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B-50/GAP43 Is Localized at the Cytoplasmic Side of the Plasma Membrane in Developing and Adult Rat Pyramidal Tract

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The neuron-specific phosphoprotein B-50/GAP43 has been implicated in axonal outgrowth, since high levels of B-50/GAP43 are found in growth cones and during development of the nervous system. In adult brain, the B-50 levels are decreased. B-50 is primarily found in axons and presynaptic terminals. It is phosphorylated by protein kinase C, and this process has been implicated in the modulation of membrane signal transduction.

During the outgrowth of the pyramidal tract, high levels of B-50 have been reported, whereas a low amount of B-50 persists into the adult stage. By immunoelectron microscopy, using immunogold labeling on cryosections and pre-embedding peroxidase labeling, we examined the distribution of B-50 in the pyramidal tract at the third cervical segment in developing 2-d-old and adult 90-d-old rats. B-50 immunoreactivity was found in axons and growth cones of the outgrowing tract. In the adult pyramidal tract, both unmyelinated and myelinated axons contained B-50 immunoreactivity. The immunogold label was predominantly located at the plasma membrane. Since the peroxidase reaction product was observed exclusively intracellularly, we conclude that the B-50 immunoreactivity is predominantly located at the cytoplasmic side of the plasma membrane of axons and growth cones. The high immunoreactivity in growth cones and axons of the outgrowing pyramidal tract further supports the hypothesis that B-50 plays a role in neurite outgrowth. The presence of B-50 in the adult pyramidal tract cannot merely be attributed to transport to the synapse. Therefore, it is suggested that B-50 plays, in addition, a local, growth-associated role in the adult tract.

During the development of the nervous system, nerve cells extend neurites that grow over long distances, navigating by means of growth cones toward their targets to establish synaptic contacts. In the peripheral nervous system, this ability is preserved throughout life and is called on in the process of regeneration. Although in the adult mammalian CNS the capacity of axonal growth is largely lost, modifications can occur during adult life at a smaller scale of the axonal terminal arbor and the synapse (Cotman and Nieto-Sampedro, 1984). Work on the molecular basis of these processes has shown an interesting convergence by the identification in different laboratories of a neuron-specific phosphoprotein, known as B-50, GAP43, F1, pp46, and P-57, which has been found to be involved in neuritogenesis, axon regeneration, and synaptic plasticity (Benowitz and Routtenberg, 1987).

B-50/GAP43 is a member of a small group of growth associated proteins (GAPs) (Skene and Willard, 1981; Willard et al., 1985) characterized by a dramatic increase in synthesis during axonal regeneration in nonmammalian nervous system and in vertebrate peripheral nervous system. Furthermore, high levels of B-50/GAP43 are correlated with neurite outgrowth during CNS development (Jacobson et al., 1986; Zwiers et al., 1987) and with NGF-induced neurite outgrowth in pheochromocytoma PC12 cells (Van Hooff et al., 1986, 1989). B-50 is also identical to pp46, a major phosphoprotein constituent of nerve growth cone fractions isolated from fetal rat brain (Katz et al., 1985; De Graan et al., 1985). By immunocytochemistry, B-50 has been localized in outgrowing neurites during CNS development (Oestreicher et al., 1983a; Oestreicher and Gispen, 1986; Gorgels et al., 1987; McGuire et al., 1988), in regenerating axons of peripheral nerve *in situ* (Verhaagen et al., 1986, 1988), and in outgrowing neurites and growth cones *in vitro* (Meiri et al., 1986). The expression of B-50 is developmentally regulated, and highest levels are found in neonatal rat brain (Jacobson et al., 1986; Zwiers et al., 1987). B-50 persists into the adult rat CNS. It is then predominantly located in neuropil-rich areas (Oestreicher and Gispen, 1986; Benowitz et al., 1988), where it appears to be restricted to presynaptic terminals (Gispen et al., 1985). In membranes of growth cones and outgrowing neurites isolated from fetal and postnatal rat brain and in synaptosomes, B-50 acts as a major substrate of protein kinase C (Oestreicher et al., 1982; De Graan et al., 1985, 1986; Van Hooff et al., 1988; Dekker et al., 1989). Phosphorylation of B-50 may serve to modulate membrane signal transduction in these structures (Gispen, 1986).

The pyramidal tract (PT) represents an adequate model system to document the relation between the expression of B-50 and the development of central nerve tracts. The ontogeny of the pyramidal tract has been widely studied, especially in rodents. The spatial and temporal outgrowth of its fibers in the spinal cord have been described (e.g., in the rat: Schreyer and Jones, 1982; Gribnau et al., 1986; in the hamster: Reh and Kalil, 1981). Concurrent with its proposed role in neurite outgrowth,

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high levels of B-50/GAP43 have been reported during the outgrowth of the PT in the rat and in the hamster (Kalil and Skene, 1986; Gorgels et al., 1987). Although low levels of B-50 persist into the adult stage, the failure of the adult PT to regenerate has been correlated with a lack of increase of B-50/GAP43 synthesis on transection of the mature tract (Kalil and Skene, 1986; Reh et al., 1987). In the present study, we analyzed by immunocytochemistry the ultrastructural localization of B50 in the outgrowing PT as well as in the adult PT.

