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Immunocytochemistry of B-50 (GAP-43) in the spinal cord and in dorsal root ganglia of the adult cat

W. NACIMIENTO[1,3*], R. TÖPPER[3], A. FISCHER[1], A. B. OESTREICHER[4], A. C. NACIMIENTO[2], W. H. GISPEN[4], J. NOTH[1] and G. W. KREUTZBERG[3]

[1] *Department of Neurology, Aachen University Medical School, Pauwelsstrasse, D-5100 Aachen, Germany*
[2] *Neurosurgical Research Laboratory, Saarland University Medical School, D-6650 Homburg, Germany*
[3] *Max-Planck-Institute for Psychiatry, Department of Neuromorphology, Am Klopferspitz 18a, D-8033 Martinsried n. Munich, Germany*
[4] *Rudolf Magnus Institute, University of Utrecht, 3584 CH Utrecht, The Netherlands*

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Summary

The distribution of the neural-specific growth associated protein B-50 (GAP-43), which persists in the mature spinal cord and dorsal root ganglia, has been studied by light and electron microscopic immunohistochemistry in the cat. Throughout the spinal cord, B-50 immunoreactivity was seen confined to the neuropil, whereas neuronal cell bodies were unreactive. The most conspicuous immunostaining was observed in the dorsal horn, where it gradually decreased from superficial laminae (I–II) toward more ventral laminae (III–V), and in the central portion of the intermediate gray (mainly lamina X). In these regions, the labelling was localized within unmyelinated, small diameter nerve fibres and axon terminals. In the rest of the intermediate zone (laminae VI–VIII), B-50 immunoreactivity was virtually absent. The intermediolateral nucleus in the thoracic and cranial lumbar cord showed a circumscribed intense B-50 immunoreactivity brought about by the labelling of many axon terminals on preganglionic sympathetic neurons. In motor nuclei of the ventral horn (lamina IX), low levels of B-50 immunoreactivity were present in a few axon terminals on dendritic and somal profiles of motoneurons. In dorsal root ganglia, B-50 immunoreactivity was mainly localized in the cell bodies of small and medium-sized sensory neurons. The selective distribution of persisting B-50 immunoreactivity in the mature cat throughout sensory, motor, and autonomic areas of the spinal cord and in dorsal root ganglia suggests that B-50-positive systems retain in adult life the capacity for structural and functional plasticity.

Introduction

The acid phosphoprotein B-50, which is specific to nervous tissue, belongs to a group of growth-associated proteins and is identical to GAP-43, GAP-48, pp46, F1, and P57 (for reviews see Gordon-Weeks, 1989; Skene, 1989; Benowitz & Perrone-Bizzozero, 1991; Gispen *et al.*, 1992). B-50 is produced at high levels in developing and regenerating neurons and accumulates after fast axonal transport in growth cones (De Graan *et al.*, 1985; Kalil & Skene, 1986; Meiri *et al.*, 1986; Skene *et al.*, 1986; Verhaagen *et al.*, 1986; Gorgels *et al.*, 1987, 1989; Dekker *et al.*, 1989; De la Monte *et al.*, 1989; Gordon-Weeks, 1989; Skene, 1989; Tetzlaff *et al.*, 1989; Van der Zee *et al.*, 1989; Van Lookeren Campagne *et al.*, 1989, 1990; Sommervaille *et al.*, 1991; Schreyer & Skene, 1991; Knyihar-Csillik *et al.*, 1992; Woolf *et al.*, 1990, 1992). The phosphorylation of B-50 by protein kinase C in growth cones is presumed to influence neurite elongation and synapse formation (Van Hooff *et al.*, 1988). When synaptic contacts are established, B-50 normally decreases in concentration or disappears (Skene & Willard, 1981; Verhaagen *et al.*, 1986; McIntosh *et al.*, 1989; Burry *et al.*, 1992; Mahalik *et al.*, 1992).

Since the presence of B-50 correlates well with nerve terminal growth and synaptogenesis during development and regeneration, it is of interest to determine where the protein persists in the mature CNS. This has been studied by immunohistochemistry in the human and rat brain (Oestreicher & Gispen 1986; Benowitz *et al.*, 1989; De la Monte *et al.*, 1989; DiFiglia *et al.*, 1990). There, normal levels of B-50 were found to be low except in distinctive areas such as the hippocampus and cortical association areas. In these regions, its persistence in adulthood has been linked to the

* To whom correspondence should be addressed (Aachen address).

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maintenance of a neuronal capacity for structural remodeling and functional plasticity (e.g. axonal sprouting, synaptogenesis, and prolonged alteration in synaptic efficacy; for reviews see Skene, 1989 and Gispen *et al.*, 1992).

In the present work, we describe the distribution of B-50 immunoreactivity (B-50-IR) persisting in the uninjured adult cat spinal cord and dorsal root ganglia (DRG). In the rat spinal cord, light microscopic observations of B-50-IR are in part controversial: using a polyclonal GAP-43 (B-50) antiserum, Woolf and colleagues (1990) described a lack in immunostaining in the dorsal horn of uninjured rats, and an intense labelling in this region after peripheral nerve lesion. In contrast, employing one of their monoclonal antibodies against GAP-43 (B-50), Schreyer and Skene (1991) found a strong immunolabelling in the normal rat in superficial laminae of the dorsal horn which was not further increased following peripheral nerve injury. This discrepancy may be related to differences in antibodies recognizing different epitopes of the protein (Schreyer & Skene, 1991). The aim of the present investigation was to extend these observations in the intact cat using a polyclonal antibody against B-50 which has been previously shown to detect neuronal plasticity in immunohistochemical experiments (for review see Gispen *et al.*, 1992). In the context of current concepts of restorative processes in response to spinal cord injury (Goldberger & Murray, 1988; Schnell & Schwab, 1990; Schwab 1990; Cadelli *et al.*, 1992), B-50 appears to be a reliable immunohistochemical marker for the further elucidation of neuronal plasticity.

Materials and methods

One female and two male adult cats, 2.8–3.6 kg in body weight, were deeply anaesthetized with pentobarbital and perfused through the heart with 500 ml 0.9% saline solution, followed by 3 l of 2% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer (PB; pH 7.4). After fixation, the following spinal cord segments were removed together with their DRG in all animals: cervical segments 2, 3, 4, and 6; thoracic 2, 5, and 11; lumbar 1, 4, and 7; sacral 1–3. The tissue blocks were postfixed for 24 h in fixative solution as used for perfusion. For light microscopy, a portion of the tissue was cryoprotected in graded sucrose (10%, 20%, and 30%) in 0.1% phosphate-buffered saline (PBS) for 48 h, frozen in liquid nitrogen, and cut in 14 μm thick transverse sections on a cryostat. For electron microscopy, the remaining tissue was transferred to 2% paraformaldehyde in 0.1 M phosphate buffer and cut in 50 μm thick transverse sections on a Vibratome.

Free-floating tissue sections were stained immunohistochemically for B-50 using the peroxidase-antiperoxidase method of Sternberger (1979). Prior to immunostaining, sections were incubated in a solution containing 0.3% hydrogen peroxide in PBS for 30 min, to block the endogenous peroxidase activity, followed by three 10 min rinses in PBS. Briefly, sections were then incubated in affinity purified polyclonal rabbit antibodies to rat B-50 (anti-B-50 IgGs of rabbit 8920; 1:2,000) for 48 h at 4° C and in secondary antisera (goat-antirabbit IgG, 1:200, Accurate Chemicals) and PAP (Accurate Chemicals, 1:500) for 1 h each at room temperature. Primary antisera, secondary antisera, and PAP were diluted in 0.1 M PBS containing 1% normal goat serum (NGS). Tissue sections were rinsed in two changes of 0.1 M PBS (10 min each) and 3% NGS (30 min) before each antisera or PAP incubation. Following the PAP incubation, tissue sections were rinsed twice in PBS and reacted in 0.05% diaminobenzidine (Sigma) and 0.01% H_2O_2 in 0.1 M PB for 10 min. Cryostat sections were mounted on L-polylysine coated slides, dehydrated in alcohols, cleared in xylene, and coverslipped with Depex. Vibratome sections were postfixed in 2.5% glutaraldehyde for 20 min and in Dalton's chrome-OsO_4 solution for 30 min, dehydrated and flat embedded in Araldite between slides coated with Repelcoat (dimethyldichlorsilane). After polymerization, regions of interest were excised from the tissue and reembedded on Araldite blocks. Ultrathin sections were counterstained with uranyl acetate and lead citrate and examined in a Zeiss EM10.

