Rapidly Transported Organelles Containing Membrane and Cytoskeletal Components: Their Relation to Axonal Growth

P. J. Hollenbeck and D. Bray

Cell Biophysics Unit, Medical Research Council, London WC2B 5RL, England

Abstract. We have examined the movements, composition, and cellular origin of phase-dense varicosities in cultures of chick sympathetic and sensory neurons. These organelles are variable in diameter (typically between 0.2 and 2 μm) and undergo saltatory movements both towards and away from the neuronal cell body. Their mean velocities vary inversely with the size of the organelle and are greater in the retrograde than the anterograde direction. Organelles stain with the lipophilic dye 1, 1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine and with antibodies to cytoskeletal components. In cultures double-stained with antibodies to α-tubulin and 70-kD neurofilament protein (NF-L), ~40% of the organelles stain for tubulin, 30% stain for NF-L, 10% stain for both tubulin and NF-L, and 40% show no staining with either antibody. The association of cytoskeletal proteins with the organelles shows that these proteins are able to move by a form of rapid axonal transport.

Under most culture conditions the predominant direction of movement is towards the cell body, suggesting that the organelles are produced at or near the growth cone. Retrograde movements continue in culture medium lacking protein or high molecular mass components and increase under conditions in which the advance of the growth cone is arrested. There is a fourfold increase in the number of organelles moving retrogradely in neurites that encounter a substratum-associated barrier to elongation; retrograde movements increase similarly in cultures exposed to cytochalasin at levels known to block growth cone advance. No previously described organelle shows behavior coordinated with axonal growth in this way.

We propose that the organelles contain membrane and cytoskeletal components that have been delivered to the growth cone, by slow or fast anterograde transport, in excess of the amounts required to synthesize more axon. In view of their rapid mobility and variable contents, we suggest that they be called "neuronal parcels."

The steady arrival of cytoskeletal components at the distal end of an axon presents an as yet unsolved problem of cellular homeostasis. In a growing axon the rate of elongation varies minute by minute according to the microenvironment of the growth cone, but transport is initiated and sustained at a location remote from the distal end. Consequently, it is hard to see how the supply of material reaching the growing axonal tip could precisely match the quantities needed to make more axon. An even more extreme imbalance may exist in mature axons where the delivery of materials is thought to continue unabated, even though elongation has ceased (Lasek et al., 1984).

One possibility is that excess material is broken down near the distal end of the axon. After the identification of a calcium-activated protease in the axoplasm of invertebrate giant axons (Gilbert et al., 1975), Lasek and Hoffman (1976) proposed that cytoskeletal components, especially neurofilaments, may be proteolyzed upon entrance to the axon terminal. Consistent with this suggestion is the finding that neurofilament polypeptides transported in guinea pig retinal axons are degraded as they reach the synaptic terminals on the lateral geniculate (Lasek and Black, 1977). Also, exposure of goldfish tectum to an inhibitor of calcium-dependent protease leads to accumulation of neurofilament "rings" in synaptic terminals (Roots, 1983).

An alternative, or perhaps additional, possibility is that cytoskeletal components might be returned to the neuronal cell body by a form of fast axonal transport operating in the retrograde direction. This notion was raised in a recent study by Koenig et al. (1985) of phase-dense particles and motile varicosities in the axons of cultured goldfish retinal ganglion cells. These authors noted that: the varicosities move rapidly both towards and away from the neuronal cell body, they contain membrane, and they can be stained with antibodies to the cytoskeletal components actin and spectrin.

We have examined chick embryonic neurons in culture and find that they also carry striking phase-dense varicosities which are transported in both directions along the neurites. As we shall show, the varicosities are nonuniform in composition since tubulin and neurofilament protein can be detected in specific subpopulations of them. When neurons are cultured under conditions that allow axonal extension to be blocked, the production and retrograde movement of these motile structures increases. This increase is consistent with

© The Rockefeller University Press, 0021-9525/87/12/2827/9 $2.00

Downloaded from http://jcb.rupress.org/jcb/article-pdf/105/6/2827/1466216/2827.pdf by guest on 23 April 2024

the view that they serve to redistribute excess material that has been delivered to the advancing growth cone. We propose that these organelles be called "neuronal parcels."

Materials and Methods

Materials

Cytochalasin E, dilauryl DL-phosphatidylcholine and *p*-phenylenediamine were obtained from Sigma Chemical Co. (Poole, United Kingdom). Monoclonal anti-α-tubulin YL1/2 was the gift of J. Kilmartin, and has been previously characterized (Kilmartin et al., 1982). Rabbit antiserum against the 70-kD chicken neurofilament protein (anti-NF-L)[1] has been characterized elsewhere (Jacobs et al., 1982). FITC-conjugated goat anti-rat Ig was obtained from Nordic Immunology (Tilburg, The Netherlands). Texas red-conjugated donkey anti-rabbit Ig and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (diI-C_{18}) were obtained from Molecular Probes Inc. (Eugene, OR). Ruled coverslip masks were prepared by Agar Aids (Cambridge, U. K.). The motion analysis software was developed by Dr. A. F. Brown (of this institution).

Cell Culture

Dorsal root ganglia or sympathetic ganglia were dissected from 9-12-d chicken embryos and cultured either whole or after dissociation into single cells as previously described (Hollenbeck et al., 1985). Cells or ganglia were placed onto glass coverslips and grown for 18-48-h in a humidified incubator in Leibovitz L-15 medium plus 10% FCS, 0.6% glucose, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 0.5% methylcellulose, and sufficient mouse nerve growth factor to support neurite outgrowth. For some experiments, coverslips were treated with conditioned medium (prepared by the method of Lander et al., 1982) for 10-30 min before use.

Time-Lapse Cinematography

Whole dorsal root ganglia from 9-11-d embryos were cultured on 22 × 22-mm coverslips for 48 h, by which time they had extended a radial array of neurites. They were then mounted on a chamber formed by a glass coverslip placed over an 18-mm hole drilled in a glass slide. Neurites in the periphery of the radial array were selected and filmed in a 37°C room using a Bolex H-16 camera, a Variotimer (Paillard-Wild S. A., St. Croix, Switzerland), a Zeiss Plan Neofluor objective of 25 and Ilford Pan F film. An exposure of 0.8 s was taken every 6.8 s. Film was analyzed to quantitate parcel movements using an Optical Data Analyzer 224-A projector (L-W Photo, Inc., Van Nuys, CA).

