Molecular Basis of Growth Cone Adhesion: Anchoring of Adheron-containing Filaments at Adhesive Loci

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Abstract. Adhesive contacts made by filopodia of neuronal growth cones are essential for proper neurite elongation and may have a role in the formation of synaptic junctions. Previously we described the appearance of filamentous materials extending from growth cone surfaces that seem to be associated with the strongly adhesive behavior of filopodia (Tsui, H.-C., K. L. Lankford, and W. L. Klein. 1985. *Proc. Natl. Acad. Sci. USA.* 82:8256–8260). Here, we have used immunogold labeling to determine whether known adhesive molecules might be localized at points of adhesion and possibly be constituents of the filamentous material. Antibodies to an adhesive molecule (neural cell adhesion molecule [N-CAM]) and to an adhesive macromolecular complex of proteins and proteoglycans (adheron) were localized at the EM level in whole mounts of cultured avian retina cells. Labeling of fixed cells showed that N-CAM and adheron molecules were both present on growth cones and on filopodia. However, filamentous materials extending from the cell surface were labeled with anti-adheron but not with anti-N-CAM. If cells were labeled before fixation, patches of anti-N-CAM labeling occurred in random areas over the growth cones, but adheron antibodies concentrated at points of apparent adhesion. Particularly dense clustering of anti-adheron occurred at individual filopodial tips and at points of contact between pairs of filopodia. The different patterns of labeling imply that N-CAMS do not associate with the main antigenic components of adheron on the membrane surface. Most importantly, the data indicate the N-CAMs were mobile in the membrane but that constituents of adherons were anchored at adhesive loci. An appealing hypothesis is that molecules found in adheron preparations have an important role in establishing the adhesive junctions formed by growth cone filopodia.

MECHANISMS which underly axonal guidance and synaptic target recognition are critical to the generation of proper neuronal connections. These mechanisms are likely to rely on disparate factors, including physical channels and barriers, preestablished axonal pathways, glial guidance, electrical field, and gradient distribution of diffusible factors (Tosney and Landmesser, 1985; Singer et al., 1979; Patel and Poo, 1984; Campenot, 1977; Gundersen and Barrett, 1979). At the molecular level, the role of specific adhesive interactions between growth cones and their environment have been of particular interest (Letourneau, 1975; Silver and Rutishauser, 1984; Bentley and Caudy, 1983; Berlot and Goodman, 1984). These studies of adhesion have focused on membrane-associated proteins and extracellular matrix molecules (Tomaselli et al., 1986; Rogers et al., 1986; Tosney and Landmesser, 1985).

The best characterized adhesive molecule is neural cell adhesion molecule (N-CAM),[1] an integral membrane glycoprotein first obtained from avian retina (Thiery et al., 1977; Thanos et al., 1984, Rutishauser, 1985, 1986; Silver and Rutishauser, 1984; Maier et al., 1986). The concentration and form of N-CAM is developmentally regulated, and this has been suggested to play a role in axon guidance (Fraser et al., 1984, Rutishauser, 1985). Adhesion mediated by N-CAM is thought to involve homophilic binding of N-CAM molecules on both adhering membranes (Rutishauser and Goridis, 1986; Rutishauser et al., 1982; Edelman et al., 1983). N-CAM also binds to heparan sulphate proteoglycan, suggesting a role in cell–extracellular matrix adhesion (Cole and Glaser, 1986). In the developing optic tract (Silver and Rutishauser, 1984) and along motor neuron routes (Tosney et al., 1986), N-CAM occurs on the surfaces of the axon as well as on the non-neuronal cells. However, antibodies against N-CAM alter only the route, but not the growth rate of retinal ganglion cell axons (Silver and Rutishauser, 1984), suggesting N-CAM is not the only essential adhesive molecule required for axon growth. The exact role of N-CAM in growth cone adhesion and axon guidance remains to be determined.

1. *Abbreviation used in this paper*: N-CAM, neural cell adhesion molecule.

Adhesive interactions also have been associated with macromolecular complexes released by both non-neuronal (Adler et al., 1981; Collins, 1978; Lander et al., 1983) and neuronal (Riopelle and Cameron, 1984) cultured cells. The effects of complexes from non-neuronal cells are mediated by components of the extracellular matrix, including laminin, fibronectin, and heparan sulphate proteoglycan, that bind to specific receptors on the neuronal cell surfaces (Tomaselli et al., 1986; Bozyczko and Horwitz, 1986; Schubert et al., 1983). While neurons generally are not thought to have an extracellular matrix, they do release macromolecular complexes possessing adhesive properties (Riopelle and Cameron, 1984; Schubert et al., 1983). For cultured chick retina neurons, such complexes can be isolated by high speed centrifugation of conditioned media (Schubert et al., 1983). These complexes, termed adherons, contain many proteoglycans and proteins, including a 20-kD retinol-binding protein that promotes cell–adheron adhesion (Schubert et al., 1986). Adheron-mediated adhesion is cell-type specific and developmentally regulated. Although isolated adherons appear as 15-nm spheres, their native configuration is not known.

Current work focuses on mechanisms of filopodia adhesion in growth cones of cultured avian retina neurons. Cells from the retina show extensive differentiation in culture, including the formation of synapses (Crisanti-Combes et al., 1977, 1978; Vogel et al., 1976; DeMello et al., 1982; Siman and Klein, 1983; Lankford et al., 1987), and they have been used in studies of both N-CAMs and adherons (Thiery et al., 1977; Schubert et al., 1983). In this study, we have used EM immunogold labeling to determine whether N-CAM or adheron might be localized at points of filopodial adhesion. Past work has indicated that adhesive junctions formed by filopodia may be mediated by filamentous materials extending from the membrane surfaces (Tsui et al., 1985). The current data show that the filamentous extensions between filopodia are labeled with anti-adheron but not anti-N-CAM, and indicate that adhesive interactions of growth cones and filopodia are mediated by adheron constituents.

Materials and Methods

Retinal Cell Culture

As described previously, retina neurons were dissociated from 8-d chicken embryo with 0.5% trypsin and mechanical disruption, and plated on polylysine-treated, formvar-coated gold grids in DME containing 10% FCS (Tsui et al., 1985). After 2 d in culture, cells were fed serum-free DME supplemented with 5 μg/ml of insulin, 100 μg/ml transferrin, 20 nM progesterone, 100 μM putrescine, and 30 nM selenium.

Antiserum against Adheron

Rabbit antiserum was obtained as described by immunizing rabbits with adherons purified from cultured 10-d embryonic chick neural retinas (Schubert et al., 1983). This antiserum blocks the adhesion of chick neural retina cells to adheron-coated substrata as well as cell–cell adhesion (Schubert et al., 1983). Western blot analysis (Burnette, 1981; Schubert et al., 1986) of anti-adheron serum was done against total serum-free growth conditioned medium (Fig. 1, lane *A*), purified adherons (lane *B*), and 100,000 *g* supernatant (lane *C*). Nitrocellulose strips with transferred proteins were incubated overnight with a 1:100–1:200 dilution of immune serum at room temperature. The nitrocellulose was then washed for 2 h in incubation buffer minus BSA, incubated for 2 h with 2 million cpm of ^{125}I-protein A, and extensively washed before drying and autoradiography. Four densely stained bands (40, 65, 185, and 230 mol wt) and at least 20 minor bands were detected in Fig. 1, lanes *A* and *B*. Bands at 65 and 185 mol wt were missing in Fig. 1, lane *C*, and the overall pattern was recognizably different.

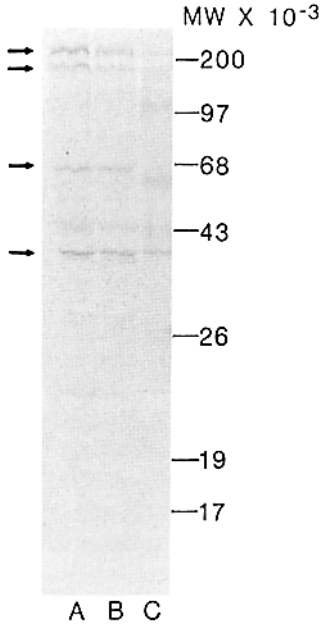


Figure 1. Specificity of anti-adheron antibody. Two aliquots of 10-d embryonic neural retina serum-free growth-conditioned media were either desalted or centrifuged at 100,000 *g* for 3 h and the supernatant desalted. The total supernatant (*A*), 100,000 *g* supernatant (*C*), or adheron pellet (*B*) were then lyophilized, run on a 15% acrylamide SDS gel, transferred to nitrocellulose, and blotted with a 1:200 dilution of rabbit anti-adheron serum. Four distinct bands at 40, 65, 185, and 230 mol wt (*arrows*) and at least 20 minor bands are recognized in the total medium (*A*) and adheron lanes (*B*). Bands at 65 and 185 mol wt were missing in 100,000 *g* supernatant (*C*), and the overall pattern was recognizably different. N-CAM is present as the band at 230 mol wt in the three lanes (Cole et al., 1985; data not shown).

No labeling was seen on Western blot of preimmune serum. Since SDS was present in the acrylamide gel to denature the protein, the molecules recognized in the immunofluorescence and immunogold experiments may only partially reflect the molecular species seen in the Western blots.

