The Osmium-Ethyl Gallate Procedure is Superior to Silver Impregnations for Mapping Neuronal Pathways

By: Esther M. Leise and Brian Mulloney

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Abstract:
Ganglia processed through the osmium-ethyl gallate procedure (OEG)[19] retain more structural integrity than those processed through various silver impregnation methods. However, the OEG method continues to be neglected by most neuroanatomists. Both types of procedures have been used to trace large neuronal tracts, but during silver impregnation the neuropils lose many of their identifying characteristics. We demonstrate here the advantages of the OEG procedure by comparing it with two silver techniques, Rowell's[11] and Holmes's[5]. The OEG method yields consistent and reliable results and is easier to carry out than silver protocols. Most importantly, the better preservation of the neuropils has led to the discovery and study of regional specializations that were previously undetected from silver preparations.
Key words: crayfish ethyl gallate — nervous system— osmium — silver impregnation

Article:

INTRODUCTION

In histological studies of neural tissues, silver impregnations are still commonly used to survey the overall anatomical organization[1-3]. A successful silver impregnation results in a brilliantly colored nervous system, in which most of the axons, larger processes and cell bodies are heavily stained, so their paths through the tissue can be mapped. Narrower processes, however, usually remain undefined,[11] Moreover, the silver impregnation procedures are capricious and cytological details are often masked or poorly preserved.[11] A superior alternative exists, the osmium-ethyl gallate (OEG) procedure[19], that darkens cell membranes more than normal osmication. It eliminates most of the shrinkage and distortion seen in silver-impregnated tissues and yields consistent and reliable results. Small neural processes are retained and as we demonstrate herein, can often be visualized with the electron microscope. Larger fibers can be traced in serial sections. The OEG procedure has been used successfully to map neuronal[12,20,21,22] and neuropils.[13]

Only a few researchers have used the OEG procedure since its first description in 1957[12,13,19-22.] This procedure was not cited in several recent reviews of neuroanatomical techniques[6,9,15-17] and remains a relatively obscure method. In this paper we compare this enhanced osmium staining procedure to two routine silver methods and demonstrate the generally superior preservation and appearance of material processed by the OEG method. We now use the OEG procedure routinely in our studies of the crayfish nervous system and encourage our colleagues to adapt this simple and dependable method to other neural preparations.

MATERIALS AND METHODS

Abdominal nerve cords were dissected from adult *Procambarus clarkii* (Girard) or *Pacifastacus leniusculus* and pinned out in Sylgard-lined petri dishes filled with cold crayfish saline.[10] Cords to be stained by Wigglesworth's[19] osmium-ethyl gallate (OEG) procedure were processed as follows: (1) fix on ice in 3% glutaraldehyde in 0.1 M sodium cacodylate, at pH 7.6, for 1 h; (2) at room temperature, wash in 0.2 M sodium cacodylate; (3) postfix in 2% OsO_4 in 0.1 M sodium cacodylate for 1 h; (4) wash for 0.5h with at least 5 changes of 0.2 M sodium cacodylate buffer; (5) place nerve cord in a fresh, saturated solution of ethyl gallate in the dark for 24 h. (Stain is made during step no. 3 by dissolving 1.2 g ethyl gallate (Biochemical Laboratories) and 0.25 ml technical cresol in 100 ml distilled water (DW). The stain may take an hour to dissolve even when stirred.); (6) wash in several changes of DW for 15 min; (7) dehydrate in ethanol and propylene oxide; (8) infiltrate and embed in soft Spurr's[14] resin, mixed from 10 g VCD, 10.5 g DER 736, 26 g NSA and 0.4 g DMAE. Cure for 16-24 h in a 60 °C oven.

Thick (10 or 20 μm) sections were cut on dry glass knives on a Sorvall JB-4 microtome, picked up with forceps, placed on slides and mounted in high viscosity immersion oil, type VH (Polysciences). Thin sections were cut on a Reichert OmU3 Ultramicrotome, picked up on Pioloform-coated grids (Mikrotechnik, Kunigundenstrasse 66, 8000 Munchen 40, F.R.G.), stained with a solution of 50% ethanol saturated with uranyl acetate for 1.5 h at 40 °C followed by lead citrate staining[18] for 45 min at room temperature. Spurr's resin is refractory to shorter staining protocols (Spurr, personal communication). The above regime results in sections of satisfactory contrast. Sections were examined on a Philips 410 electron microscope.

