

Axonal and Dendritic Endocytic Pathways in Cultured Neurons

Robert G. Parton, Kai Simons, and Carlos G. Dotti

European Molecular Biology Laboratory, Postfach 10.2209, D-6900 Heidelberg, Germany

Abstract. The endocytic pathways from the axonal and dendritic surfaces of cultured polarized hippocampal neurons were examined. The dendrites and cell body contained extensive networks of tubular early endosomes which received endocytosed markers from the somatodendritic domain. In axons early endosomes were confined to presynaptic terminals and to varicosities. The somatodendritic but not the presynaptic early endosomes were labeled by internalized transferrin. In contrast to early endosomes, late endosomes and lysosomes were shown to be predominantly located in the cell body. Video microscopy was used to follow the transport of internalized markers from the periphery of axons and dendrites back to the cell body. Labeled structures in both domains moved

unidirectionally by retrograde fast transport. Axonally transported organelles were sectioned for EM after video microscopic observation and shown to be large multivesicular body-like structures. Similar structures accumulated at the distal side of an axonal lesion. Multivesicular bodies therefore appear to be the major structures mediating transport of endocytosed markers between the nerve terminals and the cell body. Late endocytic structures were also shown to be highly mobile and were observed moving within the cell body and proximal dendritic segments. The results show that the organization of the endosomes differs in the axons and dendrites of cultured rat hippocampal neurons and that the different compartments or stages of the endocytic pathways can be resolved spatially.

MEMBRANE proteins, lipids, and solutes internalized by animal cells enter endosomes. Within these structures selective targeting to the recycling, degradative, and, in polarized epithelial cells, transcytotic pathways occurs (Hubbard, 1989; Rodman et al., 1990). The exact sequence of events leading to the delivery of a ligand to the degradative compartments is not yet defined. Different stations on the pathway to lysosomes have been identified and their protein compositions described (Beaumelle et al., 1990; Schmid et al., 1988). However, the boundaries between early and late endocytic structures and their interrelationships are not yet clear (e.g., see Hopkins et al., 1990; Griffiths and Gruenberg, 1991; Murphy, 1991). Several features of the endocytic pathway make *in vivo* analysis difficult. First, the compartments are extremely plastic and their form can vary between different cell types (Hubbard, 1989) or even within the same cell under different conditions (Heuser, 1989a; Parton et al., 1991; Lippincott-Schwartz et al., 1991; Wood et al., 1991). Second, there is asynchrony in the endocytic pathways so that the wave of a ligand reaching late compartments may be broad (Kielian et al., 1986). Third, early and late endocytic structures are not well-segregated in a fibroblast-like cell; early endosomes appear more peripherally located than late structures which are close to the microtubule-organizing center but at least some early structures are also clustered close to the centrioles (Hopkins, 1983; Yamashiro et al., 1984; Tooze and Hollinshead, 1991) and in some cell types endosomes form an extensive reticulum spread throughout the cell (Hopkins et al., 1990). At least

some of these problems could be overcome in a cell with spatially separated early and late endosomes. In this respect, the neuronal cell offers many advantages.

Past studies of neuronal endocytosis have largely concentrated on the retrieval of membrane after neurotransmitter release at presynaptic terminals. Synaptic vesicle fusion with the axolemma is immediately followed by recycling via clathrin-coated vesicles (Heuser, 1989b). The retrieval of synaptic vesicle components and the formation of synaptic vesicles may involve a modification of the endocytic process occurring in nonneuronal cells (for recent reviews see Kelly, 1991; Südhof and Jahn, 1991). Whether an endosomal intermediate is involved in the recycling pathway as in nonneuronal cells is not yet clear. Endosomal structures have been observed in nerve terminals (e.g., Teichberg et al., 1975; Sulzer and Holtzman, 1989) and these may be involved in the delivery of internalized components to the cell body where lysosomal compartments are located (Holtzman, 1989).

In the present study we have characterized endocytosis in a cultured neuronal system, the rat hippocampal neuron. These cells mature *in vitro* in a well-defined sequence (Dotti et al., 1988) to produce dendrites and axons and to form synaptic contacts (Bartlett and Banker, 1984). The two plasma membrane domains have different compositions and viral proteins are differentially sorted to the two domains (Dotti and Simons, 1990; Dotti et al., 1991). The spatial separation of these domains makes the hippocampal neuron an excellent system to study the pathways of membrane traffic; in mature

cells dendrites reach lengths of up to 400 μm and axons several millimeters. This system offers the possibility to study the overall organization of neuronal endocytic pathways for the first time. Here in a combined video microscopy and EM study we describe the endocytic circuits from the axonal and dendritic domains of hippocampal neurons. In addition we characterize the traffic between early and late endocytic stations and show that this is mediated by large multivesicular body (mvb)-like carrier vesicles which are transported by fast retrograde transport along the axon or within the dendrites.

Materials and Methods

Cell Culture

Rat hippocampal cultures were prepared exactly as described (Goslin and Banker, 1991). For all experiments cells were cultured for 7–14 d in vitro (stage 5 cells; Dotti et al., 1988) before use.

Endocytic Markers

HRP was obtained from Sigma Chemical Co. (St Louis, MO) (type II HRP) or from Serva Biochemicals (Heidelberg, Germany) (high activity HRP, i.e., 1,362 U/mg) and was used at a concentration of 10 mg/ml in internalization medium (neuronal medium, as described by Dotti and Simons [1990] but with 10 mM Hepes and 4 mM NaHCO_3 , pH 7.35) containing 0.1% chicken ovalbumin to reduce nonspecific binding. In some experiments HRP was dialyzed before use and identical results were obtained.

Ovalbumin-gold (ovalb-gold) was prepared by addition of excess ovalbumin to a sol of 9 nm gold at pH 7.0 prepared by the tannic acid method (Slot and Geuze, 1985). The gold was concentrated by centrifugation and before use was dialyzed versus internalization medium. The gold was added to the cells at an OD_{520} of 20–40 in the presence of 0.1% ovalbumin to reduce nonspecific binding to the plasma membrane. All washes were with internalization medium containing ovalbumin.

Transferrin-HRP (Tf-HRP) was kindly provided by Dr B. Hoflack (European Molecular Biology Laboratory [EMBL], Heidelberg, Germany). Cells were washed over 30 min with serum-free medium containing 0.1 mg/ml ovalbumin. They were then incubated with 50 $\mu\text{g}/\text{ml}$ Tf-HRP in the same medium for 30–90 min at 37°C.

In some experiments, markers were internalized during and after membrane depolarization to induce neurotransmitter release. This was achieved by using an isotonic depolarizing solution (Rosa et al., 1985) with the following composition: 10 mM Hepes, 2.2 mM CaCl_2 , 0.33 mM Na_2HPO_4 , 0.44 mM KH_2PO_4 , 4.2 mM NaHCO_3 , 5.6 mM glucose, 77 mM NaCl, 55 mM KCl, pH 7.35. Cells were incubated with HRP in this solution for 5

min and then with HRP in standard internalization medium for a further 25 min at 37°C.

Video Microscopy

Cells cultured on 35-mm dishes were incubated with fluid-phase markers at 37°C, washed with internalization medium, and then the dishes were mounted on a Zeiss Axiovert 10 microscope (Carl Zeiss, Inc., Thornwood, NY) fitted with a temperature-controlled stage maintained at $37 \pm 0.2^\circ\text{C}$. The microscope was fitted with a LD 0,55H/4-mm-diameter air condenser with a long working distance and a $32\times$ LD Achroplan objective. Cells were first located with Nomarski using Köhler illumination. The polarizers were then removed to obtain the bright-field image. The signal was detected with a Hamamatsu CCD camera using a Hamamatsu Argus 10 image processor and the video signal was recorded with a Panasonic AG-6720 time-lapse video recorder.

Fields containing well-isolated axons or dendrites were recorded and phase-dense vesicles undergoing transport were followed. Analysis of the movement of transported organelles was performed using the Macintosh Image 1.4 program with a Macintosh CI and a Panasonic NVW1 video player. The movement of each vesicle was followed over a distance of at least 30 μm .

In experiments in which the morphology of transported organelles were examined by EM, cells were fixed by addition of 2.5% glutaraldehyde in 50 mM cacodylate buffer directly to the culture dish during observation. This caused immediate cessation of transport. Reference marks close to the area of interest were scratched on the plastic dish. The fixative was then replaced with fresh fixative solution and left a further 30 min at room temperature before processing for flat embedding in Epon.

Immunofluorescence

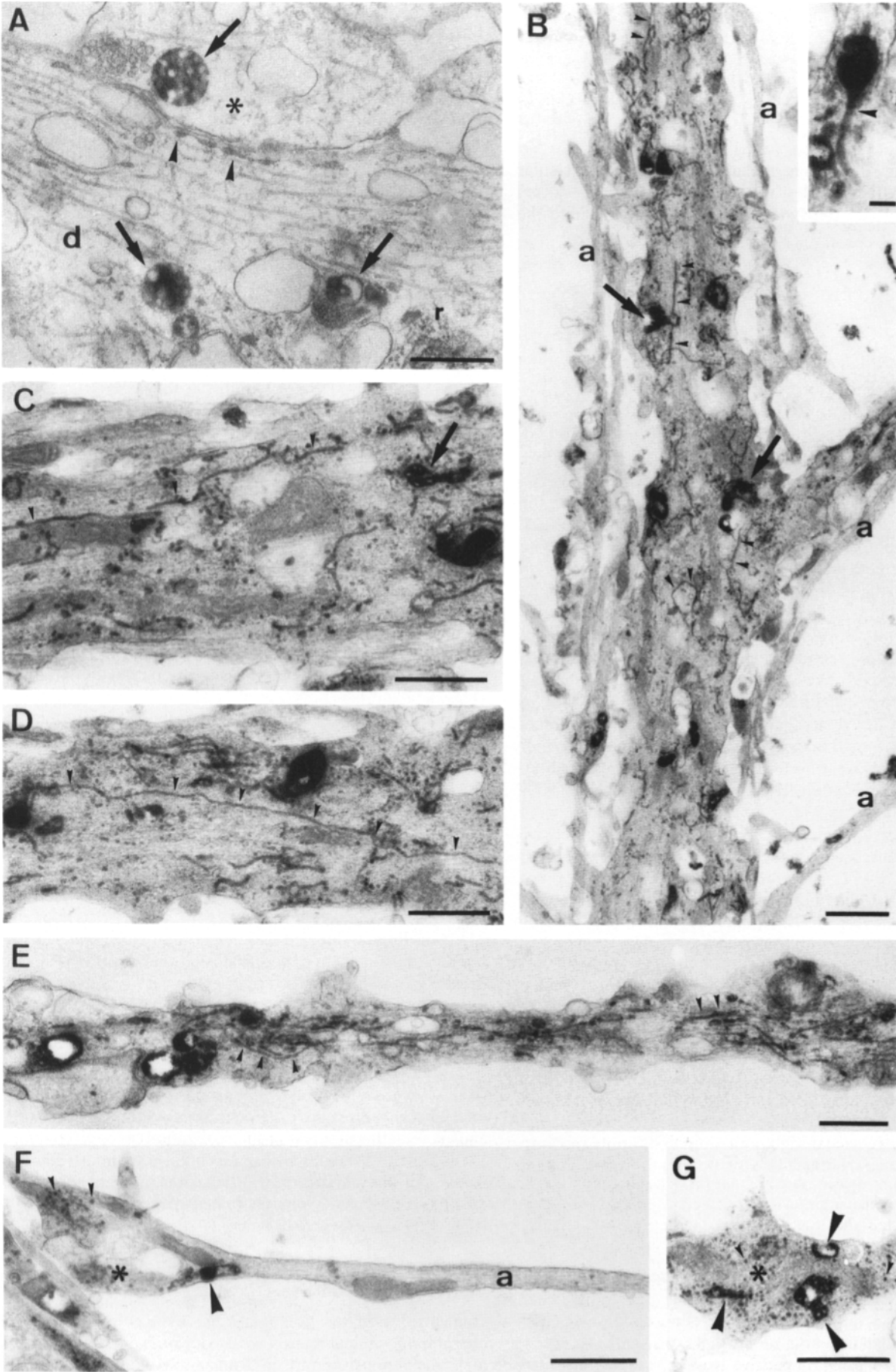
The intracellular distribution of late endocytic structures was analyzed by indirect immunofluorescence. The following reagents were used: antibodies raised against the chicken cation-independent mannose-6-phosphate receptor (Parton et al., 1989) and against a COOH-terminal peptide of rab7 (Chavrier et al., 1990) were kindly provided by Drs. B. Hoflack and M. Zerial, respectively (EMBL, Heidelberg, Germany). An antibody against the lysosomal glycoprotein, lgp 120 (Lewis et al., 1985), was kindly provided by Dr I. Mellman (Yale University, New Haven, CT). Cells were either fixed in saponin/paraformaldehyde as described previously or fixed for 6 min in freezing cold methanol. Indirect immunofluorescence was then continued as previously described (Dotti and Simons, 1990).

