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GAP-43 distribution is correlated with development of growth cones and presynaptic terminals

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Summary

GAP-43 (F1, B-50, pp46) has been associated with neuronal development and regeneration, but precise localization within neurons is not known. Pre-embedding electron microscopic immunocytochemistry using silver-enhanced 1 nm gold particles was used to localize GAP-43 label in cell cultures of cerebellar neurons. In the plasma membranes of early cultures, high levels of GAP-43 were seen in all parts of the neuron. In older cultures, consistent with previous reports, the first loss of GAP-43 label was seen in the soma and then the axon. Growth cones had high levels of GAP-43 label on the plasma membrane, with increased distribution over unattached relative to attached filopodia. The amount of GAP-43 seen over the plasma membrane of forming presynaptic terminals is lower than over growth cones, indicating a possible correlation between the presence of GAP-43 and the stage of presynaptic terminal development. Intracellular GAP-43 in axons and growth cones was highest in membranes of smooth cisternae. The levels of GAP-43 in smooth cisternae in axons fell by seven days in culture while the levels of GAP-43 in smooth cisternae of growth cones fell at 14 days. When mini-explant cerebellar cultures were examined with light microscopic immunocytochemistry, GAP-43 label of plasma membrane was highest at the periphery of the radial axonal outgrowth, suggesting that addition of GAP-43 to the plasma membrane can occur in the distal axon or at the growth cone.

Introduction

The growth-associated protein GAP-43 has been found to be present in growing axons and growth cones associated with both developing and regenerating axons (Skene & Willard, 1981a, b; Benowitz *et al.*, 1981; Benowitz & Routtenberg, 1987; Skene, 1989). GAP-43 has been associated with membrane fractions from regenerating axons (Skene & Willard, 1981b), isolated membranes from developing axons (Meiri *et al.*, 1986) and isolated growth cone particles (Katz *et al.*, 1985). Several functions for GAP-43 have been proposed (Gordon-Weeks, 1989). In a study of fraction isolated from growth cones, Meiri and Gordon-Weeks (1990) have found GAP-43 associated with the cytoskeleton, indicating that GAP-43 could possibly be involved in loss of growth cone motility. Baetge and Hammang (1991) have analyzed PC12 cells deficient in GAP-43 protein and found these cells can initiate neurite extension even without GAP-43. Strittmatter and colleagues (1990) have found that GAP-43 can bind to the α subunit of G_0 and increase binding of GTP to the G protein, indicating a potential involvement of GAP-43 in signal transduction. In addition, Meiri and colleagues (1991) have found that kinase C phosphorylated GAP-43 is highest in the distal parts of growing axons, suggesting GAP-43 may be related to interactions of the growth cone with its environment.

To understand more about GAP-43, it is important to determine the subcellular distribution during axonal elongation and synapse formation. Neurons are highly polarized cells and the concentration of GAP-43 has been shown to be non-homogeneous (Goslin *et al.*, 1988; Burry *et al.*, 1991). Recent studies have examined the distribution within neurons by light microscopic immunocytochemistry (van der Neut *et al.*, 1990; Burry *et al.*, 1991) and determined that GAP-43 is restricted to axons and thus lost from cell bodies of developing neurons. Results have also shown that GAP-43 is associated with PC12 cell growth cone filopodia (Van Hooff *et al.*, 1989) and is lost from growth cones of cerebellar neurons after they form presynaptic terminals (Burry *et al.*, 1991). In cultured hippocampal neurons, GAP-43 is present in the cell

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body, and found in the axon and growth cone (Goslin *et al.*, 1988). Hippocampal neurons in culture for 30 days do not lose GAP-43 labelling (Goslin & Banker, 1990), consistent with the finding of GAP-43 in adult hippocampal neurons. The details of subcellular distribution of the protein have not been examined in those neurons in which GAP-43 levels fall during development. Experiments reported here used cerebellar neurons because the cerebellum shows marked developmental changes in levels of GAP-43 and its mRNA (Neve *et al.*, 1987). In the adult cerebellum only very low levels of the protein and mRNA have been found (Jacobson *et al.*, 1986; Oestreicher & Gispen, 1986; Neve *et al.*, 1987; Benowitz *et al.*, 1988). Cell cultures of the cerebellum have been shown by light microscopic immunocytochemistry to down-regulate GAP-43 during development (Burry *et al.*, 1991).

The subcellular distribution of GAP-43 label within developing axons and growth cones has been difficult to determine because of technical limitations (van Lookeren Campagne *et al.*, 1989). We have developed a high resolution EM immunocytochemistry method with silver enhanced 1 nm gold-labelled antibodies (Lah *et al.*, 1990), which allows particles to be assigned to specific organelles. This pre-embedding technique also allowed the quality of morphological preservation necessary for the examination of growth cones in cell cultures. Results presented here show that GAP-43 can be localized to specific membranous organelles and that changes in the distribution of label occur during development of synaptic contacts. Some of these results have been presented in abstract form (Burry & Hayes, 1990).

Materials and methods

Cell culture methods

The enriched neuronal cell cultures of the neonatal rat cerebellum were prepared as previously described (Burry & Hayes, 1989). Mini-explant cultures of 2–3 day rat cerebellum were prepared as enriched neuronal cell cultures, except for the tissue dissociation procedure. After dispersing the cells with a glass Pasteur pipette, the supernatant was removed after 20 s and allowed to set 4 min. Cell clusters that settled to the bottom of the tube were plated on polylysine-coated dishes. Treatment with cytosine arabinoside was as described above. Light microscopic (LM) immunocytochemistry for GAP-43 cultures was done as previously reported (Burry *et al.*, 1991).

EM immunocytochemistry

For EM immunocytochemistry, cultures were fixed with 4% paraformaldehyde and 0.1/% glutaraldehyde in 0.1 M phosphate buffer with 0.05 M sucrose and 0.4 mM $CaCl_2$. All rinses and incubations were done in phosphate buffered saline (PBS) with 5% calf serum, 0.1% saponin, 50 mM glycine, 0.1% gelatin, 1 mg ml^{-1} bovine serum albumin and 0.02% NaN_3. Following three rinses in the buffer, the cultures were incubated in the buffer with 1:500 anti-GAP-43 (generously donated by Drs Larry Benowitz and Nora Perrone-Bizzozero) for 3 h. The generation and characterization of this antibody has been described (Benowitz *et al.*, 1988). Cultures were incubated in rabbit anti-sheep antibody 1:5000 for 3 h. Because 1 nm gold was only available bound to anti-rabbit antibody and anti-mouse antibody, this step is required to bridge the anti-GAP generated in sheep to the 1 nm gold-labelled goat anti-rabbit antibody. Following PBS rinses, the cultures were incubated in anti-rabbit labelled with 1 nm gold (AuroProbe One, Janssen) overnight. Following rinses in PBS, the cultures were fixed with 1.6% glutaraldehyde for 15 min, rinsed in PBS and three times in 20 mM HEPES with 280 mM sucrose at pH 6.8. The 1 nm gold was developed with the neutral pH developer (Lah *et al.*, 1990) for 5 min (with gum arabic prepared at 500 g per litre), and fixed in 250 mM sodium thiosulphate in 20 mM HEPES at pH 6.8 for 10 min. Following buffer rinses, cultures were osmicated, dehydrated and embedded. Thin sections parallel to the substrate were stained with uranyl acetate and lead citrate, and examined. Particles of silver-enhanced 1 nm gold, unlike colloidal gold, have a range of sizes which result from the enhancement procedure. Control cultures incubated with preimmune serum or treated by elimination of an antibody gave no labelling.