Immunofluorescence

Retina cells plated on polylysine-coated coverslips were labeled with antiserum against adheron or affinity-purified rabbit antibodies against chick N-CAM provided by Covault and Sanes (1985). Labeling was carried out on live cells as well as on lightly fixed cells. In either case, cultures were washed gently but extensively (six times) with culture media before the fixation and antibody incubation procedure to remove N-CAM and adheron from the media. This prevented artifactual complexes which might form between the antibody and the antigen in the media. For postfixation labeling, the cultures were fixed with 1% paraformaldehyde in PBS for 10 min at room temperature, washed with PBS, preincubated in 10% normal goat serum, 1% BSA, and PBS and incubated with rabbit anti-adheron serum at 1:20 or 1:50 dilution or rabbit anti-N-CAM at a concentration of 5 μg/ml in NGS/BSA/PBS solution for 1 h. The cultures were washed and incubated with FITC goat anti-rabbit IgG (1:200; ICN K&K Laboratories, Inc., Plainview, NJ) for 1 h. Live cell labeling was done in similar procedure except the initial fixation was omitted and all the incubations were carried out in DME at 37°C in carbon dioxide incubator.

Preparation for Whole Mount Electron Microscopy

Cultures were prepared for EM examination by the critical point drying procedure as described (Tsui et al., 1985). Solutions were freshly made and microfiltered before use. Cells were fixed in 2.5% glutaraldehyde in 0.1 M Hepes buffer, postfixed in 0.1% osmium tetroxide, stained in 1% uranyl acetate, dehydrated in ethanol, then critical point dried through carbon dioxide. Whole mounts were examined with the 1 MeV microscope at the High Voltage Electron Microscope (HVEM) facility at the University of Wisconsin-Madison or with a JOEL 100 CX electron microscope at 100 kV.

Immunogold Labeling

Immunogold labeling was carried out on live cells as well as on lightly fixed cells. Cultures were washed extensively (10 times) with culture media before the fixation and antibody incubation procedure. For postfixation labeling, the cultures were fixed with 0.1% glutaraldehyde and 1% paraformaldehyde

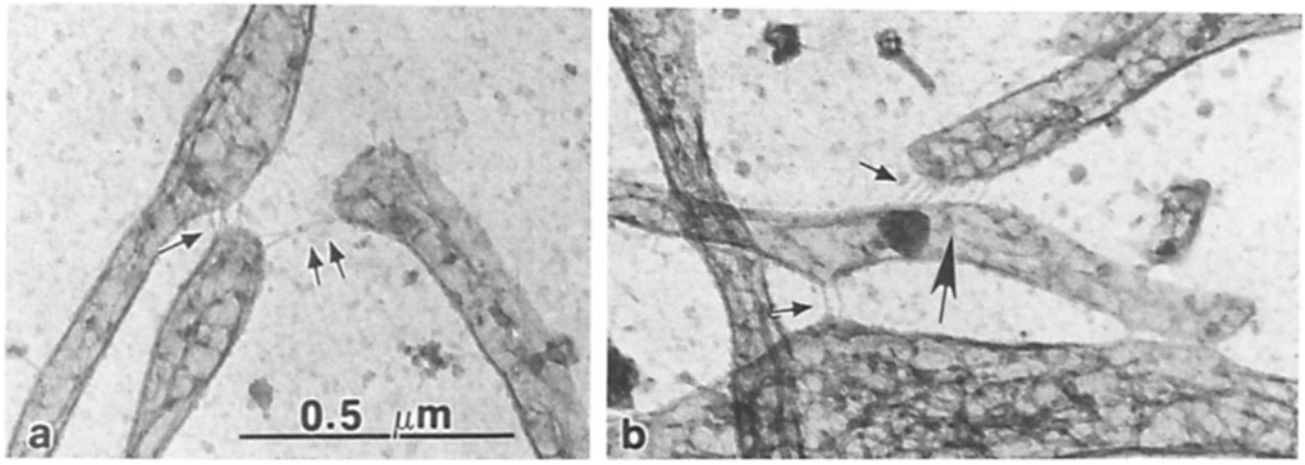


Figure 2. Electron microscopic examination of retina cultures showing that gaps between filopodial tips are bridged by filamentous structures (*small arrows*). Filamentous links between filopodia were usually 10–13 nm in diameter and 30–100 nm long. *a* shows the tip of a filopodium connected to two other filopodia via distinct filaments. In *a*, the filaments appear to emanate from different points of filopodia tips and are in contact with the membrane surface on the other filopodia. The appearance of the membranous surface near the contact areas suggests that the filaments adhere to the opposing membranes. At areas of contact, usually one or both membranes bulge outward toward an apex marked by the filaments. At points where the shaft of a filopodium was contacted by filaments, the whole shaft (*large arrow* in *b*) was seen to bend at the contact point. The bulging and bending at contact areas give the impression that the cell surfaces were held together under tension by the filaments.

in 0.1 M Hepes buffer for 10 min at room temperature. The cultures were then treated with 1 mg/ml of sodium borohydride in Hepes buffer twice for 5 min each and finally washed three times with Hepes buffer. After preincubation with 1% BSA in Hepes buffer, fixed cells were incubated with rabbit anti-adheron sera at a 1:20 or 1:50 dilution or rabbit anti-N-CAM antibody at a concentration of 5 μg/ml for 1 h. The antibody reaction was followed by three 10-min washes with Hepes buffer for 10 min each and then incubated with 5 or 15 nm gold-conjugated goat anti-rabbit IgG (1:5 or 1:10 dilution; Jannsen Life Sciences Products, Piscataway, NJ) for 1 h. The cells then were washed with Hepes buffer, postfixed in 1% glutaraldehyde, osmicated, dehydrated, and critical point dried as with unlabeled preparations. The experimental conditions for live cell labeling were similar to the above fixed cell procedure except that the inital fixation and sodium borohydride procedure were omitted and all the incubations were carried out in DME at 37°C in a carbon dioxide incubator.

Results

Electron Microscopy of Whole Mount Cultures

Electron microscopic examination of retina cells was done using whole mount specimens rather than sections. This permitted rapid localization of the regions of interest and enabled us to detect details of structural organization that otherwise might be obscured by embedding and sectioning.

Filopodial tips in whole mount preparations were frequently seen to contact one another. Inspection at high magnification revealed that filopodial tips were bridged by filamentous structures (Fig. 2). Filamentous links between

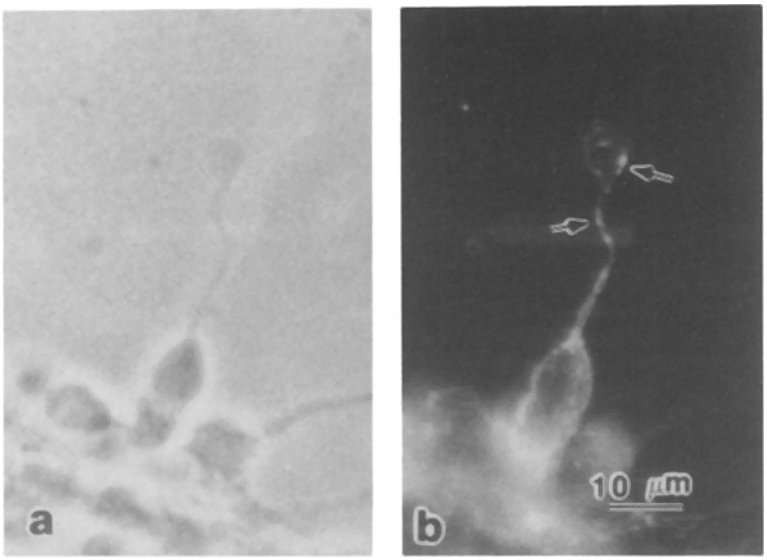
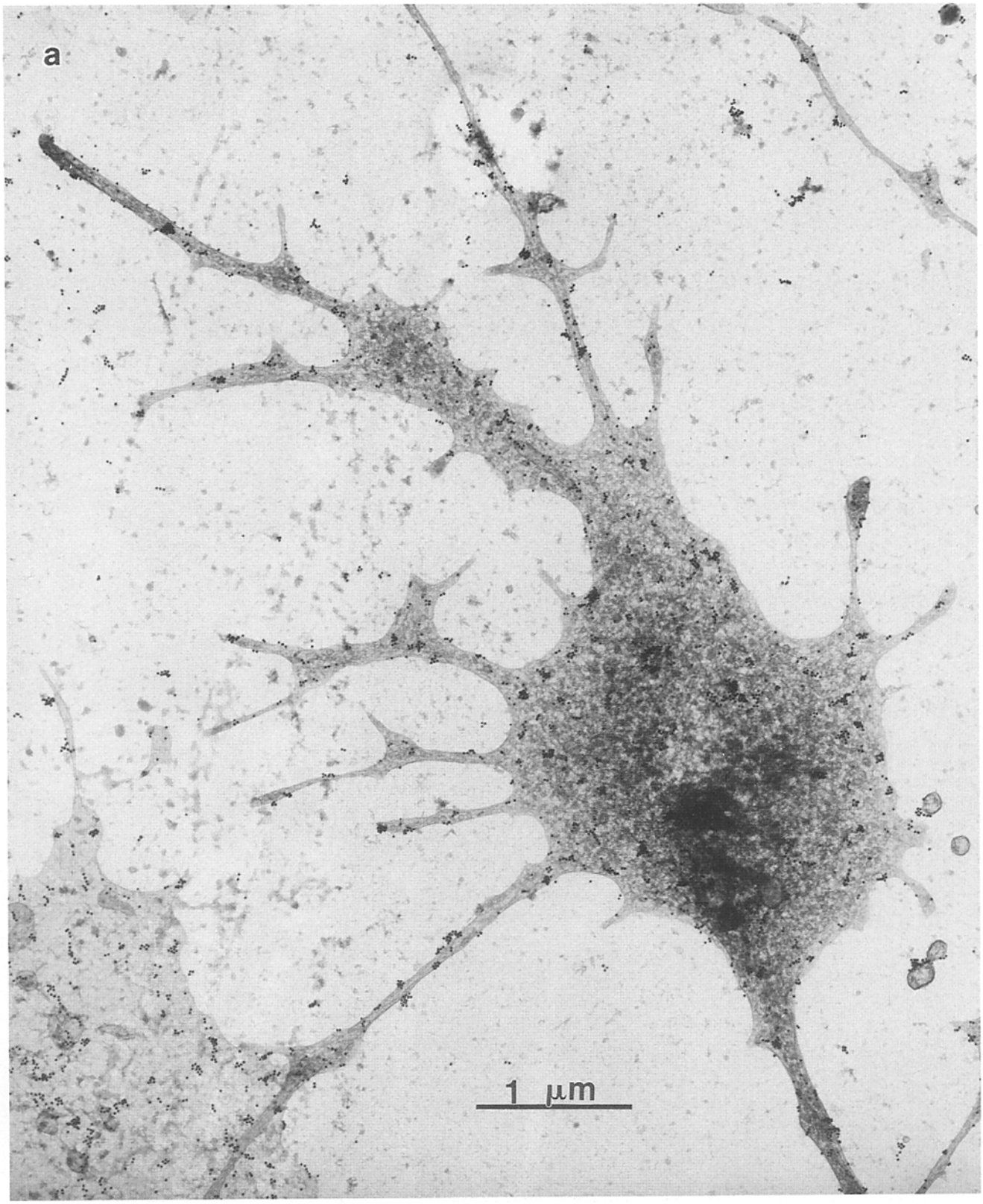


