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Ultrastructural immunocytochemical localization of B-50/GAP43, a protein kinase C substrate, in isolated presynaptic nerve terminals and neuronal growth cones

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Summary

Accumulating evidence indicates that the neuron-specific B-50/GAP43, a substrate for protein kinase C, plays a role in neuronal differentiation and neuritogenesis during nervous tissue development and axonal regeneration. An ultrastructural immunocytochemical study on the localization of B-50 in presynaptic terminals (synaptosomes) and neuronal growth cones was carried out by means of cryoultramicrotomy with affinity-purified B-50 antibodies. Detection was accomplished with colloidal gold, conjugated either to protein-A or goat anti-rabbit immunoglobulins. In synaptosomes, isolated from the frontal cortex of 6-week-old rats, and in neuronal growth cones, isolated from forebrains of 5-day-old rats, the majority of B-50 is detected at the surrounding neuronal plasma membrane. In both neuronal growth cones and synaptosomes, a relatively small fraction of B-50 in the cytoplasm was not evidently associated with internal membranes. Our results indicate that B-50 is mainly located at the cytoplasmic face of the synaptosomal and neuronal growth cone plasma membrane. The similar B-50 localization in neuronal growth cones and synaptosomes suggests that, both in extending axons and mature synaptic terminals, B-50 may exert identical functions as a protein kinase C substrate at the plasma membrane.

Introduction

The neuron-specific phosphoprotein B-50 is characterized as an endogenous substrate of protein kinase C in synaptosomal plasma membranes (Aloyo *et al.*, 1983; Eichberg *et al.*, 1986; De Graan *et al.*, 1988) and in membranes of neuronal growth cones isolated from foetal and neonatal rat brain (De Graan *et al.*, 1985; Van Hooff *et al.*, 1988). Several laboratories have provided evidence that B-50 is identical to GAP43 (Basi *et al.*, 1987; Benowitz & Routtenberg, 1987; Karns *et al.*, 1987; Nielander *et al.*, 1987; Zwiers *et al.*, 1987), to GAP48 (Neve *et al.*, 1987), to F1 (Gispen *et al.*, 1986; Rosenthal *et al.*, 1987) and to pp46 (Meiri *et al.*, 1986).

The role of B-50 in neuronal differentiation and development has been established in light and electron microscopic studies. The developmental changes in B-50 immunoreactivity in the rat hippocampal formation as reported by Oestreicher and Gispen (1986), revealed that the localization of B-50 in neuronal growth cones of growing axons changed on maturation to a more restricted localization in the synapse. In the neonatal rat spinal cord, high B-50 immunoreactivity is found in outgrowing pyramidal tract fibres. As the fibres mature and undergo myelination, a remarkable reduction in B-50 immunoreactivity is found (Gorgels *et al.*, 1987). Correspondingly, in human intramuscular nerve fibres a decrease in B-50-positive end plates was observed with increasing age (Hesselmans *et al.*, 1989).

The role of B-50 in differentiation is also demonstrated by studies on pheochromocytoma PC12 cells. On differentiation, for instance induced by nerve growth factor (NGF), PC12 cells extend neurites bearing motile neuronal growth cones which manifest strong B-50 immunofluorescence (Van Hooff *et al.*, 1986). Ultrastructural localization studies of B-50 in cryosectioned PC12 cells showed that on NGF-

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induced differentiation, B-50 is relocated from cytosol to the plasma membrane and is enriched in the plasma membrane of lamellipodia and filopodia (Van Hooff *et al.*, 1989).

Electron microscopic studies by Gispen and co-workers (1985) showed a presynaptic localization of B-50 in the hippocampal stratum radiatum as well as in nerve terminals isolated from rat brain. Since we are interested in the function of B-50 during neural differentiation and neurite outgrowth, we made a comparison of the ultrastructural localization of B-50 in developing and mature presynaptic structures isolated from rat CNS. We applied immunocytochemistry to cryosections of isolated neuronal growth cones and synaptosomes, using affinity-purified anti-B-50 immunoglobulins (IgGs; Oestreicher *et al.*, 1983; Oestreicher & Gispen, 1986) combined with two types of colloidal gold conjugates. Using this technique, we obtained a satisfactory compromise between ultrastructural preservation and accessibility of the B-50 antigen for the antibodies. Our results indicate that B-50 is predominantly localized at the inner face of the plasma membrane of the neuronal growth cones and the adult counterparts, the presynaptic terminals.

Materials and methods

Preparation of synaptosomes and neuronal growth cones

Synaptosomes were prepared from 6-week-old male Wistar rats according to Dunkley and co-workers (1987). Synaptosomes were obtained from the 15–23% Percoll (v/v) interface and mitochondria from the bottom of the 23% Percoll in the four-step gradient. Neuronal growth cones were isolated from 5-day-old rat forebrains (day of birth is day 1) as described by Gordon-Weeks & Lockerbie (1984). However, as suggested by Gordon-Weeks (1987), the second Ficoll step was omitted and thus the neuronal growth cones were obtained directly from the sucrose/Ficoll interface.

Preparation of cryosections

The synaptosome and neuronal growth cone suspensions were washed once in Krebs Ringer buffer (KRB) and then fixed for 30 min at room temperature in a large volume of 0.5% glutaraldehyde/2% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4. Previous studies have shown that this fixative is optimal with respect to both ultrastructural preservation and accessibility of antigens in ultrathin cryosections (Gispen *et al.*, 1985). For the postsectioning immunoincubation, fixed suspensions were washed twice in PBS and incubated in 5% gelatin for 30 min at 37° C. Next, gelatin was solidified on ice and fixed overnight at 4° C in the above mentioned fixative. Following a modification of Tokuyasu (1986), gelatin-embedded specimens were immersed in a stepwise increasing concentration of polyvinylpyrollidone-10 (PVP-10, Sigma) and sucrose, both dissolved in PBS, with a final concentration of 10% PVP-10 and 1.15 M sucrose. Specimens were plunged into liquid nitrogen and cryosectioned at −90° C on a Reichert Ultracut E provided with FC4 cryoattachment according to Tokuyasu (1984). Cryosections, 70–100 nm thick, were mounted on nickel grids, covered with carbon-coated parlodion film. Grids with sections were placed upside down on droplets during the subsequent immunoincubation procedure.

