

# Trafficking of Cell Surface $\beta$ -Amyloid Precursor Protein: Retrograde and Transcytotic Transport in Cultured Neurons

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**Abstract.** Amyloid  $\beta$ -protein ( $A\beta$ ), the principal constituent of senile plaques seen in Alzheimer's disease (AD), is derived by proteolysis from the  $\beta$ -amyloid precursor protein ( $\beta$ PP). The mechanism of  $A\beta$  production in neurons, which are hypothesized to be a rich source of  $A\beta$  in brain, remains to be defined. In this study, we describe a detailed localization of cell surface  $\beta$ PP and its subsequent trafficking in primary cultured neurons. Full-length cell surface  $\beta$ PP was present primarily on perikarya and axons, the latter with a characteristic discontinuous pattern. At growth cones, cell surface  $\beta$ PP was inconsistently detected. By visualizing the distribution of  $\beta$ PP monoclonal antibodies added to intact cultures,  $\beta$ PP was shown to be internalized from distal axons or terminals and retrogradely transported back to perikarya in organelles which colocalized with fluid-phase endocytic

markers. Retrograde transport of  $\beta$ PP was shown in both hippocampal and peripheral sympathetic neurons, the latter using a compartment culture system that isolated cell bodies from distal axons and terminals. In addition, we demonstrated that  $\beta$ PP from distal axons was transcytotically transported to the surface of perikarya from distal axons in sympathetic neurons. Indirect evidence of this transcytotic pathway was obtained in hippocampal neurons using antisense oligonucleotide to the kinesin heavy chain to inhibit anterograde  $\beta$ PP transport. Taken together, these results demonstrate novel aspects of  $\beta$ PP trafficking in neurons, including retrograde axonal transport and transcytosis. Moreover, the axonal predominance of cell surface  $\beta$ PP is unexpected in view of the recent report of polarized sorting of  $\beta$ PP to the basolateral domain of MDCK cells.

**A**LZHEIMER'S disease (AD)<sup>1</sup> is a progressive, neurodegenerative disorder characterized by the extracellular deposition of amyloid protein in the brain parenchyma and in the walls of meningeal and cortical blood vessels (38). These amyloid deposits are composed principally of the 39–43-amino acid residue amyloid  $\beta$ -protein ( $A\beta$ ) (13).  $A\beta$  is derived from proteolytic cleavages of the  $\beta$ -amyloid precursor protein ( $\beta$ PP) (24), a ubiquitously expressed membrane-spanning glycoprotein (46). Several  $\beta$ PP isoforms are produced by alternative splicing although one isoform, designated  $\beta$ PP695, is predominantly expressed by neurons (42). Cleavage of  $\beta$ PP by an endoprotease designated " $\alpha$ -secretase" results in the secretion of soluble  $\beta$ -amy-

loid precursor protein ( $\beta$ PPs), a  $\sim$ 100-kD soluble  $NH_2$ -terminal fragment of  $\beta$ PP (46), and membrane retention of a 10-kD  $COOH$ -terminal fragment (39). The  $\alpha$ -secretase cleavage occurs within the  $A\beta$  region and therefore precludes formation of an intact  $A\beta$  peptide (10, 41). Although present in all cell types studied to date, the  $\alpha$ -secretase pathway may be used to only a limited extent in neurons (16, 21).

In addition to the secretory pathway,  $\beta$ PP is also processed and degraded within an endosomal/lysosomal pathway. Full-length  $\beta$ PP can be internalized from the cell surface and targeted to endosomes and lysosomes, where  $COOH$ -terminal fragments containing the entire  $A\beta$  sequence have been detected (14, 17). Similar fragments have also been identified in human brain and vasculature (11, 45). Whether these  $COOH$ -terminal fragments are the precursors to  $A\beta$  has not been determined (11, 14).

Recently,  $A\beta$  has been shown to be a product of normal cellular metabolism (11, 19, 40), in that  $A\beta$  and a variety of closely related low molecular weight peptides (19) are constitutively produced and released into the medium of cultured cells. The pathway resulting in  $A\beta$  production and release has not been defined. One recent study suggested that

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1. *Abbreviations used in this paper:*  $A\beta$ , amyloid  $\beta$ -protein; AD, Alzheimer's disease; BFA, brefeldin A;  $\beta$ PP,  $\beta$ -amyloid precursor protein;  $\beta$ PPs, soluble  $\beta$ -amyloid precursor protein; GAP-43, growth-associated protein 43; MAP2, microtubule-associated protein 2; NCAM, neuronal cell adhesion molecule.

the receptor-mediated endocytic pathway may be important in the constitutive production and release of A $\beta$  in cultured CHO cells (28). In these cells, full-length  $\beta$ PP is recycled after internalization, a step which may contribute to A $\beta$  release. In contrast to nonneuronal cells, intracellular A $\beta$  is readily detected in cultured neuronal cells and appears before its release in medium (47). How the mechanism of A $\beta$  production is different between neuronal and nonneuronal cells is unknown.

In MDCK cells, a model system for polarized epithelial cells, sorting and secretion of  $\beta$ PPs are highly polarized (18). In these cells, 90% of  $\beta$ PPs, A $\beta$ , and 3 kD COOH-terminal peptide of A $\beta$  (p3) are released into the basolateral compartment (18). This polarized secretion of  $\beta$ PP products is associated with a 90% preferential localization of full-length  $\beta$ PP on the basolateral membrane surface. Because neurons are highly polarized cells, an analogy has been suggested between the apical surface of MDCK cells and the axons of cultured hippocampal neurons (8). However, the assessment of membrane distribution in many of the studies was made by analyzing viral glycoproteins after infection (23). Thus, the analysis of  $\beta$ PP provides an opportunity whereby the membrane distribution of an endogenous protein can be determined in neurons.

Localization of  $\beta$ PP in cultured rat hippocampal neurons and PC12 cells has recently been reported (5, 12). However, these studies described only the intracellular distribution of  $\beta$ PP. In addition, because only COOH-terminal antibodies were used, whether  $\beta$ PP was present as a full-length or truncated molecule was not determined. Therefore, the trafficking of cell surface  $\beta$ PP in neurons, an issue that may be central to cerebral A $\beta$  production, has not been addressed. In this report, we describe the localization and trafficking pathways of cell surface full-length  $\beta$ PP in cultured rat hippocampal and sympathetic neurons. In addition, using a compartment culture system, we show that  $\beta$ PP is internalized and retrogradely transported to the perikaryon from distal axonal membrane sites. Finally, we present evidence showing the transcytosis of cell surface  $\beta$ PP from axonal to somatic compartments in cultured neurons.

## Materials and Methods

### Cultures

**Rat Hippocampal Neurons.** Hippocampal cultures were prepared from embryonic day 18 rats as previously described (2). In brief, cells from the dissected hippocampi were dissociated by trypsin (0.25% for 15 min at 37°C) followed by trituration with fire-polished Pasteur pipettes. The cells were plated at a density of 100,000 cells/60-mm culture dish on glass coverslips coated with poly-L-lysine (1 mg/ml) in MEM with 10% horse serum. After 2–4 h, the medium was changed to 1 ml of MEM with N2 supplements, ovalbumin (0.1%), and pyruvate (0.01 mg/ml) that had been conditioned in cultures of astroglial cells for 24 h. Coverslips plated with neurons were cocultured with astroglia.

**Compartment Culture.** Dissociated sympathetic neurons from superior cervical ganglia of newborn rats were cultured in the center chamber of a three-compartment culture system, as described (4). Briefly, collagen-coated glass coverslips were scored with 18 parallel scratches to remove dried collagen. In this way, neurites advanced along the collagen channels bounded by the scratches. A Teflon divider similar to the original model (Tyler Research Instruments, Edmonton, Alberta) was seated on the coverslips to create three compartments that were isolated from each other by silicone grease and oriented so that the scratches connected all three compartments. The divider was placed on prewetted coverslips to prevent the

silicone grease from adhering to the collagen. The resultant system was such that there was no diffusion of medium between compartments but penetrable to neurites advancing along the collagen channels beneath the silicone grease dam. Dissociated neurons were plated into the center compartment at  $\sim$ 2 ganglia per dish. Neurons were cultured in serum free media (20) and treated with 10  $\mu$ M cytosine arabinoside at the time of plating to eliminate nonneuronal cells. In control experiments using iodinated monoclonal  $\beta$ PP antibody and [ $^3$ H]Inulin, less than 1% of the radiolabel diffused into the adjacent chamber after 6 h.