Quantitative analysis of EM immunocytochemistry

Two types of quantitative analysis were used. The first type tested the null hypothesis that particles were randomly distributed over the tissue (Salpeter *et al.*, 1969; Salpeter & McHenry, 1973). If the null hypothesis were true, then the particle densities over all structures would be the same as a random set of points. In this type of analysis, all cells and cellular structures in a section must be analyzed. The analysis determined the density of silver-enhanced particles over the culture, which was divided into three different types of structures: soma, neuropil or swellings. The term 'swelling' was chosen because it is not possible to determine in all cases the nature of an axonal swelling seen in random sections, and we wished to include all particles in the analysis. Many presynaptic terminals were sectioned so that no cluster of synaptic vesicles was seen, and growth cones were sectioned in a plane that did not include filopodia or characteristic vesicles. Swellings were elliptical, generally larger than 1 μm across and connected to an axon. Within each of the three structures noted above, the particles were next assigned to one of 10 different organelles: cytoplasm, Golgi apparatus, plasma membrane, polysomes, mitochondria, rough endoplasmic reticulum, smooth cisternae, vesicles-synaptic, vesicles-larger (than synaptic). For the data in Fig. 6, a total of 7162 particles were counted over 996 μm^2 of cells, and data for each experiment were normalized. Statistical significance test was determined with a $P < 0.05$. Background labelling of 1.23 particles μm^{-2} was determined by counting particles over glial cells.

The second type of analysis focused on one type of structure, the swellings. Analysis determined particle density via the unit density method of analysis (Burry, 1982). Here, swellings were examined individually and the swellings that could be classified as either growth cones or presynaptic terminals were further analyzed. After the

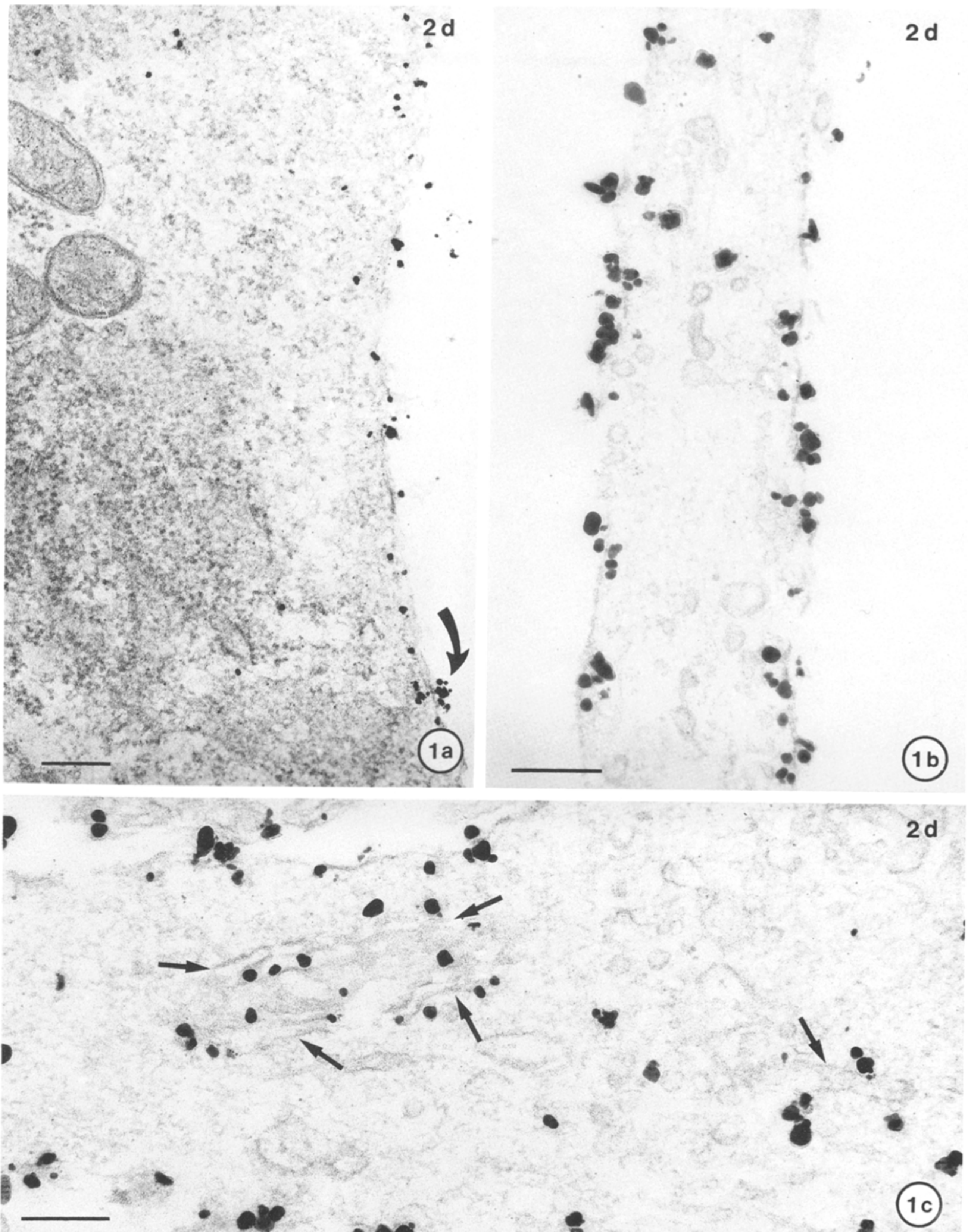


Fig. 1. GAP-43 labelling in neurons of two day cultures. (a) Soma demonstrating the level of plasma membrane labelling and an example of particle clustering (arrow). Low levels of GAP-43 label were seen over cytoplasm, and did not appear to be associated with any specific organelle. (b) Axon at two days with heavy labelling of the plasma membrane was seen both as clusters of particles and as individual particles. Labelling levels for internal structures were lower. (c) In favorable sections, the smooth cisternae within an axon show labelling for GAP-43 (arrows). Scale bars: 0.2 μm.

number of particles and the area of each structure was determined, its total particle density was calculated.