Nerve cords to be impregnated by the Holmes[5] silver method were similarly dissected, and treated as follows: (1) fix in Bouin's fluid for 48 h; (2) dehydrate in ethanol and clear in methyl benzoate; (3) embed in paraffin; (4) cut 10 μm sections and mount on slides; (5) immerse slides in a solution of 20% silver nitrate for 4 h in the dark; (6) rinse in distilled water (DW) for 5 min; (7) incubate in a solution of 32 ml of 0.2 M boric acid, 8 ml of 0.05 M borax, 20 ml of 1% silver nitrate, and 10 ml of 2.6 lutidine in DW, total volume 250 ml, for 48 h at 54 °C; (8) wash sections for 3 min in running tap water (RTW); (9) tone in 0.2% sodium chloroaurate for 5 mins; (10) rinse 3 times in DW; (11) reduce in 2% oxalic acid for 5 mins; (12) wash for 3 min.

The toning, rinsing, reducing, and washing sequence was repeated 3 more times. In each repetition, slides were left in the gold toner for 5 min. During the last two sequences the gold toner concentration was increased to 2%. Finally, the slides were fixed in 5% sodium thiosulfate for 3 mins, washed to 5 min, dehydrated, cleared and mounted in Permount.

Nerve cords to be impregnated by Rowell's[11] method were dissected and processed as follows: (1) fix in isosmotic glutaraldehyde solution, 0.48 M glucose plus 6% glutaraldehyde overnight (~16 h); (2) dehydrate in ethanol, clear in xylene, and embed in paraffin; (3) cut 10 μm sections and mount on slides (4) immerse for 1 h in the dark in 20% silver nitrat; (5) rinse in distilled water (DW); (6) incubate at 50 °C for 15 h in a solution of 40 ml Tris/maleate at pH 7.2, 20 ml of 1% $AgNO_3$, 10 ml of 2,6 lutidine, total vol. 250 ml; (7) wash in DW; (8) immerse in 2% sodium sulfate for 2 min; (9) wash in DW; (10) develop at room temperature for 8 min in 9 ml of 5% silver nitrate, 300 ml of 9% sodium sulfite plus 20 ml of 0.5% hydroquinone; (11) rinse in DW; (12) rinse in RTW for 5 min; (13) rinse in DW; (14) tone for 5 min in 0.2% gold chloride at pH 2; (15) rinse in DW; (16) reduce in 2% oxalic acid for 5 min; (17) rinse in DW; (18) fix in 5% sodium thiosulfate for 2 min; (19) wash in RTW and DW; (20) counterstain in 0.15% gallocyanin chrome alum overnight; (21) wash for 10 min in RTW; (22) stain in 5% phosphotungstic acid for 30 min; (23) stain in 0.1 g aniline blue, 0.5 g fast green, 2 g orange G, and 0.8 ml of glacial acetic acid in 92 ml DW; (24) differentiate in 70% ethanol; (25) dehydrate, clear and mount in Permount.

RESULTS

Light microscopical appearance

Nerve cords display the same general anatomical features if they have been processed by either the osmium ethyl gallate (OEG) procedure or a silver impregnation, but the cytological appearances of the cords are significantly different (Fig. 1). In general the OEG method yields nerve cords that are round and expanded. The axons are quite cylindrical Fig. 1) and if any are collapsed, it is usually only the largest (Fig. 2a), so their identification is not obscured. Of the plastic embedding media we used (Epon, Araldite and Spurr's), Spurr's[14] low viscosity resin caused the collapse of the fewest axons (Leise and Mulloney, unpublished data). In the OEG preparations even the connective tissue spaces are open giving the impression of a well-preserved tissue (Figs. 2a, 3a). There are relatively few empty spaces within the connectives (Fig. la) or in the body of the ganglion (Figs. 2. 3), This is unlike the silver-impregnated tissue; in which unexplainable transparent gaps often occur

(Figs. 1b, 3b). In transverse sections through the nerve cord (Fig. 1b) the faint outlines and lack of central staining in these gaps makes it impossible to decide if they are axons, connective tissue spaces or processing artifacts. Likewise, in frontal sections through the ganglia (Fig. 3b) some of the round areas may be unstained axons and other artifacts. The ‘foamy’ appearance of the neuropils (Fig. 3b) makes it difficult to distinguish definitively between the two possibilities.

