

## Novel Effects of FCCP [Carbonyl Cyanide *p*-(Trifluoromethoxy)phenylhydrazone] on Amyloid Precursor Protein Processing

Bruce P. Connop, \*Robert L. Thies, †Konrad Beyreuther, †Nobuo Ida, and Peter B. Reiner

Kinsmen Laboratory of Neurological Research, Department of Psychiatry; and \*Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, British Columbia, Canada; and †Center for Molecular Biology, University of Heidelberg, Heidelberg, Germany

**Abstract:** Amyloidogenic processing of the  $\beta$ -amyloid precursor protein (APP) has been implicated in the pathology of Alzheimer's disease. Because it has been suggested that catabolic processing of the APP holoprotein occurs in acidic intracellular compartments, we studied the effects of the protonophore carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) and the H<sup>+</sup>-ATPase inhibitor bafilomycin A1 on APP catabolism in human embryonic kidney 293 cells expressing either wild-type or "Swedish" mutant APP. Unlike bafilomycin A1, which inhibits  $\beta$ -amyloid production in cells expressing mutant but not wild-type APP, FCCP inhibited  $\beta$ -amyloid production in both cell types. Moreover, the effects of FCCP were independent of alterations in total cellular APP levels or APP maturation, and the concentrations used did not alter either cellular ATP levels or cell viability. Bafilomycin A1, which had no effect on  $\beta$ -amyloid production in wild-type cells, inhibited endocytosis of fluorescent transferrin, whereas concentrations of FCCP that inhibited  $\beta$ -amyloid production in these cells had no effect on endosomal function. Thus, in wild-type-expressing cells it appears that the  $\beta$ -amyloid peptide is not produced in the classically defined endosome. Although bafilomycin A1 decreased  $\beta$ -amyloid release from cells expressing mutant APP but not wild-type APP, it altered lysosomal function in both cell types, suggesting that in normal cells  $\beta$ -amyloid is not produced in the lysosome. Although inhibition of  $\beta$ -amyloid production by bafilomycin A1 in mutant cells may occur via changes in endosomal/lysosomal pH, our data suggest that FCCP inhibits wild-type  $\beta$ -amyloid production by acting on a bafilomycin A1-insensitive acidic compartment that is distinct from either the endosome or the lysosome. **Key Words:**  $\beta$ -Amyloid precursor protein— $\beta$ -Amyloid—Carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone—Acidic compartment—Endosome—Lysosome. *J. Neurochem.* **72**, 1457–1465 (1999).

Alzheimer's disease (AD) pathology is characterized by the widespread distribution of intracellular neurofibrillary tangles and the presence of extracellular senile plaques (Hardy, 1997; Selkoe, 1997). The principal com-

ponent of the senile plaque is the 4-kDa  $\beta$ -amyloid peptide (A $\beta$ ), which is between 39 and 43 amino acids in length and is formed by endoproteolysis of the  $\beta$ -amyloid precursor protein (APP) (Kitaguchi et al., 1988; Ponte et al., 1988; Tanzi et al., 1988; Weidemann et al., 1989). Alternative splicing of APP mRNA generates several different isoforms of APP, and in neurons the predominant isoform is 695 amino acids in length (APP<sub>695</sub>) (Kitaguchi et al., 1988; Ponte et al., 1988; Tanzi et al., 1988). After APP is synthesized, it traverses the endoplasmic reticulum and *trans*-Golgi network, where it becomes *N*- and *O*-glycosylated as well as tyrosine-sulfated (Weidemann et al., 1989). The mature APP holoprotein can then be catabolized in several cellular compartments to produce either amyloidogenic fragments containing the intact A $\beta$  sequence or nonamyloidogenic fragments cleaved within the A $\beta$  sequence (Sisodia and Price, 1995; Selkoe et al., 1996).

APP is expressed and constitutively catabolized in most cells (Sisodia and Price, 1995; Selkoe et al., 1996). The dominant catabolic pathway appears to be cleavage of APP within the A $\beta$  sequence by an enzyme provisionally termed  $\alpha$ -secretase, leading to release of a soluble ectodomain fragment known as APPs $\alpha$  ( $\alpha$ -secretase-cleaved N-terminal ectodomain of APP) (Esch et al., 1990; Sisodia, 1992). In contrast to this nonamyloido-

---

Resubmitted manuscript received November 5, 1998; accepted November 19, 1998.

Address correspondence and reprint requests to Dr. P. B. Reiner at Kinsmen Laboratory of Neurological Research, Department of Psychiatry, 2255 Westbrook Mall, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3.

**Abbreviations used:** A $\beta$ ,  $\beta$ -amyloid peptide; AD, Alzheimer's disease; APP,  $\beta$ -amyloid precursor protein; APPs $\alpha$  and APPs $\beta$ ,  $\alpha$ - and  $\beta$ -secretase-cleaved N-terminal ectodomain of  $\beta$ -amyloid precursor protein, respectively; baf A1, bafilomycin A1; C99, C-terminal fragment that contains the entire  $\beta$ -amyloid peptide sequence; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; HEK, human embryonic kidney; NaA/DG, sodium azide and 2-deoxy-D-glucose; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tf-F, transferrin–fluorescein; Tf-Tx, transferrin–Texas red.

genic pathway, APP can also be cleaved by unidentified enzymes known as  $\beta$ - and  $\gamma$ -secretases at the N and C termini of A $\beta$ , respectively, followed by release of A $\beta$  into the extracellular space (Haass and Selkoe, 1993; Seubert et al., 1993). Akin to the release of APP $\alpha$  following  $\alpha$ -secretase cleavage of APP,  $\beta$ -secretase cleavage of APP results in the release of a truncated soluble ectodomain fragment known as APP $\beta$  ( $\beta$ -secretase-cleaved N-terminal ectodomain of APP). In addition to the release of APP $\alpha$ , APP $\beta$ , and A $\beta$ , several different C-terminal fragments are generated as processing intermediates (Caporaso et al., 1992; Estus et al., 1992; Golde et al., 1992; Haass et al., 1992; Knops et al., 1992), including an intracellular  $\sim$ 12-kDa C-terminal fragment that contains the entire A $\beta$  sequence (C99) produced following  $\beta$ - but not  $\gamma$ -secretase activity (Checler, 1995).