Video-enhanced Microscopy and Motion Analysis

Cultures were prepared as for cinematography except that they were mounted on a 22 × 64-mm coverslip with spacers made of double-sided tape and attached to a milled aluminum holder on a heated microscope stage. Parcel movements were observed by darkfield microscopy using a Zeiss apochromatic objective of 100 with an adjustable aperture, a Zeiss darkfield condensor, and a Zeiss tungsten light source. A video image of the microscope field was obtained with an RCA SIT video camera using an Arlunya TF6000 image processor (Dindima Ltd., Victoria, Australia) to reduce noise by real-time frame averaging. This image was mixed with that of a computer-generated cross-hair, the position of which was controlled manually by moving the cursor of a Bit Pad 2 digitizing tablet (Summagraphics Corp., Fairfield, CT). Parcels were tracked by following them with the cross-hair and storing their *x* and *y* coordinates automatically at 1-s intervals. These data were then used to calculate the velocity and direction of movement of the tracked parcels. Still photographs were obtained from the video monitor using the image processor frame store and Kodak Tech Pan film at ASA 100.

Studies on Pattern-shadowed Coverslips

Ruled masks consisted of a 10 × 10-mm metallic film electroformed to a specific design comprising an array of fine slots of varying length (see Fig. 3 *A*). Glass coverslips were coated with silane, overlayed with masks, and shadowed with silicon monoxide, resulting in a surface on which the only adhesive areas were strips ~7 µm wide and 50, 100, 200, 400, or 800 µm long. Coverslips thus prepared were fixed to the bottoms of 60-mm culture dishes that had 18-mm holes drilled through them. They were then treated briefly with conditioned medium diluted to a concentration which promoted neurite extension on the adhesive strips but did not permit growth on the surrounding silane-treated areas. Dissociated dorsal root ganglion neurons at a concentration of one-fourth ganglion per coverslip were plated onto the treated coverslips and incubated for 18 h before use.

Cytochalasin Treatment

Whole dorsal root ganglia from 9-11-d embryos were grown on glass coverslips fixed to culture dishes as described above for 18-24 h, by which time they had extended a radial array of neurites. Peripheral regions of the neuritic array were selected and the number of parcels moving in each direction along one or more chosen neurites was recorded five times at 5-min intervals before the addition of cytochalasin E to the culture medium. After drug addition, the same neurites were relocated and again the number of parcels moving in each direction was scored five times at 5-min intervals.

Immunofluorescent Staining of Culture

Whole dorsal root or sympathetic ganglia from 9-12-d embryos were grown for 36-48 h on either conditioned medium-treated or plain glass coverslips. The cultures were examined to ensure that the neurites contained abundant moving parcels and were then fixed by layering a warm solution of 0.12 M sucrose, 0.5 mM EGTA, 75 mM phosphate buffer, pH 7.4, 2% glutaraldehyde (Luduena, 1973) under the culture medium and allowing 5 min before washing. This fixation technique was selected by observing cultures before, during, and after fixation using various fixatives and solution osmolarities. It preserves all visible organelles, growth cones, and neuritic attachment to the substratum without producing osmotically generated varicosities or other artifacts visible with the light microscope. In our experience this is not true of most other fixation techniques applied to cultured neurons. After fixation, the cultures were extracted for 5 min in PBS, pH 7.4, plus 0.1% Triton X-100; then autofluorescence was reduced by five 3-min washes in 3 mg/ml $NaBH_4$ in 50% PBS/50% methanol (Weber et al., 1978). For extraction experiments, cultures were treated as described above except that, before fixation, they were incubated for 2 min in 0.1 M Pipes, 2.5 mM $MgSO_4$, 1 mM EGTA, 0.05% or 0.1-0.5% Triton X-100, pH 6.9, at 37°C. After fixation and reduction, cultures were washed in PBS plus 0.1% Triton X-100, incubated for 45 min in antitubulin or anti-NF-L, washed, and incubated for 60 min in the appropriate secondary antibody. In double-labeling studies, both primary antibodies were applied simultaneously and the secondary antibodies sequentially. Coverslips were mounted using 1 mg/ml *p*-phenylenediamine in 90% glycerol/10% 10× PBS (Johnson and Araujo, 1981). For scoring the number of antigen-positive parcels, cultures were observed with phase-contrast optics to locate parcels and then with epifluorescence illumination and Zeiss fluorescein or Texas red filter sets for immunofluorescence. Parcels were scored only if the neurite on which they were located stained brightly and evenly with antitubulin and/or anti-NF-L. Parcels were considered to be positive for an antigen if they stained as bright or more brightly than the neurite, and negative if they stained dimly or not at all; the distinction was usually obvious. All of the parcels meeting the above criteria were scored in three peripheral fields on each ganglion (25-95 parcels per ganglion). Photographs were taken using a Zeiss Planapochromatic objective of 63 and Kodak Tech Pan film, ASA 200.

diI-C_{18} Staining of Live Cultures

Whole dorsal root ganglia from 9-12-d embryos were grown on glass coverslips overnight and were then injected with an emulsion of diI-C_{18} made by mixing 6 mg diI-C_{18} with 9 mg dilauryl DL-phosphatidylcholine, adding 200 µl PBS, and sonicating. After 24-48 h, the ganglia produced an array of neurites, some of which were stained with diI-C_{18}. The dye was excited and observed in cells using epifluorescence illumination and a Zeiss rhodamine filter set. Parcels were scored as positive or negative for diI-C_{18} in live cells by observing moving ones with phase-contrast optics and then switching to epifluorescence illumination to evaluate their staining. In cells that had taken up the dye, parcels were either intensely fluorescent or very faint; the former were scored as diI-C_{18} positive, the latter as negative. Parcels on neurites that had not taken up diI-C_{18} were not scored. Stained cells were photographed using Kodak Tri-X film at ASA 400.

1. *Abbreviations used in this paper*: diI-C_{18}, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine; NF-L, 70-kD neurofilament protein.

Downloaded from http://jcb.rupress.org/jcb/article-pdf/105/6/2827/1466216/2827.pdf by guest on 23 April 2024

Results

Phase-Dense Organelles

When cultured chick neurons were observed using phase-contrast or darkfield optics, three types of motile organelles were apparent: mitochondria, small round or elliptical organelles, and extremely refractile, usually hemispherical organelles that protruded from the side of the neurite. The latter distinctive organelles, which we will refer to as parcels, were extremely refractile, phase dense (see Fig. 1), and variable in size; those chosen for study had diameters between 0.2 and 2 μm. They were present in neurons regardless of the age of the embryonic source or the nature of the culture substratum, and at culture densities ranging eightfold from completely isolated neurons to dense networks.