### Antibodies

The monoclonal antibodies 5A3 and IG7 (28) and goat polyclonal antibody 207 (gift from Dr. B. Greenberg) (40) made against  $\beta$ PPs from transfected CHO or baculovirus-infected cells, respectively, were used in the studies. 5A3 and IG7 recognize nonoverlapping epitopes in the mid-region of  $\beta$ PP and these two monoclonal antibodies were used together to obtain higher signal (28). Monovalent Fab fragments of 5A3 and IG7 were obtained by papain digestion and purified by protein A-Sepharose chromatography to remove Fc fragments and undigested IgGs. The monoclonal antibody 13G8 (gift from Dr. D. Schenk) and polyclonal antibody C7 (33) were raised against the carboxy terminus of  $\beta$ PP. At the immunocytochemical level, these anti- $\beta$ PP antibodies, except for C7, did not recognize amyloid precursor like protein 2 (APLP2) expressed in transfected CHO cells (data not shown)(43). Polyclonal antibodies 1963 (19) and  $\alpha$ 1 (gift from Dr. Y. Ihara) were raised against 21–37 and 17–28 of A $\beta$  sequence, respectively. Additional monoclonal antibodies included transferrin receptor (gift from Dr. I. Trowbridge), GAP-43, synaptophysin (Boehringer Mannheim, Indianapolis, IN), MAP2 (Sigma Chem. Co., St. Louis, MO), and kinesin heavy chain (Sigma), and polyclonal antibodies to  $\beta$ -tubulin (Sigma) and neuronal cell adhesion molecule (NCAM) (Chemicon, Temecula, CA).

### Immunocytochemistry

Cultured cells were fixed for 20 min with warm 4% formaldehyde in PBS containing 0.12 M sucrose. As necessary, cultures were treated with 0.3% Triton X-100 for 5 min at room temperature after fixation to permeabilize the cell membranes. Without detergent permeabilization, intracellular structures, such as tubulin, were not immunoreactive, demonstrating that only surface  $\beta$ PP was visualized in untreated cells. After blocking in 10% BSA for 1 h at 37°C, the fixed cultures were exposed to primary antibodies overnight at 4°C. After several PBS washes, the cells were incubated for 1 h with rhodamine or FITC-conjugated secondary antibodies (Jackson Immunoresearch Labs, West Grove, PA).

In some experiments, double-labeling immunocytochemistry was carried out with two monoclonal antibodies. In this case, the cells were first labeled with one monoclonal antibody, washed extensively, and incubated with an excess of Fab fragments of goat anti-mouse IgG (1:10) after manufacturer's instructions (Jackson Immunoresearch) so that all the primary antibodies (usually anti- $\beta$ PP monoclonal antibodies) were sterically covered by goat Fab fragments. Subsequently, the cells were incubated with the second monoclonal antibody, followed by anti-goat and anti-mouse secondary antibodies to visualize the two different primary antibodies. Two control experiments confirmed the effectiveness of this approach. First, a control FITC-conjugated anti-mouse antibody was not immunoreactive in the presence of excess Fab fragments of goat anti-mouse antibody, the latter used to sterically mask the primary mouse monoclonal antibody. Second, the staining pattern of the double-labeled cultures is identical to immunostaining with each of the respective monoclonal antibodies alone.

### Internalization Study in Rat Hippocampal Neurons

3- and 10-d-old rat hippocampal cultures were incubated with  $\beta$ PP monoclonal antibodies for 30 min at 37°C together with ovalbumin or dextran conjugated to Texas red (1 mg/ml) (Molecular Probes, Eugene, OR). In some experiments, cultures were incubated with whole IgG or monovalent Fab fragments of  $\beta$ PP antibodies for 1 h at 4°C, washed extensively, and then incubated at 37°C to exclude fluid phase internalization. After fixation, cells were permeabilized and incubated with FITC-conjugated secondary antibody to visualize the  $\beta$ PP monoclonal antibodies. Control studies included the addition of nonimmune mouse IgG or Fab fragments added under identical incubation conditions and at the same concentration as  $\beta$ PP antibodies.

## Internalization Study in Compartment Culture System

After the sympathetic neurons have extended their neurites into the lateral chambers (usually 4–6 d after plating), monoclonal antibodies (5A3/IG7) were added only to lateral compartments and incubated for 1 h at 37°C. After fixation with or without permeabilization, the chambers were removed and cells were then incubated with FITC-conjugated secondary antibodies for visualization of primary antibodies. Control studies included the addition of either nonimmune mouse IgG at identical concentrations in the lateral chamber or  $\beta$ PP antibodies in the central chamber.

## Antisense Oligonucleotides

Kinesin heavy chain was inhibited by the addition of antisense oligonucleotides as described (12). The 16-mer antisense phosphorothioate modified oligonucleotide used in the study consisted of the sequence GGTCGCCA-TCTTCT, complementary to bases -6 to +10 of the rat kinesin cDNA sequence (12). A 16-mer sense oligonucleotide corresponding to the bases at -6 to +10 was used as control. Three days after plating of hippocampal neurons, either oligonucleotide was added in serum free medium at 50  $\mu$ M, followed by 25  $\mu$ M 12 h later. After 36 h, the cells were fixed and cell surface  $\beta$ PP detected with the monoclonal antibodies 5A3 and IG7 as described above. In addition, uptake of Texas red-conjugated human transferrin (20  $\mu$ g/ml) (Molecular Probes) was determined in cultures treated with oligonucleotides. Finally, washout experiments were performed in some cultures for 48 h after withdrawal of oligonucleotides.

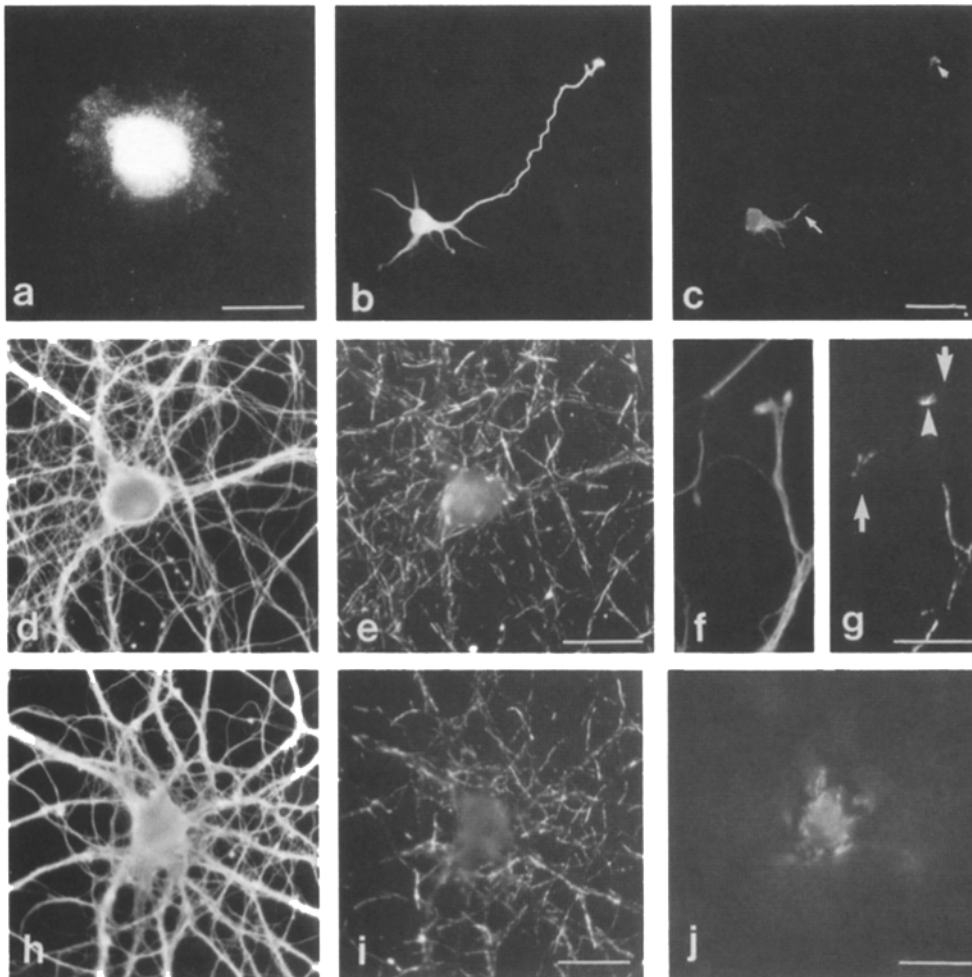
## Cell Surface Biotinylation

Surface biotinylation was carried out on  $1 \times 10^6$  (5-d-old) hippocampal neurons with hydroxysulfosuccinimide-biotin (Pierce, Rockford, IL). The reagent was dissolved in PBS (pH 8) at 1 mg/ml and added to the cultures at 4°C. After thorough washing with PBS supplemented with 50 mM glycine, the cells were lysed with 1% NP-40. Immunoprecipitation was carried out with C7 polyclonal antibody recognizing the COOH terminus of  $\beta$ PP. The immunoprecipitated biotinylated  $\beta$ PP was fractionated on SDS-PAGE, transferred to nitrocellulose, incubated with an anti-biotin monoclonal antibody (Jackson ImmunoResearch), and then detected with  $^{125}$ I-labeled goat anti-mouse antibody. After the autoradiogram was exposed and developed, the blot was incubated with C7 antibody followed by detection with ECL (Amersham Corp., Arlington Heights, IL) to visualize all  $\beta$ PP species. As a control, the immunoblotting step was also carried out with CHO cells stably transfected with  $\beta$ PP695.