Figure 3. Immunofluorescence with antiserum to adheron showing labeling on cell bodies, neurites, and growth cones. A subpopulation (30%) of cultured retina cells was brightly labeled with antiserum to adheron. The fluorescence pattern appeared punctate on the cell bodies. neurites, and growth cones (*arrows*). The punctate appearance indicated that there were areas of high concentrations of adheron immunoreactivity on the growth cone.


a
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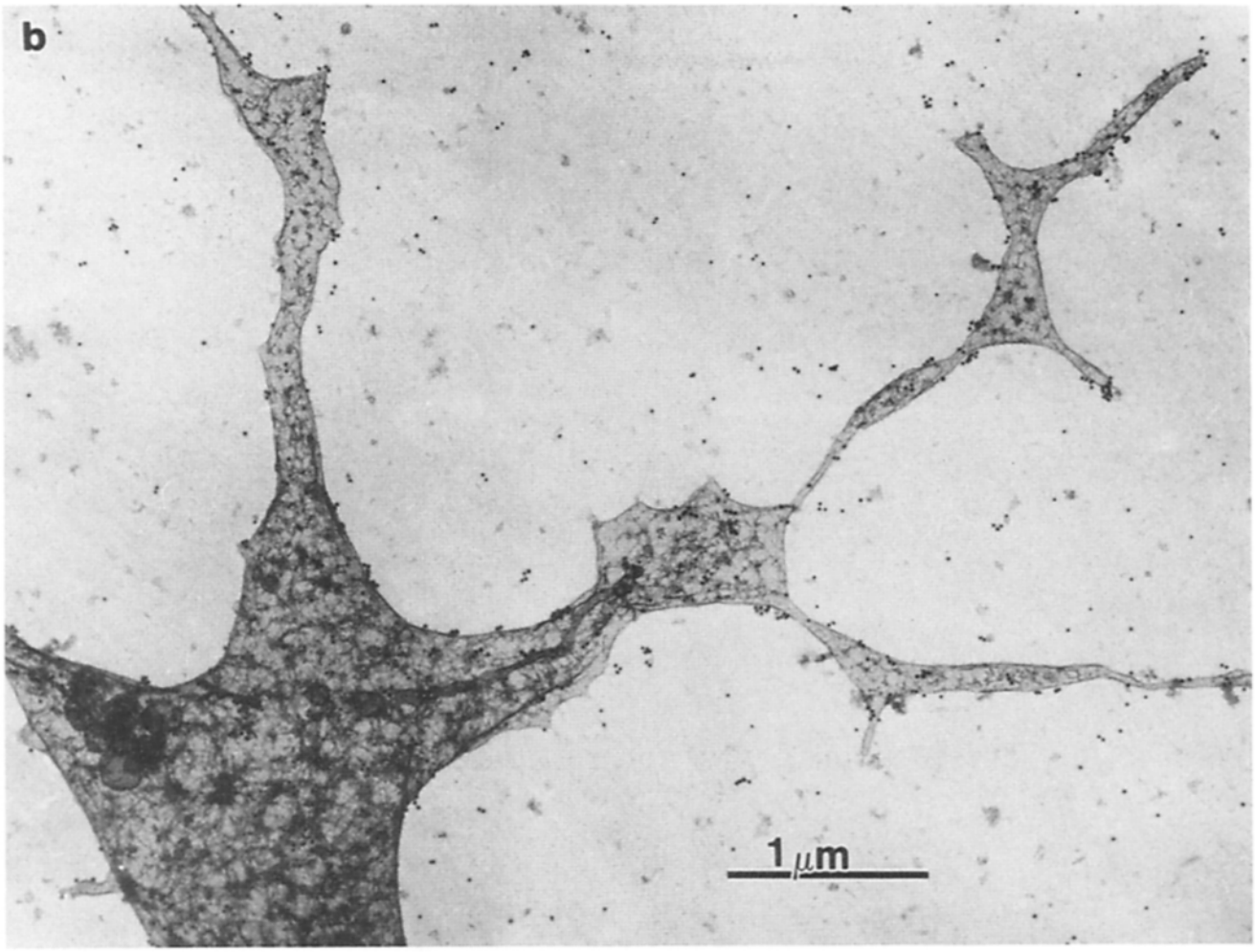


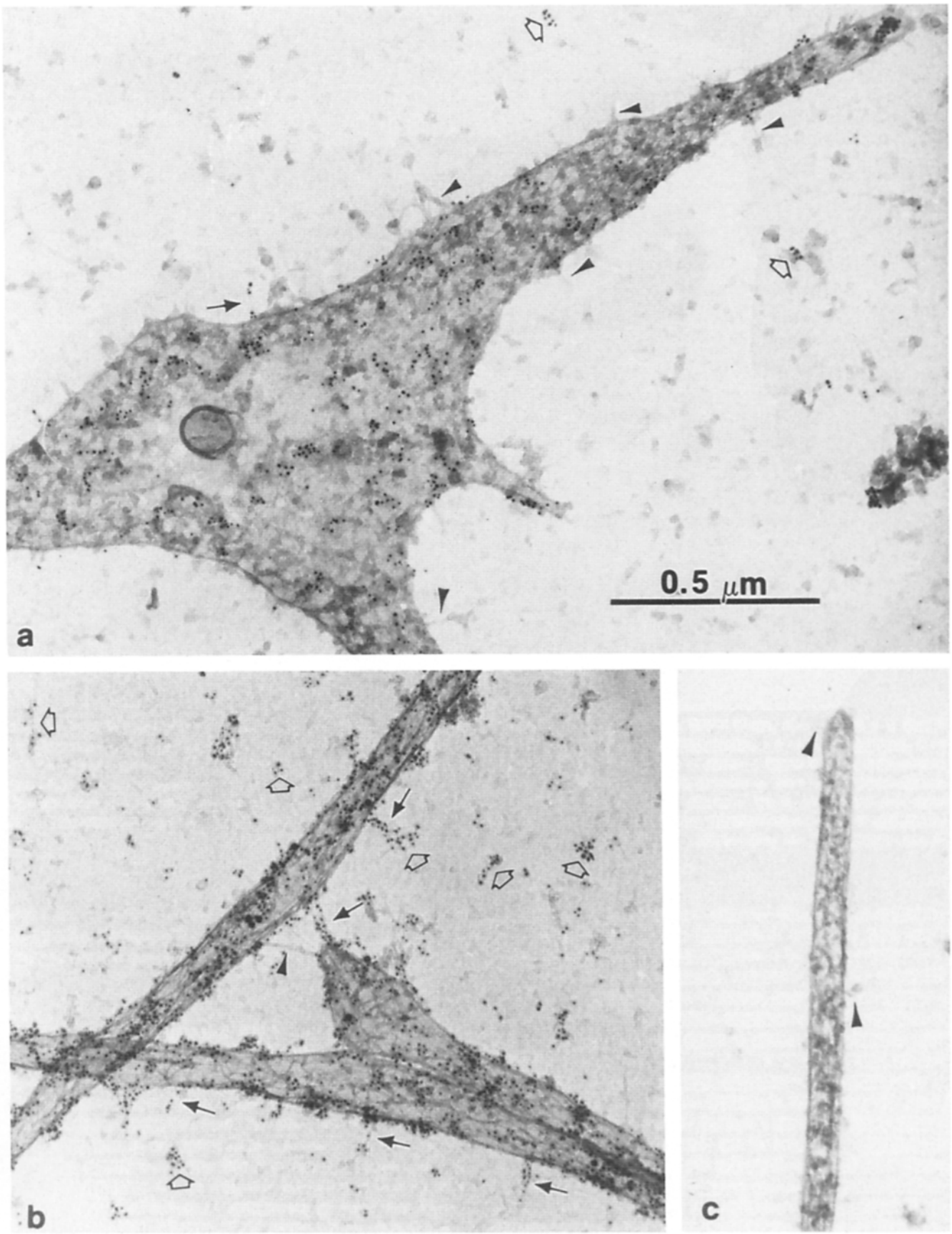
Figure 4. Labeling with anti-N-CAM and anti-adheron on growth cones and filopodia of fixed cells. The culture was lightly fixed with 0.1% glutaldehyde and 1% paraformaldehyde, rinsed with sodium borohydride, and then incubated with the antibodies. 15-nm gold particles were used to provide better visibility at low magnification. Although the density of the labels due to anti-N-CAM (*a*) varied among different growth cones, there were no obvious differences in label densities on the palms of growth cones versus the filopodia. Note also that there were very few labels on the substrate. Anti-adheron (*b*) label was found on both growth cones and filopodia and did not show obvious differences in the densities between growth cones and filopodia. However in contrast to anti-N-CAM, many more anti-adheron labels were on the substrate (15-nm gold labels).

filopodia were slightly variable in appearance, but they typically were 10–13 nm in diameter and 30–100 nm long. Fig. 2 *a* shows the tip of a filopodium connected to two other filopodia via these distinct filaments. Filaments appeared to emanate from different points of the filopodial tip and were in contact with the membrane surfaces of the other filopodia. The number of filaments between tips was variable; seven can be seen in Fig. 2 *b*.