Parcels exhibited a broad range of velocities, but when the data were divided into classes by diameter, it became apparent that parcel behavior depended in part upon their size (Fig. 2). Both their mean velocity and their duty cycle (the

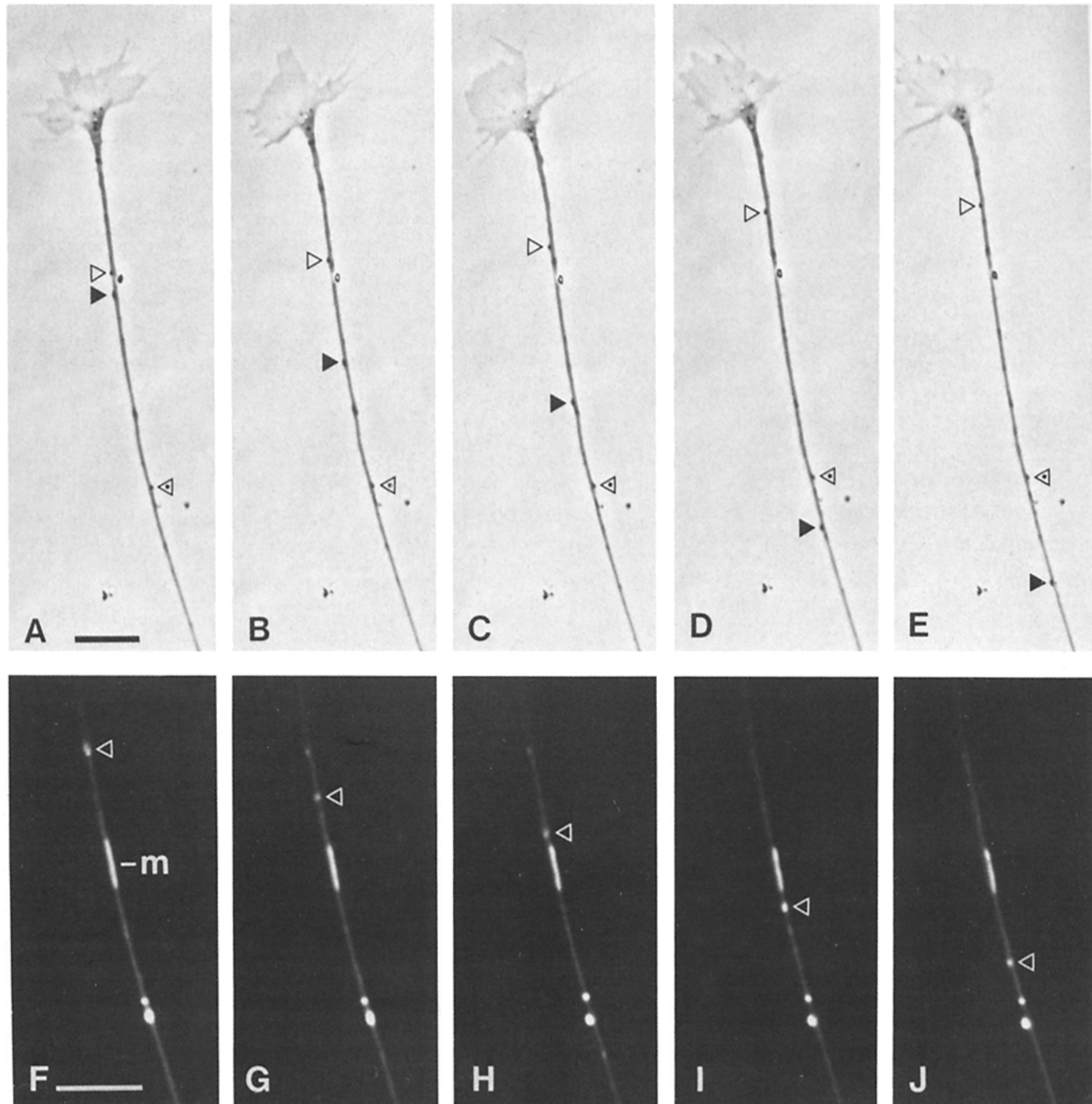

Figure 1. Parcel movements. Phase-contrast micrographs taken at 10-s intervals (*A–E*) show three small parcels on the distal portion of a sensory neuron in culture. One moves anterograde (▷), one retrograde (▶), and one remains stationary (◬). Several parcels are visible in the base of the growth cone. Video-enhanced darkfield images (*F–J*) shot from the monitor at 10-s intervals show a parcel (*arrowhead*) moving retrogradely past a mitochondrion (*m*) and toward two stationary parcels. Bars, 10 μm.

Downloaded from http://jcb.rupress.org/jcb/article-pdf/105/6/2827/1466216/2827.pdf by guest on 23 April 2024

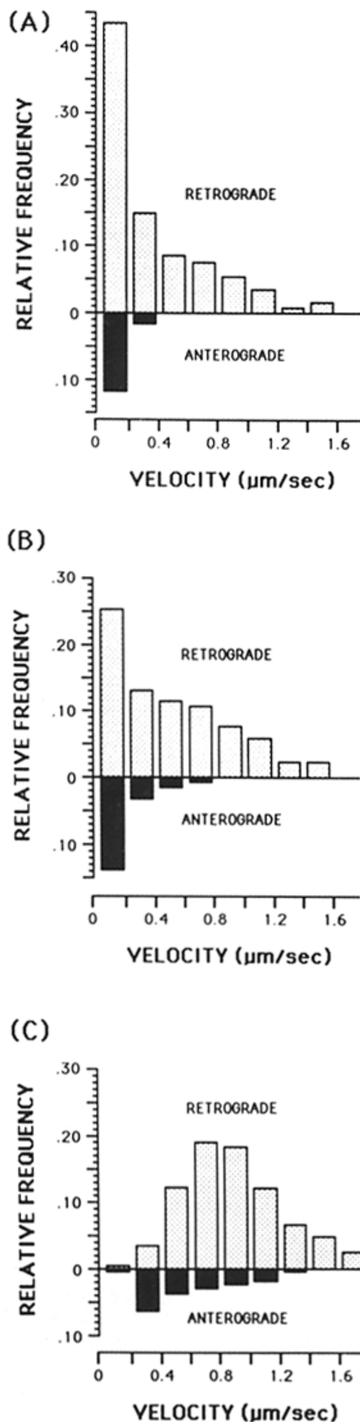

Figure 2. Velocity distributions for parcels divided into size classes. Velocities in each direction are plotted versus relative frequency for parcels of diameter ≥1 µm (*A*), 0.5–0.9 µm (*B*), and <0.5 µm (*C*). Measurements for *A* and *B* come from analysis of phase-contrast time-lapse cinematography; those for *C* are from video-enhanced darkfield analysis as described in Materials and Methods.