## Results

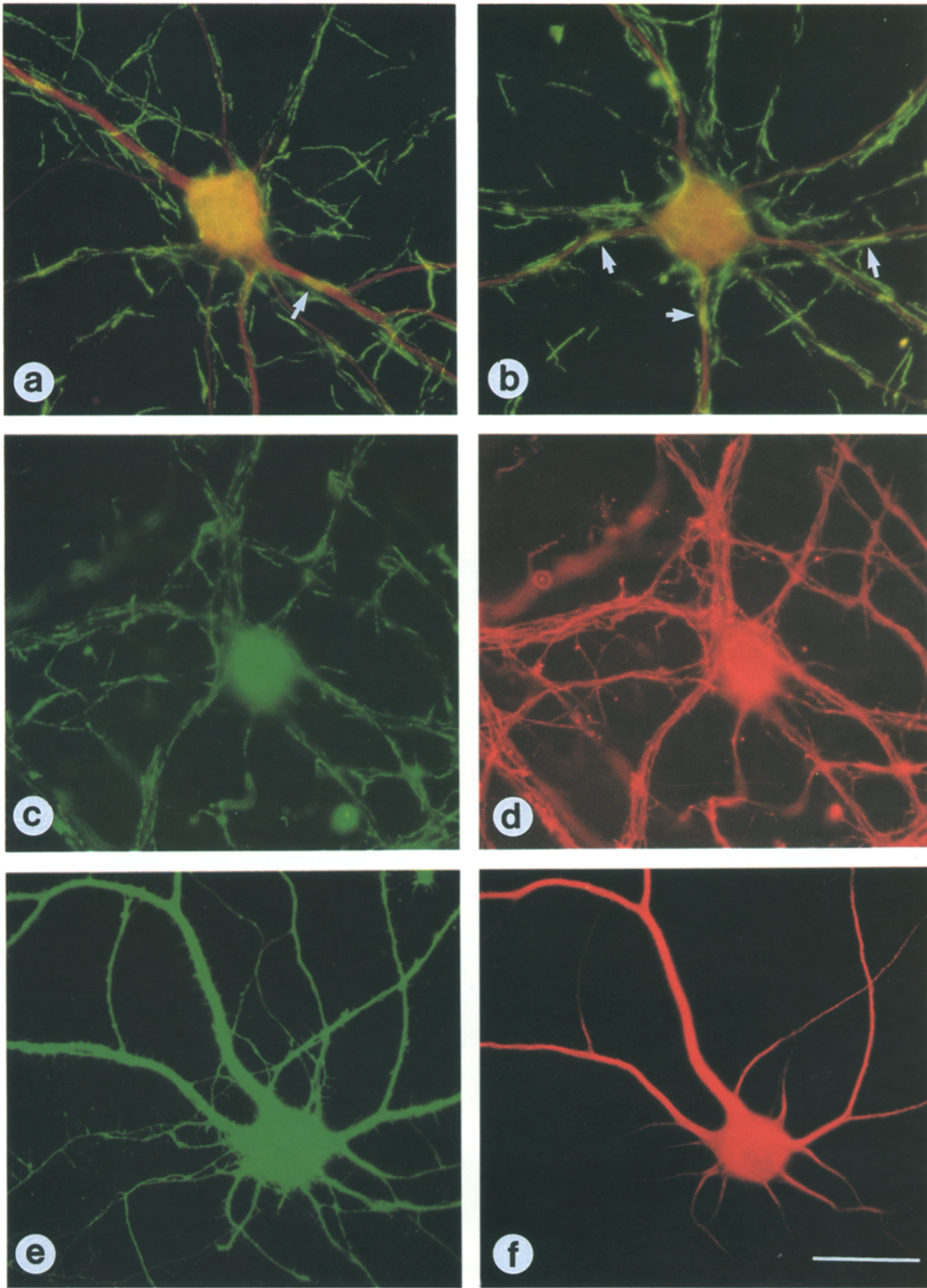
### Cell Surface $\beta$ PP Localization during Maturation of Cultured Hippocampal Neurons

Shortly after plating, hippocampal neurons extended a lamellipodial veil consisting of multiple short processes around the cell body (stage 1) (9). At this stage, immuno-



**Figure 1.** Cell surface expression of  $\beta$ PP in cultured hippocampal neurons of varying ages. At stage 1 shortly after plating (a),  $\beta$ PP was seen on the surface of perikaryon and lamellipodial veil using 5A3/IG7 antibodies. Paired double-labeling images using tubulin antibody (b, d, f, and h) and 5A3/IG7  $\beta$ PP antibodies (c, e, g, and i) were used to demonstrate the cellular localization of  $\beta$ PP from immature to mature neurons in culture. At stage 3 (c), a fine granular staining on the surface of soma, minor neurites, proximal portion of an axon (arrow), and a growth cone (arrowhead) was seen. A neuron cultured for 14 d (e) showed a characteristic discontinuous and patchy distribution of  $\beta$ PP on the surface of neurites. Higher magnification of stage 5 neurons seen in e is illustrated in g. In this view, one growth cone was immunoreactive to  $\beta$ PP monoclonal antibodies (arrowhead) but two other growth cones were negative (arrows). The patchy staining pattern of  $\beta$ PP on neurites remained after 28 d of culture (i). Perikaryal  $\beta$ PP is illustrated at higher magnification in j. However, this mi-

crograph is not truly representative because much of the surface of the cell body as well as the neurites were not within the focal plain of the photograph. Bars: (a and g) 10  $\mu$ m; (c, e, i, and j) 20  $\mu$ m.



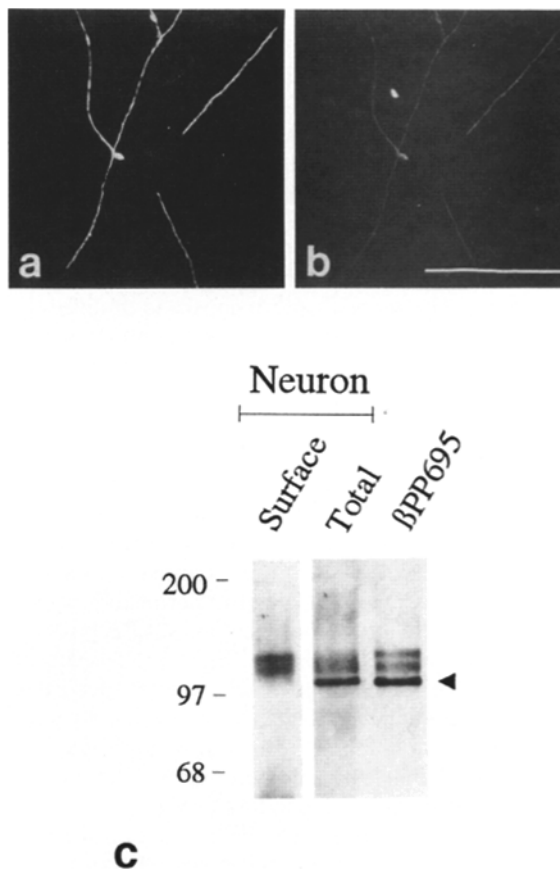
**Figure 2.** Polarized distribution of cell surface  $\beta$ PP in mature neurons. Hippocampal neurons cultured for 10–14 d (stage 5) were double labeled with 5A3/1G7  $\beta$ PP (FITC) and MAP2 (rhodamine). The two photomicrographs in *a* and *b* are representative of the immunofluorescence images obtained from double-labeling experiments captured in simultaneous exposure to both fluorophores. Surface  $\beta$ PP consistently showed a characteristic granular discontinuous staining pattern (*green*) on fine nontapering axons and appeared to be excluded from MAP2

