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Trafficking of Cell-Surface β-Amyloid Precursor Protein: Evidence that a Sorting Intermediate Participates in Synaptic Vesicle Recycling

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We recently demonstrated that the Alzheimer's β-amyloid precursor protein (APP) is internalized from the axonal cell surface. In this study, we use biochemical and cell biological methods to characterize endocytotic compartments that participate in trafficking of APP in central neurons. APP is present in presynaptic clathrin-coated vesicles purified from bovine brain, together with the recycling synaptic vesicle integral membrane proteins synaptophysin, synaptotagmin, and SV2. In contrast, APP is largely excluded from synaptic vesicles purified from rat brain. In primary cerebellar macroneurons, cell-surface APP is internalized with recycling synaptic vesicle integral membrane proteins but is subsequently sorted away from synaptic vesicles and transported retrogradely to the neuronal soma. Internalized APP partially co-localizes with rab5a-containing compartments in axons and with V-ATPase-containing compartments in both axons and neuronal soma. These results provide direct biochemical evidence that an obligate sorting compartment participates in the regeneration of synaptic vesicles during exo/endocytotic recycling at nerve terminals but do not preclude concurrent "kiss-and-run" recycling. Moreover, APP is now, to our knowledge, the first demonstrated example of an axonal cell-surface protein that is internalized with recycling synaptic vesicle membrane proteins but is subsequently sorted away from synaptic vesicles.

Key words: Alzheimer's disease; amyloid precursor; axons; cell surface; cerebellar macroneurons; clathrin-coated vesicles; endocytosis; protein trafficking; synaptic vesicle recycling

An invariant feature in the cerebral cortex of patients with Alzheimer's disease is the extraneuronal deposition of the β-amyloid peptide (Aβ), derived by proteolytic processing from type I transmembrane glycoproteins, called β-amyloid precursor protein or APP (Kang et al., 1987). APP exists as three principal isoforms derived by alternative splicing; predominant expression of the 695 amino acid residue isoform is seen in neurons (Sisodia et al., 1993). Constitutive α-secretase processing of APP results in the release of a 100–110 kDa soluble N-terminal fragment, with membrane retention of a 10 kDa C-terminal fragment (Sisodia et al., 1990). Cleavage by α-secretase occurs within the Aβ sequence and thereby precludes the formation of intact Aβ (Wang et al., 1991). Data from *in vitro* studies suggest that the α-secretase pathway may be used to only a limited extent by neural cells (Hung et al., 1992).

In addition to processing by the secretory pathway, APP is processed by internalization from the cell surface, apparently by receptor-mediated endocytosis (Haass et al., 1992; Koo and Squazzo, 1994; Yamazaki et al., 1996). Several lines of evidence, derived largely from non-neuronal systems, suggest that the APP internalization pathway contributes to the production and extracellular release of Aβ. First, a portion of the internalized APP is delivered to the degradative late endosomal–lysosomal compartment, where a series of potentially amyloidogenic fragments (i.e., contain the entire Aβ sequence) can be detected (Golde et al., 1992; Haass et al., 1992). Second, cell-surface APP is a precursor to Aβ that is constitutively secreted by cultured cells, and cells that express internalization-deficient, C-terminal-deleted APP secrete significantly less Aβ than cells that express full-length protein (Koo and Squazzo, 1994).

It is not known whether APP internalization by central neurons results in increased secretion of Aβ and, hence, contributes to cerebral amyloid burden and the pathogenesis of Alzheimer's disease. To address this issue, we have chosen to study endocytotic trafficking of full-length, cell-surface APP in neurons. In previous studies (Yamazaki et al., 1995), we demonstrated that neuronal APP is internalized selectively from the axonal cell surface and delivered by retrograde transport to the neuronal soma, where a portion is sorted transcytotically to the somatic cell surface. Transcytotic sorting to the somatic cell surface has been confirmed in other studies (Simons et al., 1995). In the present study, we use biochemical and cell biological methods to characterize endocytotic compartments that participate in neuronal APP trafficking. We demonstrate that APP is endocytosed together with recycling synaptic vesicle membrane proteins but is subsequently sorted away from synaptic vesicles for retrograde transport to neuronal soma. We discuss the implications of our data to the synaptic

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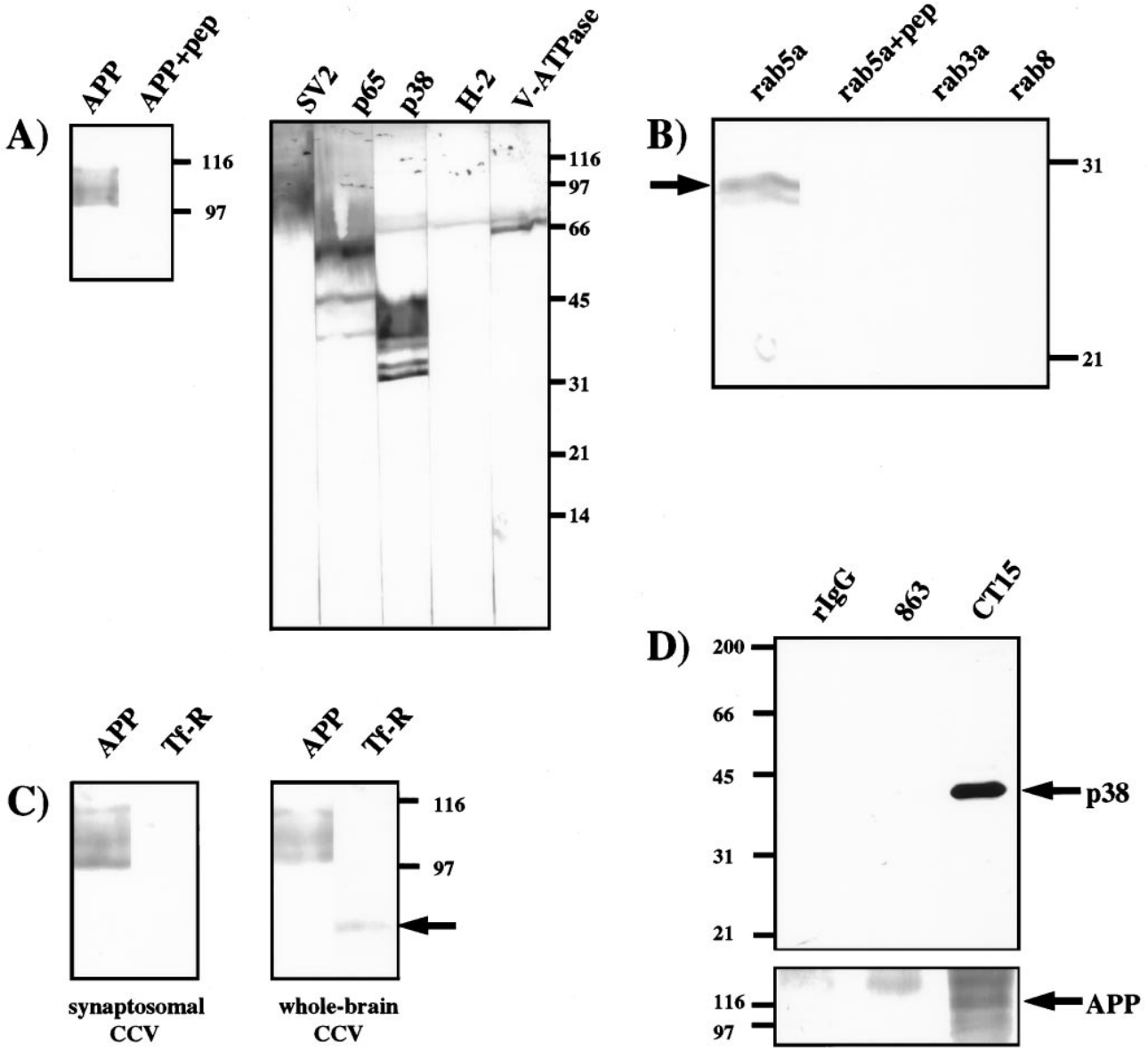