proportion of elapsed time spent in movement) increased with decreasing organelle diameter, and for all sizes the mean velocity was greater in the retrograde than the anterograde direction (Table I). Those < 0.5 µm had an average

Table I. Behavior of Neuronal Parcels of Different Sizes

Size	<0.5 µm	0.5–0.9 µm	≥1.0 µm
Retrograde velocity (*µm/s*)	1.05 ± 0.03 (*n* = 332)	0.51 ± 0.04 (*n* = 105)	0.39 ± 0.02 (*n* = 327)
Anterograde velocity (*µm/s*)	0.75 ± 0.05 (*n* = 79)	0.23 ± 0.04 (*n* = 25)	0.17 ± 0.03 (*n* = 46)
Duty cycle	77%	72%	62%

Moving parcels were tracked using video-enhanced darkfield microscopy and motion analysis software (<0.5 µm) or phase-contrast microscopy and time-lapse cinematography (≥0.5 µm) as described in Materials and Methods. Mean velocities are expressed ±SEM with the number of measurements shown in parentheses. Duty cycle equals the percentage of the total observation time during which parcels were moving.

retrograde velocity of 1.05 ± 0.03 µm/s, comparable to that of the small membrane-bounded organelles moving by fast transport in these neurons (Hollenbeck et al., 1985). Larger parcels were more common in cells that had been in culture for more than 24 h. Parcels sometimes appeared in previously clear regions of the growth cone (Fig. 1); retrogradely moving ones often entered the cell body, after which their progress was impossible to follow.

Behavior of Parcels in Growing Versus Blocked Neurons

When neurons were cultured on pattern-shadowed substrata, as described in Materials and Methods, they were constrained to grow on ~7-µm-wide adhesive tracks. This resulted in simple bipolar shapes in which most cell bodies bore two neurites at 180° to each other, each elongating toward the end of the shadowed segment (Fig. 3). Neurons growing under these conditions were observed at intervals and the number of moving parcels and their direction of movement were scored. We compared these values for neurons in which one neurite had reached the end of the shadowed segment and stopped while the other was still growing. As summarized in Table II, the blocked neurites had a nearly three-fold increase in the number of moving parcels over their unblocked partners on the same cell. Moreover, the number of retrogradely moving parcels increased fourfold in blocked neurites while the number of anterogradely moving ones decreased by 50%, increasing the ratio of retrograde/anterograde movements from 1.6 to 11.3.

Behavior of Parcels in Cytochalasin-treated Neurons

Growing neurons treated with 0.3–1.0 µg/ml cytochalasin E showed a cessation of growth cone activity and stopped elongating, but did not retract for 45–60 min; nor did the transport of mitochondria or other organelles cease. During this interval in pharmacologically blocked neurons, we again saw an effect on the number and direction of moving parcels: for each neurite examined, the total number of parcels increased after drug treatment. The number moving retrogradely rose 2.3- to 3-fold and the number moving anterogradely dropped by 50% (Table III).

Downloaded from http://jcb.rupress.org/jcb/article-pdf/105/6/2827/1466216/2827.pdf by guest on 23 April 2024

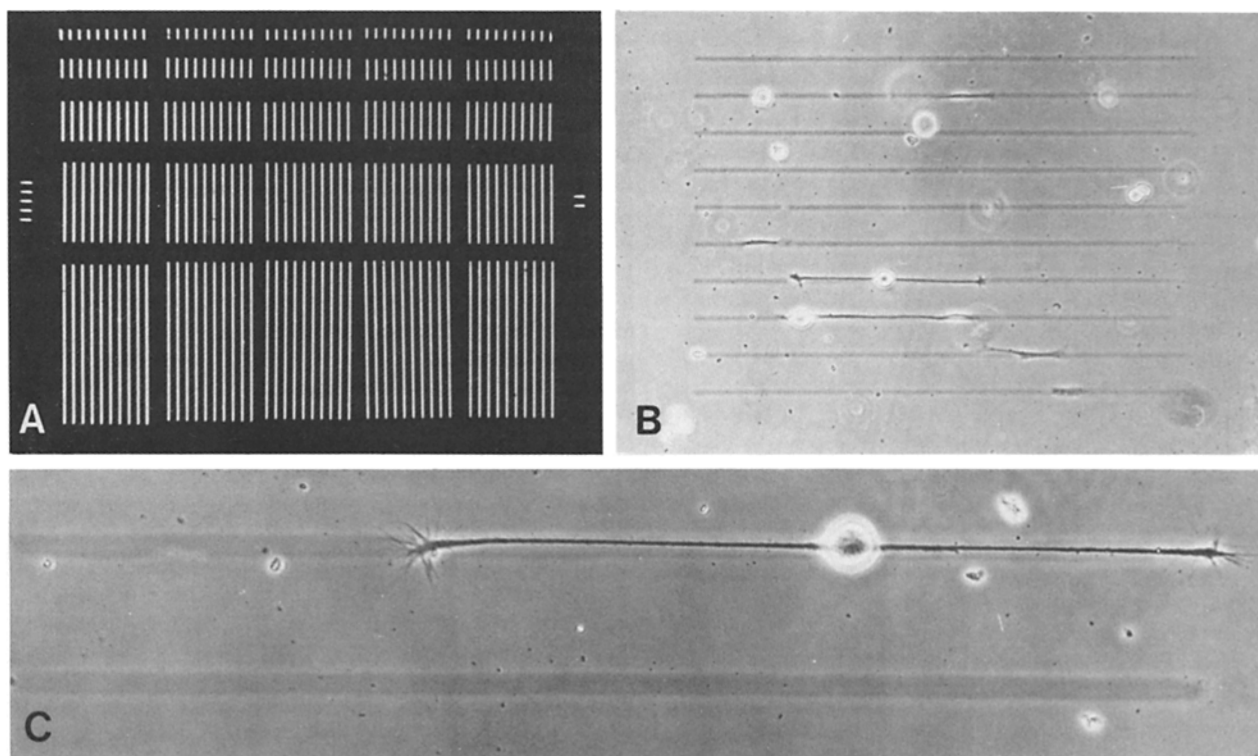

Figure 3. Substratum-associated barriers to neurite outgrowth. The pattern of fine slots electroformed onto metallic film is shown in *A* illuminated from underneath. Each mask carried a three by three array of the pattern shown, on which the longest slots are 800 μm in length. When silicon monoxide was shadowed onto silane-coated coverslips through a mask, the pattern of adhesive strips on the substratum defined the area on which neurons could attach and grow. The low power phase-contrast micrograph in *B* shows several neurons and nonneuronal cells growing on 800-μm-long strips. The higher power micrograph in *C* shows a typical neuron that has extended two neurites, only one of which has reached the end of a 400-μm strip; an adjacent empty strip is also visible.