The filamentous extensions appear to serve adhesive functions. At regions of filopodial contact, the surface membranes usually showed a modest convex or outward bowing appearance. The apex of the curves occurred where filaments linked the two filopodia. (Fig. 2). At points where the shaft of a filopodium was contacted by filaments, the whole shaft often appeared bent (Fig. 2 *b*, *large arrow*). The bowing and bending at cross-bridging areas gives the impression that the cell surfaces were held together under tension by the filaments. The appearance of adhesion, as shown by curving of filpodia at contact areas, could also be seen in living cells as well as in specimens prepared by different EM techniques (Tsui et al., 1985), and thus appears not due to the preparation procedures.

Immunofluorescence of N-CAM and Adheron on Cells First Fixed, Then Labeled

To start investigating the molecular identity of the adhesive filaments, we have labeled chick retina cultures with antibodies to N-CAM and adheron. Immunofluorescence experiments were first carried out to obtain an overall pattern of the distribution of anti-N-CAM and anti-adheron labeling. E9C4 retina cultures were fixed with 1% paraformaldehyde before the labeling procedure. Anti-N-CAM fluorescence was present on all phase-bright, process-bearing cells (not shown). There was no obvious difference in the intensity of labeling among the different neuronlike cells. N-CAM label-


0.5 μm
a
b
c

ing was found on cell bodies, neurites, and growth cones. In contrast to the relatively uniform N-CAM labeling on all neuronlike cells, anti-adheron very brightly labeled only 30% of the phase-bright cells. Flat cells and the rest of the phase-bright cells were dimly labeled. The cell bodies of the brightly labeled cells were larger in diameter (9.23 ± 1.44 μm compared with 6.4 ± 0.3 μm of dimly labeled cells; n = 12 in each case). It therefore appears that the antiserum to adheron selectively labels a subpopulation of retina cells in culture. Fluorescent labels were present as punctate areas on cell bodies, neurites, and growth cones (Fig. 3). The punctate appearance indicated that there were areas of high concentrations of adheron immunoreactivity on the growth cone.

EM Immunogold Labeling of Anti-N-CAM and Anti-Adheron on Cells First Fixed, Then Labeled

To obtain a more detailed picture of the distribution of N-CAM and adheron immunolabeling, we have labeled cultured neurons with the immunogold method and examined the cultures as whole mounts with the electron microscope. In Fig. 4, *a* and *b*, the cultures were first lightly fixed (0.1% glutaldehyde and 1% paraformaldehyde for 10 min), labeled with anti-N-CAM or anti-adheron, incubated with secondary antibodies conjugated to 15 nm gold particles, and then processed by critical point drying. Examination at low magnification showed that both N-CAM (Fig. 4 *a*) and adheron (Fig. 4 *b*) were present on growth cones and filopodia. Close examination at high magnification showed that there was a major difference in the distribution of N-CAM and adheron immunoreactivity on fixed preparations. When the cultures were labeled with the smaller 5 nm gold particles and examined at 20,000×, the filamentous extensions from membrane surfaces were found to label with antiserum to adheron (Fig. 5 *b*) but not anti-N-CAM (Fig. 5 *a*). N-CAM labeling showed abundant gold particles on the surfaces of growth cones and filopodia (Fig. 5 *a*). However, little or no labeling occurred on the filamentous materials extending from the membrane surfaces. In contrast, antibodies against adheron prominently labeled these filamentous structures (Fig. 5 *b*). Typically the labels were present all along the filaments which extended from the membrane surfaces and were in contact with other membrane surfaces or with the substratum. Some of the filements, however, remained unlabeled (Fig. 5 *c*, *arrowhead*).

Anti-adheron also labeled filamentous materials on the substratum (Fig. 5 *b*, *open arrows*). The filaments were similar in dimension to those attached to cell surfaces. These background materials were not components of the serum in the media since they were also found in cultures that were plated directly in serum-free media (Fig. 2). They were present even with the use of meticulously clean glassware and freshly made and microfiltered solutions, and they were not present on control processed grids that did not contain retina cells. These materials could be reduced, although not eliminated, by extensive washing (10 times) of the cultures before fixation. The results show that the background materials labeled by anti-adheron were produced by the cultured retina cells and adhered to the polylysine-coated culture surface.

The labeling of the filamentous extensions and substrate surfaces was specific to adheron antiserum, with little labeling occurring when either anti-N-CAM (compare the background labeling on Fig. 5 *a* with 5 *b*) or nonimmune sera were used (Fig. 5 *c*). Nonimmune serum at the same dilution as immune sera showed very low labeling of the cells and the substrate (Fig. 5 *c*).

Patterns of Anti-N-CAM and Anti-Adheron on Cells First Labeled, Then Fixed

To investigate whether these molecules played a role in growth cone or filopodia adhesion, we have incubated cells with primary antibodies prior to fixation. Under these circumstances, the bivalent antibodies can induce patching of mobile membrane antigens. For completely mobile membrane proteins, the location of these patches may be random. However, if some of the antigens are specifically anchored at adhesive regions, the antibodies would show patching at these points.

Immunofluorescence labeling showed patching of fluorescence labeling with antibodies to N-CAM and adheron on cell bodies, neurites, and growth cones. However, because of the limit of resolution of light microscopy, we have analyzed in detail the patching phenomenon with the EM immunogold method.

When live cultures were labeled with anti-N-CAM, gold labels were found in dense patches at random areas of the cell body (detected with immunofluorescence), neurites or growth cones but very rarely at filopodia or cell–cell contact regions. Fig 6 *a* shows a large patch of label at the center of the growth cone while the periphery and the filopodia were almost devoid of labeling. The pattern of anti-N-CAM labeling on live cells gives the impression that the patches were formed at random areas of the membrane and were not clustered at areas of adhesion.

A strikingly different pattern of labeling was found with antiserum directed against adheron. In contrast to anti-N-CAM labeling, label due to anti-adheron was concentrated on filopodia and at the edges of growth cones. This is illustrated at low magnification in Fig. 6 *b* in which very high concentrations of 15 nm particles line the filopodia and the edges of the growth cone. The center of the growth cone, in comparison, has much less label. At higher magnification (Fig. 7), the labels were seen clustered as small patches around areas where the membrane bowed out towards the

Figure 5. Labeling with anti-N-CAM (*a*), anti-adheron (*b*), and nonimmune serum (*c*) on fixed cells with the smaller size 5-nm gold particles showing a major difference in the labeling pattern of N-CAM molecules and adheron molecules. For N-CAM (*a*), although many labels were found on the surfaces of growth cones and filopodia, very little labeling occurred on the filamentous materials (*arrows* point to labeled filaments, *arrowheads* point to unlabeled filaments) extending from the membrane surfaces. Occasional labeling was found on background materials (*open arrows*), but this is rare. In contrast to the anti-N-CAM–labeling patterns, anti-adherons (*b*) were found on the filamentous materials extending from the cell surfaces as well as on the background materials on the substratum. Nonimmune serum used at the same dilution as the immune serum showed very low labeling both on the cells and on the culture substrate (*c*).

