

Immunocytochemical studies on the occurrence of gamma-aminobutyric acid in the nervous system of the nematodes *Panagrellus redivivus*, *Meloidogyne incognita* and *Globodera rostochiensis*

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Summary – Indirect immunofluorescence was used to probe *Panagrellus redivivus* (mixed stages) and second-stage juveniles (J2) of *Meloidogyne incognita* with a polyclonal antiserum reactive to gamma-aminobutyric acid (GABA), the putative nematode inhibitory neuromuscular transmitter. In *P. redivivus*, GABA-like immunoreactivity was demonstrated in the dorsal nerve cord, in the ventral nerve cord including some neuron cell bodies, and in commissure processes running around the nematode between the nerve cords. Immunoreactivity was also observed in processes around the terminal bulb of the pharynx and in the nerve ring. In J2 of *M. incognita*, immunoreactivity was detected in processes and cell bodies in the ventral nerve cord. Immunogold postembedding staining of osmicated, epoxy-embedded J2 of *Globodera rostochiensis* showed GABA-like immunoreactivity in neuronal processes in both the dorsal and ventral nerve cords and in the cytoplasm and nuclei of some cell bodies in the ventral cord. The location of these neurons within the nerve cords indicates that they are probably the inhibitory motor neurons. In the nerve ring, immunoreactivity was observed in neuronal processes and in a cell body, probably the ventral ring motor neuron.

Résumé – Étude immunocytochimique de la présence d'acide gamma-aminobutyrique dans le système nerveux des nématodes *Panagrellus redivivus*, *Meloidogyne incognita* et *Globodera rostochiensis* – Un antisérum polyclonal a été utilisé en immunofluorescence indirecte pour démontrer la présence d'acide gamma-aminobutyrique – un inhibiteur possible de la transmission de l'influx nerveux aux muscles – chez *Panagrellus redivivus* et chez les juvéniles de deuxième stade (J2) de *Meloidogyne incognita*. Chez *P. redivivus*, il a été observé une immunoréactivité, présumée GABA, dans la corde nerveuse dorsale, dans la corde ventrale ainsi que dans certaines cellules nerveuses et les commissures qui ceignent le nématode entre les cordes nerveuses. Une immunoréactivité a été également observée autour du pharynx et dans l'anneau nerveux. Chez les J2 de *M. incognita*, on a détecté cette immunoréactivité dans les nerfs et les cellules de la corde nerveuse ventrale. La coloration à l'immunogold après inclusion de J2 de *Globodera rostochiensis* traités à l'osmium a montré une immunoréactivité, présumée GABA, dans les procès des neurones des cordes nerveuses ventrale et dorsale, et dans le cytoplasme et le noyau de certaines cellules de la corde ventrale. La position de ces neurones dans les cordes nerveuses indique qu'ils sont probablement des neurones moteur inhibiteurs. Dans l'anneau nerveux, l'immunoréactivité a été observée dans les procès neuronaux et dans un corps cellulaire, probablement le neurone moteur de l'anneau ventral.

Key-words : Immunofluorescence, immunogold, neurotransmitters, *Globodera*, *Meloidogyne*, *Panagrellus*, *Nemata*.

Current methods of chemical control against plant parasitic nematodes depend on the use of highly toxic compounds whose continued commercial viability is doubtful (Sayre, 1986) and more selective compounds are urgently required (Wright, 1981). Since novel chemical control agents must prevent nematode invasion or feeding on plant roots, they are ideally fast acting and are likely to be neuroactive. A more detailed understanding of the nematode nervous system, including that of plant parasitic species, is therefore important.

Gamma-aminobutyric acid (GABA) has long been implicated as an inhibitory neuromuscular transmitter in nematodes exerting a hyperpolarizing action on the muscle cells of the animal parasite, *Ascaris suum*, by opening chloride ion channels (del Castillo *et al.*, 1964;

Martin, 1980). The application of GABA to cut individuals of the free-living species, *Caenorhabditis elegans*, caused a flaccid paralysis (Lewis *et al.*, 1980) while avermectins, an anthelmintic group of compounds associated in part with GABA-gated chloride ion channels (Wright, 1987), caused a non-flaccid paralysis in *C. elegans* and *A. suum* (Kass *et al.*, 1980). Recent evidence suggests that GABA is also an excitatory neurotransmitter in *C. elegans* (McIntire *et al.*, 1993).

