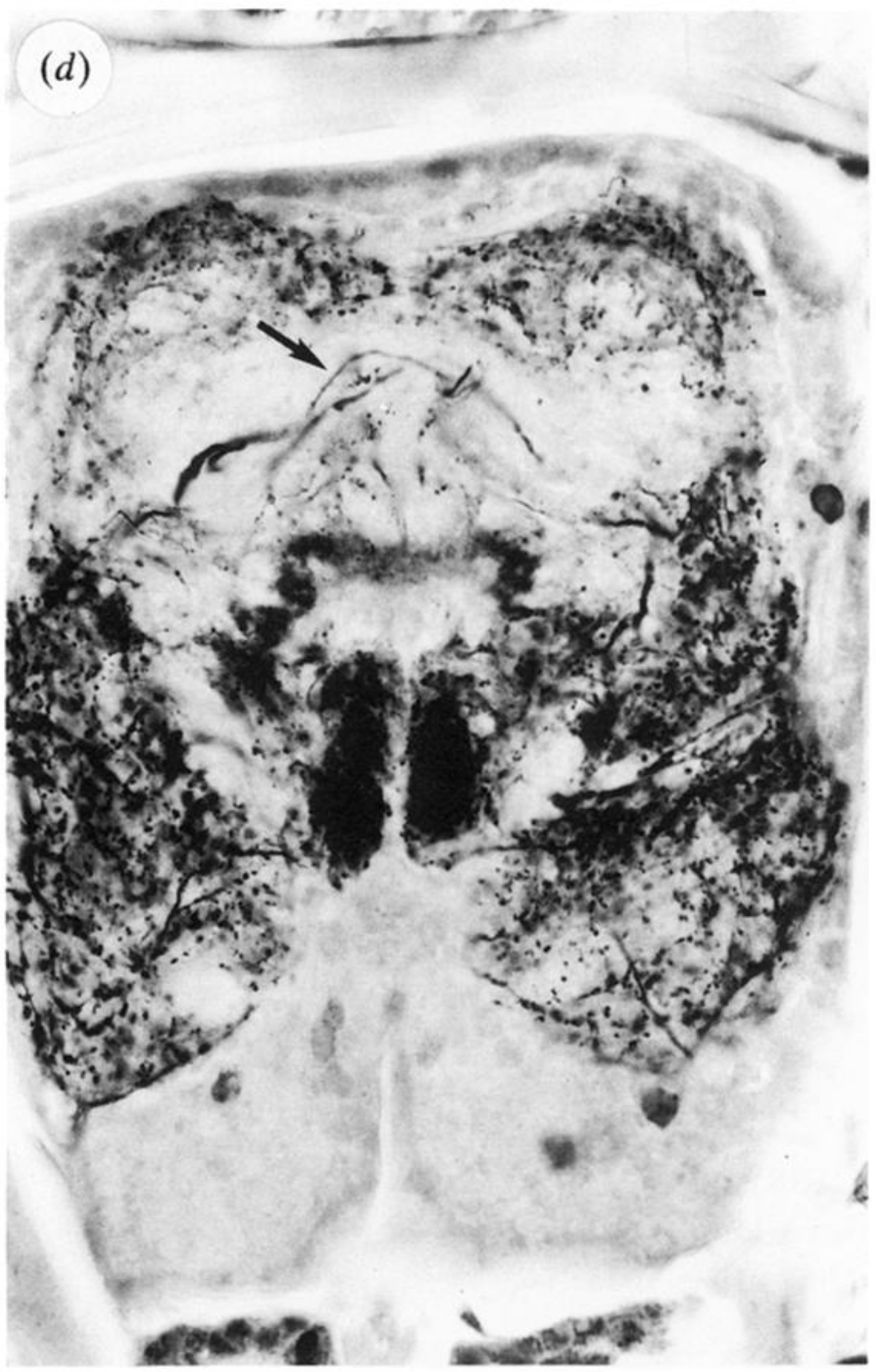


Figure 9. LomTK-LI neurons in the suboesophageal ganglion. (*a*) Sagittal section slightly off the midline. Immunoreactive fibres are seen in all three neuromers. (*b*) Anterior portion of the ganglion. (*c*) Midportion of the ganglion. (*d*) Slightly more posterior position. Scales: (*a*)–(*d*) 50 μm.