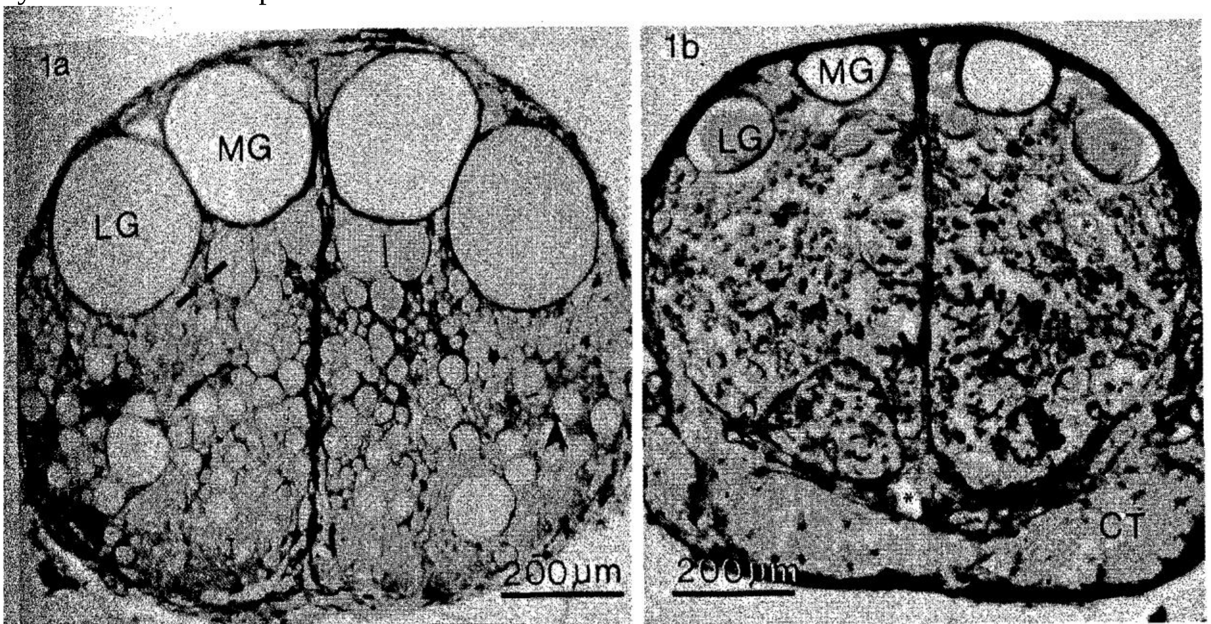

Fig. 1. Transverse, 10 μm thick sections through the connectives between abdominal ganglia 4 and 5 of *P. clarkii*. Dorsal is up. a: osmium-ethyl gallate procedure (OEG). A small (~6 μm diameter) axon lies between the arrows. Note glial cell nuclei (arrowheads). A gradient of fiber diameters is readily apparent. The apparent fuzziness of the image stems from the thickness and transparency of the section ×180. b: Holmes[5] silver technique. Note the differential staining of lateral (LG) and medial giant (MG) axons and that the stain incompletely fills the LG and many of the smaller axons (arrowheads). Many gaps occur throughout the tissue (asterisks) (CT, connective tissue). ×180.