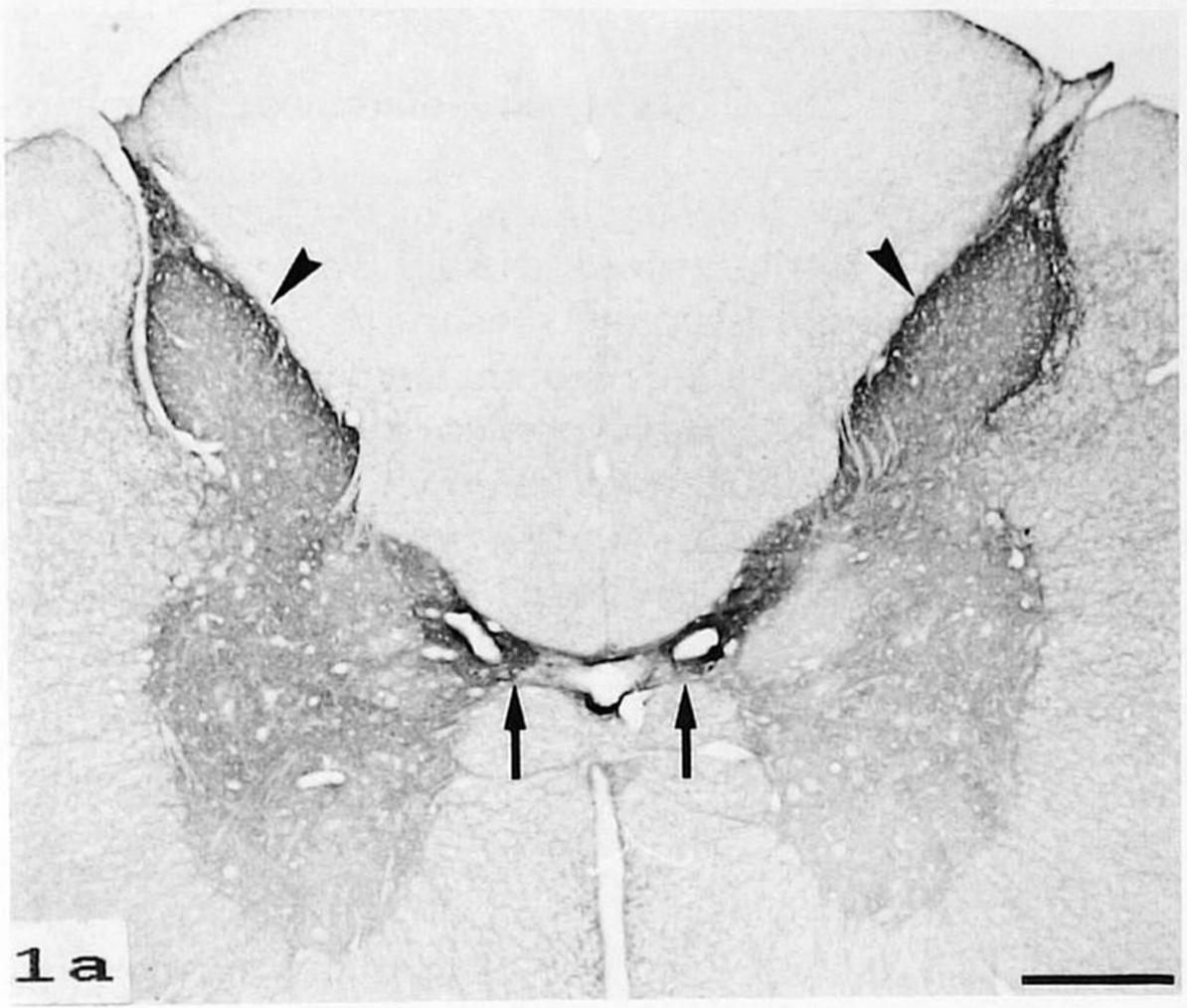
Control sections for both light and electron microscopic immunohistochemistry were incubated in PBS/NGS or preimmune-rabbit IgG instead of primary antisera, and resulted in no staining of the tissue. The specificity and characteristics of the rabbit polyclonal antibody against B-50 have been reported previously (Oestreicher *et al.*, 1983, 1986; Gorgels *et al.*, 1989; Mercken *et al.*, 1992). Various dilutions of B-50 antibody (in the range from 1:100 to 1:10 000) were tried out in pilot experiments. Optimum conditions for immunohistochemical labelling were found at a dilution of 1:2000.