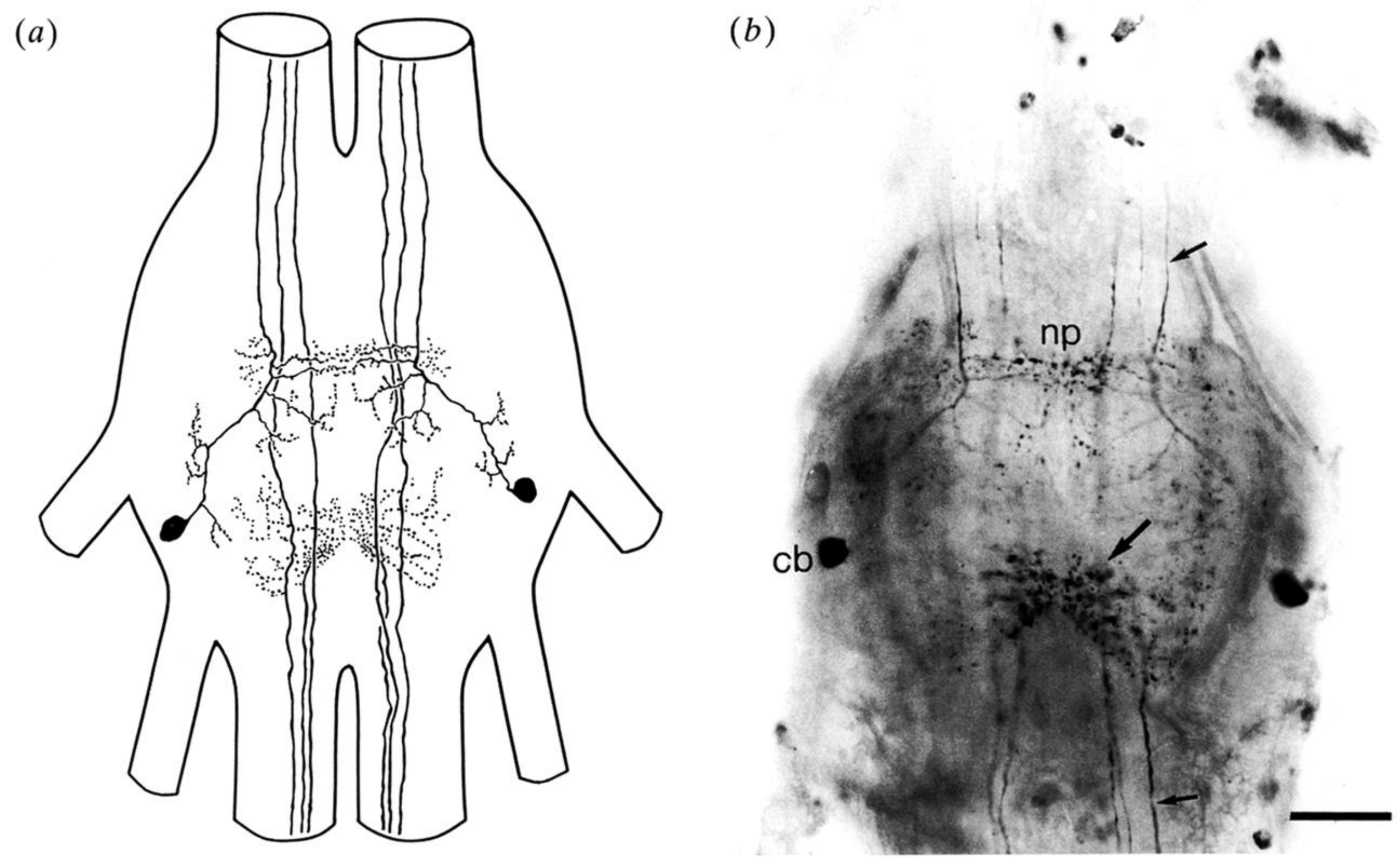
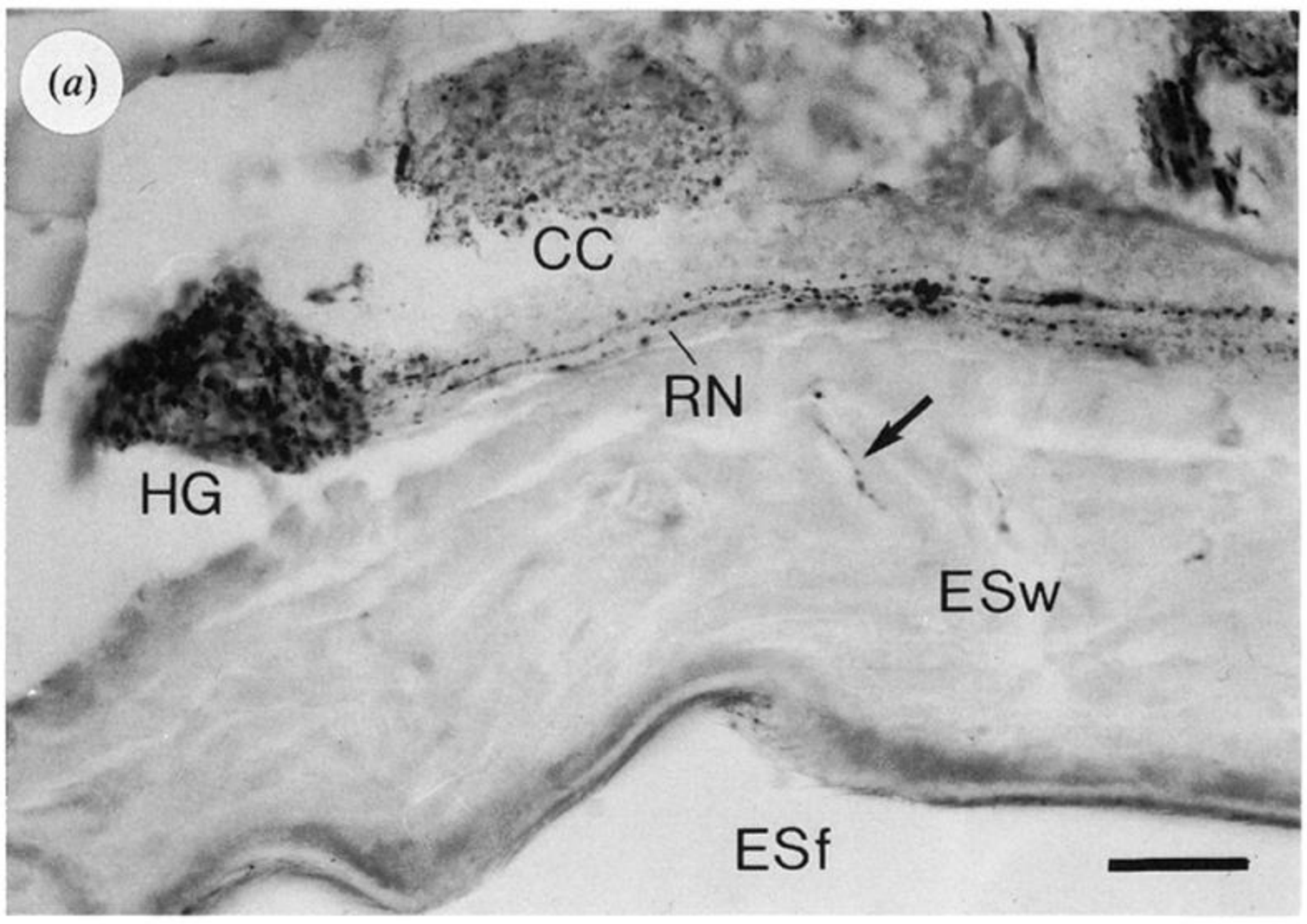
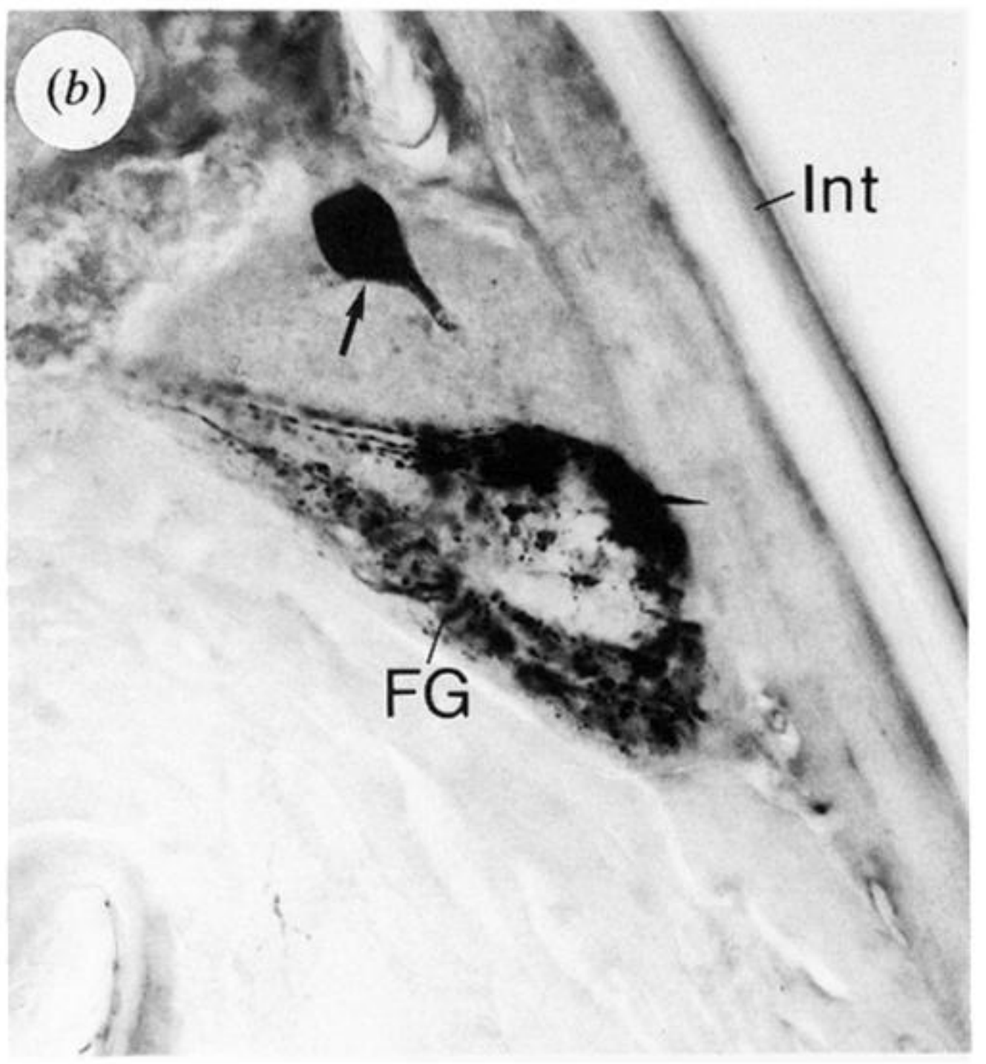
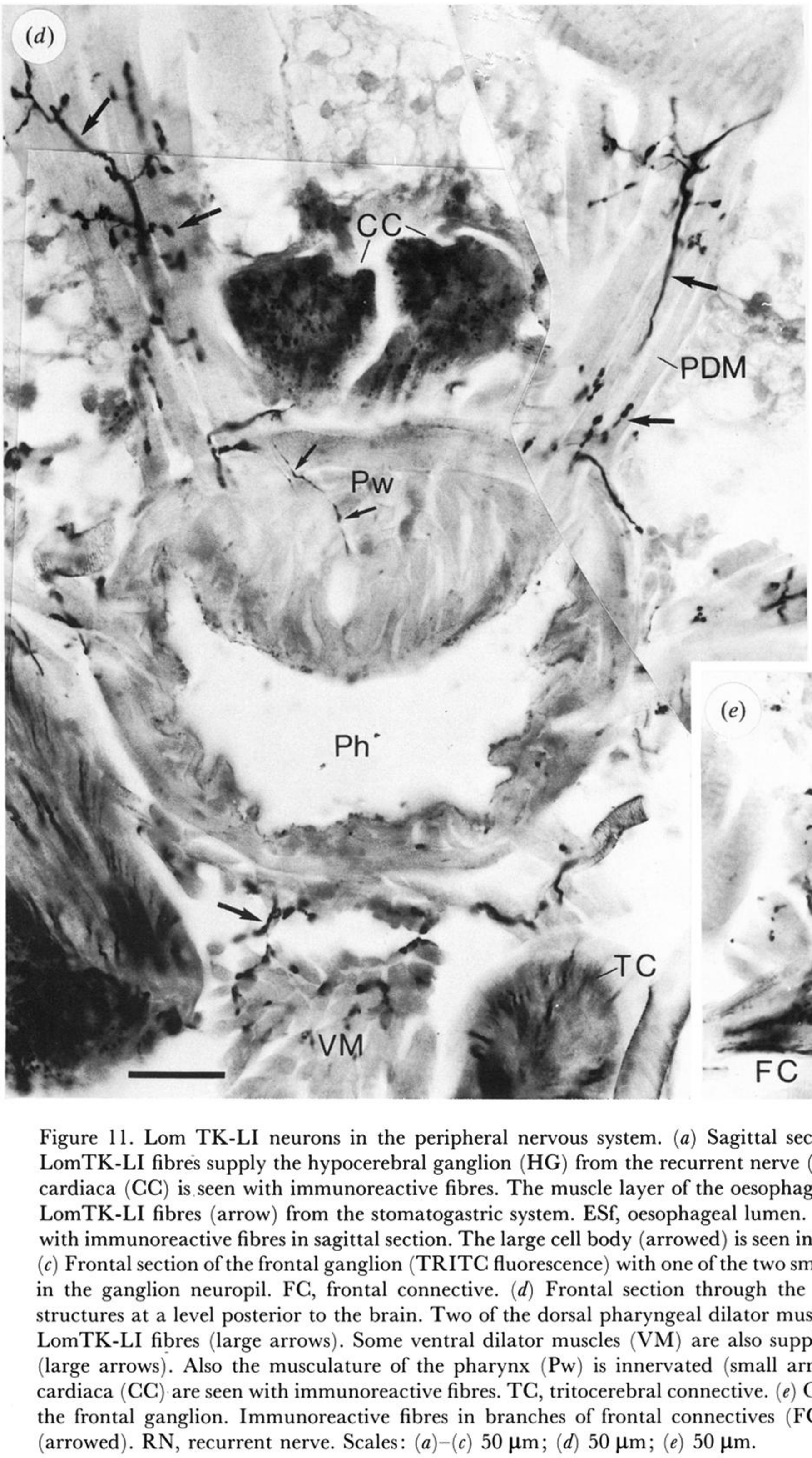
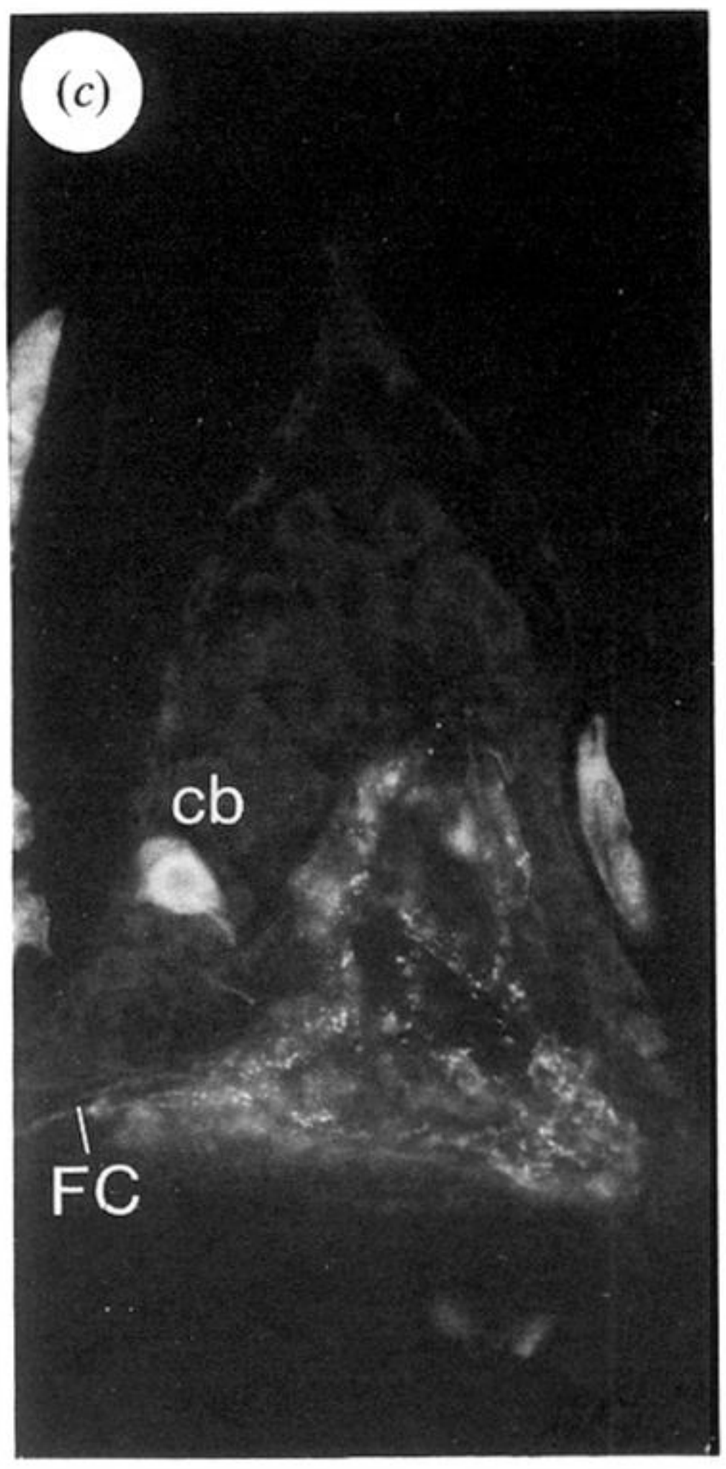


Figure 10. LomTK-LI neurons in an unfused abdominal ganglion. (*a*) Tracing of the pair of neurons located in the third unfused abdominal ganglion. The axons of this pair of neurons project anteriorly and