Each axon the OEG nerve cord has a well-defined border glial wrappings can increase this effect (Fig. 1). The axoplasm is quite translucent (Figs. 1a, 2a). The density of the axoplasm may vary (Fig. la) but the intensity of staining throughout the cord is relatively uniform (Fig. la). In contrast, axons in the silvered material occur either with or without dense centers and most axons are incompletely filled with stain (Figs 1b, 2b). Many axons, especially the larger ones with glial wrappings, have dense outlines (Fig. 1b). Small axons are difficult to distinguish at low magnifications because their edges are often indistinct (Figs. lb, 2b). Axons as small as 6 μm in diameter are visible in low-power OEG micrographs (Fig. 1). In the OEG preparation, it is obvious that there are gradients of axonal diameters and clusters of similarly-sized axons within the connectives (Fig. la) and ganglionic tracts (Fig. 2a). This is much less apparent from the silvered sections (Figs. lb, 2b).

Within the body of the ganglion discrete axonal tracts and commissures are much more prominent in OEG preparations than in silver impregnations (Fig. 3). In part, this distinction occurs because the OEG method preserves small neuronal processes relatively well, allowing one to distinguish easily the longitudinal and vertical tracts and commissures from the surrounding neuropil or processing artifacts. In frontal sections of silver preparations, longitudinal axonal tracts are visible, but the vertical tracts resemble tissue artifacts and it is not always possible to distinguish between the two (Fig. 3b).

Because the OEG method preserves most small neuronal processes, one can detect several types of neuropils based on their cytological appearances in optical sections[12,13]. The horseshoe and lateral neuropils differ in position within the abdominal ganglia but also have distinctive ultrastructural features[13]. The horseshoe neuropil contains many synaptic glomeruli (Figs. 2a. 4a) and because it has more of the very tiny fibers[13] stains more darkly than the lateral neuropil in OEG material (Figs. 2a, 3a). The differential staining and glomeruli are not evident in the silver impregnation (Figs, 2b, 3b).

Of the two silver methods used, Rowell's[11] method produces neuropils with a distinctly 'foamy' appearance (Fig. 3b), but the irregularity of the stain makes it impossible to assign any differential characteristics to the various neuropils. Holmes's[5] method yields more fine-grained neuropils, in which lie some distinct dark strands (Fig. 2b). These may be neuronal processes of large diameter. The difference in staining quality between the horseshoe and lateral neuropils does not occur in every Holmes section and is considered to be a processing artifact. The staining quality does not reflect the real differences in these neuropils[12,13].

Ultrastructural preservation

To determine if the OEG method is suitable for studies of tissue ultrastructure, we examined part of the horseshoe neuropil by electron microscopy (Fig. 4), As expected, large axons are discernible (Fig. 4b), although their limiting membranes may be obscured, in some areas. Smaller processes are harder to identify (Fig. 4c); the loss of membranous structures appears to be more common in such processes. Large lucent areas occur that are processing artifacts. Nonetheless, many cellular organelles are visible. Cell nuclei (not illustrated) are preserved, as are mitochondria, free ribosomes, and arrays of microtubules within the neurons (Fig. 4b, c). The neuronal cytoplasm is generally lucent, although there is much fine flocculent material that appears to be connecting the arrays of microtubules (Fig. 4b). Some neuronal profiles also contain round structures between 50 and 80 nm in diameter that may be synaptic vesicles. The incomplete preservation of the membranes that occurs in the smaller fibers makes the positive identification of synapses and synaptic vesicles difficult.

DISCUSSION

For many reasons, we find the osmium-ethyl gal-late (OEG) method[19] to be a histological technique superior to either the Holmes[5] or Rowell" silver impregnation procedures. The OEG method is far simpler, requiring fewer solutions and less experimentation with times and temperatures to obtain satisfactory and reliable results. All the staining is done en bloc and the tissue is suitable for embedding in any plastic or wax medium. In combination with plastic embedding and sectioning techniques results can be obtained within 3 days, as opposed to at least 5 days for the combined paraffin and silver techniques. The OEG procedure is carried out with common electron microscopical fixatives, and for light microscopy the tissue looks well preserved. However, the preservation of the tissue is not satisfactory for routine electron microscopy.