Materials and Methods

Animals and tissue processing. Two-d-old and adult (90-d-old) Wistar rats were anesthetized by intraperitoneal injection of an aqueous solution of sodium pentobarbital and perfused intracardially with a fixative consisting of 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The third cervical spinal cord segment was then removed and immersed overnight in the fixative at 4°C. Experiments were also carried out with a fixative containing 0.5% glutaraldehyde instead of 2% glutaraldehyde, without apparent change in immunoreactivity. For pre-embedding peroxidase immunocytochemistry, 50 μm thick transverse Vibratome sections were cut of this segment into chilled Tris-buffered saline (TBS: 0.1 M Tris, 0.9% NaCl, pH 7.6). For cryoultramicrotomy, the tissue segments were stored up to 4 d in 1% paraformaldehyde in PBS (0.1 M phosphate buffer, containing 0.9% NaCl, pH 7.4) until further processing.

Pre-embedding peroxidase labeling. Free-floating Vibratome sections were incubated in 5% normal goat serum in TBS containing 0.1% BSA (TBS/BSA) for 30 min. Next, the sections were rinsed in TBS/BSA (3 times 5 min) and incubated for 48 hr at 4°C in affinity-purified rabbit anti B-50 immunoglobulins (a-B-50 IgGs of rabbit 8420) (Oestreicher et al., 1983b; Oestreicher and Gispen, 1986), diluted 1:8000 in TBS/BSA. Control sections were incubated in TBS/BSA or in preimmune IgGs diluted in TBS/BSA. Immunodetection was performed using the avidine biotin procedure (ABC kit, Vector). Sections were rinsed in TBS/BSA (3 times 5 min) and incubated for 90 min in biotinylated goat antirabbit antibodies in TBS/BSA and subsequently, after rinsing in buffer, in avidine-HRP complex in TBS/BSA for 90 min. Next, the sections were rinsed in buffer and incubated in 0.05% diaminobenzidine tetrahydrochloride (DAB) in 0.05 M Tris-HCl buffer, pH 7.6, containing 0.01% hydrogen peroxide. The staining reaction was terminated after 5 min by rinsing the sections in TBS. Some sections were washed in bidistilled water, mounted on glass slides, and embedded in Entellan for light microscopic analysis. Other sections were postfixed for 1 hr in 1% OsO_4, 1.5% K_4FeCN_6 in 0.1 M phosphate buffer, pH 7.4. After rinsing in phosphate buffer and in bidistilled water, the sections were stained en bloc in 0.5% aqueous uranyl acetate for 1 hr. Sections were dehydrated in an ascending series of ethanol and in propylene oxide, transferred into a mixture of propylene oxide and Epon (1:1) for 2 hr, and left overnight in Epon. The sections were flat embedded in Epon between slides coated with Repelcoat (dimethyldichlorsilane). After polymerization, the tissue was reembedded on Epon blocks, and ultrathin sections were cut. The sections were not counterstained and were analyzed in a Philips EM 300 electron microscope at an accelerating voltage of 60 kV.

Immunogold labeling of cryosections. The tissue segments were rinsed in PBS and incubated for 1 hr in 5% gelatin in PBS in small Eppendorf cups at 37°C. The gelatin containing the tissue blocks was fixed overnight in 2% paraformaldehyde, 2% glutaraldehyde in PBS, and the PT was dissected out using a razor blade. After immersion in 1.15 M sucrose, 15% polyvinyl-pyrrolidone-10 (PVP) in PBS, the tissue was rapidly frozen in liquid nitrogen. Transverse ultrathin cryosections were cut at −100°C on a Reichert Ultracut microtome (Tokuyasu, 1984). Sections were picked up from the knife and mounted on nickel grids using a drop of 2.3 M sucrose in PBS. The grids were rinsed on drops of PBS containing 50 mM glycine and on drops of PBG (0.1% gelatin, 0.5% BSA in PBS) and incubated overnight at 4°C in affinity-purified rabbit anti-B-50 IgGs (of rabbit 8613; Oestreicher et al., 1983b; Oestreicher and Gispen, 1986) diluted 1:300 in PBG. Control sections were incubated in PBG or in preimmune IgGs diluted in PBG. Next, the grids were rinsed in PBG and incubated in goat antirabbit conjugated gold (GAR-gold, diameter 9.3 nm; Van Bergen en Henegouwen and Leunissen, 1986) in PBG for 1 hr. After rinsing in bidistilled water, the grids were incubated for 10 min in 1.1% tylose, 0.5% uranyl acetate, pH 4.0, on ice. The grids were air-dried and examined in a Philips EM 300 electron microscope at 60 kV.

For both immunolabeling procedures, at least 2 animals per age group were examined, giving similar results.

Results

Light microscopic examination of the Vibratome sections confirmed the developmental pattern in B-50 expression in the spinal cord as has been described previously using different techniques (Gorgels et al., 1987). In the 2-d-old rat, immunoreactivity was high in the gray and even higher in the white matter, whereas in the adult rat, the immunoreactivity was greatly reduced, especially in the white matter. In this study, we focused on the expression of B-50 in the PT during its outgrowth phase (at P2) and at the mature stage (at 90 d of age). In the 2-d-old rat, high immunoreactivity was present in the dorsal funiculus, including the PT (Fig. 1*A*). The posterior median septum consisting of glial cells was not stained. In the 3-month-old rat, the immunoreactivity was greatly reduced in the dorsal funiculus, but in contrast to the ascending tracts, the PT still displayed a reduced but distinct immunoreactivity (Fig. 1*B*).

Immunoelectron microscopy of the outgrowing pyramidal tract

The ultrastructure of the developing cervical PT in the rat has been described by De Kort et al. (1985). The bundle consists of small, unmyelinated axons and interlaced glial elements. At their distal ends, the axons bear growth cones characterized by their larger diameter, the presence of vesicles, and a meshwork of smooth endoplasmic reticulum. Distally, these growth cones taper into filopodia.