appear to terminate three ganglia further up the cord. Thus three axons are seen in the connectives between the abdominal ganglia. In the posterior connective one axon (from three ganglia down the cord) terminates posteriorly in the ganglion neuropil, another is derived from two ganglia down the cord and the final from the previous posterior ganglion (the last two continue past the ganglion). (*b*) Micrograph of an unfused abdominal ganglion with the LomTK-LI cell bodies (cb) and axons in connective (arrows) and neuropil arborizations (np). Scale: 50 μm.









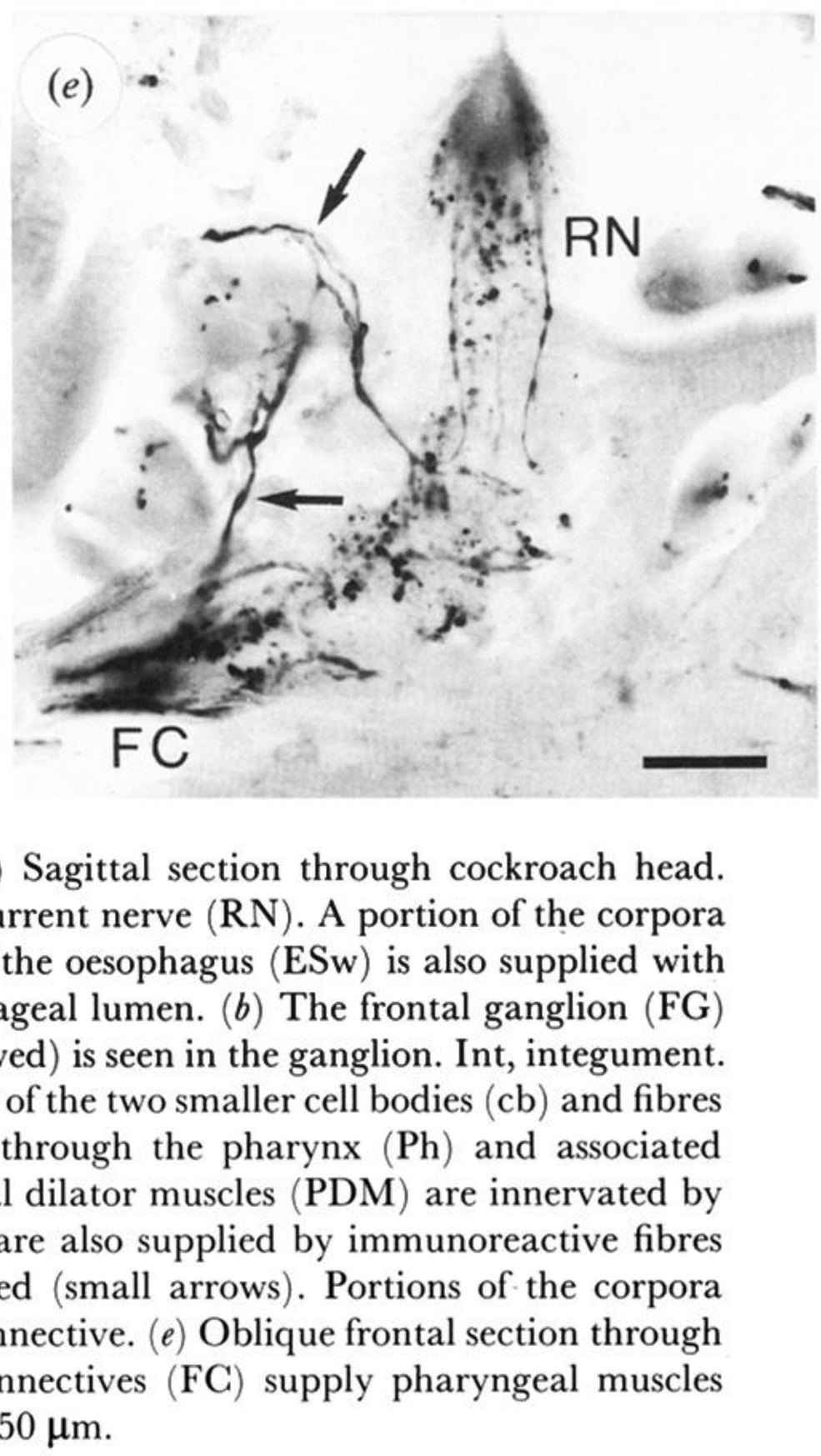


Figure 11. Lom TK-LI neurons in the peripheral nervous system. (*a*) Sagittal section through cockroach head. LomTK-LI fibres supply the hypocerebral ganglion (HG) from the recurrent nerve (RN). A portion of the