Double fixation of tissue for electron microscopy is now the standard procedure'. Several published protocols using glutaraldehyde or a mixture of glutaraldehyde and formaldehyde in a cacodylate buffer followed by osmication in the same buffer give satisfactory preservation of crayfish neural tissue[7,8,13,17]. Although the relative quality of the ultrastructural preservation in any fixation should be weighed against that produced by other fixation procedures, there are standards by which an individual protocol can be judged[4]. Possibly the most important criterion is the continuity of membranes[4], which is not met by the OEG procedure (Fig. 4c). The particles that may be synaptic vesicles lack surrounding membranes and there are also few vesicles of smooth endoplasmic reticulum that normally appear in this tissue[13]. Also the cytoplasmic ground substance should be a fine granular precipitate, showing no empty spaces[4]. The empty spaces produced by the OEG procedure (Fig. 4c) indicate again that the structures are incompletely preserved. We expected to find well-preserved cell membranes because of their dark appearance in optical sections. It seems likely that the glial wrappings and dense extracellular matrix[13] account for the enhanced cell borders. However, it is clear that further refinement of this technique will be necessary before it can be used routinely for studies of neuronal fine structure.

At the light level, one can use the OEG method to reconstruct large tracts and commissures accurately[12,20-22]. The fixation preserves enough of the fine structure in the smaller processes (Fig. 4c) to enable one to discern the cytological differences between neuropils. This is perhaps its most significant advantage over both silver preparations. Skinner's[13] use of the OEG procedure has led to the discovery of structures, such as the synaptic glomeruli that may be sensory integration centers, and that are not preserved in the silver impregnations.

We have found that, once we locate the basic tracts and neuropil areas in our OEG preparations, we can study silver preparations and locate the same features. However, we feel that the OEG sections contain more data, as well as being more aesthetically pleasing. The OEG sections are of high contrast and make excellent photographic subjects.

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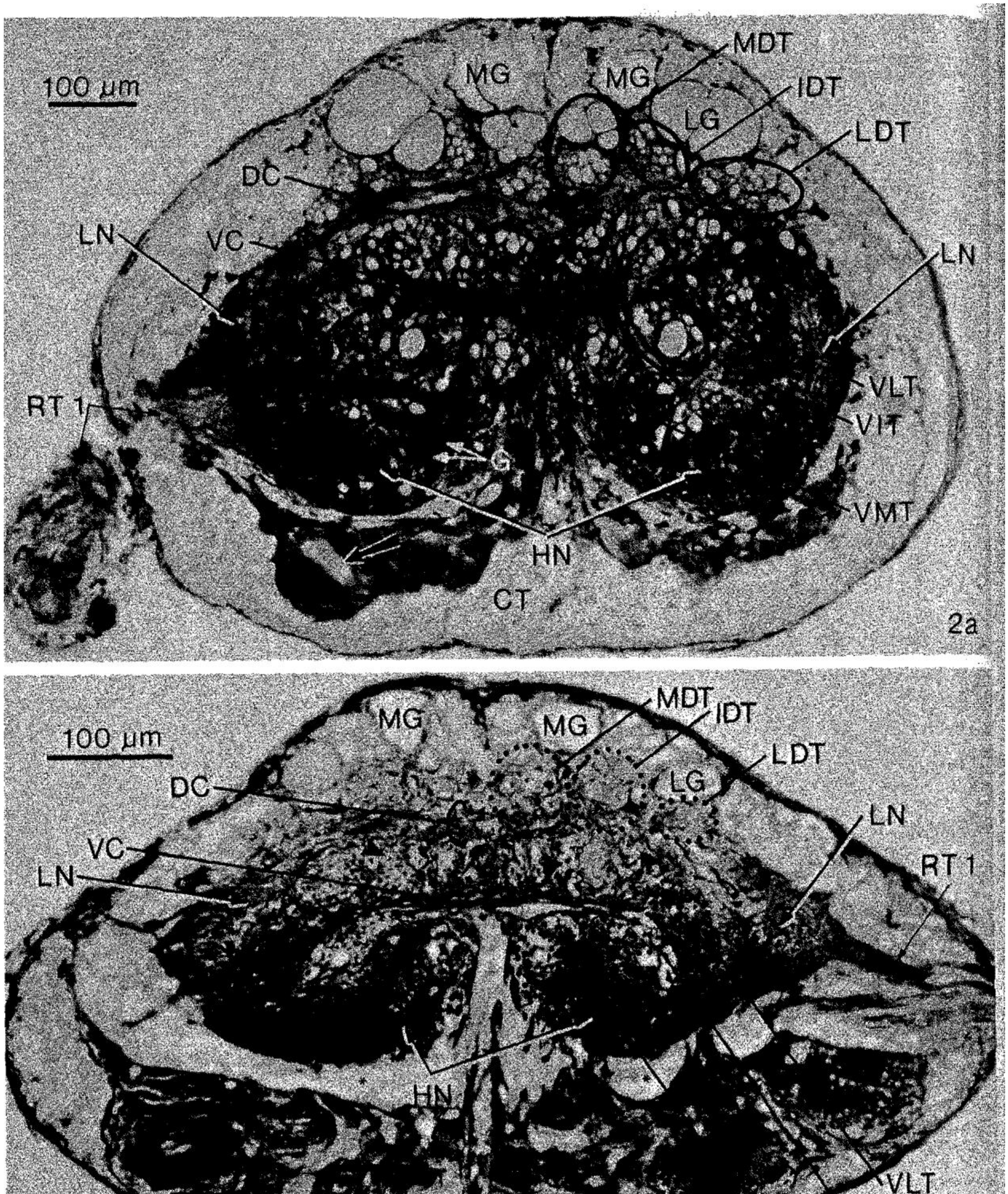