At the electron microscopic level, the immunoreaction for B-50 in the sections stained with the pre-embedding technique was revealed by the presence of the electron-dense osmicated DAB reaction product. The intensity of immunolabeling was greatest on the surface of the Vibratome sections and decreased with depth. The maximal penetration of the immunoreaction was 4–5 μm. In ultrathin sections close to the surface of the Vibratome sections of the 2-d-old rat, practically all PT axons were heavily stained, whereas glial cell bodies and their processes were not stained (Fig. 2*A*). Growth cones and filopodia were more readily identified in less superficial sections, since in these sections, the intensity of the immunoreaction was reduced and more intracellular details could be discerned (Fig. 2, *B, C, D*). In these tissue sections containing slices of labeled growth cones, the reaction product was predominantly deposited near the plasma membrane, whereas the meshwork of smooth endoplasmic reticulum in the central region of the growth cone showed no immunoreaction (Fig. 2, *B, C*). In filopodia, the reaction product was deposited throughout the whole cytosolic compartment (Fig. 2*D*). No reaction product was detected extracellularly.

In the cryosections stained with the immunogold technique, all axons in the PT of the 2-d-old rat were labeled (Fig. 3*A*). To

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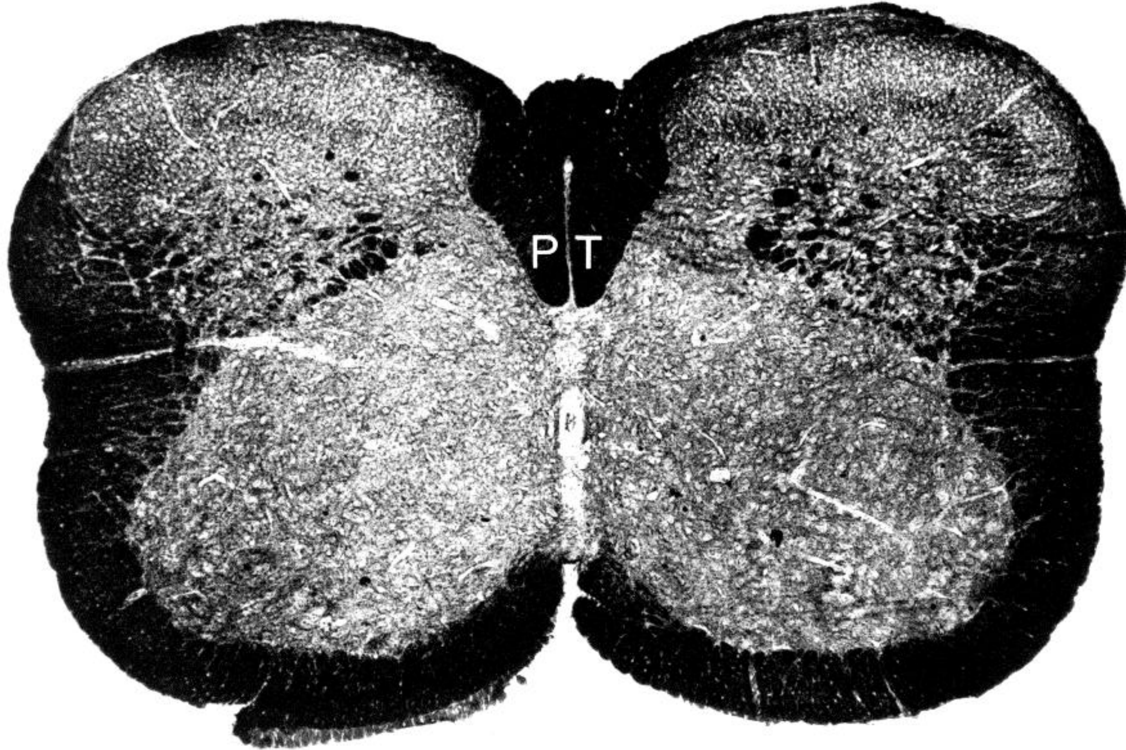
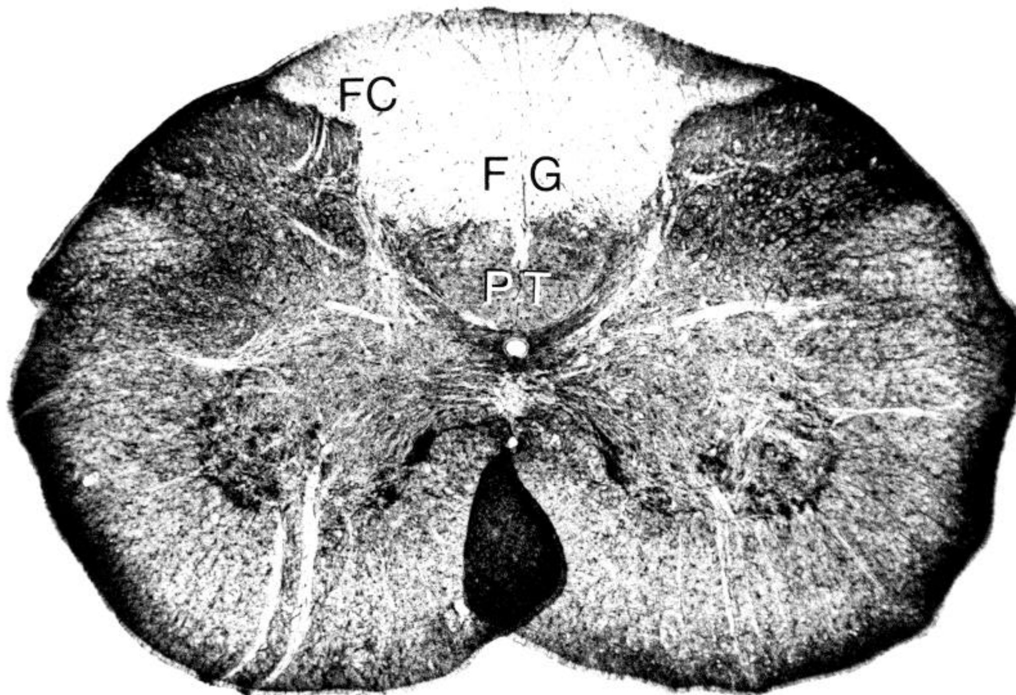
Figure 1. Distribution of B-50 immunoreactivity as detected with the PAP method in transverse Vibratome sections of the third cervical segment in the 2-d-old rat (*A*) and in the 90-d-old rat (*B*). In the 2-d-old rat, intense immunoreactivity was found in the dorsal funiculus, where the *PT*