Based on the inhibition of APP catabolism by agents that are known to disrupt intracellular pH and/or acidic organelles, it has been suggested that proteolytic cleavage of APP occurs within acidic compartments of the cell (Caporaso et al., 1992; Golde et al., 1992; Haass et al., 1992, 1993, 1995; Knops et al., 1992, 1995; Shoji et al., 1992; Schrader-Fischer and Paganetti, 1996). For example, exposure of cells to either the monovalent ionophore monensin or high concentrations of NH<sub>4</sub>Cl decreases APP proteolytic processing accompanied by concomitant alterations in full-length cellular APP (Caporaso et al., 1992; Golde et al., 1992; Haass et al., 1993). Similarly, the vacuolar H<sup>+</sup>-ATPase inhibitor bafilomycin A1 (baf A1) has been reported to produce alterations in APP catabolism that are both cell type- and APP mutation-specific (Haass et al., 1995; Knops et al., 1995; Schrader-Fischer and Paganetti, 1996). However, interpretation of these data is complicated by the fact that these manipulations appear to alter both maturation and cleavage of APP. In preliminary experiments we found that the protonophore carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) had no effect on APP maturation and reasoned that this compound might be a useful tool to address the issue of APP catabolism in acidic cellular compartments. Toward that end, we have now carried out a detailed analysis of the effects of FCCP and baf A1 on both amyloidogenic and nonamyloidogenic APP catabolism in human embryonic kidney (HEK) 293 cells expressing either wild-type or "Swedish" mutant APP. Our data suggest that production of A $\beta$  from wild-type APP occurs in an acidic intracellular compartment that is distinct from the endosome or the lysosome yet sensitive to the actions of FCCP.

## MATERIALS AND METHODS

### Cell lines and drug treatments

HEK 293 cells stably transfected with either wild-type APP<sub>695</sub> (K695 cells) or APP carrying the AD-linked double "Swedish" mutation (K695sw cells) (Citron et al., 1996) were cultured in Dulbecco's modified Eagle's medium supplemented with sodium pyruvate (1 mM) and 10% fetal calf serum as

previously described (Mills et al., 1997). Cells were plated at  $1 \times 10^6$  cells per well in 35-mm<sup>2</sup> culture dishes (Corning) 24 h before drug exposure. Preceding the addition of drugs, cultures were washed once with warm phosphate-buffered saline (37°C) and then exposed to various treatments in Dulbecco's modified Eagle's medium supplemented with sodium pyruvate (1 mM). Cultures were then exposed to control medium, vehicle, or varying concentrations of FCCP (5  $\mu$ M, 500 nM, or 50 nM in 0.05% ethanol) or baf A1 (1  $\mu$ M in dimethyl sulfoxide). Four hours after addition of drugs, the medium was removed, and the cultures were washed once with phosphate-buffered saline and then harvested in 100  $\mu$ l of ice-cold lysis buffer containing 20 mM MOPS (pH 7.2), 5 mM EDTA, 0.01% Nonidet P-40, 75 mM  $\beta$ -glycerol phosphate, 1 mM dithiothreitol, 1 mM sodium vanadate, 0.5 mM phosphatase substrate, 1 mM phenylmethylsulfonyl fluoride, and 20  $\mu$ g/ml each of leupeptin, aprotinin, and pepstatin.

### Detection of intra- and extracellular APP catabolic fragments

To examine extracellular APP fragments, the medium was retained and centrifuged at 4°C for 10 min at 16,000 *g* followed by precipitation of extracellular protein by 10% trichloroacetic acid (Mills et al., 1997). Extracellular APP $\alpha$  and A $\beta$  were quantified, respectively, by 10% Tris-glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and 16% Tris-tricine SDS-PAGE western blot analysis using the monoclonal antibody WO-2 generated against the first 16 amino acids of the N-terminal region of A $\beta$  (Ida et al., 1996). Similarly, extracellular wild-type APP $\beta$  was quantified by 10% Tris-glycine SDS-PAGE western blot analysis using the polyclonal antibody 192wt generated against amino acids 590–596 of wild-type APP (Knops et al., 1995). For examination of intracellular APP fragments, cultures were harvested in ice-cold lysis buffer and sonicated on ice for 8 s. Intracellular C99 and total APP were quantified, respectively, by 16% Tris-tricine western blot analysis using the WO-2 antibody and 10% Tris-glycine western blot analysis using an anti-APP N-terminal antibody (22C11; Boehringer Mannheim, Laval, QC, Canada). Immunoreactive bands were visualized using ECL detection (Amersham, Oakville, ON, Canada) and analyzed by standard densitometric techniques as described (Mills et al., 1997).

### Quantification of cellular ATP levels and assessment of cell viability

K695sw cells were maintained, exposed to drugs, and harvested as described above with the exception that cells were plated at 500,000 cells per plate and the lysis buffer used consisted of 25 mM Tricine (pH 7.8), 270 mM sucrose, and 1 mM EDTA. After harvesting, samples were centrifuged at 4°C for 10 min at 16,000 *g* and assayed for ATP content using a luciferase/luciferin ATP determination kit (A-6608; Molecular Probes, Eugene, OR, U.S.A.). ATP-dependent luminescence was quantified using an Optocomp II luminometer (MGM Instruments, Hamden, CT, U.S.A.), and ATP levels were determined by extrapolation from a standard curve. Cellular protein levels were quantified using the BCA protein assay (Pierce, Rockford, IL, U.S.A.), and cellular ATP levels were expressed as micromoles per microgram of protein. As a positive control, the effect of combined treatment with various concentrations of sodium azide and 2-deoxy-D-glucose (NaA/DG) was also examined in these studies.

Cell viability was quantified based on the exclusion of the membrane-impermeant probe YO-PRO (Molecular Probes).

K695sw cells were maintained and exposed to vehicle or various concentrations of FCCP as mentioned above with the exception that cells were plated at a density of 20,000 cells per well in 96-well plates (Falcon). Twenty-four hours after plating, cells were exposed to various treatments in Dulbecco's modified Eagle's medium supplemented with sodium pyruvate (1 mM). At the same time as drug exposures, YO-PRO (4  $\mu$ M) was added to each well, and its uptake was quantified every 30 min for 1 day at 37°C using a Cytofluor 2350 fluorometric plate reader (Millipore, Mississauga, ON, Canada). As a positive control, all wells were exposed to 0.1% Triton X-100 at the end of the experiment.

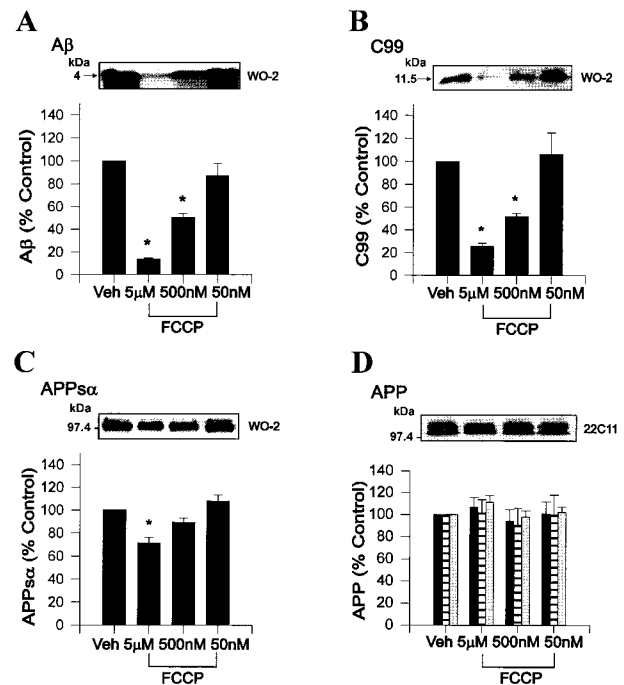
### Quantification and visualization of endosomal and lysosomal function