Electron Microscopy

Cells were routinely grown on plastic dishes for EM analysis. After incubations with fluid-phase markers, the cells were fixed either using 2.5% glutaraldehyde in 50 mM cacodylate or with 0.1% glutaraldehyde in 100 mM cacodylate buffer. Cells were then viewed by light microscopy and selected cells to be sectioned were marked on the dish surface. After embedding in Epon the culture dish was removed and the marked cells were sectioned parallel to the substratum. Conventional ultrathin or semi-thick (~ 250 nm) sections were prepared and were viewed without further contrasting at an accelerating voltage of 60 kV. In experiments where single

Abbreviations used in this paper: mvb, multivesicular body; ovalb-gold, ovalbumin-gold; Tf-HRP, transferrin-HRP.

Figure 1. Early endocytic structures in mature rat hippocampal neurons. Mature rat hippocampal neurons were incubated with HRP (10 mg/ml) for various times at 37°C. Thin (A) or semi-thick sections (B–G) were then cut parallel to the substratum. In A, HRP internalized for 10 min labels vesicular structures (arrows) in both the dendrites (recognizable by the presence of ribosomes, r) and in a presynaptic terminal (asterisk) containing synaptic vesicles. Possible HRP reaction product is also evident in tubular structures within the dendrites (small arrowheads). Using high activity HRP, semi-thick sections (~ 250 nm), and a longer incubation time (30 min), the full extent of the endosomal compartment within the dendrites is evident (B–E). B shows a low magnification view of a dendrite surrounded by axons (a). Numerous tubular structures (small arrowheads) of constant diameter (~ 45 nm) and varying lengths are labeled. Tubules run both down the length of the dendrites and across the dendrite. Large vesicular structures (examples indicated by large arrows) are also visible at fairly regular intervals along the dendrite. These structures resemble mvbs (arrow in C) and in fortuitous sections appear to be connected to tubules (B, inset). In D a tubule extending over several microns is evident (small arrowheads). Tubules are present even in the most peripheral regions of the dendrite as shown in E; this region was chosen after examination of the light micrograph of the cell and was ~ 100 μm from the cell body. (F and G) Cells were incubated with high activity HRP as above but were also treated with high potassium medium for 5 min to induce neurotransmitter release as described in Materials and Methods. Axon shafts (a) are free of reaction product although labeled tubulovesicular structures (large arrowheads) are evident in presynaptic terminals and in varicosities (asterisks). HRP labeling is also apparent in putative synaptic vesicles (small arrowheads). Bars: (A) 0.5 μm ; (B–G) 1 μm .



vesicles were relocated, serial semi-thick sections were collected and picked up on slot grids.

Axon Lesioning

Cells on plastic culture dishes were incubated with fluid-phase markers for 30 min at 37°C and then were washed. The dish was transferred to the stage of an automatic microinjection system (Ansorge and Pepperkok, 1988). Well-isolated cells in which the axon was clearly distinguishable were chosen for lesioning. Lesioning was achieved by drawing the tip of a glass needle across the axon using the micromanipulator. Reference marks were then made on the dish to allow subsequent relocation. The cells were incubated for a further 20 min at 37°C before fixation and flat embedding in Epon. Semi-thick sections parallel to the culture substratum were prepared and viewed as described above.

Results

Morphological Characterization of the Endocytic Apparatus of Mature Rat Hippocampal Neurons

Rat hippocampal neurons maintained for 10–14 d *in vitro* (stage 5 cells, see Dotti et al., 1988) were incubated with HRP as a fluid-phase marker for varying times at 37°C. The cells were then fixed and embedded in Epon. Selected cells to be sectioned were photographed after fixation and the light micrographs were used to distinguish axons and dendrites based on gross morphological characteristics before sectioning parallel to the culture substratum. HRP-labeled structures in the different domains of the cell were then examined.

After a 10-min incubation with HRP and preparation of conventional thin sections, labeled structures, consisting of tubules and mvb-like structures were evident in the cell body, nerve terminals, and dendrites (Fig. 1 *A*). To try to visualize the true extent of the early endosomal compartment in these cells, a high activity HRP and relatively thick sections (~250 nm) were used (Tooze and Hollinshead, 1991). After a 5-min incubation, HRP was shown to label vesicular and tubular structures within dendrites and presynaptic nerve terminals (not shown). After a 30-min incubation extensive tubular structures within the dendrites were labeled with HRP (Fig. 1, *B–E*). These branching tubules of 40–50-nm diameter were sometimes seen to extend over a distance of several microns (Fig. 1 *D*). At fairly regular intervals larger spherical structures, with a mvb-like structure, appeared to be associated with the tubules (Fig. 1, *B–D*, inset Fig. 1 *B*). Tubules extended to the distal ends of the dendrites (Fig. 1 *E*). Similar, but less extensive endosomal tubules were observed in the cell body (not shown; see Fig. 2 *A*). Tubulovesicular structures within the presynaptic nerve terminals also contained HRP reaction product. As it has previously been shown that increased uptake of fluid-phase markers occurs after synaptic vesicle exocytosis (e.g., Heuser and Reese, 1973) we used a high potassium-containing medium to induce neurotransmitter release. After a 5-min incubation with HRP under depolarizing conditions followed by a further 25-min incubation with HRP both tubulovesicular endosomal structures and synaptic vesicles in the nerve terminals contained HRP reaction product (Fig. 1, *F* and *G*), consistent with previous observations in other systems (e.g., Heuser and Reese, 1973; Teichberg et al., 1975; Schacher et al., 1976). Even under these labeling conditions HRP labeling was not observed along the axon shaft, except in areas containing synaptic vesicles (Fig. 1 *F*). In contrast, in

immature neurons (stage 3 cells) labeling of endosomal structures was observed throughout the cell, including the already identifiable axon (results not shown).

Previous *in vivo* studies have shown the existence of an extensive endosomal reticulum labeled by internalized transferrin in Hep2 cells (Hopkins et al., 1990). As the transferrin receptor has been localized to the dendritic surface of stage 4 (Cameron et al., 1991) and stage 5 (results not shown) rat hippocampal neurons, we investigated whether the tubular endosomal structures observed in the dendrites and cell body in the present study could be labeled by internalized transferrin-HRP (Tf-HRP). After internalization of 50 µg/ml Tf-HRP for times between 30 and 90 min, long tubular structures identical to those observed after fluid-phase HRP uptake within the cell body (Fig. 2 *A*) and dendrites (Fig. 2, *B–D*) were labeled. No tubules were labeled after internalization of such a low concentration of HRP as a fluid-phase marker (results not shown) showing that uptake was via a receptor-mediated process. Interestingly, negligible labeling was observed in presynaptic terminals (Fig. 2 *D*) consistent with the proposed polarized distribution of the transferrin receptor (Cameron et al., 1991). No large vesicular endocytic structures in the cell body were labeled even after a 60-min incubation with Tf-HRP.

To characterize the late compartments in the endocytic pathways, high activity HRP was internalized for 30 min as above and then the cells were incubated in the absence of the tracer for various times at 37°C. After a 30-min chase time, HRP reaction product was rarely observed within tubular structures; the principal labeled elements at this time were mvb-like structures of 300–450-nm diameter in the dendrites (Fig. 3, *A* and *C*). Labeling of endosomal structures in the nerve terminals was also reduced but labeling was observed within mvb-like structures within the axon shaft (Fig. 3, *B* and *D*). As we generally observed no internalization from the plasma membrane in the shaft, we conclude that these structures must have moved into the axon either from the cell body, or more likely, from the presynaptic terminals. To investigate whether the multivesicular structures within the dendrites were distinct from the early endosomes, cells were incubated for 30 min at 37°C with ovalb-gold. After washing to remove unbound gold a 30-min pulse of HRP was applied. Many ovalb-gold-labeled structures within the dendrites remained unlabeled by the subsequent pulse of HRP even though HRP-labeled structures were in very close proximity (e.g., Fig. 3 *C*).

After a 60- (Fig. 3 *E*) or 90-min (Fig. 3 *F*) chase-time HRP reaction product was mainly observed within large (up to 1 µm diameter) spherical structures in the cell body and the proximal segment of the dendrites. Occasionally, labeled mvb-like structures were also observed within the dendrites and axons at this time. After internalization of ovalb-gold followed by an 8-h chase labeling was present within structures in the cell body and proximal dendritic segment that were electron dense and often contained arrays of membranes (Fig. 3, *G* and *H*).

