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Research Article

Expression of nerve growth factor-like polypeptides and immunoreactivity related to the two types of neurotrophin receptors in earthworm tissues

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Abstract. Nerve growth factor (NGF) belongs by sequence homology to the neurotrophins, a family of proteins binding the same p75 receptor and closely related members of the Trk family of receptor tyrosine kinases. Fundamental in the vertebrate nervous system, neurotrophin signals have also been suggested as essential for relatively complex nervous systems occurring in invertebrate species that live longer than *Caenorhabditis elegans* and *Drosophila melanogaster*. Mammalian neurotrophins have been found to influence invertebrate neuronal growth. However, there are only a few data on the presence of molecules related to neurotrophin signalling components in invertebrates. Our studies provide evidence that analogues of neurotrophins and neurotrophin receptors are expressed in *Eisenia foetida* earthworms. In particular, NGF-like and Trk-like immunoreactive proteins are both expressed in the nervous system, whereas p75-like positivity identifies tubular structures associated with dorsal pores that are involved in the earthworm response to mechanical irritation or stress.

Key words. Nerve growth factor; neurotrophin; p75 neurotrophin receptor; Trk receptor; evolution; earthworm; annelid; invertebrate.

Nerve growth factor (NGF), the first discovered neurotrophic factor, is the prototype member of a family of structurally and functionally related proteins, termed neurotrophins. Since its identification, our understanding of the structure, function and signalling mechanisms of this expanding family of vertebrate proteins continues to increase [reviewed in refs 1–4]. Nevertheless, NGF remains pre-eminent in terms of knowledge of both its pleiotropic functions and structural biology, and continues to provide a reference point in the field of neurotrophin studies [5–7].

In the male mouse submandibular gland, the traditional source of NGF, the mature active form (indicated as 2.5S, β- or simply NGF) consists of a tightly associated homodimer of two identical non-covalently linked polypeptide chains. Each monomer, of about 13 kDa, contains three intrachain disulphide bonds that are essential for the three-dimensional structure and the biological activity of the molecule [8]. Crystal structure analyses of murine NGF revealed a particular clustering of these three disulphide bonds, termed a 'cystine knot', successively detected in the topology of different proteins [6, 9, 10].

To date, four members of the neurotrophin family have been identified in mammals: NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) (also called NT-4/5 or NT-5) [11, 12]. These neurotrophins share about 50% amino acid identity, with the major regions of structural similarity mainly

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arranged around six strictly conserved cysteine residues [13], which have been shown to form the three intrachain disulphide bonds and the characteristic 'cystine knot' structure in NGF. They appear to have similar tertiary structures [14, 15] and common epitopes, since polyclonal antibodies to individual neurotrophins have been found to cross-react with other members of the neurotrophin family [12, 16, 17].

Homologous sequences to NGF, BDNF, NT-3 and NT-4 (named, as species variants of the same factor, orthologues) have been found in a wide range of vertebrates, from fishes to mammals, except for NT-4 which has so far not been found in birds and fishes [reviewed in ref. 18]. Two additional members, denoted neurotrophin-6 (NT-6) [19] and neurotrophin-7 (NT-7) [20, 21], have been found in bony fish species and are structurally and functionally more related to NGF than to any other neurotrophin.

A primitive neurotrophin homologue has been recently detected in lamprey. This *Lampetra fluvialis* neurotrophin (*Lf*-NT) displays the typical neurotrophin structure with the six conserved cysteine residues and no close similarity to all other known neurotrophins [18].

Individual neurotrophins are able to interact independently with two distinct classes of transmembrane receptors, the shared p75 neurotrophin receptor and specific members of the Trk family of receptor tyrosine kinases. Although multimeric receptor complexes and functional interactions between both receptors have been detected, each can also display independent signalling properties with distinct signalling pathways [reviewed in refs 2, 4, 7, 22, 23]. The signalling pathways activated by the two types of neurotrophin receptor in vertebrate cells have been characterised and a number of intracellular p75-interacting proteins have been detected [24–26]. However, the function of the p75 neurotrophin receptor is less well defined than that of the Trk receptors that mediate specificity and several of the neurotrophic functions of neurotrophins.

The two types of cell surface receptors are structurally unrelated in either their ligand-binding or cytoplasmic regions. The p75 receptor, which binds all neurotrophins with similar affinity, is the founding member of the tumour necrosis factor (TNF) receptor superfamily [27]. However, it is mostly regarded as a stress receptor, similar in behaviour to the TNF receptor family members which serve to modulate the effects of cytokines following inflammation, infection or injury [4, 23, 28]. On the other hand, p75 is known to modulate Trk receptors as a positive regulator of cell survival and differentiation. Interestingly, for neurotrophin-responsive neuronal populations, particularly in the vertebrate peripheral nervous system, p75 and Trk members are frequently co-expressed, while p75 is expressed widely during development in non-neuronal cells and also following nerve injury or trauma [29].

The Trk receptors (TrkA, TrkB and TrkC) are closely related proteins that display selective neurotrophin-dependent activation of the intracellular tyrosine kinase domain. TrkA binds NGF, TrkB binds BDNF and NT-4, and TrkC binds NT-3. In addition to these preferred interactions, TrkA can also be activated to a lesser degree by NT-3, NT-6 and NT-7 [20, 21], and TrkB by NT-3 [22].

Recent phylogenetic analyses of the vertebrate neurotrophin and Trk receptor gene families suggest that both evolved during two major gene duplication events, one early in chordate history and another prior to the appearance of cartilaginous fish [18].

The existence of functional NGF-related neurotrophins in invertebrate lineages is supported by studies in *Drosophila* [30] and *Lymnaea* [31]. Despite attempts performed in some laboratories [32, 33], no homologous sequences to vertebrate neurotrophins have been detected in such animals and no neurotrophin homologue has so far been cloned from an invertebrate. A protein purification approach in *Lymnaea* only resulted in the discovery of a novel cysteine-rich neurotrophic factor (CRNF) not similar in sequence to neurotrophins, although able to interact with the p75 neurotrophin receptor [33]. However, a Trk receptor homologue displaying binding capacity for human NT-3 has been recently found in *Lymnaea* [34].

Because the NGF family of neurotrophins are involved in regulating neuronal numbers and plasticity in the vertebrate nervous system, neurotrophin signals may be essential for the evolution of relatively complex nervous systems in invertebrate species that live longer than *Caenorhabditis elegans* or *Drosophila melanogaster* [35].

Mammalian neurotrophins have been found to have effects on neuronal cells from several invertebrate species [reviewed in ref. 36], indicating the presence in invertebrates of receptors, and possibly putative ligands, with functional groups shared by the components of the neurotrophin signalling system. However, further studies and suitable invertebrate models are needed to increase the information on structural homologues and/or functional analogues of neurotrophins and their receptors in invertebrates.

Our study provides evidence that polypeptides immunologically related to at least one member of the neurotrophin family are expressed in the earthworm *Eisenia foetida*. In addition, we report here findings on the presence and localisation of immunoreactivity related to neurotrophin receptor sequences and we discuss the possible role of the p75-like immunoreactive structures in earthworms.

Materials and methods

Animals and tissue specimens

Sexually mature *E. foetida* earthworms (more than 100 worms in clitellum) kept under controlled laboratory conditions [37] were used.

Before experimentation, the animals were maintained for 3 days in Petri dishes at 25 °C on filter paper humidified with tap water.

The tissue specimens were removed from worms anaesthetised by exposure to cold (5 min at –20 °C). The pieces sectioned for immunohistochemistry were mostly from the cephalic portion of the body (approximately the first 10–15 segments) which includes the buccal cavity, pharynx and oesophagus as well as brain, commissures and some segmental ganglia of the ventral nerve cord [38, 39]. Serial fragments were also obtained from the whole body or from central and caudal portions of several worms.

The central nervous system, consisting of the cerebral ganglion (brain) connected by a pair of commissures (circumpharyngeal connectives) to the segmentally ganglionated ventral nerve cord, was removed from animals anaesthetised by exposure to cold and was processed for electron microscopic studies.

Mouse submandibular glands were used for control purposes.

Antibodies and antisera

Two antisera raised in rabbits against 2.5S NGF (mNGF) from mouse submandibular glands [40] were kindly provided by Dr. Delio Mercanti (Institute of Neurobiology, CNR, Rome, Italy). A commercially available anti-mNGF serum from rabbit was purchased from Sigma (St. Louis, MO.). The first anti-mNGF serum, obtained from Dr. Mercanti together with affinity-purified antibodies and mNGF for the antigen-preabsorbed serum, was used only in preliminary immunohistochemical experiments [41]. The second and the commercial antisera were tested on Western blots for reactivity to human recombinant (hr)β-NGF, hrBDNF, hrNT-3, hrNT-4 (PeproTech, London, UK) and to mNGF (Sigma), as described below. Moreover, the second anti-mNGF obtained from Dr. Mercanti was used to perform preabsorption with hrβ-NGF immobilised on blotting filter, and the commercial anti-mNGF serum was employed for immunoprecipitation of NGF-like immunoreactive proteins from earthworm extracts.