staining of cell surface  $\beta$ PP using antibodies recognizing the extracellular region of  $\beta$ PP strongly labeled the perikaryon and the short lamellipodia (Fig. 1 *a*). Within the first 24 h after plating, neurons elongate a number of neurites, of which the longest one becomes the axon and the remaining processes differentiate into dendrites (stage 3). At this stage, both kinds of neurites and cell body demonstrated abundant surface  $\beta$ PP immunoreactivity (Fig. 1 *c*). Of note, the fine punctate immunostaining pattern was never present along the entire length of the neurite. Minor neurites were less consistently labeled. This distribution of cell surface  $\beta$ PP shares some similarities with the pattern of intracellular  $\beta$ PP reported previously (12). However, growth cones in contrast were only occasionally positively stained (Fig. 1 *c*). At 14–28 d after plating, neurites in the mature stage (stage 5) demonstrated a characteristic discontinuous and patchy pattern of fine granularity on the cell surface (Fig. 1, *e* and *h*). As before, neuronal tips remained inconsistently labeled for surface  $\beta$ PP (Fig. 1 *g*). At all stages,  $\beta$ PP on the surface of the perikaryon showed a fine punctate pattern (Fig. 1 *j*). Identical results described above were obtained with polyclonal (207) and monoclonal (1G7/5A3) antibodies, both of which recognize the extracellular domain of  $\beta$ PP.

Double-labeling experiments with the somatodendritic marker protein, MAP2 (microtubule-associated protein 2) (1), and the axonal marker protein, growth-associated protein 43 (GAP-43) (15), were carried out to identify the neuritic processes in mature neurons. In these stage 5 cells,  $\beta$ PP was generally confined to thin processes that were MAP2 negative (Fig. 2, *a* and *b*). In contrast, the processes immunostained by  $\beta$ PP antibodies were consistently GAP-43 positive (Fig. 2, *c* and *d*). While we could not exclude any dendritic localization of  $\beta$ PP, the pattern of  $\beta$ PP immunoreactivity was distinctly different from proteins that are distributed in both axons and dendrites such as tubulin (not shown) or NCAM (Fig. 2 *e*). From the double-labeling studies, occasional areas of MAP2 positive dendrites that showed apparent  $\beta$ PP immunoreactivity appeared to represent immunopositive axons overlapping with those dendritic segments. The staining patterns of  $\beta$ PP, GAP-43, and NCAM with respect to MAP2 were absolutely consistent and could be reliably distinguished from each other. This was determined in a blinded study of photomicrographs taken from 25 random fields from each set of double-labeled cultures. Although qualitatively much less intense than the axonal staining, cell surface  $\beta$ PP was consistently present on the perikarya in mature neurons. In sum, these findings suggested that cell surface  $\beta$ PP, like intracellular  $\beta$ PP (12), was predominantly localized to axons.

### Cell Surface $\beta$ PP Molecules Are Full-Length

To determine whether cell surface  $\beta$ PP immunoreactivity represented full-length molecules, neurons were double



**Figure 3.** Cell surface  $\beta$ PP represents full-length molecules. Neurites of mature neurons were double labeled with  $\beta$ PP antibodies raised against mid-region (5A3/1G7) (*a*) and the ectodomain region between the  $\alpha$ -secretase cleavage site and the cell surface (1963) (*b*). Although the signal intensity differed, the staining pattern of the two antibodies was identical. See text for explanation. (*c*) Hippocampal neurons were biotinylated at 4°C followed by immunoprecipitation with COOH-terminal  $\beta$ PP antibody. Biotinylated surface  $\beta$ PP species (*Surface*) migrating at  $\sim$ 100–110 kD consistent with full-length  $\beta$ PP695 isoform can be seen (*left lane*). This first image was obtained by  $^{125}$ I secondary antibody to antibiotin primary antibody. When the same nitrocellulose blot was reincubated with COOH-terminal  $\beta$ PP antibody and developed by chemiluminescence, a lower migrating band (*arrowhead*) was observed (*middle lane, Total*). This higher intensity nonbiotinylated  $\beta$ PP band is consistent with immature  $\beta$ PP species. The  $\beta$ PP species from hippocampal neurons comigrated with  $\beta$ PP precipitated from CHO cells stably transfected with  $\beta$ PP695 analyzed in parallel (*right lane, betaPP695*). Bar in *b*, 10  $\mu$ m.

labeled with  $\beta$ PP monoclonal antibodies and antibodies directed against either the ectodomain region of  $\beta$ PP between the  $\alpha$ -secretase cleavage site and the cell surface (antibodies 1963 and  $\alpha\beta$ 1) or the carboxy terminus (antibody C7).

positive dendrites (*red*). In cultured neurons, axons are frequently associated with dendrites but the two different processes can generally be identified. Occasionally,  $\beta$ PP-stained axons would cross over dendrites; at these short segments (*arrows*), dendrites would appear to be positively stained for  $\beta$ PP (*in yellow*) as a result of the overlap of the two fluorophores. The impression that  $\beta$ PP was generally confined to axons was confirmed by double labeling with  $\beta$ PP (*c*) and GAP-43 (*d*). Aside from the characteristic patchy distribution of  $\beta$ PP, the two markers otherwise showed virtual overlap in staining. In contrast, the patterns obtained from  $\beta$ PP and GAP-43 immunostaining are quite distinct from proteins distributed in both axons and dendrites, as demonstrated by double labeling for NCAM (*e*) and MAP2 (*f*). Bar, 20  $\mu$ m.

The former antibodies were selected because constitutive  $\alpha$ -secretase cleaved  $\beta$ PPs does not contain this region (10, 41) and thus would exclude the possibility that the staining reflected secreted  $\beta$ PPs attached to the cell surface. In mature neurons, there was a close correlation between the immunoreactivity obtained by 5A3/1G7 (Fig. 3 *a*) and 1963 (residues 692-708 of  $\beta$ PP770) (Fig. 3 *b*) or  $\alpha\beta$ 1. In contrast, COOH-terminal  $\beta$ PP antisera (C7 and 13G8) showed three different patterns by indirect immunofluorescence. The first pattern in these permeabilized cells consisted of punctate granular staining. The second pattern consisted of diffusely labeled neurites. These constituted the most common staining patterns, as was shown previously (12). Finally, the third pattern was infrequently seen and consisted of discontinuous patchy labeling reminiscent of the cell surface pattern described above and overlapped with cell surface  $\beta$ PP visualized by 5A3/1G7  $\beta$ PP monoclonal antibodies. Immunostaining of total  $\beta$ PP by 5A3/1G7 (Fig. 4, *a* and *b*) and 207 in permeabilized hippocampal neurons showed essentially the same pattern as that seen with COOH-terminal antibodies (C7 and 13G8). It is noteworthy that after permeabilization,  $\beta$ PP immunoreactivity could be seen in MAP2 positive processes with all the  $\beta$ PP antibodies (Fig. 4). However, in mature neurons, the intensity in MAP2 positive dendrites was substantially less than that seen in axons and did not have the distinctive granularity (Fig. 4, *b* and *c*). As expected, immunoreaction of C7 and 1963 antibodies were abolished by preabsorption with their corresponding peptides.

For additional confirmation that  $\beta$ PP present on the cell surface consisted of full-length molecules, hippocampal neurons were surface biotinylated at 4°C followed immediately by immunoprecipitation with COOH-terminal specific antibody. With this approach, full-length biotinylated  $\beta$ PP was recovered from the labeled cell lysates (Fig. 3 *c*). In addition, when the nitrocellulose blot was then immunoblotted with the same antibody, a lower molecular weight nonbiotinylated  $\beta$ PP species of greater abundance, consistent with immature  $\beta$ PP, was seen (Fig. 3 *c*). This latter experiment suggested that only cell surface molecules were biotinylated and that the more abundant immature species present intracellularly were not labeled. Taken together, these observations from surface  $\beta$ PP immunostaining and biotinylation suggested that cell surface  $\beta$ PP represents full-length molecules, rather than secreted molecules that may have adhered to the cell surface.