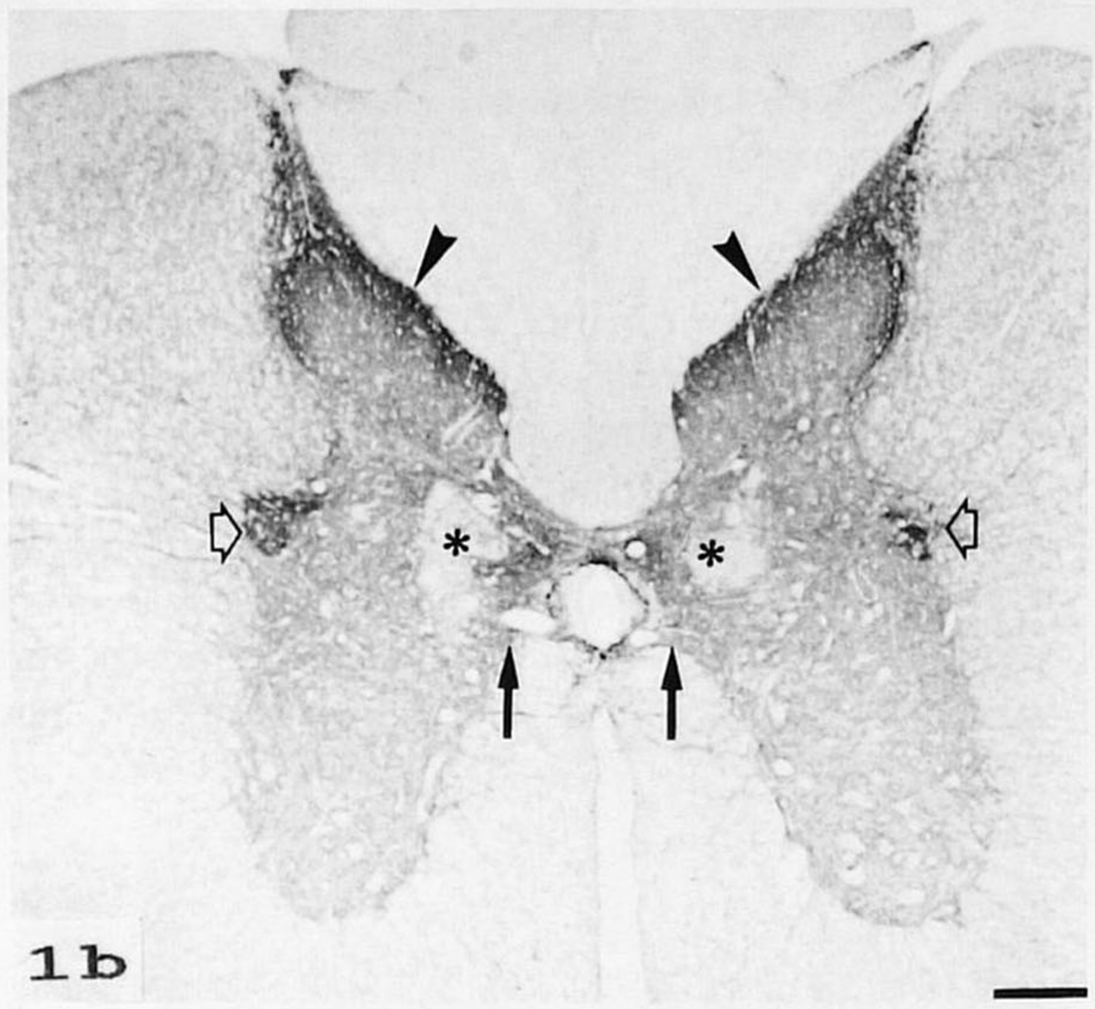
Results

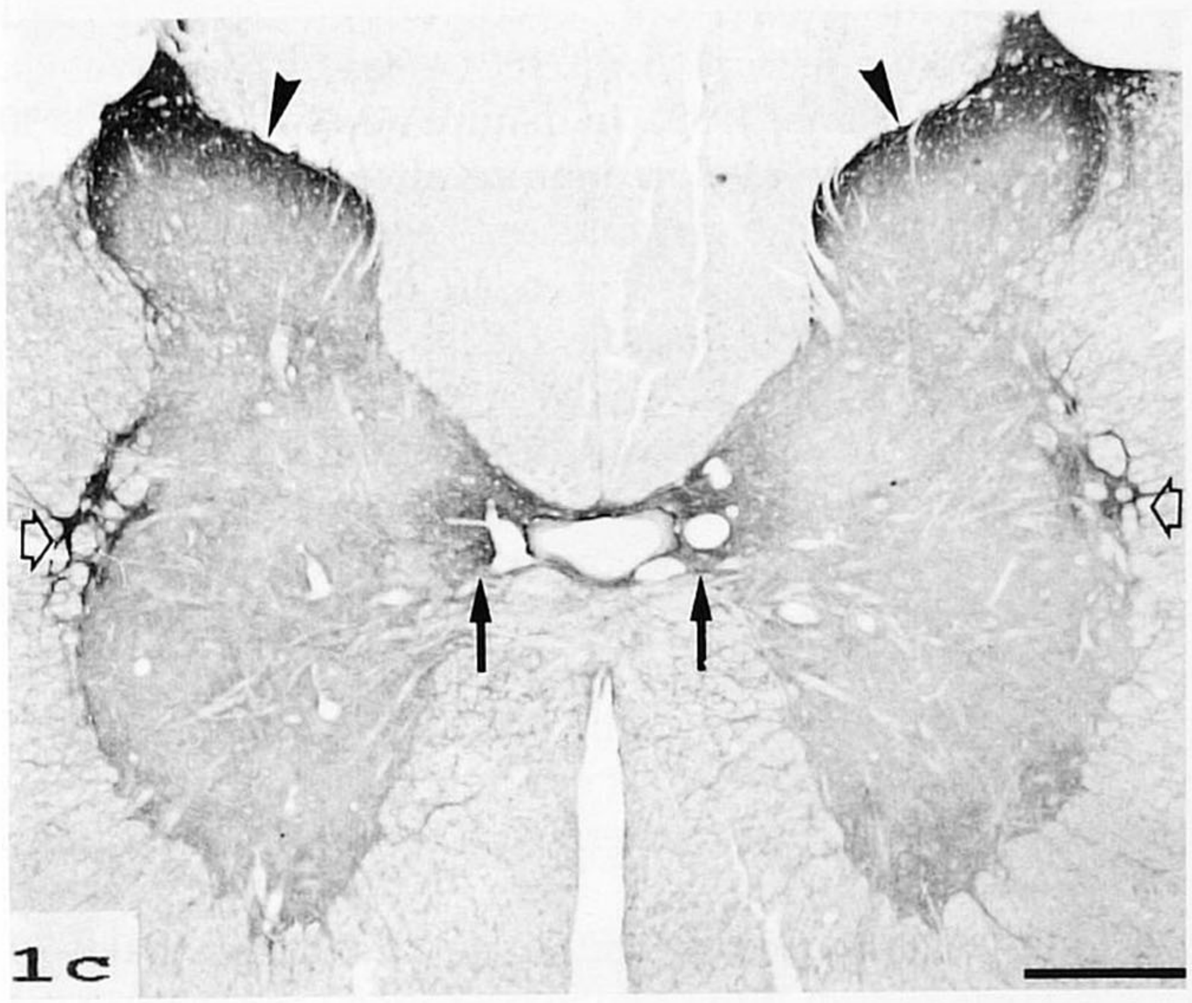
Light microscopy

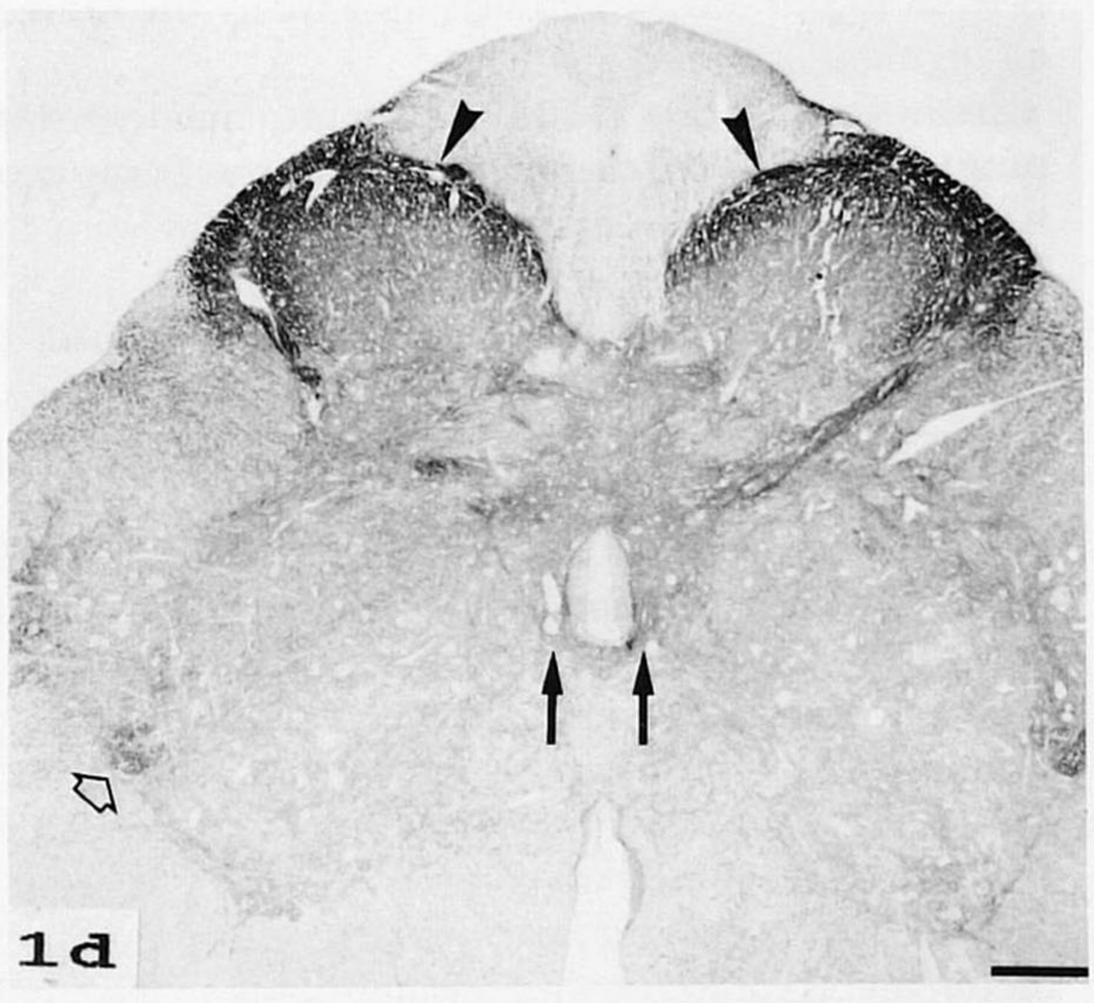
The distributions of B-50 immunolabelling in the spinal cord and DRG were identical in all animals.

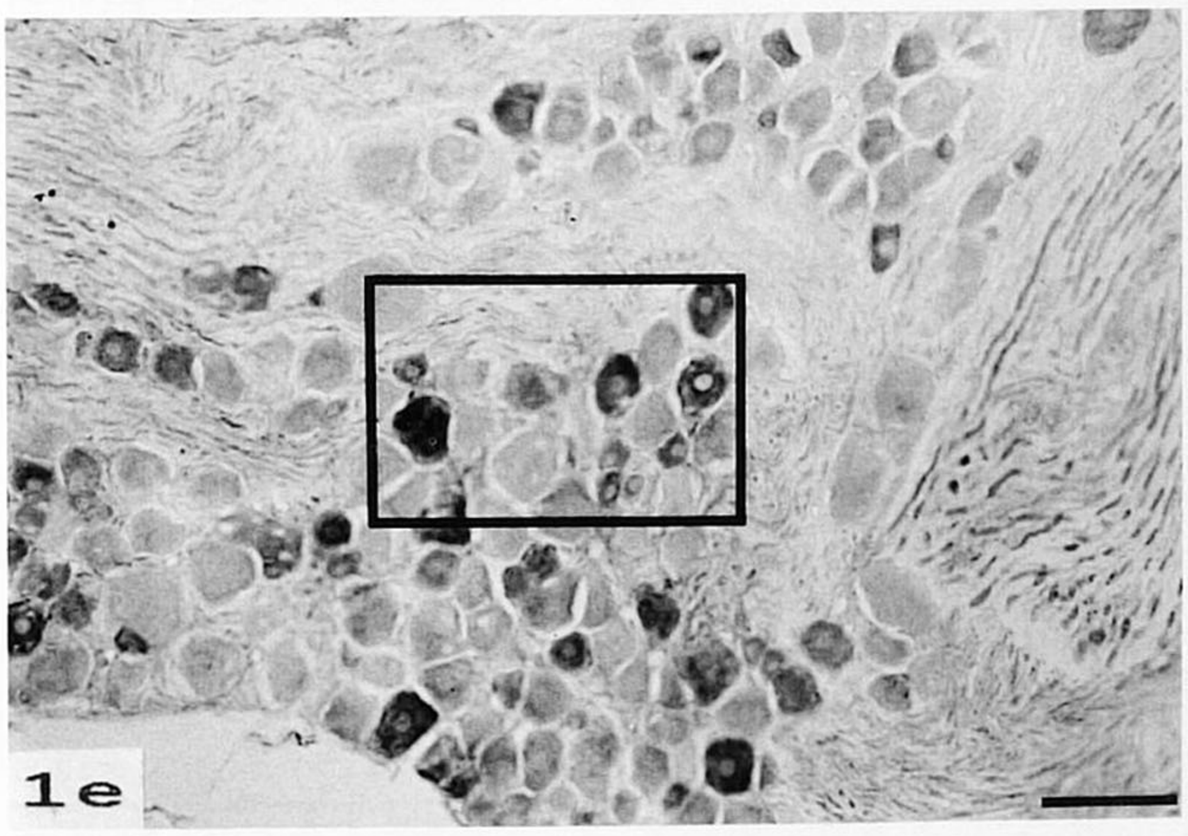
In the spinal cord tissue, most of the bilaterally symmetrical granular staining indicative of B-50-IR accumulated in the neuropil of the gray matter, leaving neuronal cell bodies unlabelled. In the white matter, only Lissauer's tract consistently displayed B-50-IR. In the dorsal horn of all spinal cord segments examined, the labelling intensity differed considerably within Rexed's laminae, being high in laminae I and II, and low in laminae III–V. An intense staining was also present in the central portion of the intermediate zone (mainly lamina X) and in nerve fibre bundles crossing in the dorsal gray commissure. Conversely, the lateral portion of the intermediate gray (lamina VI–VIII) was nearly devoid of immunostaining. In thoracic and cranial lumbar segments, an intense immunoreactivity was found in the neuropil of