The anti-p75 antiserum (Chemicon International, Temecula, Calif.) was generated in rabbits against an extracellular fragment corresponding to amino acids 43–161 of the deduced mouse p75 sequence.

The affinity-purified rabbit antibodies reactive with TrkA, TrkB and TrkC were supplied by Santa Cruz Biotechnology (Santa Cruz, Calif.). These Trk (C-14) antibodies map within the highly conserved carboxyl-terminal region corresponding to amino acids 777–790 of the deduced human TrkA sequence.

The secondary anti-rabbit immunoglobulins were goat fluorescein isothiocyanate (FITC)-conjugated affinity-purified antibodies (Cappel Research Products, ICN Biomedicals, Opera MI, Italy) or 10-nm gold-conjugated goat $F(ab)_2$ (British BioCell International, Cardiff, UK) for immunocytochemical studies, and affinity-purified goat alkaline phosphatase (AP)-conjugated antibodies (Bio-Rad, Richmond, Calif.) or donkey horseradish peroxidase (HRP)-conjugated antibodies (Amersham, Little Chalfont, UK) for Western blot analysis.

Immunoblots

Electrophoresis was performed in a 15% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) according to Laemmli [42], using as sample buffer 75 mM Tris pH 7.8 containing 9 M urea, 2% SDS, 30 mM dithiothreitol and 2% β-mercaptoethanol.

To detect the reactivity of anti-mNGF sera to hr-neurotrophins, individual neurotrophin samples (from 250 ng to 1 µg/well) and the molecular size standards (Bio-Rad), resolved by electrophoresis, were transferred to polyvinylidene difluoride (PVDF) membrane. After blocking for 1 h at room temperature with 5% non-fat dry milk in 10 mM Tris, 160 mM NaCl (TBS), the membrane was incubated in anti-mNGF serum (1:1000–1:2000) and in AP-conjugated secondary antibodies (1:3000) for 1 h each at room temperature and then visualised by the chromogenic detection system using alkaline phosphatase substrata (NBT/BCIP).

The immunoadsorption of anti-mNGF serum with hrβ-NGF (20 µg/ml of diluted antiserum) on PVDF filters was carried out by performing electrophoresis of a single sample, hrβ-NGF or bovine serum albumin (BSA) used as a control, along the entire length of the gel. After transfer to PVDF membrane, the filter strip containing the resolved protein was incubated for 1 h at room temperature in blocking buffer consisting of 0.01 M phosphate-buffered saline (PBS), 3% BSA, 0.02% sodium azide, and then for 5 h at 25–27 °C in anti-mNGF serum diluted (1:500) in blocking solution.

Immunoprecipitation of NGF-like immunoreactive proteins from earthworm extracts was performed as described for NT-3 detection in tissue extracts with few modifications [43]. Whole-body tissues from animals anaesthetised by cold were rapidly minced on ice and mildly homogenised in 3 vol (w/v) of 10 mM Tris buffer pH 7.4 containing protease inhibitors (0.1 µg/ml pepstatin, 5 µg/ml aprotinin, 0.5 µg/ml antipain, 175 µg/ml benzamidine and 17 µg/ml phenylmethylsulphonylfluoride). The homogenate was centrifuged at 30,000 g for 30 min and at 100,000 g for 1 h. Aliquots of soluble extract (4 ml/1.3 g of tissue, 36 mg of total proteins) were preabsorbed for 2 h at 4 °C with protein A-agarose

(3.5 mg in 35 µl Tris buffer) and then with non-immune rabbit IgG bound to protein A-gel to remove, after centrifugation, non-specific binding. The supernatant was successively incubated for 2 h at 4 °C with protein A-agarose gel binding IgG from anti-mNGF serum (3.5 mg protein A-agarose/1 µl serum) or, as a control, from non-immune rabbit serum. After centrifugation, the pellet was washed in Tris buffer three times and then boiled for 4 min in 20 µl of sample buffer containing SDS, urea, dithiothreitol and β-mercaptoethanol. The proteins in the supernatant and the mNGF (250 ng) were separated in 15% SDS-PAGE and then transferred to PVDF membrane. The membrane, blocked for 1 h at room temperature in TBS containing 10% dry milk and 0.3% Tween 20, was incubated in the same anti-mNGF serum (1:4000 in blocking solution), or in non-immune rabbit serum as a control, and then in anti-rabbit immunoglobulin HRP-conjugated antibodies (1:5000 in blocking solution) for 1 h each. The bands were visualised by the enzyme-chemiluminescence (ECL) detection system (Amersham).

Immunocytochemistry

For immunohistochemistry in light microscopy, the specimens were fixed in Bouin's solution and routinely embedded in paraffin. Serial sagittal or frontal sections 5 µm thick were cut from each block. Deparaffinised tissue sections were processed by indirect immunofluorescence techniques. Primary antisera from rabbit were applied to the sections for 24 h at 4 °C, after a 10-min treatment with 1:10 normal goat serum. The sections were then rinsed in PBS and incubated for 1 h at room temperature with goat anti-rabbit immunoglobulin FITC-conjugated antibodies. The working dilutions of primary antisera in PBS-1% BSA were 1:500 for anti-mNGF sera, 1:200 for anti-p75 serum and 20 µg/ml for anti-Trk antibodies. The secondary fluoresceinated antibody was diluted 1:30. Tissue sections, mounted in buffered glycerol, were examined on a Leitz Orthoplan microscope equipped for epifluorescence.

Paraffin-embedded 10-µm sections processed for immunohistochemistry using anti-p75 serum were counterstained with propidium iodide (working dilution 1 µg/ml) and observed on a Leica TCS 4D confocal laser scanning microscope supplemented with an Argon/Krypton laser and equipped with 40 × 1.00–0.5 and 100 × 1.3–0.6 oil immersion lenses. The excitation and the emission wavelengths employed were 488 and 510 nm for fluorescein and 568 and 590 nm for propidium iodide, respectively.

For ultrastructural localisation, the dissected nervous systems were fixed in 4% paraformaldehyde and 0.05% glutaraldehyde in Millong's buffer and embedded in LR White acrylic resin (London Resin, UK). Ultra-thin sections (0.5 µm) were collected on Formvar-coated grids and processed by the indirect immunogold technique. After preincubation for 10 min at room temperature with PBS containing 10% normal goat serum (NGS) and 1% BSA, the sections were incubated overnight at 4 °C with anti-mNGF sera (1:2000) or purified antibodies (10 µg/ml) diluted in PBS-1% BSA-1% NGS. The samples were rinsed with the diluent solution and post-incubated for 1 h at room temperature with the gold-conjugated secondary antibodies from goat diluted 1:20. Finally, the sections were counterstained with uranyl acetate and lead citrate, and observed on a Philips CM12 transmission electron microscope (TEM) at 100 kV.

To test the specificity of the immunoreactions, each experiment in light and electron microscopy included negative controls obtained by omission of the primary antiserum and by substitution of primary antiserum with non-immune (or pre-immune) rabbit serum.

Software application used

Digital confocal fluorescence images were obtained directly from the LEICA TCS 4D confocal laser-scanning microscope, while digital TEM and optical microscopy images were attained from micrographs using a Hewlett Packard ScanJet 4C scanner. Photographic tables from digital images were mounted using the Corel PHOTO-PAINT 8 and CorelDRAW 8 Microsoft software and printed on photographic paper using a Hewlett Packard DeskJet 895 Cxi professional printer.

Results

NGF-like immunoreactivity

Using different anti-mNGF sera, bright fluorescent cells and fibres were detected by light immunocytochemistry in the central nervous system of *E. foetida* earthworms (fig. 1). In particular, numerous immunoreactive perikarya and some positive nerve fibres were observed in the cerebral ganglion (fig. 1A). These cells were located in the dorsal region of the brain, showing their positive axons projected toward the neuropil (fig. 1A). Immunoreactive plexiform nerve fibres and clusters of positive perikarya were also observed in the cephalic portion of the circumpharyngeal connectives (fig. 1B, C).

In the subpharyngeal ganglion, only one pair of immunoreactive nerve cells, located in the ventral portion, were detectable (fig. 1D). In the subsequent ganglia of the ventral nerve cord, a few positive perikarya were located at the roots of segmental nerves (fig. 1E) and scattered along ganglia (fig. 1F) where filiform immunoreactive fibres were also detected (fig. 1G).