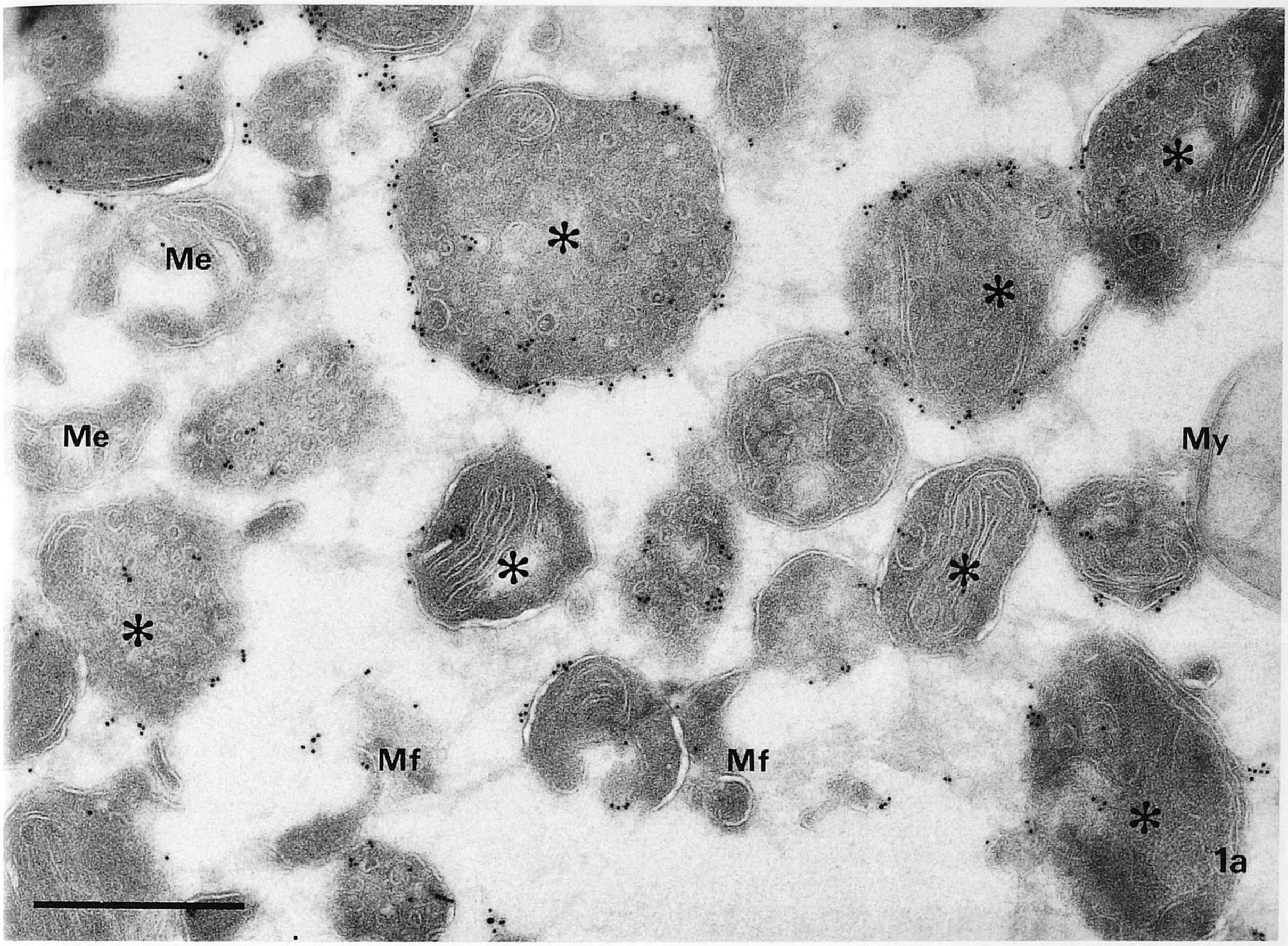
Postsectioning immunoincubation of synaptosomes and neuronal growth cones

Grids with cryosections were washed twice in PBS containing 50 mM glycine (PBS/glycine) for 10 min and twice in PBS containing 0.2% gelatin and 0.5% bovine serum albumin for 10 min. Sections were incubated overnight at 4° C with affinity-purified anti-B-50 IgGs (rabbit antiserum 8613; dilution 1:300; Oestreicher *et al.*, 1983; Oestreicher & Gispen, 1986). Control sections were incubated in identical concentrations of preimmune IgGs (preimmune serum 8613) or in anti-B-50 IgGs preabsorbed with excess purified B-50. The immunoincubation step was followed by a 2 h incubation at room temperature with protein A–gold complexes (PA–gold; diameter: 10.5 nm) or goat anti-rabbit IgG–gold complexes (GAR–gold; diameter: 9.3 nm; Van Bergen en Henegouwen & Leunissen, 1986). Each incubation was followed by four washes in PBS containing 0.2% gelatin and 0.5% bovine serum albumin for 40 min. Sections were rinsed four times in bidistilled water, followed by incubation in 1.1% Tylose/0.5% uranyl acetate, pH 4.0, on ice for 10 min. Sections were air dried and examined in Philips EM-201 or EM-301 electron microscopes.

Presectioning immunoincubation of synaptosomes

To determine if B-50 was localized at the external surface of the synaptosomes, isolated synaptosomes were washed in KRB and fixed as described before. Fixed synaptosomes were washed for 30 min in PBS containing 0.2% gelatin and 0.5% bovine serum albumin and incubated overnight at 4° C with an 1:10 dilution of anti-B-50 IgGs or of preimmune IgGs (as control). Following removal of excess antibodies, the synaptosomes were incubated for 4 h at room temperature with PA–gold of 10.5 nm diameter. The subsequent preparation was processed for cryoultramicrotomy and electron microscopic analysis as described above.

Fig. 1. Low power micrograph of ultrathin cryosectioned synaptosomes (a) and neuronal growth cones (b). The synaptosome-enriched fraction consists of synaptosomes (asterisks), free extrasynaptosomal mitochondria (Me), membrane fragments (Mf) and myelin (My), shown in (a). In (b) the growth cone preparation (GC) contains unidentified membrane-bound organelles (arrowhead) and membrane fragments (Mf). B-50 antibodies were detected with goat anti-rabbit conjugated to colloidal gold (9.5 nm). Scale bars: 0.5 μm.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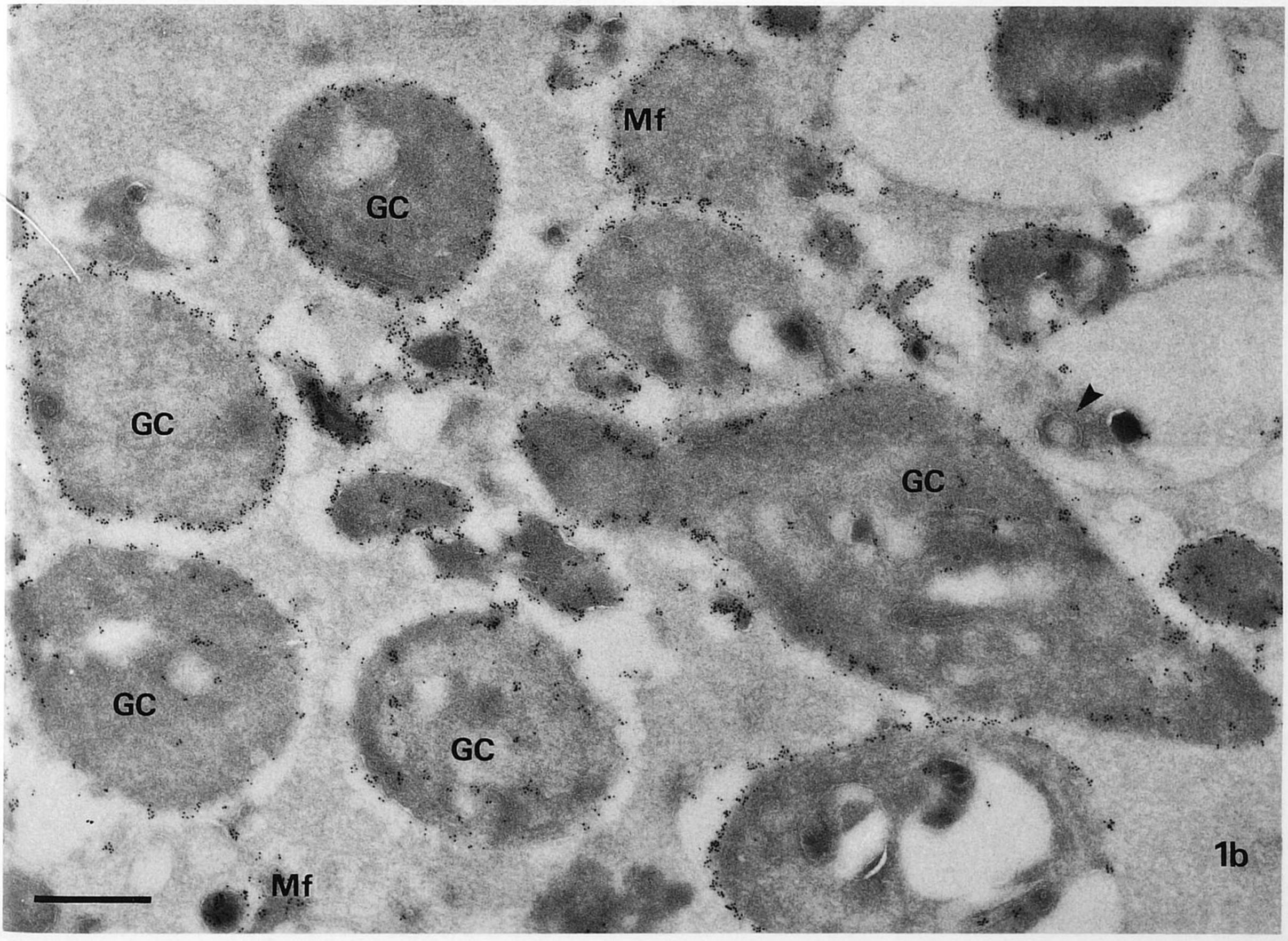
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Silver enhancement