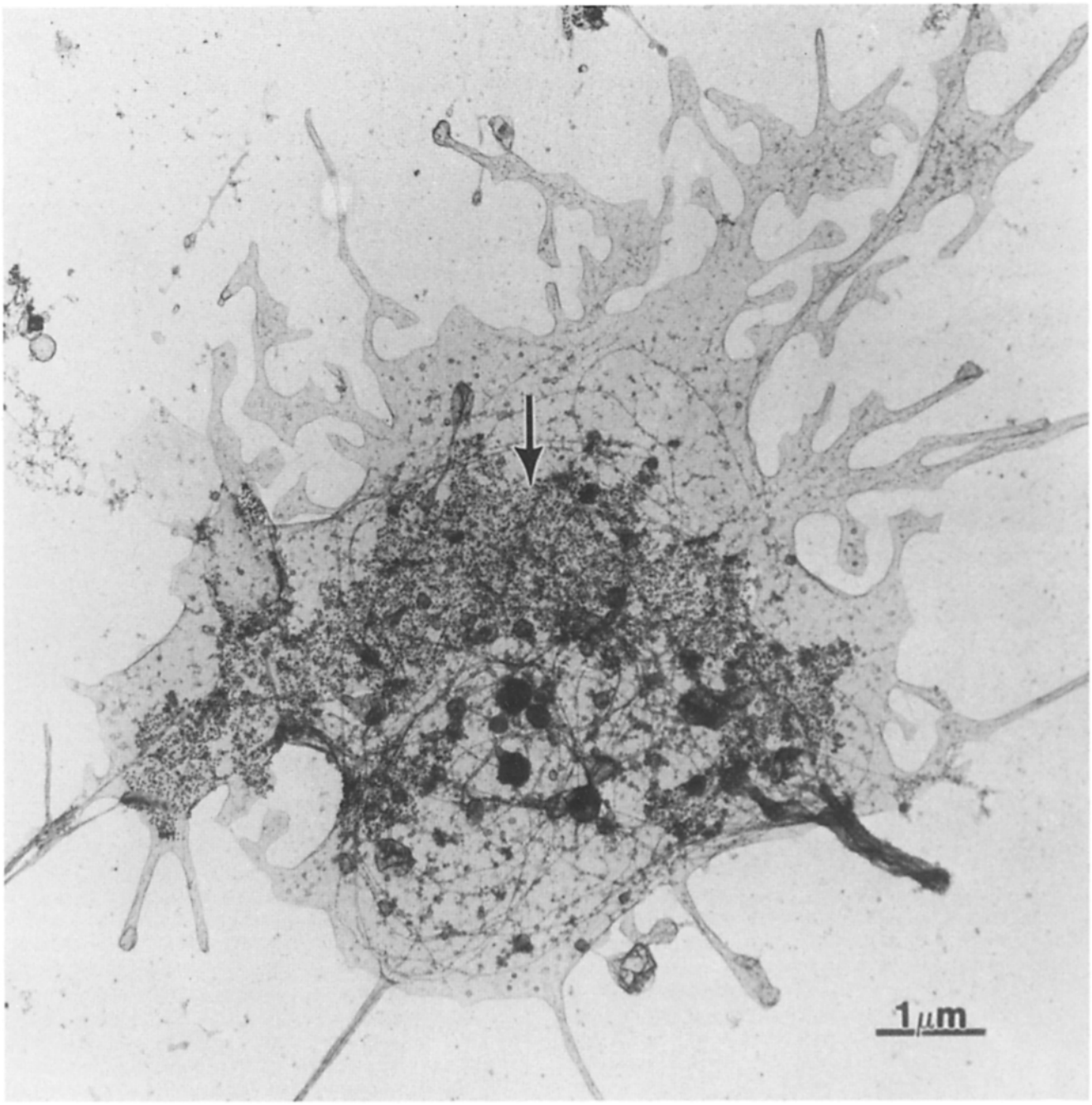
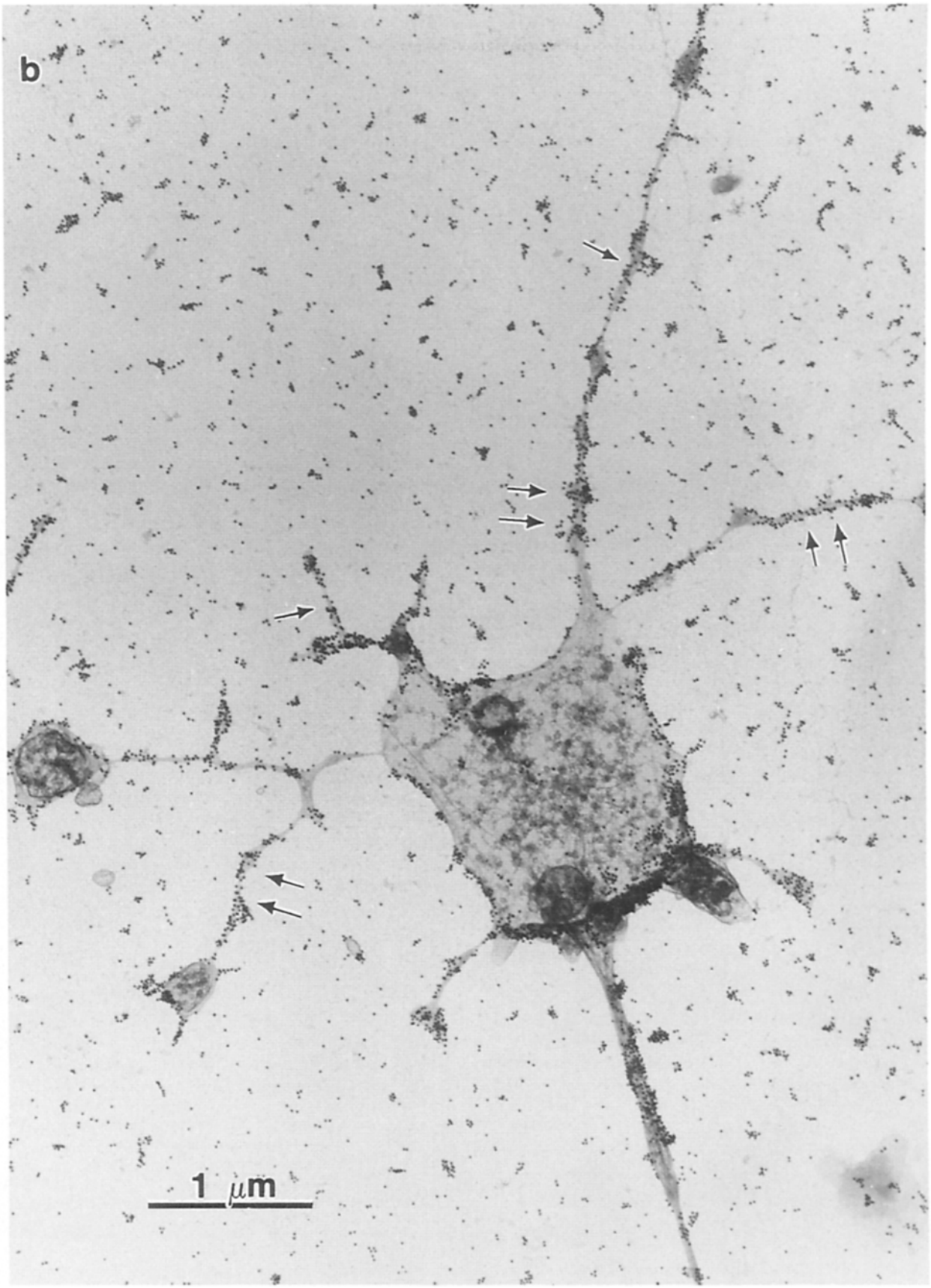


Figure 6. Labeling of anti-N-CAM and anti-adheron on live cells. In *a*, a large patch of labels due to anti-N-CAM was concentrated at the center of the growth cone while the periphery and the culture substrate are almost devoid of labels. In *b*, labeling of anti-adheron showed high concentration of labels at edges of growth cones and filopodia. This pattern of labeling is in sharp contrast to the pattern of N-CAM labeling in *a*. Arrows point to the dense labels on filopodia.

substrate (*black arrows*). Antibodies were present on the membrane and on the filamentous extensions that connected the membrane to the substrate. The pattern of anti-adheron labeling thus showed an association with adherent regions of the growth cone.

We found particularly high concentrations of anti-adheron labeling at the tips of filopodia (Fig. 8). As above, label was present on the filamentous extensions as well as on the membrane surface around the tip. Fig. 8, *a* and *b* shows two stereopairs of filopodia attached to the substrate. The filopodia tips were covered with the 5-nm labels, and these were continuous with the labels on the filamentous extensions. Examination of the filopodia as stereopairs shows the labeled filaments joined the surface of the filopodia to the culture substrate.

Where filopodia tips contacted other membrane surfaces, there again was a high concentration of label (Fig. 8 *c*). Here the labels formed a continuous patch from the surface of the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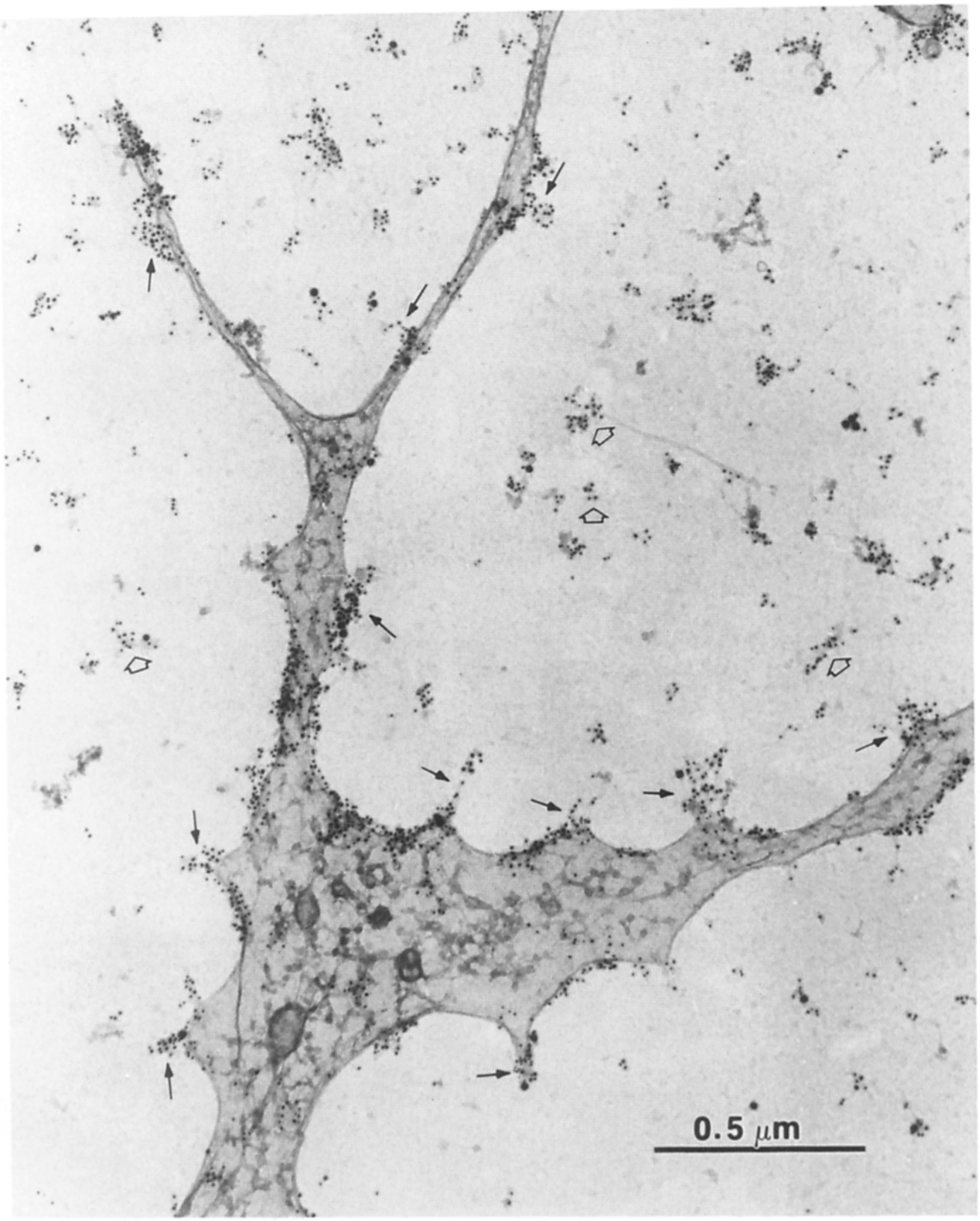