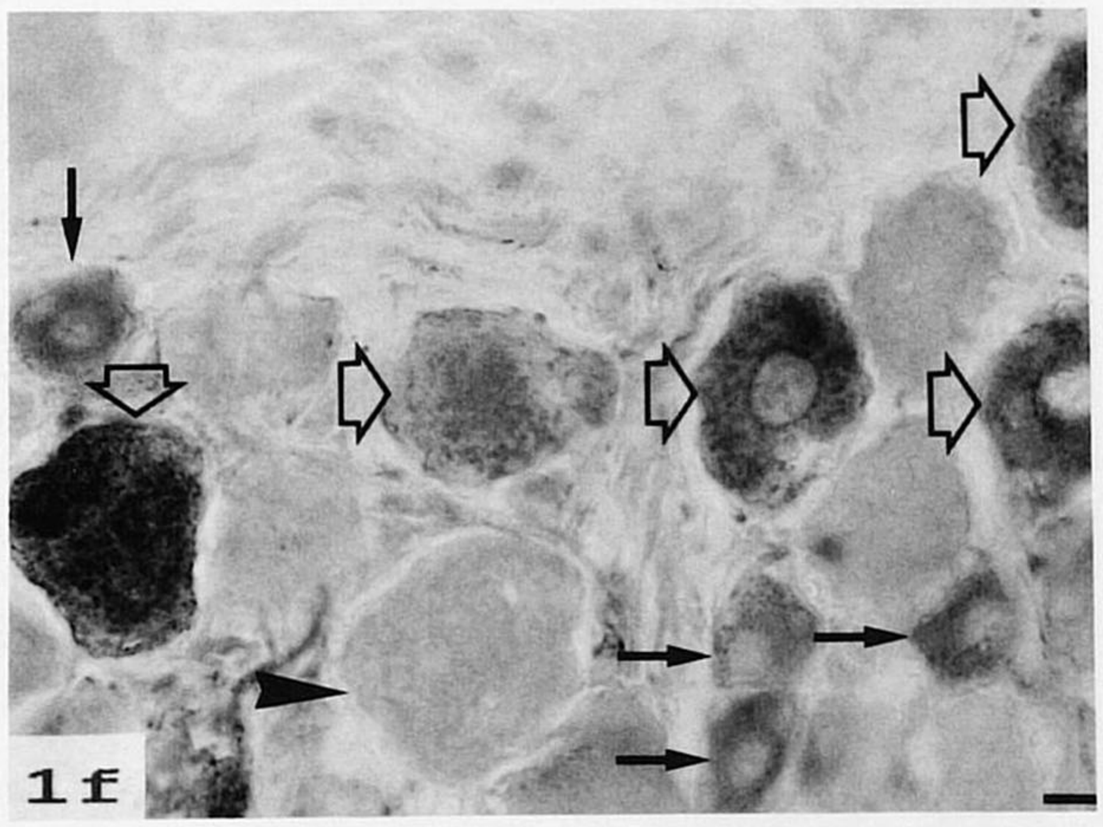


Fig. 1. (a–d) Light micrographs showing B-50 immunostaining in spinal cord segments cervical 3 (a), thoracic 5 (b), lumbar 4 (c), and sacral 2 (d). Note the dense immunolabelling in superficial laminae of the dorsal horn (arrowheads), in the medial portion of the intermediate gray (arrows), and in the intermediolateral nucleus (open arrows in (b)(c)). Clarke's nucleus (asterisks in (b)) is unlabelled. A faint labelling is found in the ventral horn except for a well-circumscribed immunoreactivity in Onuf's nucleus (open arrows in (d)). Scale bars = 500 μm. (e–f) Light micrographs showing B-50 staining in a dorsal root ganglion (segment L4). Immunolabelling predominates within small (arrows) and medium sized (open arrows) neurons. The majority of large neurons (arrowhead) are unlabelled. (f) shows a higher magnification of the inset indicated in (e). Scale bars = 500 μm (e); 30 μm (f).

the intermediolateral column, whereas Clarke's nucleus was unstained. Low levels of B-50 labelling were observed in the ventral horn around motoneurons (lamina IX) in all segments, except for an intense circumscribed staining of the neuropil surrounding motoneurons of Onuf's nucleus in sacral segments 1 and 2. Typical examples of the pattern of B-50-IR distribution are shown in Fig. 1a–d.

In DRG, B-50-IR was present within the somata of numerous small and medium-sized sensory neurons, whereas most of the large sensory neurons were unstained (Fig. 1e,f). Additionally, an irregular staining appeared within nerve fibre bundles of DRG.

Electron microscopy

The following ultrastructural distribution pattern of B-50-IR in the spinal cord was essentially the same in all segments studied.

In the dorsal horn, B-50-IR was found mainly within unmyelinated small diameter nerve fibres. Some axon terminals were also labelled. In agreement with the light microscopic observations, most of the labelled structures were found in Rexed's laminae I and II, indicating that they mainly represent small diameter primary afferents (C-fibres) and some of their terminals (Fig. 2a,b).

In the central portion of the intermediate gray (lamina X), conspicuous B-50-IR occurred in numerous axon terminals (Figs 3, 4). Labelling was also found in many unmyelinated axons in the immediate vicinity of the central canal (Fig. 3). In accordance with the light microscopic findings, the lateral portion of the intermediate gray was unstained.

Intense immunoreactivity was present in the intermediolateral column within numerous axon terminals on preganglionic sympathetic neurons (Fig. 5).

In the ventral horn, some axon terminals contacting motoneuron dendrites (lamina IX) were labelled (Fig. 6a,b). Occasionally, immunoreactivity was found within small diameter axons in this region. The ultrastructural distribution of B-50 in Onuf's nucleus will be described elsewhere (Nacimiento *et al.*, 1993).

Myelinated axons, dendritic and cell body profiles of neurons, as well as glial and vascular cells were devoid of immunoreactivity throughout the spinal cord. The ultrastructural appearance of B-50-positive axon terminals could not be defined consistently because subcellular structures were frequently masked by the histochemical diaminobenzidine reaction product.

In DRG, B-50-IR was found in the soma of some small and medium-sized sensory neurons, whereas the majority of the large sensory neurons were unlabelled. The labelling predominated in the cytoplasm of the most peripheral portions of the perikaryon, particularly within small neuronal processes which protrude into the surrounding unlabelled satellite cells (Fig. 7a,b). In regions where these protrusions were absent, the immunolabelling was only occasionally found just inside the plasma membrane of neuronal somata.