In order to improve detection of the PA–gold/anti-B-50 IgG complexes, possibly bound to synaptosomes during the immunoincubation prior to cryosectioning, the procedure of Holgate and co-workers (1983) was applied on cryosections derived from such a preparation. This procedure increases the size of the particles by precipitating silver around them. Sections were washed thoroughly with doubly distilled water and incubated for 1–2 min in silver enhancer (Intense II, Janssen Pharmaceutica, Beerse/Belgium) resulting in precipitation of silver on available gold particles, thus increasing their size. Excess reagent was removed by washing four times with doubly distilled water. To demonstrate B-50 that was unaccessible in the intact synaptosome, the silver-treated sections were subjected to a second incubation with anti-B-50 IgGs. This immunoreaction was visualized with PA–gold of 10.5 nm diameter as described above. The silver enhancement procedure was also applied on immunoincubated cryosections of a mitochondria-enriched fraction to reveal a minor contamination of B-50 positive synaptosomes.

Morphometric analysis

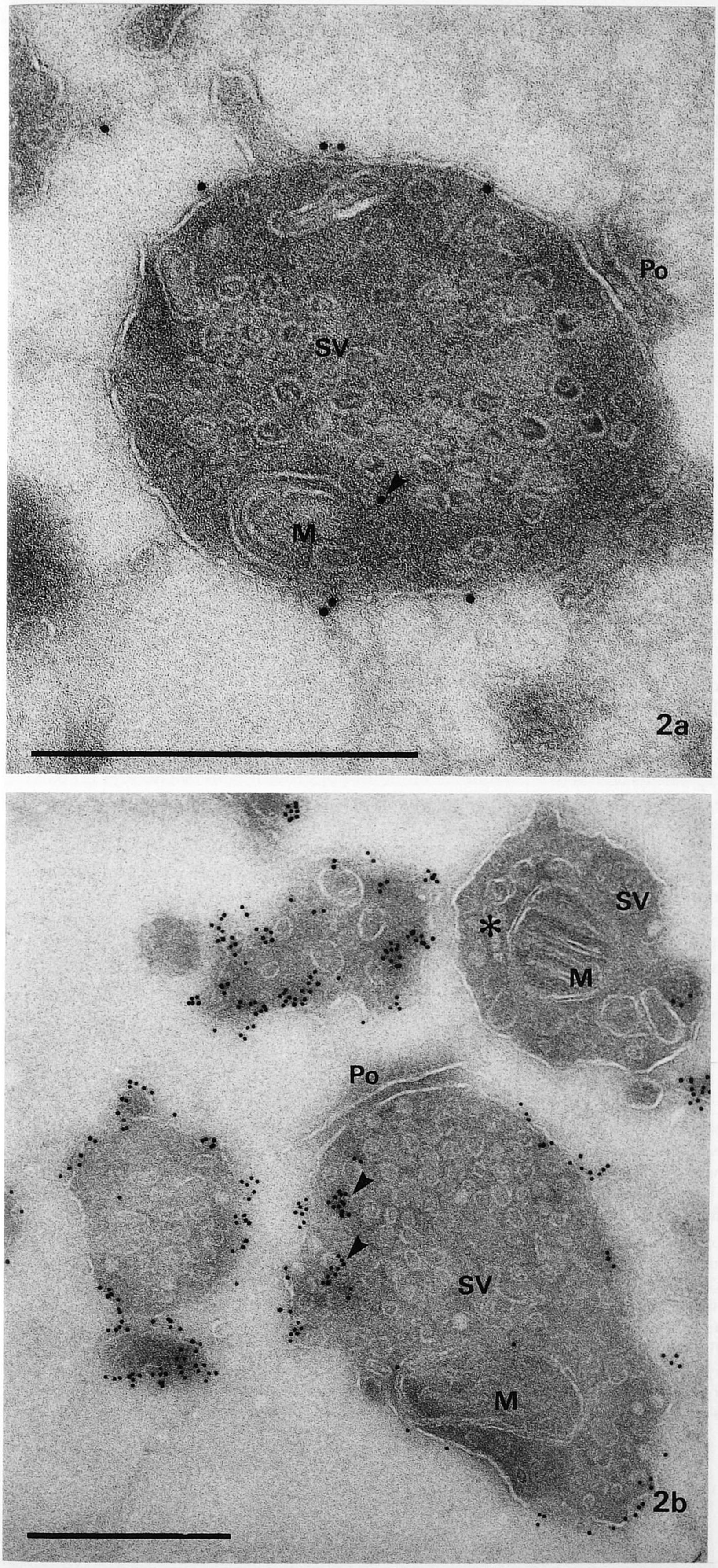
The quantitative analysis of gold particle density was performed only on sections incubated with PA–gold conjugates, since several GAR–gold complexes may bind to one IgG–B-50 complex. For the various quantitative comparisons, postsectioned immunoincubated synaptosomes and neuronal growth cones with well-preserved ultrastructure were selected. Gold particle density on control sections was estimated as less than 0.1 particle per μm^2 and therefore not further counted. Electron micrographs were taken at random until a sample of 200 synaptosomes and 50 neuronal growth cones were obtained from one experimental immuno condition and from two separate isolations. The magnification of the final printed micrographs of synaptosomes and neuronal growth cones was × 63 500 and × 47 200, respectively. Each synaptosome and neuronal growth cone was examined for the total number of gold particles in the cytoplasm, the surface area of synaptosome and neuronal growth cone sections, the length of the surrounding plasma membrane and the total number of gold particles counted within 20 nm distance of the surrounding plasma membrane. The result of this last count was defined as the total number of gold particles bound to the neuronal plasma membrane. Areas were measured with a Calcomp 2000 graphics tablet linked to an IBM XT personal computer, supplied with a digitizer program. For each synaptosome and neuronal growth cone, the following ratios were estimated: (a) total number of gold particles/total length of surrounding plasma membrane, (b) total number of gold particles/cytoplasmic surface area of synaptosome and neuronal growth cone section, and (c) gold particles bound to the neuronal plasma membrane/total number of gold particles.