No immunoreactivity was observed outside the nervous system. Essentially the same pattern of distribution of NGF-like immunoreactivity in earthworm tissues was obtained with all antisera used during our studies, including preliminary experiments [41]. In particular, a similar pattern of staining was obtained from two sera that displayed

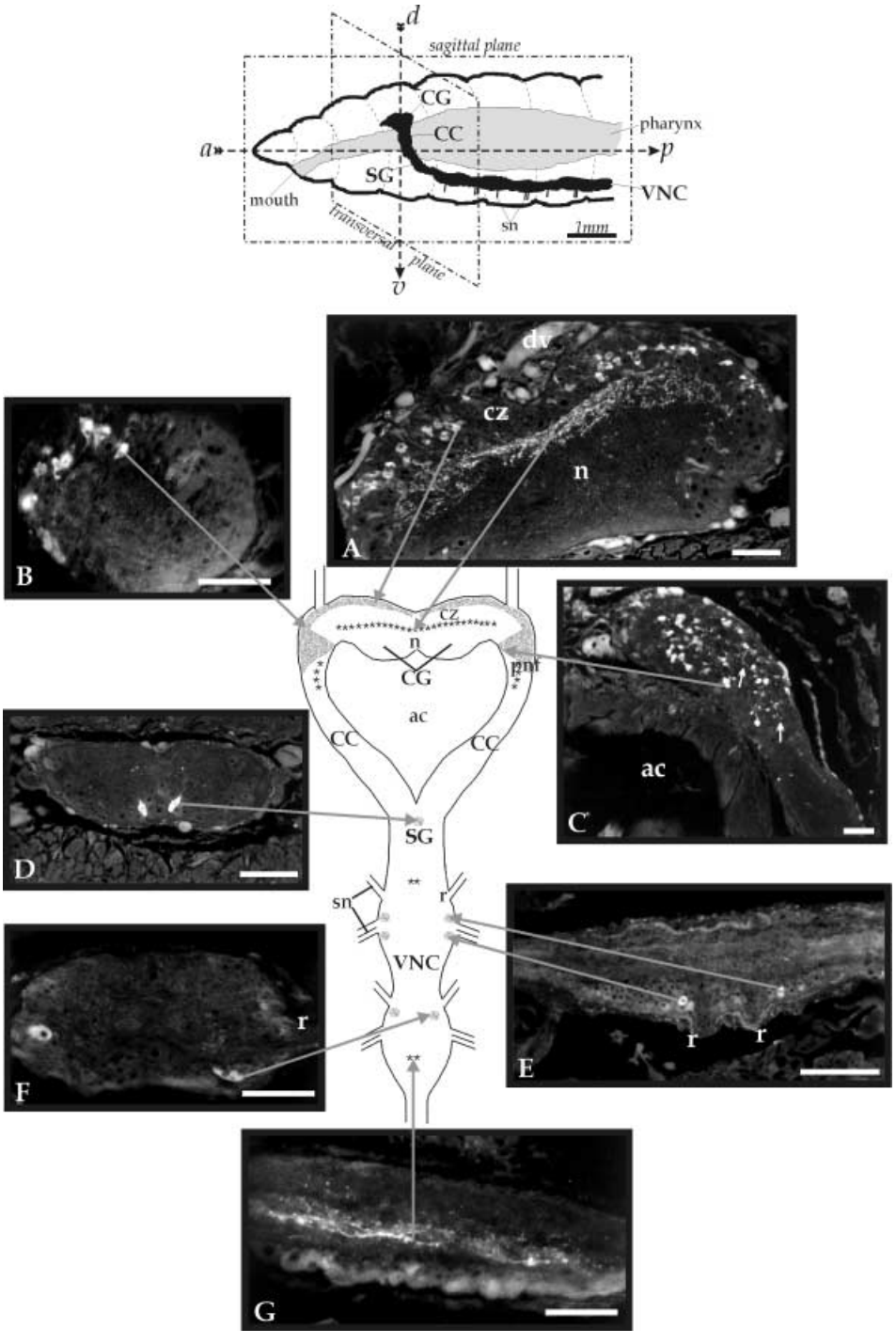

Figure 1. Nerve growth factor (NGF)-like immunoreactivity by light microscopy. Schematic drawings of the central nervous system (CNS) showing section planes and orientation in *Eisenia foetida* anterior part from the side (upper), and a reconstruction of the CNS with the localisation of NGF-like immunoreactive cells (dotted) and nerve fibres (asterisks) (lower). Arrows indicate the zones where the corresponding positive cells and nerve fibres are located in the pictures (*A–G*). *a*, anterior side; ac, alimentary canal; *d*, dorsal side; dv, dorsal vessel; *p*, posterior side; *v*, ventral side. Scale bars, 100 µm. (*A*) Transverse section through the cerebral ganglion (CG) showing numerous positive cells in the cortical zone (cz) and immunoreactive nerve fibres in the dorsal layer of the neuropil (n). Note that some cells display positive axons. (*B, C*) Circumpharyngeal connective (CC), transversal sections. *B* shows clusters of immunoreactive neurons and axons. *C* shows fluorescent cell bodies mixed with thin, less bright, plexiform nerve fibres (pnf) (double-headed arrows). (*D*) Subpharyngeal ganglion (SG), transversal section. (*E–G*) Ventral nerve cord (VNC). *E*, sagittal section showing positive cells located at the roots (r) of segmental nerves (sn); *F*, transversal section; *G*, sagittal section showing immunoreactive nerve fibres.

different cross-reactive properties to hr-neurotrophins on Western blots. In fact, by immunoblot analyses, the commercial and the second anti-mNGF serum recognised hrBDNF or hrNT-4, respectively, only at a higher concentration (1 µg), but reacted strongly with hrNGF (250 ng) and to lesser extents with equivalent amounts of hrNT-3 and of either hrNT-4 or hrBDNF, respectively (fig. 2). Such cross-reactivities are not unexpected, since neurotrophins share common epitopes [12, 16, 17] and orthologous NGFs, particularly mouse and human NGFs [44], display highly conserved sequences across species. Moreover, the specificity of the immunohistochemical reactions in earthworms, evaluated by using either an antigen-preabsorbed antiserum or preimmune and normal sera, was also confirmed by the hrNGF-preabsorbed serum displaying completely abolished or drastically reduced immunoreactivity in the tissues from different worms.

Given the ability of protein A to bind to the Fc region of immunoglobulins, especially IgGs, from several species [45], protein A covalently coupled to agarose was used at first as an affinity support to isolate from both anti-mNGF and, as control, non-immune sera all rabbit IgGs and successively for immunoprecipitation of target proteins from earthworm extracts. The samples obtained after immunoprecipitation, also containing the immunoglobulin fractions dissolved by the denaturing and reducing conditions from protein A-agarose, in Western blotting showed intense spots at higher molecular weights than the 13-kDa mNGF band, used as a control; in addition, in the sample immunoprecipitated with the anti-mNGF serum, an NGF-like protein fraction showing electrophoretic properties congruent with those of mNGF was identified (fig. 3). This immunoreactive electrophoretic fraction was not detected from samples immunoprecipitated with non-immune rabbit serum (fig. 3). The specificity of the NGF-like immunoreactive band was ascertained by incubating the membranes with non-immune rabbit serum and with the secondary antibody alone.

In ultrastructural studies, performed on isolated nervous systems using anti-NGF sera, the immunogold reactions were almost exclusively distributed within cells and fibres, being essentially localised on secretory granules (fig. 4). The background labelling density of the 10-nm gold particles was ≤ 1 gold particle/µm^2 [see also ref. 41]. In the cerebral ganglion, positive cells showing the typical features of neurosecretory cells were detected in the dorsal layer of the cortical zone. They displayed immunogold labelling on numerous electron-dense granules, ranging from 100 to 200 nm in diameter, within the perikarya (fig. 4A). Similar findings were also obtained with purified antibodies [41]. No labelling was detected in the cell nucleus, in the endoplasmic reticulum or in the Golgi apparatus, whereas gold-labelled electron-dense material associated with the omega figure indicating exocytosis [46] was also observed close to the extracellular space adjacent to perikaryal profiles (fig. 4B). In the most superficial layer of the neuropil, the ultrastructural

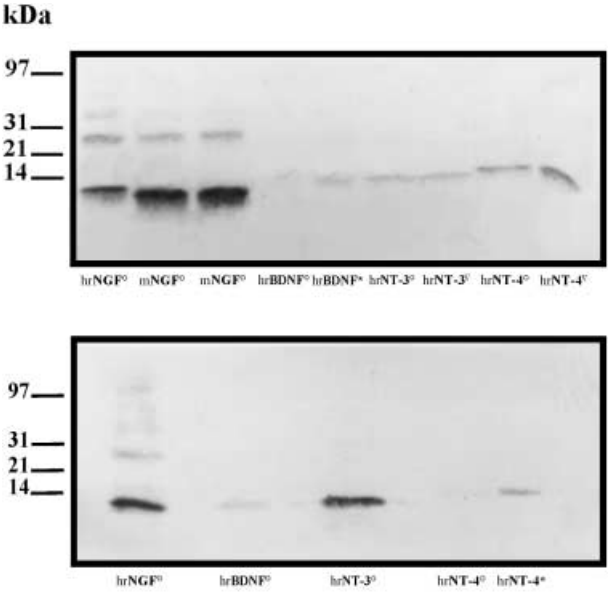

Figure 2. Cross-reactivity of rabbit antisera to mouse 2.5S NGF (mNGF) with human recombinant (hr)-neurotrophins by Western blotting analyses. Upper, commercial antiserum; lower, second anti-mNGF serum. °250 ng; ▽500 ng; *1 µg.