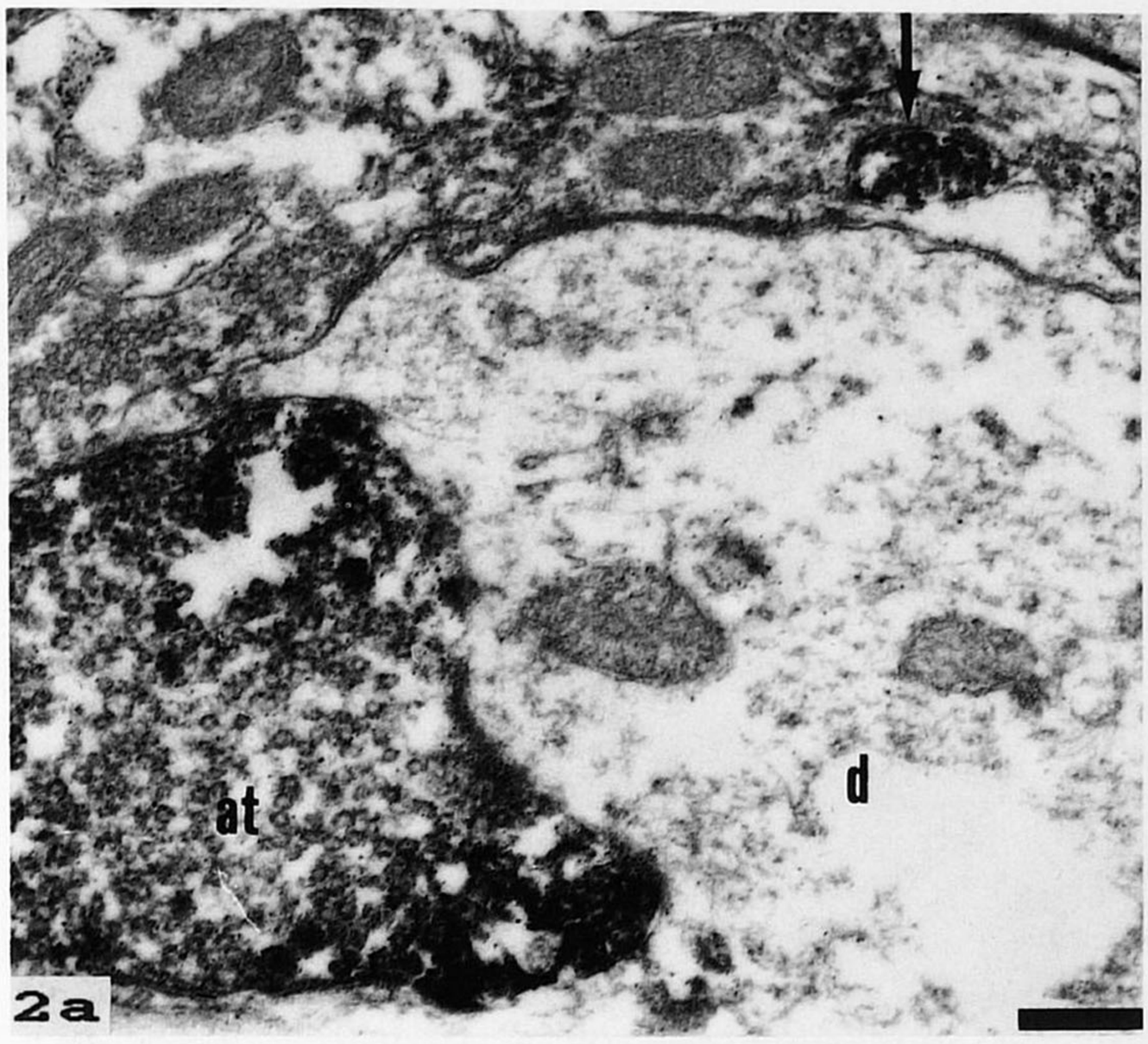
Discussion

The growth-associated protein B-50 is believed to be critical for axonal outgrowth in regenerating and developing neurons (for reviews see Gordon-Weeks, 1989 and Skene, 1989). In mature nervous tissue, B-50 is rapidly expressed in neurons after peripheral nerve injury as a part of as transient regenerative response (Verhaagen *et al.*, 1986; Tetzlaff *et al.*, 1989; Van der Zee *et al.*, 1989; Mashliah *et al.*, 1991; Schreyer & Skene, 1991; Sommervaille *et al.*, 1991; Woolf *et al.*, 1990, 1992). In the adult human and rat CNS, B-50 persists in a distinctive set of neurons, as shown in previous light microscopic studies (Oestreicher *et al.*, 1986; Benowitz *et al.*, 1989; De la Monte *et al.*, 1989) and in recent electron microscopic investigations (DiFiglia *et al.*, 1990; Knyihar-Csillik *et al.*, 1992). The function of this persisting protein is unknown, and its ultrastructural distribution pattern has only been partially described.

In the present investigation we have found that B-50 persists in thin unmyelinated axons in the mature cat spinal cord. The same ultrastructural localization of the protein has been recently described in the uninjured rat spinal cord (Averill *et al.*, 1992; Knyihar-Csillik *et al.*, 1992). Interestingly, oligodendrocyte- and myelin-associated inhibitory proteins play a crucial role in the lack of axonal outgrowth within the mature mammalian CNS (Schnell & Schwab 1990; Schwab, 1990; Cadelli *et al.*, 1992). Thus, B-50 prevails within a subpopulation of nerve fibres which are surrounded by a favourable environment for axonal outgrowth due to the lack of these inhibitory components (Schwab, 1990). In the light of current concepts of CNS regeneration (Schwab, 1990; Cadelli *et*

Fig. 2. Electron micrographs of the dorsal horn (superficial laminae, segment L4). B-50 labelling is present within unmyelinated small diameter nerve fibres (arrows) and some axon terminals (at). The latter are frequently found in synaptic contact with a dendrite (d) as shown in (a). Scale bars = 0.3 μm.

Fig. 3. Medial portion of the intermediate gray in the immediate vicinity of the central canal (segment Th5). B-50-stained axon terminals (at) in close proximity to unstained axon terminals (asterisks) on dendrites (d). Labelling is also present within unmyelinated nerve fibres (arrows). Numerous astrocytic processes (as) are found in this region. Scale bar = 0.3 μm.



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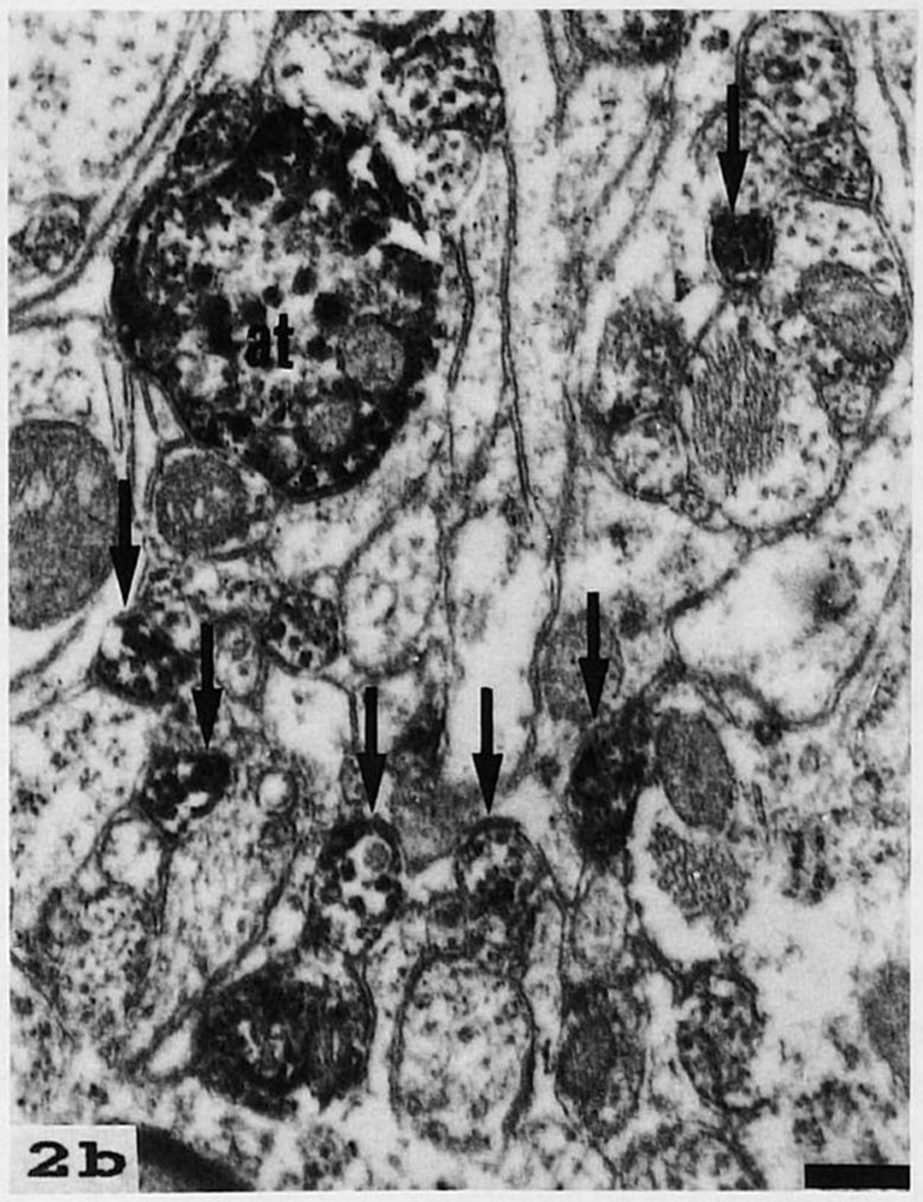