Results

Synaptosome and nerve growth cone morphology

An overview of the material used for cryoultramicrotomy is presented in Fig. 1a and 1b showing, respectively, ultrathin cryosections of the synaptosome-enriched fraction and neuronal growth cones. Synaptosomes (asterisks, Fig. 1a) are visible as membrane-surrounded bodies 0.3–1.0 μm in diameter, containing synaptic vesicles (SV, Fig. 2a, 2b) of 40–60 nm diameter and occasional mitochondria (M, Fig. 2a, 2b). Some synaptosomes contained an attached postsynaptic membrane (Po, Fig. 2a, 2b). Synaptosome morphology as shown in the cryosections is in agreement with previous observations (Dunkley *et al.*, 1987, 1988). In the synaptosome fraction, a low level of other structures was found, namely myelin (My), extrasynaptosomal mitochondria (Me) and plasma membrane fragments (Mf, Fig. 1a). Neuronal growth cones (GC, Fig. 1b) were defined as membrane-bound structures, containing a characteristic system of smooth endoplasmic reticulum (SER, Fig. 3a, 3b), mitochondria (M, Fig. 3a, 3b) and vesicles of various sizes (V, Fig. 3a, 3b; Gordon-Weeks & Lockerbie, 1984). The diameter of the growth cones was approximately twice the diameter of the synaptosomes (Fig. 1b, 3a, 3b). The morphological appearance of the isolated neuronal growth cones agreed well with cryosections of neuronal growth cones in the outgrowing rat pyramidal tract (Gorgels *et al.*, 1989). Additional components in the neuronal growth cone fraction were membrane fragments and unidentified organelles (arrowhead, Fig. 1b). The length of the surrounding plasma membrane and surface areas of the sectioned synaptosomes and neuronal growth cones were quantified and results are reported in Table 1.

Fig. 2. Electron micrographs of ultrathin cryosections of synaptosomes (Fig. 2a–d) and mitochondria (Fig. 2e) enriched fractions. The sections were treated and immunoincubated with affinity-purified anti-B-50 IgGs (a, b, e) or pre-immune rabbit IgGs (c, d) as described under Materials and Methods. Both PA–gold (a, c) and GAR–gold (b, d, e) were used as detection agents. Mitochondria (M), various synaptic vesicles (SV) and attached postsynaptic membranes with a postsynaptic density (Po) are illustrated. Particles of PA–gold (a) and GAR–gold (b) are mainly localized at the synaptic plasma membrane. A few gold particles are found in the cytosol (arrowheads). No B-50 immunoreactivity is found in mitochondria or at the postsynaptic plasma membrane (Po). Note that B-50 immunoreactivity in one of the synaptosome sections appears to be almost absent (asterisk, Fig. 2b). Control sections, incubated in pre-immune serum and subsequently treated with PA–gold (c) as well as GAR–gold (d), are devoid of B-50 immunoreactivity. There is no significant label found in mitochondria (asterisks, Fig. 2e), whereas the synaptosomes (S) are heavily labelled. Scale bars: (a–c) 0.5 μm; (d, e) 1.0 μm.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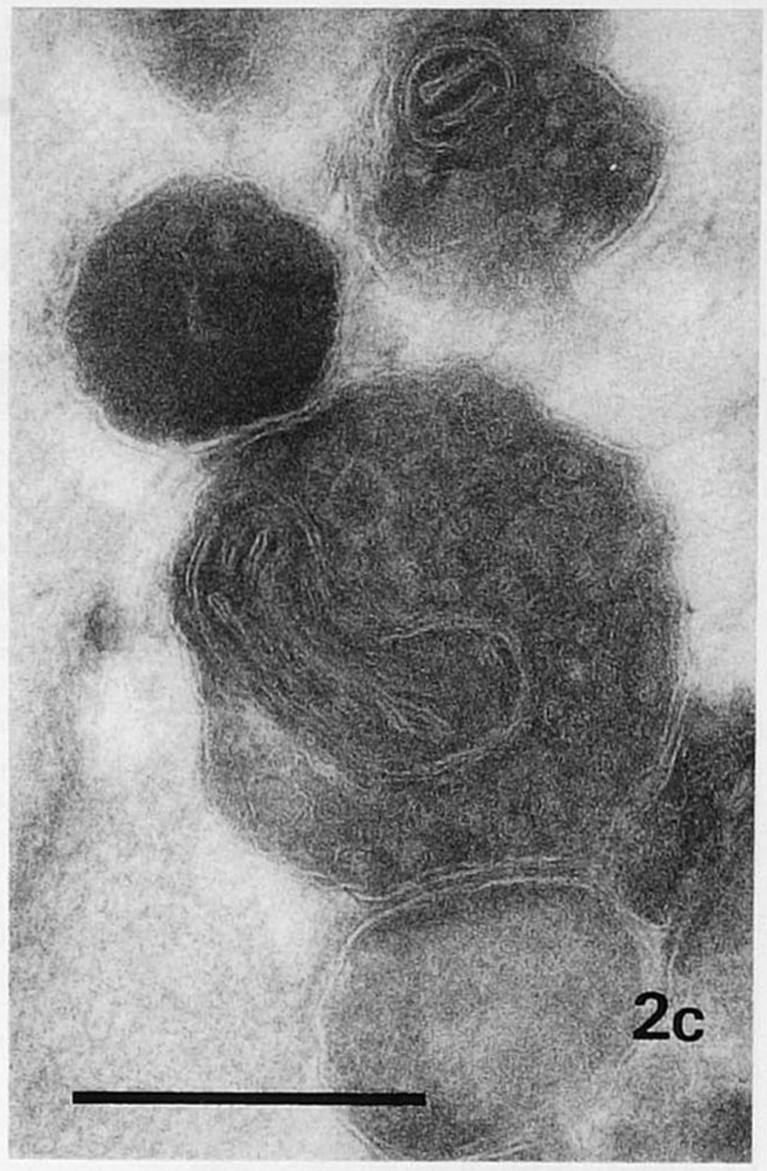
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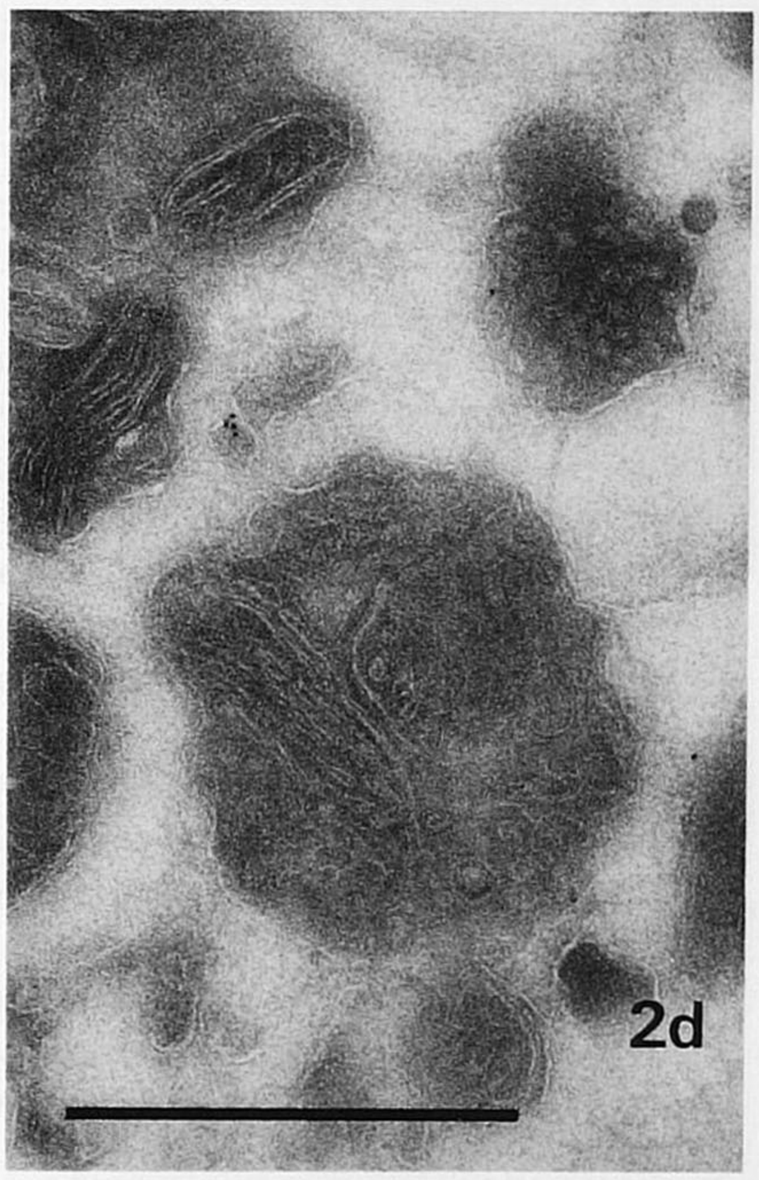
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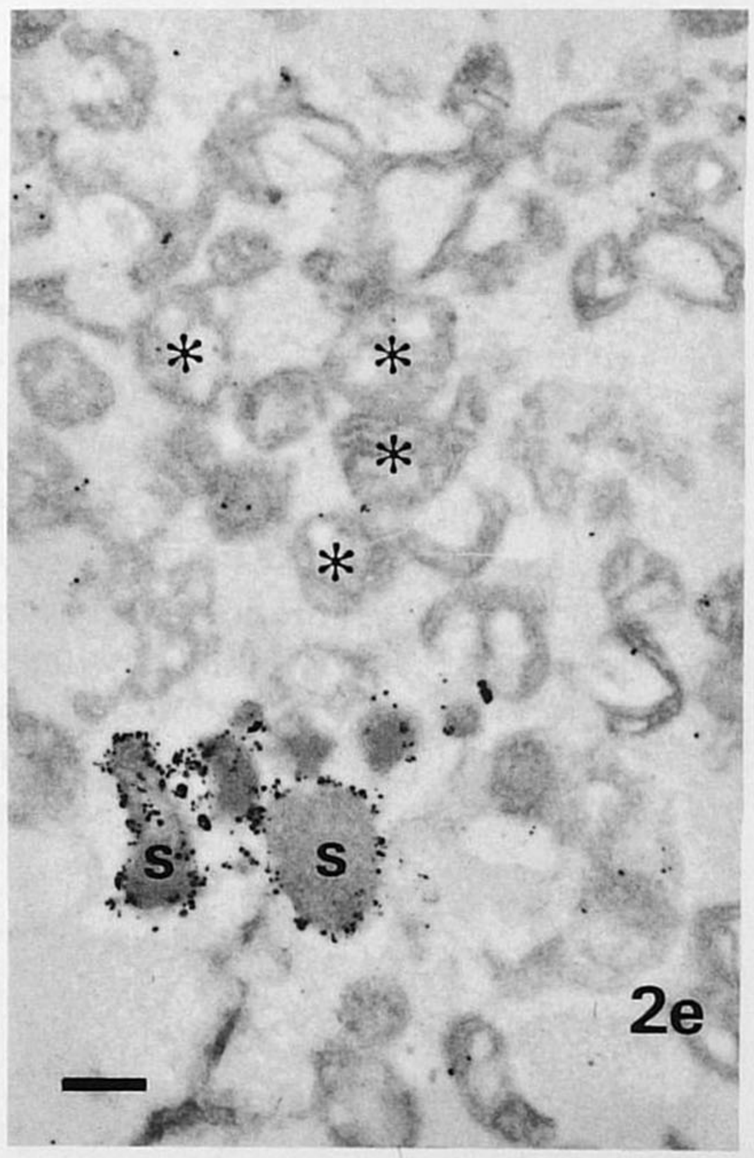
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Table 1 Morphometric analysis of B-50 stained cryosections of neuronal growth cones and synaptosomes (values in mean ± SEM)