corpora cardiaca (CC) is seen with immunoreactive fibres. The muscle layer of the oesophagus (ESw) is also supplied with LomTK-LI fibres (arrow) from the stomatogastric system. ESf, oesophageal lumen. (*b*) The frontal ganglion (FG) with immunoreactive fibres in sagittal section. The large cell body (arrowed) is seen in the ganglion. Int, integument. (*c*) Frontal section of the frontal ganglion (TRITC fluorescence) with one of the two smaller cell bodies (cb) and fibres in the ganglion neuropil. FC, frontal connective. (*d*) Frontal section through the pharynx (Ph) and associated structures at a level posterior to the brain. Two of the dorsal pharyngeal dilator muscles (PDM) are innervated by LomTK-LI fibres (large arrows). Some ventral dilator muscles (VM) are also supplied by immunoreactive fibres (large arrows). Also the musculature of the pharynx (Pw) is innervated (small arrows). Portions of the corpora cardiaca (CC) are seen with immunoreactive fibres. TC, tritocerebral connective. (*e*) Oblique frontal section through the frontal ganglion. Immunoreactive fibres in branches of frontal connectives (FC) supply pharyngeal muscles (arrowed). RN, recurrent nerve. Scales: (*a*)–(*c*) 50 μm; (*d*) 50 μm; (*e*) 50 μm.