Figure 1. Distribution of APP in clathrin-coated vesicles from bovine brain. Clathrin-coated vesicles (*CCV*) were purified from homogenates of cerebral cortex (*whole-brain*), unless otherwise indicated, and incubated with low-ionic strength buffer to dissociate clathrin coats before analysis by SDS-PAGE and immunoblotting (20 μg protein/gel lane). Position and relative mass (in kilodaltons) of protein standards are indicated. *A*, Mature, full-length APP (*APP*, *inset*) is present in whole-brain clathrin-coated vesicles, together with the recycling synaptic vesicle integral membrane proteins SV2 (*SV2*), synaptotagmin I (*p65*), and synaptophysin (*p38*). APP reactivity can be eliminated when anti-APP is applied together with its cognate peptide (*APP+pep*, *inset*). *H-2*, Murine H-2 complex cell-surface antigen (negative control); *V-ATPase*, 70 kDa subunit of vacuolar ATPase from bovine brain (positive control). Laemmli gel, 4–20% (*inset*, 6%). *B*, Rab5a (*rab5a*, *arrow*) is present in whole-brain clathrin-coated vesicles, but neither rab3a (*rab3a*) nor rab8 (*rab8*) can be detected. Rab5a reactivity can be eliminated when anti-rab5a is applied together with its cognate peptide (*rab5a+pep*). Laemmli gel, 15%. *C*, To demonstrate definitively that APP is present in the presynaptic subpopulation, clathrin-coated vesicles were additionally purified from hypotonically lysed synaptosomes (*synaptosomal*). Full-length APP (*APP*) is present in whole-brain and synaptosomal clathrin-coated vesicles. Transferrin receptor (*Tf-R*) can only be detected in whole-brain clathrin-coated vesicles (*arrow*). Laemmli gel, 6%. *D*, To demonstrate that APP and recycling synaptic vesicle integral membrane proteins are present in the same subpopulation of clathrin-coated vesicles, whole-brain clathrin-coated vesicles were immunoprecipitated with antibody directed to the cytoplasmic tail of APP (*CT15*), resuspended, and analyzed by SDS-PAGE and immunoblotting for synaptophysin (*top panel*). Synaptophysin (*p38*, *arrow*) is readily detected when CT15 is used but cannot be detected when antibody directed to the midregion of the extracellular domain of APP (*863*) or nonimmune IgG (*rIgG*), respectively, is used. The membrane was subsequently stripped and reprobed for APP (*bottom panel*) to demonstrate that immunoprecipitation with CT15 selectively enriches for APP-containing clathrin-coated vesicles (*APP*, *arrow*). Laemmli gel, 12%.

vesicle exo/endocytotic cycle and present a model for trafficking of cell-surface APP in central neurons.

MATERIALS AND METHODS

Antibodies. Monoclonal antibodies 5A3 and 1G7 (Koo and Squazzo, 1994) recognize nonoverlapping epitopes in the midregion of APP and were used in combination (designated 5A3,1G7) for APP trafficking studies (Yamazaki et al., 1995). Polyclonal antibody CT15 recognizes the 15 C-terminal residues in the cytoplasmic tail of APP (Sisodia et al., 1993). 5A3,1G7 and CT15 are specific for APP and do not cross-react with the related protein APLP-2 (see Slunt et al., 1994). Polyclonal antibody 863 was generated against a bacterial fusion protein encompass-

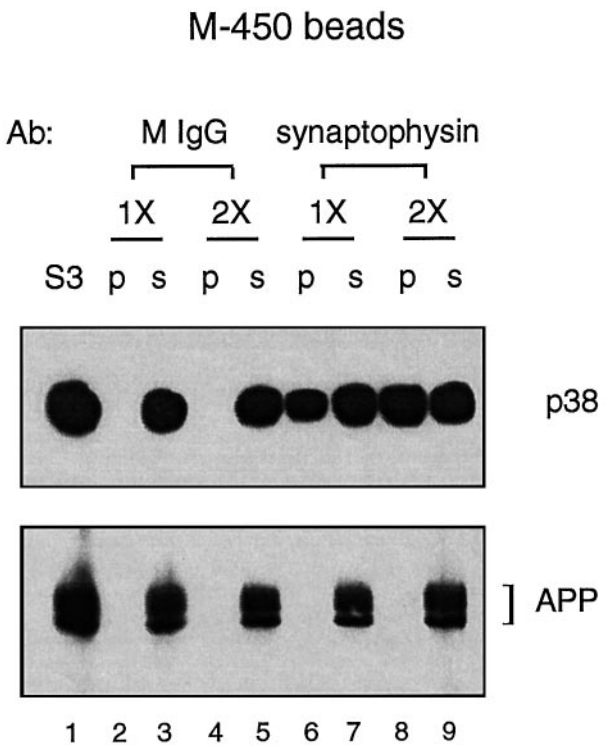

Figure 2. Distribution of APP in synaptic vesicles immunoisolated from rat brain synaptosomal lysates (*S3*). Control (*MIgG*) or anti-synaptophysin-coated magnetic beads were used for immunoisolation. *2×*, Twice the amount of control or anti-synaptophysin beads used relative to *1×*. After separation with a magnetic rack, equal amounts of immunobeads (*p*) and supernatant (*s*) fractions were analyzed by SDS-PAGE and immunoblotting. The membrane was incubated with anti-synaptophysin to detect synaptic vesicles, then stripped and reprobed with CT15 to detect APP. APP (*APP*) is not detected in the immunobeads fraction using either amount of anti-synaptophysin beads. No synaptophysin-reactive material (*p38*) is detected in the immunobeads fraction when control beads are used. Laemmli gel, 8%.

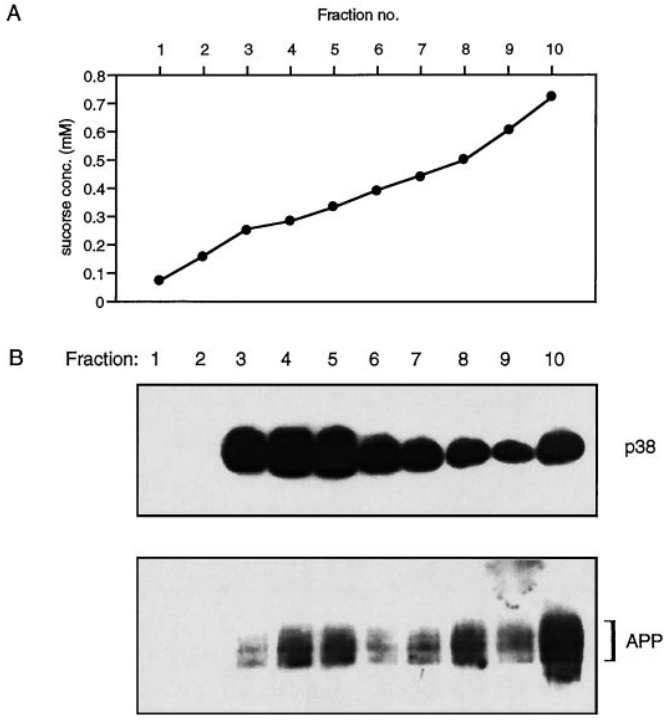

Figure 3. Distribution of APP in synaptic vesicles purified from rat brain by differential and sucrose gradient centrifugation as described by Huttner et al. (1983). Gradient fractions were analyzed by SDS-PAGE and immunoblotting. Anti-synaptophysin was applied first to detect synaptic vesicles. The membrane was subsequently stripped and reprobed for APP. Synaptophysin (*p38*) reactivity peaks at gradient fractions 3–5 (250–350 mM sucrose), whereas APP (*APP*) reactivity peaks at fraction 10 (>700 mM sucrose) at the bottom of the gradient. Laemmli gel, 8%.

ing ~280 amino acid residues in the midregion of APP (from *Xho*I to *Bgl*II restriction sites); the transmembrane and cytoplasmic domains are excluded from this construct. Polyclonal antibody Syt_{lum}–Abs (Matteoli et al., 1992) (a gift from Dr. P. De Camilli) recognizes the small intraluminal domain of rat synaptotagmin I. Monoclonal anti-synaptotagmin I (gift from Dr. R. Jahn) recognizes the large cytoplasmic domain of synaptotagmin I. Additional monoclonal antibodies include anti-clathrin heavy chain (clone X22, a gift from Dr. F. Brodsky), anti-SV2 (a gift from Dr. K. Buckley), anti-transferrin receptor (a gift from Dr. I. Trowbridge), anti-70 kDa subunit of V-ATPase from bovine brain (clone 3.2-F1, a gift from Dr. M. Forgac), anti-murine H-2 complex cell-surface antigen (a gift from Dr. R. Melvold), anti-synaptophysin (clone SY38, Boehringer Mannheim, Indianapolis, IN), anti-MAP-2 (clone HM-2, Sigma, St. Louis, MO), and anti-α-tubulin (clone DM-1A, Sigma). Polyclonal antibodies to the 70 and 60 kDa subunits of V-ATPase were obtained from Dr. B. Bowman. Antibodies to rab3a, 5a, and 8 were purchased from Santa Cruz Biotech (Santa Cruz, CA).

Cell culture. Primary cerebellar macroneurons were prepared from embryonic day 14 rat using the protocol described for hippocampal neurons by Goslin and Banker (1991). Briefly, cells from the dissected cerebellar anlage (Altman and Bayer, 1978, 1985) were dissociated by trypsinization (15 min at 37°C) followed by trituration with flame-polished Pasteur pipettes. The cells were plated at a density of 150,000 or 450,000 per 60 mm tissue culture dish on polylysine-coated (1 mg/ml) glass coverslips in MEM supplemented with 10% horse serum. After 3–4 hr to allow attachment of neurons, the coverslips were transferred to tissue culture dishes containing glial cell monolayers and serum-free neuronal medium consisting of MEM containing the N2 supplements of Bottenstein and Sato (1979), 0.1% ovalbumin, and 0.01% pyruvate. Glial cultures for conditioning of neuronal medium were prepared as described by Goslin and Banker (1991).

Immature cerebellar macroneurons were typically used at day 4 in culture (day 0 = day that cultures are prepared); no difference in APP distribution was seen between days 4 and 6 in culture. Mature cerebellar macroneurons were used at day 14 in culture. Polarity for the somatodendritic marker MAP-2 (Caceres et al., 1986) was established at day 5–6 in culture.