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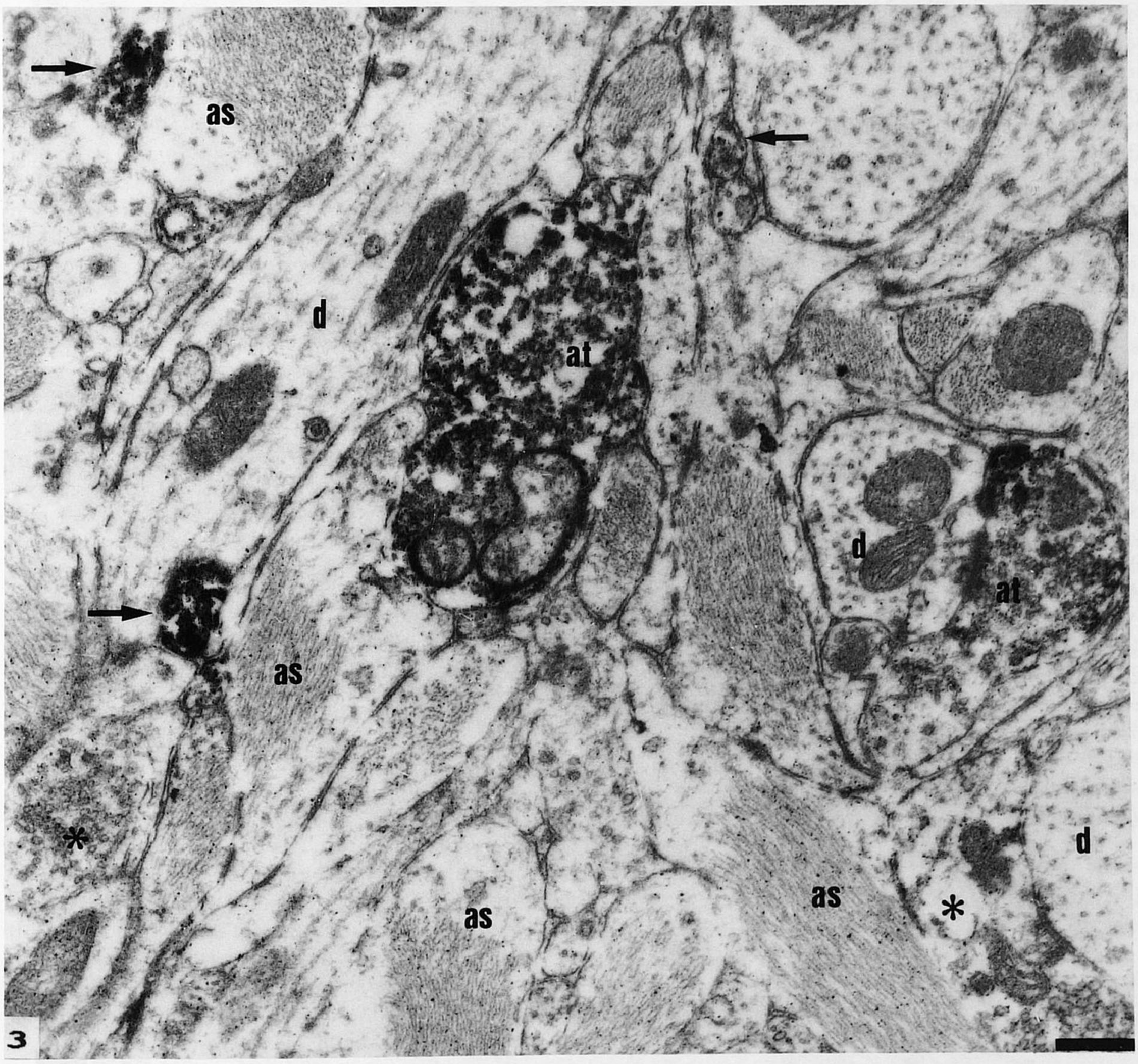

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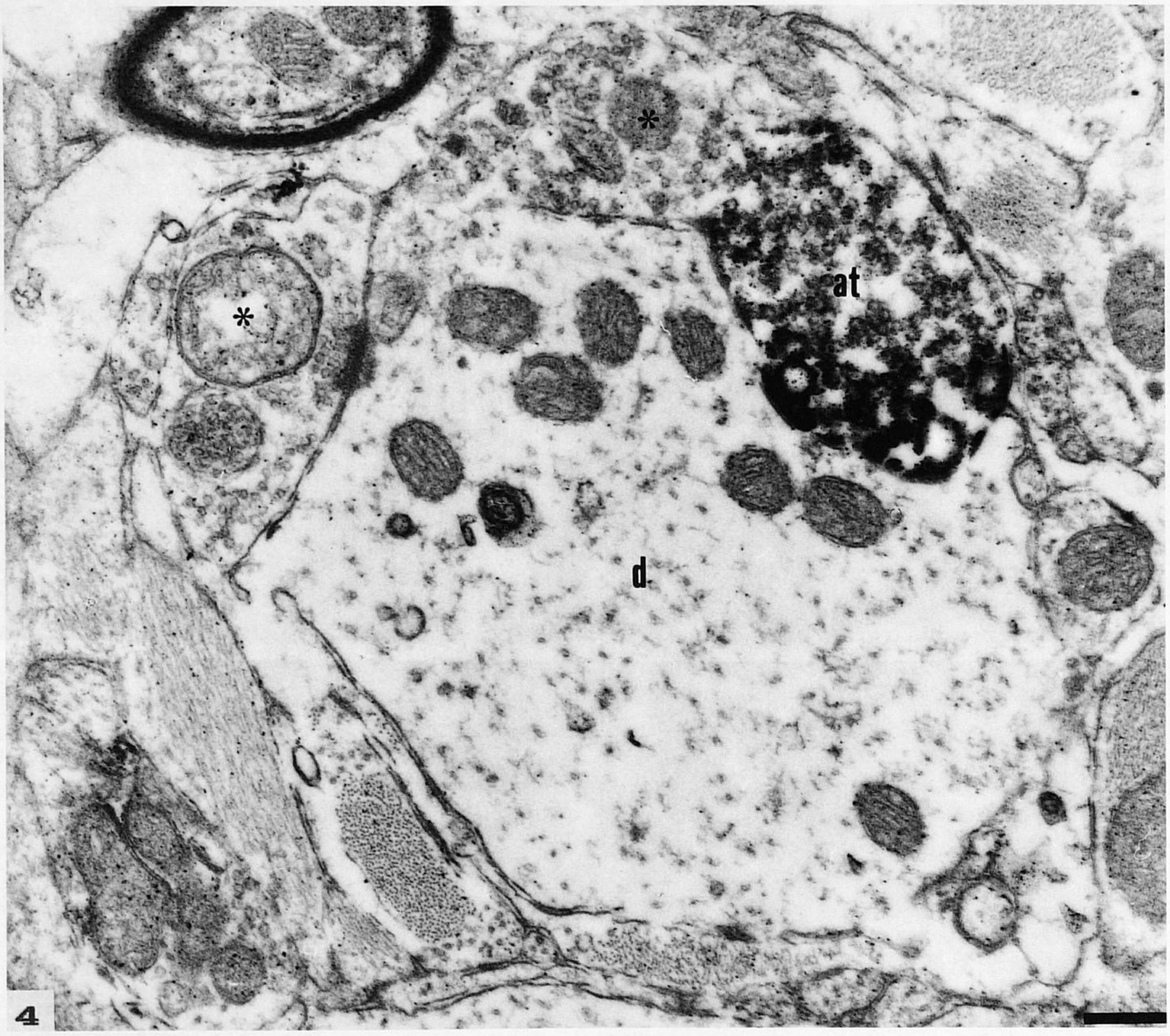