Endosomal function was assessed by fluorometric quantification of the receptor-mediated uptake of both Texas red- and fluorescein-labeled transferrin (Molecular Probes) (Johnson et al., 1993; van Weert et al., 1995; Presley et al., 1997). K695 and K695sw cells were maintained and exposed to various treatments as described with the exception that cells were plated at a density of 250,000 cells per well in 24-well plates (Corning). Twenty-four hours after plating, cells were exposed to various treatments in Dulbecco's modified Eagle's medium supplemented with sodium pyruvate (1 mM) and 10% fetal bovine serum. Coincident with exposure to drugs, both transferrin-Texas red (Tf-Tx) and transferrin-fluorescein (Tf-F) (10  $\mu$ l/ml of medium of 5 mg/ml stocks in phosphate-buffered saline) were added to each well and incubated for 4 h at 37°C. Each well was then gently washed three times with warm medium, and uptake of fluorescent probes was quantified at 37°C using a Cytofluor 2350 fluorometric plate reader (Millipore). The non-pH-dependent Tf-Tx fluorescence and the pH-dependent Tf-F fluorescence were quantified using excitation filters of 590 (bandwidth = 20 nm) and 485 nm (bandwidth = 20 nm) with emission filters of 645 (bandwidth = 40 nm) and 530 nm (bandwidth = 25 nm), respectively.

Similarly, lysosomal function was assayed by uptake of the fluorescent probe Lysosensor Yellow/Blue DND-160 (Molecular Probes) (Hurwitz et al., 1997). Assay of Lysosensor uptake was performed in an identical manner to that of the fluorescently labeled transferrin conjugates with the exception that addition of 7  $\mu$ l of Lysosensor/ml of medium was obtained from a 1 mM stock solution in dimethyl sulfoxide. The low-pH-dependent blue fluorescence and the high-pH-dependent yellow fluorescence were quantified using an excitation filter of 360 nm (bandwidth = 40 nm) and emission filters of 460 (bandwidth = 40 nm) and 530 nm (bandwidth = 25 nm), respectively. At the end of the experiment, Triton X-100 (0.1%) and the membrane-impermeant probe propidium iodide were added to each well to standardize fluorescence measurements to total cell number. Propidium iodide fluorescence was determined using an excitation filter of 530 nm (bandwidth = 25 nm) and an emission filter of 620 nm (bandwidth = 40 nm). For all three probes examined, relative fluorescence was obtained by subtraction of values from sister wells that had not been exposed to fluorophores. Exposure of blank wells (no cells) to probes and subsequent washing produced less signal than that observed from cellular autofluorescence.

As positive controls in both transferrin and Lysosensor uptake experiments, sister wells were exposed to NH<sub>4</sub>Cl (20 mM), which is known to affect acidic cellular compartments.

For the direct visualization of the cellular distribution of the Tf-Tx, Tf-F, and Lysosensor probes, K695 cells were grown, treated, and exposed to fluorophores as above with the excep-



**FIG. 1.** FCCP inhibits processing of mutant APP. K695sw cells were exposed to various concentrations of FCCP for 4 h, and APP catabolic fragments were quantified by western blot analysis. A representative western blot is shown at the top of each panel with the probing antibody indicated on the right and quantification given below. The effects of FCCP on (A) A $\beta$  release, (B) cellular C99, (C) APPs $\alpha$  release, and (D) total cellular APP are depicted. In D, solid, hatched, and shaded columns represent total, mature, and immature APP, respectively. Data are mean  $\pm$  SEM (bars) values ( $n = 3-5$ ). \* $p < 0.05$ . Veh, vehicle.

tion that cells were plated in 35-mm<sup>2</sup> plates (Corning) at a density of 750,000 cells per dish. Cultures were visualized and photographed through a water immersion lens (63 $\times$  magnification) using an Axiophot fluorescent microscope (Zeiss, Germany) with the same excitation and emission filters as described above.

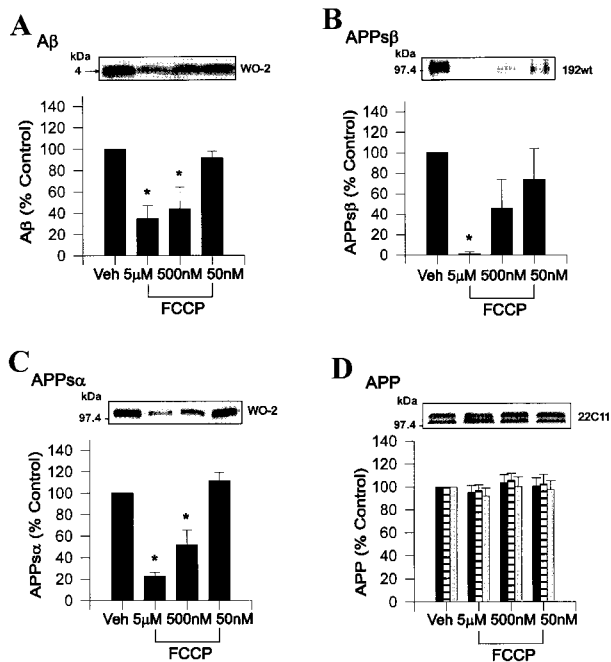
### Statistical analysis

Statistical significance was determined using an ANOVA with Tukey's post hoc analysis. Values of  $p < 0.05$  were considered significant. Data are expressed as mean  $\pm$  SEM values. Sequential western blots are representative of between three and five separate samples of which at least two were taken from a different trial. Four to six repetitions were used for each treatment group in experiments quantifying cellular ATP levels, cell viability, and endosomal/lysosomal function.

## RESULTS

### FCCP inhibits APP catabolism but not maturation

We began our study using HEK 293 cells stably transfected with APP bearing the "Swedish" mutation (K695sw cells), as detection of the APP fragments A $\beta$  and C99 is relatively straightforward in this cell line (Citron et al., 1996). Exposure of K695sw cells to the protonophore FCCP resulted in a concentration-depen-



**FIG. 2.** FCCP inhibits processing of wild-type APP. K695 cells were exposed to various concentrations of FCCP for 4 h, and APP catabolic fragments were quantified by western blot analysis. A representative western blot is shown at the top of each panel with the probing antibody indicated on the right and quantification given below. The effects of FCCP on (A) Aβ release, (B) APPsβ release, (C) APPsα release, and (D) total cellular APP are depicted. In D, solid, hatched, and shaded columns represent total, mature, and immature APP, respectively. Data are mean ± SEM (bars) values (n = 3–5). \*p < 0.05. Veh, vehicle.