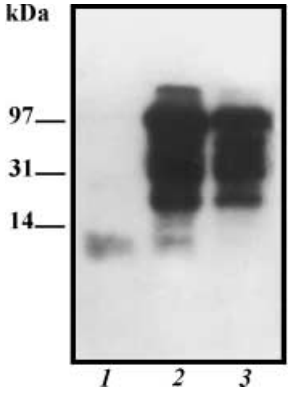

Figure 3. Western blotting analyses after immunoprecipitation of NGF-like proteins from extracts by protein A-agarose. Denatured and reduced samples for electrophoresis were mNGF used as control (lane 1) and soluble proteins dissociated from protein A-agarose after immunoprecipitation using protein A-agarose-binding immunoglobulin molecules, especially IgG populations either from anti-mNGF serum (lane 2) or, as control, from non-immune serum (lane 3). Shown are immunoblots in the chemiluminescent system at 2 min, using anti-mNGF serum as primary and anti-rabbit immunoglobulins as secondary antibodies. Note a supplementary band, showing electrophoretic mobility similar to that of the ca 13-kDa mNGF, in the sample obtained after immunoprecipitation with anti-mNGF serum (lane 2) not detected in the sample from immunoprecipitation with non-immune serum (lane 3). Neither the NGF-like immunoreactive band nor mNGF was observed when either non-immune serum as primary antibodies or the secondary antibodies alone were used to detect the immunoreactions.

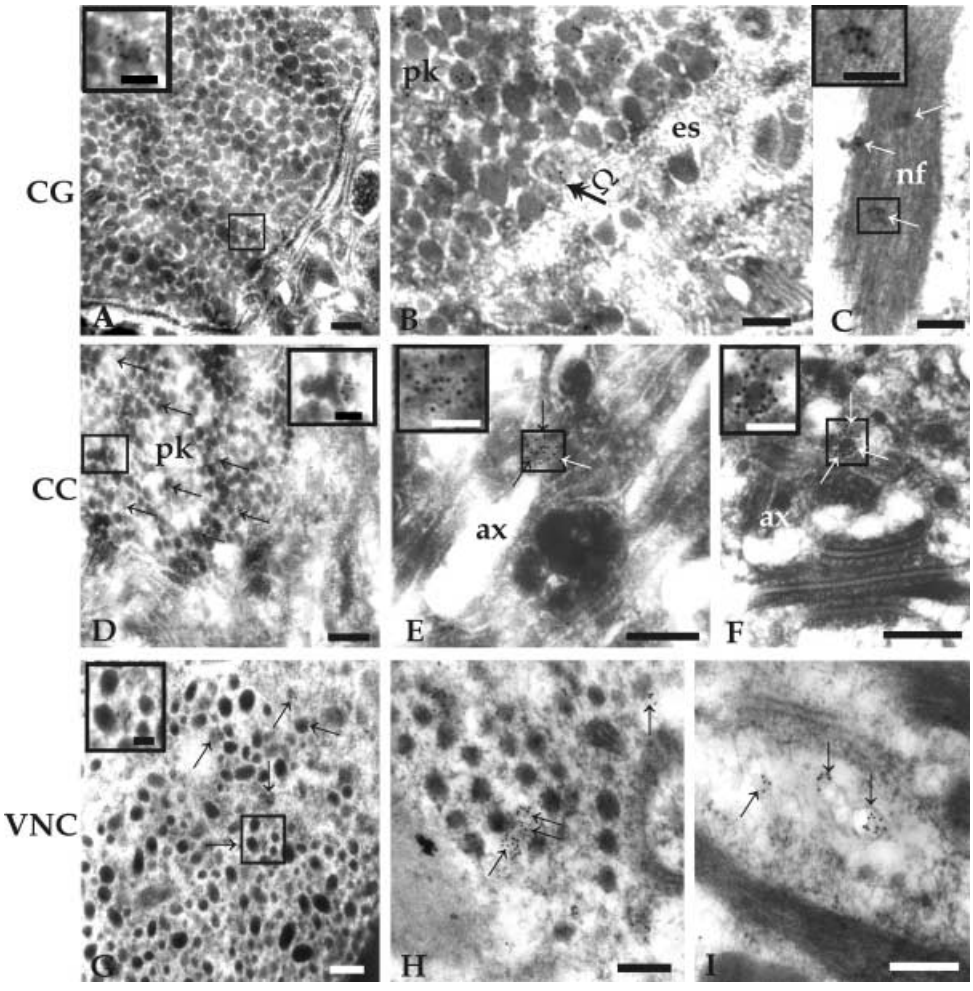

Figure 4. Electron micrographs of NGF-like positive cells and nerve fibres in the CNS. The background labelling density of 10 nm gold particles used to detect immunocytochemical reactions was ≤ 1 gold particle/µm^2. (*A–C*) Cerebral ganglion (CG) (scale bars, 200 nm). *A*, immunogold-labelled secretory granules within a positive cell; *B*, gold-labelled electron-dense material (double-headed arrow) associated with omega figure (Ω) in the extracellular space (es) adjacent to the perikaryal (pk) profile of a positive neurosecretory cell; *C*, gold-labelled electron-dense granules (arrows) associated with neurofilaments (nf) in a positive nerve fibre of the neuropil. (*D–F*) Circumpharyngeal connectives (CC). Immunolabelled granules (arrows) located within the perikaryon (pk) (*D*) and the axons (ax) of positive neurons (*E, F*) (scale bars, 300 nm). (*G–I*) Ventral nerve cord (VNC). Gold-labelled slightly electron dense granules (arrows) within a positive cell (*G*) and fibres (*H, I*), enclosed in vesicle structures (*I*) (scale bars, 300 nm). *Insets:* particulars at higher magnification of neurosecretory cell (*A, D, G*) and nerve fibres (*C, E, F*) (scale bars, 100 nm).

observation of positive fibres showed a few electron-dense immunolabelled granules, which were frequently associated with bundled microfilaments (fig. 4C).

In the cephalic portion of circumpharyngeal connectives, a positive immunogold reaction was observed on both moderately and highly electron dense granules located within the perikarya and the axons of secretory neurons (fig. 4D–F).

In the ventral nerve cord, the few positive cells and fibres showed immunogold labelling almost exclusively localised on slightly electron dense granules (fig. 4G–I). Furthermore, along the fibres, these immunoreactive granules were in some cases enclosed in vesicle structures (fig. 4I).

Neurotrophin-receptor-like immunoreactivity

In immunohistochemical studies using purified polyclonal antibodies to pan-Trk sequences and anti-p75 serum, immunoreactivity related to the two types of neurotrophin receptors was detected in different *E. foetida* tissues.

The immunohistochemical reactions to Trk antibodies were located in some neuronal cell bodies and in many nerve fibres in the nervous system (fig. 5A–C), showing particularly numerous immunoreactive fibres in the neuropil of the cerebral ganglion (fig. 5A) and in circumpharyngeal connectives (fig. 5B). Positive cells scattered in the gut wall were also detected (fig. 5B, D). In particular, Trk-like immunoreactive cells were mainly observed

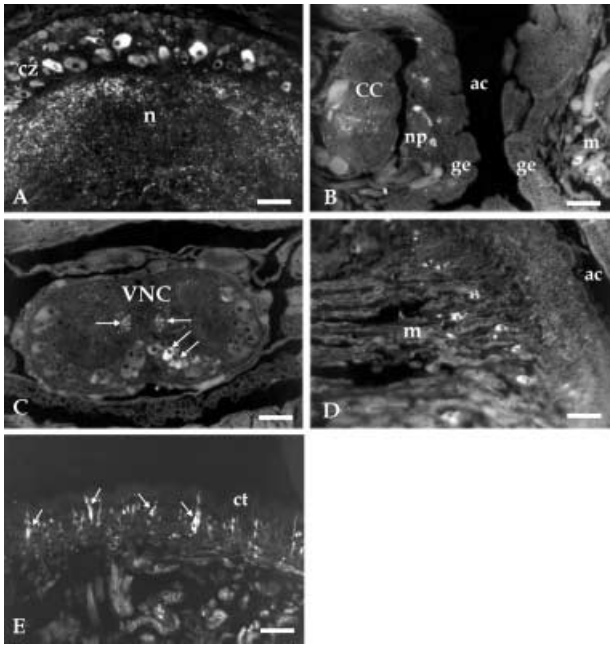

Figure 5. Immunohistochemical localisation of pan-Trk-like immunoreactivity in transversal sections from the *E. foetida* earthworm. (*A*) Cerebral ganglion, positive cells in the cortical zone (cz) and immunoreactive fibres in the neuropil (n) (scale bar, 60 µm). (*B*) Immunoreactivity at the level of circumpharyngeal connectives (CC) showing positive cells under the gut epithelium (ge), among the muscle fibres (m) and in the pharyngeal neuronal plexus (np) (ac, alimentary canal) (scale bar, 100 µm). (*C*) Ventral nerve cord (VNC), with immunoreactive cells and fibres (arrows) (scale bar, 60 µm). (*D*) The anterior region of the alimentary canal (ac), with positive cells among the muscle fibres (m) (scale bar, 60 µm). (*E*) Through the epithelial layer of the body wall, showing positive ciliate sensory cells (arrows) in the cuticle (ct) (scale bar, 60 µm).