occupies the ventralmost position. In the adult rat, the immunoreactivity in the dorsal funiculus was greatly reduced. Some immunoreactivity persisted, however, in the PT, whereas the ascending sensory tracts in the dorsal funiculus, the fasciculus gracilis (*FG*), and the fasciculus cuneatus (*FC*) were essentially negative. Scale bars, 0.25 mm (*A*) and 0.5 mm (*B*).

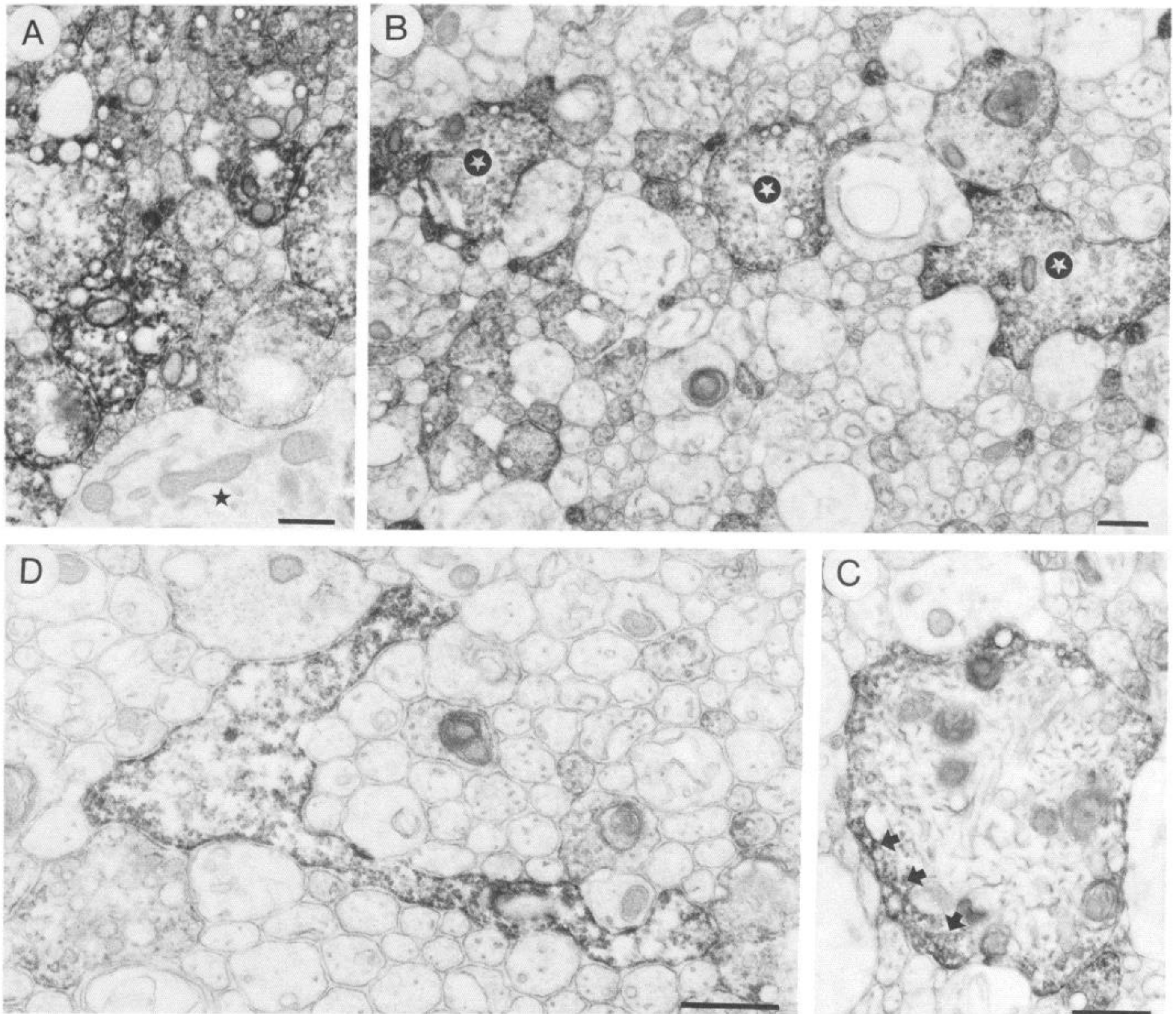


Figure 2. Immunoelectron microscopic localization of B-50 in the PT at the third cervical segment in the 2-d-old rat, using pre-embedding peroxidase labeling. In ultrathin sections taken close to the surface of the Vibratome section (*A*), essentially all axons were intensely labeled, whereas the glial elements (*black asterisk*) were devoid of label. In less superficial sections, the intensity of the immunoreaction was reduced, and labeled growth cones (*B, white asterisk*; *C*) and filopodia (*D*) could be identified. The reaction product was deposited in growth cones near the plasma membrane (*C*), whereas in the filopodia, it was detected also in the cytosol (*D*). No label was observed extracellularly. Scale bars, 0.5 μm.