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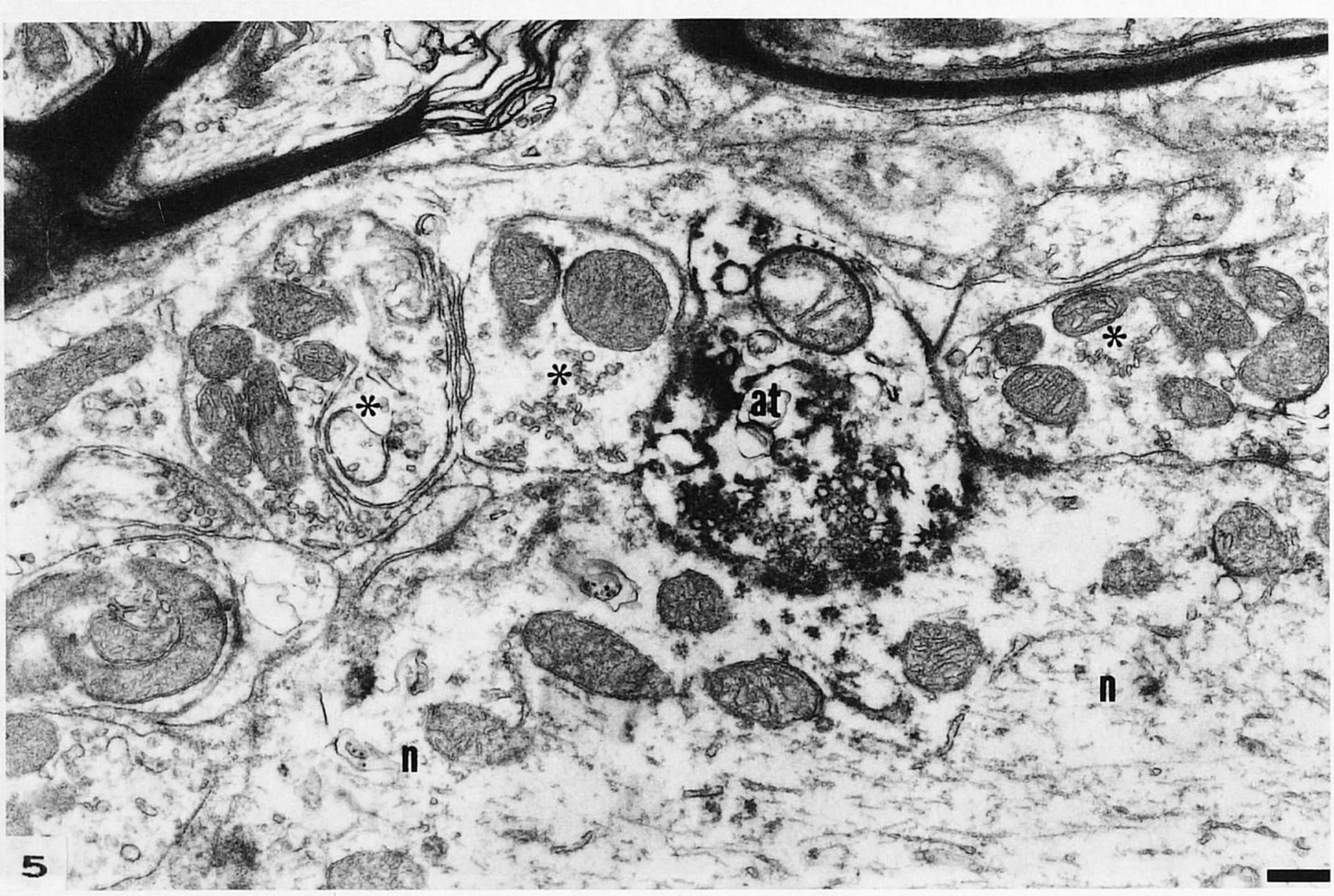

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al., 1992), this preferential location of the protein is in keeping with the idea that B-50-positive systems in the mature spinal cord maintain the capacity to engage in functional plasticity and structural remodeling (Benowitz *et al.*, 1989; De la Monte *et al.*, 1989; Gordon-Weeks, 1989; Skene, 1989; Mashliah *et al.*, 1991). B-50 immunocytochemistry may therefore reveal those systems in the intact mature spinal cord that are in a state of readiness for participating in plasticity. This view is supported by our observation that a high amount of B-50 is present in thin unmyelinated axons in the dorsal horn, which are known to possess an outstanding efficiency for remodeling, as demonstrated in numerous experiments (Hulsebosch & Coggeshall, 1981; Woolf *et al.*, 1990; Yasunobu & Tessler, 1990; McNeill *et al.*, 1991).

We found some B-50-labelled axon terminals in different regions of the spinal gray in close proximity to numerous unlabelled boutons. This staining pattern may indicate that B-50-positive axon terminals are in a state of remodeling. Alternatively, since B-50 has been strongly implicated in the noradrenaline release from synaptosomes (Dekker *et al.*, 1989) and even in long-term potentiation (Lovinger *et al.*, 1985; Nelson *et al.*, 1987), the presence of the protein in boutons may be related to a prolonged increase in synaptic efficacy. A possible function of B-50 in synaptic transmitter release is suggested by our observation that, in some labelled axon terminals, immunoreactivity tends to accumulate close to the presynaptic plasma membrane. Due to the frequent masking of subcellular structures by diaminobenzidine, however, we were not able to determine consistently the vesicle shape and type of synapse, and hence the possible origin of B-50-positive terminals.

The relatively dense GAP-43 (B-50) immunostaining in the neuropil of the cat ventral horn described by Arvidsson and colleagues (1992) is in contrast to the low staining we found in the same region. Antibody differences may account for this discrepancy (Schreyer & Skene, 1991). In a very recent study, Stewart and colleagues (1992) described high levels of GAP-43 (B-50) in the normal peripheral autonomic nervous system of the adult rat using immunohistochemical and immunoblotting techniques. Our demonstration of an intense B-50-IR in the intermediolateral nucleus of the spinal cord suggests that the protein is also associated with intact central autonomic nervous tissue.

The presence of B-50-IR in a sub-population of small- and medium-diameter DRG sensory neurons of the cat is in keeping with previous light microscopic findings in the rat (Schreyer & Skene, 1991; Stewart *et al.*, 1992). Our ultrastructural observation that B-50 preferentially accumulates in the DRG in small neuronal processes protruding into the surrounding satellite cells suggests a functional role for the protein in the outgrowth of these neuronal protrusions. This notion is supported by the fact that the neuronal protrusions are transient structures, even in adulthood (Pannese, 1981). Yet, it has been proposed that the surface of mature sensory neurons in DRG may usually be in a highly dynamic state (Pannese, 1981). In fact, the protrusions show microfilaments and undulating plasmalemma at the ultrastructural level, both of which are characteristic of unstable and growing structures. These protrusions, called *paraphytes* by Nageotte (1907), may be of functional significance by increasing the surface area available for metabolic exchange in the otherwise adendritic cell bodies of DRG neurons (Lieberman, 1976). The presumptive role of B-50 in the outgrowth of paraphytes may explain the abundance of the protein in the perikaryon of primary sensory neurons. The presence of B-50-IR in neuronal somata is a rather surprising finding, since B-50 normally enters fast axonal transport immediately after its neuronal production (Gordon-Weeks, 1989; Skene, 1989), thus escaping accumulation in the perikaryon. At the light microscopic level, the staining in B-50-positive neurons of DRG is detectable throughout the cytoplasm, whereas ultrastructurally it appears confined to the peripheral portion of the soma. This discrepancy may be explained by a relatively low concentration of B-50 in the central portion of neuronal somata as compared to the peripheral part. If so, the cryostat sections would be thick enough to reveal B-50-IR throughout the cell body, whereas in ultrathin sections the staining would not be detectable in the central portion of the soma. Satellite cells grown with sensory neurons in rat DRG cell cultures transiently express GAP-43 (B-50) (Woolf *et al.*, 1990). In contrast, we could not reveal B-50-IR within satellite cells in cat DRG *in situ*.

B-50 immunohistochemistry represents a valuable tool for the reliable demonstration of structural plasticity in nervous tissue (for reviews see Benowitz & Perrone-Bizzozero, 1991 and Gispen *et al.*, 1992). Plasticity may derive from systems in which B-50 persists in adulthood or from systems in which the protein is newly expressed in response to injury.

Fig. 4. Medial portion of the intermediate gray (lamina X segment Th5). B-50-IR is located within an axon terminal (at). Note that the labelling tends to accumulate near the presynaptic membrane. Other axon terminals are in contact with the same dendrite (d) are unstained (asterisks). Scale bar = 0.3 μm.

Fig. 5. B-50 labelling in the intermediolateral nucleus (segment L1) is situated within an axon terminal (at) in contact with the neuronal soma (n). Unstained axon terminals (asterisks) are in close vicinity. Scale bar = 0.3 μm.