among the muscle fibres of the anterior regions of the alimentary canal (fig. 5D), in the pharyngeal neuronal plexus and below the gut epithelial cell layer (fig. 5B). Moreover, positive reactions to Trk antibodies were detected in ciliate sensory cells of the epidermis (fig. 5E).

p75-like immunoreactivity was only expressed in a restricted area, providing a marker to identify tubular structures in a narrow zone of the longitudinal muscles along the body wall (fig. 6). This zone, which we named the 'mid-dorsal block', was indistinguishable from the adjacent muscular tissue by either histochemical or immunohistochemical methods when other antisera not directed to p75 were used.

In earthworms, the body wall musculature consists of two concentric layers: a thin circular muscle layer extending around the circumference of the body, except at the intersegmental positions, and a thicker inner layer of longitudinal muscles. These longitudinal muscles are continuous throughout the length of the body, and are arranged in groups or blocks around the body [38]. Our results show that the two dorsal blocks reported in the anatomical books [see ref. 38] are in reality three, and include the small 'mid-dorsal block' which contains on the mid-line, the openings of each segmental dorsal pore. Each single dorsal pore communicates with the coelom cavity filled with coelomic fluid and opens in the intersegmental groove of the body wall.

In *E. foetida,* the position of the first dorsal pore, used as a systematic character at the species level, corresponds to the 4/5 segmental groove [38].

The p75-like immunoreactive structures were detected from the fourth to the last (caudal) segment, and were located close to and inside the particular block of longitudinal muscles, which we called 'the mid-dorsal block' since it lies in the mid-dorsal region of the body wall (fig. 6).

By conventional fluorescence microscopy, cross-sections through this small muscular block, at the level of intersegmental dorsal pores (fig. 6A) and within the segments (fig. 6B), showed irregularly distributed positive structures which appeared, in frontal sections, as convolute tubules (fig. 6C).

These features were confirmed by a confocal microscopic study (fig. 7). In the cross-sections counterstained with propidium iodide (PI), the p75-like immunoreactivity was located on empty structures, negative for PI, which occurred inside and near the edge of the 'mid-dorsal block' of longitudinal muscles, being differently distributed along the segment (fig. 7A–D). Moreover, by three-dimensional reconstruction of confocal imagines obtained through 10-µm transversal sections, different cross-sectioned profiles of positive tubular structures were detectable, including those of longitudinal immunoreactive tubules (fig. 7E, F).

In addition, analysis of serial confocal sections through the dorsal pore showed a clear anatomical correlation between positive tubular structures and the pore wall (fig. 7G, H).

Discussion

The invertebrates have played a pivotal role in the development of the field of neurosecretion, providing useful model systems for studying unifying organisational principles among metazoans and eukaryotes [46–48].

Ultrastructural aspects of neurosecretion have been carefully investigated in annelids [46]. Moreover, among annelids, the earthworms have long been taken as a model for regeneration studies [49]. In addition, recent data suggest that particular cytokine-related molecules may be common chemical mediators in immune and neuroendocrine systems of the earthworm as observed in vertebrate systems [50–52].

In vertebrates, the nervous, endocrine and immune systems are known to be interrelated and may integrate responses to stress, diseases or inflammation through the

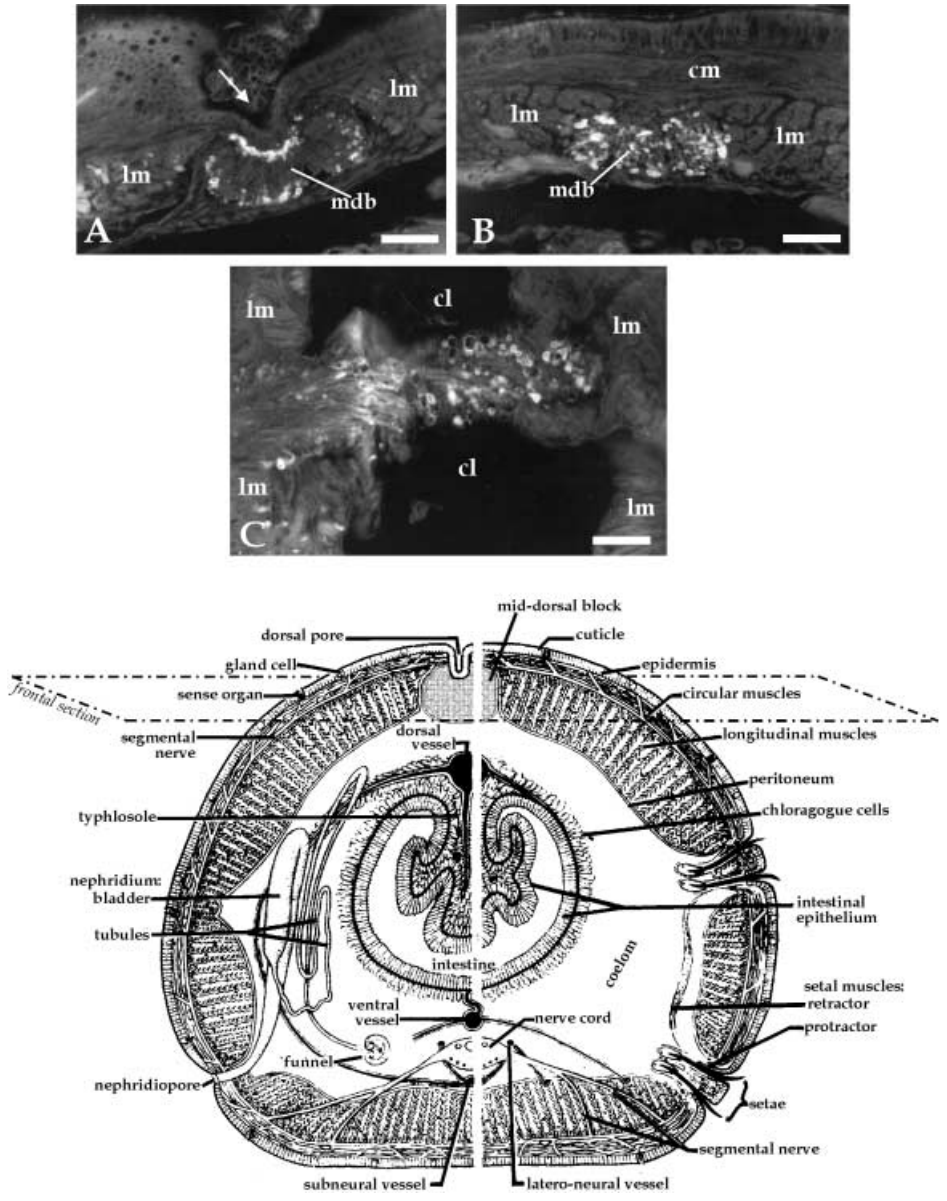

Figure 6. Diagrammatic cross-section in the central region of a Lumbricidae earthworm, illustrating the 'mid-dorsal block' of longitudinal muscles in the body wall. The left half shows a nephridium and the dorsal pore; the right half shows setae but omits the nephridium. The level of the frontal section illustrated in *C* is also reported. The micrographs (*A–C*) show the localisation of p75-like immunoreactivity in *E. foetida* (scale bars, 60 µm). (*A, B*) Cross-sections through the body wall, at the level of a dorsal pore (arrow) in the intersegmental groove (*A*) and within a segment (*B*), showing p75-like immunoreactivity in the 'mid-dorsal block' (mdb) of longitudinal muscles (lm) (cm, circular muscles). (*C*) Frontal section in the 'mid-dorsal block' at the level of the coelomic cavity (cl).

use of humoral agents that have multiple and complex functions. NGF appears to be one of these putative integrating messengers, displaying a broader distribution and a wider spectrum of biological functions than its name implies [53–56].