Fig. 2. Transverse, 10 μm thick sections through similar regions of two abdominal ganglia, through the exit of a first root (RT 1). a: OEG preparation of ganglion 5 of *P. leniusculus*. Medial and lateral giant axons (MG, LG) are dorsal, neuronal somata (S) are ventral. The base of one neurite (arrow) is visible within a large soma. The entire ganglion is surrounded by a connective tissue (CT) sheath. Both medial giants collapsed, probably during infiltration. Synaptic glomeruli (G) (cf Fig. 4a) occur frequently within the horseshoe neuropil (HN), which always stains more densely than the lateral neuropils (LN). Four layers of axonal tracts occur in this section. Their axons are cut in cross-section. Tracts in the first and third layers are circled (MDT, median dorsal tract; IDT, intermediate dorsal tract; LDT, lateral dorsal tract; VMT, ventral median tract; VIT, ventral intermediate tract; VLT, ventral lateral tract, as in Skinner[12]). Two layers of commissures (DC, dorsal commissures; VC, ventral commissures) run below the first and second layers of tracts, respectively. ×144. b: Holmes[5] silver impregnation of ganglion 4 of *P. clarkii*. Labels and orientation as in a. Individual tracts or bundles of axons in the first and third layers are encircled with dots and labeled as in a. Their identification is based on a comparison with the tracts in a. Note the indistinct outlines of most small axons (arrowheads) and that the stain incompletely fills them. The large gaps (asterisks) in the ganglion between the neuronal somata and neuropil may be artifactual. ×205.