	Neuronal growth cones (n = *50*)	*Synaptosomes* (n = *200*)
Surrounding plasma membrane length (μm)	4.39 ± 0.27	1.99 ± 0.05
Surface area of sections (μm^2)	1.50 ± 0.21	0.28 ± 0.01
Gold particles per μm surrounding plasma membrane	4.75 ± 0.42	2.29 ± 0.15
Gold particles per μm^2 cytoplasmic section area	6.82 ± 1.47	11.03 ± 0.88
Ratio membrane bound gold particles vs. total gold particles	0.75 ± 0.02	0.60 ± 0.02

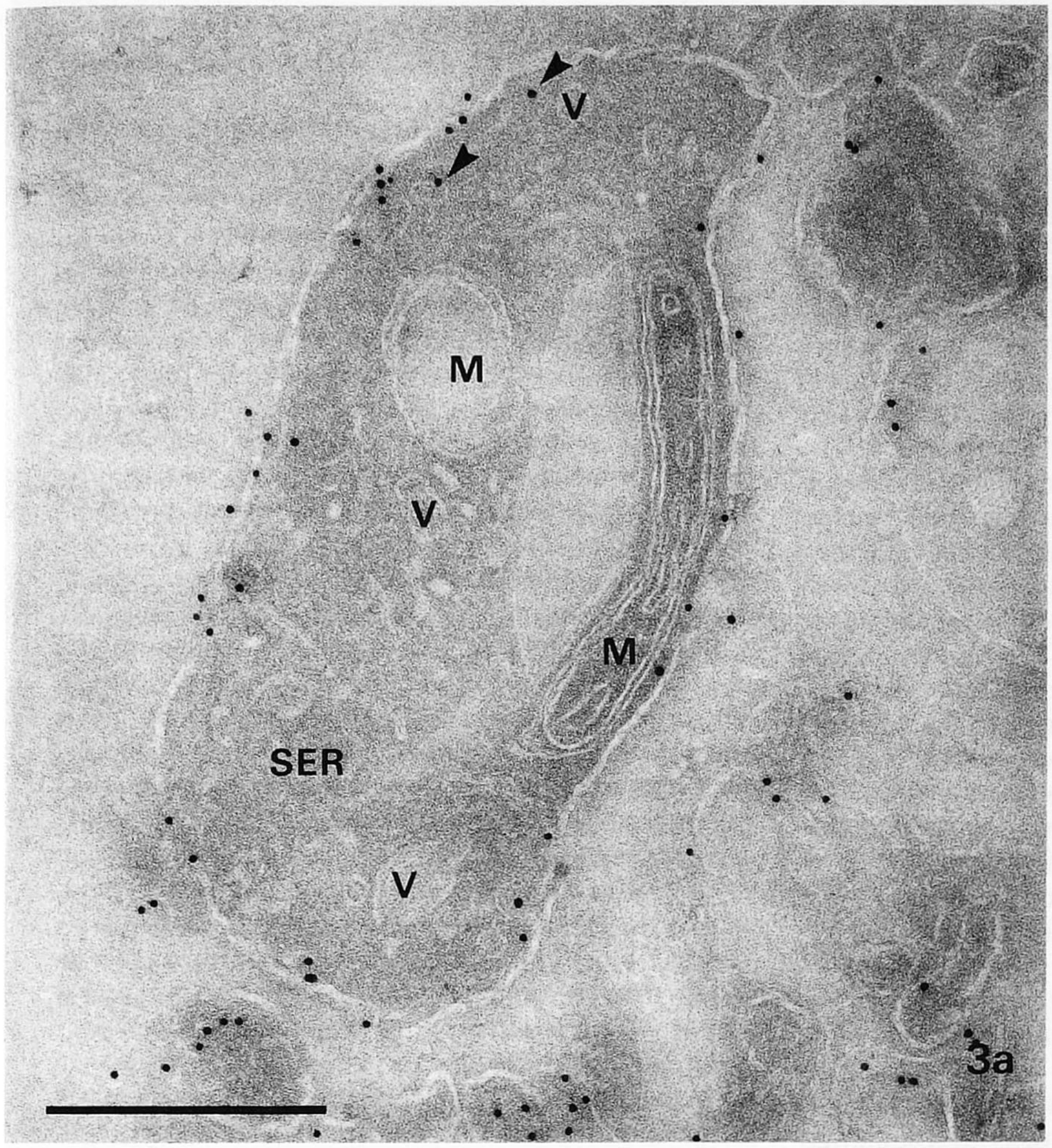
Localization of B-50 in postsectioned immunoincubated synaptosomes and neuronal growth cones

Cryosections of synaptosomes and neuronal growth cones, immunoincubated with GAR–gold (Figs 1a, 1b, 2b, 3b) showed a higher labelling density than sections immunoincubated with PA–gold (Figs 2a, 3a). However, due to clustering of GAR–gold particles bound to IgGs around antigenic sites, the gain in labelling intensity might be accompanied by a loss in resolution of labelling.