Preparation of vesicular fractions from bovine brain. Clathrin-coated vesicles were prepared from homogenates of bovine cerebral cortex (designated "whole-brain coated vesicles") by differential centrifugation followed by equilibrium centrifugation in linear Ficoll/D_2O gradients as described by Forgac and Cantley (1984). Whole-brain and synaptosomal (see below) coated vesicles were incubated with low-ionic-strength buffer containing 5 mM Tris, pH 8.5, 150 mM sucrose, and 0.5 mM EGTA to dissociate clathrin coats (designated "stripped vesicles"), as described (Forgac and Cantley, 1984) before analysis by SDS-PAGE.

Clathrin-coated vesicles were additionally purified from hypotonically lysed synaptosomes (designated "synaptosomal coated vesicles") using the Maycox et al. (1992) protocol adapted for bovine brain. Briefly, synaptosomes were prepared from cerebral cortical tissue (obtained from whole calf brains) by differential centrifugation followed by Ficoll gradient centrifugation, washed with tartrate buffer to remove bound vesicular material, and lysed by hypotonic shock. The synaptosomal lysate was quickly returned to isotonicity by the addition of 1/10 volume of 10× buffer A (Forgac and Berne, 1986) and subfractionated by equilibrium centrifugation in linear Ficoll/D_2O gradients as described (Forgac and Cantley, 1984). Synaptosomal clathrin-coated vesicles were collected as a small but distinct band from the linear gradients, resuspended in buffer A, homogenized with a Dounce glass–glass homogenizer, and snap-frozen for storage at −80°C.

Immunoprecipitation of whole-brain clathrin-coated vesicles with polyclonal antibody CT15 (directed to the cytoplasmic tail of APP) was carried out as follows. Clathrin coats were dissociated as described above,

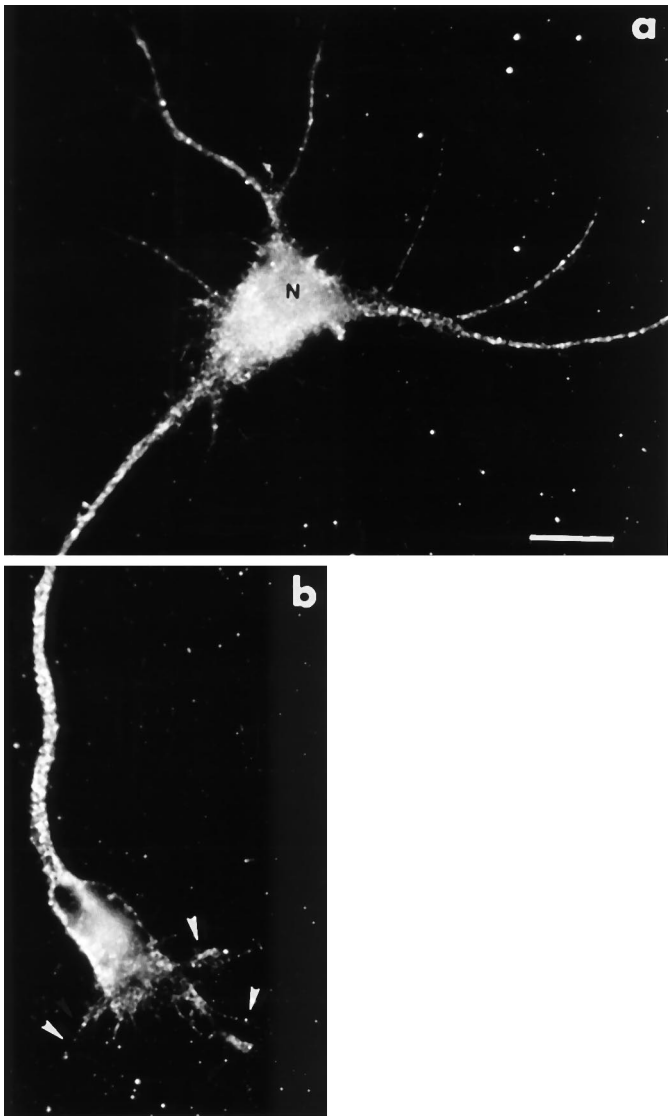

Figure 4. Immature cerebellar macroneuron (day 6) fluorescently labeled for total cellular APP using antibody 5A3,1G7. The somatodendritic domain and proximal axonal shaft are shown in *a*, and the distal axonal shaft and growth cone of the same neuron are shown in *b*. Diffuse, punctate immunoreactivity for APP is observed in the somatodendritic and axonal domains, including the axonal growth cone and growth cone filopodia, several of which are indicated (*arrowheads*). Juxtanuclear enrichment of APP reactivity is observed in the neuronal soma. *N*, Nuclear region. Scale bar, 10 μm.

and the resulting stripped vesicles (0.4 mg total protein) were diluted with Tris–saline buffer (10 mM Tris, pH 8.0, 140 mM NaCl) containing 5 mg/ml BSA to a final volume of 0.5 ml. Stripped vesicles were precleared for 1 hr at 4°C using 30 μl of a 50:50 slurry of protein A-Sepharose (Pharmacia Biotech, Piscataway, NJ) in Tris–saline/BSA and incubated with polyclonal antibody CT15 overnight at 4°C using end-to-end rotation. Overnight incubation with an equivalent amount of polyclonal antibody 863 (directed to the midregion of the extracellular domain of APP) or nonimmune rabbit IgG was used as a negative control. A second aliquot (30 μl) of protein A-Sepharose was added, and the incubation was continued for an additional 2 hr. The immunobeads fraction was obtained by sedimentation, washed five times with Tris–saline/BSA, resuspended in Laemmli (1970) sample buffer, and heated at 85°C for 10 min to dissociate bound proteins. One-half of the resuspended immunobeads fraction was used for analysis by SDS-PAGE and immunoblotting.

Preparation of vesicular fractions from rat brain. Rat brain synaptosomes were prepared using the following protocol provided by R. Jahn (personal communication). The cerebral hemispheres from one adult rat brain were homogenized in ice-cold buffer (0.32 M sucrose, 5 mM HEPES, pH 7.4, 1 mM EDTA, containing freshly added 0.5 mM PMSF, 10 μg/ml aprotinin, and 5 μg/ml each leupeptin and pepstatin A) using a glass/Teflon homogenizer (10 strokes at 600 rpm). Remaining steps were carried out at 4°C. The homogenate was centrifuged at 800 × *g* for 10 min, and the resulting supernatant was centrifuged at 11,000 × *g* for 12 min. The pellet fraction from the 11,000 × *g* centrifugation step was resuspended in 4 ml of homogenization buffer and layered onto discontinuous Ficoll gradients (3 ml of 13% Ficoll, 0.75 ml of 9% Ficoll, and 3 ml of 6% Ficoll in homogenization buffer). The Ficoll gradients were centrifuged for 30 min at 65,000 × *g* (23,000 rpm) in a TH641 rotor. After centrifugation, synaptosomes were collected by pooling the bands present at the 6–9% and 9–12% Ficoll interfaces.

Biochemical purification of synaptic vesicles was carried out using the protocol described by Huttner et al. (1983) with minor modifications. The cerebral hemispheres from two adult rat brains were used. Ten fractions were obtained from the linear sucrose gradients. Controlled-pore glass chromatography was not performed.

Immunoisolation of synaptic vesicles with anti-synaptophysin beads was carried out as follows. Crude synaptosomes (P2 fraction) obtained as described (Huttner et al., 1983) were resuspended in ice-cold homogenization buffer (see above) and lysed by hypotonic shock. HEPES/NaOH was immediately added to a final concentration of 7.5 mM, pH 7.4, and the suspension was kept on ice for 30 min. NaCl (150 mM final concentration) was added to restore isotonicity, and the lysate was cleared by centrifugation (11,000 × *g* for 12 min). The resulting supernatant (S3 fraction) was used as the starting material for immunoisolation. Anti-synaptophysin beads were prepared as follows. Magnetic beads coated with sheep-anti-mouse IgG (Dynal, Great Neck, NY) were incubated with monoclonal anti-synaptophysin (2 mg antibody/mg beads) in PBS overnight at 4°C. The beads were washed three times and resuspended in 150 mM NaCl, 10 mM HEPES, pH 7.4, and 1 mM EDTA (resuspension buffer). Anti-synaptophysin beads (2 mg of bound antibody) were incubated with the S3 fraction (0.5 mg total protein) for 2 hr at 4°C with end-to-end rotation. After the incubation, the beads were retrieved with a magnetic rack (Dynal), washed three times for 10 min each with resuspension buffer, and resuspended in Laemmli (1970) sample buffer. The supernatant from the immunoisolation was centrifuged for 30 min at 97,000 rpm in an AT4 rotor, and the resultant pellet was resuspended in sample buffer. Control beads were prepared by incubation with an equivalent amount of nonimmune mouse IgG.

Immunocytochemistry. Antibodies 5A3,1G7 and Syt_{lum}–Abs were applied to the neuronal culture media for 60 min at 37°C to detect internalized APP and internalized synaptotagmin I, respectively. Mouse or rabbit nonimmune IgG was applied to the culture media (same concentration as used for 5A3,1G7) as a negative internalization control. Rhodamine-conjugated wheat germ agglutinin (WGA, Vector Labs, Burlingame, CA) was applied to the culture media at 10 μg/ml. Goat serum (5%) was added to the culture media to reduce background.