EM immunocytochemistry of whole mounts

Cultures to be examined as whole mounts were grown on grids (Tsui *et al.*, 1983, 1985) and processed as described above. For immunocytochemistry, the cover slip with cells growing on coated grids was processed as described above for GAP-43, but after osmication the cultures were dehydrated in ethanol and critical point dried. The cultures were examined in a Philips CM12 (120 kV; tilted 40°). Because many of the growth cones seen in the EM were partially dislodged in processing, criteria were established to identify attached growth cones. Video studies of live growth cones had previously shown that filopodia were found only in certain orientations to the substrate. Either filopodia were attached to the substrate along their entire length, or filopodia were detached along their length and attached at the tip. If the tip was unattached, then the entire filopodium was unattached as well (Goldberger & Burmeister, 1986). In addition, filopodia that were unattached were usually curved or rounded and not straight along their entire length. Therefore, straight and unattached filopodia were considered to be displaced during processing for EM.

Results

Initial distribution of GAP-43

The plasma membrane of neuronal cell bodies was labelled for GAP-43, but the label was frequently distributed in patches (Fig. 1a, arrow). By comparison cytoplasmic labelling was sparse. At 2 days, axons showed heavy labelling of the plasma membrane (Fig. 1b) but the labelling was frequently patchy. Within the cytoplasm of axons, GAP-43 was associated with membrane-bound organelles. Smooth cisternae were labelled, and in favourable sections several adjacent smooth cisternae were labelled (Fig. 1c, arrows). Within axons, most of the label was seen associated with irregular elongate cisternae but not with vesicles.

Growth cones at two days were heavily labelled with a high density of gold particles on both the plasma membrane and internal structures. The highest concentration of growth cone labelling occurred on the plasma membrane, but some label appeared to be associated with smooth cisternae similar to those seen in axons. The cisternae could be followed, in favourable sections, over considerable distances (Fig. 2a, arrows). Labelled smooth cisternae were frequently close to the plasma membrane (Fig. 2b, arrows), but it could not be determined if they were continuous with the plasma membrane. In some cases, both labelled smooth cisternae and labelled large vesicles were seen near the plasma membrane of growth cones. Labelled smooth cisternae (Fig. 2c, arrows) were frequently localized close to clusters of labelled large vesicles (Fig. 2c, V) and close to the plasma membrane.

To enable further evaluation the distribution of GAP-43 on growth cones, cells were grown on grids, labelled and examined using EM stereopair analysis. Properly preserved growth cones had been attached and unattached filopodia (Fig. 3a & 3b). The unattached filopodia were labelled at a level similar to or higher than the rest of the growth cone (Fig. 3a & 3b; arrowheads). Note in Fig. 3c (arrowhead) the high number of particles on the plasma membrane of the unattached filopodia. In contrast, the attached filopodia had levels of label similar to or lower than the rest of the growth cone (Fig. 3d; arrow). This observation suggests that in addition to growth cone filopodia labelling for GAP-43, the distribution of an unattached and attached filopodia may differ.

GAP-43 distribution at later times

At 14 and 21 days a significant drop in labelling of the soma was noted, although axons and growth cones were still labelled heavily. By 14 days the number of growth cones seen had decreased, but the growth cones still present were heavily labelled (Fig. 4a). Label was associated predominantly with the filopodial plasma membrane (Fig. 4a & 4b, stars) and with smooth cisternae (Fig. 4a, arrowheads).

Presynaptic terminals were labelled to a variable extent but the label was predominantly localized to the plasma membrane. Some of the presynaptic terminals at 14 days still had plasma membrane labelling at levels similar to growth cones (Fig. 4c). Labelling within the presynaptic terminals was lowest in the areas of synaptic vesicles (Fig. 4c, stars), and higher in regions of larger vesicles and smooth cisternae. At 21 days the majority of presynaptic elements had only background levels of GAP-43 label associated with the plasma membrane (Fig. 5).

Quantitative analysis of GAP-43 distribution

The changes seen above indicate a loss of GAP-43 from the plasma membrane with increased time in culture, but the changes in distribution of GAP-43 associated with organelles were not always clear. The cellular distribution of GAP-43 label was further examined quantitatively to determine patterns of change over specific cellular organelles with time in culture.

A comparison of plasma membrane particle density showed the soma, axons and swellings (see Materials and Methods) were all labelled at high densities after two days in culture (Fig. 6a). After seven days labelling of swellings (Fig. 6a) remained high while the labelling of soma and axons had dropped significantly. At 14 days, plasma membranes from all three areas showed significant decrease in labelling in comparison to the seven day values (Fig. 6a). Even at 14 days the labelling of the plasma membrane was higher in the axons and swellings than in the soma (Fig. 6a). These results show that the plasma membrane of swellings