GABA and its anabolic and catabolic enzymes, glutamic acid decarboxylase (GAD) and GABA transaminase, have been detected in homogenates of *C. elegans* (Schaeffer & Bergstrom, 1988) and GABA and GAD have been detected in the nerve cords of *A. suum* (Chalfie & White, 1988). Johnson and Stretton (1987) de-

monstrated a specific GABA-like immunoreactivity associated with the 13 VI and 6 DI inhibitory motor neurons of *A. suum*. GABA-like immunoreactivity has also been localized in eleven other neurons in females of *A. suum* (Guastella *et al.*, 1991). The latter include four RME-like neurons, two ventral ganglion neurons, two lateral ganglion neurons at the level of the amphid commissures and two at the level of the deirid commissures. Autoradiographic studies on the uptake of GABA in *A. suum* have defined three classes of GABA-associated neurons: neurons that contain endogenous GABA and have a GABA uptake system; neurons that contain endogenous GABA but lack a detectable GABA uptake system; and neurons that lack endogenous GABA but possess a GABA uptake system (Guastella & Stretton, 1991). In *C. elegans*, out of 302 neurons in the adult hermaphrodite, 26 react with antisera raised against GABA. These 26 GABAergic neurons comprise 13 VD, six DD (analogs of VI and DI in *A. suum*), four RME, one RIS, one AVL and one DVB neurons (McIntire *et al.*, 1993). Immunocytochemical studies by Leach *et al.* (1987) have also demonstrated GABA-like immunoreactivity in two cell bodies located in the anterior head region of the first juvenile stage of the free-living nematode *Goodeyus ulmi*. Studies are now required to determine how highly conserved the GABAergic nervous system is between free-living and plant parasitic nematodes.

In the present study, an indirect immunofluorescence technique was used to demonstrate the presence of GABA-like immunoreactivity in mixed stages of the free-living nematode, *Panagrellus redivivus* and in J2 *M. incognita*. In addition, GABA-like immunoreactivity was also demonstrated in the nervous system of J2 of the potato cyst nematode, *Globodera rostochiensis* Ro1, using an indirect immunogold technique for transmission electron microscopy. This is the first study to localise GABA at the electron microscope level in nematodes.

Materials and methods

M. incognita was cultured on tomato, *Lycopersicon esculentum* cv. Pixie. Mature egg masses were removed from the roots and infective J2 were hatched in water at 25 °C for 24 h before collection and immediate use. Cysts of *G. rostochiensis*, cultured on potato, *Solanum tuberosum* cv. Désirée, were extracted and soaked for one week in glass-distilled water before being treated with tomato root diffusate (Wright *et al.*, 1989) to obtain freshly hatched juveniles. *P. redivivus* (mixed stages) were extracted from multixenic cultures on wholewheat flour. The primary antiserum was an affinity isolated rabbit polyclonal antibody specific to GABA developed by using GABA-bovine serum albumin (BSA) as the immunogen and was obtained from Sigma Chemical Company Ltd., Dorset, U. K.