Table II. Effect of a Substratum-associated Barrier to Neurite Elongation on the Number of Moving Parcels

State of neurite	Mean neurite length	Anterogradely moving parcels	Retrogradely moving parcels	Ratio retro/ antero
	μm			
Unblocked	254 ± 13.7	37 (2.38 ± 0.52/mm)	59 (3.80 ± 0.62/mm)	1.6
Blocked	284 ± 13.4	21 (1.21 ± 0.25/mm)	237 (13.66 ± 1.56/mm)	11.3

Dissociated dorsal root ganglia neurons were grown on pattern-shadowed coverslips as described in Materials and Methods. Cells with one neurite elongating and the other blocked by the end of the adhesive zone were selected for analysis ($n = 61$). At 60-min intervals, the length of each neurite was recorded along with the number of parcels moving in each direction. The data on moving parcels have been normalized for the length of each neurite, averaged, and expressed ±SEM in parentheses beneath the number of parcels.

Table III. Effect of Cytochalasin E Addition on the Number of Moving Parcels

Movement	Cytochalasin E	No. moving before treatment	No. moving after treatment	Ratio after/ before
	μg/ml			
Retrograde	0.3	5.00 ± 0.63	9.80 ± 1.02	2.0
	0.5	1.87 ± 0.27	5.67 ± 0.56	3.0
	1.0	4.13 ± 0.31	9.60 ± 1.04	2.3
Anterograde	0.3	2.20 ± 0.37	1.40 ± 0.24	0.63
	0.5	1.07 ± 0.27	0.47 ± 0.16	0.44
	1.0	1.47 ± 0.31	0.67 ± 0.25	0.46

Moving parcels were scored in the neuritic arrays of cultured dorsal root ganglia as described in Materials and Methods. Data from three cultures were averaged for each cytochalasin E concentration shown in the table; these averages are expressed ±SEM.

Downloaded from http://jcb.rupress.org/jcb/article-pdf/105/6/2827/1466216/2827.pdf by guest on 23 April 2024

Protein Composition of Parcels

When neurons were fixed and immunofluorescently stained for the presence of tubulin and NF-L, images like those in Fig. 4 were obtained. Although fixation and extraction reduced the phase density of the parcels, they were still clearly visible (Fig. 4, *A* and *D*). The neurites stained uniformly for both antigens, and a certain proportion of the parcels was also positive. As shown in Table IV, antitubulin labeled 39.2% of the parcels, while anti-NF-L labeled 30.7%. Double-label immunofluorescent staining with these two antibodies revealed that 9.4% of the parcels were positive for both antigens while 36.7% stained for neither antigen. These results did not vary significantly with the size of the parcels, the age of the embryonic source of the culture, or with the culture substratum (conditioned medium-treated versus plain glass). In addition, variation between cultures was very small, as illustrated by the standard errors in Table IV.

When neurons were gently detergent-extracted (2 min in 0.05% Triton X-100) before fixation and then double stained, the percent of parcels staining positively for tubulin was reduced fourfold while the percent staining for NF-L was not significantly changed (Table IV). This occurred despite the retention of normal antitubulin staining in the neurites and growth cones, as well as in nonneuronal cells in extracted cultures (Fig. 4 *E*). Detergent extraction sufficient to remove all cellular components but the cytoskeleton (2 min in 0.1–0.5% Triton X-100) completely eliminated parcels from the neurites (data not shown).

The possibility that parcel immunostaining was due to cytoskeletal proteins released into the culture medium during