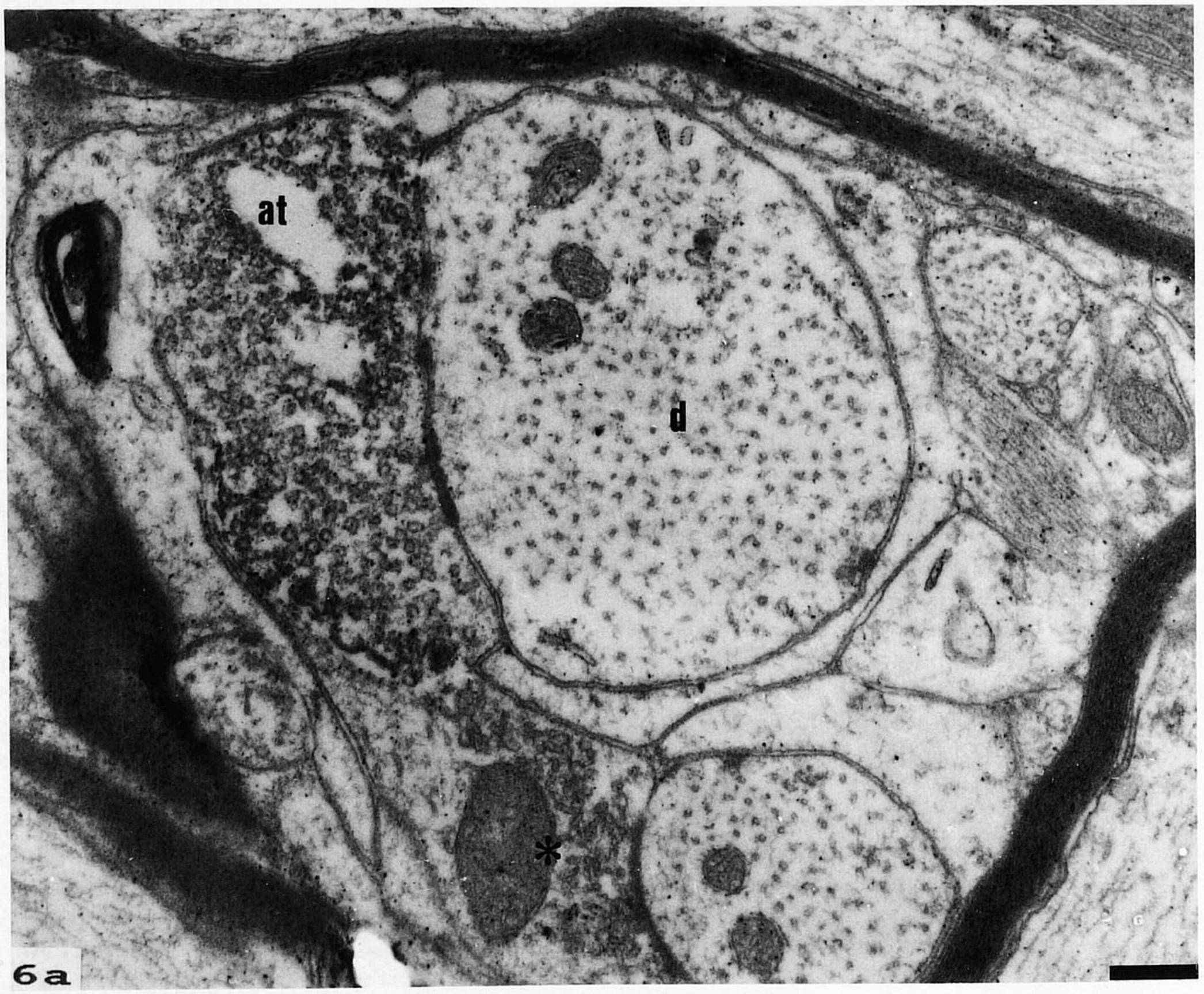

Fig. 6. Ventral horn (segment L4). B-50 staining is located in some axon terminals (at) on dendrites (d). (a) An unstained axon terminal (asterisk) in close proximity to the B-50-positive bouton. The arrow in (b) indicates an unmyelinated axon. Scale

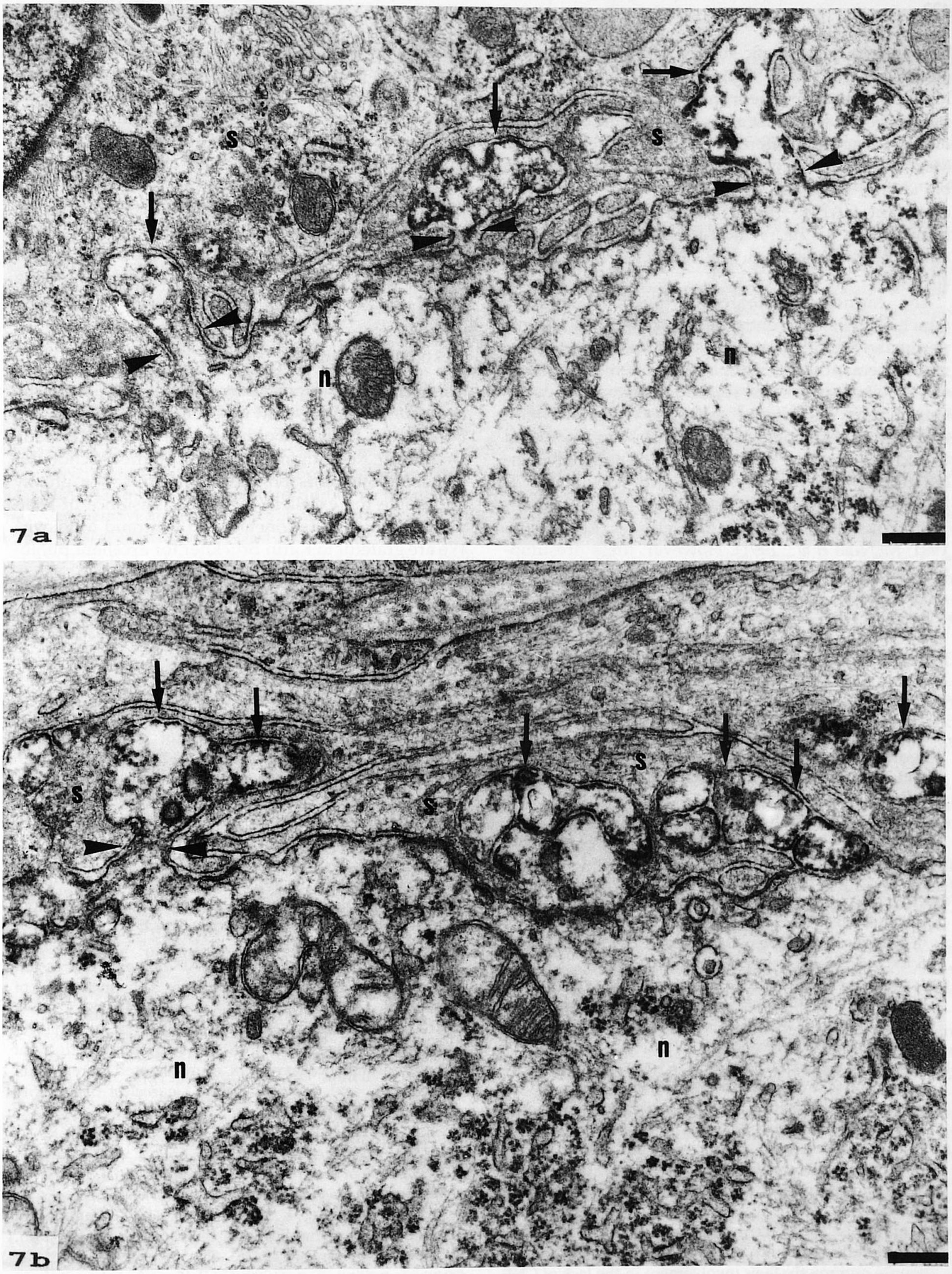

Fig. 7. Electron micrograph of a small dorsal root ganglion neuron (segment L4). B-50 staining predominates in the peripheral portions of the soma and in small projections (arrows) of the neuron (n) which protrude into the surrounding satellite cells (s). These protrusions are continuous with the perikaryon (arrowheads). Frequently, they appear as isolated entities which are completely surrounded by the satellite cells because they arise from the neuron at another level. Scale bar = 0.3 μm.

Recent light microscopic studies revealed a transient increase in GAP-43 (B-50) immunoreactivity in the rat hippocampus during neuronal sprouting and reactive reinnervation by intact remaining systems after removal of specific afferents (Benowitz *et al.*, 1990; Mashliah *et al.*, 1991). Additionally, growth cones can be identified at the ultrastructural level by their abundant GAP-43 (B-50) immunolabelling, as has been shown in the rat dorsal horn after peripheral nerve injury (Coggeshall *et al.*, 1991; Knyhar-Csillik *et al.*, 1992).

The considerable degree of functional recovery which follows different types of spinal cord lesions, particularly in cats, has been attributed to anatomical rearrangement of intact remaining systems (for review see Goldberger & Murray, 1988). Reinnervation by sprouting of undamaged pathways and/or strengthening of pre-existing synapses have been postulated to occur in response to hemisection or partial deafferentation of the mature spinal cord (Goldberger & Murray, 1988; Goshgarian *et al.*, 1989; LaMotte *et al.*, 1989; Polistina *et al.*, 1990; McMahon & Kett-White, 1991; McNeill *et al.*, 1991). However, these presumptive mechanisms of regeneration are a matter of controversy (Rodin *et al.*, 1983; Rodin & Kruger, 1984; Goldberger & Murray, 1988). Moreover, recent experiments have provided evidence that foetal CNS tissue grafts transplanted into lesions of the adult spinal cord induce a considerable remodeling of synaptic circuits in the spinal cord which even appears to restore locomotion (for review see Reier *et al.*, 1992). B-50 immunohistochemistry would be a particularly useful tool for detecting and following such changes. The present data provide a basis for a reliable assessment of structural plasticity in the cat spinal cord following injury.

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Note added in proof

The following articles on GAP-43 (B-50) in the spinal cord and dorsal ganglia of rat and cat have been published after this report was submitted for publication:

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