Localization of Late Endosome/Lysosome-specific Antigens

To determine the location of late endocytic compartments in the rat hippocampal neuron we used immunofluorescence to localize a number of antigens known to be enriched in these

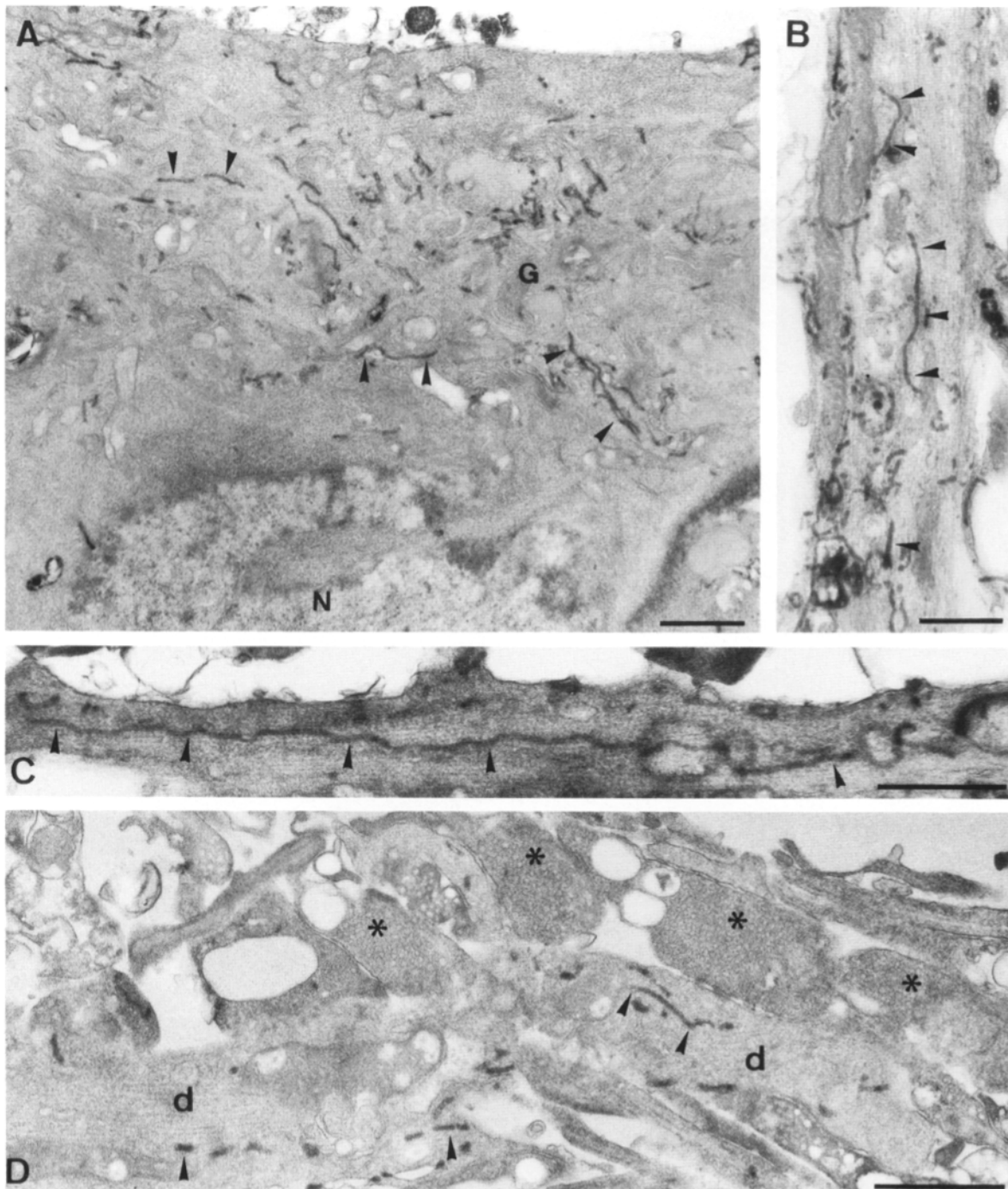
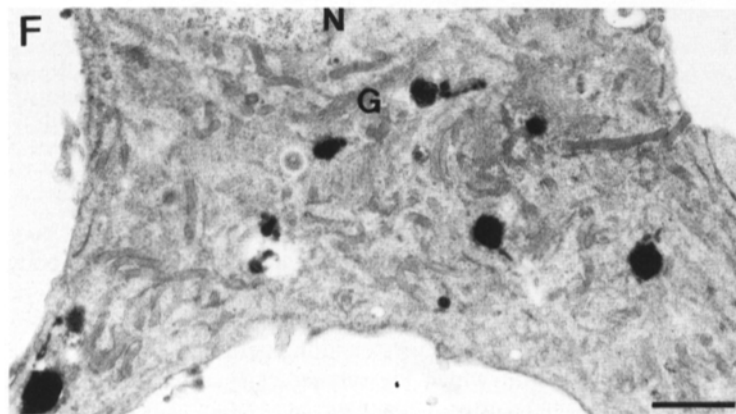
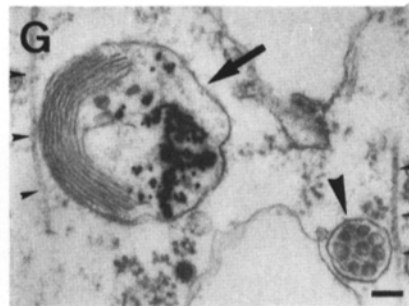
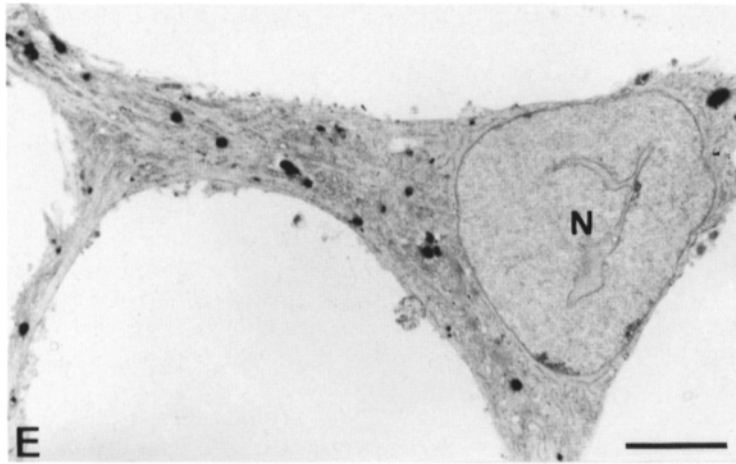
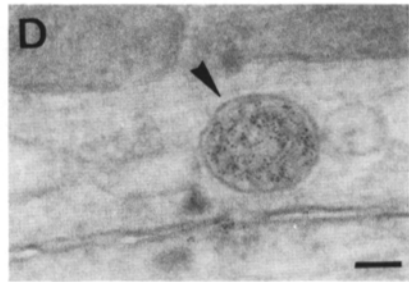
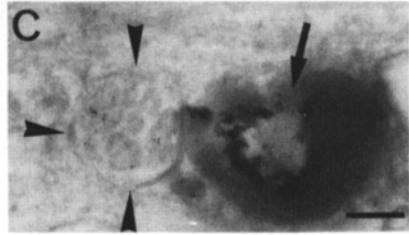
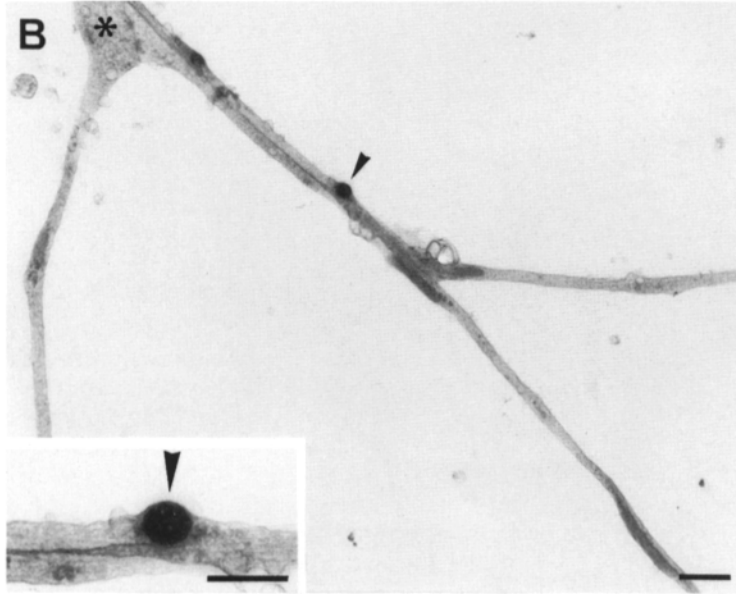
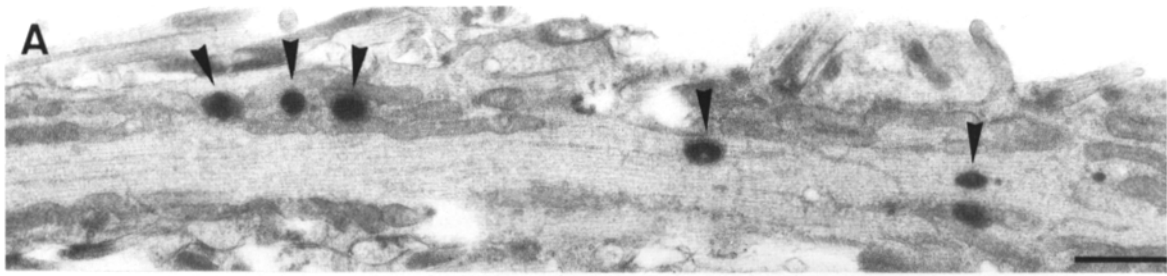


Figure 2. Internalization of transferrin-HRP. Cells were incubated with 50 $\mu\text{g/ml}$ Tf-HRP for 60 min. Semi-thick sections were then prepared. In *A*, HRP reaction product is present within tubules (*arrowheads*) in the Golgi region (*G*) of the cell body. Tubules are also apparent within dendrites (*B-D*) and appear identical to those visualized using HRP (see Fig. 1). Note that few vesicular structures are labeled. *D* shows a thinner section (which therefore shows lower HRP labeling) to show the morphology of the presynaptic terminals (*asterisks*). The latter are unlabeled whereas labeled tubular structures (*arrowheads*) are evident within the dendrite (*d*). Bars, 1 μm .

structures. These included antibodies to the cation-independent mannose-6-phosphate receptor (CI-MPR), which is predominantly associated with late endosomes and the TGN (Griffiths et al., 1988; Geuze et al., 1988), to a lysosomal glycoprotein (lgp) enriched in late endosomes and lysosomes (Lewis et al., 1985; Griffiths et al., 1990) and to rab7, a small GTP-binding protein associated with late endosomes (Chavrier et al., 1990). As shown in Fig. 4, *A-F*, the pattern

of immunofluorescence labeling with each of these antibodies was similar with the predominant labeling in the cell body and proximal dendritic segments (20–30 μm from the cell body). No detectable labeling was observed within the axons or presynaptic terminals or in the peripheral regions of the dendrite into which the tubular early endosomal profiles were seen to extend. Since the late endocytic compartments are believed to be the principal sites of lysosomal enzyme ac-



tivity it appears that the major degradative compartments are located in the cell body close to the Golgi as in fibroblast-like cells.