Cultured neurons were fixed for 10 min with 4% formaldehyde (prepared freshly from paraformaldehyde powder) in PBS, pH 7.4, containing 0.12 M sucrose. All steps were carried out at room temperature. The cells were then washed with PBS, quenched with 0.1 M glycine (in PBS), and permeabilized with 0.1% Triton X-100. To detect APP confined to the cell surface, permeabilization with detergent was omitted. Cytoskeletal proteins could not be detected without previous permeabilization with Triton X-100, indicating that cell-surface APP only was detected when detergent was omitted. After blocking with 5% goat serum for 20 min, cells were incubated with additional primary antibodies for 1 hr (where applicable), washed, and incubated with fluorescein- or rhodamine-conjugated secondary antibodies (Boehringer Mannheim). For double-labeling with two monoclonal primary antibodies, the antibodies were applied sequentially and the first antibody was visualized using a large excess of affinity-purified, rhodamine-conjugated secondary antibody as the Fab fragment as described (Yamazaki et al., 1995). For labeling of the Golgi compartment, fixed, permeabilized cells were incubated for 1 hr with fluorescein-conjugated lentil lectin (10 μg/ml, Vector Labs). Cells were viewed with a Zeiss axiophot microscope equipped with epifluorescence optics and photographed with T-MAX 400 film (Eastman Kodak, Rochester, NY).

Other methods. SDS-PAGE was performed using the method of Laemmli (1970). Immunoblotting was performed as described by Towbin et al. (1979) using an enhanced alkaline phosphatase detection kit purchased from BioRad (Hercules, CA). For immunoblotting of subcellular

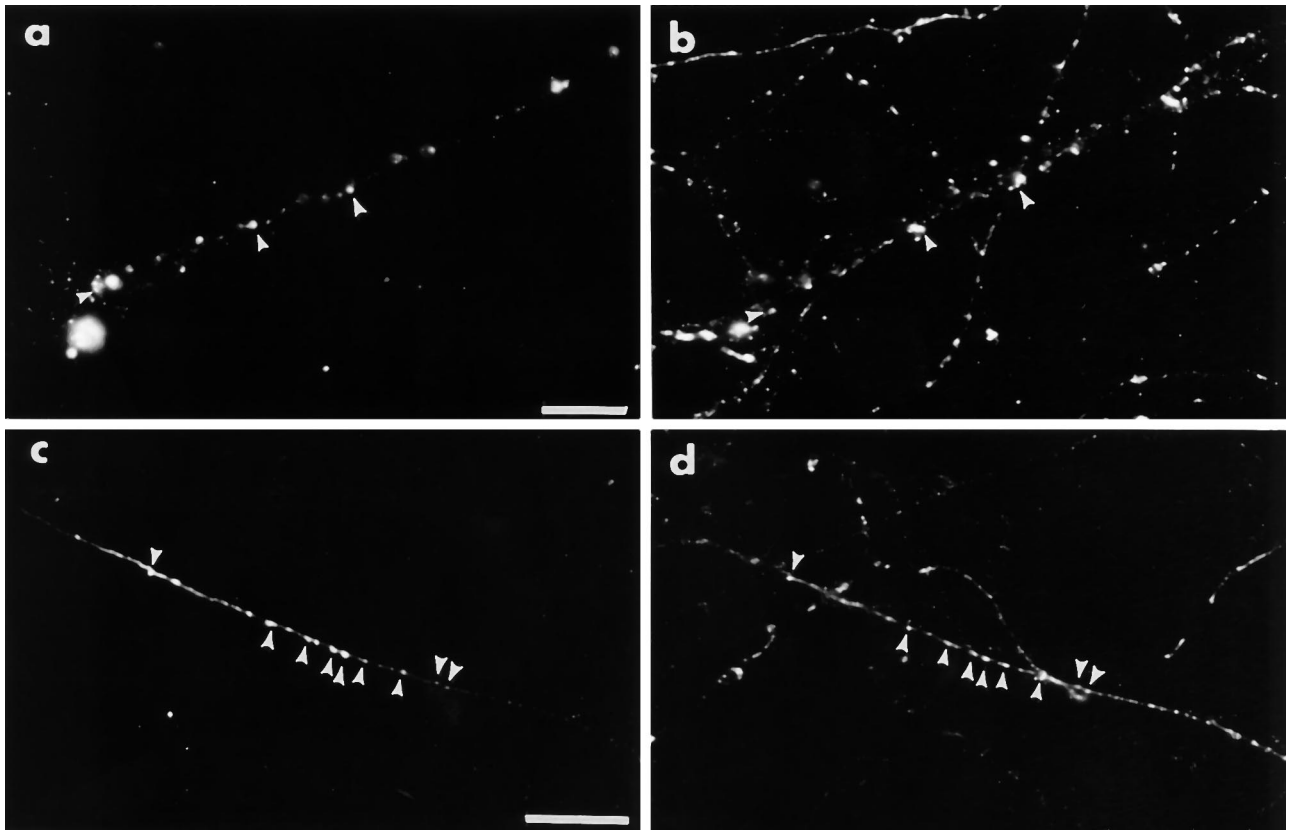

Figure 5. Mature cerebellar macroneurons (day 14) fluorescently labeled for internalized APP (*a*, *c*) and either total cellular clathrin (*b*) or total cellular rab5a (*d*). Partial co-localization of internalized APP and clathrin or rab5a is observed in axons (*arrowheads*). Stronger co-localization for rab5a is observed. Scale bars, 10 μm.

fractions from rat brain, an enhanced chemiluminescence kit purchased from Amersham (Arlington Heights, IL) was used. For reprobing of immunoblots, antibodies bound to the nitrocellulose membrane were removed by stripping with 62.5 mM Tris, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol for 30 min at 70°C. Membranes were rinsed twice in PBS then incubated with a second primary antibody. Protein concentration was determined using the method of Bradford (1976).

RESULTS

Full-length APP is present in presynaptic clathrin-coated vesicles

Nerve terminal (presynaptic) clathrin-coated vesicles are felt to participate in synaptic vesicle recycling (Mundigl and DeCamilli, 1994). APP has been shown previously to be enriched in clathrin-coated vesicles (Nordstedt et al., 1993), but it is not known whether the protein is enriched in *presynaptic* clathrin-coated vesicles. To determine whether APP is present selectively in the presynaptic subpopulation, coated vesicles were purified from homogenates of bovine cerebral cortex by differential and equilibrium centrifugation as described by Forgac and Cantley (1984) and immunoblotted for APP. The Forgac and Cantley method yields a very homogeneous clathrin-coated vesicle population that is essentially devoid of synaptic vesicles. By electron microscopy, >95% of vesicles obtained using this method are coated, and virtually all of the clathrin coat assemblages actually contain a membrane vesicle (Forgac and Cantley, 1984). In addition, coated vesicles purified from homogenates of cerebral cortex ("whole-brain" coated vesicles) are highly enriched for the presynaptic subpopulation, as demonstrated by Maycox et al. (1992).

As predicted, immunoblotting of purified whole-brain coated vesicles with polyclonal antibody CT15 directed to the cytoplasmic tail of APP demonstrates the presence of full-length APP in this preparation (Fig. 1*A*, *inset*). Adsorption of CT15 with its cognate peptide results in elimination of APP signal. In addition, the synaptic vesicle integral membrane proteins synaptophysin, synaptotagmin, and SV2 can be detected in this preparation (Fig. 1*A*). The small GTP-binding protein rab5a can also be detected in this preparation, but rab3a and rab8 cannot be detected (Fig. 1*B*). Adsorption of anti-rab5a with its cognate peptide results in elimination of rab5a signal.

The precise fraction, albeit small, of whole-brain coated vesicles that is derived from sites other than nerve terminals (e.g., neuronal soma or glia) is not known. To demonstrate unequivocably that APP is present in the presynaptic subpopulation, coated vesicles were additionally purified by equilibrium centrifugation from hypotonic lysates of bovine brain synaptosomes ("synaptosomal" coated vesicles) and immunoblotted for APP. Full-length APP can be detected in synaptosomal as well as whole-brain coated vesicles (Fig. 1*C*). In contrast, transferrin receptor, an endocytotic marker for the somatodendritic domain (Cameron et al., 1991), can only be detected in whole-brain coated vesicles.