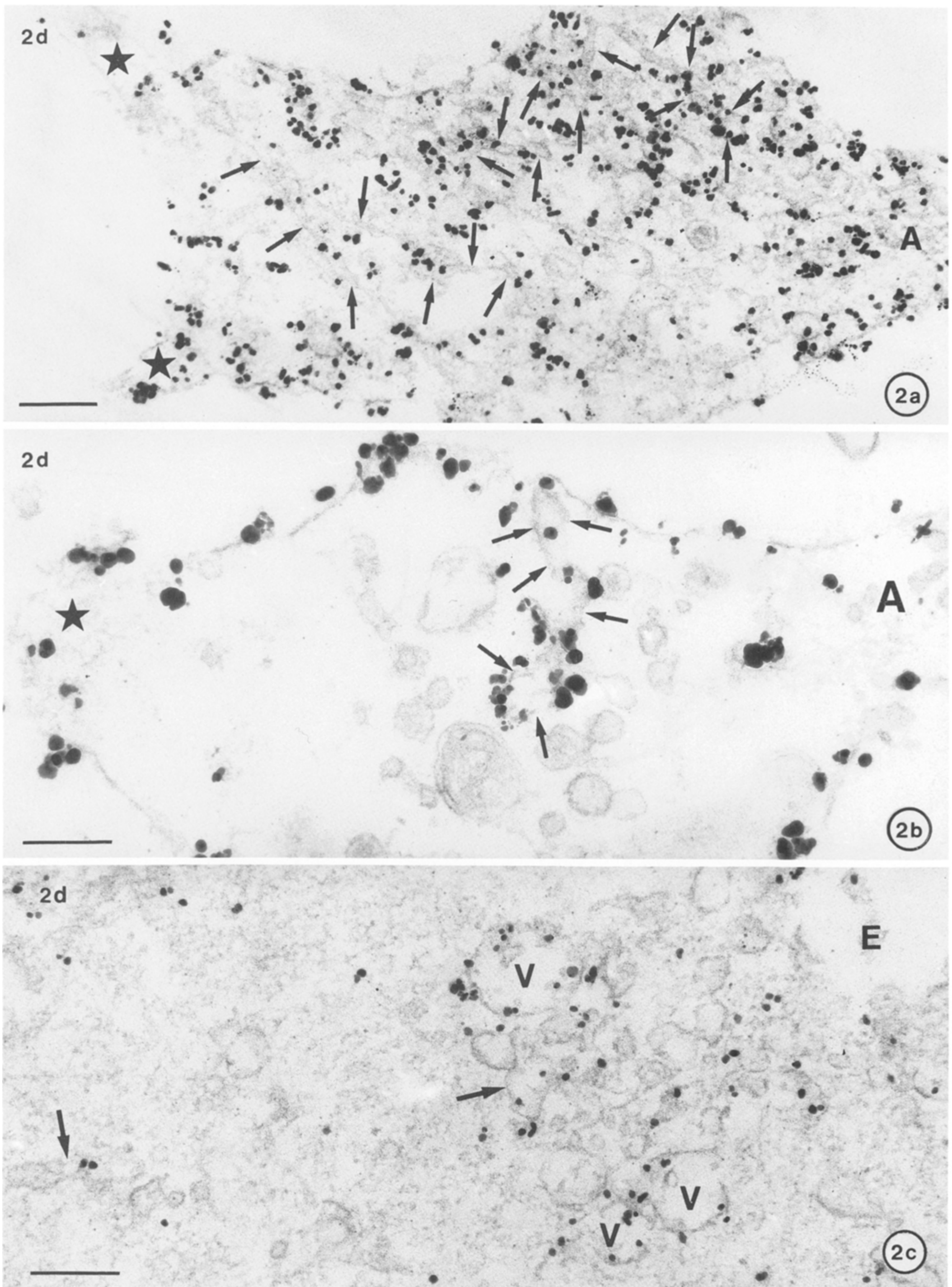


Fig. 2. Growth cones at two days are heavily labelled with GAP-43. (a) In addition to the heavy plasma membrane labelling, many growth cones were labelled extensively over internal membranes. Most of this internal label appeared to be associated with membranes, and cisternal membranes were frequently identified as labelled (arrows). The axon (A) and the filopodia (stars) are indicated. (b) Smooth cisternae labelled for GAP-43 were seen close to the plasma membrane (arrows), but it was difficult to determine if they were continuous with the plasma membrane. The axon (A) and a filopodium (star) are indicated. (c) Labelled vesicles (V) were seen in growth cones, close to labelled smooth cisternae (arrows). The extracellular space is indicated (E). Scale bars 0.2 μm.

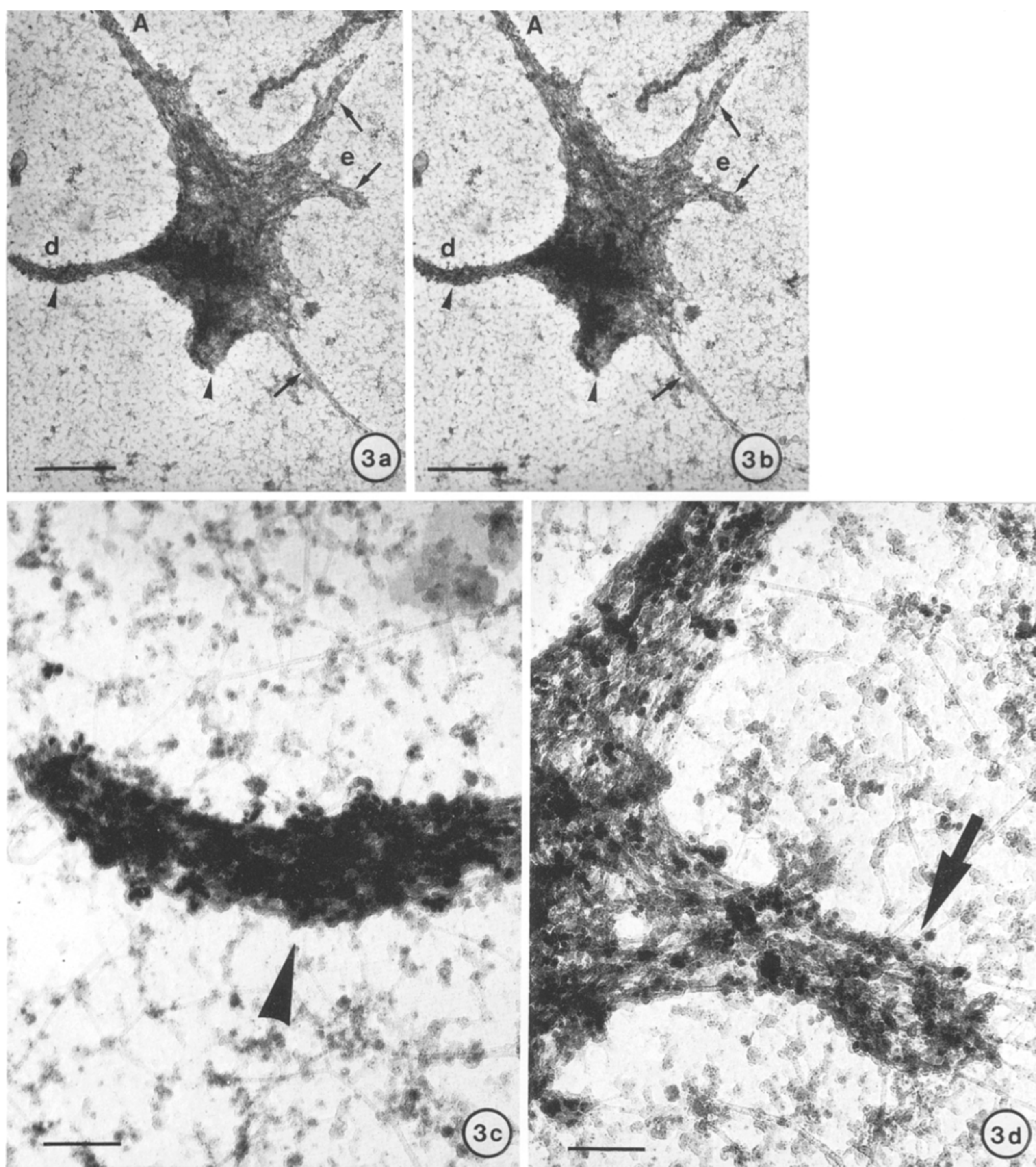
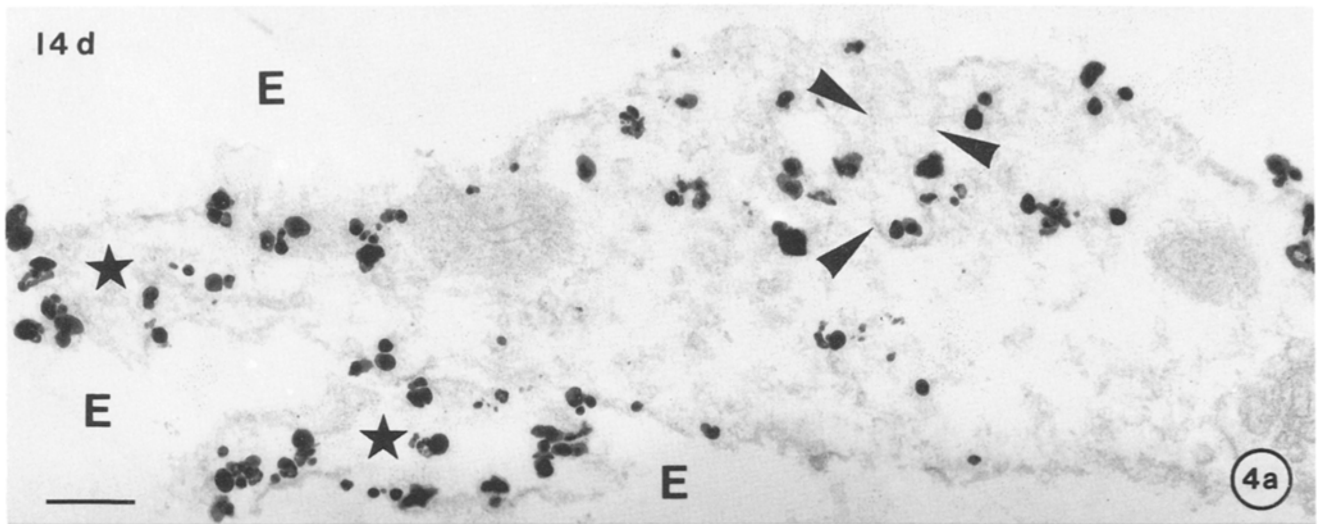