Figure 7. Anti-adheron labels associated with adherent regions of growth cones in live cell-labeling preparations. The labels mostly appear as small patches around areas where the membrane convexed out towards the substrate (*black arrows*). The labels are present on the membrane and on the filamentous extensions that connected the membrane to the substrate. Filamentous and particulate materials on the substrate are also labeled (*open arrows*).

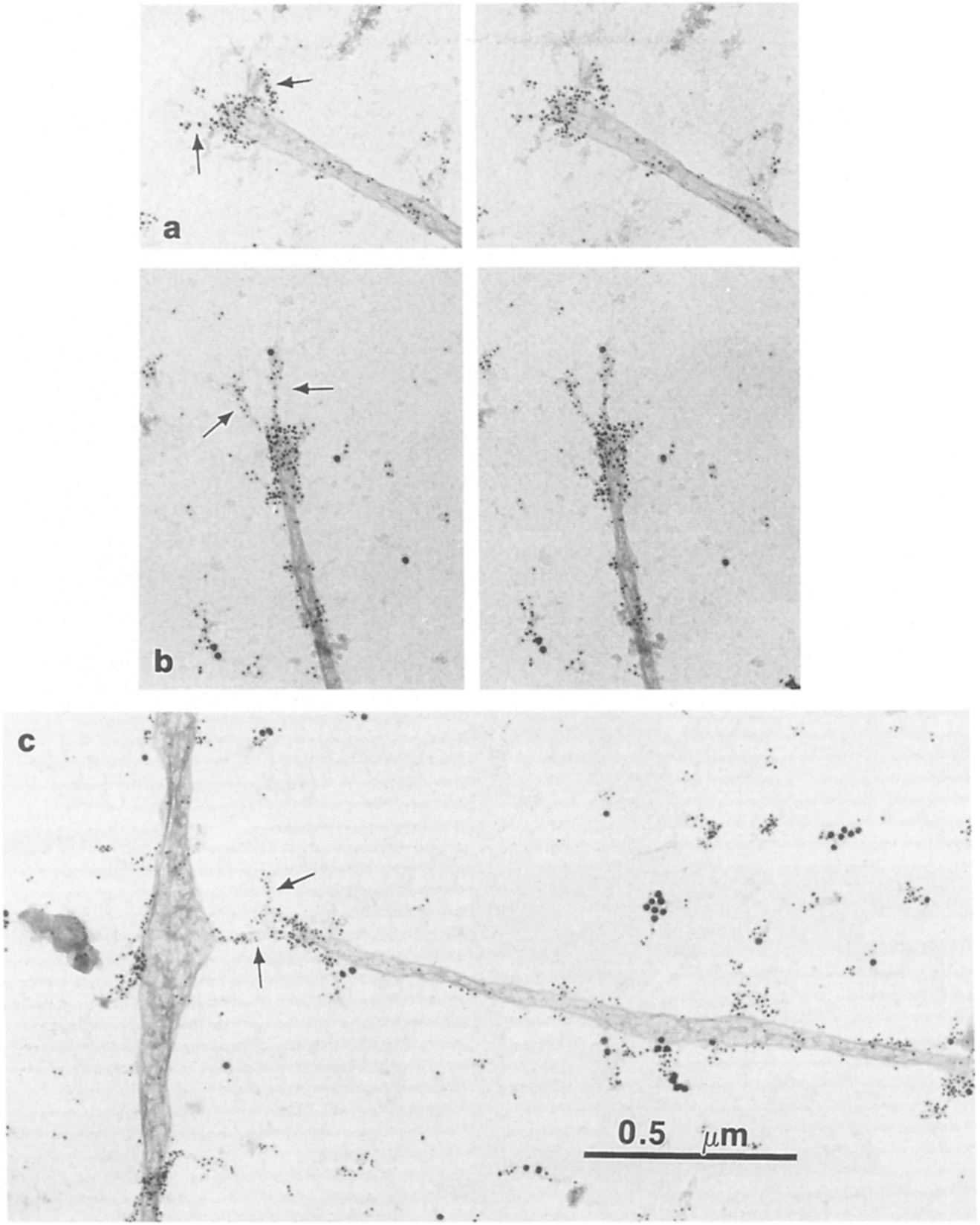

Figure 8. High concentration of anti-adheron labels at the tips of filopodia. The filopodia tips are covered with the 5-nm labels that are continuous with the labels on the filamentous extensions. Examination of the filopodia as stereo pairs (*a* and *b*) shows the labeled filaments (*arrows*) join the surface of the filopodia to the culture substrate and that some of the labels extend upwards from the membrane surface. In *c*, the labels formed a continuous patch from the surface of the filopodia tip, through the filamentous extensions, to the opposite membrane surface.

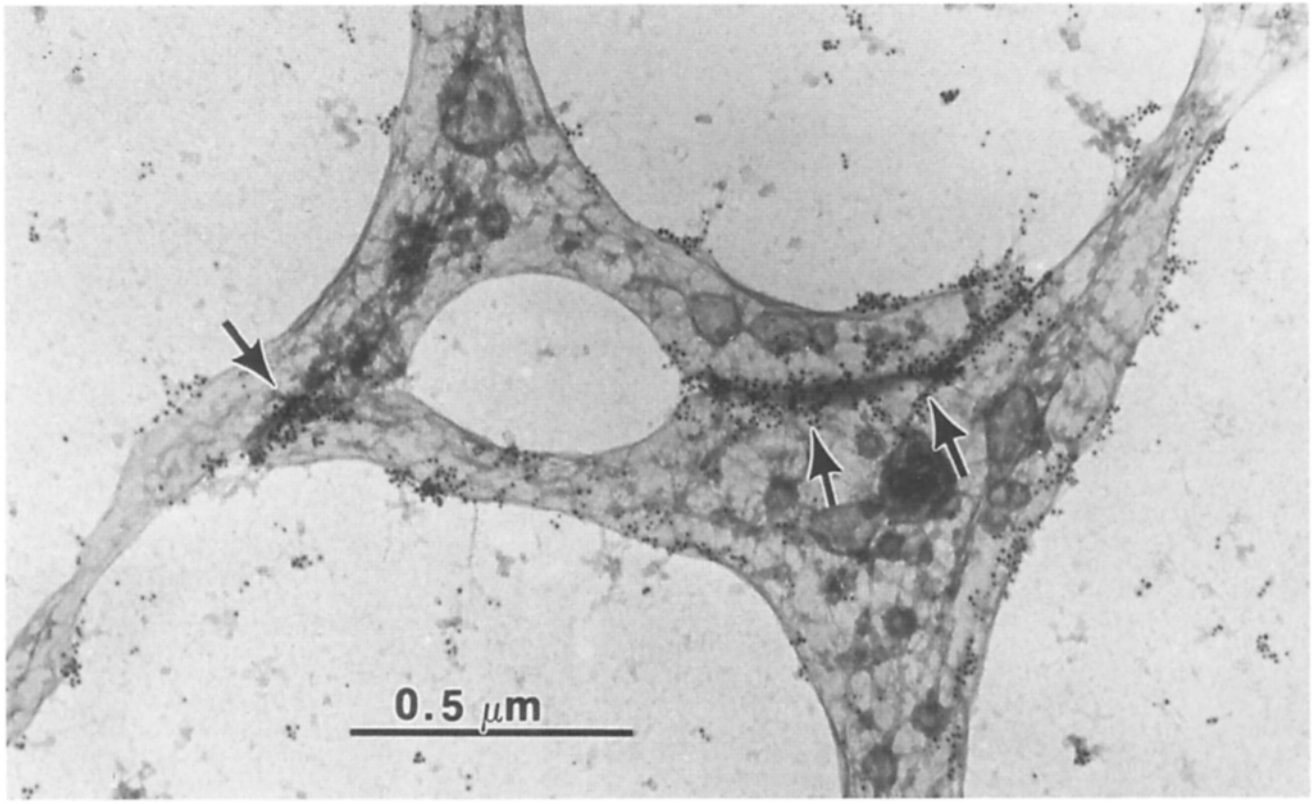

Figure 9. Anti-adheron labeling on live cells showing high concentrations of anti-adheron at membrane contact areas. The labels are extensive with the entire membrane contact areas while the neighboring noncontacting areas are devoid of labels.

filopodia tip, through the filamentous extensions, to the opposite membrane surfaces. Other filaments joining the filopodia to the substratum were also labeled.