For indirect immunofluorescence studies, mixed stages of *P. redivivus* and J2 of *M. incognita* were washed in 0.1 M sodium phosphate buffer (SPB), pH 7.4, and fixed in 4% (v/v) glutaraldehyde in SPB to a temperature of 50 °C (20–30 sec.) using a Matsui 170 TC microwave oven (Jones & Ap Gwynn, 1991). After rinsing with SPB, the nematodes were cut with a scalpel to improve antisera penetration and incubated in 0.1% (w/v) trypsin (Sigma) in 0.01 M phosphate buffered saline (PBS), pH 7.4, with 10 mM calcium chloride for 15 min at 37 °C. The trypsin solution was then removed and the nematodes were frozen to –20 °C before being placed in methanol for 1 min and acetone for 2 min at –20 °C (Okamoto & Thomson, 1985). The acetone was then replaced with 0.2 mM Na-p-tosyl-L-lysine chloromethyl ketone in PBS for 10 min at 20 °C to inhibit any remaining enzyme. After rinsing in PBS, the nematodes were incubated in 1:20 normal swine serum (Dako Ltd., High Wycombe, Buckinghamshire, U. K.) diluted in PBS containing 0.5% (w/v) Triton X-100 and 0.01% (w/v) sodium azide for 1 h at 20 °C. The normal swine serum was then replaced with primary antiserum diluted 1:1000 in PBS/Triton/sodium azide with 0.1% (w/v) BSA and incubated overnight at 4 °C. After thorough rinsing in PBS/Triton/BSA, fluorescein isothiocyanate-conjugated swine anti-rabbit immunoglobulins (Dako Ltd.) diluted 1:40 in PBS/Triton/BSA/sodium azide was applied for 2 h at 20 °C. Finally, the nematodes were rinsed in PBS, mounted in Citifluor (Agar Aids Ltd., Stansted, Essex, U. K.) and viewed with a Leitz Dialux 20 epifluorescence microscope. Several hundred individuals were examined for each species.

Specificity controls involved omitting the primary antiserum, substituting the primary antiserum for a non immune rabbit-serum and liquid phase pre-absorption of the primary antiserum with 100 µg ml⁻¹ of GABA-glutaraldehyde-BSA conjugate for 2 h at 20 °C.

For indirect immunogold studies, J2 of *G. rostochiensis* were prepared for transmission electron microscopy by the method of Jones and Ap Gwynn (1991). Freshly hatched (< 24 h) J2 were fixed in 4% (v/v) glutaraldehyde in 0.05 M potassium phosphate buffer (PPB), pH 7.2, to a temperature of 50 °C (20–30 s) using a Matsui 170 TC microwave oven and immediately rinsed in PPB at 4 °C. The nematodes were post-fixed in 1% (w/v) osmium tetroxide in PPB using the microwave oven and rinsed in cold buffer. The anterior portions of the nematodes were excised and embedded in 1% (w/v) agar blocks which were then dehydrated in acidified 2,2 dimethoxypropane (Muller & Jacks, 1975) for 5 min. Following rinsing for 5 min in absolute acetone, the nematode tissue was infiltrated with Emix resin (Fisons Scientific Equipment, Loughborough, U. K.) at 40 °C for 2 h and then embedded in fresh resin and polymerised at 60 °C overnight.

Silver-gold sections were cut and picked up on formvar-coated 150 mesh nickel grids. The grids were incubated in a saturated solution of sodium metaperiodate for 12 min (Bendayan & Zollinger, 1983; Causton, 1984) and rinsed in three changes of 0.2 M Tris buffered saline (TBS), pH 7.2, containing 1% (w/v) Triton X-100, and in six changes of TBS. The grids were then incubated for 1 h at 20 °C on drops of 1 : 10 normal goat serum (Sigma) diluted in TBS containing 1% (w/v) BSA and 0.2% (w/v) sodium azide. The grids were drained and transferred to the primary antiserum diluted 1 : 1200 in TBS/BSA/sodium azide for 24 h at 4 °C. After rinsing in six changes of TBS and three changes of TBS/BSA, the grids were incubated for 2 h at 20 °C in 1 : 40 goat anti-rabbit IgG gold conjugate (10 nm) (Sigma) diluted in TBS/BSA/sodium azide. The grids were rinsed in six changes of TBS/BSA and six changes of TBS and then fixed in 2% (v/v) glutaraldehyde in TBS for 15 min. Following a final rinse in distilled water the sections were counterstained with uranyl acetate and lead citrate and viewed with a Philips 300 transmission electron microscope at 80 kV. Sections from four individual nematodes were examined. The particle density of colloidal gold marker was calculated for neuronal cell bodies in the ventral cord and for muscle cells and cuticle. Specificity controls were as described for indirect immunofluorescence.