Fig. 3. Stereopairs of a growth cone (a,b) demonstrating that some filopodia were flattened and firmly attached to the substrate (arrows) while others were rounded and extended away from the substrate (arrowheads). The lamellipodium between the two extending filopodia is also extended above the substrate. The axon (A) is indicated, and filopodia indicated by lower case letters (c & d) are shown at higher magnification. The GAP-43 labelling over the rounded and extended filopodia (c, arrowhead) is high while the labelling over the flattened and attached filopodia (d, arrow) is low. Scale bars: (a,b) 1.0 μm; (c,d) 0.2 μm.

Fig. 4. GAP-43 labelling in neuronal swellings in older cultures. (a-b) Growth cones at 14 days (a) and 21 days (b) still showed high levels of labelling more concentrated over the filopodia (stars). The extracellular space is indicated (E). While the structure in (b) has filopodia, it also has a cluster of vesicles in lower left side, which may be synaptic vesicles. Cisternal labelling for GAP-43 was also seen in these older growth cones (a, arrowheads). (c) Presynaptic terminals generally had lower levels of label than growth cones, but this terminal with high levels shows clearly the distribution of label. The majority of GAP-43 label is associated with the plasma membrane rather than with the synaptic vesicles (stars). Scale bars: 0.2 μm.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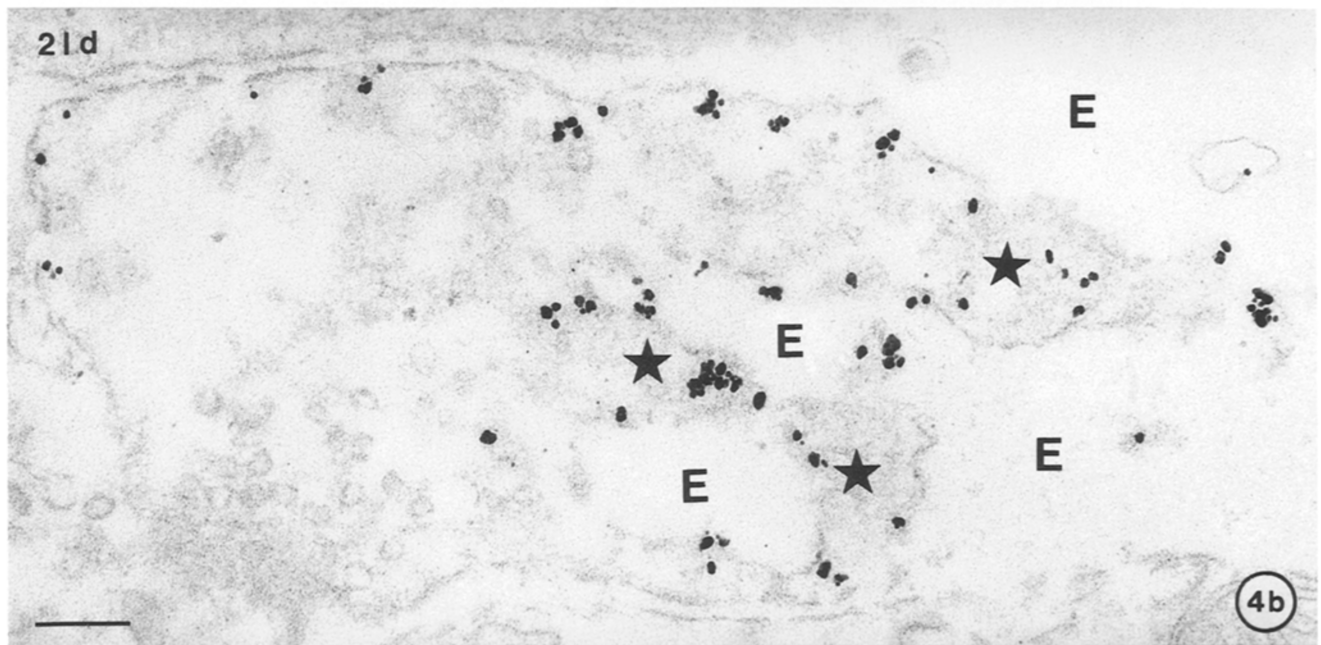
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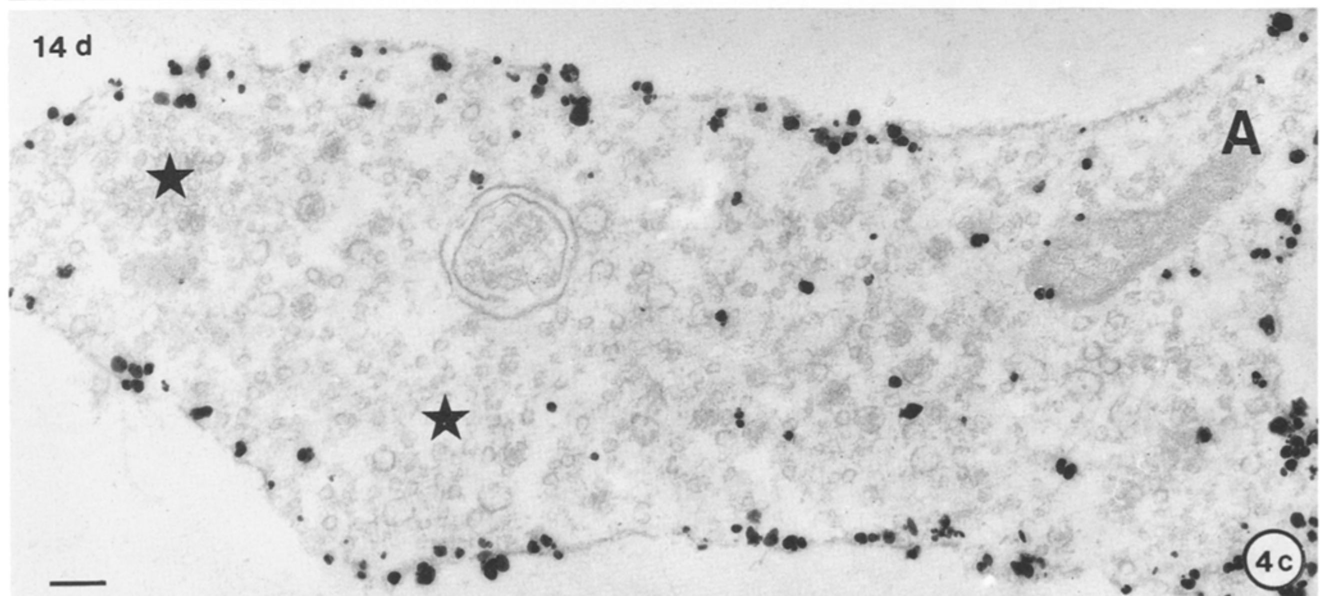
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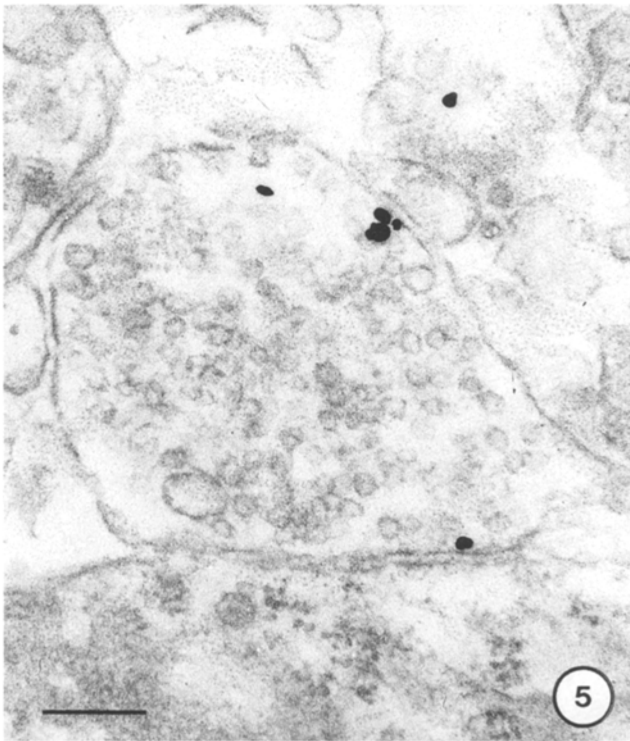