a great extent, the label was located at the axolemna. In growth cones similarly, the majority of the gold particles were located at the plasma membrane (Fig. 3*C*). Occasionally, some label was encountered in the cytoplasm of axons and growth cones. Although this label sometimes was found close to vesicles, no consistent labeling of vesicles was observed. Mitochondria were mostly free of immunolabeling. The meshwork of agranular reticulum in the central part of the growth cone showed hardly any label. Filopodia could not unambiguously be identified in the cryosections. Control sections incubated in the preimmune serum (Fig. 3*B*) or in buffer showed only a few scattered gold particles.

Immunoelectron microscopy of the adult PT

The mature PT contains, in addition to myelinated axons, unmyelinated axons (Leenen et al., 1982; Joosten and Gribnau, 1988; Gorgels et al., 1989). In the mature PT stained with the pre-embedding technique, the intensity of the immunoreaction

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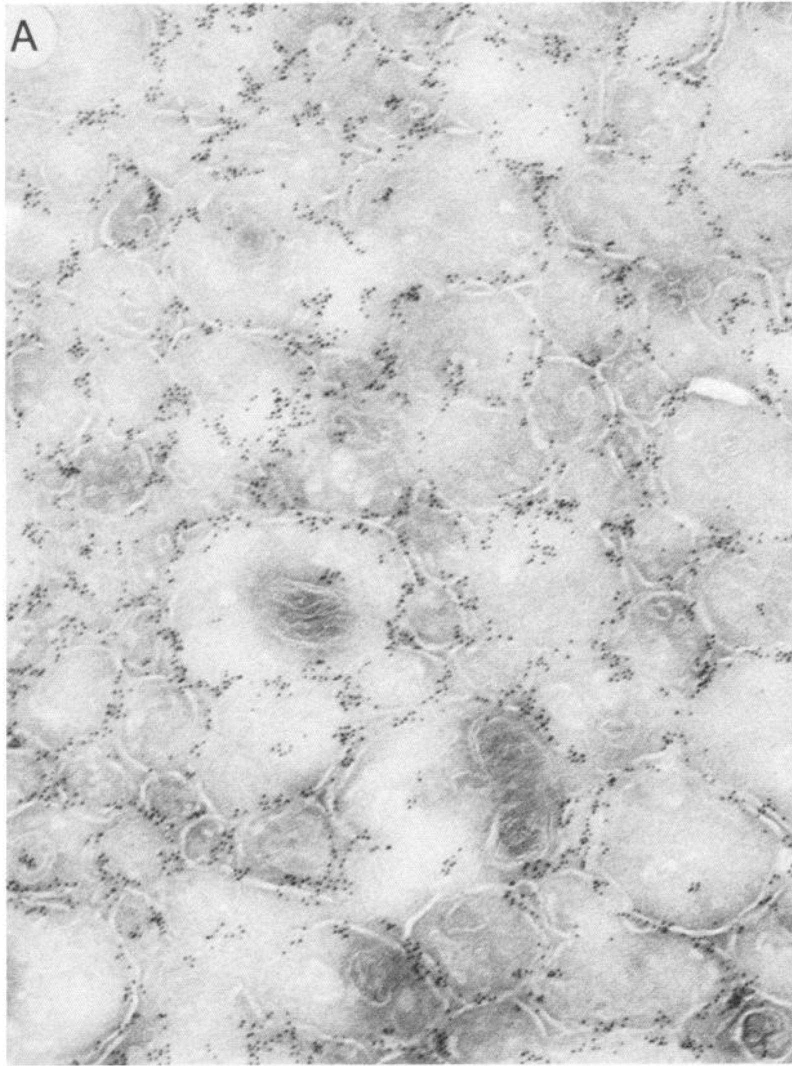
Figure 3. Immunogold labeling of cryosections of the 2-d-old PT, using GAR-gold particles (9.3 nm) following incubation with affinity purified anti-B-50 IgGs (*A,C*) or with preimmune IgGs as negative control (*B*). The gold particles were mainly located at the axolemna of the axons (*A*). Note the absence of label in sections stained with preimmune IgGs (*B*). In growth cones (*C, asterisks*) similarly, the majority of gold particles was found at the plasma membrane. Some label was observed in the cytoplasm of axons and growth cones. Scale bars, 0.5 μm.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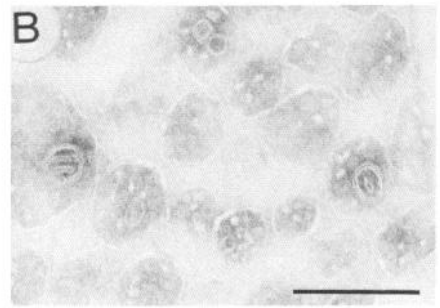
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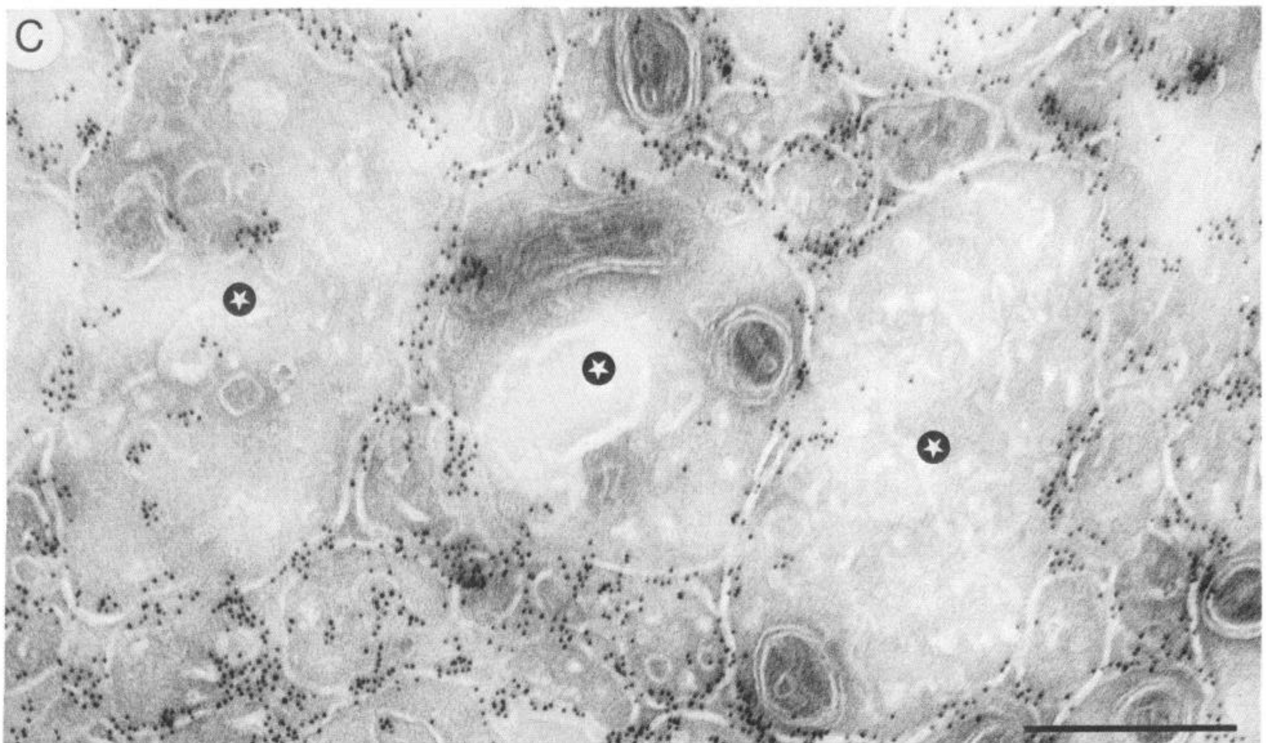
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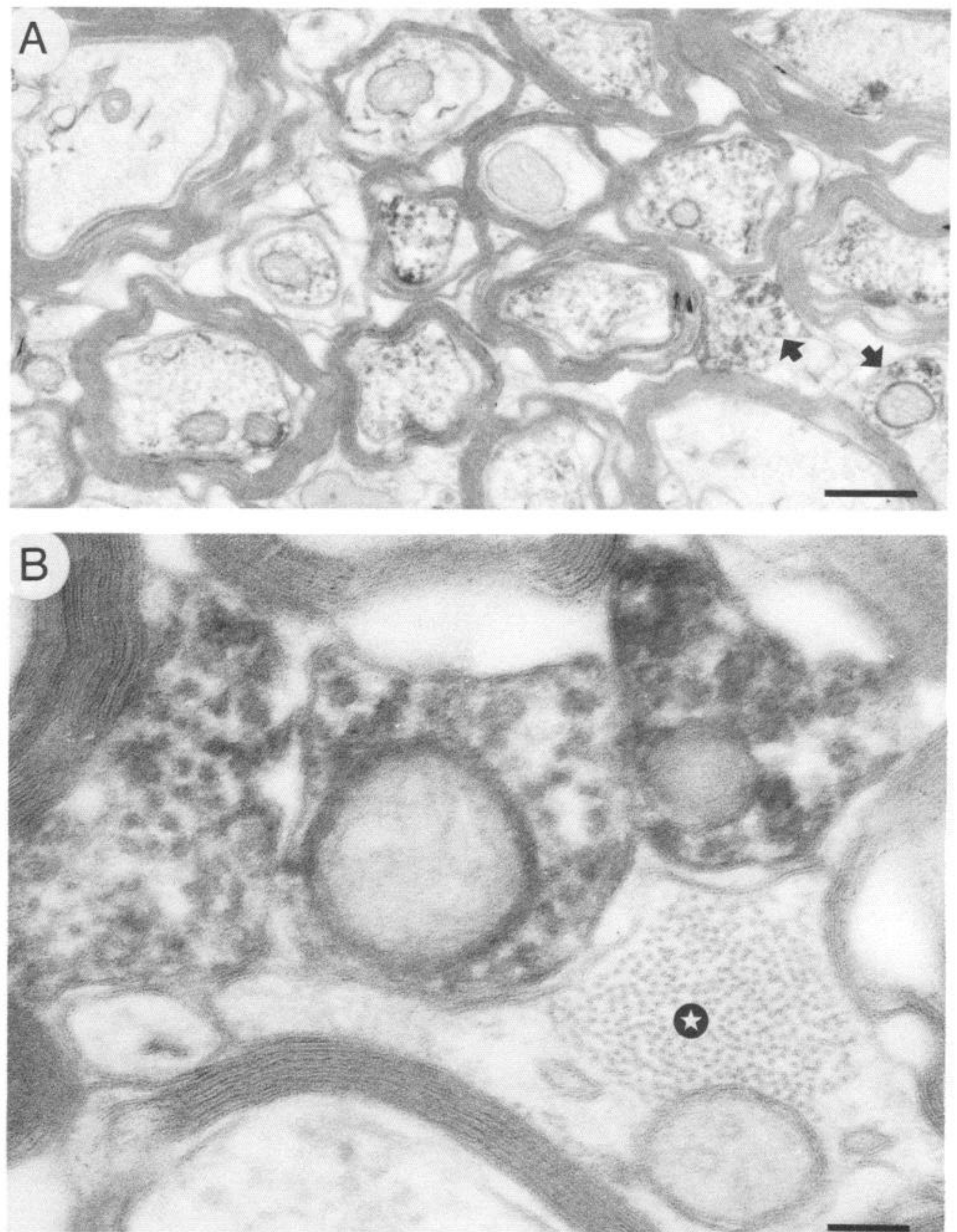