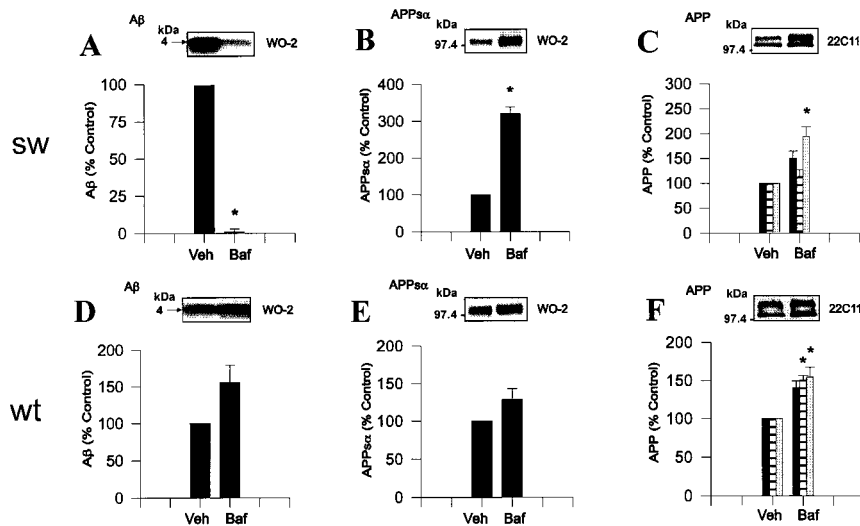
dent decrease in both Aβ release (Fig. 1A) and the formation of the cell-associated C99 fragment (Fig. 1B). Production of the ectodomain fragment APPsα was only affected by exposure to the highest concentration of FCCP (5 μM; Fig. 1C), whereas neither total cellular

levels of APP nor the maturation of APP via N/O'-linked glycosylation appeared to be affected by any concentration of FCCP used (Fig. 1D).

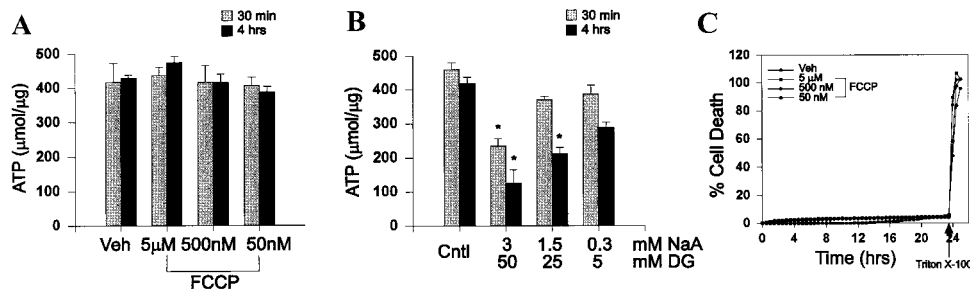
In previous studies it was shown that the H<sup>+</sup>-ATPase inhibitor baf A1 reduced Aβ production in K695sw cells but not in cells expressing wild-type APP (Haass et al., 1995; Knops et al., 1995; Schrader-Fischer and Paganetti, 1996). As both baf A1 and FCCP alter the pH of acidic compartments, albeit via different mechanisms, we tested the effects of FCCP on APP catabolism in HEK 293 cells stably transfected with wild-type APP<sub>695</sub> (K695 cells). In contrast to the reported lack of effect of baf A1 on Aβ production in these cells, treatment with FCCP clearly inhibited Aβ and APPsβ production in a concentration-dependent manner (Fig. 2A and B). Moreover, in contrast to the modest effects of FCCP on APPsα release from K695sw cells, treatment of K695 cells with FCCP also resulted in a marked concentration-dependent decrease in APPsα production (Fig. 2C). The effect of FCCP on Aβ, APPsβ, and APPsα production did not arise secondary to effects on protein maturation, as both total levels of cellular APP and maturation of APP appeared to be unaffected (Fig. 2D). Because of the low levels of C99 found in K695 cells, we were unable to quantify accurately the effects of FCCP on this APP catabolic fragment in wild-type cells.

**Baf A1 decreases Aβ production from mutant but not wild-type APP**

To confirm previous reports that treatment of HEK 293 cells with baf A1 results in selective reductions in amyloidogenic processing of “Swedish” mutant but not wild-type APP (Haass et al., 1995; Knops et al., 1995; Schrader-Fischer and Paganetti, 1996), we examined the effects of treatment with baf A1 (1 μM) on these two cell lines (Fig. 3). Consistent with previous reports, exposure of K695sw cells to baf A1 led to a potent reduction in Aβ production (>95%; Fig. 3A) and increased APPsα in the medium by >200% (Fig. 3B). In contrast, treatment of



**FIG. 3.** Baf A1 decreases Aβ release from cells expressing mutant but not wild-type APP. K695sw (SW; A–C) or K695 cells (wt; D–F) were exposed to 1 μM baf A1 for 4 h, and levels of APP catabolic fragments were quantified by western blot analysis. A representative western blot is shown at the top of each panel with the probing antibody indicated on the right and quantification given below. The effects of baf A1 on (A and D) Aβ release, (B and E) APPsα release, and (C and F) total cellular APP are depicted. In C, solid, hatched, and shaded columns represent total, immature, and mature APP, respectively. Data are mean ± SEM (bars) values (n = 3–4). \*p < 0.05. Veh, vehicle.



**FIG. 4.** FCCP does not deplete cellular ATP levels or decrease cell viability. K695sw cells were exposed to various concentrations of FCCP for either 30 min (shaded columns) or 4 h (solid columns), and cellular ATP levels were quantified by luciferin/luciferase bioluminescence assay (**A**). Veh, vehicle. **B**: As a positive control, the ability of NaA/DG to deplete cellular ATP levels can be seen. Cntl, control. **C**: K695sw cells were exposed to Veh (circles) or 5  $\mu$ M (squares), 500 nM (diamonds), or 50 nM (hexagons) FCCP for 24 h, and cell viability was assessed by the YO-PRO exclusion method. At the end of the experiment, 0.1% Triton X-100 was added to each well as a positive control. Data are mean  $\pm$  SEM (bars) values ( $n = 4-6$ ). \* $p < 0.05$ .

K695 cells with baf A1 did not significantly alter either A $\beta$  or APP $\alpha$  release (Fig. 3D and E). Analysis of cellular APP levels in both cell types revealed that exposure to baf A1 led to increases in the amount of “mature” APP and in wild-type cells to an increase in “immature” APP levels as well (Fig. 3C and F).