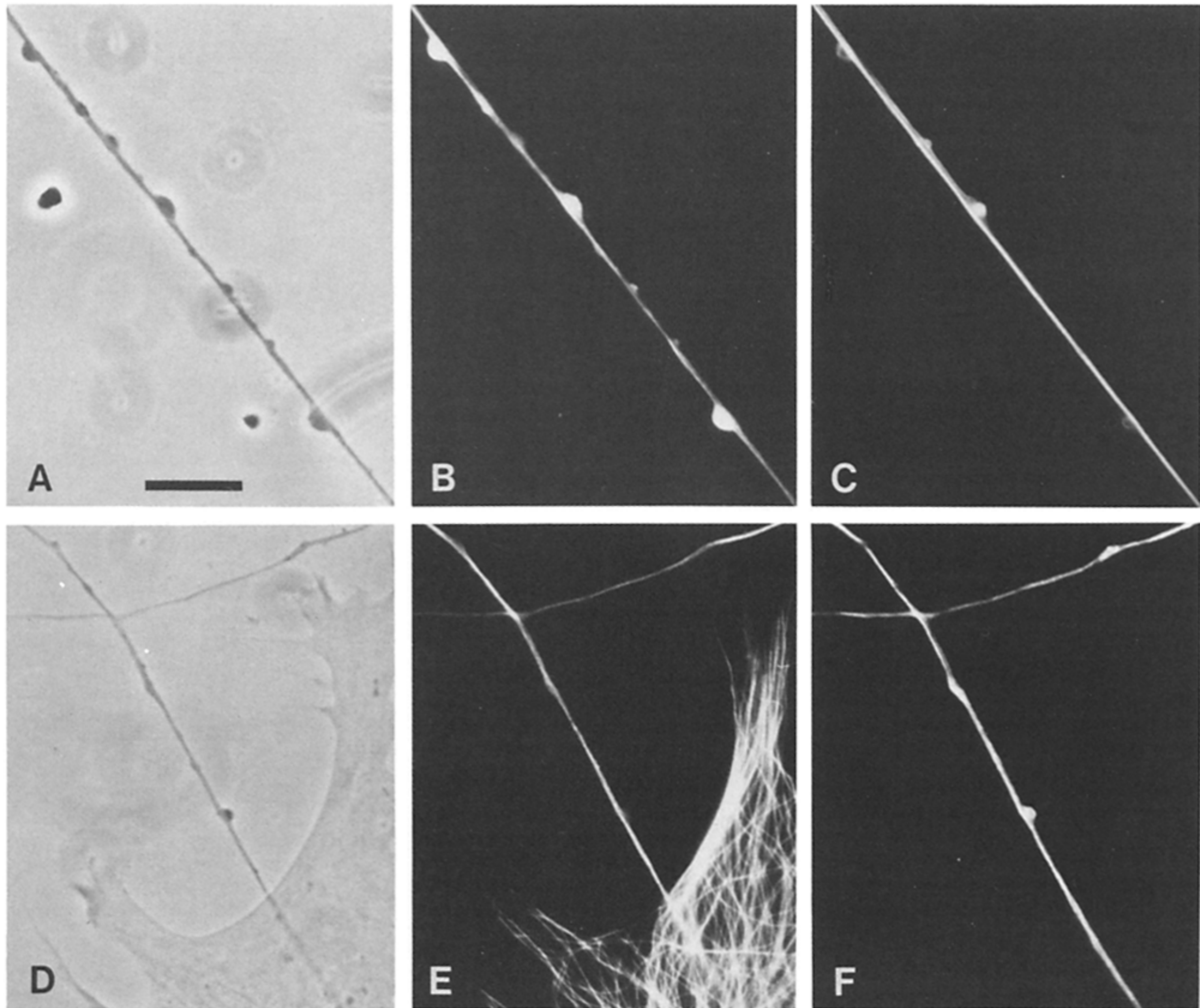

Figure 4. Phase-contrast images (*A* and *D*) and double immunofluorescent staining of parcels in sensory neurons with antitubulin (*B* and *E*) and anti-NF-L (*C* and *F*). Parcels in neurons that have been fixed and processed for immunofluorescence are less phase-dense than in live cells, but are still clearly visible. A normally fixed neurite with a large number of parcels (*A–C*) illustrates their nonuniform composition. Comparison of the phase image (*A*) with antitubulin (*B*) and anti-FN-L staining (*C*) shows that some parcels are positive for only one antigen, some for both antigens, and some for neither. A typical image from a culture that has been detergent-extracted before fixation (*D–F*) shows the persistence of the tubulin cytoskeleton (*E*) and of anti-NF-L staining of a parcel (*F*) under conditions that greatly reduce antitubulin staining of parcels. Bar, 10 μm.

Downloaded from http://jcb.rupress.org/jcb/article-pdf/105/6/2827/1466216/2827.pdf by guest on 23 April 2024

Table IV. Percent of Parcels Staining for Tubulin and Neurofilament Protein

Treatment	Tubulin⁺	NF = L⁺	Both⁺	Neither⁺
	%	%	%	%
Normal fixation	39.2 ± 1.1 (n = 14)	30.7 ± 1.2 (n = 13)	9.4 ± 1.0 (n = 8)	35.7 ± 2.2 (n = 8)
Detergent extracted	8.2 ± 1.9 (n = 7)	27.0 ± 3.3 (n = 7)	1.5 ± 1.0 (n = 7)	68.0 ± 3.8 (n = 7)

Ganglia were cultured, fixed, and immunofluorescently stained for tubulin and 70-kD neurofilament protein, and parcels were scored as positive or negative for each antigen as described in Materials and Methods. The percentages in this table are means of several ganglia ±SEM. The numbers in parentheses refer to the number of ganglia scored; on each ganglion 25–95 parcels were scored, so each average represents 175–650 parcels scored.

fixation associating nonspecifically with the surface membrane of the organelles is made highly unlikely by: (*a*) the intense staining by the two antibodies of separate subpopulations of parcels; (*b*) the failure of other agents that stain the neurons brightly (antivimentin, antiactin, antispectrin, and rhodamine–phalloidin, data not shown) to stain parcels; (*c*) the persistence of all of the anti–NF-L reactivity, and a fraction of the anti–α-tubulin reactivity, in cultures that were extracted before fixation.

Lipid Staining of Parcels

When injected as an emulsion into live ganglia, the lipophilic fluorescent carbocyanine dye diI-C_{18} (Honig and Hume, 1986) was taken up by many neurons and concentrated in parcels, as shown by their intense fluorescence relative to the axolemma (Fig. 5). In neurons that took up the dye, 63% of the moving parcels showed intense staining 24 h after treatment (n = 274).

Discussion

The phase-dense particles we have called neuronal parcels evidently represent a significant route for the redistribution of material within cultured neurons. Although individually they are small, their abundance and rapidity of movement allow them to deliver surprisingly large amounts of membrane and cytoskeletal materials in a short space of time. Thus, a convoy of parcels each 0.5 μm in diameter, moving retrogradely at a rate of 1 μm/s, and spaced at intervals of 20 μm along a neurite, would deliver material to the cell body at a rate of ~10 μm^3/h. This may be compared with the volume increase of a typical growing axon: 1 μm in diameter and extending at 40 μm/h, which is close to 30 μm^3/h. Our hypothetical convoy of parcels would then carry away from the growth cone one-third of the volume delivered to it, and probably an even greater proportion of the mass, considering their apparent density.

Although parcels move in either direction along a neurite, the majority of those we examined moved retrogradely, i.e., toward the cell body (Tables II and III, Fig. 2). Since, moreover, parcels moving retrogradely have a greater velocity than those moving anterogradely (Table I), it follows that there is a net flux of phase-dense material from the growing tip of the neurite back to its cell body.

Where has this material come from? It is unlikely to consist of proteins or other high molecular mass substances taken up from the surrounding culture medium by endocytosis or phagocytosis for two reasons. First, the appearance and transport of parcels continues unabated in the absence of protein, methylcellulose, or other macromolecular constituents from the medium. Second, the parcels stain brightly with diI-C_{18} that has been applied specifically to the cell body rather than to the culture medium as a whole and which therefore has presumably diffused in the plane of the axolemma (Schlessinger et al., 1977). The simplest conclusion appears to be that the material present in parcels was originally delivered to the growing tip via the neurite itself by the mechanisms of fast or slow transport. Consistent with this suggestion is the finding that stationary parcels are often seen in the web of the growth cone and sometimes appear from previously phase-transparent regions (Fig. 1). Furthermore, if the progress of the growth cone is arrested, an increase in the number of retrogradely moving parcels follows, as expected if they contain material delivered by internal transport.

We have shown that blocking the elongation of neurites in two different ways causes a pronounced increase in the number of moving parcels per unit length of neurite (Tables II and III). More important, the number moving in the retrograde

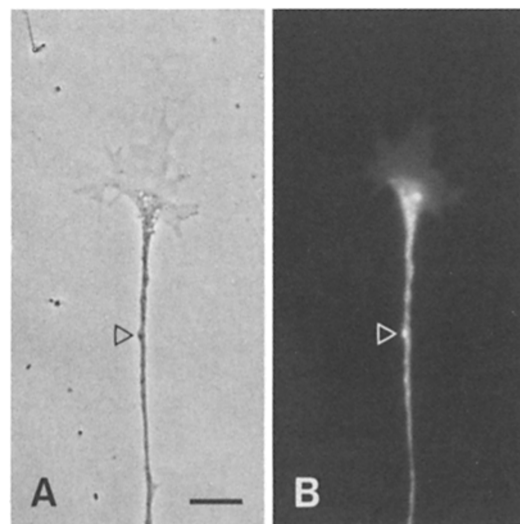

Figure 5. diI-C_{18} staining of a live neuron. A sensory ganglion in culture has been injected with diI-C_{18} emulsion as described in Materials and Methods and viewed 24 h after injection. The distal region of a neuron examined in phase-contrast (*A*) shows a small parcel (*arrowhead*) on the left side of the neurite. Epifluorescent illumination (*B*) reveals that the parcel stains brightly with the lipophilic dye. Bar, 10 μm.