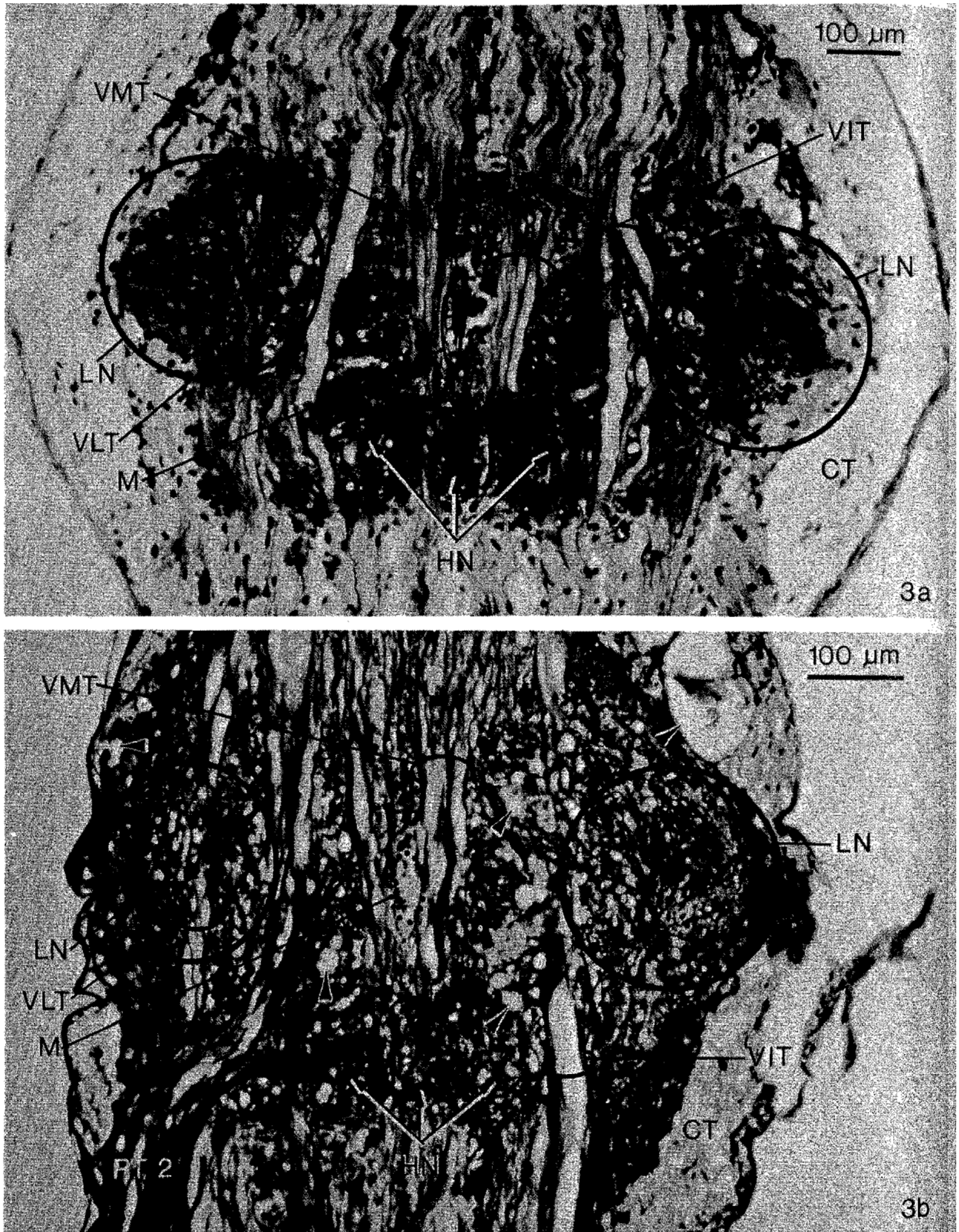

Fig. 3. a: frontal section, 20 μm thick, prepared by the OEG method, passing through the horseshoe neuropil (HN) and lateral neuropils (LN) of abdominal ganglion 5 of *P. leniusculus*. Approximate extent of the LNs is indicated by the large circles. Two pairs of longitudinal tracts (VMT and VIT), the left VLTs (abbreviations as in Fig. 2), and the central vertical tract, M, are visible here. Anterior is up. ×125. b: frontal section, 10 μm thick, prepared by Rowell's[11] silver method, through abdominal ganglion 5 of *P. clarkii*. This section is somewhat more dorsal than the one in a, and is also tilted. The left side is higher than the right, as evinced by the asymmetry of the LNs and the exiting of the left second root (RT2) from the ganglion. Labels as above. Gaps (arrowheads) of various sizes occur throughout the ganglion. Some may be vertical tracts of unstained axons, others tissue artifacts. ×166.