Transport between Presynaptic Terminals and the Cell Body

To examine how transport between the peripherally located endocytic compartments and the putative degradative compartments in the cell body might occur we used video microscopy. Ovalb-gold particles were used as a fluid-phase marker to allow visualization of transported vesicles by light microscopy. Cells were incubated with ovalb-gold and HRP together for 30 min at 37°C. They were then washed and transferred to the warmed stage of the microscope in the bright field mode connected to a video display system.

Essentially all mobile phase-dense (gold-labeled) structures within the axons showed movement only in the retrograde direction. Observations of >50 cells were made. The labeled structures moved in a saltatory fashion with a mean velocity (observations of labeled vesicles averaged over a total distance of ~650 μm) of $0.32 \pm 0.13 \mu\text{m/s}$. However, individual vesicles reached velocities of up to 1.8 $\mu\text{m/s}$ during the saltations. These rates are comparable with those reported for fast axonal transport of larger organelles (Breuer et al., 1975; Grafstein and Forman, 1980; Allen et al., 1982). Pauses between saltations varied between 1 s (the shortest resolvable time period in this study) to over 20 s. Fig. 5 *A* shows the distance vs time plots of a number of labeled vesicles. The mean velocities of the vesicles range between 0.1 and 0.5 $\mu\text{m/s}$ but as described above these velocities represent the average of saltations and pauses of varying length. Fig. 5 *B* shows a detailed analysis of the movement of a single organelle. Brief pauses are evident between saltations of ~0.7 $\mu\text{m/s}$.

To determine the nature of typical retrogradely transported organelles, gold-labeled structures moving towards the cell body were observed by video microscopy and then were fixed by addition of fixative during the observation (Fig. 6). Using glutaraldehyde-containing fixative an immediate cessation of transport was observed. After processing for EM, semi-thick sections were cut parallel to the substratum and the same structures were relocated. As shown in Fig. 6 *B* the structures observed moving retrogradely by light microscopy were shown to be almost spherical or ovoid vesicles of between 300 and 450 nm in diameter. As expected they were labeled with both the internalized gold and with HRP.

The use of the HRP and the thick sections allows us to rule out that these structures have attached tubules; under the same experimental conditions extensive tubules were observed in the dendrites (e.g., see Fig. 1 *B*).

Although the majority of the labeled structures in the axons showed these characteristics, occasionally, smaller vesicles were also observed moving along the axon. Whether these structures were labeled was difficult to ascertain by light microscopy.

Axonal Lesioning to Study Retrograde Transport

To determine the relative contribution of different types of organelles (including any small or weakly labeled structures) to retrograde transport of endocytosed material in axons, an independent technique was used. The axons of a number of cells were cut with the aid of a micromanipulator system and accumulation of internalized markers distal to the lesion was examined by EM. Fig. 7 shows an example of such an experiment in which the cell was incubated with HRP for 30 min at 37°C before lesioning the axon. After a further 20-min incubation the cells were fixed, and processed for EM. Semi-thick sections were cut parallel to the substratum. At the distal border of the lesion numerous HRP-labeled structures accumulated (Fig. 7, *A* and *B*). These structures were predominantly mvb-like structures 300–450 nm in diameter, similar to those which accumulated at the distal side of a block in axonal transport *in vivo* (Hirokawa et al., 1990; Tsukita and Ishikawa, 1980). There was no accumulation in the segment of the axon proximal to the lesion (Fig. 7, *B* and *C*). Again, there was no evidence for tubular structures in association with the vesicles. The results strongly suggest that these structures are the major organelles involved in transport of endocytosed markers from the nerve terminals to the cell body.

Dendritic Transport

We investigated whether transport of endocytosed material in dendrites showed the same properties as retrograde axonal transport by video microscopy. As the dendritic microtubules of rat hippocampal neurons show mixed polarity (Baas et al., 1988), it was of particular interest to determine whether transport of individual vesicles within the dendrites was unidirectional as observed in axons.

Cells incubated for 60 min with ovalb-gold as a fluid-phase marker were washed and observed by bright-field micros-

Figure 3. Late endocytic structures in mature rat hippocampal neurons. Cells were incubated with either HRP (*A, B, E,* and *F*) or ovalb-gold (*C, D, G,* and *H*) for 30 min at 37°C and then washed and incubated for a further 30 min (*A, C,* and *D*), 60 min (*B* and *E*), or 90 min (*F*) in marker-free medium. In *C* and *D* HRP was included in the medium for the last 30 min of the chase period. All panels except *G* and *H* show unstained semi-thick sections. *A* shows HRP labeling in a dendrite after a 30 min incubation with HRP and a 30 min chase. Labeling of tubular structures is barely detectable (compare with Figs. 1 and 2) but reaction product is evident within relatively large mvb-like structures (*arrowheads*). *B* shows a group of axons after a 60-min chase. A mvb-like structure is evident within the axon shaft as shown at higher magnification in the inset. Little labeling is evident within presynaptic terminals (*asterisk*). In *C* and *D*, cells were incubated with ovalb-gold for 30 min and then incubated for a further 30 min with HRP but without ovalb-gold. Spherical vesicles (*arrowheads*) in a dendrite (*C*) and axon (*D*) contain ovalb-gold but no HRP reaction product (*arrow*). *E* and *F* show late endocytic compartments within the cell body after a 30-min incubation with HRP followed by a 60-min (*E*) or 90-min (*F*) chase in marker-free medium. HRP labeling is mainly observed within large vesicular structures. (*G* and *H*) After an 8-h chase to label the final stages of the endocytic pathway gold-labeled structures (*arrows*) are evident within the cell body (*G*) and proximal dendritic segment (*H*). These stained thin sections show the morphology of the late compartments. A possible carrier vesicle (mvb) which is unlabeled is indicated in *G*. Small arrowheads indicate microtubules. *N*, nucleus; *G*, Golgi. Bars: (*A, B,* and *F*) 1 μm ; (*B*, inset) 0.5 μm ; (*C, D, G,* and *H*) 100 nm; (*E*) 5 μm .

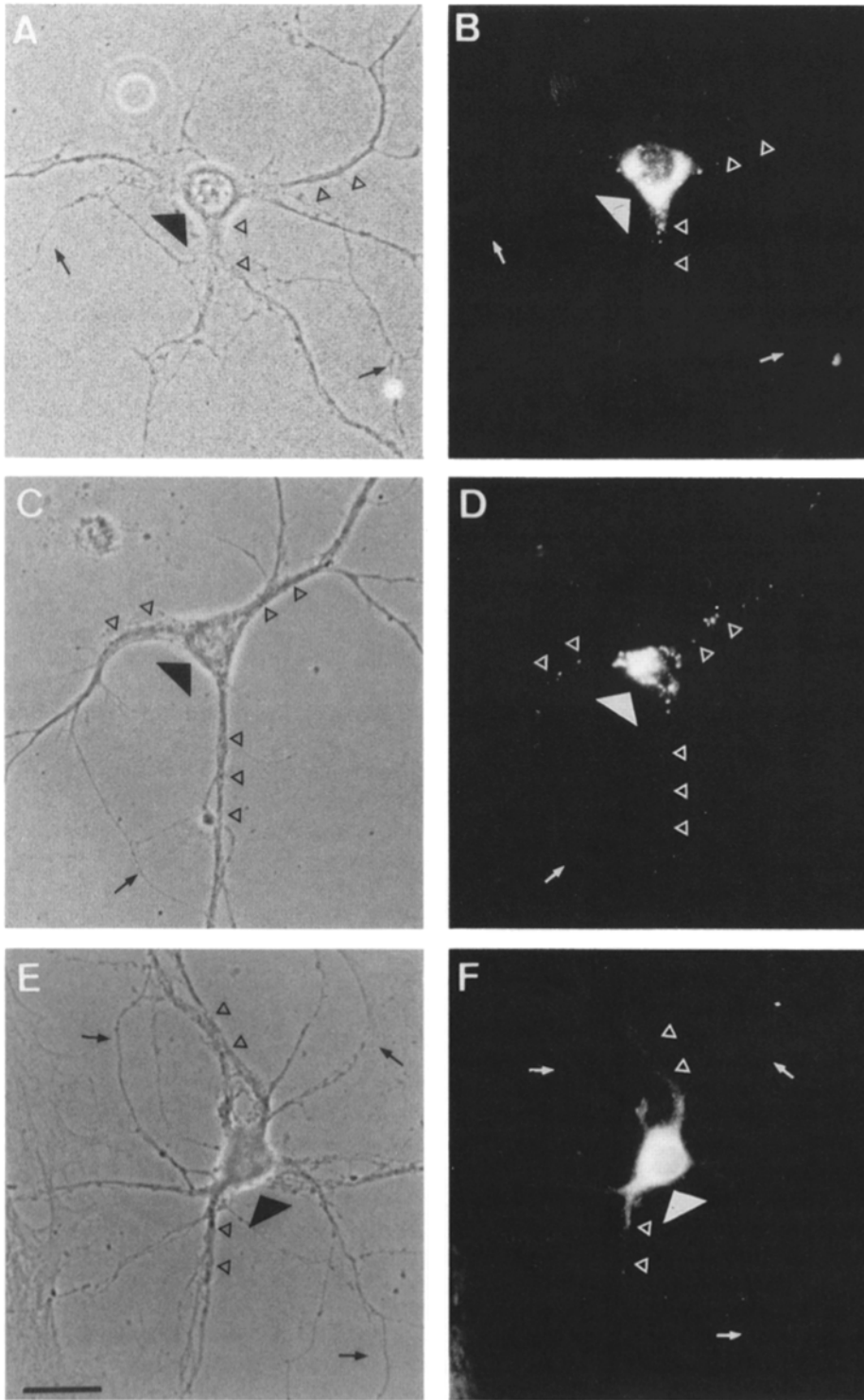


Figure 4. Immunofluorescent localization of late endosome/lysosome specific antigens. Cells were fixed, permeabilized, and incubated with antibodies to the cation-independent mannose-6-phosphate receptor (*A* and *B*), lysosomal membrane glycoprotein (lgp120) (*C* and *D*) or rab7 (*E* and *F*). Labeling is predominantly in the cell body (*large arrowhead*) and proximal dendritic segments. No labeling is evident in axons (*arrows*). Small arrowheads indicate dendrites. Bar, 10 μm .

copy. Gold-labeled structures of similar size (by light microscopy) to the axonally transported vesicles described above moved retrogradely from peripheral regions of the dendrites (Fig. 8). The mean velocity of $0.41 \pm 0.18 \mu\text{m/s}$ (observations of labeled vesicles over a total distance of $\sim 800 \mu\text{m}$) is similar to that for axonal transport. From observations of ~ 30 different cells it was apparent that while transport in peripheral regions was always unidirectional, in

the 30- μm segment closest to the cell body some vesicles changed direction (Fig. 8 *D*). This is consistent with the finding that in distal areas microtubules are of uniform polarity but closer to the cell body dendritic microtubules show mixed polarity (Baas et al., 1988). Nevertheless many vesicles ($\sim 80\%$ of those observed) moved without change of direction into the cell body.