Figure 4. B-50 immunoreactivity in the adult PT detected with pre-embedding peroxidase labeling. Staining was observed in unmyelinated (*arrows*) as well as in myelinated axons (*A*). Large myelinated axons were not stained. The reaction product was contained within the axon (*B*). Astrocyte process (*asterisk*) was not stained. Scale bars, 0.5 μm (*A*) and 0.1 μm (*B*).

even in superficial layers of the Vibratome sections was reduced as compared to the early postnatal PT. Even so, many unmyelinated axons as well as myelinated axons were stained (Fig. 4). The reaction product was exclusively contained within the axons, indicating that the antigen is located intracellularly. The intensity of the immunoreaction varied greatly. In general, however, large myelinated axons (diameter >1 μm) (Gorgels et al., 1989) were hardly stained. Processes of astrocytes and oligodendrocytes were not labeled.

The immunogold technique applied to the cryosections of the adult PT showed B-50 immunoreactivity predominantly at the axolemma of unmyelinated and myelinated axons. In addition, some label was encountered in the axoplasm (Fig. 5). Although occasionally the cytoplasmic label was found close to vesicles, in general no specific association with organelles was apparent. Practically all axons were labeled. The intensity of the immunolabeling was quite variable. On screening many sections, it appeared that the largest myelinated axons (>1 μm) were less intensely labeled.

Discussion

Previous studies on B-50 expression in the PT have shown that during the outgrowth of its fibers high levels of B-50 are present in the tract, and some B-50 persists into the adult stage (Kalil and Skene, 1986; Gorgels et al., 1987). In the present study, this general developmental pattern was corroborated, and in addition, the ultrastructural localization of B-50 was established using immunogold labeling on cryosections and pre-embedding peroxidase labeling. The results of both techniques demonstrate B-50 immunoreactivity in axons and growth cones of the outgrowing PT, as well as in unmyelinated and myelinated axons

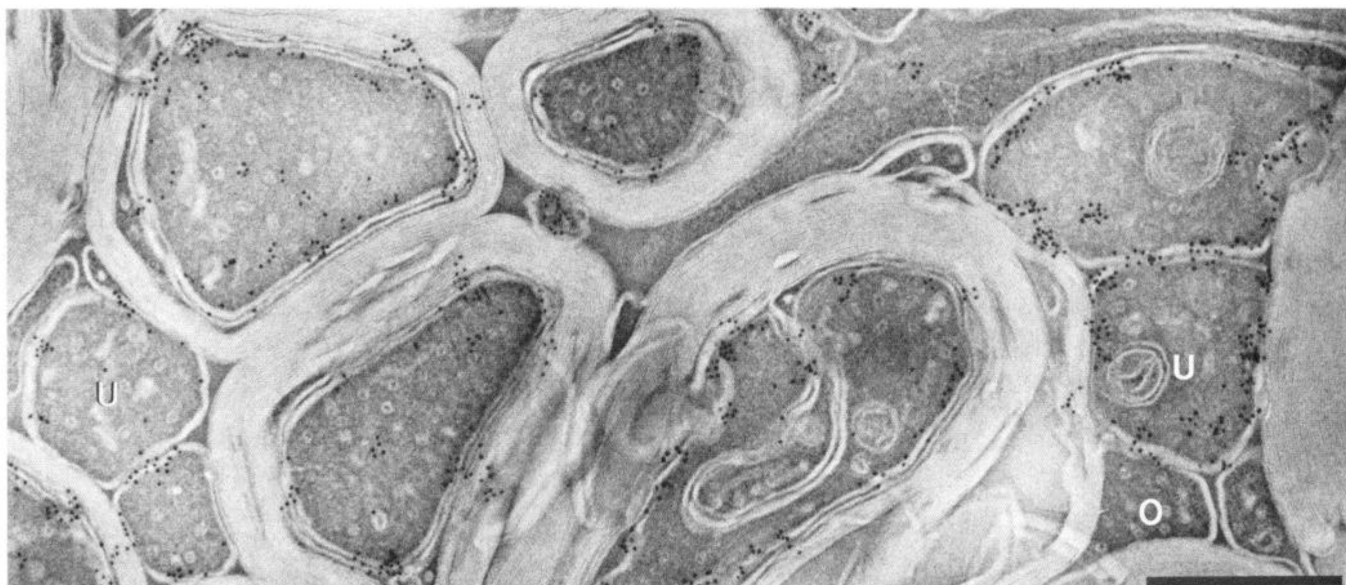

Figure 5. Immunogold labeling of B-50, using GAR-gold particles (9.3 nm) in cryosections of the adult rat PT at the third cervical segment. Unmyelinated as well as myelinated axons were stained predominantly, although not exclusively at the plasma membrane. Virtually no label was observed on oligodendrocyte processes. *O,* outer tongue process. Scale bar, 0.5 μm.