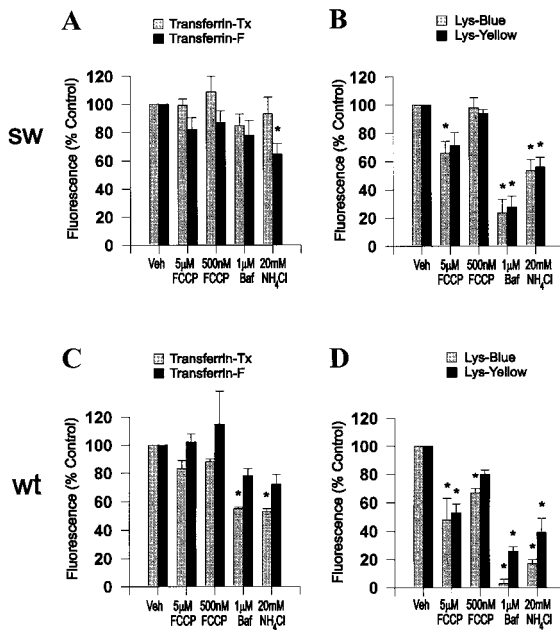
#### FCCP does not inhibit ATP formation and is nontoxic

Because mitochondria utilize the proton gradient as a driving force for ATP generation, it has long been thought that FCCP, and the class of mobile ionophores of which it is a member, may uncouple oxidative phosphorylation by acting as protonophores in mitochondrial membranes (Benz and McLaughlin, 1983; Luvisetto et al., 1987). Indeed, it has been shown that low concentrations of FCCP (1  $\mu$ M) are able to alter mitochondrial membrane potential (Maechler et al., 1997) but that prolonged application (2 h) of high concentrations (30  $\mu$ M) are required to decrease cellular ATP levels modestly (Luo et al., 1997). To determine whether the concentrations of FCCP that altered APP catabolism were sufficient to change ATP levels in K695sw cells, we measured ATP levels following exposure of these cells to various concentrations of FCCP (Fig. 4A). As a positive control, the effect of a combination treatment with NaA/DG on cellular ATP levels was also evaluated (Fig. 4B). As expected, treatment of K695sw cells with NaA/DG resulted in a concentration-dependent decrease in cellular ATP levels. In contrast, treatment with FCCP did not alter cellular ATP levels at any of the concentrations measured. In addition to the lack of effect of FCCP on ATP levels, the question as to whether FCCP was toxic was also addressed by using YO-PRO exclusion staining. It was observed that a 24-h exposure of K695sw cells to any concentration of FCCP did not reduce cell viability (Fig. 4C). Taken together, these data suggest that the effects of FCCP on APP catabolism are independent of secondary effects on oxidative phosphorylation or the result of reduced cell viability.

#### Effects of FCCP and baf A1 on endosomal and lysosomal function

Based on the lack of effect of FCCP on cellular ATP levels, we sought to ascertain the specific acidic compartment(s) through which deacidification by FCCP could account for a decrease in APP catabolism. Endosomal function was assessed by the receptor-mediated uptake of both Tf-Tx and Tf-F, which are non- and pH-sensitive endosomal probes, respectively. The use of these probes to measure endosomal function has been well characterized (Johnson et al., 1993; van Weert et al., 1995; Presley et al., 1997), and the pH dependency of the Tf-F signal is based on the known sensitivity of fluorescein to low pH, whereby its fluorescent signal is diminished. Thus, decreases in Tf-Tx uptake represent decreased receptor-mediated internalization of transferrin and/or total endosomal number, whereas increases in Tf-F fluorescence may be indicative of endosomal deacidification. FCCP did not exert a significant effect on endosomal function in either K695 or K695sw cells (Fig. 5A and C). In contrast, treatment of K695 cells with baf A1 and of both cell lines with NH<sub>4</sub>Cl significantly reduced endosomal function as measured by the uptake of Tf-Tx/Tf-F. Representative photomicrographs of FCCP-, baf A1-, and NH<sub>4</sub>Cl-induced changes in Tf-Tx and Tf-F cellular fluorescence in K695 cells are depicted in Fig. 6.

Through the use of a similar technique, putative lysosomal function in both cell lines was assessed by quantification of Lysosensor Yellow/Blue DND-160 uptake. Through the use of this fluorophore, the number of lysosomes that possess both low and high acidity can be quantified based on the ability of this probe to exhibit a predominantly blue fluorescent emission spectrum in less acidic lysosomes and a yellow emission spectrum in more highly acidic lysosomes. Although it is probable that a proportion of this probe may partition into other acidic compartments, it is thought that Lysosensor predominantly accumulates in lysosomes of somatic cells, and its staining pattern has been reported to resemble that of LAMP-1 and LAMP-2, markers for lysosomes (Hurwitz et al., 1997). A slight drawback with this technique



**FIG. 5.** Effects of FCCP and baf A1 on endosomal and lysosomal function. K695sw (sw) and K695 (wt) cell lines were exposed to vehicle (Veh), FCCP (5  $\mu$ M or 500 nM), baf A1 (1  $\mu$ M), or NH<sub>4</sub>Cl (20 mM) for 4 h, and endosomal and lysosomal function was assessed. **A** and **C**: Endosomal function was evaluated by fluorescent quantification of both the non-pH-dependent probe, Tf-Tx (shaded columns), and the pH-sensitive conjugate, Tf-F (solid columns). **B** and **D**: Similarly, lysosomal function was measured by fluorescent quantification of both the blue (shaded columns) and yellow (solid columns) emission spectra of Lysosensor, which are more predominant in less and more acidic lysosomes, respectively. Data are mean  $\pm$  SEM (bars) values ( $n = 4-6$ ). \* $p < 0.05$ .

is the presence of a small amount of overlap between the excitation and emission spectra, which are originating from the same parent molecule. However, as with the effects of baf A1 and NH<sub>4</sub>Cl on endosomal function, it was observed that both of these agents produced profound reductions in lysosomal function as seen by reductions in the number and/or function of both low and highly acidic lysosomes (Fig. 5B and D). Similarly, FCCP also led to reductions in lysosomal function, albeit not as profound as those observed with baf A1 or NH<sub>4</sub>Cl. Representative photomicrographs of FCCP-, baf A1-, and NH<sub>4</sub>Cl-induced changes in Lysosensor blue and yellow cellular fluorescence in K695 cells are depicted in Fig. 6.