Fig. 5. Typical presynaptic terminal at 21 days labelled for GAP-43. The level of label is 4.40 particles per μm^2. Scale bar: 0.2 μm.

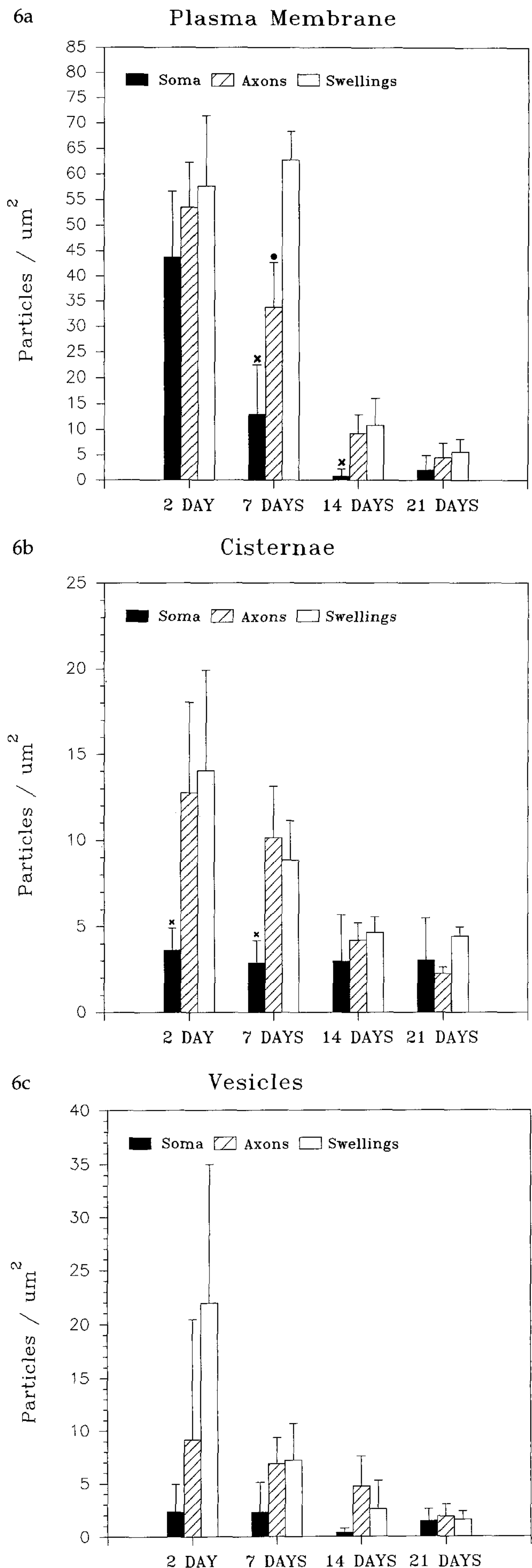

Fig. 6. Quantitation of GAP-43 particle distribution in developing neurons. (a) Density of particles over the plasma membrane in soma, axons and swellings at 2, 7, 14 and 21 days. The swellings had the highest density of label at all times in culture. At seven days, the swellings had significantly greater GAP-43 label than axons; also at seven days, axons had significantly more label than soma. At 14 days the levels of GAP-43 in all structures dropped greatly from seven days, but the axons and swellings were still labelled at significantly greater levels than the soma. The x over the standard deviation bar indicates that soma value is significantly different ($P < 0.05$) than values for axons and swellings. The solid circle over the standard deviation bar indicates that axons value is significantly different ($P < 0.05$) than value for swellings. (b) Density of particles at 2, 7, 14 and 21 days within the smooth cisternae compartment over soma, axons and swellings. At two days and seven days the values within the soma were significantly lower than values for the axons and swellings. The x over the standard deviation bar indicates that soma value is significantly different ($P < 0.05$) than values for axons and swellings. (c) Density of particles at 2, 7, 14 and 21 days within the vesicle compartment of soma, axons and swellings. High standard deviations with no statistically significantly differences for the values at two days indicate considerable diversity in the labelling of the vesicle compartment. The diversity of labelling within the morphologically similar vesicles indicates the compartment may be functionally heterogeneous.

remained highly labelled for longer time than the soma or axons.