of the adult tract. By immunogold labeling, we found that immunoreactivity was located predominantly at the plasma membrane of axons as well as growth cones. Since with the preembedding peroxidase technique no label was detected extracellularly and because the primary structure of B-50 contains no membrane spanning domain (Nielander et al., 1987), we conclude that B-50 is predominantly located at the cytoplasmic side of the plasma membrane.

Localization of the antigen by immunocytochemistry depends on the antibody and the method specificity. The latter was tested by incubation with the preimmune serum and with buffer. The specificity of the affinity-purified antibodies has been characterized in previous studies (Oestreicher et al., 1983a, b; Oestreicher and Gispen, 1986; Van Hooff et al., 1988, 1989; Van Lookeren Campagne et al., 1989). The polyclonal antibodies used in the present study recognize B-50 irrespective of its phosphorylation state (Oestreicher et al., 1986; De Graan et al., 1989) and irrespective of its binding to calmodulin (A. B. Oestreicher and P. N. E. De Graan, unpublished observations). We, therefore, think that the distribution of B-50 immunoreactivity corresponds to the actual distribution of B-50 in the rat PT (Oestreicher et al., 1986).

By immunocryoultramicrotomy, B-50 has been localized at the cytoplasmic face of the plasma membrane in presynaptic terminals, synaptosomes, and nerve growth cone particles (Gispen et al., 1985; Van Lookeren Campagne et al., 1989). The present *in situ* study indicates that this localization also holds true for growth cones and myelinated and unmyelinated axons in the PT. B-50 also is present in filopodia, as shown by peroxidase labeling. Since the DAB reaction product generated by the peroxidase technique can diffuse away from the site of generation (Courtoy et al., 1983), the subcellular localization of B-50 within the filopodia could not be determined. Van Hooff et al. (1989) showed that in filopodia of outgrowing neurites of PC12 cells, B-50 is also predominantly associated with the inner side of the plasma membrane.

Apart from the localization at the plasma membrane, a small fraction of the B-50 immunoreactivity was found in the cytoplasm of growth cones and axons. Although some of this cytoplasmic label might be associated with vesicles, in general, no specific association with organelles could be determined. Similar observations were made by Van Lookeren Campagne et al. (1989), studying isolated growth cones and synaptosomes. A possible explanation for this cytosolic B-50 comes from a recent study by Skene and Virág (1989), who showed that B-50/GAP43 maintains reversible interactions with the growth cone membrane by fatty acylation, probably of the cysteine residues at the amino terminus. On the other hand artifactual dissociation of the highly hydrophilic B-50 (Nielander et al., 1987) during the fixation and the immunocytochemical procedure cannot be ruled out.

The high immunoreactivity in the outgrowing PT supports the hypothesis that B-50 is important for axon growth. Furthermore, the B-50 localization in the growth cone suggests a role for B-50 in neurite elongation and target-directed navigation (Lockerbie, 1987). Biochemical experiments on synaptosomes and growth cone particles have indicated that B-50 is a prominent substrate of protein kinase C, which is shown to be activated as a result of receptor-mediated hydrolysis of phosphatidyl inositol bisphosphate (Van Hooff et al., 1988; Dekker et al., 1989). In this neuronal membrane signal transduction, phosphorylation of B-50 is supposed to serve as a negative feedback control by inhibition of the membrane-associated PIP kinase (Gispen, 1986). The localization of B-50 at the cytoplasmic side of the plasma membrane agrees well with this model. Another mechanism of B-50 function is suggested by the identity of B-50 to P-57, an atypical calmodulin binding protein, which releases calmodulin and then can be phosphorylated (Cimler et al., 1987). The importance of calmodulin and local Ca^{2+} levels on nerve growth cone functioning has been well documented (Lockerbie, 1987; Kater et al., 1988).

Previous studies on the localization of B-50 in the adult rat brain have emphasized its localization in neuropil-rich areas (Oestreicher and Gispen, 1986; Oestreicher et al., 1986; Be-

nowitz et al., 1988; McGuire et al., 1988), particularly in presynaptic terminals (Gispen et al., 1985). Nonetheless, in the adult PT, some B-50 immunoreactivity remains present in unmyelinated and myelinated axons. Part of this immunoreactivity might reflect transport of B-50 to the synapse. In addition, however, the predominant localization of B-50 at the axolemma of unmyelinated and myelinated axons suggests a local role for B-50 within the adult PT. Interestingly, the distribution of protein kinase C in the dorsal funiculus of the rat cervical spinal cord is very similar to that of B-50, the ascending fiber tracts being essentially devoid of immunoreactivity and the PT being moderately densely stained (Saito et al., 1988). The function of B-50 in the adult rat PT is unknown. The implication of B-50 in axonal growth and our observation that the largest myelinated axons are less B-50 immunoreactive suggest that PT axons retain a neuroplastic capacity for increase in diameter, elongation, or possibly sprouting.

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