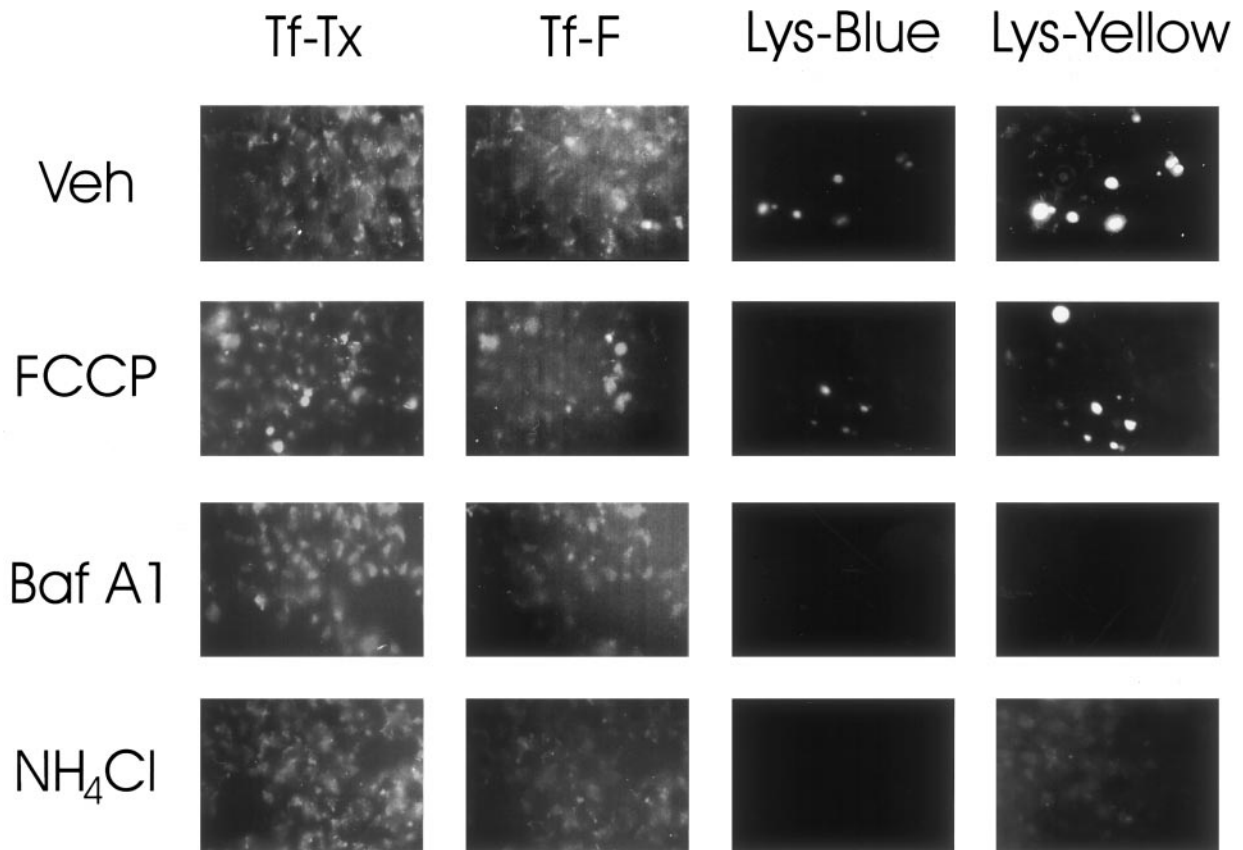
## DISCUSSION

The two major findings of this study are (a) that FCCP is able to inhibit APP catabolism independent of overt alterations in either total APP synthesis or maturation and (b) that  $\beta$ -amyloid production from wild-type APP appears to occur in an FCCP-sensitive acidic compartment that is not what is classically defined as either the endosome or the lysosome. Although other agents capa-

ble of altering intracellular pH gradients have been shown to alter APP catabolism (Caporaso et al., 1992; Golde et al., 1992; Haass et al., 1992, 1993, 1995; Knops et al., 1992, 1995; Shoji et al., 1992; Schrader-Fischer and Paganetti, 1996), to the best of our knowledge FCCP is the only compound that does so at concentrations that do not overtly affect either synthesis or maturation of APP. As such, the use of FCCP represents an opportunity to better define the intracellular compartments in which catabolism of APP occurs.

A large body of evidence suggests that production of A $\beta$  occurs in an acidic intracellular compartment. Thus, the macrolide antibiotic baf A1, which inhibits vacuolar H<sup>+</sup>-ATPases (Bowman et al., 1988), the monovalent ionophore monensin, which depletes H<sup>+</sup>/Na<sup>+</sup>/K<sup>+</sup> gradients and inhibits Golgi/lysosomal function (Tartakoff, 1983), and the ammonia donor NH<sub>4</sub>Cl all inhibit A $\beta$  production (Caporaso et al., 1992; Golde et al., 1992; Haass et al., 1992, 1993, 1995; Knops et al., 1992, 1995; Shoji et al., 1992; Schrader-Fischer and Paganetti, 1996). Although each of these compounds has effects on APP maturation, the fact that three agents capable of altering intracellular pH reduce A $\beta$  production is consistent with the hypothesis that A $\beta$  is generated in an intracellular acidic compartment. Our finding that the protonophore FCCP reduces A $\beta$  production without altering APP maturation or synthesis further strengthens the hypothesis that the enzymes  $\beta$ - and/or  $\gamma$ -secretase require an acidic environment in which to operate. With respect to APPs $\alpha$ , both monensin and NH<sub>4</sub>Cl reduce production of this APP catabolite (Caporaso et al., 1992; Golde et al., 1992; Haass et al., 1992; Knops et al., 1992; Shoji et al., 1992), whereas baf A1 does not (Knops et al., 1995; Haass et al., 1995; Schrader-Fischer and Paganetti, 1996), leaving open the question of whether  $\alpha$ -secretase is localized to an acidic compartment. Our observation that FCCP decreases APPs $\alpha$  release from cells expressing both wild-type and mutant APP, again without affecting synthesis or maturation of APP, suggests that this enzyme is indeed localized to an acidic compartment. Because FCCP but not baf A1 inhibited APPs $\alpha$  production from both cell types, we propose that  $\alpha$ -secretase cleavage of wild-type APP and to a lesser degree mutant APP occurs in a baf A1-insensitive acidic compartment. Given the potential of the enzymes  $\alpha$ -,  $\beta$ -, and  $\gamma$ -secretase as therapeutic targets in AD, identifying the acidic compartments in which they act is of considerable importance.

Previous studies have reported that the actions of baf A1 are APP type-specific insofar as inhibition of H<sup>+</sup>-ATPase does not decrease either APPs $\alpha$  or A $\beta$  release in cells expressing wild-type APP yet selectively inhibits amyloidogenic processing in cells expressing the "Swedish" mutant form of APP (Haass et al., 1995; Knops et al., 1995; Schrader-Fischer and Paganetti, 1996). Our findings confirm these observations and for the first time show that baf A1 impairs the integrity of acidic compartments in both mutant and wild-type APP-expressing cells even though it only alters A $\beta$  production in the former. The observation that both baf A1 and FCCP



**FIG. 6.** Photomicrographs represent treatment groups from Fig. 5C and D. Representative photographs of each treatment group from Fig. 5C and D are illustrated with each column of panels depicting each of the four quantification groups. Rows are representative of each of the four cellular treatments: Veh, vehicle; FCCP, 5  $\mu$ M FCCP; Baf A1, 1  $\mu$ M baf A1; and NH<sub>4</sub>Cl, 20 mM NH<sub>4</sub>Cl. Lys, Lysosensor.  $\times$ 630.

impair lysosomal function as well as decrease the formation of C99, A $\beta$ , and other  $\beta$ -secretase cleavage products from cells expressing mutant APP (Haass et al., 1995; Knops et al., 1995; Schrader-Fischer and Paganetti, 1996) is in agreement with the notion that the  $\beta$ -secretase(s) that cleaves mutant APP is localized to a baf A1-sensitive acidic compartment such as the lysosome.

It has been suggested that baf A1 decreases the formation of A $\beta$  starting at Asp<sup>1</sup> in cells expressing both "Swedish" mutant and wild-type APP but that this decrease is masked in cells expressing wild-type APP by a compensatory increase in the formation of A $\beta$ -like peptides starting at N termini such as Val<sup>3</sup> (Haass et al., 1995). Based on these findings, it was suggested that the  $\beta$ -secretase responsible for cleavage of A $\beta$  at Asp<sup>1</sup> is localized in a baf A1-sensitive acidic compartment (Haass et al., 1995). Our data demonstrating that FCCP decreases the formation of both APPs $\beta$  and A $\beta$  in K695 cells suggest that  $\beta$ -secretase cleavage of wild-type APP does indeed occur in an acidic compartment but that this compartment is FCCP-sensitive and baf A1-insensitive.