To demonstrate that APP and recycling synaptic vesicle integral membrane proteins are endocytosed via a common pool of clathrin-coated vesicles, purified whole-brain coated vesicles were immunoprecipitated with polyclonal antibody CT15 (directed to the cytoplasmic tail of APP) and immunoblotted for synaptophysin. Synaptophysin is readily detected in coated vesicles immunoprecipitated with CT15 but cannot be detected when an equiva-

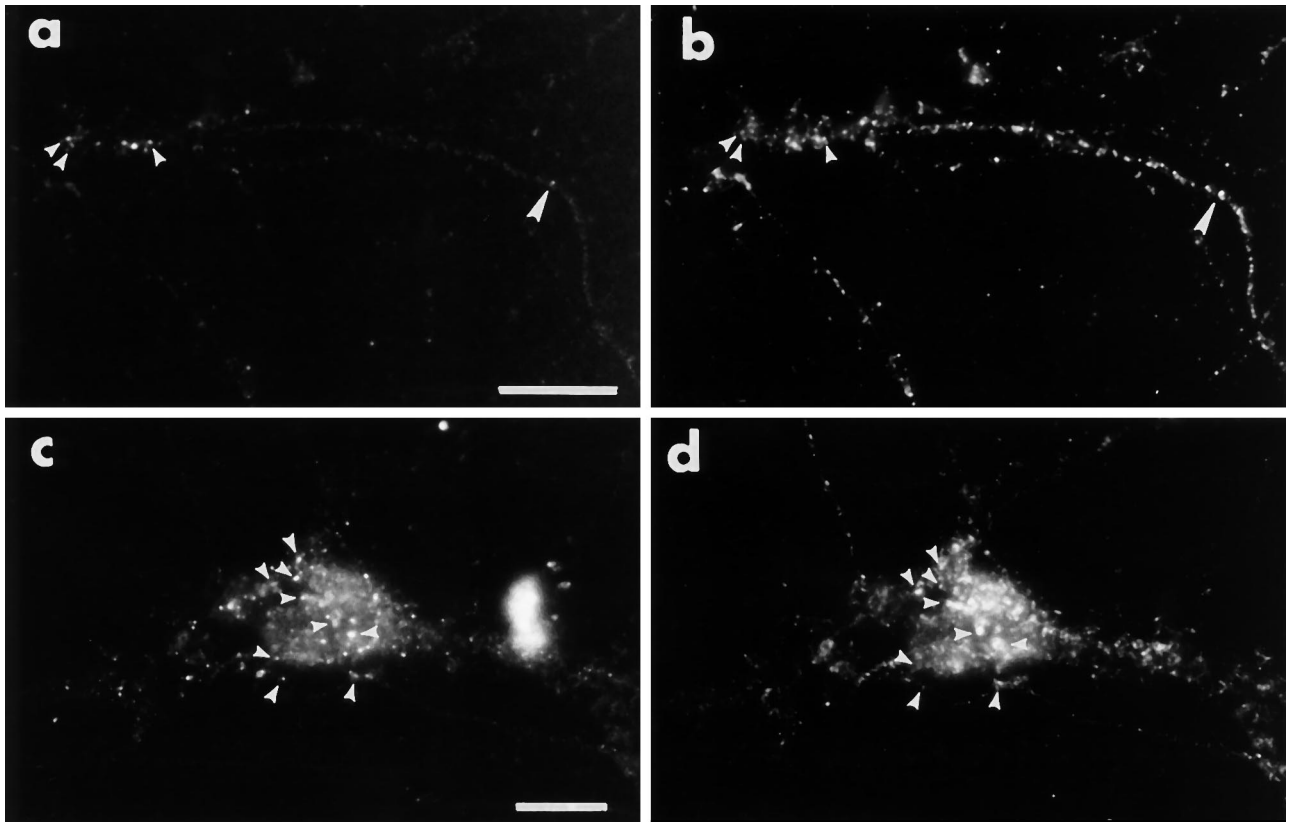

Figure 6. Mature cerebellar macroneurons (day 14) fluorescently labeled for internalized APP (*a*, *c*) and total cellular V-ATPase (*b*, *d*), the enzyme principally involved in maintaining an acidic luminal state in intracellular compartments. Two adjacent distal axonal segments (including small growth cones) are shown in *a* and *b*. Partial co-localization of internalized APP and V-ATPase is observed in axons and neuronal soma (*arrowheads*). Scale bars, 10 μm.

lent amount of polyclonal antibody 863 (directed to the midregion of the extracellular domain of APP) or nonimmune IgG is used (Fig. 1*D*, *top panel*). Reprobing for APP (Fig. 1*D*, *bottom panel*) demonstrates that immunoprecipitation with CT15 selectively enriches for APP-containing coated vesicles.

APP is largely excluded from synaptic vesicles

The above results indicate that APP is enriched in presynaptic clathrin-coated vesicles. To determine whether APP is specifically excluded from synaptic vesicles, the latter were purified from rat cerebral cortex by magnetic bead immunoisolation and immunoblotted for APP. Both APP and synaptic vesicles are selectively enriched in synaptosomes prepared from rat cerebral cortex (data not shown). Synaptosomes were then lysed by hypotonic shock and incubated with magnetic beads coated with anti-synaptophysin (anti-synaptophysin beads) (Fig. 2) to immunoisolate synaptic vesicles. After immunoisolation, equal portions of immunobeads ("pellet") and supernatant fractions were analyzed by SDS-PAGE and immunoblotting. Approximately 25% of starting synaptophysin-reactive material is recovered with the anti-synaptophysin beads and doubles when twice the amount of beads is used (Fig. 2, *top panel*, compare lanes 6 and 8.). All of the APP immunoreactivity, however, remains in the supernatant fraction (i.e., is not precipitated with the anti-synaptophysin beads). No synaptophysin-reactive material is recovered when nonimmune beads are used.

Synaptic vesicles were also purified from rat cerebral cortex using the method of Huttner et al. (1983). By immunoblotting with anti-synaptophysin, synaptic vesicles are enriched in fractions 3–5 near the top of the gradient (Fig. 3). A second, smaller peak of synaptophysin immunoreactivity is present in fraction 10 at the bottom of the gradient. These results are comparable with those obtained by Huttner et al. (1983). In contrast, APP is enriched in fraction 10 at the bottom of the gradient, consistent with its presence in denser vesicular fractions, including presynaptic clathrin-coated vesicles.

APP is internalized with recycling synaptic vesicle membrane proteins then sorted away from synaptic vesicles in cerebellar macroneurons

To identify compartments that participate in trafficking of neuronal cell-surface APP, immunofluorescent labeling of primary cerebellar macroneurons with monoclonal antibodies 5A3,1G7 (Koo and Squazzo, 1994; Yamazaki et al., 1995) that recognize the midregion of the ectodomain of native APP was carried out. Antibodies 5A3,1G7 were first applied to fixed, permeabilized immature macroneurons to detect total cellular APP, which appears as diffuse puncta throughout the somatodendritic and axonal domains (Fig. 4). Axonal growth cones and growth cone filopodia are consistently labeled, as described in primary hippocampal neurons (Ferreira et al., 1993), and neuronal soma reveal prominent juxtanuclear labeling that corresponds to the Golgi region fluorescently labeled using lentil lectin (data not shown), as described previously (Caporaso et al., 1994). Next, the antibodies were applied to fixed, nonpermeabilized immature macroneurons to detect cell-surface APP, which has a more limited distribution. Axons are consistently labeled, with signal