Results

The indirect immunofluorescence studies on *P. redivivus* demonstrated specific GABA-like immunoreactivity in both the dorsal and ventral nerve cord. The ventral nerve cord included highly immunoreactive cell bodies along its length (Fig. 1 A) which were absent in the dorsal nerve cord. Immunoreactive commissures ran from one nerve cord around the nematode to the other nerve cord (Fig. 1 B) and these were observed running in both right handed and left handed directions (Fig. 1 C). Despite partial enzymatic digestion, the penetration of the antisera through the nematode tissue was limited so immunoreactivity was only observed in short lengths of the specimens or near to the cut ends. Immunoreactive processes were also observed around the terminal bulb of the pharynx and running away from it in a posterior direction (Fig. 1 D, E). A complex arrangement of immunoreactive processes was regularly observed in the nerve ring but the low intensity of the fluorescence precluded successful photography. In J2 of *M. incognita*, immunoreactivity was observed in processes along the ventral nerve cord and in cell bodies which appeared as small loops irregularly spaced down the cord length (Fig. 1 F).

The immunoelectron micrographs of J2 of *G. rostochiensis* showed GABA-like immunoreactivity in some

neurons of the ventral nerve cord, particularly in the cytoplasm of the cell bodies (particle density [PD] = 20 particles/ μm^2) and nuclei (PD = 33) (Fig. 2 A, B). Other neuronal cell bodies (including nuclei) in the ventral cord exhibited much lower levels of staining (PD = 3) similar to those observed in other tissues, for example in muscle cells (PD = 4) and in the cuticle (PD = 1.5). In general, the processes of the immunoreactive neurons were poorly stained with immunogold label but occasionally they could be identified and were nearly always situated on the outside of the process bundle adjacent to the basement membrane. Immunoreactivity was also observed in neuronal processes down the dorsal nerve cord although at certain localized points along its length the reactivity was more pronounced (Fig. 2 C). These areas of strongest immunoreactivity were always situated on the outside of the process bundle. The ultrastructural preservation was good, allowing visualization of individual neurons but despite this it was not possible to identify synaptic regions. Fig. 2 D is an immunoelectron micrograph of the nerve ring of a J2 of *G. rostochiensis* and demonstrates the presence of specific GABA-like immunoreactivity in a neuronal cell body and its nucleus and in a number of neuronal processes.

All specificity controls for the immunofluorescence and the immunogold (PD < 2) were negative.

Discussion

The general pattern of GABA-like immunoreactivity demonstrated in the ventral and dorsal nerve cords and interconnecting commissures of *P. redivivus* was similar to that shown in *A. suum* (Johnson & Stretton, 1987) and in *C. elegans* (Chalfie & White, 1988; McIntire *et al.*, 1993). However, limited penetration of the antisera through the nematode tissue resulted in the staining of only small portions of the nervous system in each cut specimen. This precluded the precise identification of the immunoreactive neurons and it was therefore difficult to compare the putatively GABAergic motor neurons of *P. redivivus* with the more extensively studied motor neurons of *A. suum* and *C. elegans*. Further investigation into the permeabilization of the nematode by digestion with other enzymes may help to provide better immunolocalization and a more complete picture of the putative GABAergic system in *P. redivivus*. The immunoreactivity observed in the ventral nerve cord of J2 of *M. incognita* demonstrated the presence of putatively GABAergic neurons in this important plant parasite. The looped appearance of immunoreactivity in the cell bodies along the ventral cord was probably due to the large nuclei in these cells.

The post-embedding immunogold technique employed to localize GABA-like immunoreactivity in J2 of *G. rostochiensis* prepared for transmission electron microscopy was successful in that specific reactivity could be identified at a cellular level and to some extent at a