In postsectioned immunoincubated synaptosomes, B-50 immunoreactivity is predominantly found at the surrounding synaptic plasma membrane (Figs 1a, 2a, 2b; Table 1). In addition, some of the synaptosome sections showed B-50 immunoreactivity in the vesicle-rich cytoplasm (arrowheads, Fig. 2a, 2b). Due to the limited resolution here, we could not determine whether these gold particles were associated with synaptic vesicles. Mitochondria and postsynaptic membrane fragments (Po) did not show significant B-50 immunoreactivity. There was a considerable variability in density of B-50 immunoreactivity in different synaptosome sections. Thus some sections of synaptosomes (about 3% of total population) were almost devoid of B-50 immunoreactivity (asterisk, Fig. 2b). Control sections of synaptosomes, immunoincubated without primary antibodies (results not shown) and with preimmune antibodies, followed by PA– or GAR–gold detection (Fig. 2c and 2d, respectively) were negative. Preabsorption of anti-B-50 IgGs with excess of purified B-50 prevented any specific labelling of cryosectioned synaptosomes (results not shown). In cryosections of the mitochondria-enriched fraction, abundant free mitochondria are present; their cristae appear swollen, probably due to the isolation procedure (asterisks, Fig. 2e). Immunoincubation and subsequent silver enhancement of gold particles on these sections revealed high B-50 immunoreactivity, exclusively in the few synaptosomes in this fraction (Fig. 2e). The specificity of the applied method is illustrated by the finding that gold particles in mitochondria were very scarce and distributed randomly. Upon application of the same immunoincubation procedure to ultrathin cryosections of neuronal growth cones, we found dense B-50 immunoreactivity near the plasma membrane of all neuronal growth cone sections (Fig. 3a, 3b). A relatively smaller fraction of gold particles was found in the cytoplasm (arrowheads, Fig. 3a, 3b). As for synaptosomes, we could not assess whether these gold particles were associated with vesicular structures. B-50 immunoreactivity was not detected in mitochondria and structures resembling components of smooth endoplasmic reticulum (Fig. 3a, 3b). Cryosections of neuronal growth cones, incubated without primary antibody or in pre-immune IgGs, were negative (results not shown).

Fig. 3. Electron micrographs of ultrathin cryosections of neuronal growth cones. Sections of the neuronal growth cones were treated and immunoreacted for B-50 as described under Materials and Methods, using PA–gold (a) or GAR–gold (b). Smooth endoplasmic reticulum (SER), a distinct feature of neuronal growth cones, is clearly illustrated as well as mitochondria (M) and various types of vesicles (V). Gold particles are found mainly associated with the surrounding plasma membrane. No B-50 immunoreactivity is detected in mitochondria and smooth endoplasmic reticulum. Scale bars: 0.5 μm.