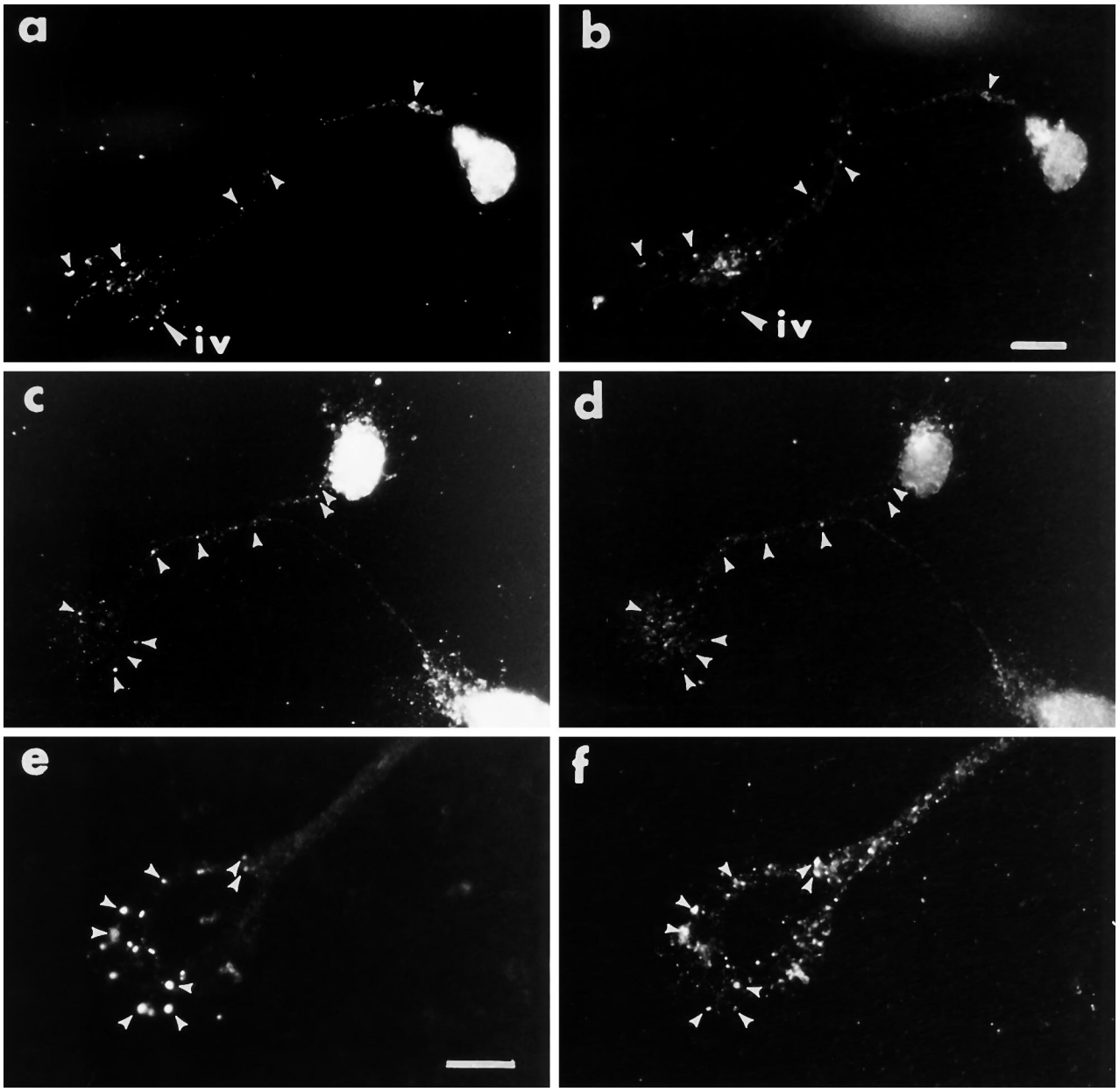

Figure 7. Immature cerebellar macroneurons (day 4) fluorescently labeled to detect recycling synaptic vesicle membrane proteins and either total cellular or internalized APP. Cells were incubated with anti-synaptotagmin I as a marker for recycling synaptic vesicle membrane proteins, then fixed, permeabilized, and labeled with 5A3,1G7. Partial co-localization of total cellular APP (*a*, *c*) and internalized synaptotagmin (*b*, *d*) is observed in axons (*a–d*, *arrowheads*; *iv* indicates four puncta of co-localization in an axonal growth cone). Juxtanuclear reactivity for internalized synaptotagmin is also present, as observed previously in hippocampal neurons (Matteoli et al., 1992). Additionally, cells were incubated simultaneously with anti-synaptotagmin and 5A3,1G7 to detect internalized synaptotagmin (*f*) and internalized APP (*e*). Strong, partial co-localization of internalized APP and internalized synaptotagmin is observed in distal axons and is particularly evident in axonal growth cones (*e*, *f*, *arrowheads*). Scale bars, 10 μm.

confined to concentrated patches, whereas minor neurites/dendrites are only rarely labeled (data not shown). These results fully confirm our earlier observation in hippocampal neurons that cell-surface APP exhibits a polarized distribution in neuritic processes (Yamazaki et al., 1995).

Antibodies 5A3,1G7 were then added to the macroneuronal culture medium for 60 min at 37°C, and the cells were subsequently fixed, permeabilized, and incubated with fluorescent secondary antibody to detect APP internalized from the neuronal cell surface. Incubation with antibodies directed to the ectodomain of APP has been used successfully in both neuronal (Yamazaki et al., 1995) and non-neuronal (Haass et al., 1992; Koo and Squazzo, 1994; Koo et al., 1996) cells to detect APP internalized from the cell surface. This approach does not result in redirection of internalized APP (Haass et al., 1992; Koo and Squazzo, 1994; Koo et al., 1996). Antibodies 5A3,1G7 are not internalized by fluid-phase (adsorptive) endocytosis and do not effect nonphysiological internalization of APP molecules via cross-linking at the cell surface (Yamazaki et al., 1995). Similarly, incubation with non-immune IgG at the same concentration used for 5A3,1G7 is uniformly negative (data not shown). In mature macroneurons, punctate immunoreactivity for internalized APP is detected seg-

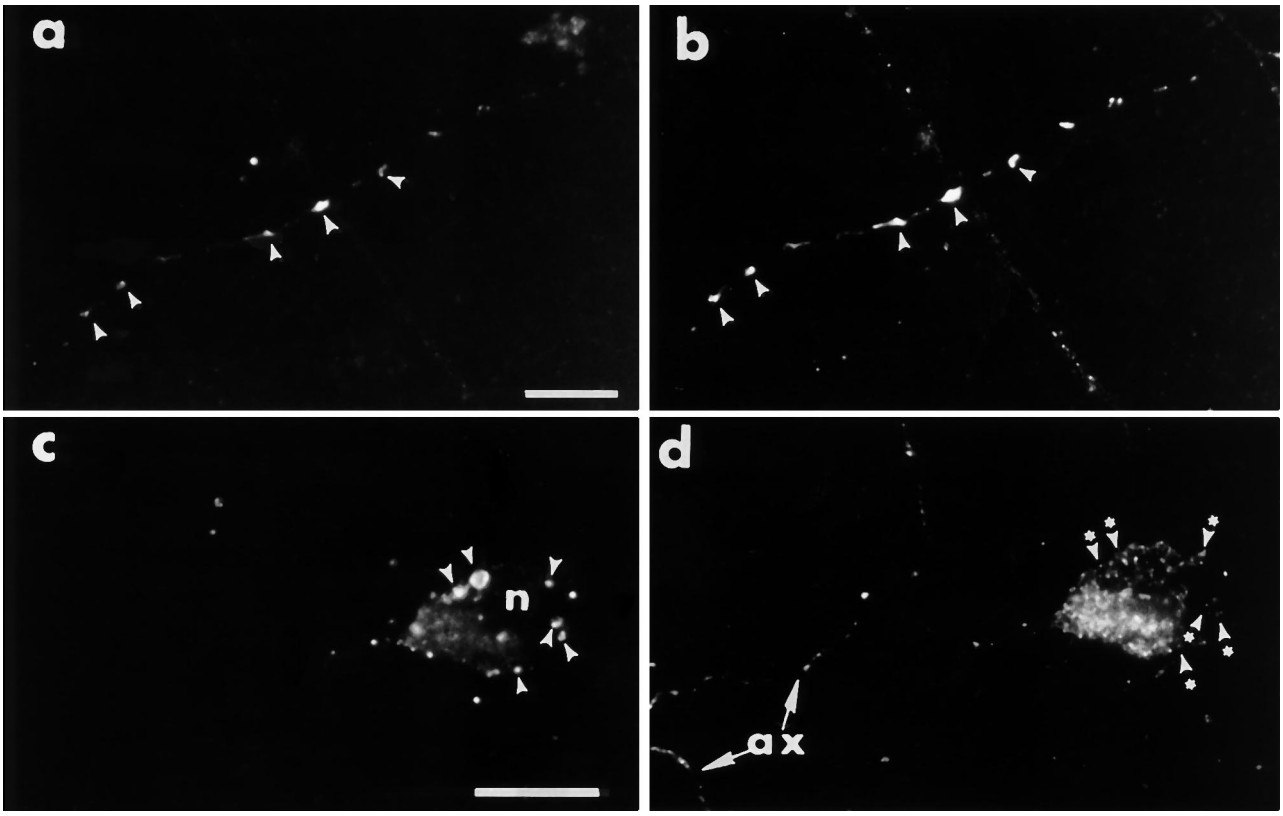

Figure 8. Mature cerebellar macroneurons (day 14) fluorescently labeled for internalized APP (*a*, *c*) and internalized synaptotagmin (*b*, *d*). Strong, partial co-localization of internalized APP and internalized synaptotagmin is observed in axonal varicosities (*arrowheads* in *a*, *b*; two intersecting axonal segments are shown). In contrast, no co-distribution of internalized APP and internalized synaptotagmin is observed in neuronal soma (*starred arrowheads* in *d* indicate site of selected puncta of internalized APP marked by *arrowheads* in *c*). In neuronal soma, internalized synaptotagmin is largely juxtanuclear, whereas internalized APP is diffusely distributed. *n*, Nuclear region; *ax*, axonal segments. Scales bars, 10 μm.

mentally in axons and is particularly evident in axonal varicosities (Fig. 5). Diffusely scattered punctate immunoreactivity is also detected in neuronal soma (see below). Partial co-localization is observed for rab5a and internalized APP in axon segments (Fig. 5*c*,*d*), consistent with the presence of APP in early endosomal compartments of the axonal domain (de Hoop et al., 1994). Partial co-localization is also observed for clathrin and internalized APP (Fig. 5*a*,*b*) but to a lesser extent than seen for rab5a. No co-localization of internalized APP and either rab5a or clathrin is observed in the somatodendritic domain (data not shown). Partial co-localization of V-ATPase and internalized APP is observed in both the axonal and somatodendritic domains (Fig. 6).