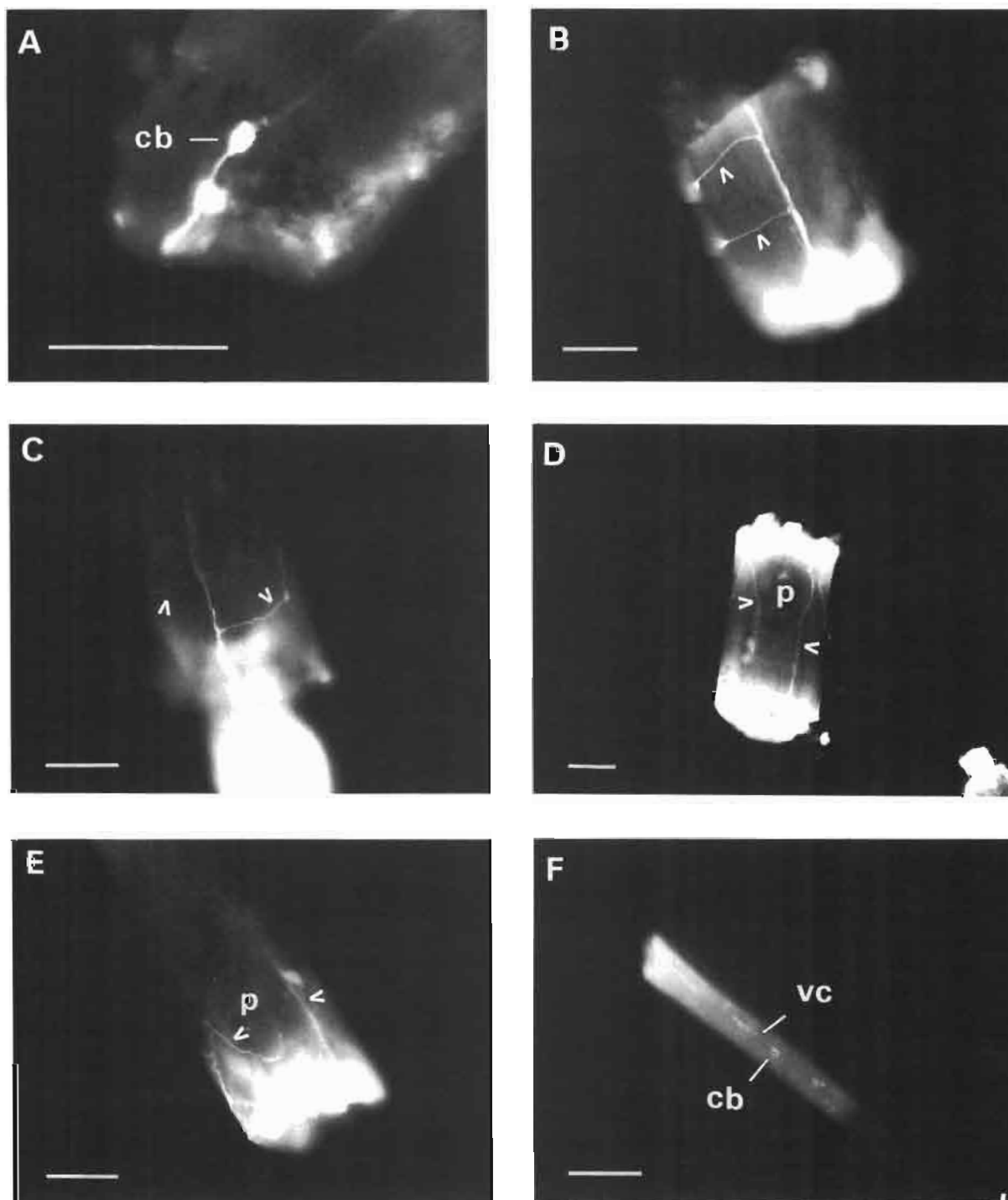


Fig. 1. Indirect immunofluorescence staining of neurons in *Panagrellus redivivus* (A-E) and *Meloidogyne incognita* (F) showing localisation of GABA in : A : Cell bodies (CB) in the ventral cord; B : Nerve cord with commissures (arrows); C : Nerve cord with left and right handed commissures (arrows); D : Neuronal processes (arrows) at the terminal bulb of the pharynx (P) and posterior to it; E : Neuronal process (arrows) running around the terminal bulb of the pharynx (P); F : Cell bodies (CB) in the ventral cord (VC). (All scale bars = 30 μ m.)