As in the axon, small structures were very occasionally

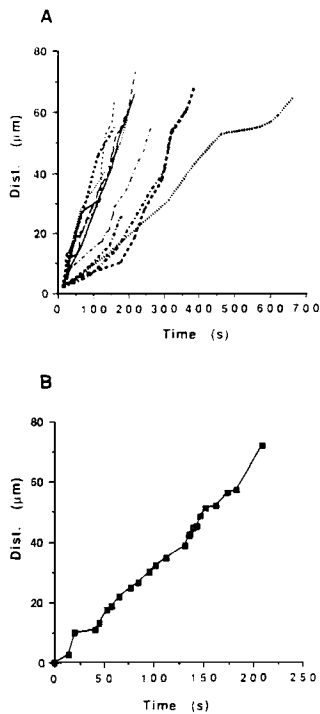


Figure 5. Kinetic analysis of retrograde axonal transport of gold-labeled organelles. Cells were incubated with oval-gold for 30 min at 37°C and then washed. They were then transferred to the warmed stage of the microscope and observed in the bright field mode. Phase-dense vesicles moving into the field were observed and their movement plotted versus time. *A* shows the variation in velocities of labeled vesicles. Note that these differences may represent variations in pause times rather than different velocities during the saltations. In *B*, a more precise analysis of the movement of one vesicle shows periods of fairly constant movement interrupted by brief pauses.

observed moving in either direction within the dendrite but by video microscopy it was difficult to ascertain whether such structures were labeled.

Observations of Late Endocytic Compartments

Our immunofluorescence studies showed that late endocytic compartments, although mainly restricted to the cell body, are also found in the proximal dendritic segment. We investigated the properties of these structures by video microscopy using the conditions described above except that oval-gold was chased for 8 h after a 1-h incubation to label only late compartments (i.e., the same conditions as in Fig. 3, *E* and *F*). Under these conditions phase-dense vesicles, which appeared to be significantly larger than those undergoing retrograde transport, were observed in the cell body and in proximal regions of the dendrites (up to 30 μm from the cell body). These structures, which we assumed to be late endosomes and/or lysosomes, were highly mobile and were frequently observed to move into proximal dendrites (Fig. 9). Periods of inactivity were broken by brief and rapid movements in either direction along the dendrite (~30 cells were observed). All vesicles which could be followed over a relatively long time period (several minutes) showed some bi-directional movement. Late endocytic structures were not observed in axons or in peripheral regions of the dendrites under these conditions.

Discussion

In the present study we have compared endocytosis from the dendritic and axonal domains of cultured neurons. Past studies have largely concentrated on endocytosis from nerve terminals as a response to neurotransmitter release or on retrograde traffic to the cell body. Here we have studied the

cell as a whole in terms of traffic from both domains back to the cell body.

As observed in other neuronal cells there are differences in endocytic activity over the neuronal cell surface (Sinclair et al., 1988). Previous studies have shown that endocytosis along the surface of neurites is very low. Here we show that the axon shaft of mature rat hippocampal neurons is largely devoid of endocytic activity; internalization only occurs at presynaptic nerve terminals and at varicosities (i.e., axonal regions without synaptic contacts but with groups of synaptic vesicles). Polarization of the sites of internalization is a property of mature cells; stage 3 cells, in which the process destined to become an axon is already distinguishable (Dotti et al., 1988), show internalization over the entire plasma membrane surface (results not shown). In contrast to the axonal shaft, the entire dendritic plasma membrane of mature cells showed high endocytic activity. Fluid internalized from the dendritic surface entered an extensive endosomal network which extended to the distal ends of the dendrites. This network is presumably analogous to the reticulum described in Hep2 cells (Hopkins et al., 1990) and to the tubular endosomes observed in a number of different cell lines (Marsh et al., 1986; Van Deurs et al., 1987; Tooze and Hollinshead, 1991). As in the latter study the true extent of the tubules was only appreciated in thick sections. Long tubules of 40–50-nm diameter were visualized by this technique. At least some of these appeared to have attached mvb-like domains. These results using a primary culture show that tubular endosomes are not just a feature of transformed cell lines. In agreement with other studies in nonneuronal cell lines (Tooze and Hollinshead, 1991) the tubular endosomes appear to be an early endosomal compartment; markers chased out of these structures relatively rapidly, and the tubules were labeled by internalized transferrin-HRP but not with antibodies against late endocytic markers. What is the function of this compartment? The major nutritional requirements of the cell (e.g., iron uptake) are likely to be mediated through the somatodendritic surface; the polarized uptake of transferrin shown here and the polarized distribution of the transferrin-receptor (Cameron et al., 1991) are consistent with this. The large surface area of the tubular endosomal compartment may allow efficient lateral segregation of membrane proteins into its different domains such as tubules for recycling proteins and into mvb-like domains for components destined for degradation (see below).

Our observations are in agreement with earlier studies suggesting the presence of endosomal structures in nerve terminals (Heuser and Reese, 1973; Teichberg et al., 1975; Schacher et al., 1976; Stieber et al., 1989; Sulzer and Holtzman, 1989). Structurally the synaptic endosomes resembled those in the dendrites, with tubular and mvb-like domains, but were much less extensive. Results from a number of studies suggest that an endosomal compartment may be involved in the formation of synaptic vesicles (reviewed by Kelly, 1991; Südhof and Jahn, 1991). The nerve terminal early endosome may therefore receive both recycling synaptic vesicle components and membrane proteins destined for degradation and would need sorting functions analogous to those of nonpolarized cells (Geuze et al., 1983). Whether the early endosomes in the nerve terminals are different at the molecular level from those in the somatodendritic domain,

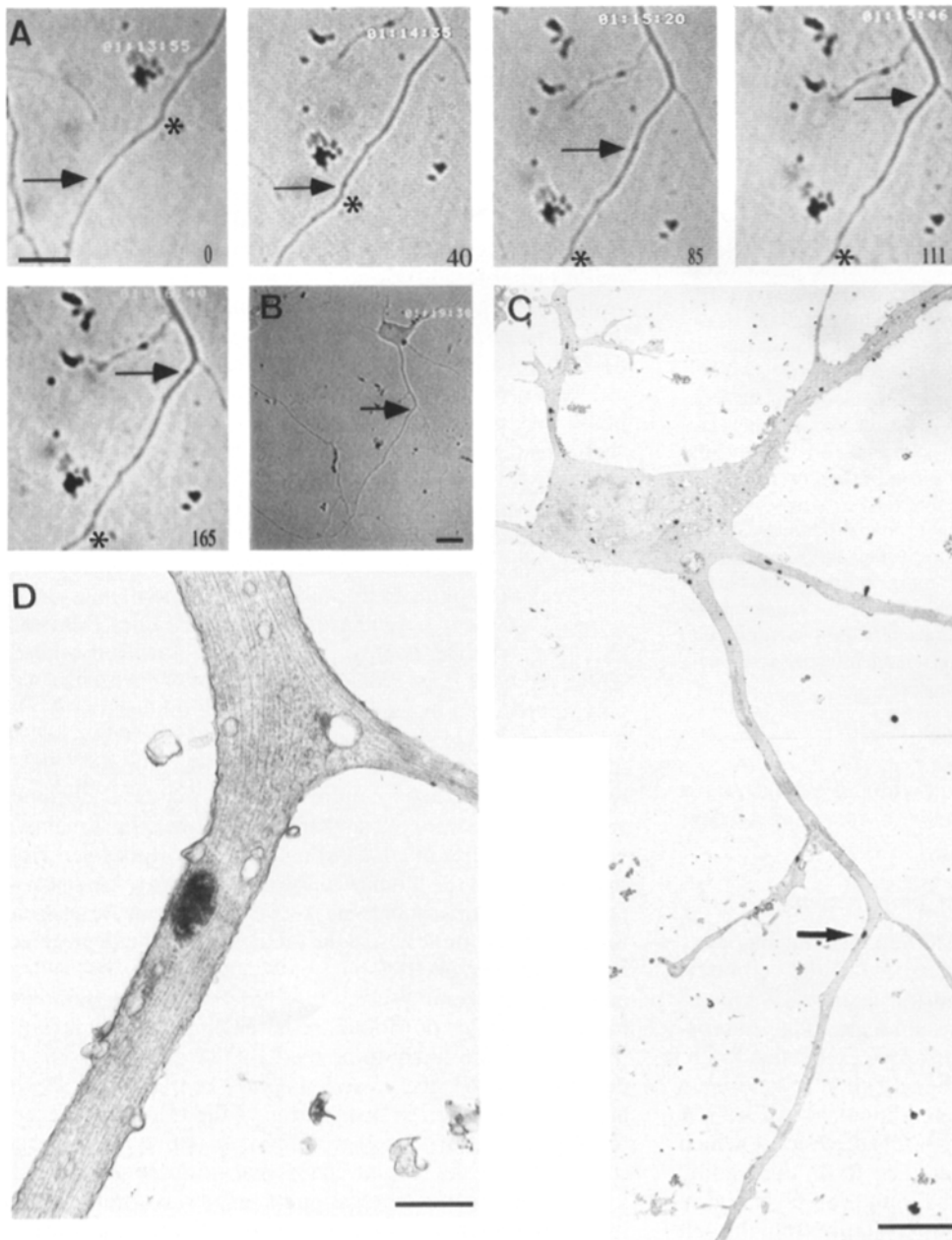


Figure 6. Bright field microscopy to follow retrograde axonal transport of gold-labeled organelles. Cells were incubated with ovalb-gold and high activity HRP for 30 min at 37°C and then washed. They were then transferred to the prewarmed stage of the microscope and observed in the bright field mode. **A** shows a sequence of images from a representative experiment. Images were recorded at the times indicated (in seconds). A phase-dense organelle (*arrows*) moves uniformly in the retrograde direction. For reference a stationary phase-dense structure is indicated (*asterisk*). The cell is shown immediately before (111 s) and after fixation (165 s) and at low power in **B**. The same organelle was relocated after Epon embedding and preparation of semi-thick sections (**C**). The organelle which underwent movement was shown to be a 450-nm-diameter vesicle as shown at higher magnification in **D**. Bars: (**A** and **B**) 10 μm ; (**C**) 5 μm ; (**D**) 0.5 μm .

as suggested in polarized epithelial cells for apical and basolateral early endosomes respectively (Bomsel et al., 1990), awaits further experimentation, for example, to localize early endosome-specific rab proteins (Chavrier et al., 1990; Van der Sluijs et al., 1991).