Occasionally, filopodia contacted each other over an extended membrane region, as shown in Fig. 9. These extended contacts may represent a transition of casual filopodial contact to the formation of synapses. In these instances, label was coextensive with the entire junctional area, while the neighboring noncontacting areas were devoid of labels.

Discussion

The adhesive contacts of motile filopodia have an essential role in determining the extent and direction of axon elongation (Letourneau, 1975; Bentley and Caudy, 1983; Berlot and Goodman, 1984). In addition, some of these contacts appear to have the potential for developing into synaptic junctions (Tsui et al., 1985). EM whole mount data in the present study demonstrated that filopodial adhesion is associated with molecules found in isolated complexes called adherons. The structure of adheron constituents at adhesive loci is filamentous.

Three lines of evidence support the conclusion that molecules extending from the cell surfaces play a critical role in filopodial adhesion. First, direct observation of living neurons with video-enhanced contrast, differential interference contrast microscopy showed that pairs of motile filopodia would form adhesive junctions despite apparent gaps between their tips; sometimes these gaps contained faint structures suggesting the two filopodia were connected via fine filaments (Tsui et al., 1985; unpublished data). Second, EM examination of whole mounts clearly showed extended filamentous materials at adherent areas of filopodia and growth cones (Fig. 2; see Tsui et al., 1985). Third, EM localization using colloidal gold-labeled antibodies showed these filamentous structures to comprise constituents of adherons, macromolecular complexes previously implicated in cellular adhesion (Schubert et al., 1983).

A different type of adhesive molecule, N-CAM, also was present on the membrane surfaces of growth cones and their filopodia, in agreement with other studies (Rutishauser, 1985; van der Pol et al., 1986). However, N-CAM molecules were not associated with the filamentous structures that appeared to be involved in adhesive contacts. Moreover, incubation of living cells with anti-N-CAM caused large patches of labels to form at random areas on membrane surfaces of growth cones and neurites. The absence of label at adhesive areas was not likely due to a recognition failure of anchored N-CAM, since a polyclonal antibody preparation was used. Our data support the conclusion that the N-CAM population was unanchored at sites of filopodial contact or adhesion and that they are freely mobile as previously described by others (Gall and Edelman, 1981; Pollenberg et al., 1986).

It is not clear which components of adherons were labeled with the antiserum. The adheron immunofluorescence experiment showed that a subpopulation of retina cultured cells was brightly labeled, suggesting that the antiserum may contain a high titer of antibodies against specific antigens. Although Western blot analysis showed several major bands, it is not clear whether these bands represent the antigens recognized in the immunofluorescence and immunogold experi-

ments since the proteins were denatured in the Western blot experiments. Antibodies against specific components of adheron will be necessary to identify the molecular species of the filaments.

The 30% of cells in culture that were brightly labeled may represent a subpopulation of cultured retina cells. Preliminary results with frozen sections of 18-d-old embryonic retinas showed that although photoreceptor cells and ganglion cells were slightly higher in labeling intensity, the difference in intensity was not as drastic as retina cells in culture (Tsui, H.-C. T., and W. L. Klein, unpublished results). It is possible that different cell types intrinsically have varying capacities for expressing adheron constituents (Berman et al., 1987). Alternatively, it may be that the brightly labeled cells represent neurons at different developmental stages, perhaps influenced by the culture condition.

Differences in antibody labeling patterns suggest that the main antigenic components of adheron and N-CAM are not closely associated. This is in contrast to the close association of N-CAM and L1, another adhesive molecule (Thor et al., 1986). Sites for anti-N-CAM were not present in extensions of the membrane surface or in particulate materials on the substratum. Labeling due to antiserum to adheron, in contrast, was present both in the filamentous extensions and in the substrate materials. Most importantly, the patching patterns of the two antibodies were strikingly different. Anti-N-CAM patches at random areas of the neuronal membrane surface while antiserum to adheron patches at adhesive loci. Since N-CAM is partially removed by high speed centrifugation of embryonic chick neural retina growth-conditioned medium, it has been suggested that it is a component of the adheron complex (Cole and Glaser, 1984; Cole et al., 1985; Cole and Glaser, 1986). Our results showed that N-CAM and the main immunogenic components of adheron were different with respect to their location on the membrane surface and their lateral mobility within the membrane.

The whole mount EM results clarify the appearance of adheron constituents in their native form. Adherons are purified as 100,000 g pellets from conditioned media of cultured chick retina neurons and contain all of the adhesion-promoting properties of the conditioned media (Schubert et al., 1983). After the preparative procedure, which includes centrifugation, sonication, and resuspension in buffer, the isolated adheron particles have a relatively homogenous 12 S value on sucrose gradients and appear as 15-nm spheres under the electron microscope. Schubert and co-workers (1983) have suggeted that the 12 S particles were aggregated in the growth conditioned medium, since a 12 S particle would not be quantitatively pelleted by centrifugation at 100,000 *g* for 3 h. Our results showed that in the native form, the adhesive properties were present as clusters of filamentous materials, 10–13 nm thick. These filamentous materials, which in some cases were as long as 100 nm, connected the membrane surfaces to one another and to the substratum.

The molecular mechanisms by which the filamentous constituents of adherons mediate adhesion remains unclear. One component of adheron is purpurin, a 20,000-mol-wt retinol-binding protein that promotes cell–adheron adhesion by interacting with a cell-surface heparan sulphate proteoglycan (Schubert and LaCorbiere, 1985*a*, *b*; Schubert et al., 1986). This protein also prolongs the survival of cultured neural retina cells. Adheron contains other glycoproteins and proteoglycans in addition to the 20,000-mol-wt protein, and it is not clear how the complex is assembled or how the complex becomes associated with the extracellular membrane surfaces. One possibility is that adherons are shed from the membrane surface, released into the culture media, and promote adhesion by binding to membrane surfaces and the culture substratum. Proteases and protease inhibitors, shown to be released by growth cones, may play a role in the regulation of adheron attachment and detachment (Patterson, 1985; Pittman, 1985).

It is not yet established whether adherons are neuronal or glial in origin. Neuron attachment to extracellular matrix deposited by non-neuronal cells has been studied extensively in the recent years (Adler et al., 1981; Collins, 1978; Lander et al., 1983). In particular, laminin and fibronectin have been implicated in neurite extension (Bozyczko and Horwitz, 1986; Tomaselli et al., 1986). In our culture system, immunofluorescence studies show that fibronectin is only associated with an occasional fibroblast in the culture and not with any of the neuronal cells (unpublished data). Laminin, which has been shown to be associated with the inner membrane of the retina (Adler et al., 1985), was not present in cultured retina cells (unpublished data). In our cultures, which were plated at low cell density on a highly adhesive surface, nearly all of the cells in the current study were neuronal in morphology. In retina cultures grown in similar conditions, 100% of the cells failed to incorporate thymidine and 95% or more were tetrodotoxin positive (Adler et al., 1982). Immunofluorescence data show that the brightly labeled cells were neuronlike in morphology. While the available data do not rule out a glial origin for adherons, it seems more likely that they are synthesized and released by neurons. Purpurin, a major component of neuronal retina adherons, is synthesized only by photoreceptor cells and deposited on the outer photoreceptor cell matrix (Berman et al., 1987). If neurons are the source of adherons, an interesting possibility is that central nervous system growth cones may release adherons to provide extracellular matrixlike complexes for adhesive guides during axon extension. Although this idea is speculative, the activity of adheron has been shown to be developmentally regulated (Schubert et al., 1983), and hence could play a role in the selective adhesion functions necessary for such guidance.

The apparent anchoring of some adheron constituents at filopodial tips and junctions may provide a new opportunity to investigate the developmental transition from growth cone to synapse. Previous results have indicated that some filopodia may have the capacity to differentiate into synapses (Tsui et al., 1985), and it was suggested that an early event in forming a nascent synapse might be the formation of a filamentous cross-linkage between the apposing membranes. The current data raise the possibility that constituents of adherons could provide the chemical adhesion that holds together nascent synaptic junctions. The whole mount EM immunolocalization of each component of the adheron complex would significantly further our understanding of growth cone differentiation and of mechanisms associated with neuronal adhesion in general.

This work was supported by National Institutes of Health grant NS21088 to Wiliam L. Klein.

Received for publication 22 April 1987, and in revised form 9 February 1988.

References

Adler, R., J. Jerdan, and A. T. Hewitt. 1985. Responses of cultured retina cells to substratum-bound laminin and other extracellular matrix molecules. *Dev. Biol.* 112:100–114.

Adler, R., P. J. Magistretti, A. G. Hyndman, and W. J. Shoemaker. 1982. Purification and cytochemical identification of neuronal and non-neuronal cells in chick embryo retina cultures. *Dev. Neurosci.* 5:27–39.

Adler, R., M. Manthorpe, S. D. Skaper, and S. Varon. 1981. Polyornithine-attached neurite promoting factors (PNPFs). Culture sources and responsive neurons. *Brain Res.* 206:129–144.

Bentley, D., and M. Caudy. 1983. Navigational substrates for peripheral pioneer growth cones: limb axis polarity cues, limb-segment boundaries and guidepost neurons. *Cold Spring Harbor Symp. Quant. Biol.* 48:573–585.

Berlot, J., and C. S. Goodman. 1984. Guidance of peripheral pioneer neurons in the grasshopper: adhesive hierarchy of epithelial and neuronal surfaces. *Science (Wash. DC).* 223:493–496.