Fig. 4. (a) Electron micrograph of cryosectioned synaptosomes, immunoincubated, prior to sectioning, in anti-B-50 IgGs and PA–gold. No B-50 immunoreactivity is found in the synaptosome with an intact surrounding plasma membrane (asterisk), whereas in the synaptosome with a partially disrupted plasma membrane, gold particles are found on the membrane fragments (arrowheads). (b) Electron micrograph of synaptosomes, immunoincubated as described for (a). Gold particles on sections of these immunoreacted synaptosomes were silver intensified, resulting in an increase of the particle diameter (large arrowheads). Following silver intensification, these cryosections were immunoincubated in anti-B-50 IgGs and PA–gold. B-50 immunoreactivity, resulting from the first immunoincubation of intact synaptosomes, is displayed by large particles (large arrowheads). These can thus be distinguished from the smaller gold particles, resulting from B-50 immunoreactivity in the second immunoincubation of sections of these synaptosomes (small arrowheads). Scale bars: 0.5 μm.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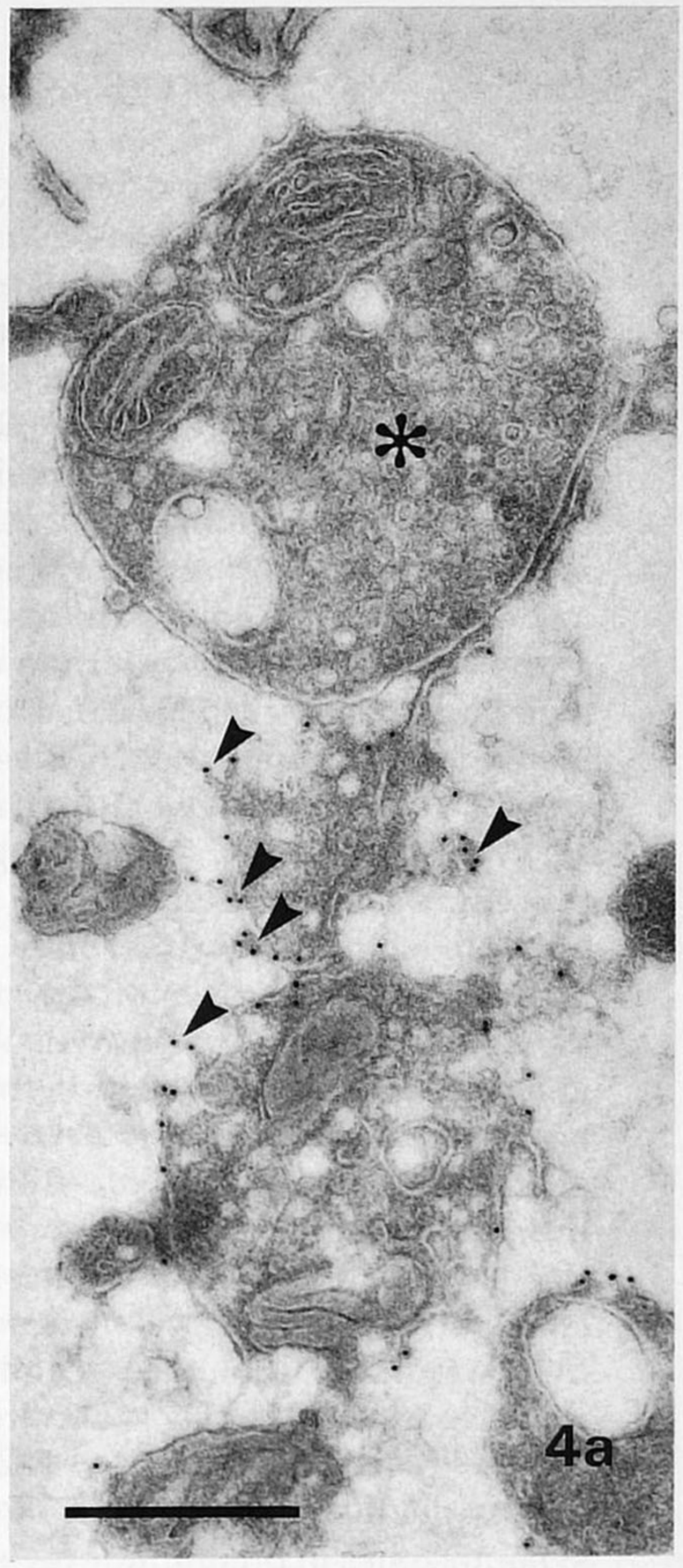
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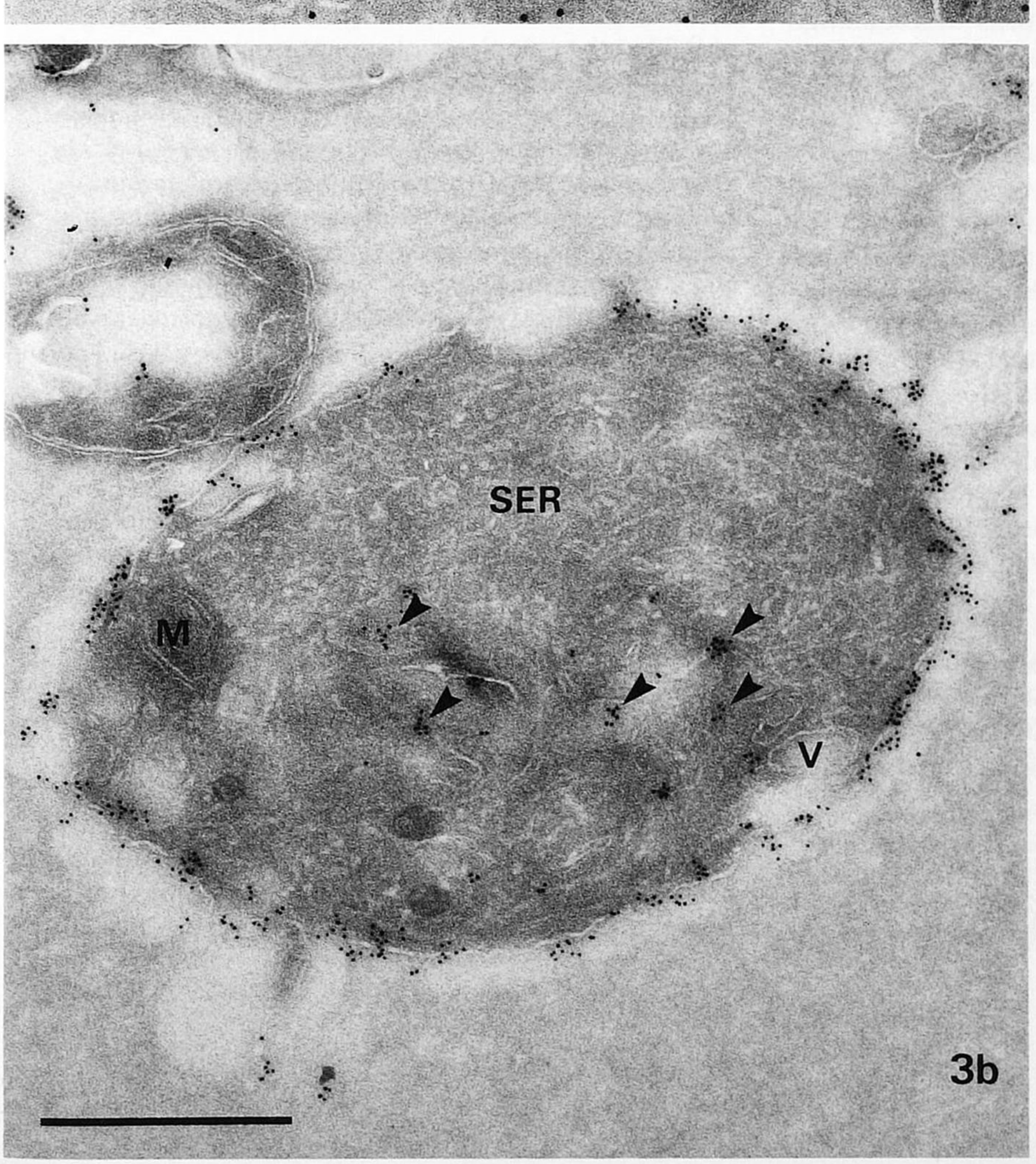
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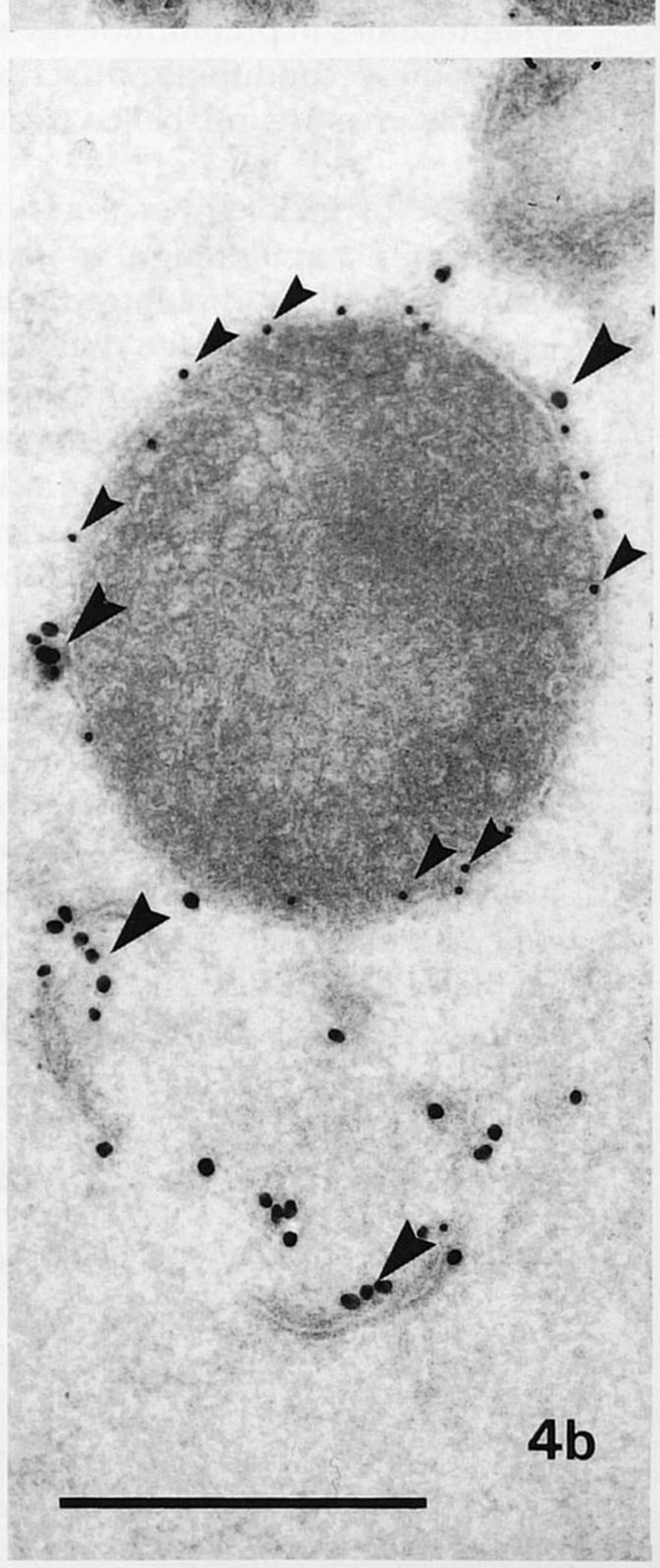
4b

Localization of B-50 in presectioned immunoincubated synaptosomes

Considering the gold particle distribution in immunoincubated cryosectioned synaptosomes and neuronal growth cones, we could not assess clearly whether the B-50 molecules were located on the outer or inner face of the surrounding plasma membrane. To examine the precise plasma membrane localization of B-50, we immunoincubated intact synaptosomes. This resulted in an absence of gold particles in cryosections of synaptosomes surrounded by an undamaged plasma membrane (asterisk, Fig. 4a). However, adjacent to membrane fragments of damaged synaptosomes, several gold particles were found (arrowheads, Fig. 4a). Application of the immunogold–silver staining procedure on cryosections of the presectioned immunoincubated synaptosomes resulted in enlargement of the gold particles on the membrane fragments (large arrowheads, Fig 4b). Following this immunogold–silver staining procedure, the same sections can now be incubated again with newly added antibodies and subsequently PA–gold, without interference of the first antibodies of the presection incubations (Bienz *et al.*, 1986). The smaller sized gold particles (small arrowheads, Fig. 4b) show B-50 immunoreactivity resulting from the second incubation. These particles are found along the entire plasma membrane of the intact synaptosome. The specificity of the applied method was checked by immunoincubating synaptosomes in preimmune IgGs prior to sectioning. Under these conditions, intact as well as ruptured synaptosomes are unlabelled (results not shown).