GAP-43 levels in smooth cisternae at two and seven days were significantly higher within axons and swellings than within soma (Fig. 6b). By 14 days the low levels over smooth cisternae showed no statistically significant difference between soma, axons or swellings. Comparing the smooth cisternae and the plasma membranes of axons, levels of GAP-43 fell sooner over smoother cisternae (Fig. 6b) than over plasma membrane (Fig. 6a, cross-hatched bar). The particle density associated with large vesicles showed variable patterns of change (Fig. 6c). At two days, large vesicles in both axons and swellings were heavily labelled but due to the large standard errors the differences were not significant. The large error for quantitative EM immunocytochemical analysis of the vesicle compartment at two days was probably a result of the heterogeneity of these vesicles.

The above analysis of GAP-43 label in the category of 'swellings' showed a decrease with time in culture. This reduction could be a result of either decreased label in both growth cones and presynaptic terminals, which make up this compartment, or decreased label in the presynaptic terminals only. To distinguish between these possibilities, the density of particles for individual growth cones and individual presynaptic terminals was determined. For this analysis, the swellings were individually classified as growth cones, presynaptic elements, or other. From two to 14 days, growth cones had a median density of about 25 particles per μm^2, and at 21 days about 20 particles per μm^2. The median density of particles in presynaptic terminals at seven and 14 days were about 18 particles per μm^2, and decreased at 21 days to about 7 particles per μm^2. The relatively high density of label density in growth cones, even at 21 days, supports the association of GAP-43 with growth cones regardless of the age of the culture. The higher levels of GAP-43 in growth cones as compared to presynaptic terminals also suggests that GAP-43 loss could begin near the time at which presynaptic terminals form.

Distribution of GAP-43 in explant cultures

Electron microscopic immunocytochemistry results with GAP-43-labelled plasma membranes indicate that levels in the axons fell at seven days while levels in the swellings did not fall until 14 days (Fig. 6a). One interpretation of these changes in label is that GAP-43 is present only at the distal ends of growing axons, while it is lost in a proximal to distal gradient. Alternatively, GAP-43 could be present along the entire length of some axons and adjacent axons could be unlabelled. To help distinguish between these possibilities, mini-explant cerebellar cultures were prepared so that a radial pattern of axonal growth could be examined for the distribution of GAP-43 with LM immunocytochemistry.

In mini-explant cultures to five days, the cell bodies and axons labelled uniformly, in a pattern predicted from results with dispersed cell cultures (Burry *et al.*, 1991). At 7–14 days the levels of GAP-43 labelling in axons begin to fall, as was seen in the dispersed cell cultures (Burry *et al.*, 1991). At 10 days, the growth cones and distal axons (Fig. 7a, arrowheads) had higher levels of GAP-43 label than proximal axons (Fig. 7a arrows), most of which were lightly labelled. The most intense labelling in Fig. 7a is over a growth cone (double arrowhead). These results show that GAP-43 labelling in axonal plasma membranes of cultured granule cells is at the highest levels near the growth cone. These results support the observation that GAP-43 is present in axons in a proximal to distal gradient.

Discussion

Distribution of GAP-43 in axons

GAP-43 is synthesized in the soma, prior to its movement via fast axonal transport in a membrane fraction to the growth cone (Skene & Willard, 1981a; b; Heacock & Agranoff, 1982; Kalil & Skene, 1986; Moya *et al.*, 1988). Our results show that, in early cultures, the entire neuronal plasma membrane is labelled. GAP-43 labelling of axons decreased at an earlier time in culture than labelling of growth cones. With LM immunocytochemistry, the radial outgrowth in mini-explant cultures showed high levels of label associated with the distal axons and the growth cones. One explanation for these results is that the distal growth cone is the site where GAP-43 is added to the plasma membrane. This site of addition to the plasma membrane is consistent with the reports of high levels of GAP-43 found in isolated growth cone particles (Katz *et al.*, 1985). While our results do not exclude the possibility that GAP-43 is added to the plasma membrane of the proximal axon, heavy labelling for GAP-43 in axons near growth cones in older cultures suggests a different primary site of addition near the growth cone.

Fast axonal transport of GAP-43 could occur, at least in part, in the smooth cisternae, and there is evidence that fast axonal transport observed in other systems also involves cisternal membranes. Fast axonal transport studied in the squid axon with video-enhanced microscopy has revealed that particles (vesicles and tubulo-vesicular elements) were moved in an anterograde direction toward the terminal (Allen *et al.*, 1982). EM analysis of squid axons showed that the particles detected with video-enhanced microscopy were mitochondria, vesicles and endoplasmic tubules (Fahim *et al.*, 1985). Axonal endoplasmic reticulum has been