Our results indicate that polypeptides of approximately 13 kDa immunologically related to NGF are synthesised in earthworms and provide evidence that molecules immunologically related to at least one member of the neurotrophin family are present in *E. foetida* neurosecretory cells.

In the cerebral ganglion of earthworms, neurosecretory granules are released by exocytosis from cell bodies as well as from axon terminals [57]. In our studies, ultrastructural evidence (Ω figure) for the release of immunoreactive granules in the extracellular space adjacent to perikaryal profiles in the *E. foetida* cerebral ganglion indicates a possible autocrine and/or paracrine role of the NGF-related molecules in the nervous system of these animals. Moreover, the presence of NGF-like immunoreactive fibres in the brain neuropil, containing a 'plexi-

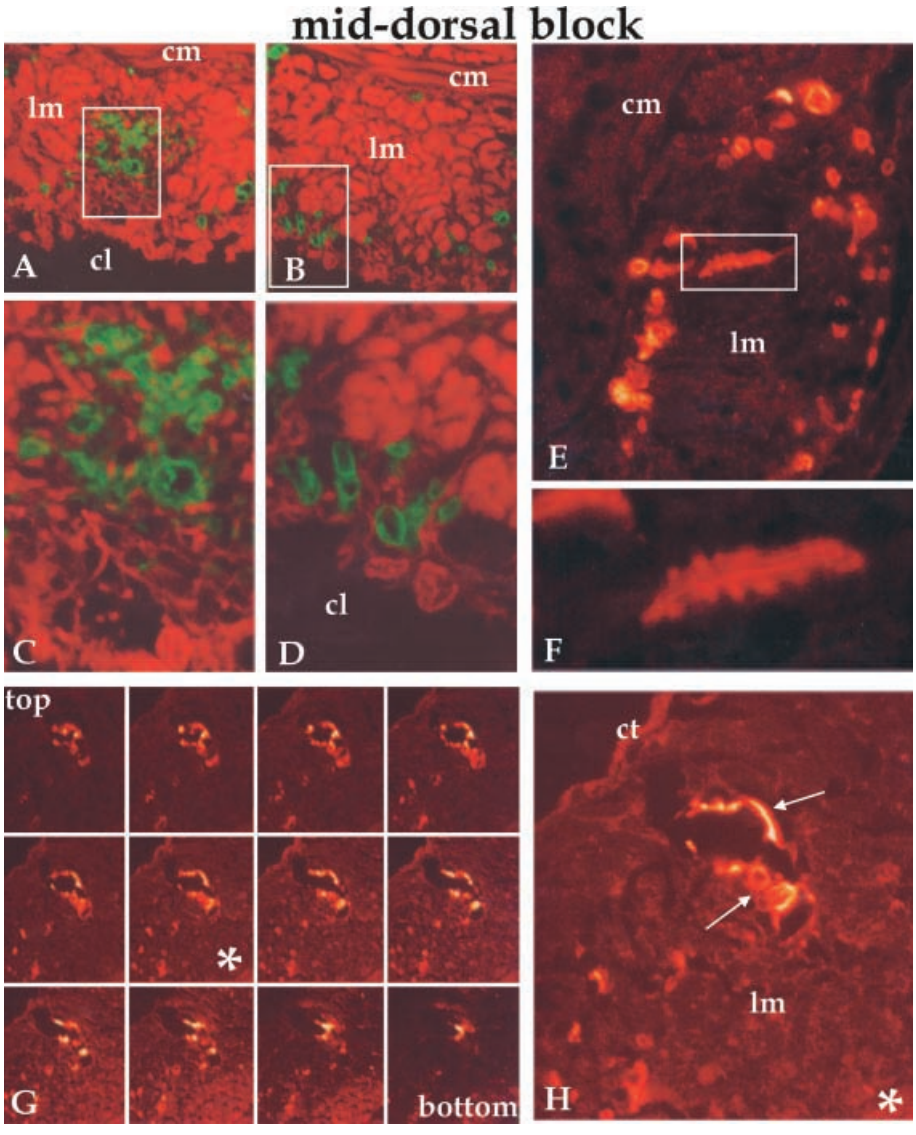

Figure 7. Confocal fluorescence micrographs of p75-like immunoreactivity in transversal sections through the 'mid-dorsal block' (original magnification: *A, B* × 40; *C–E, G, H* × 100; F × 200). (*A–D*) Combined images showing the localisation of p75-like proteins (green) in intrasegmental sections, through circular (cm) and longitudinal (lm) muscles, counterstained with propidium iodide (red). *C*, and *D* particulars at higher magnification indicated by the *inset* in *A* and *B*, respectively. Note the p75-like immunoreactive tubular structures, negative for propidium iodide, near the coelomic cavity (cl) and their different localisation along a body segment (*A, B*). (*E–H*) p75-like-deriving fluorescence signal (bright red and yellow) in transversal sections at the end (*E, F*) and at the level (*G, H*) of a dorsal pore. *E,* a three-dimensional reconstruction revealing that the positive cross-sectioned tubular structures were convoluted with different profiles; a longitudinal tubular structure in the *inset* is detailed in *F*. *G,* a sequence of confocal sections from the top to the bottom; the confocal image indicated by a white asterisk is showed in detail in *H*, where arrows point to positive structures (ct, cuticle).

form neurohaemal complex' in the most superficial layer [58], could suggest other possible functions, such as hormonal and/or neurotransmitter roles.

A different composition and changes due to component processing are likely responsible for most of the electron-dense heterogeneity observed at the ultrastructural level in positive secretory granules inside the neurosecretory cells of the cerebral ganglion, the circumpharyngeal connectives and the ventral nerve cord. Moreover, some fibres of the ventral nerve cord displayed immunoreactive slightly electron dense granules enclosed in large vesicle structures. Since these multivesicular bodies could indicate a retrograde axonal transport of NGF-like molecules [22, 59–61], of interest would be to determine whether, in that eventuality, both NGF- and Trk-like immunoreactive proteins are co-expressed in the same vesicle, thus revealing ligand-receptor interaction.

In our study, the presence and distribution in *E. foetida* tissues of pan-Trk-like immunoreactive sequences were investigated by light microscopy, using polyclonal anti-

bodies which map a TrkA highly conserved carboxyl-terminal region shared by TrkB and TrkC. Our results are in accordance with the data obtained by other authors using antibodies specific for the catalytic domain of each individual receptor of the Trk family [62]. In particular, the presence of pan-Trk-like immunoreactivity in numerous cells and fibres of the central nervous system further supports the data indicating an early evolutionary origin of the Trk family as neuronal receptor tyrosine kinases [34]. Phylogenetic analyses of vertebrate neurotrophin and Trk receptor gene families show a remarkable parallel in their evolutionary histories and suggest the presence of primitive ancestors in invertebrates [6, 18]. Several neural receptor tyrosine kinases have been found in *Drosophila* and a novel member of the Trk family, showing a sequence-related tyrosine kinase domain and the ability to bind human NT3, has been recently detected in *Lymnaea stagnalis* [34]. The amino acid or gene sequence of a member of the neurotrophin family in an invertebrate is still lacking. However, the suggestion has been made that the invertebrate clotting protein coagulogen isolated from the horseshoe crab hemocytes, could be evolutionarily related to the vertebrate neurotrophin family (http://www.cryst.bbk.ac.uk/~ubcg09j/neurotrophins/nt_new.html).

The clotting cascade system of this invertebrate (*Limulus*) is involved in both homeostasis and host defence. The three-dimensional structure of coagulogen exhibits the characteristic 'cystine knot motif', displaying in the C-terminal half of the molecule a striking topological similarity to NGF [10].

Similarities have also been described among coagulogen, NGF and the *Drosophila* Spätzle protein. The putative receptor of this last protein is Toll, which in *Drosophila* participates in establishing the dorsoventral pattern in the embryo and inducing an antifungal immune response in the adult fly. Interestingly, Toll and Trk receptors share a similar arrangement in their extracellular domain [10, 63, 64].

Thus, proteins displaying the NGF-type 'cystine knot' motif typical of vertebrate neurotrophins and able to activate similar receptors may well exist in invertebrates.

In our study, by using polyclonal antibodies to extracellular fragment sequences of p75 neurotrophin receptor, empty tubular structures never before detected were identified in the 'mid-dorsal block' of longitudinal muscles, along the body wall of *E. foetida* worms. During our long experience studying *E. foetida* studies [see ref. 58], we have never before detected either such tubular structures or the anatomic area where they are located. This area, which we have named the 'mid-dorsal block', was absolutely indistinguishable from the adjacent muscular tissue by either histochemical or immunohistochemical methods when other antisera not directed to p75 were used.