Downloaded from http://jcb.rupress.org/jcb/article-pdf/105/6/2827/1466216/2827.pdf by guest on 23 April 2024

direction increases fourfold while their anterograde transport is reduced considerably, resulting in a shift in the ratio of retrogradely moving to anterogradely moving parcels from 3:2 to >11:1. Thus parcel transport is greatest, and biased most toward the retrograde direction, under conditions where the supply of cytoskeletal components and membrane to the distal tip by fast and slow anterograde transport is most likely to exceed the region's requirements. No previously described organelle or component of axonal transport shows behavior coordinated with local conditions in this way.

The intense staining of parcels in live cells with the lipophilic carbocyanine dye diI-C_{18} (Fig. 5) indicates that they contain membrane (Axelrod, 1979). This probably explains in part their refractility and phase density, and is consistent with the electron microscopic evidence of Koenig et al. (1985), which showed that mobile neuronal varicosities contain reticulated membrane. The presence of lipid bilayer in parcels suggests that they may retrieve excess membrane delivered to the neuronal terminal by fast anterograde transport in the absence of elongation. A similar function, in mature nongrowing axons, has been attributed to large membranous bodies of varying sizes that accumulate just distal to a region of damming or local cooling (Tsukita and Ishikawa, 1980; Grafstein and Forman, 1980). The relationship between these multivesiculated and lamellated structures and the organelles we have examined is presently unclear.

Although some parcels were unstained with diI-C_{18} under the conditions we used, this does not necessarily mean that they do not contain membrane components. In contrast to immunostaining procedures, which register the presence of antigen regardless of when or where it was formed, diI-C_{18} acts as a "tracer" that follows a complex route through the various membrane-containing compartments of the cell (e.g., Jacobson et al., 1981). Unstained organelles may therefore simply be older, or may for some reason be inaccessible to the dye.

It was not possible to probe simultaneously for the presence of lipid and of cytoskeletal proteins, but the figures obtained from separate staining procedures (62% contain membrane, 63% contain α-tubulin and/or NF-L) imply that membrane and cytoskeletal proteins coexist in at least some parcels.

The immunocytochemical evidence (Fig. 4, Table IV) shows that subpopulations of parcels contain α-tubulin and 70-kD neurofilament protein. As shown in Table IV, 39.2% of parcels stain for α-tubulin, 30.7% stain for NF-L, and 9.4% stain for both α-tubulin and NF-L; the frequency of coincidence of the two proteins in individual parcels is thus no higher than would be expected if they were packaged independently and at random. Neither protein in parcels is stably associated with the cytoskeleton, since the organelles and their immunoreactivity disappear under conditions of detergent extraction of the sort routinely used to prepare cytoskeletons (Brown et al., 1976). The tubulin in parcels is in a particularly soluble form, as it can be extracted under very mild conditions that leave the 70-kD neurofilament protein and most of the phase density of the parcels behind (Fig. 4, Table IV); these conditions also preserve the labile microtubules of nonneuronal cells (Fig. 4 *D*) and of neuronal growth cones (data not shown).

In their study of motile varicosities in cultured goldfish retinal ganglion cells, Koenig and co-workers (1985) showed that reticulated membrane in the varicosities is embedded in an amorphous matrix, and they have suggested that the cytoskeletal proteins contained within the varicosities are responsible for packaging the membranes. The relative arrangement of membranes and cytoskeletal proteins within the neuronal parcels we have studied has yet to be determined.

A large body of published work indicates that tubulin and neurofilament protein are transported, sometimes coordinately, in the slowest component of anterograde transport, and that their steady delivery to the distal end of the axon continues in mature, nonelongating neurons (e.g., Hoffman and Lasek, 1975; Lasek and Hoffman, 1976; Black and Lasek, 1980; Heriot et al., 1985). This body of evidence has been interpreted to mean that cytoskeletal proteins move slowly down the axon in the form of an assembled framework of interconnected polymers (Lasek and Brady, 1982; Lasek et al., 1984). However, more recent studies have indicated that a major part of the mature axonal cytoskeleton may in fact be stationary, while newly made neurofilament protein, and perhaps also tubulin, travels down the axon in an unassembled or partially assembled form (Nixon and Logvinenko, 1986; Nixon, 1987; Tashiro and Komiya, 1987). The theoretical possibility has been raised that this movement might be driven by an interaction with the stable assemblage of microtubules through mechanisms similar to those responsible for fast transport (Tashiro and Komiya, 1987).

In light of this recent work, our finding, and that of Koenig and co-workers, that organelles containing cytoskeletal proteins move within neurons by a form of rapid transport, is therefore not entirely heretical. Since the overall direction of the organelles we have examined is unambiguously toward the cell body, they cannot be directly responsible for the anterograde movements of cytoskeletal structures in the studies just mentioned. But it seems to us unlikely that such a radical mobilization of cytoskeletal structures would be confined to rapidly growing embryonic neurites; or that it would not be capable, under some physiological conditions, of conveying material away from the cell body, towards the axon terminal.

Cell types other than cultured neurons show forms of motility similar to parcel transport. Heliozoan axopodia (Fitzharris et al., 1972) and the reticulopodial network of foraminifera (McGee-Russell, 1974) are two examples of other systems in which dense particles show saltatory, bidirectional movement at velocities of 1 μm/s or more (Bowser, 1983). Like cultured neurons, both of these systems consist of long cellular processes containing a cylindrical core of cytoskeletal elements and having a closely apposed plasma membrane which is deformed by moving organelles. It is possible that parcel transport is a phenomenon general to cells which, for reasons of asymmetry, require extensive redistribution of materials in their distal regions.

The original observations of the phase-dense organelles we have studied were made by Gerry Shaw and Richard Adams in the course of their graduate work in this department. We thank them, along with Kevin Chapman, for excellent technical assistance. We thank Dr. A. F. Brown (Medical Research Council Cell Biophysics Unit) for developing motion analysis programs.

P. J. Hollenbeck was supported by a fellowship from the Jane Coffin Childs Memorial Fund for Medical Research.

Received for publication 19 June 1987, and in revised form 21 July 1987.