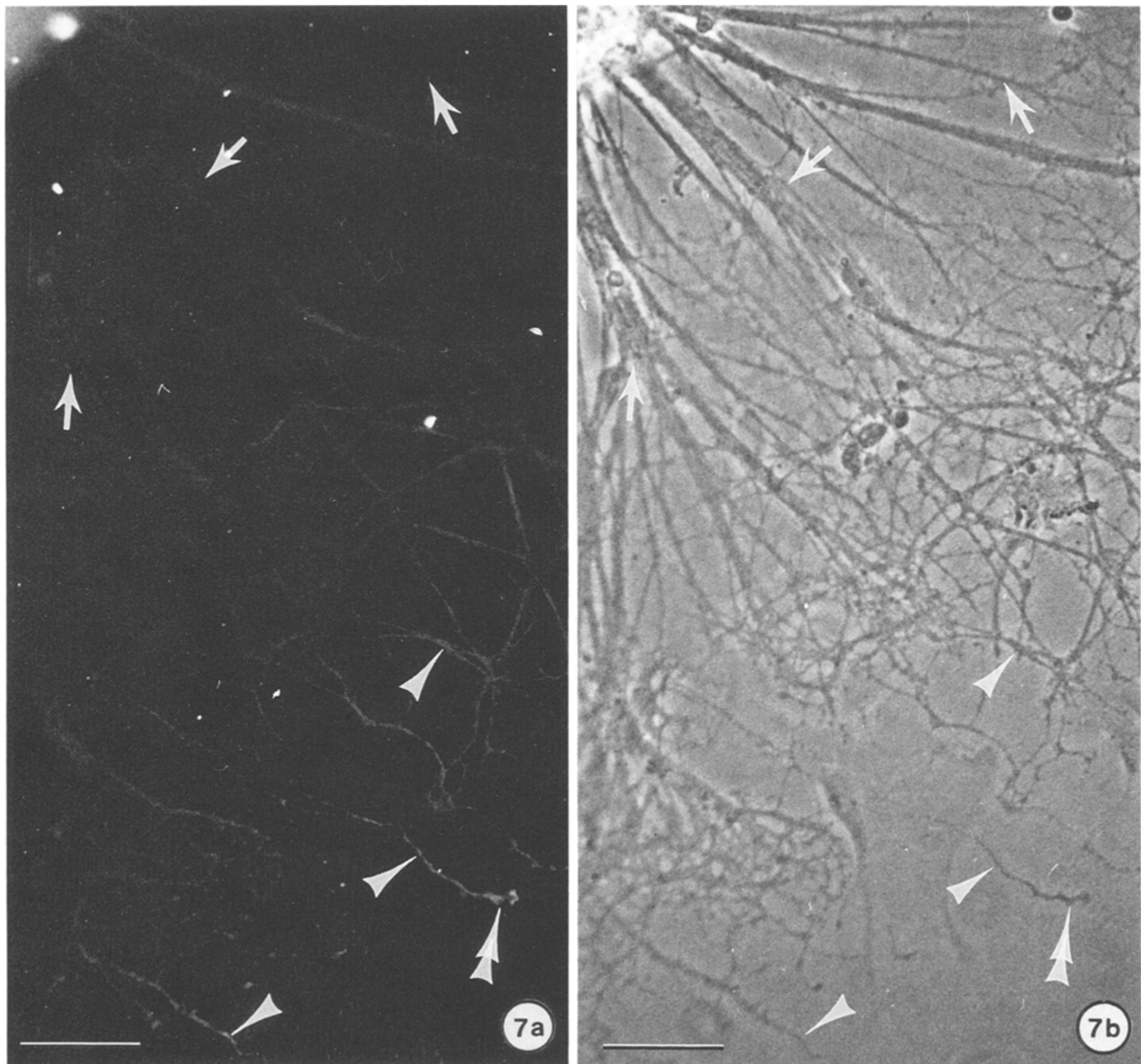

Fig. 7. Cerebellar explant cultures examined for GAP-43 light microscopic immunocytochemistry at 10 days. (a) Immunofluorescence of GAP-43 with the explant at the top left of the micrographs. The distal axons (arrowheads) had higher levels of immunofluorescence than proximal axons (arrows). Intense labelling was seen over growth cones (double arrowheads) at the distal edge of the outgrowth. (b) Phase optics micrograph of the culture demonstrating the extent of axonal outgrowth. Scale bar 20 μm.

identified as an organelle of fast axonal transport in studies with EM autoradiography (Byers, 1974; Droz *et al.*, 1973, 1975). These studies also suggest that synaptic vesicles are formed in the terminal from the axonal reticulum (Droz *et al.*, 1975). Other workers have indicated that a tubulo-vesicular, but not the axonal reticulum system present in axons and terminals, is involved in rapid anterograde axonal transport (Ellisman & Lindsey, 1982). The relationship of the smooth cisternae compartment seen in our cultures to the membrane structures defined by others is not clear. However, we have observed GAP-43 particles labelling intracellular cisternal membranes. This observation suggests that parts of the intracellular membrane may be associated with transport of GAP-43, consistent with the previously reported tubulo-vesicular transport system.

GAP-43 distribution in growth cones and presynaptic terminals

In this study, the level of GAP-43 associated with growth cones at all times is greater than that for presynaptic terminals. With increased time in culture, the levels of GAP-43 over presynaptic terminals

dropped by 21 days to near-background levels. Growth cones at 21 days, however, maintained higher levels of GAP-43. These results suggest that GAP-43 levels begin to fall only after growth cone differentiation into presynaptic terminals.

The time course for the appearance of a synaptic vesicle protein (p65) in growth cones has been compared to that of GAP-43 with a light microscopic double label technique (Burry *et al.*, 1991). The earliest growth cones were GAP-43 positive and p65 negative. After two days in culture, many structures became GAP-43 and p65 positive. As growth cones developed presynaptic terminal characteristics (became p65 positive), they became GAP-43 negative. It is possible that the mechanism which triggers presynaptic terminal formation may also initiate the loss of GAP-43.

GAP-43 is distributed primarily on the plasma membranes of growth cones and immature presynaptic terminals. In a recent report on adult and neonatal hippocampus, GAP-43 was seen associated with the plasma membrane of immature and adult presynaptic terminals (van Lookeren Campagne *et al.*, 1990). These authors found virtually no GAP-43 associated with the cell bodies and dendrites of these pyramidal neurons. In both the present results and those of van Lookeren Campagne and colleagues (1990), virtually no GAP-43 was seen associated with the synaptic vesicles of the presynaptic terminals.

GAP-43 distribution in growth cone filopodia

Motile growth cones have filopodia that move in the culture medium (Nakai & Kawasaki, 1959) and flattened filopodia that adhere to the substrate, and anchor the growth cone (Bray & Chapman, 1985). Previous studies have suggested that GAP-43 in growth cone plasma membranes is found at high levels in filopodia (Gorgels *et al.*, 1989; Van Hooff *et al.*, 1989; Zuber *et al.*, 1989). In our present results, whole growth cones were examined in the EM for the distribution of GAP-43. Filpodia that were not attached to the substrate were probably motile, and had high levels of GAP-43, while lower levels of GAP-43 were seen in filopodia that were flattened and attached to the substrate. This high density of GAP-43 on filopodia plasma membrane is consistent with the suggestion that GAP-43 is involved with growth cone function. Zuber and colleagues (1989) showed that GAP-43 may contribute directly to growth cone activity based on observations of transfected fibroblasts expressing GAP-43. However, Meiri and Gordon-Weeks (1990) found high levels of GAP-43 in a 'membrane cytoskeletal' fraction of growth cone particles, and observed that at least some of the GAP-43 in growth cones may be associated with areas of growth cone membranes that are tightly attached to the substrate. LaBate and Skene (1989) have suggested a membrane-cytoskeletal complex involving GAP-43. The recent report that GAP-43 deficient PC12 mutant cells can still initiate axonal out growth (Baetge & Hammang, 1991), and the finding that high levels of kinase C phosphorylated GAP-43 is found in distal growing axons (Meiri *et al.*, 1991), suggests that GAP-43 has a role in growth cone interactions with its environment.

The presence of GAP-43 on filopodia may indicate a role based on possible signal transduction through the growth cone membrane (Van Hooff *et al.*, 1989). This idea was supported by Strittmatter and colleagues (1990) who reported that GAP-43 from growth cones can stimulate GTP-γ-S binding to G_0, a G protein. G proteins have been identified as transducers for extracellular receptors in a variety of systems (Gilman, 1987; Neer & Clapham, 1988). The first 25 amino acids of GAP-43 were responsible for the binding of GAP-43 to a subunit of G_0 and stimulation of GTP-γ-S binding. High concentrations of GAP-43 and high concentrations of G_0 in growth cones combined with GAP-43 stimulation of G_0 suggests that GAP-43 may be a regulator of G_0 (Strittmatter *et al.*, 1990). Thus, it is possible that GAP-43 in growth cones may be involved in regulation of some signal transduction events. The high concentration of GAP-43 on motile filopodia is consistent both with a function in filopodial motility, with a function in regulation of transduction on these motile filopodia, or some combination of the two.

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