Although the clathrin coated-pit endocytic pathway has been implicated in production of A $\beta$  following in-

ternalization of cell surface APP (Koo and Squazzo, 1994; Lai et al., 1995), our data suggest that the endosome is an unlikely site for wild-type A $\beta$  synthesis. Although FCCP potentially attenuated A $\beta$  synthesis, even the highest concentration used did not have an effect on endosomal function as measured using fluorescently labeled transferrin. As with its effect on lysosomes, baf A1 reduced receptor-mediated uptake of transferrin in cells expressing wild-type APP, presumably via altering endosomal pH, yet baf A1 had no significant effect on A $\beta$  production in these cells. Thus, FCCP reduces A $\beta$  production without affecting endosomal function, whereas baf A1 reduces endosomal function without altering production of A $\beta$ . Such a double dissociation implies that normal production of A $\beta$  does not occur in the endosome, as classically defined by transferrin uptake.

The evidence against a lysosomal site of A $\beta$  synthesis in wild-type cells is convincing. Irrespective of the ability of baf A1 and, to a lesser degree, FCCP to alter lysosomal function in both cell lines, the observation that FCCP reduced A $\beta$  production from both cells expressing the mutant and those expressing wild-type APP, whereas baf A1 did not decrease A $\beta$  formation from the latter,

clearly indicates that wild-type production of A $\beta$  occurs in a cellular compartment that is insensitive to the acidifying actions of baf A1. Given the evidence that lysosomes express the vacuolar H<sup>+</sup>-ATPase (Nelson, 1991), which is inhibited by baf A1 (Bowman et al., 1988), and our data demonstrating that application of baf A1 drastically reduces lysosomal function as measured using the Lysosensor probe, it seems fair to conclude that wild-type production of A $\beta$  does not occur in the lysosome.

One interpretation of our data is that FCCP inhibits catabolic processing of wild-type APP by acting on a baf A1-insensitive acidic compartment that is distinct from what is classically defined as either the endosome or the lysosome. As the endoplasmic reticulum and *trans*-Golgi compartments have been implicated in the generation of A $\beta$  (Xu et al., 1995, 1997; Hartmann et al., 1997; Wild-Bode et al., 1997), it seems reasonable to hypothesize that this represents the site of FCCP action. However, a direct test of this hypothesis is limited because current techniques do not permit the quantification of the pH and functional activity of these compartments in living cells. Moreover, even though FCCP is well known to be a protonophore, our data do not allow us to rule out other mechanisms of action. For example, it has recently been reported that in erythrocyte membranes FCCP stimulates the activity of aminophospholipid translocase (Sokal and Bartosz, 1998), a member of the P-type ATPases (Tang et al., 1996). These enzymes are thought to act as "flip-flops," moving aminophospholipids between the inner and outer leaflets of cellular membranes (Williamson and Schlegel, 1994; Dolis et al., 1997). Given the fact that both  $\alpha$ - and  $\beta$ -secretases act in close proximity to the membrane, it is entirely possible that their activity is altered by subtle changes in membrane composition. Identifying the mechanism by which FCCP alters APP processing may provide valuable insight into potential therapeutic strategies for AD.

**Acknowledgment:** This work was supported by the grants from the Alzheimer Society of Canada, the Medical Research Council (MRC) of Canada, and the Louise Brown Chair in Neuroscience. B.P.C. was supported by a postdoctoral fellowship from the Alzheimer Society of Canada. P.B.R. is an MRC Scientist. We thank Rouzbeh Shooshtarian and Anna Bahyrycz for technical assistance, Peter Hochachka for the use of the luminometer, Dennis Selkoe for the generous donation of K695 and K695sw cell lines, and Sukanto Sinha (Athena Neurosciences) for providing the 192wt antibody.

## REFERENCES

- Benz R. and McLaughlin S. (1983) The molecular mechanism of action of the proton ionophore FCCP (carbonylcyanide *p*-trifluoromethoxyphenylhydrazone). *Biophys. J.* **41**, 381–398.
- Bowman E. J., Siebers A., and Altendorf K. (1988) Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. *Proc. Natl. Acad. Sci. USA* **85**, 7972–7976.
- Caporaso G. L., Gandy S. E., Buxbaum J. D., and Greengard P. (1992) Chloroquine inhibits intracellular degradation but not secretion of Alzheimer  $\beta$ /A4 amyloid precursor protein. *Proc. Natl. Acad. Sci. USA* **89**, 2252–2256.
- Checler F. (1995) Processing of the  $\beta$ -amyloid precursor protein and its regulation in Alzheimer's disease. *J. Neurochem.* **65**, 1431–1444.
- Citron M., Diehl T. S., Capell A., Haass C., Teplow D. B., and Selkoe D. J. (1996) Inhibition of amyloid  $\beta$ -protein production in neural cells by the serine protease inhibitor AEBSF. *Neuron* **17**, 171–179.
- Dolis D., Moreau C., Zachowski A., and Devaux P. F. (1997) Aminophospholipid translocase and proteins involved in transmembrane phospholipid traffic. *Biophys. Chem.* **68**, 221–231.
- Esch F. S., Keim P. S., Beattie E. C., Blacher R. W., Culwell A. R., Oltersdorf T., McClure D., and Ward P. J. (1990) Cleavage of amyloid  $\beta$  peptide during constitutive processing of its precursor. *Science* **248**, 1122–1124.
- Estus S., Golde T. E., Kunishita T., Blades D., Lowery D., Eisen M., Usiak M., Qu X. M., Tabira T., Greenberg B. D., and Younkin S. G. (1992) Potentially amyloidogenic, carboxyl-terminal derivatives of the amyloid protein precursor. *Science* **255**, 726–728.
- Golde T. E., Estus S., Younkin L. H., Selkoe D. J., and Younkin S. G. (1992) Processing of the amyloid protein precursor to potentially amyloidogenic derivatives. *Science* **255**, 728–730.
- Haass C. and Selkoe D. J. (1993) Cellular processing of  $\beta$ -amyloid precursor protein and the genesis of amyloid  $\beta$ -peptide. *Cell* **75**, 1039–1042.
- Haass C., Koo E. H., Mellon A., Hung A. Y., and Selkoe D. J. (1992) Targeting of cell-surface  $\beta$ -amyloid precursor protein to lysosomes: alternative processing into amyloid-bearing fragments. *Nature* **357**, 500–503.
- Haass C., Hung A. Y., Schlossmacher M. G., Teplow D. B., and Selkoe D. J. (1993)  $\beta$ -Amyloid peptide and a 3-kDa fragment are derived by distinct cellular mechanisms. *J. Biol. Chem.* **268**, 3021–3024.
- Haass C., Capell A., Citron M., Teplow D. B., and Selkoe D. J. (1995) The vacuolar H(+)-ATPase inhibitor bafilomycin A1 differentially affects proteolytic processing of mutant and wild-type  $\beta$ -amyloid precursor protein. *J. Biol. Chem.* **270**, 6186–6192.
- Hardy J. (1997) Amyloid, the presenilins and Alzheimer's disease. *Trends Neurosci.* **20**, 154–159.
- Hartmann T., Bieger S. C., Bruhl B., Tienari P. J., Ida N., Allsop D., Roberts G. W., Masters C. L., Dotti C. G., Unsicker K., and Beyreuther K. (1997) Distinct sites of intracellular production for Alzheimer's disease A $\beta$  40/42 amyloid peptides. *Nat. Med.* **3**, 1016–1020.
- Hurwitz S. J., Terashima M., Mizunuma N., and Slapak C. A. (1997) Vesicular anthracycline accumulation in doxorubicin-selected U-937 cells: participation of lysosomes. *Blood* **89**, 3745–3754.
- Ida N., Hartmann T., Pantel J., Schroder J., Zerfass R., Forstl H., Sandbrink R., Masters C. L., and Beyreuther K. (1996) Analysis of heterogeneous A4 peptides in human cerebrospinal fluid and blood by a newly developed sensitive Western blot assay. *J. Biol. Chem.* **271**, 22908–22914.
- Johnson L. S., Dunn K. W., Pytowski B., and McGraw T. E. (1993) Endosome acidification and receptor trafficking: bafilomycin A1 slows receptor externalization by a mechanism involving the receptor's internalization motif. *Mol. Biol. Cell* **4**, 1251–1266.
- Kitaguchi N., Takahashi Y., Tokushima Y., Shiojiri S., and Ito H. (1988) Novel precursor of Alzheimer's disease amyloid protein shows protease inhibitory activity. *Nature* **331**, 530–532.
- Knops J., Lieberburg I., and Sinha S. (1992) Evidence for a non-secretory, acidic degradation pathway for amyloid precursor protein in 293 cells. Identification of a novel, 22-kDa,  $\beta$ -peptide-containing intermediate. *J. Biol. Chem.* **267**, 16022–16024.
- Knops J., Suomensaaari S., Lee M., McConlogue L., Seubert P., and Sinha S. (1995) Cell-type and amyloid precursor protein-type specific inhibition of A $\beta$  release by bafilomycin A1, a selective inhibitor of vacuolar ATPases. *J. Biol. Chem.* **270**, 2419–2422.
- Koo E. H. and Squazzo S. L. (1994) Evidence that production and release of amyloid  $\beta$ -protein involves the endocytic pathway. *J. Biol. Chem.* **269**, 17386–17389.
- Lai A., Sisodia S. S., and Trowbridge I. S. (1995) Characterization of sorting signals in the  $\beta$ -amyloid precursor protein cytoplasmic domain. *J. Biol. Chem.* **270**, 3565–3573.