In addition, p75-like immunoreactive tubules were found to be correlated to the dorsal pores, which open in the inter-segmental groove on the mid-dorsal line of the body wall. These pores communicate with the body cavity, allowing the expulsion of coelomic fluid and coelomocytes, free cells present in the coelomic fluid, at times of either chemical and mechanical irritation or stress. These cells are involved in immune responses [50] and are currently under investigation in our laboratory for the relevant presence of NGF-like polypeptides [65].

The presence and the particular location of the p75-like immunoreactive tubular structures detected in the worms suggest a putative role for p75-like immunoreactive proteins in earthworm defence mechanisms. Further multidisciplinary investigations, however, are needed to substantiate this hypothesis.

Moreover, we need to increase our understanding of the molecular basis underlying the similarities between the immunoreactive proteins detected in *E. foetida* and the vertebrate components of the neurotrophin signalling system. Such similarities might be vestiges of a common evolutionary origin or may represent convergence upon a parsimonious solution to a common problem. Such is the case for the *E. foetida* defence molecule coelomic cytolitic factor-1 and the mammalian TNF-α, which share a lectin-like domain that appears to have been functionally conserved as a recognition mechanism during innate defence reactions in invertebrates and vertebrates [66].

The cross-reactivity of the anti-mNGF sera used in our study with the human recombinant neurotrophins is very likely related to genetic homology [17, 44], whereas no sufficient data are available concerning the role and the structural properties of the epitopes shared by neurotrophins and the NGF-like molecules detected in the annelid *E. foetida*.

However, the current studies are corroborated by our preliminary results confirming the expression of NGF-like proteins in coelomocytes by Western blot analysis of cell protein extracts and by reverse transcription-polymerase chain reaction (RT-PCR), performed on individual samples of coelomocyte RNA using as primers highly conserved sequences in neurotrophins [65]. In particular, since PCR amplification product lengths from several individual samples conforming to expected lengths, the results corroborate the existence of NGF-like polypeptides in earthworms, indicating the presence of substantial regions of conservation in its correspondent cDNA.

The expression of precursors (proneurotrophins) preferentially activating the p75 receptor has very recently been detected in vertebrate tissues and cells [67, 68].

In our experimental conditions, using immunoprecipitation with protein A and anti-immunoglobulin as secondary antibody to detect the immunoreactions, identify NGF-like fractions at a higher molecular weight than that of the NGF monomer is difficult. However, protease inhibitors were always used during the protein extractions, and in Western blotting analysis of coelomocyte extracts

(obtained directly without immunoprecipitation), the NGF-like immunoreactive fractions were only localised in the zone corresponding to about 13 kDa and no other bands were detectable (unpublished data).

Nevertheless, we cannot exclude the possibility that different physiological conditions (i. e. development and repair processes) forms not cleaved intracellularly could also be expressed at levels detectable by Western blotting, as recently reported for vertebrate tissues and cells [67, 68].

In conclusion, our results in this earthworm provide new information and new ideas for testable hypotheses to obtain insights, at least at the evolutionary level, into the puzzling picture of the neurotrophin signalling system, representing a starting point for in-depth studies in non-controversial, readily available and inexpensive animal model.

Finally, the suggestion has been made recently that neurotrophic signalling mechanisms might be one of the prerequisites for the evolution of complex nervous systems and a search for them should be made in invertebrate models displaying a relatively long life cycle and a complex nervous system [35]: earthworms may well represent one such candidate.

1 Thoenen H. (1991) The changing scene of neurotrophic factors. Trends Neurosci. **14:** 165–170
2 Lewin G. R. and Barde Y. A. (1996) Physiology of the neurotrophins. Annu. Rev. Neurosci. **19:** 289–317
3 Tonra J. R. (1999) Classical and novel directions in neurotrophin transport and research: anterograde transport of brain-derived neurotrophic factor by sensory neurons. Microsc. Res. Techn. **45:** 225–232
4 Kaplan D. R. and Miller F. D. (2000) Neurotrophin signal transduction in the nervous system. Curr. Opin. Neurbiol. **10:** 381–391
5 Levi-Montalcini R., Dal Toso R., Della Valle F., Skaper S. D. and Leon A. (1995) Update of the NGF saga. J. Neurol. Sci. **130:** 119–127
6 Ibáñez C. F. (1998) Emerging themes in structural biology of neurotrophic factors. Trends Neurosci. **21:** 438–444
7 Wiesmann C. and Vos A. M. de (2001) Nerve growth factor: structure and function. Cell. Mol. Life Sci. **58:** 748–759
8 Fahnestock M. (1991) Structure and biosynthesis of nerve growth factor. Curr. Top. Microbiol. Immunol. **165:** 1–26
9 Murray-Rust J., McDonald N. Q., Blundell T. L., Hosang M., Oefner C., Winkler F. et al. (1993) Topological similarities in TGF-β2, PDGF-BB and NGF define a superfamily of polypeptide growth factors. Structure **1:** 153–159
10 Bergner A., Muta T., Iwanaga S., Beisel H.G., Delotto R. and Bode W. (1997) Horseshoe crab coagulogen is an invertebrate protein with a nerve growth factor-like domain. Biol. Chem. **378:** 283–287
11 Hallböök F., Ibáñez C. F. and Persson H. (1991) Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in *Xenopus* ovary. Neuron **6:** 845–858
12 Ip N. Y., Ibáñez C. F., Nye S. H., McClain J., Jones P. F., Gies D. R. et al. (1992) Mammalian neurotrophin-4: structure, chromosomal localization, tissue distribution, and receptor specificity. Proc. Natl. Acad. Sci. USA **89:** 3060–3064
13 Kullander K. and Ebendal T. (1994) Neurotrophin-3 acquires NGF-like activity after exchange to five NGF amino acid residues: molecular analysis of the sites in NGF mediating the specific interaction with the NGF high affinity receptor. J. Neurosci. Res. **39:** 195–210
14 Narhi L. O., Rosenfeld R., Talvenheimo J., Prestrelski S. J., Arakawa T., Lary J. W. et al. (1993) Comparison of the biophysical characteristics of human brain-derived neurotrophic factor, neurotrophin-3, and nerve growth factor. J. Biol. Chem. **268:** 13309–13317
15 Robinson R. C., Radziejewski C., Stuart D. I. and Jones E.Y. (1995) Structure of the brain-derived neurotrophic factor/neurotrophin-3 heterodimer. Biochemistry **34:** 4139–4146
16 Acheson A., Barker P. A., Alderson R. F., Miller F. D. and Murphy R. A. (1991) Detection of brain-derived neurotrophic factor-like activity in fibroblasts and Schwann cells: inhibition by antibodies to NGF. Neuron **7:** 265–275
17 Murphy R. A., Acheson A., Hodges R., Haskins J., Richards C., Reklow E. et al. (1993) Immunological relationships of NGF, BDNF, and NT-3: recognition and functional inhibition by antibodies to NGF. J. Neurosci. **13:** 2853–2862
18 Hallböök F. (1999) Evolution of the vertebrate neurotrophin and Trk receptor gene families. Curr. Opin. Neurobiol. **9:** 616–621
19 Götz R., Köster R., Winkler C., Raulf F., Lottspeich F., Schartl M. et al. (1994) Neurotrophin-6 is a new member of the nerve growth factor family. Nature **372:** 266–269
20 Lai K. O., Fu W. Y., Ip F. C. F. and Ip N. Y. (1998) Cloning and expression of a novel neurotrophin, NT-7, from carp. Mol. Cell. Neurosci. **11:** 64–76
21 Nilsson A. S., Fainzilber M., Falck P. and Ibáñez C. F. (1998) Neurotrophin-7: a novel member of the neurotrophin family from zebrafish. FEBS Lett. **424:** 285–290
22 Bothwell M. (1995) Functional interactions of neurotrophins and receptor. Annu. Rev. Neurosci. **18:** 223–253
23 Casaccia-Bonnefil P., Gu C., Khursigara G. and Chao M. V. (1999) p75 neurotrophin receptor as a modulator of survival and death decisions. Microsc. Res. Tech. **45:** 217–224
24 Casademunt E., Carter B. D., Benzel I., Frade J. M., Dechant G. and Barde Y. A. (1999) The zinc finger protein NRIF interacts with the neurotrophin receptor $p75^{NTR}$ and participates in programmed cell death. EMBO J. **18:** 6050–6061
25 Chittka A. and Chao M. V. (1999) Identification of a zinc finger protein whose subcellular distribution is regulated by serum and nerve growth factor. Proc. Natl. Acad. Sci. USA **96:** 10705–10710
26 Khursigara G., Orlinick J. R. and Chao M. V. (1999) Association of the p75 neurotrophin receptor with TRAF6. J. Biol. Chem. **274:** 2597–2600
27 Mallett S. and Barclay A. N. (1991) A new superfamily of cell surface proteins related to the nerve growth factor receptor. Immunol. Today **12:** 220–223
28 Raoul C., Pettmann B. and Henderson C. (2000) Active killing of neurons during development and following stress: a role for $p75^{NTR}$ and Fas? Curr. Opin. Neurobiol. **10:** 111–117
29 Chao M., Casaccia-Bonnefil P., Carter B., Chittka A., Kong H. and Yoon S. O. (1998) Neurotrophin receptors: mediators of life and death. Brain Res. Rev. **26:** 295–301
30 Hayashi I., Perez-Magallanes M. and Rossi J. M. (1992) Neurotrophic factor-like activity in *Drosophila.* Biochem. Bioph. Res. Commun. **184:** 73–79
31 Ridgway R. L., Syed N. I., Lukowiak K. and Bulloch A. G. M. (1991) Nerve growth factor (NGF) induces sprouting of specific neurons of the snail, *Lymnaea stagnalis.* J. Neurobiol. **22:** 377–390
32 Barde Y. A. (1994) Neurotrophic factors: an evolutionary perspective. J. Neurobiol. **25:** 1329–1333
33 Fainzilber M., Smit A. B., Syed N. I., Wildering W. C., Hermann P. M., Schors R. C. van der et al. (1996) CRNF, a mol-