The degradative endocytic compartments, comprising late endosomes and lysosomes, showed a centralized distribution within the cell body and proximal dendritic segment of mature rat hippocampal neurons. Traffic between the peripheral early endosomal compartments and the cell body must be an important process involved in the turnover and degradation of presynaptic and postsynaptic components. By video microscopy and by axonal lesioning we showed that this traffic is mediated by large mvb-like structures which move retrogradely via fast axonal transport to the cell body. These results are consistent with earlier *in vivo* studies of retro-

gradely transported organelles (La Vail and La Vail, 1974; Tsukita and Ishikawa, 1980). More recent studies showed that mvbs accumulated at the distal side of an axonal lesion *in vivo* and were labeled by antibodies to the microtubule minus end-directed motor dynein (Hirokawa et al., 1990). The similarity in size and morphology of the retrogradely transported organelles to the vesicular domains of endosomes in both the nerve terminals and in the dendrites leads us to speculate that these structures may accumulate membrane components destined for degradation, bud from the early endosomes and then undergo translocation along microtubules towards the cell body. In the dendrites transferrin-HRP, which recycles rather than being targeted to degradative compartments, appeared to be at least partially excluded from these domains and preferentially label the tubules. Our observations strongly suggest that the mechanisms of trans-

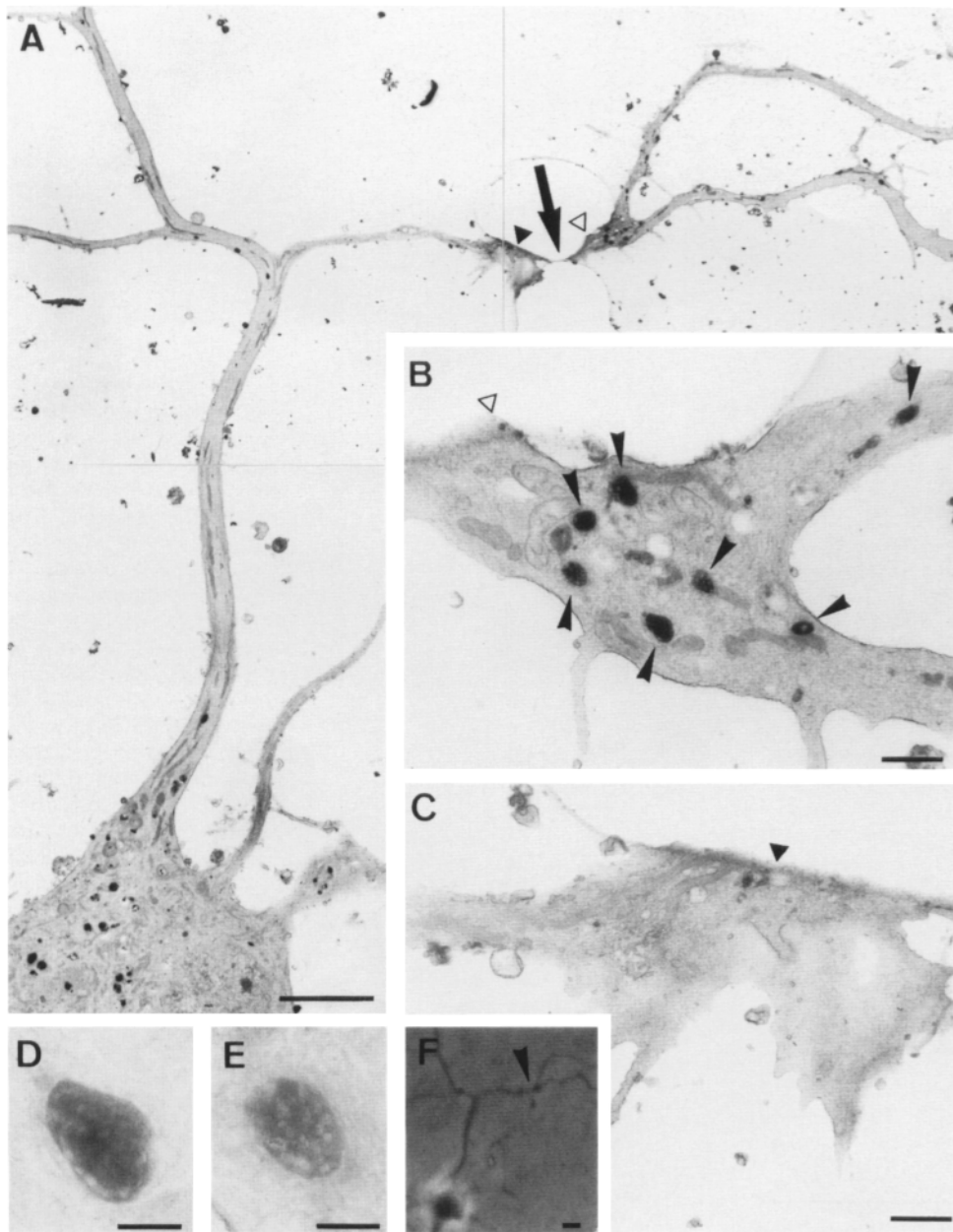


Figure 7. Study of retrogradely transported organelles by axonal lesioning. Cells were incubated with HRP for 30 min at 37°C. They were then washed and transferred to a micromanipulator stage where the axon was lesioned. After a further 20-min incubation at 37°C the cells were fixed, processed for Epon embedding and the same axon was relocated. *A* shows a low magnification semi-thick section of the cell. The corresponding light micrograph is shown in *F*. The lesion is indicated by a large arrow and an arrowhead in *A* and *F*, respectively, and is also recognizable by the mark on the Epon section in *A*. The areas indicated by the triangles in *A* are shown at higher magnification in *B* and *C*. At higher magnification an accumulation of HRP-labeled vesicles is evident on the distal side of the cut (*B*). No such accumulation is evident on the proximal side (*C*). At higher magnification and printed more lightly, the accumulated structures are clearly multivesicular body-like (*D* and *E*). Bars: (*A* and *F*) 10 μm ; (*B* and *C*) 1 μm ; (*D* and *E*) 250 nm.

port between early and late endosomes in the dendrites and axons are similar. This is suggested by the morphology of the early endosomes and putative carrier vesicles in the dendrites and by the characteristics of the retrograde transport between peripheral dendritic regions and the cell body. The results are also consistent with studies in nonneuronal cells. In both fibroblasts and in epithelial cells transport between early and late endosomes was shown to be microtubule-dependent (Gruenberg et al., 1989; Bomsel et al., 1990). After microtubule-depolymerization internalized markers accumulated in mvb-like carrier vesicles which were proposed to mediate the microtubule-dependent transport to late endosomes. Together with these studies, the results presented here in which the vesicles undergoing fast axonal transport were later sectioned, suggest that such structures move along microtubules. However, we cannot completely rule out the involvement of actin filaments in view of recent

studies showing actin-based movement in squid axoplasm (Kuznetsov et al., 1992).

In the axon, the transported structures appeared to have no connections to endosomal tubules even when thick sections were used. Similar structures were observed in the dendrites after internalization of a fluid-phase marker followed by a chase and were not accessible to a subsequent administered pulse of a second marker. It has been proposed that mvb-like structures move within a continuous reticulum in Hep2 cells (Hopkins et al., 1990). While our results do not rule out a similar process in the dendrites of rat hippocampal neurons, they are more compatible with a subsequent budding of the mvb-like carrier vesicle before being transported. An open question is whether these structures then mature into late endosomes through acquisition of lysosomal enzymes or instead fuse with late endosomes.

Although dynein is the most likely candidate for the

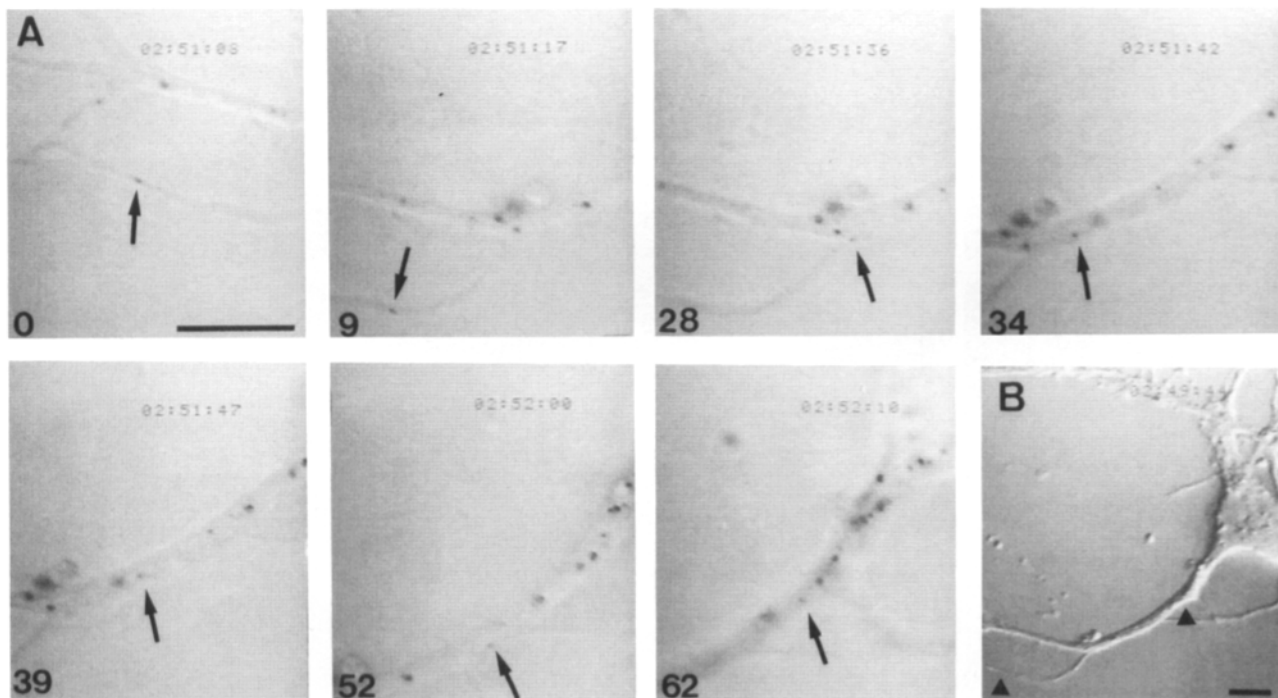


Figure 8. Bright field microscopy to follow dendritic transport of gold-labeled organelles. Cells were incubated with ovalb-gold for 1 h at 37°C. After washing the cells, the movement of labeled organelles within dendrites was followed by bright-field microscopy. The sequence shown in *A* shows a gold-labeled organelle (arrow) moving uniformly towards the cell body. Images were recorded at the times indicated (in seconds). The organelle moves without changes of direction between the points indicated by arrowheads on the Nomarski image of the same cell shown in *B*. *C* shows the distance versus time plots of representative vesicles undergoing dendritic transport. All vesicles shown were followed over at least 50 μm within clearly distinguishable dendrites into the cell body. Note that the kinetics are similar to those for axonally transported vesicles (Fig. 5). Few changes of direction occur. *D* shows a plot of the movement of two vesicles towards the cell body and is representative of the two types of movement observed. One vesicle moves uniformly into the cell body whereas the second shows some bidirectional movement in the 20 μm of the dendrite closest to the cell body. Bars, 10 μm .

microtubule-dependent motor responsible for axonal transport of carrier vesicles towards the cell body (Hirokawa et al., 1990), the mechanism of dendritic transport is less clear. In mature neurons the mid-region of the dendrite (measured 75 μm from the cell body) shows mixed microtubule polarity whereas in peripheral regions all microtubules show the same polarity with the plus ends distal (Baas et al., 1988).