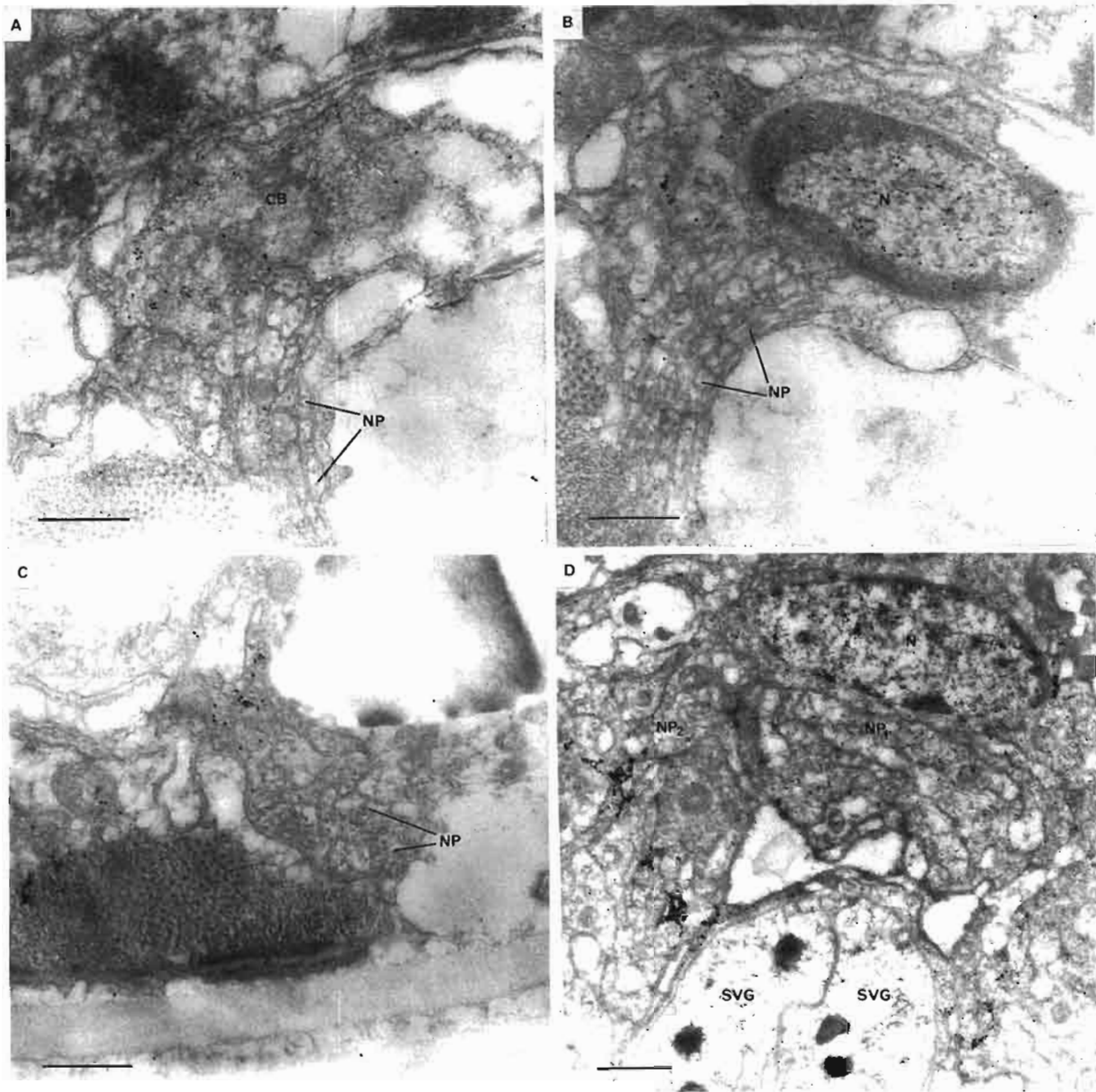


Fig. 2. Immunoelectron micrographs of *Globodera rostochiensis* showing localisation of GABA in : A, B : Ventral nerve cord (approximately 150 μm from the anterior tip); C : Anterior dorsal nerve cord (approximately 150 μm from the anterior tip); D : Nerve ring. CB : neuronal cell body; N : nucleus; NP : neuronal process; NP₁ neuronal process showing negative reactivity; SVG sub-ventral gland extension. (All scale bars = 500 nm.)