Berman, P., P. Gray, E. Chen, K. Keysen, D. Ehrlich, H. Karten, M. LaCorbiere, F. Esch, and D. Schubert. 1987. Sequence analysis, cellular localization and expression of a neuroretina adheron and cell survival molecule. *Cell.* 51:135–142.

Bozyczko, D., and A. F. Horwitz. 1986. The participation of a putative cell surface receptor for laminin and fibronectin in peripheral neurite extension. *J. Neurosci.* 6:1241–1251.

Burnette, W. N. 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112:195–203.

Campenot, R. B. 1977. Local control of neurite development by nerve growth factor. *Proc. Natl. Acad Sci. USA.* 74:4516–4519.

Cole, G. J., and L. Glaser. 1984. Cell-substratum adhesion in embryonic chick central nervous system is mediated by a 170,000-mol-wt neural-specific polypeptide. *J. Cell Biol.* 99:1605–1612.

Cole, G. J., and L. Glaser. 1986. A heparan-binding domain from N-CAM is involved in neural cell-substratum adhesion. *J. Cell Biol.* 102:403–412.

Cole, G. J., D. Schubert, and L. Glaser. 1985. Cell-substratum adhesion in chick neural retina depends upon protein-heparan sulfate interactions. *J. Cell Biol.* 100:1192–1199.

Collins, F. 1978. Induction of neurite outgrowth by a conditioned medium factor bound to the culture substratum. *Proc. Natl. Acad. Sci. USA.* 75:5210–5213.

Covault, J., and J. R. Sanes. 1985. Neural cell adhesion molecule (N-CAM) accumulates in denervated and paralysed skeletal muscles. *Proc. Natl. Acad. Sci. USA.* 82:4544–4548.

Crisanti-Combes, P., B. Pessac, and G. Calothy. 1978. Choline acetyl transferase activity in chick embryo neuroretina during development in ovo and in monolayer cultures. *Dev. Biol.* 65:228–232.

Crisanti-Combes, P., A. Privat, B. Pessac, and G. Calothy. 1977. Differentiation of chick embryo neuroretina cells in monolayer cultures. An ultrastructural study. *Cell.* 185:159–173.

DeMello, M. C. F., A. L. M. Ventura, R. P. DeCarvalho, W. L. Klein, and F. G. DeMello. 1982. Regulation of dopamine- and adenosine-dependent adenylate cyclase systems of chick embryo retina cells in culture. *Proc. Natl. Acad. Sci. USA.* 79:5708–5712.

Edelman, G. M., S. Hoffman, C.-M. Chuong, J.-P. Thiery, R. Brackenburg, W. J. Gallin, M. Grumet, M. E. Greenberg, J. J. Hemperly, C. Cohen, and B. A. Cunningham. 1983. Structure and modulation of neural cell adhesion molecules in early and late embryogenesis. *Cold Spring Harbor Symp. Quant. Biol.* 58:515–526.

Fraser, S. E., B. A. Murray, C.-M. Chuong, and G. M. Edelman. 1984. Alteration of the retinaltectal map in Xenopus by antibodies to neural cell adhesion molecules. *Proc. Natl. Acad. Sci. USA.* 81:4222–4226.

Gall, W. E., and G. M. Edelman. 1981. Lateral diffusion of surface molecules in animal cells and tissue. *Science (Wash. DC).* 213:903–905.

Gundersen, R. W., and J. N. Barrett. 1979. Neuronal chemotaxis: chick dorsal-root axon turn toward high concentration of nerve growth factor. *Science (Wash. DC).* 206:1079–1980.

Lander, A. D., K. Tomaselli, A. L. Calof, and L. F. Reichardt. 1983. Studies on extracellular matrix components that promote neurite outgrowth. *Cold Spring Harbor Symp. Quant. Biol.* 58:611–623.

Lankford, K., F. G. DeMello, and W. L. Klein. 1987. A transient embryonic dopamine receptor inhibits growth cone motility and neurite outgrowth in a subset of avian retina neurons. *Neurosci. Lett.* 75:169–174.

Letourneau, P. C. 1975. Cell-to-substratum adhesion and guidance of axonal elongation. *Dev. Biol.* 44:92–101.

Maier, C. E., M. Watnanbe, M. Singer, I. G. Mcquarrie, J. Sunshine, and U. Rutishauser. 1986. Expression and function of neural cell adhesion molecules during limb regeneration. *Proc. Natl. Acad. Sci. USA.* 83:8395–8399.

Patel, N. B., and M.-M. Poo. 1984. Perturbation of the direction of neurite growth by pulsed and focal-electric fields. *J. Neurosci.* 4:2939–2947.

Patterson, P. H. 1985. On the role of proteases, their inhibitors and the extracellular matrix in promoting neurite outgrowth. *J. Physiol. (Paris).* 80:207–211.

Pittman, R. N. 1985. Release of plasminogen activator and a calcium-dependent metalloprotease from cultured sympathetic and sensory neurons. *Dev. Biol.* 110:91–101.

Pollenberg, G. E., M. Schachner, and J. Davoust. 1986. Differentiation state-dependent surface mobilities of two forms of the neural cell adhesion molecules. *Nature (Lond.).* 324:462–465.

Riopelle, R. J., and D. A. Cameron. 1984. Neurite-promoting factors from embryonic neurons. *Dev. Brain Res.* 15:265–274.

Rogers, S. L., K. J. Edson, P. C. Letourneau, and S. C. McLoon. 1986. Distribution of laminin in the developing peripheral nervous system of the chick. *Dev. Biol.* 113:429–435.

Rutishauser, U. 1985. Influences of the neuronal cell adhesion molecules on axon growth and guidance. *J. Neurosci. Res.* 13:123–131.

Rutishauser, U. 1986. Differential cell adhesion through spatial and temporal variations of NCAM. *Trends Neurosci.* 9:374–378.

Rutishauser, U., and C. Goridis. 1986. N-CAM: the molecules and its genetics. *Trends Genetics.* 2:72–76.

Rutishauser, U., S. Hoffmann, and G. M. Edelman. 1982. Binding properties of a cell adhesion molecules from neural tissue. *Proc. Natl. Acad. Sci. USA.* 79:685–689.

Schubert, D., and M. LaCorbiere. 1985*a*. Isolation of a cell-surface receptor for chick neural retina adherons. *J. Cell Biol.* 100:56–63.

Schubert, D., and M. LaCorbiere. 1985*b*. Isolation of an adhesion-mediating protein from chick neural retina adherons. *J. Cell Biol.* 101:1071–1077.

Schubert, D., M. LaCorbiere, and F. Esch. 1986. A chick neural retina adhesion and survival molecule is a retinol-binding protein. *J. Cell Biol.* 102: 2295–2301.

Schubert, D., M. LaCorbiere, F. G. Klier, and C. Birdwell. 1983. A role for adherons in neural retina cell adhesion. *J. Cell Biol.* 96:990–999.

Silver, J., and U. Rutishauser. 1984. Guidance of optic axons in vivo by preformed adhesive pathway on neuroepithelial endfeet. *Dev. Biol.* 106: 485–499.

Siman, R. G., and W. L. Klein. 1983. Differential regulation of muscarinic and nicotinic receptors by cholinergic stimulation in cultured avian retina cells. *Brain Res.* 262:99–108.

Singer, M., N. Nordlander, and N. Egar. 1979. Axonal guidance during embryogenesis and regeneration in the spinal cord of the newt. The blueprint hypothesis of neuronal pathway patterning. *J. Comp. Neurol.* 185:1–22.

Thanos, S., F. Bonhoeffer, and U. Rutihauser. 1984. Fiber-fiber interaction and tectal cues influence the development of the chicken retinatectal projection. *Proc. Natl. Acad. Sci. USA.* 81:1906–1910.

Thiery, J. P., R. Brackenburg, U. Rutishauser, and G. M. Edelman. 1977. Adhesion among neuronal cells of the chick embryo. II. Purification and characterization of a cell adhesion molecule from neural retina. *J. Biol. Chem.* 252:6841–6845.

Thor, G., E. G. Pollerberg, and M. Schachner. 1986. Molecular association of two neural cell adhesion molecules within the surface membrane of cultured mouse neuroblastoma cells. *Neurosci. Lett.* 66:121–126.

Tomaselli, K. J., L. F. Reichardt, and J. L. Bixby. 1986. Distinct molecular interactions mediate neuronal process outgrowth on non-neuronal cell surfaces and extracellular matrices. *J. Cell Biol.* 103:2659–2672.

Tosney, K. W., and L. T. Landmesser. 1985. Development of the major pathways for neurite outgrowth in chick hindlimb. *Dev. Biol.* 109:193–214.

Tosney, K. W., M. Watanabe, L. Landmesser, and U. Rutishauser. 1986. The distribution of NCAM in the chick hindlimb during axon outgrowth and synaptogenesis. *Dev. Biol.* 114:437–452.

Tsui, H.-C. T., K. L. Lankford, and W. L. Klein. 1985. Differentiation of neuronal growth cones: specialization of filopodial tips for adhesive interactions. *Proc. Natl. Acad. Sci. USA.* 82:8256–8260.

van der Pol, A. N., U. di Porzio, and U. Rutishauser. 1986. Growth cone localization of neural cell adhesion molecules on central nervous system neurons in vitro. *J. Cell Biol.* 102:2281–2294.

Vogel, Z., M. P. Daniels, and M. Nirenberg. 1976. Synapse and acetylcholine receptor synthesis by neurons dissociated from retina. *Proc. Natl. Acad. Sci. USA.* 73:2370–2374.