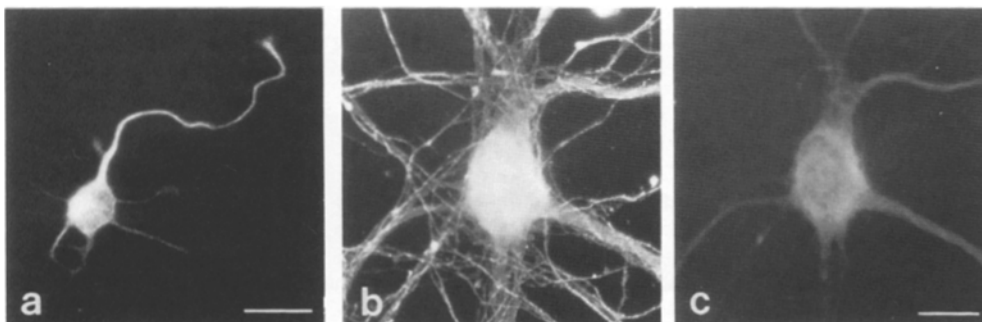
### Internalization of Cell Surface $\beta$ PP

We have previously shown in  $\beta$ PP-transfected CHO cells that

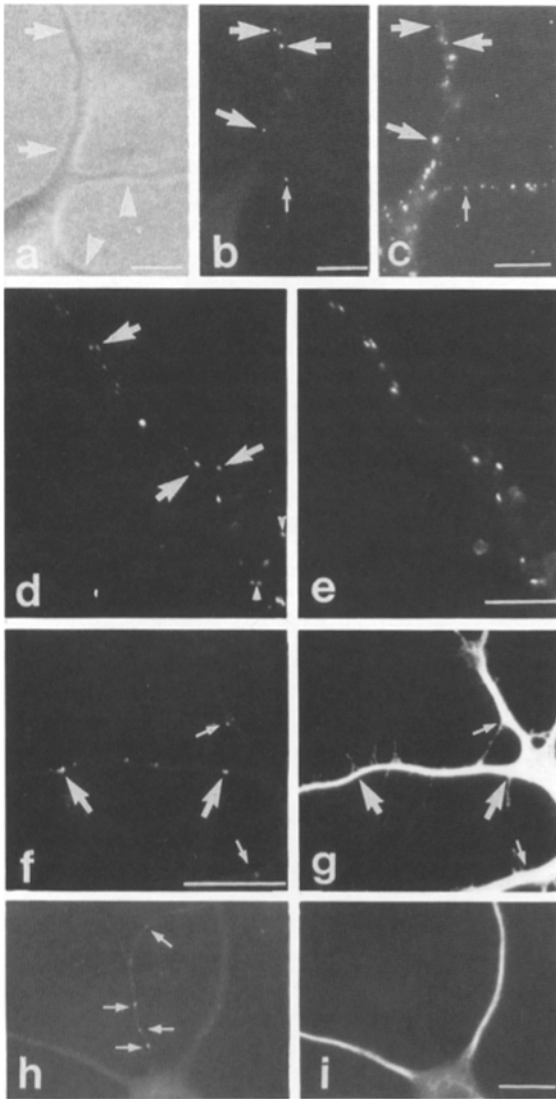
cell surface  $\beta$ PP is rapidly internalized via coated pits and subsequently recycled or targeted to late endosomes and lysosomes (Yamazaki, T., E. H. Koo, E. T. Hedley-Whyte, and D. J. Sekoe. 1993. *Soc. Neurosci. Abstr.* 19:396. To determine if  $\beta$ PP is similarly internalized from the cell surface of primary hippocampal neurons, cells were incubated with  $\beta$ PP monoclonal antibodies 5A3/1G7 for 30 min at 37°C. The cells were then fixed, permeabilized, and incubated with secondary antibodies. In premature (stage 3) neurons, internalized  $\beta$ PP was detected in small granular structures located both in axons and in minor neurites and soma (Fig. 5, *b* and *d*), indicating the internalization and transport of surface  $\beta$ PP to these sites. When the cells were incubated with both  $\beta$ PP antibodies and fluid phase markers,  $\beta$ PP was found to be colocalized with these markers within the same compartments (Fig. 5, *b-e*). As expected, the signal of the fluid phase markers was considerably more intense and more abundant than that of internalized  $\beta$ PP. To exclude the possibility that  $\beta$ PP antibodies were internalized via nonspecific (fluid phase) endocytosis, the cultures were first incubated with whole IgG or Fab fragments of 5A3/1G7  $\beta$ PP antibodies at 4°C before internalization was carried out at 37°C. Regardless of the conditions of antibody incubation, the staining patterns were identical (Fig. 5, *f* and *h*), although the signal intensity from Fab fragments was weaker than from whole IgG. In stage 5 neurons, when axon and dendritic polarity is established, internalized  $\beta$ PP was observed only in axons, i.e., MAP2-negative processes (Fig. 5, *h* and *i*), suggesting that  $\beta$ PP was predominantly internalized from axonal but not dendritic sites. This is consistent with the observation that cell surface  $\beta$ PP was principally located on axons, rather than dendrites. When the cells were incubated with the secondary antibody without permeabilization or with nonimmune mouse IgG at a concentration identical to 5A3/1G7, no staining was observed. In a few experiments, internalization of  $\beta$ PP monoclonal antibodies was carried out during and after membrane depolarization to accelerate synaptic vesicle recycling (32). However, the results did not change qualitatively after depolarization (data not shown). In summary, these findings indicated that  $\beta$ PP was rapidly internalized from the surface of axons into intracellular compartments which also contained fluid phase endocytic markers.

### Retrograde and Transcytotic Transport of Cell Surface $\beta$ PP

The above findings demonstrated that in mature hippocampal neurons, full-length  $\beta$ PP is primarily present on the surface of axons and perikarya. The presence of full-length  $\beta$ PP

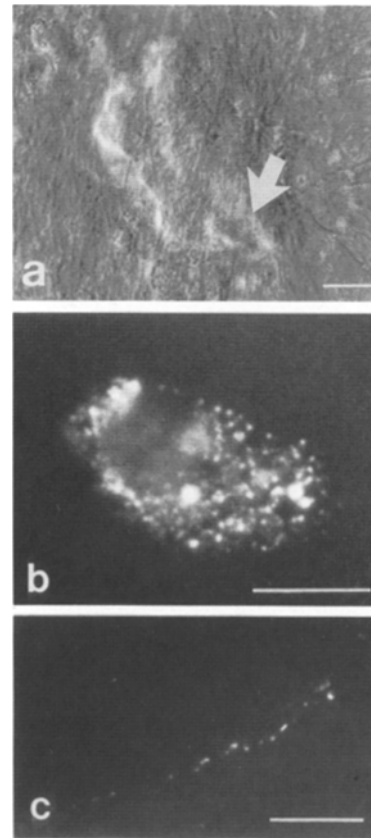


**Figure 4.** Intracellular localization of full-length  $\beta$ PP. Both in premature (*a*) and mature (*b*) neurons, intracellular  $\beta$ PP, as detected by 5A3/1G7 antibodies, was located throughout the cell bodies and neurites. Unlike cell surface  $\beta$ PP, MAP2 positive dendrites (*c*) were slightly immunoreactive for intracellular  $\beta$ PP (*b*). Bars: (*a*) 20  $\mu$ m; (*c*) 10  $\mu$ m.



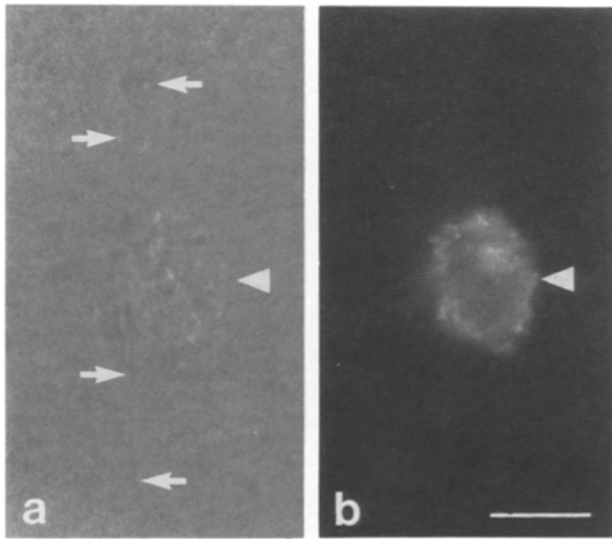
**Figure 5.** Internalization of cell surface  $\beta$ PP in cultured hippocampal neurons. Living cells were incubated with 5A3/1G7  $\beta$ PP antibodies and dextran-Texas red for 30 min. In a stage 3 neuron, phase contrast photomicrograph (a) shows one long axon (arrows) and minor neurites (arrowheads). The internalized  $\beta$ PP (b) was detected in immunostained vesicles within an axon (large arrows) and in the proximal portion of a minor neurite (small arrow). Another example of a premature neuron (d) showed internalized  $\beta$ PP within an axon (arrows) and perikaryon (arrowheads). Some of these labeled vesicles were colocalized with dextran-Texas red (c and e). Fab fragments of  $\beta$ PP antibodies labeled at 4°C showed similar pattern after internalization (f and h). In a premature neuron (f) internalized  $\beta$ PP were located both in an axon (large arrows) and minor neurites (small arrows). Panel g shows the same neuron from f stained with tubulin. In a mature neuron cultured for 7 d (h), internalized  $\beta$ PP (arrows) were localized only in MAP2 negative neurite (i), suggesting an axonal localization. Panels a, b, and c; d and e; f and g; h and i are paired images from double-labeled cells. Bars, 10  $\mu$ m.