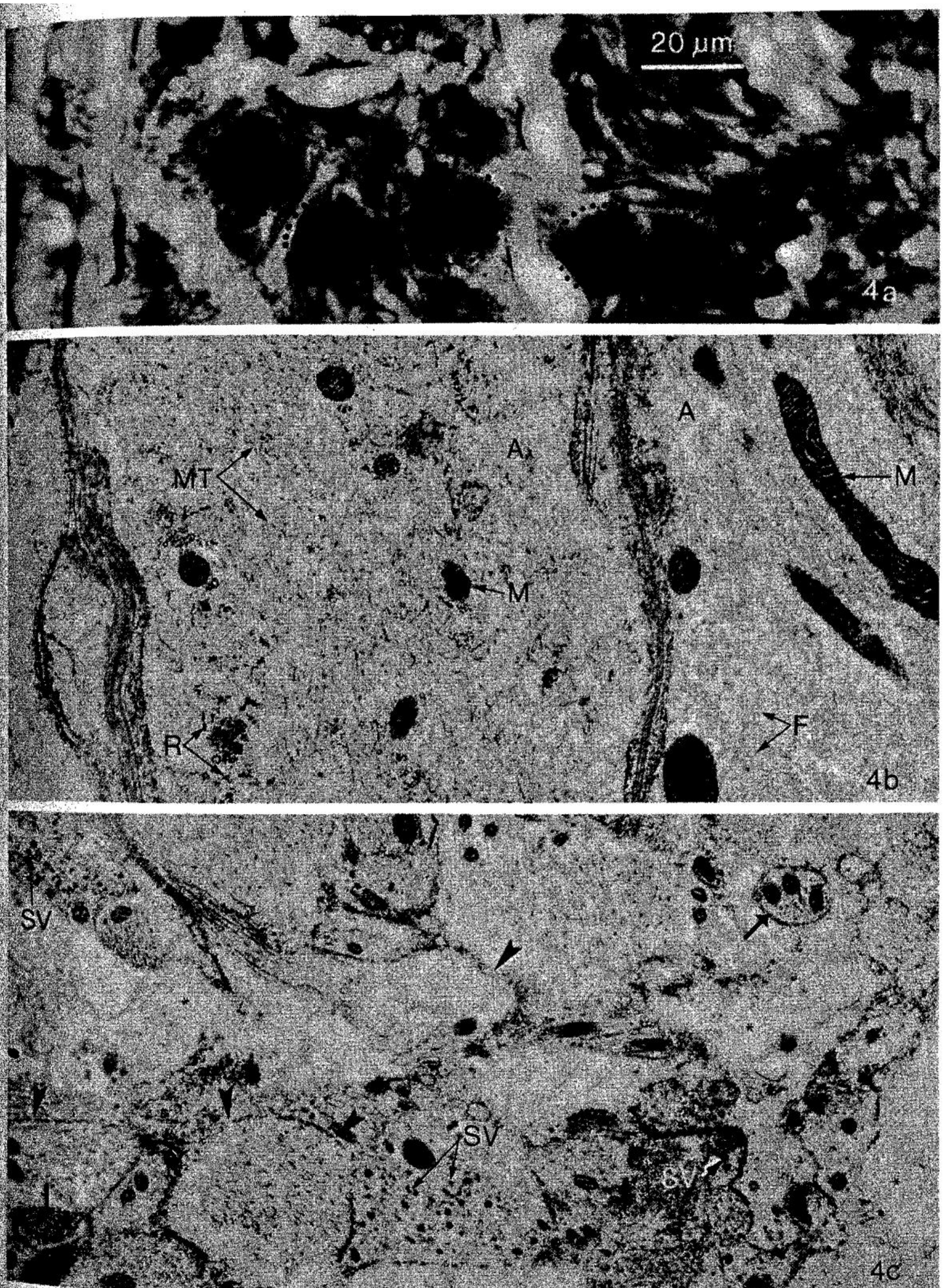

Fig. 4. a: frontal section, 20 μm thick, prepared by the OEG method, through the horseshoe neuropil of abdominal ganglion 5 of *P. lenusculus* demonstrating 5 synaptic glomeruli (encircled). ×866. b and c: electron micrographs from the tissue in a. b: two large axons (A) contain microtubules (MT), ribosomes (R) and mitochondria (M). Fine flocculent material (F) appears along with the microtubules. ×18,730. c: a section through many small neural processes within a glomerulus. Some axonal profiles are distinct and have clearly defined cell membranes (arrows), but many do not (arrowheads). In many areas, the cytoplasm is exceedingly electron lucent (*), indicating the probable loss of intracellular components. Three regions contain numerous round particles between 50 and 80 nm in diameter, the size of synaptic vesicles (SV), although the vesicles lack encircling membranes. ×16,970.