Morphometric analysis

The mean diameter length of neuronal growth cones was calculated by dividing the surrounding plasma membrane length (Table 1) by 3.14 (π). Thus we found that this mean diameter of growth cones is approximately twice that of synaptosomes. This is in line with findings of Gordon-Weeks & Lockerbie (1984). Quantitative analysis of gold particle density in the cryosections (presented in Table 1) showed that neuronal growth cones contained about twice as many gold particles per unit membrane length as synaptosomes. Whereas most gold particles in the synaptosome and neuronal growth cone sections were located at the inner face of the plasma membrane (respectively 60% and 75%), the cytoplasm of both structures also contained gold particles. Expressing gold particle density per unit cytoplasmic section area, we found almost twice as many gold particles located in the cytoplasm of synaptosomes as in neuronal growth cones (Table 1).

Discussion

Since recent studies have shown that B-50 is involved in neuritogenesis and neuronal differentiation (Van Hooff, 1988), we thought it of importance to compare the immunocytochemical ultrastructural localization of the growth-associated phosphoprotein B-50 in isolated synaptosomes and neuronal growth cones. Immunocytochemical localization studies of B-50 in developing and mature rat brain, using anti-B-50 IgGs, have shown that the antibody reaction is specific for B-50 (Oestreicher *et al.*, 1983; Oestreicher & Gispen, 1986). The following results of control experiments performed by us demonstrated that the procedure adapted for ultrastructural immunolocalization of B-50 is specific. (1) Preabsorption of anti-B-50 IgGs with excess of purified B-50 was effective in preventing any specific labelling of cryosectioned synaptosomes. (2) Cryosections of synaptosomes and neuronal growth cones, incubated in preimmune IgGs, never displayed immunoreactivity (Fig. 2c, 2d). (3) Cryosectioning of a fraction enriched in mitochondria showed no significant immunoreactivity at mitochondrial membranes, whereas occasional synaptosomes were heavily labelled (Fig. 2e).

By quantitation of the gold particle density per unit plasma membrane, we demonstrated a higher B-50 content at the plasma membrane of neuronal growth cones than at the synaptosomal plasma membrane (Table 1). This is in line with former studies comparing B-50 content in isolated membranes of neuronal growth cones and synaptosomes by biochemical means (Skene *et al.*, 1986). However, the total amount of B-50 in the cytoplasm of synaptosomes (expressed as gold particle density per unit cytoplasmic section area) is twice as great as in growth cones. So far we have no evidence for B-50 being associated with synaptic vesicles. In other ultrastructural immunocytochemical studies of rat nerve terminals which are reinnervating neuromuscular junctions (Verhaagen *et al.*, 1988) and in synapses of the mature rat brain (Norden *et al.*, 1987), B-50/GAP43 is reported to be associated with membranes of synaptic vesicles. The use of peroxidase labelling methods in these studies may account for differences from our observations. There is a tendency of the peroxidase reaction products to accumulate in the cytoplasmic space. There, the products are non-specifically adsorbed to any adjacent particle structure (De Camilli *et al.*, 1983).

Part of the cytosolic B-50 in synaptosomes and growth cones, found in our study, might represent B-50 released from the neuronal plasma membrane during fixation. Purification studies have shown that B-50 can be extracted from the membrane by detergent and salt (Zwiers *et al.*, 1979, 1980) or with alkali (Oestreicher *et al.*, 1983; Zwiers *et al.*, 1985). This indicates that both ionic forces and covalent bonds play a role in the association of B-50 with the membrane. Recently, more insight has been gained into how this hydrophilic protein (Nielander *et al.*, 1987) is

anchored to the membrane. There is evidence for an dynamic linkage of fatty acid moieties on B-50 molecules. These fatty acids could provide a hydrophobic domain for the association of B-50 with the membrane (Skene & Virág, 1989).

We did not detect B-50 immunoreactivity in presectioned immunoincubated synaptosomes, which confirms findings of a former study (Gispen *et al.*, 1985). Our negative labelling results on sections of synaptosomes surrounded by an intact plasma membrane suggest that the B-50 antigen is located at the cytoplasmic face of the neuronal membrane. Therefore, B-50 is not accessible for the applied antibodies. The positive result in sections of synaptosomes with damaged or ripped off plasma membranes can be explained by an enhanced accessibility. This agrees with the findings of Meiri *et al.* (1988), who showed that in a culture of sympathetic neurons the only cells that were immunoreactive were those which were made permeable before treatment with GAP43 antibodies. A localization of B-50 at the inner face of the plasma membrane is in line with the suggested role of this phosphoprotein as a protein kinase C substrate in transmembrane signal transduction. The amount of protein kinase C activity at synaptic plasma membranes is directly correlated with the *in vitro* phosphorylation of protein F1, a protein shown to be identical to B-50 (Gispen *et al.*, 1986; Akers & Routtenberg, 1987).

The question arises whether B-50 in synaptosomes and neuronal growth cones exerts similar functions related to signal transduction mechanisms. There is evidence that signal transduction mechanisms related to growth-associated processes in the neuronal growth cone (Van Hooff *et al.*, unpublished findings) as well as to neurotransmitter release in hippocampal slices (Dekker *et al.*, 1989) might work along common reaction pathways involving phosphorylation of B-50.

Recently, it came to our attention that B-50/GAP43 is identical to P-57, an unusual calmodulin binding protein (Cimler *et al.*, 1987). A regulating function for P-57/calmodulin-complex, has been suggested in the local release of calmodulin in response to, for example, depolarization, increased calcium levels and phosphorylation of P-57/B-50 by protein kinase C (Alexander *et al.*, 1987). Our findings that B-50 is predominantly localized at the inner face of the presynaptic plasma membrane and absent on postsynaptic membranes, fits well with a possible role of B-50 releasing calmodulin locally as a regulator of intracellular calcium levels and vesicle fusion processes (Goldenring *et al.*, 1986; Schiebler *et al.*, 1986; Petrucci & Morrow, 1987). Vesicle fusion processes could serve both to insert membrane at the growing tip of the axon and to release transmitter in the presynaptic terminal.

In conclusion, the abundant presence of B-50 at the plasma membrane of neuronal growth cones suggests that B-50 is required for growth-associated mechanisms. The association of B-50 with the cytoplasmic face of the neuronal plasma membrane agrees with the involvement of B-50 in the second messenger effector system of membrane signal transduction. The decrease in content of membrane bound B-50 in synaptosomes compared to neuronal growth cones suggests that the B-50 involvement in signal transduction mechanisms related to neurite outgrowth persists during maturation. The function of B-50, distributed in the cytosol of synaptosomes and neuronal growth cones, remains unknown.

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