on the surface of axons is consistent with the finding that  $\beta$ PP is anterogradely transported in axons and subsequently integrated into the axolemma (27, 30, 35, 42). As demonstrated above,  $\beta$ PP appeared to be internalized from the axonal or presynaptic terminal membranes. To directly dem-



**Figure 6.** Retrograde transport of  $\beta$ PP internalized from distal axons. In the central chamber of the compartment culture system, sympathetic neurons were aggregated in clusters as seen in a bright-field phase contrast photomicrograph (a). Only one neuron within the same cluster (arrow in a) showed punctate staining in the perikaryon (b) after addition of 5A3/1G7  $\beta$ PP antibodies to the lateral chamber. Occasional proximal axons also demonstrated intracellular vesicular immunostaining (c). See Materials and Methods and text for detail. Bars: (a) 50  $\mu$ m; (b and c) 25  $\mu$ m.

onstrate the internalization of  $\beta$ PP from distal axonal sites and retrograde transport back to perikarya, neurons were grown in a compartment culture system (4) that effectively isolated the perikarya from distal axons and terminals. Dissociated rat sympathetic neurons were cultured in the middle chamber of this three compartment system such that neurites gradually extended underneath the dividing wall into the two lateral chambers, usually 4–6 d after plating. The uniqueness of this system is that aside from the neuritic growth, there is no mixing of the media within the different compartments. Sympathetic neurons are known to extend only axons when cultured alone without glia in serum-free medium, thereby excluding dendrites from the culture system (20). In these experiments,  $\beta$ PP monoclonal antibodies were added only to the lateral chambers, into which axons had penetrated. 6–8 d after plating, the distal axons and terminals were then incubated with 5A3/1G7  $\beta$ PP antibodies at 37°C for 3 h followed by fixation, permeabilization, and incubation with secondary antibody. Sympathetic neurons were usually found to be distributed in small aggregates (Fig. 6 a). In the central compartment, one or two cells in such a cluster of neurons demonstrated abundant vesicular  $\beta$ PP staining in



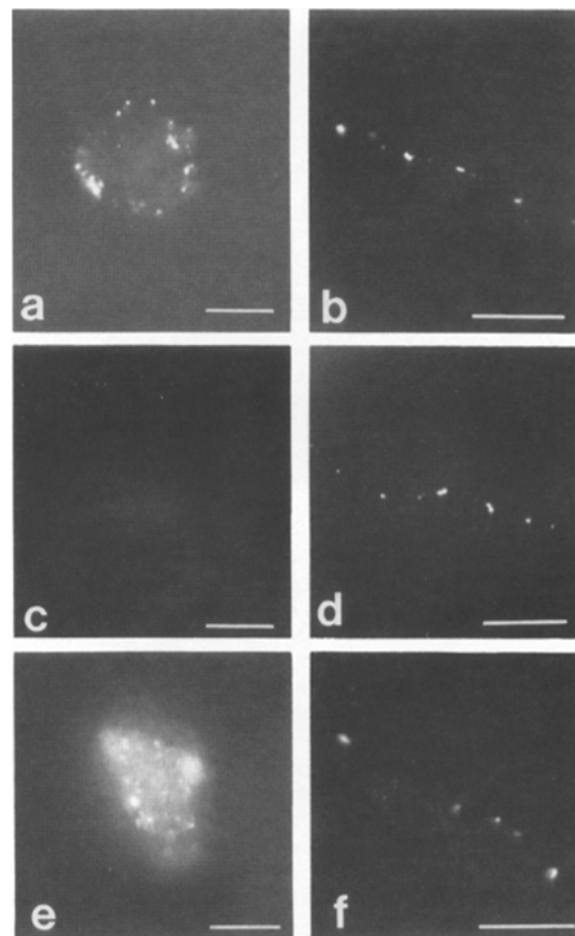
**Figure 7.** Transferrin receptors are excluded from axons. Polarity of sympathetic neurons was demonstrated by the presence of transferrin receptors only on the perikaryon (arrowhead) but not in axons (b). Phase contrast photomicrograph (a) of the corresponding cell seen in b shows the perikaryon of a neuron (arrowhead) within a network of axons (arrows). Bar, 50  $\mu\text{m}$ .

their cytoplasm, similar to that seen in hippocampal neurons above (Fig. 6 b), suggesting that only these neurons have internalized the  $\beta\text{PP}$  antibodies from their distal axons. In addition, small vesicles in proximal axons in the central chamber were occasionally immunoreactive (Fig. 6 c). On average,  $\sim 20\%$  of neurons were immunopositive, suggesting that only a fraction of neurons have successfully extended their axons into one of the two lateral chambers. In contrast, when the central, rather than lateral, compartment containing the cell bodies was incubated with  $\beta\text{PP}$  antibodies, all the neuronal perikarya and processes were immunostained for intracellular  $\beta\text{PP}$  (data not shown). This result provided confirmation that  $\beta\text{PP}$  antibodies added in the lateral chambers had not leaked into the central chamber; otherwise, all cells, not just a few cells within a cluster, would have been positive. As additional negative controls, cells incubated with either nonimmune mouse IgG instead of  $\beta\text{PP}$  antibodies or without primary antibody showed no reaction. In sum, these observations provided convincing evidence that  $\beta\text{PP}$  molecules were internalized from the surface of distal axonal sites and carried by retrograde axonal transport back to the cell body.

Because  $\beta\text{PP}$  is present not only on the axonal but also the perikaryal surface, we speculated that  $\beta\text{PP}$  may also be transported transcytotically from the axonal to the perikaryal surface compartment. To demonstrate the  $\beta\text{PP}$  transcytotic pathway, we first showed that sympathetic neurons consisted of two different compartments, akin to cultured hippocampal neurons and epithelial cells. Accordingly, when sympathetic neurons were incubated with transferrin conjugated to Texas red, uptake was observed only in perikarya (Fig. 7 b) but not axons. Therefore, in the absence of dendrites, cultured sympathetic neurons nevertheless consisted of distinct axonal and somatic compartments (3).

To examine the transcytosis of cell surface  $\beta\text{PP}$  from axons to perikarya, the same compartment culture experiment performed above was repeated. However, after the incubation of  $\beta\text{PP}$  monoclonal antibodies in the lateral chamber, secondary antibodies were added in the central chamber without permeabilization. With this approach, peripheral punctate staining was observed on the perikaryal surface of neurons in the central compartment (Fig. 8 a), indicating transcytosis of  $\beta\text{PP}$  from axonal to somatic sites. Compared to intracellular  $\beta\text{PP}$  in the cell body derived by retrograde transport (Fig. 6 c), the intensity was significantly less and the staining pattern was more fine and less vesicular. As before, only a minority of neurons in the central chamber was positively stained. Interestingly a few proximal axons within the central compartment also showed small punctate surface staining, indicating the recycling of internalized  $\beta\text{PP}$  to the axonal surface.