Our observations suggest that transport within the dendrite is unidirectional up to $\sim 20\text{--}30\ \mu\text{m}$ from the cell body. One possible explanation for this finding is that the microtubules of mixed polarity only extend 30 μm into the dendrites of hippocampal neurons under our culture conditions. Alternatively, the microtubules of mixed polarity may extend further into the dendrite but a vesicle may interact with a microtu-

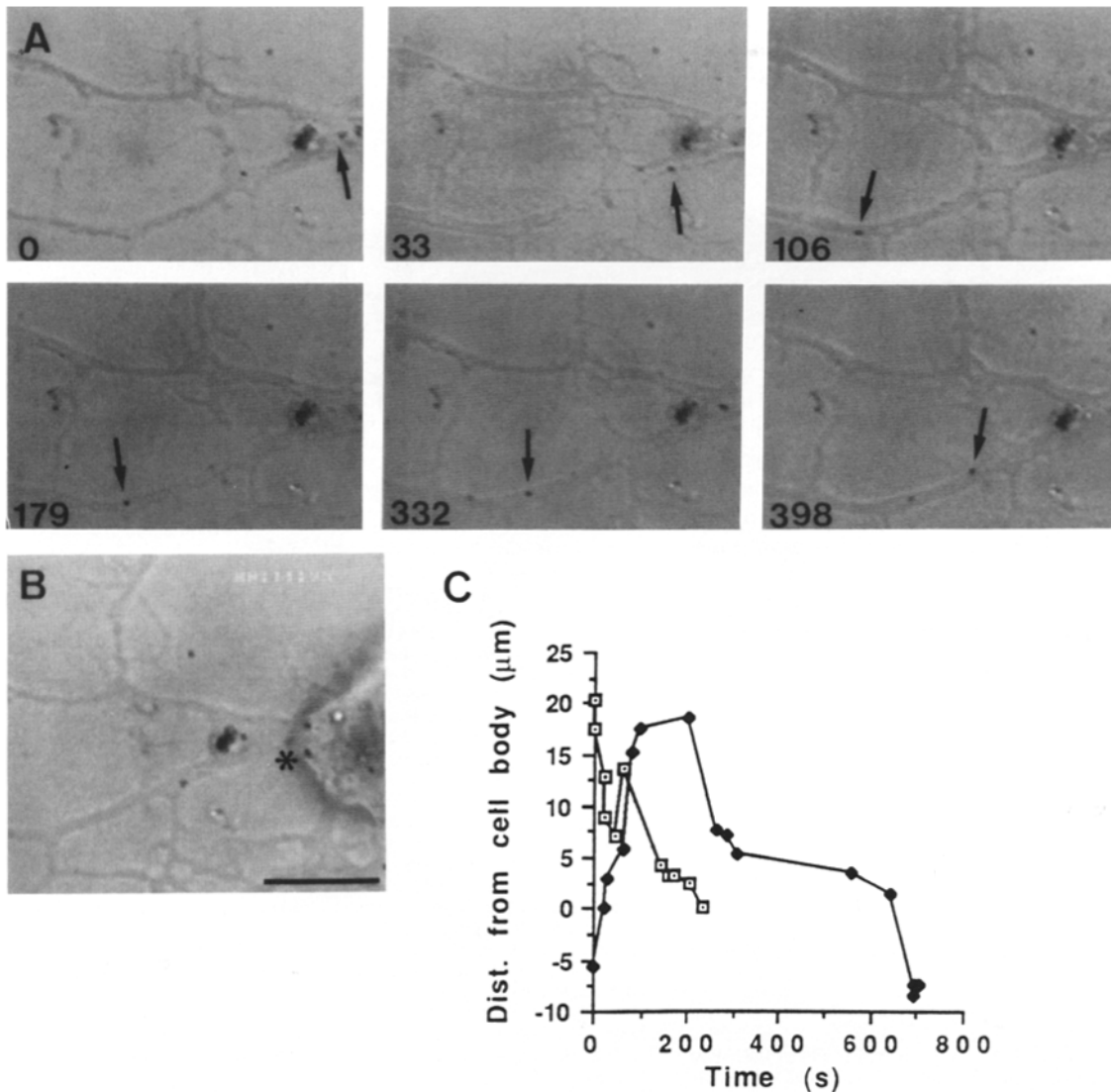


Figure 9. Bright field microscopy to follow movement of late endocytic organelles. Cells were incubated with ovalb-gold for 1 h and then incubated for a further 8 h in tracer-free medium. Movement of gold-labeled organelles was then followed by bright-field microscopy. *A* shows typical movement of a gold-labeled late endocytic structure (arrow). Times are indicated in seconds. The large labeled structure moves from the cell body $\sim 20 \mu\text{m}$ into the dendrite and then returns to the cell body. *B* shows an overview of the same region of the cell. In *C*, the distance versus time plots of two representative vesicles in the same cell are shown. Results are expressed as the distance from the dendritic origin (asterisk in *B*) at different times. Negative values indicate movement past this point into the cell body whereas positive values indicate anterograde movement along the dendrite. Bar, $10 \mu\text{m}$.

bule in the periphery and remain on this track until it reaches the cell body. One population of axonal microtubules was previously shown to be preferred for transport in lobster axons (Miller et al., 1987). Both these possibilities would be consistent with our observations of late endocytic structures which move into the proximal segment of the dendrites where they show bidirectional movement. No movement into more distal segments of the dendrites or into the axon was observed, as would be expected if the late endosomes have only active minus end-directed motors. A third possibility is that some modification of the dendritic microtubules renders them functionally unidirectional. If so, the bidirectional movement of late endosomes within dendrites must involve both plus and minus end-directed movement. Experimental manipulations of cytosolic pH were shown to cause a redistribution of late endosomes towards the distal regions of

axons and dendrites of hippocampal neurons consistent with plus-end directed movement (Parton et al., 1991). In vitro reconstitution experiments should provide insights into the mechanisms involved.

Previous studies have shown similarities between MDCK epithelial cells and hippocampal neurons in sorting on the exocytic pathway (Dotti and Simons, 1990; Dotti et al., 1991). Hippocampal neurons also show similarities to MDCK cells in the organization of their endocytic circuits. In the latter cells there are two distinct early endocytic circuits underlying the apical and basolateral plasma membrane domains (Bomsel et al., 1989; Parton et al., 1989). Transferrin receptors are restricted to the basolateral circuit and interestingly, as shown here and previously (Cameron et al., 1991), are restricted to the somatodendritic plasma membrane domain of cultured neurons. Mixing of internal-

ized contents in MDCK cells occurs first after delivery to late endosomes. Further work is necessary to find out whether the late endocytic compartments in the neuronal cell body and the proximal dendrites receive endocytosed material from both the axon and the dendrites or whether the late endocytic compartments are separated into axonal and dendritic subclasses.

Although our results implicate large carrier vesicles as the major structures involved in the transport of fluid-phase markers to the cell body, other pathways may also exist. Studies of membrane-bound markers such as tetanus toxin and lectins have provided evidence for a role of small vesicles or tubules in retrograde axonal transport (Stoeckel et al., 1977; Schwab and Thoenen, 1978; Schwab et al., 1979). More work will be required to determine whether these membrane-associated proteins are sorted within presynaptic early endosomes into a different class of carriers destined for the Golgi complex or for postsynaptic sites. By analogy to epithelial cells it is possible that neurons also have transcytotic pathways allowing direct communication between presynaptic and postsynaptic endocytic circuits by transport carriers moving in both directions between the axon and the dendrites.

We are particularly grateful to Liane Meyn for preparation of primary neuronal cultures and to Bernard Hoflack and Ulrike Bauer for preparing the transferrin-HRP. We would also like to thank Ernst Stelzer and Jan de Mey for their help with the video microscopy, Rainer Pepperkok for his advice regarding the use of the microinjection system, Ira Mellman, Marino Zerial, and Bernard Hoflack for antibodies, and Gareth Griffiths, Jean Gruenberg, Leo Thomas, John Tooze, and Marino Zerial for helpful discussions and comments on the manuscript.

Received for publication 27 April 1992 and in revised form 2 July 1992.