To confirm our finding that cell-surface APP is internalized together with recycling synaptic vesicle membrane proteins (see first part of Results), polyclonal antibody Syt_{lum}–Abs (Matteoli et al., 1992), which recognizes the intraluminal domain of the synaptic vesicle integral membrane protein synaptotagmin I (Perin et al., 1991), was added to the macroneuronal culture medium for 60 min at 37°C. The intraluminal domain of synaptotagmin is exposed at the cell surface during fusion of synaptic vesicles with the presynaptic plasma membrane and is thus available to bind Syt_{lum}–Abs from the neuronal culture medium. Internalization of Syt_{lum}–Abs has been used previously to detect recycling synaptic vesicles in hippocampal neurons (Matteoli et al., 1992; Mundigl et al., 1993). In immature macroneurons, internalized synaptotagmin appears as scattered puncta in axons, including axonal growth cones (Fig. 7*b*,*d*,*f*). Partial co-localization is seen for internalized synaptotagmin and total cellular APP (Fig. 7*a*–*d*). When both 5A3,1G7 and Syt_{lum}–Abs are added to the culture medium to detect internalized APP and internalized synaptotagmin, respectively, strong, partial co-localization is seen in distal axons and is particularly evident in expanded axonal growth cones (Fig. 7*e*,*f*). In expanded axonal growth cones of immature macroneurons, ~50% of internalized APP co-localizes with internalized synaptotagmin. In mature macroneurons, strong, partial co-localization of internalized APP and internalized synaptotagmin is observed in axon segments and is again particularly evident in axonal varicosities (Fig. 8*a*,*b*), where ~70% co-localization of internalized APP with internalized synaptotagmin is observed. In contrast, internalized APP and internalized synaptotagmin are not co-distributed in macroneuronal soma (essentially 0% co-localization of internalized APP with internalized synaptotagmin) (Fig. 8*c*,*d*). Internalized synaptotagmin is largely juxtanuclear, whereas internalized APP is diffusely scattered. Juxtanuclear immunoreactivity, in addition to punctate immunoreactivity in axons, was observed previously in immature and mature hippocampal neurons with Syt_{lum}–Abs (Matteoli et al., 1992).

To determine whether neuronal cell-surface APP and lectins (bound to cell-surface glycoproteins) use a common internalization pathway, WGA was added to the macroneuronal culture medium for 60 min at 37°C. Internalized WGA appears as diffuse, strong puncta and includes large vesicular profiles in the axonal and somatodendritic domains of immature (data not shown) and mature (Fig. 9*b*,*d*) neurons. When both WGA and 5A3,1G7 are added to the culture medium, no co-distribution of internalized

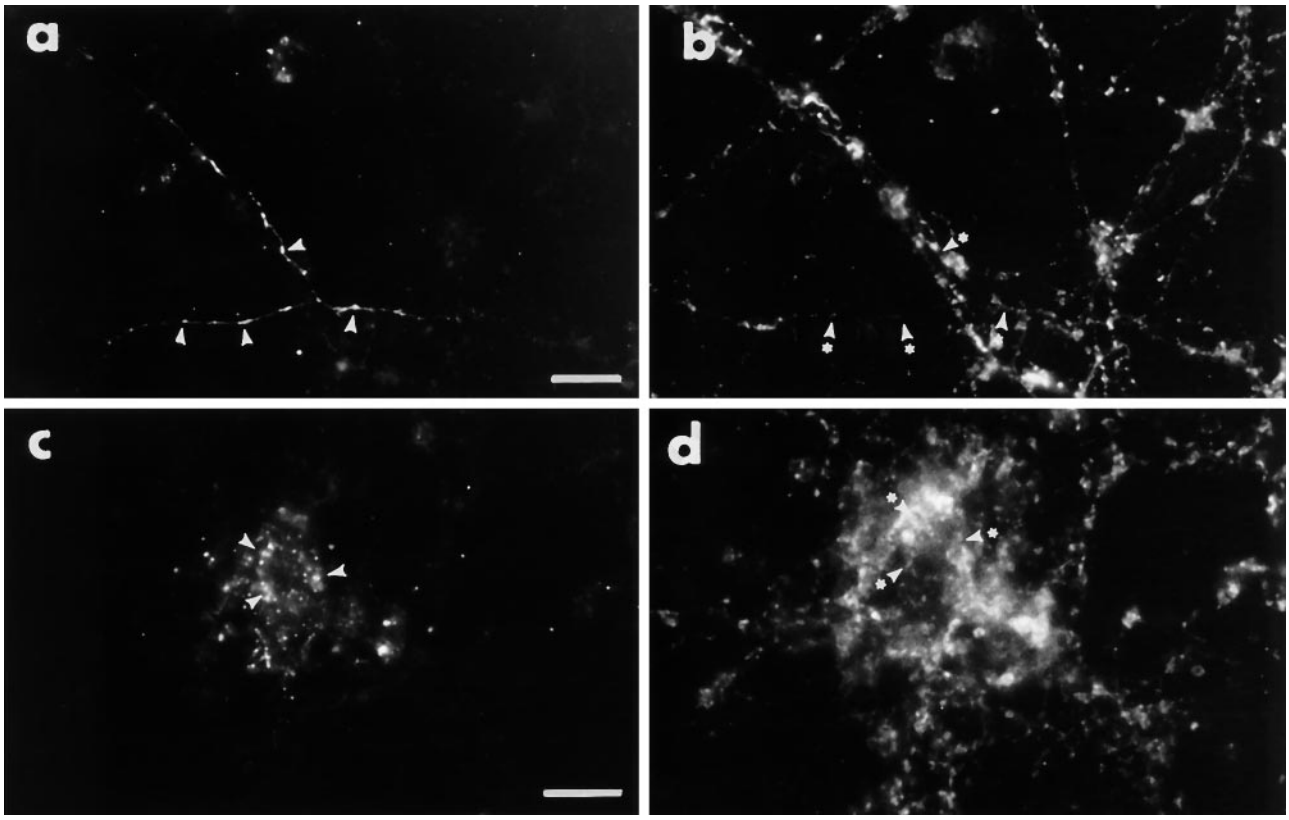

Figure 9. Mature cerebellar macroneurons (day 14) fluorescently labeled for internalized APP (*a*, *c*) and internalized WGA (*b*, *d*). No co-distribution of internalized APP and internalized WGA is observed in axons (*starred arrowheads* in *b* indicate site of selected puncta of internalized APP marked by *arrowheads* in *a*) or in neuronal soma (*starred arrowheads* in *d* indicate site of selected puncta of internalized APP marked by *arrowheads* in *c*). Scale bars, 10 μm.

lectin and internalized APP is observed either in axons or in neuronal soma.

DISCUSSION

We reported previously (Yamazaki et al., 1995) that full-length APP is internalized from the axonal cell surface and transported retrogradely to the neuronal soma. In the present study, we use biochemical and cell biological methods to characterize endocytotic compartments that participate in neuronal APP trafficking. We demonstrate that APP is present in presynaptic clathrin-coated vesicles purified from bovine brain, together with recycling synaptic vesicle membrane proteins. However, APP is largely excluded from synaptic vesicles purified from rat brain. In primary cerebellar macroneurons, cell-surface APP is internalized with recycling synaptic vesicle membrane proteins but is subsequently sorted away from synaptic vesicles for retrograde transport to neuronal soma. Internalized APP partially co-localizes with rab5a-containing compartments in axons and with V-ATPase-containing compartments in both axons and neuronal soma.

Neurons are believed to maintain distinct subpopulations of clathrin-coated vesicles and early endosomes in their axonal and somatodendritic domains (Parton and Dotti, 1993). In this view, axonal (largely presynaptic) coated vesicles and early endosomes participate primarily in synaptic vesicle recycling. We demonstrate that full-length APP is endocytosed via presynaptic coated vesicles, together with the recycling synaptic vesicle integral membrane proteins synaptophysin, synaptotagmin, and SV2. Previous reports that APP is enriched in clathrin-coated vesicles (Ferreira et al., 1993; Nordstedt et al., 1993; Sapirstein et al., 1994) did not conclusively demonstrate that the full-length protein is present in the presynaptic coated vesicle subpopulation. We also demonstrate that APP is not enriched in synaptic vesicle fractions obtained by sucrose gradient centrifugation (Huttner et al., 1983) or in synaptic vesicles immunoisolated with anti-synaptophysin beads. These findings indicate that although APP is transported anterogradely to distal axonal sites (Koo et al., 1990; Sisodia et al., 1993), it is largely excluded from synaptic vesicles at nerve terminals. The small peak of APP immunoreactivity observed in sucrose gradient fractions 4–5 (i.e., peak synaptic vesicle fractions) originates largely from presynaptic clathrin-coated vesicles that were stripped of their clathrin coats ("stripped vesicles") during hypotonic lysis of synaptosomes. Stripped vesicles are similar in buoyant density to synaptic vesicles and co-migrate with the latter during sucrose gradient centrifugation (see Huttner et al., 1983). Similarly, the small peak of synaptophysin immunoreactivity in fraction 10 (bottom of the gradient) originates largely from intact (nonstripped) presynaptic coated vesicles. Notably, the bulk of APP immunoreactivity is present in this fraction.