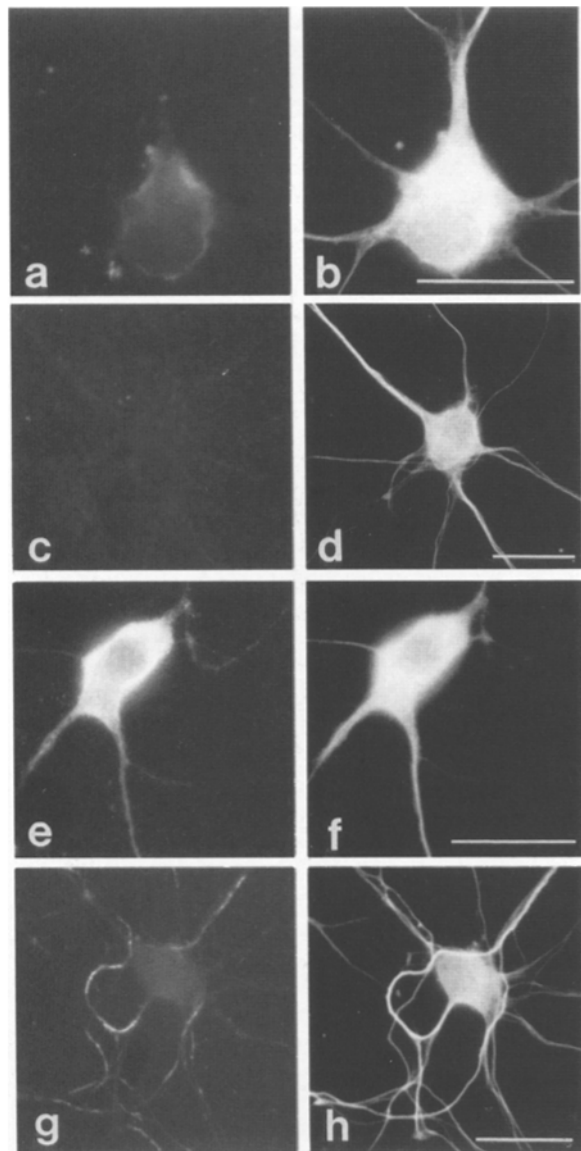
To determine whether this transport pathway is dependent



**Figure 8.** Transcytosis of cell surface  $\beta\text{PP}$  from axons to perikarya in compartment culture system. In the central chamber of the compartment culture system,  $\beta\text{PP}$  antibodies added to the lateral chamber can be seen on the surface of neuronal cell body (a) or proximal axon (b). Internalized  $\beta\text{PP}$  was absent from the surface of perikaryal membrane (c) but present on proximal axons (d) after BFA treatment. However, addition of BFA did not impair transport of  $\beta\text{PP}$  to perikarya (e) or in axons (f) as detected by secondary antibody after permeabilization. Bars, 25  $\mu\text{m}$ .



on microtubule based motors, the cells were treated with nocodazole to depolymerize microtubules (48). With this treatment, no reaction was seen within either the perikarya or proximal axons (data not shown), although cell surface  $\beta$ PP and internalized  $\beta$ PP remained at distal axons in the lateral compartment. In addition, cells were treated with brefeldin A (BFA), an agent which has been shown to inhibit



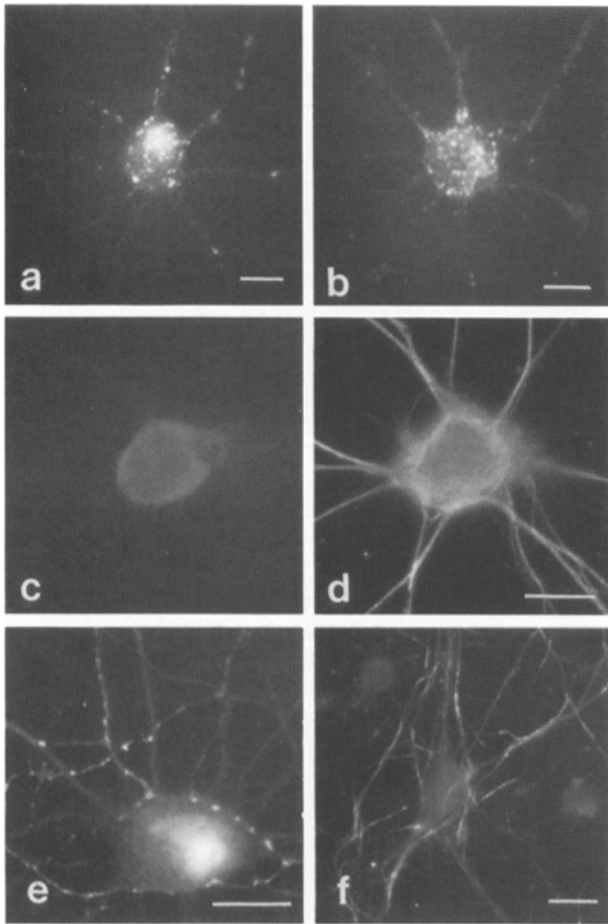
**Figure 9.** Cell surface  $\beta$ PP in hippocampal neurons after kinesin antisense oligonucleotide. Hippocampal neurons cultured for 5 d were incubated for 36 h with kinesin heavy chain antisense (*a-d*) or sense (*e-h*) oligonucleotides. Cells were double labeled with kinesin antibody (*a* and *e*) or 5A3/1G7  $\beta$ PP antibodies (*c* and *g*) and tubulin antibody (*b*, *d*, *f*, and *h*). With antisense oligonucleotide treatment,  $\beta$ PP was absent from the surface of neurites and cell body (*c*). Immunostaining of kinesin in the cell body was dramatically diminished with antisense oligonucleotide (*a*). With sense oligonucleotide treatment, cell surface  $\beta$ PP showed the typical patchy pattern on neurites (*g*) and kinesin was normally distributed throughout the cell (*e*). In *g*, the plain of focus was on the neurites, therefore, perikaryal staining cannot be appreciated in this photomicrograph. Bars, 20  $\mu$ m.

transcytosis in MDCK cells (22). After BFA pretreatment, no signal could be detected on the perikaryal surface (Fig. 8 *c*), although occasional proximal axons showed punctate immunoreactivity (Fig. 8 *d*). Moreover, if the neurons were permeabilized before the addition of secondary antibody, vesicular  $\beta$ PP staining was present within the perikarya (Fig. 8 *e*). This suggested that BFA did not alter internalization, recycling, or retrograde transport of  $\beta$ PP but was selective for the transcytotic pathway (22).

To extend the study to neurons of the central nervous system, an alternative experimental approach was used because hippocampal neurons could not be successfully grown in the same compartment culture system. In these experiments, kinesin heavy chain synthesis was inhibited by the addition of antisense oligonucleotide to the medium of hippocampal neurons (12). The rationale is that the anterograde axonal transport of  $\beta$ PP should be mediated by a kinesin based rapid organelle transport system. Thus, the impairment of kinesin should retard not only the transport of  $\beta$ PP to distal axonal sites as shown before (12) but also inhibit delivery of  $\beta$ PP to the retrograde axonal and transcytotic pathways, if  $\beta$ PP in the latter trafficking steps originated from the anterograde axonal pool. The absence of  $\beta$ PP on the axonal and perikaryal surfaces would be consistent with this scenario. After treatment with antisense oligonucleotide, neurons showed markedly reduced intracellular immunostaining for kinesin (Fig. 9 *a*), and an absence of cell surface  $\beta$ PP immunoreactivity on both the axon and cell body (Fig. 9 *c*). Similarly, synaptophysin immunostaining in axons was abolished (Fig. 10 *c*). Importantly, immunoreactivity of treated cells for transferrin receptor (Fig. 10 *a*) or transferrin uptake (Fig. 10 *b*) was not altered, demonstrating that the exocytic pathway to the surface of the perikaryon was not impaired. Control cultures treated with sense oligonucleotide showed essentially no change from untreated cells (Fig. 9, *e* and *g* and Fig. 10 *e*). Finally, to determine whether this inhibition of  $\beta$ PP transcytosis is reversible, cells treated with antisense oligonucleotide were cultured for two additional days in the absence of oligonucleotide. After this washout period, the characteristic patchy pattern of cell surface  $\beta$ PP was observed (Fig. 10 *f*). Taken together, these results suggested that retrograde axonal and transcytotic transport of  $\beta$ PP from distal axonal sites may also occur in neurons of the central nervous system.