References

- Allen, R. D., J. Metzuzal, I. Tasaki, S. T. Brady, and S. P. Gilbert. 1982. Fast axonal transport in squid axon. *Science (Wash. DC)*. 218:1127-1128.
- Anson, W., and R. Pepperkok. 1988. Performance of an automated system for capillary microinjection into living cells. *J. Biochem. Biophys. Methods*. 16:283-292.
- Baas, P. W., J. S. Deitch, M. M. Black, and G. A. Banker. 1988. Polarity orientation of microtubules in hippocampal neurons: uniformity in the axon and nonuniformity in the dendrite. *Proc. Natl. Acad. Sci. USA*. 85:8335-8339.
- Bartlett, W. P., and G. A. Banker. 1984. An electron microscopic study of the development of axons and dendrites by hippocampal neurons in culture. I. Cells which develop without intercellular contacts. *J. Neurosci.* 4:1944-1953.
- Beaumelle, B. D., A. Gibson, and C. R. Hopkins. 1990. Isolation and preliminary characterization of the major membrane boundaries of the endocytic pathway in lymphocytes. *J. Cell Biol.* 111:1811-1823.
- Bomsel, M., K. Prydz, R. G. Parton, J. Gruenberg, and K. Simons. 1989. Endocytosis in filter-grown Madin-Darby canine kidney cells. *J. Cell Biol.* 109:3243-3258.
- Bomsel, M., R. G. Parton, S. A. Kuznetsov, T. A. Schroer, and J. Gruenberg. 1990. Microtubule- and motor-dependent fusion in vitro between apical and basolateral endocytic vesicles from MDCK cells. *Cell*. 62:719-731.
- Breuer, A. C., C. N. Christian, M. Henkart, and P. G. Nelson. 1975. Computer analysis of organelle translocation in primary neuronal cultures and continuous cell lines. *J. Cell Biol.* 65:562-576.
- Cameron, P. L., T. C. Südhof, R. Jahn, and P. de Camilli. 1991. Colocalization of synaptophysin with transferrin receptors: implications for receptor biogenesis. *J. Cell Biol.* 115:151-164.
- Chavrier, P., R. G. Parton, H. P. Hauri, K. Simons, and M. Zerial. 1990. Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments. *Cell*. 62:317-329.
- Dotti, C. G., C. A. Sullivan, and G. A. Banker. 1988. The establishment of polarity by hippocampal neurons in culture. *J. Neurosci.* 8:1454-1468.
- Dotti, C. G., R. G. Parton, and K. Simons. 1991. Polarized sorting of glypiated proteins in hippocampal neurons. *Nature (Lond.)*. 349:158-161.
- Dotti, C. G., and K. Simons. 1990. Polarized sorting of viral glycoproteins to the axon and dendrites of hippocampal neurons in culture. *Cell*. 62:63-72.
- Geuze, H. J., J. W. Slot, and G. J. A. M. Strous. 1983. Intracellular site of asialoglycoprotein receptor-ligand uncoupling: double-label immunoelectron microscopy during receptor-mediated endocytosis. *Cell*. 32:277-287.
- Geuze, H. J., W. Stoorvogel, G. J. Strous, J. W. Slot, J. E. Bleekemolen, and I. Mellman. 1988. Sorting of mannose-6-phosphate receptors and lysosomal membrane proteins in endocytic vesicles. *J. Cell Biol.* 107:2491-2501.
- Goslin, K., and G. Banker. 1991. Rat Hippocampal Neurons in Low-density Culture. In *Culturing Nerve Cells*. G. Banker and K. Goslin, editors. The MIT Press, Cambridge, MA. 251-281.
- Grafstein, B., and D. S. Forman. 1980. Intracellular transport in neurons. *Physiol. Rev.* 60:1167-1283.
- Griffiths, G., and J. Gruenberg. 1991. Arguments for pre-existing early and late endosomes. *Trends Cell Biol.* 1:5-9.
- Griffiths, G., B. Hoflack, K. Simons, I. Mellman, and S. Kornfeld. 1988. The mannose-6-phosphate receptor and the biogenesis of lysosomes. *Cell*. 52:329-341.
- Griffiths, G., R. Matteoni, R. Back, and B. Hoflack. 1990. Characterization of the cation-independent mannose-6-phosphate receptor-enriched prelysosomal compartment. *J. Cell Sci.* 95:441-461.
- Gruenberg, J., G. Griffiths, and K. E. Howell. 1989. Characterization of the early endosome and putative endocytic carrier vesicles in vivo and with an assay of vesicle function in vitro. *J. Cell Biol.* 108:1301-1316.
- Heuser, J. 1989a. Changes in lysosome shape and distribution correlated with changes in cytoplasmic pH. *J. Cell Biol.* 108:855-864.
- Heuser, J. E. 1989b. The role of coated vesicles in recycling of synaptic vesicle membrane. *Cell Biol. Int. Rep.* 13:1063-1076.
- Heuser, J. E., and T. S. Reese. 1973. Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. *J. Cell Biol.* 57:315-344.
- Hirokawa, N., Y. Yoshida, and R. Sato-Yoshitake. 1990. Brain dynein (MAP1C) localizes on both anterogradely and retrogradely transported organelles in vivo. *J. Cell Biol.* 111:1027-1037.
- Holtzman, E. 1989. Lysosomes. P. Siekevitz, editor. Plenum, New York. 439.
- Hopkins, C. R. 1983. Intracellular routing of transferrin and transferrin receptors in epidermoid carcinoma A431 cells. *Cell*. 35:321-330.
- Hopkins, C. R., A. Gibson, M. Shipman, and K. Miller. 1990. Movement of internalized ligand-receptor complexes along a continuous endosomal reticulum. *Nature (Lond.)*. 346:335-339.
- Hubbard, A. L. 1989. Endocytosis. *Curr. Opin. Cell Biol.* 1:675-683.
- Kelly, R. B. 1991. Secretory granule and synaptic vesicle formation. *Curr. Opin. Cell Biol.* 3:654-660.
- Kielian, M. C., M. Marsh, and A. Helenius. 1986. Kinetics of endosome acidification detected by mutant and wild-type Semliki Forest virus. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:3103-3109.
- Kuznetsov, S. A., G. M. Langford, and D. G. Weiss. Actin-dependent organelle movement in squid axoplasm. *Nature (Lond.)*. 356:722-725.
- La Vail, M., and J. La Vail. 1974. Organelles involved in retrograde axonal transport in chick ganglion cells. *J. Comp. Neurol.* 157:303-358.
- Lewis, V., S. A. Green, M. Marsh, P. Vihko, A. Helenius, and I. Mellman. 1985. Glycoproteins of the lysosomal membrane. *J. Cell Biol.* 100:1839-1847.
- Lippincott-Schwartz, J., L. Yuan, C. Tipper, M. Amherdt, L. Orci, and R. D. Klausner. 1991. Brefeldin A's effects on endosomes, lysosomes, and the TGN suggest a general mechanism for regulating organelle structure and membrane traffic. *Cell*. 67:601-616.
- Marsh, M., G. Griffiths, G. E. Dean, I. Mellman, and A. Helenius. 1986. Three-dimensional structure of endosomes in BHK-21 cells. *Proc. Natl. Acad. Sci. USA*. 83:2899-2903.
- Miller, R. H., R. J. Lasek, and M. J. Katz. 1987. Preferred microtubules for vesicle transport in lobster axons. *Science (Lond.)*. 235:220-222.
- Murphy, R. F. 1991. Maturation models for endosome and lysosome biogenesis. *Trends Cell Biol.* 1:77-82.
- Parton, R. G., K. Prydz, M. Bomsel, K. Simons, and G. Griffiths. 1989. Meeting of the apical and basolateral endocytic pathways of the Madin-Darby canine kidney cell in late endosomes. *J. Cell Biol.* 109:3259-3272.
- Parton, R. G., C. G. Dotti, R. Bacallao, I. Kurtz, K. Simons, and K. Prydz. 1991. pH-induced microtubule-dependent redistribution of late endosomes in neuronal and epithelial cells. *J. Cell Biol.* 113:261-274.
- Rodman, J. S., R. W. Mercer, and P. D. Stahl. 1990. Endocytosis and transcytosis. *Curr. Opin. Cell Biol.* 2:664-672.
- Rosa, P., A. Hille, R. W. H. Lee, A. Zanini, P. de Camilli, and W. B. Huttner. 1985. Secretogranins I and II: two tyrosine-sulfated secretory proteins common to a variety of cells secreting peptides by the regulated pathway. *J. Cell Biol.* 101:1999-2011.
- Schacher, S., E. Holtzman, and D. C. Hood. 1976. Synaptic activity of frog retinal photoreceptors; a peroxidase uptake study. *J. Cell Biol.* 70:178-192.
- Schmid, S. L., R. Fuchs, P. Male, and I. Mellman. 1988. Two distinct subpopulations of endosomes involved in membrane recycling and transport to lysosomes. *Cell*. 52:73-83.
- Schwab, M. E., and H. Thoenen. 1978. Selective binding, uptake and retrograde transport of tetanus toxin by nerve terminals in the rat iris. *J. Cell Biol.* 77:1-13.
- Schwab, M. E., K. Suda, and H. Thoenen. 1979. Selective retrograde transsynaptic transfer of a protein, tetanus toxin, subsequent to its retrograde transport. *J. Cell Biol.* 82:798-810.
- Sinclair, G. I., P. W. Baas, and S. R. Heidemann. 1988. Role of microtubules

- in the cytoplasmic compartmentation of neurons. II. Endocytosis in the growth cone and neurite shaft. *Brain Res.* 450:60-68.
- Slot, J. W., and H. J. Geuze. 1985. A novel method to make gold probes for multiple labelling cytochemistry. *Eur. J. Cell Biol.* 38:87-93.
- Stieber, A., S. D. Erulkar, and N. K. Gonatas. 1989. A hypothesis for the superior sensitivity of wheat germ agglutinin as a neuroanatomical probe. *Brain Res.* 495:131-139.
- Stoeckel, K., M. Schwab, and H. Thoenen. 1977. Role of gangliosides in the uptake and retrograde axonal transport of cholera and tetanus toxin as compared to nerve growth factor and wheat germ agglutinin. *Brain Res.* 132:273-285.
- Sulzer, D., and E. Holtzman. 1989. Acidification and endosome-like compartments in the presynaptic terminals of frog retinal photoreceptors. *J. Neurocytol.* 18:529-540.
- Südhof, T. C., and R. Jahn. 1991. Proteins of synaptic vesicles involved in exocytosis and membrane recycling. *Neuron.* 6:665-677.
- Teichberg, S., E. Holtzman, S. M. Crain, and E. R. Peterson. 1975. Circulation and turnover of synaptic vesicle membrane in cultured fetal mammalian spinal cord neurons. *J. Cell Biol.* 67:215-230.
- Tooze, J., and M. Hollinshead. 1991. Tubular endosomes in AtT20 and other cells. *J. Cell Biol.* 115:635-654.
- Tsukita, S., and H. Ishikawa. 1980. The movement of membranous organelles in axons. *J. Cell Biol.* 84:513-530.
- Van der Sluijs, P., M. Hull, A. Zahraoui, A. Tavitian, B. Goud, and I. Mellman. 1991. The small GTP-binding protein rab4 is associated with early endosomes. *Proc. Natl. Acad. Sci. USA.* 88:6313-6317.
- Van Deurs, B., O. W. Petersen, S. Olsnes, and K. Sandvig. 1987. Delivery of internalized ricin from endosomes to cisternal Golgi elements is a discontinuous, temperature-sensitive process required for optimal intoxication. *Exp. Cell Res.* 171:137-152.
- Wood, S. A., J. E. Park, and W. J. Brown. 1991. Brefeldin A causes a microtubule-mediated fusion of the trans-Golgi network and early endosomes. *Cell.* 67:591-600.
- Yamashiro, D. J., B. Tycko, S. R. Fluss, and F. R. Maxfield. 1984. Segregation of transferrin to mildly acidic (pH 6.5) para-Golgi compartment in the recycling pathway. *Cell.* 37:789-800.