Downloaded from http://jcb.rupress.org/jcb/article-pdf/105/6/2827/1466216/2827.pdf by guest on 23 April 2024

References

Axelrod, D. 1979. Carbocyanine dye orientation in red cell membrane studied by microscopic fluorescence polarization. *Biophys. J.* 26:557–574.

Black, M. M., and R. J. Lasek. 1980. Slow components of axonal transport: two cytoskeletal networks. *J. Cell Biol.* 86:616–623.

Bowser, S. S. 1983. Fully correlative LM and HVEM analysis of motile fibrils and particles in the reticulopods of Allogromia sp. (Protozoa, Foraminiferida). *Annu. Meet. Electron Microsc. Soc. Am.*, 41st. 538–539.

Brown, S., W. Levinson, and J. A. Spudich. 1976. Cytoskeletal elements of chick embryo fibroblasts revealed by detergent extraction. *J. Supramol. Struct.* 5:119–130.

Fitzharris, T. P., R. A. Bloodgood, and J. R. McIntosh. 1972. Particle movement in the axopodia of Echinosphaerium: evidence concerning the role of the axoneme. *J. Mechanochem. Cell Motil.* 1:117–124.

Gilbert, D. S., B. J. Newby, and B. H. Anderton. 1975. Neurofilament disguise, destruction and discipline. *Nature (Lond.).* 256:686.

Grafstein, B., and D. S. Forman. 1980. Intracellular transport in neurons. *Physiol. Rev.* 60:1167–1283.

Heriot, K., P. Gambetti, and R. J. Lasek. 1985. Proteins transported in the slow components a and b of axonal transport are distributed differently in the transverse plane of the axon. *J. Cell Biol.* 100:1167–1172.

Hoffman, P. N., and R. J. Lasek. 1975. The slow component of axonal transport: identification of major structural polypeptides of the axon and their generality among mammalian neurons. *J. Cell Biol.* 66:351–366.

Hollenbeck, P. J., D. Bray, and R. J. Adams. 1985. Effects of the uncoupling agents FCCP and CCCP on the saltatory movements of cytoplasmic organelles. *Cell Biol. Int. Rep.* 9:193–199.

Honig, M. G., and R. J. Hume. 1986. Fluorescent carbocyanine dyes allow living neurons of identified origin to be studied in long-term cultures. *J. Cell Biol.* 103:171–187.

Jacobs, M., Q. L. Choo, and C. Thomas. 1982. Vimentin and 70K neurofilament protein co-exist in embryonic neurones of spinal ganglia. *J. Neurochem.* 38:969–977.

Jacobson, K., Y. Hou, Z. Derzko, J. Wojcieszyn, and D. Organisciak. 1981. Lipid lateral diffusion in the surface membrane of cells and in multibilayers formed from plasma membrane lipids. *Biochemistry.* 20:5268–5275.

Johnson, G. D., and G. M. de C. Nogueira Araujo. 1981. A simple method for reducing the fading of immunofluorescence during microscopy. *J. Immunol. Methods.* 43:349–350.

Kilmartin, J. V., B. Wright, and C. Milstein. 1982. Rat monoclonal antitubulin antibodies derived by using a new nonsecreting rat cell line. *J. Cell Biol.* 93:576–582.

Koenig, E., S. Kinsman, E. Repasky, and L. Sultz. 1985. Rapid mobility of motile varicosities and inclusions containing α-spectrin, actin, and calmodulin in regenerating axons. *J. Neurosci.* 5:715–729.

Lander, A. D., D. K. Fujii, D. Gospodarowicz, and L. F. Reichardt. 1982. Characterization of a factor that promotes neurite outgrowth: evidence linking activity to a heparin sulfate proteoglycan. *J. Cell Biol.* 94:574–585.

Lasek, R. J., and M. M. Black. 1977. How do axons stop growing? Some clues from the metabolism of the proteins in the slow component of axonal transport. *In* Mechanisms, Regulation and Special Functions of Protein Synthesis in the Brain. S. Roberts, A. Lathja, and W. H. Gisben, editors. Elsevier Science Publishing Co., Inc., New York. 161–169.

Lasek, R. J., and S. T. Brady. 1982. The structural hypothesis of axonal transport: two classes of moving elements. *In* Axoplasmic Transport. D. G. Weiss, editor. Springer-Verlag, Berlin. 397–405.

Lasek, R. J., and P. N. Hoffman. 1976. The neuronal cytoskeleton, axonal transport and axonal growth. *Cold Spring Harbor Conf. Cell. Prolif.* 3(Book A):1021–1049.

Lasek, R. J., J. A. Garner, and S. T. Brady. 1984. Axonal transport of the cytoplasmic matrix. *J. Cell Biol.* 99:212s–221s.

Luduena, M. A. 1973. The growth of spinal ganglion neurons in serum-free medium. *Dev. Biol.* 33:470–476.

McGee-Russell, S. M. 1974. Dynamic activities and labile microtubules in cytoplasmic transport in the marine foraminiferan Allogromia. *Symp. Soc. Exp. Biol.* 28:157–189.

Nixon, R. A. 1987. The axonal transport of cytoskeletal proteins: a reappraisal. *In* Axonal Transport. R. S. Smith and M. A. Bisby, editors. Alan R. Liss, Inc., New York. 175–200.

Nixon, R. A., and K. B. Logvinenko. 1986. Multiple fates of newly synthesized neurofilament proteins: evidence for a stationary neurofilament network distributed non-uniformly along axons of retinal ganglion cell neurons. *J. Cell Biol.* 102:647–659.

Roots, B. I. 1983. Neurofilament accumulation induced in synapses by leupeptin. *Science (Wash. DC).* 221:971–972.

Schlessinger, J., D. Axelrod, D. E. Koppel, W. W. Webb, and E. L. Elson. 1977. Lateral transport of a lipid probe and labelled proteins on a cell membrane. *Science (Wash. DC).* 195:307–309.

Tashiro, T., and Y. Komiya. 1987. Organization of cytoskeletal proteins transported in the axon. *In* Axonal Transport. R. S. Smith and M. A. Bisby, editors. Alan R. Liss, Inc., New York. 201–221.

Tsukita, S., and H. Ishikawa. 1980. The movement of membranous organelles in axons. Electron microscopic identification of anterogradely and retrogradely transported organelles. *J. Cell Biol.* 84:513–530.

Weber, K., P. Rathke, and M. Osborne. 1978. Cytoplasmic microtubular images in glutaraldehyde-fixed tissue culture cells by electron microscopy and by immunofluorescence microscopy. *Proc. Natl. Acad. Sci. USA.* 75:1820–1824.

Downloaded from http://jcb.rupress.org/jcb/article-pdf/105/6/2827/1466216/2827.pdf by guest on 23 April 2024