subcellular level (it should be noted that this nematode is only a little larger in transection than the nucleus of a motor neuron of *A. suum*). Contrary to the appearance during immunofluorescence studies of the looped cell bodies of the ventral cord in J2 of *M. incognita*, the immunogold studies showed that the cell bodies of reactive neurons in J2 of *G. rostochiensis* exhibited immunoreac-

tivity in the cytoplasm (Fig. 2 A) and in the nuclei (Fig. 2 B). The negative staining of other neuronal cell bodies and nuclei demonstrated the absence of detectable GABA in different classes of neuron. GABA-like immunoreactivity was also found homogeneously distributed throughout reactive neurons, including in the nuclei, of *A. suum* (Guastella *et al.*, 1991). If GABA is

truly present in the nuclei of these cells and has not simply leached there during processing, then its significance is unapparent. Autoradiographic studies to investigate the distribution of ^3H -GABA uptake sites have shown heavy labelling of the nuclei of RMEV-like and RMED-like neurons in *A. suum* (Guastella & Stretton, 1991).

The distribution of GABA-like immunoreactivity throughout neurons does indicate that GABA is not localised solely at synaptic regions. Indeed, even in some mammalian neurons, GABA has been localised not just in vesicles at neuronal terminals but non-vesicle bound throughout the cytoplasm of the cell (Merighi *et al.*, 1989). The position of the immunoreactive processes on the outside edge of the bundles of neurons which form the nerve cords is in accordance with the position of the GABA immunoreactive inhibitory motor neurons of *A. suum* (Guastella *et al.*, 1991) and also with the inhibitory motor neurons in *C. elegans* (White *et al.*, 1976). As GABA has been strongly implicated as the nematode inhibitory neuromuscular transmitter (del Castillo *et al.*, 1964; Martin, 1980; Kass *et al.*, 1980), it is likely that the immunoreactive neurons observed in the ventral and dorsal nerve cords in the present study are the inhibitory motor neurons analogous to the putatively GABAergic VD and DD motor neurons of *C. elegans* and the VI and DI motor neurons of *A. suum*. Although it was not possible to recognize pre-synaptic specialisations, the presence of more intense immunoreactivity in neurons at specific localized points along the outside edge of the dorsal nerve cord may indicate the presence of GABAergic synaptic regions. This study had necessarily favoured good ultrastructural preservation at the expense of antigenicity in order to visualize the small neurons of this diminutive nematode. Any further improvements in preservation and/or membrane contrast, to visualize for example the characteristic clear vesicles of GABAergic neurons terminals, would not be possible without the loss of antigen reactivity. Conversely, any reduction in the harshness of tissue fixation and embedding procedures, in order to improve antigenicity, has been shown in preliminary experiments to compromise preservation such that individual neurons could barely be distinguished from one another.

The GABA-like immunoreactivity demonstrated in a neuron cell body and processes in the nerve ring of J2 of *G. rostochiensis* (Fig. 2 D) and the low intensity immunofluorescence observed in the nerve ring of *P. redivivus* is in agreement with the GABA-like immunoreactivity found in the RME, RIS, AVL and DVB neurons of *C. elegans* (Chalfie & White, 1988; McIntire, 1993) and with the immunoreactivity found in RME-like neurons and ventral and lateral ganglia neurons of *A. suum* (Guastella *et al.*, 1991). The immunoreactive cell body in the nerve ring of *G. rostochiensis* was situated on the ventral side and was probably the ring motor neuron analogous to the RMEV neuron of *C. elegans*. Future

work to investigate in more detail the localisation of GABA in the cephalic region of *G. rostochiensis* may demonstrate the presence of GABA-like immunoreactivity in the remaining ring motor neurons and confirm the apparent close similarity with the GABAergic nervous system of *C. elegans*.

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