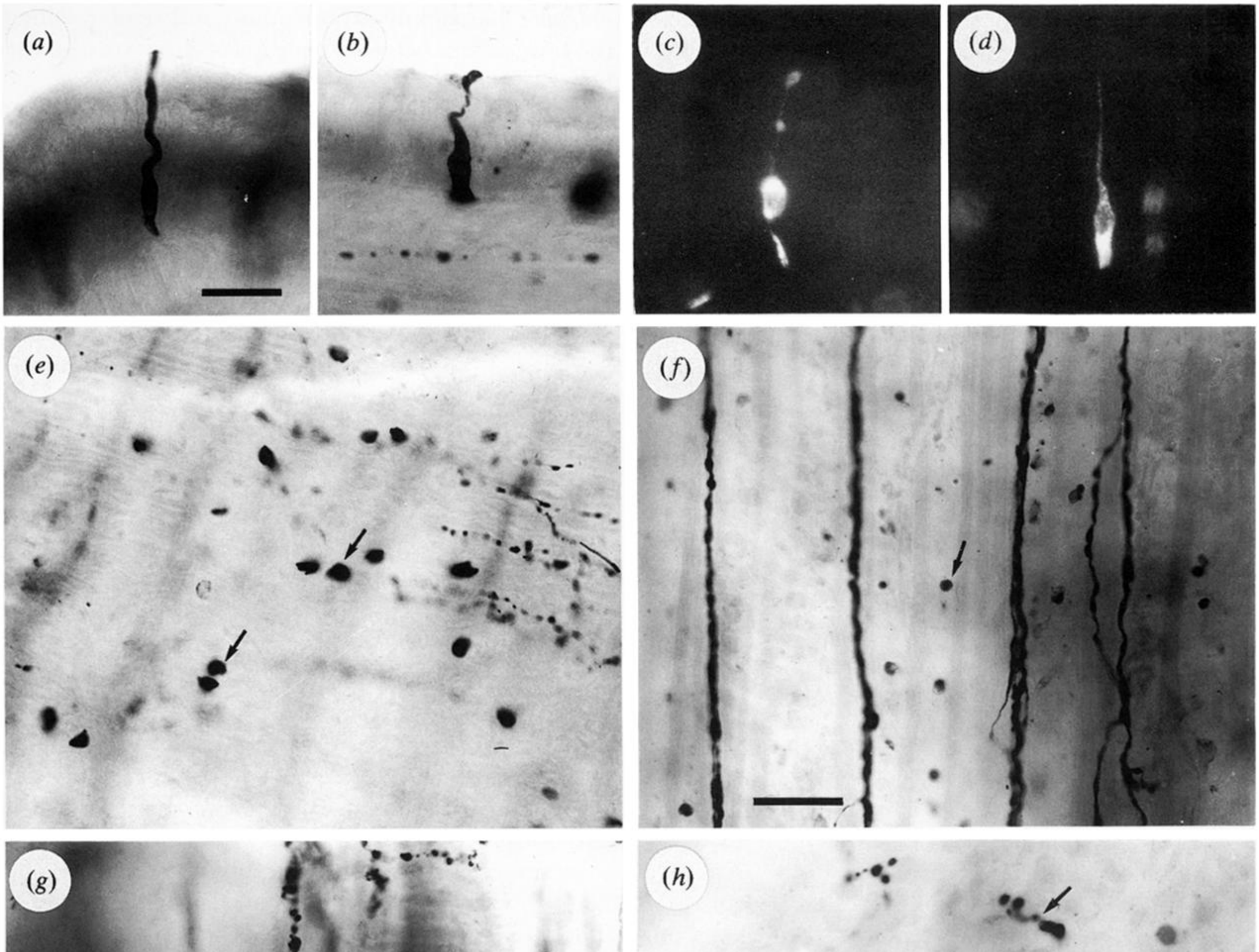


Figure 12. Lom TK-LI immunoreactive cells and fibres in the midgut. (*a*)–(*d*) Immunoreactive endocrine cells in the midgut epithelium (cryostat sections; (*a*), (*b*) PAP, (*c*), (*d*) TRITC). It is not clear whether the morphological variation represents different cell types. (*e*) Endocrine cells (arrowed) seen from the surface of a wholemounted midgut. The cells do not appear to be distributed in a geometric fashion. (*f*) LomTK-LI fibres and endocrine cells (arrowed) in surface view of wholemounted midgut. (*g*) At regular intervals the LomTK-LI fibres arborize and form varicose terminals (arrowed) in the muscle layer of the midgut. (*h*) Detail of varicose terminals (arrowed) in the midgut. Scales: (*a*)–(*e*) 25 μm; (*f*), (*g*) 50 μm; (*h*) 25 μm.