## Discussion

In this report, we described a detailed localization of cell surface  $\beta$ PP and its subsequent trafficking in primary cultured neurons. The study was undertaken for several reasons: (*a*) neurons have long been hypothesized to be a principal source of A $\beta$  in brain, (*b*) the internalization pathway of  $\beta$ PP in neurons, which may be an important contributor to A $\beta$  formation (28), has not been examined, and (*c*) we recently described the polarized sorting and secretion of  $\beta$ PP in MDCK cells (18). Therefore, trafficking of  $\beta$ PP must be understood within the context of previous studies on the localization of  $\beta$ PP in neurons and the cellular trafficking pathway of  $\beta$ PP in MDCK cells. We report here that in mature primary hippocampal neurons, full-length  $\beta$ PP was located principally on the membrane surface of axons and perikarya. From distal axons or terminals, cell surface  $\beta$ PP, as deter-



**Figure 10.** Kinesin antisense oligonucleotide did not inhibit the exocytic transport of transferrin receptor (a) or transferrin uptake (b). Antisense oligonucleotide inhibited the axonal localization of synaptophysin (c), although with addition of sense oligonucleotide, synaptophysin could be seen in axons (e). Panel d shows the same neuron seen in c double labeled with tubulin. After washout of kinesin antisense oligonucleotide for 2 d, normal cell surface  $\beta$ PP was seen on the perikaryon and neurites (f). Bars, 10  $\mu$ m.

mined by the binding of monoclonal antibodies to  $\beta$ PP, was internalized and transported retrogradely back to the perikaryon in organelles which in part colocalized with fluid phase markers. Internalization and retrograde transport of  $\beta$ PP were shown in both hippocampal and peripheral sympathetic neurons, the latter using an established compartment culture system. Unexpectedly, in addition to targeting of internalized molecules to the cell body, presumably for degradation,  $\beta$ PP was also sorted transcytotically from the axonal to the somatic surface compartments.

Initial published results of  $\beta$ PP trafficking in neurons demonstrated the anterograde axonal transport of full-length  $\beta$ PP (27). Subsequent studies reported that  $\beta$ PP may be inserted into the axolemma (35) or transported distally to presynaptic nerve terminals (30, 37). Our studies now add to this trafficking pathway by showing that at distal sites,  $\beta$ PP is actively internalized from the axolemmal surface and retrogradely transported back to the cell body as full-length molecules. At growth cones,  $\beta$ PP was inconsistently detected on the membrane surface, a result that is distinct from the invariant localization of intracellular  $\beta$ PP at the same

sites (12). Therefore, we speculate that  $\beta$ PP is actively endocytosed and possibly recycled at growth cones. In addition, along the surface of the axon, recycling of  $\beta$ PP appears to take place. Finally, the compartment culture system used in this study is only able to isolate cell bodies from distal axons and terminals, and cannot differentiate between the latter two sites. Consequently, whether the turnover rate of  $\beta$ PP along the surface of the axolemma is different than at growth cones cannot be determined.

Previous reports describing the neuronal localization of  $\beta$ PP focused on the intracellular distribution of the molecule using antibodies specifically recognizing the carboxy terminus of  $\beta$ PP (5, 12). Therefore, those studies could not determine whether the distribution of  $\beta$ PP represented predominantly full-length or  $NH_2$  terminally truncated species. We found that in mature neurons, the predominant axonal localization of  $\beta$ PP is preserved for both surface and intracellular populations of  $\beta$ PP molecules. Along the axonal surface,  $\beta$ PP is distributed in a characteristically discontinuous pattern. At these patchy surface sites and at growth cones,  $\beta$ PP is closely associated with the  $\beta$ -integrins (Yamazaki, T., D. J. Selkoe, and E. H. Koo, manuscript in preparation), a feature that may be related to the putative role of  $\beta$ PP in adhesion (29, 36). However, the distinctive discontinuous pattern of cell surface  $\beta$ PP immunostaining along the axonal shaft was lost when the cells were first permeabilized with detergent. We hypothesize that the intracellular pool of  $\beta$ PP is either considerably larger than the surface pool of molecules or the latter is rapidly turned over so that only a few molecules are present on the surface at steady state. Evidence for the former is seen in the appearance of minor amounts of intracellular, but not surface,  $\beta$ PP in dendrites. Furthermore, we did not observe a correlation between cell surface  $\beta$ PP and structures immunoreactive for clathrin (data not shown), as was reported previously (12). This suggested that the colocalization of COOH-terminal  $\beta$ PP antibodies with clathrin structures represented primarily truncated  $\beta$ PP species. This interpretation is consistent with both the detection of the 10-kD COOH-terminal membrane retained  $\beta$ PP fragments in clathrin-coated vesicles isolated from PC12 cells (31) and the redistribution of  $\beta$ PP immunoreactive organelles after acidification of the culture medium (12).

Upon maturation, neurons exhibit striking polarity manifested by the long slender axon and the extensive dendritic arborizations. To maintain these functional differences, axons and dendrites are differentially separated into distinct plasma membrane domains (25, 34). Based on initial studies of the surface distribution of viral glycoproteins in neurons and epithelial cells, it was suggested that axonal/apical and somatodendritic/basolateral membranes may represent analogous domains to the extent that polarized targeting in neurons and epithelial cells may share similar signals or pathways (7, 8, 23). We have recently examined the sorting of  $\beta$ PP in MDCK cells and found that both the distribution of cell surface  $\beta$ PP and the secretion of  $\beta$ PP proteolytic products favored the basolateral compartment (18). This finding might be expected, in view of the putative role of  $\beta$ PP in adhesion and by its association with integrins and laminin (26). By analogy, one would therefore predict a somatodendritic sorting pattern of  $\beta$ PP in cultured hippocampal neurons. However, our results demonstrated that cell surface

$\beta$ PP is localized primarily to axons and perikarya but not dendrites, an observation that is consistent with the previous demonstration of intracellular  $\beta$ PP in axons and at growth cones (12, 37). Therefore,  $\beta$ PP joins a list of molecules and other cellular properties that do not fit this apical/axonal association, and the extent to which the polarity of neurons resembles epithelial cells remains unclear.

The transport of molecules in epithelial cells from one plasma membrane (apical or basolateral) to the other is known as transcytosis. This is an important cellular pathway because in epithelial cells, the two compartments are effectively separated from each other by tight junctions (34). Based on the transport of exogenous molecules such as viruses and toxins, both transcytotic and transsynaptic pathways have been described in neurons. Recently, using a viral expression system, the polymeric immunoglobulin receptor was shown to redistribute from somatodendritic to axonal domains with the addition of dimeric IgA, a result that implies transcytosis of this receptor molecule (23). Our result showing the transcytotic transport of  $\beta$ PP from axons to perikaryal surface is, to our knowledge, the first demonstration of the use of a constitutive transcytotic pathway by an endogenous neuronal protein. This pathway was demonstrated directly in sympathetic neurons by the transport of monoclonal  $\beta$ PP antibodies from distal axonal sites to the perikaryal surface in a compartment culture system. In hippocampal neurons, addition of antisense oligonucleotides to kinesin heavy chain resulted in virtually no staining of cell surface  $\beta$ PP in either axons or perikarya. The absence of  $\beta$ PP on the axolemmal surface is consistent with the known anterograde transport of  $\beta$ PP (12, 27). More importantly, the concomitant loss of  $\beta$ PP on the perikaryal surface suggests that the molecules targeted to the somatodendritic compartment may be derived principally from the transcytotic route. This interpretation assumes that transcytotic transport from the soma is not mediated by kinesin because exocytic sorting of transferrin receptor was not affected. As such, the evidence that a similar  $\beta$ PP transcytotic pathway exists in hippocampal neurons is indirect. In addition, we found that this transcytotic pathway is sensitive to BFA treatment, an agent that has been shown to selectively block transcytosis in MDCK cells without impairing internalization or recycling (22). Thus, the inhibition of  $\beta$ PP transcytosis by BFA is particularly interesting and suggests that a sorting step required for transcytosis is functionally similar between MDCK cells and neurons.

Our study did not identify the organelle that transports  $\beta$ PP retrogradely to the cell body. It has previously been shown that axonally transported organelles containing fluid phase markers consisted mostly of multivesicular body-like structures (32). Thus, if  $\beta$ PP were carried in these organelles, as suggested by the colocalization of  $\beta$ PP and fluid phase markers, then it would imply that in the perikaryon, these vesicles can still be sorted transcytotically to the perikaryal surface and not necessarily committed to the late endosome/lysosomal system for degradation. It is not clear from our studies whether internalized and/or retrogradely transported  $\beta$ PP molecules can be redirected anterogradely into the axon.

Finally, the significance of this trafficking pathway of  $\beta$ PP to either constitutive A $\beta$  production or amyloid deposition in brains of Alzheimer's disease subjects is unclear at this

time. Recent studies have implicated the internalization of cell surface  $\beta$ PP in cultured cells as an important contributor to A $\beta$  subsequently released into the medium (27). Whether A $\beta$  generation in neurons involves the endocytic pathway is unknown. However, it should be noted that the abnormal distribution of lysosomes in neurons is a consistent finding in Alzheimer's disease, especially within dystrophic neurites surrounding deposits of  $\beta$ -amyloid (6, 44). Therefore, whether this abnormal displacement of the lysosomal system alters the normal  $\beta$ PP trafficking pathway defined in this study, thereby contributing to the pathogenesis of AD, remains an interesting speculation.

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