luscan neurotrophic factor that interacts with the p75 neurotrophin receptor. Science **274:** 1540–1543
34 Kesteren R. E. van, Fainzilber M., Hauser G., Minnen J. van, Vreugdenhil E., Smit A. B. et al. (1998) Early evolutionary origin of the neurotrophin receptor family. EMBO J. **17:** 2534–2542
35 Jaaro H., Beck G., Conticello S. G. and Fainzilber M. (2001) Evolving better brains: a need for neurotrophins? Trends Neurosci. **24:** 79–85
36 McKay S.E., Purcell A. L. and Carew T. J. (1999) Regulation of synaptic function by neurotrophic factors in vertebrates and invertebrates: implications for development and learning. Learn. Memory **6:** 193–215
37 Falugi C. and Davoli C. (1993) Localization of putative biochemical messengers during *Eisenia foetida* (Annelida, Oligochaeta) development. Tissue Cell **25:** 311–323
38 Edwards C. A. and Lofty J. R. (1977) Biology of Earthworms, Chapman & Hall, London
39 Jamieson B. G. M. (1992) Oligochaeta. In: Microscopic Anatomy of Invertebrates, vol. 7, Annelida, pp. 217–322, Harrison F. W. and Gardiner S. L. (eds), Wiley-Liss, NewYork
40 Angeletti R. H., Mercanti D. and Bradshaw R. A. (1973) Amino acid sequences of mouse 2.5S nerve growth factor. I. Isolation and characterization of the soluble tryptic and chymotryptic peptides. Biochemistry **12:** 90–100
41 Davoli C., Serafino A., Marcheggiano A., Iannoni C., Marconi A. and Ravagnan G. (1996) Neurotrophin-like immunoreactivity in the nervous system of the earthworm *Eisenia foetida* (Annelida, Oligochaeta). In: The peptidergic neuron, pp. 177–183, Krisch B. and Mentlein R. (eds), Birkhäuser, Basel
42 Laemmli E. K. (1970) Cleavage of structural proteins during assembly of the head bacteriophage T4. Nature **227:** 680–685
43 Zhou X. F. and Rush R. A. (1993) Localization of neurotrophin-3-like immunoreactivity in peripheral tissues of the rat. Brain Res. **621:** 189–199
44 Ullrich A., Gray A., Berman C. and Dull T. J. (1983) Human β-nerve growth factor gene sequence highly homologous to that of mouse. Nature **303:** 821–825
45 Lindmark R., Thoren-Tolling K. and Sjoquist J. (1983) Binding of immunoglobulins to protein A and immunoglobulin levels in mammalian sera. J. Immunol. Methods **62:** 1–13
46 Golding D. W. and Pow D. V. (1988) The new neurobiology – ultrastructural aspects of peptide release as revealed by studies of invertebrate nervous systems. In: Neurohormones in invertebrates, pp. 7–18, Thorndyke M. C. and Goldsworthy G. J. (eds), Cambridge University Press, Cambridge, UK
47 Golding D. W. (1996) Patterns of peptide discharge – implications for Dale's principle. In: The Peptidergic Neuron, pp. 7–12, Krisch B. and Mentlein R. (eds), Birkhäuser, Basel
48 Pevsner J. (1996) Molecular mechanisms of neurotransmitter and neuropeptide release. In: The Peptidergic Neuron, pp. 55–72, Krisch B. and Mentlein R. (eds), Birkhäuser, Basel
49 Dales R. P. (1978) Defence mechanisms. In: Physiology of Annelids, pp. 479–507, Mill P. J. (ed.), Academic Press, London
50 Cooper E. L. (1996) Earthworm immunity. In: Invertebrate Immunology, pp. 10–45, Rinkevich B. and Müller W. E. G. (eds), Springer, Berlin
51 Takeuchi N. (1999) Localization of cytokine-like and cytokine receptor-like molecules in the central nervous system of earthworms. In: Recent Progress in Molecular and Comparative Endocrinology, pp. 271–275, Kwon H. B., Joss J. M. P. and Ishii S. (eds), Hormone Research Center, Kwangju
52 Salzet M., Vieau D. and Day R. (2000) Crosstalk between nervous and immune systems through the animal kingdom: focus on opioids. Trends Neurosci. **23:** 550–555
53 Ehrhard P. B., Erb P., Graumann U. and Otten U. (1993) Expression of nerve growth factor and nerve growth factor receptor tyrosine kinase Trk in activated CD4-positive T-cell clones. Proc. Natl. Acad. Sci. USA **90:** 10984–10988
54 Patterson J. C. and Childs G. (1994) Nerve growth factor in the anterior pituitary: regulation of secretion. Endocrinology **135:** 1697–1704
55 Garaci E., Caroleo M. C., Aloe L., Aquaro S., Piacentini M., Costa N. et al. (1999) Nerve growth factor is an autocrine factor essential for the survival of macrophages infected with HIV. Proc. Natl. Acad. Sci. USA **96:** 14013–14018
56 Pica F., Volpi A., Serafino A., Fraschetti M., Franzese O. and Garaci E. (2000) Autocrine nerve growth factor is essential for cell survival and viral maturation in HHV-8-infected primary effusion lymphoma cells. Blood **95:** 2905–2912
57 Al-Yousuf S., Konishi A., Nomura S. and Mizuno N. (1992) An electron microscopic study of neurosecretion in the cerebral ganglion of the earthworm. Neurosci. Lett. **140:** 189–191
58 Davoli C., Marcheggiano A., Ravagnan G., Minù M., Serafino A. and Iannoni C. (1991) Iodination activity in *Eisenia foetida* (Annelida, Oligochaeta). Cell Tissue Res. **264:** 9–14
59 Parton R. G. and Dotti C. G. (1993) Cell biology of neuronal endocytosis. J. Neurosci. Res. **36:** 1–9
60 Altar C. A. and DiStefano P. S. (1998) Neurotrophin trafficking by anterograde transport. Trends Neurosci. **21:** 433–437
61 Neet K. E. and Campenot R. B. (2001) Receptor binding, internalization, and retrograde transport of neurotrophic factors. Cell. Mol. Life Sci. **58:** 1021–1035
62 Lucini C., Castaldo L., Lamanna C., Maruccio L., Vega J. A. and Gargiulo G. (1999) Neuronal and non-neuronal Trk neurotrophin receptor-like proteins in *Eisenia foetida* (Annelida Oligochaeta). Neurosci. Lett. **261:** 163–166
63 DeLotto Y. and DeLotto R. (1998) Proteolytic processing of the *Drosophila* Spätzle protein by Easter generates a dimeric NGF-like molecule with ventralising activity. Mech. Dev. **72:** 141–148
64 Yang R. B., Mark M. R., Gray A., Huang A., Xie M. H., Zhang M. et al. (1998) Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. Nature **395:** 284–288
65 Davoli C., Marconi A., Serafino A., Pierimarchi P., Mariani F., Andreola F. et al. (2001) A putative role for NGF- and p75-like immunoreactive proteins in earthworm defence mechanisms. In: Perspectives in Comparative Endocrinology: Unity and Diversity, pp. 879–885, Goos H. J. T., Rastogi R. K., Vaudry H. and Pierantoni R. (eds), Monduzzi, Bologna
66 Beschin A., Bilej M., Torreele E. and De Baetselier P. (2001) On the existence of cytokines in invertebrates. Cell. Mol. Life Sci. **58:** 801–814
67 Fahnestock M., Michalski B., Xu B. and Coughlin M. D. (2001) The precursor pro-nerve growth factor is the predominant form of nerve growth factor in brain and is increased in Alzheimer's disease. Mol. Cell. Neurosci. **18:** 210–220
68 Lee R., Kermani P., Teng K. K. and Hempstead B. L. (2001) Regulation of cell survival by secreted proneurotrophins. Science. **294:** 1945–1948