We demonstrate further that full-length APP is endocytosed together with recycling synaptic vesicle membrane proteins in primary cerebellar macroneurons but is subsequently sorted away from synaptic vesicles. Endocytosis of APP and recycling synaptic vesicle membrane proteins occurs predominantly from nerve terminals, as is evident in axonal growth cones in immature neurons and in axonal varicosities (Fletcher et al., 1991) in mature neurons. The distribution of endocytosed APP in clathrin- or rab5a-containing compartments in axons suggests that APP is probably

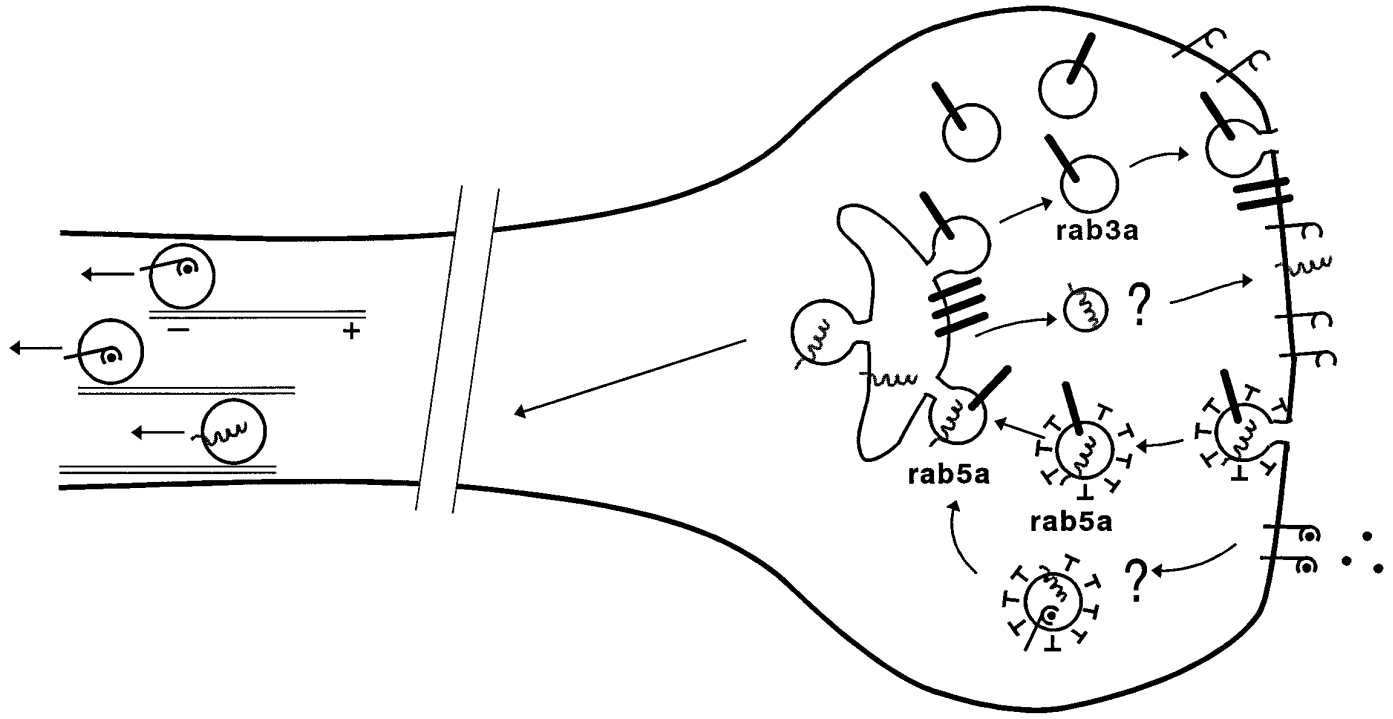

Figure 10. Proposed model for endocytotic trafficking of APP in central neurons (see Discussion for additional details). Full-length APP is internalized from the presynaptic plasmalemma via clathrin-coated vesicles, together with recycling synaptic vesicle integral membrane proteins. Presynaptic clathrin-coated vesicles deliver internalized APP and recycling synaptic vesicle membrane proteins to axonal early endosomes, where sorting takes place. Internalized APP is subsequently delivered by retrograde vesicular transport to the neuronal soma. APP does not appear to use the WGA/lectin internalization pathway for retrograde transport to the neuronal soma, but it is not clear whether APP and lectins (bound to cell-surface glycoproteins) are internalized initially via a common pool of clathrin-coated vesicles. ⦚, APP; |, synaptotagmin I; ⦍, cell-surface glycoproteins; •, lectins; ⊥, clathrin triskelions; + and − ends of axonal microtubules are indicated.

delivered from presynaptic clathrin-coated vesicles to axonal early endosomes. After sorting, APP is apparently transported retrogradely to neuronal soma, consistent with our findings in hippocampal neurons (Yamazaki et al., 1995). Endocytosed APP and WGA/lectins (Trojanowski and Gonatas, 1983) do not appear to use a common compartment for retrograde transport to neuronal soma, but it is not clear from our data whether APP and WGA are internalized initially via a common pool of clathrin-coated vesicles. No co-distribution of internalized Syt_{lum}–Abs and internalized WGA was detected previously in hippocampal neurons (Matteoli et al., 1992), consistent with our observations. In future studies, confirmation of our immunofluorescence findings, particularly our finding that recycling synaptic vesicle membrane proteins and endocytosed APP co-localize in axons, will be obtained by quantitative immunoelectron microscopy. Currently, we are preparing adenoviral expression vectors for human APP to facilitate future trafficking studies using cultured neurons. Our finding that endocytosed APP is apparently delivered, at least in part, to potentially acidic, V-ATPase-containing compartments will also be explored in future studies using transiently transfected cultured neurons. This last finding raises the possibility that vacuolar acidification participates in sorting or proteolytic processing of APP endocytosed from the neuronal cell surface (see Myers and Forgac, 1993). Selective V-ATPase inhibitors (e.g., bafilomycin A1) have been shown to affect proteolytic processing of wild-type and mutant APP isoforms in non-neuronal cells (Haass et al., 1995; Perez et al., 1996).

Our results provide additional biochemical evidence that an obligate sorting compartment, presumably the axonal early endosome, participates in the regeneration of synaptic vesicles during exo/endocytotic recycling at nerve terminals (see Mundigl and DeCamilli, 1994). Biochemical evidence for a sorting endosomal intermediate in the synaptic vesicle recycling pathway also derives from the finding by Fischer von Mollard et al. (1994) that the early endosomal marker rab5a is present in a subpopulation of synaptic vesicles purified from rat brain. However, our results do not preclude concurrent "kiss-and-run" recycling (Fesce et al., 1994), whereby synaptic vesicles deliver their contents via transient fusion pores. Moreover, APP is now, to our knowledge, the first demonstrated example of an integral plasmalemmal protein that is internalized with recycling synaptic vesicle membrane proteins but is subsequently sorted away from synaptic vesicles. Two other plasmalemmal proteins, namely SNAP-25 and syntaxin I, are enriched in presynaptic clathrin-coated vesicles and thus appear to also recycle with synaptic vesicle membrane proteins (Walch-Solimena et al., 1995). However, SNAP-25 and syntaxin I are components of the synaptic vesicle exocytotic fusion apparatus (Sudhof, 1995) and are also enriched in synaptic vesicles (Walch-Solimena et al., 1995). APP, despite its enrichment in presynaptic clathrin-coated vesicles, is apparently neither a synaptic vesicle protein nor a known component of the synaptic vesicle exocytotic fusion apparatus. Other axonal cell-surface proteins besides APP, such as neurotrophin receptors (Ehlers et al., 1995), may use a similar pathway for retrograde transport to neuronal soma. Endocytosis from the presynaptic plasmalemma via a common pool of clathrin-coated vesicles, followed by sorting away from synaptic vesicles, may actually represent the principal route for internal-

ization and retrograde transport of axonal cell-surface receptors, but this remains an interesting speculation.

On the basis of these as well as previous (Yamazaki et al., 1995) studies, we propose the following model for endocytotic trafficking of APP in central neurons (Fig. 10). Full-length APP and recycling synaptic vesicle integral membrane proteins are internalized together from the presynaptic plasmalemma via clathrin-coated vesicles. Presynaptic clathrin-coated vesicles deliver APP and recycling synaptic vesicle membrane proteins to the axonal early endosomal compartment, where sorting takes place. It is not presently known whether a portion of the APP delivered to the early endosomal compartment recycles to the presynaptic plasma membrane. After sorting, APP is transported retrogradely to the somatodendritic domain. The organelle that transports APP retrogradely is currently unknown but may correspond to the multivesicular body-like structure described previously in hippocampal neurons (Parton et al., 1992). The fate of APP delivered retrogradely to the somatodendritic domain is also poorly understood. A portion of this APP is sorted transcytotically to the somatic surface (Simons et al., 1995; Yamazaki et al., 1995), but delivery to degradative late endosomal/lysosomal compartments probably also occurs. APP does not appear to use the WGA/lectin internalization pathway (Trojanowski and Gonatas, 1983) for retrograde transport to the somatodendritic domain, but it is not known whether APP and lectins (bound to cell-surface glycoproteins) are internalized initially via a common pool of clathrin-coated vesicles.

The relationship between neuronal APP endocytosis and the molecular pathogenesis of Alzheimer's disease remains unclear. Internalization of cell-surface APP contributes to the extracellular release of Aβ in non-neuronal cells (Koo and Squazzo, 1994). Moreover, amyloidogenic processing of APP including release of Aβ have been demonstrated recently in cultured neurons (Simons et al., 1996; Turner et al., 1996). In light of these findings, we argue that further elucidation of endocytotic APP trafficking and processing in neurons will contribute to our understanding of the pathogenesis of this disease.

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