- Luo Y., Bond J. D., and Ingram V. M. (1997) Compromised mitochondrial function leads to increased cytosolic calcium and to activation of MAP kinases. *Proc. Natl. Acad. Sci. USA* **94**, 9705–9710.
- Luvisetto S., Pietrobon D., and Azzone G. F. (1987) Uncoupling of oxidative phosphorylation. I. Protonophoric effects account only partially for uncoupling. *Biochemistry* **26**, 7332–7338.
- Maechler P., Kennedy E. D., Pozzan T., and Wollheim C. B. (1997) Mitochondrial activation directly triggers the exocytosis of insulin in permeabilized pancreatic  $\beta$ -cells. *EMBO J.* **16**, 3833–3841.
- Mills J., Charest D. L., Lam F., Beyreuther K., Ida N., Pelech S. L., and Reiner P. B. (1997) Regulation of amyloid precursor protein catabolism involves the mitogen-activated protein kinase signal transduction pathway. *J. Neurosci.* **17**, 9415–9422.
- Nelson N. (1991) Structure and pharmacology of the proton-ATPases. *Trends Pharmacol.* **12**, 71–75.
- Ponte P., Gonzalez-DeWhitt P., Schilling J., Miller J., Hsu D., Greenberg B., Davis K., Wallace W., Lieberburg I., Fuller F., and Cordell B. (1988) A new A4 amyloid mRNA contains a domain homologous to serine proteinase inhibitors. *Nature* **331**, 525–527.
- Presley J. F., Mayor S., McGraw T. E., Dunn K. W., and Maxfield F. R. (1997) Bafilomycin A1 treatment retards transferrin receptor recycling more than bulk membrane recycling. *J. Biol. Chem.* **272**, 13929–13936.
- Schrader-Fischer G. and Paganetti P. A. (1996) Effect of alkalinizing agents on the processing of the  $\beta$ -amyloid precursor protein. *Brain Res.* **716**, 91–100.
- Selkoe D. J. (1997) Alzheimer's disease: genotypes, phenotypes, and treatments. *Science* **275**, 630–631.
- Selkoe D. J., Yamazaki T., Citron M., Podlisny M. B., Koo E. H., Teplow D. B., and Haass C. (1996) The role of APP processing and trafficking pathways in the formation of amyloid  $\beta$ -protein. *Ann. NY Acad. Sci.* **777**, 57–64.
- Seubert P., Oltersdorf T., Lee M. G., Barbour R., Blomquist C., Davis D. L., Bryant K., Fritz L. C., Galasko D., Thal L. J., Lieberburg I., and Schenk D. B. (1993) Secretion of  $\beta$ -amyloid precursor protein cleaved at the amino terminus of the  $\beta$ -amyloid peptide. *Nature* **361**, 260–263.
- Shoji M., Golde T. E., Ghiso J., Cheung T. T., Estus S., Shaffer L. M., Cai X. D., McKay D. M., Tintner R., Frangione B., and Younkin S. G. (1992) Production of the Alzheimer amyloid  $\beta$  protein by normal proteolytic processing. *Science* **258**, 126–129.
- Sisodia S. S. (1992)  $\beta$ -Amyloid precursor protein cleavage by a membrane-bound protease. *Proc. Natl. Acad. Sci. USA* **89**, 6075–6079.
- Sisodia S. S. and Price D. L. (1995) Role of the  $\beta$ -amyloid protein in Alzheimer's disease. *FASEB J.* **9**, 366–370.
- Sokal A. and Bartosz G. (1998) Uncouplers of mitochondrial oxidative phosphorylation are not substrates of the erythrocyte glutathione-S-conjugate pump. *Arch. Biochem. Biophys.* **349**, 113–121.
- Tang X., Halleck M. S., Schlegel R. A., and Williamson P. (1996) A subfamily of P-type ATPases with aminophospholipid transporting activity. *Science* **272**, 1495–1497.
- Tanzi R. E., McClatchey A. I., Lamperti E. D., Villa-Komaroff L., Gusella J. F., and Neve R. L. (1988) Protease inhibitor domain encoded by an amyloid protein precursor mRNA associated with Alzheimer's disease. *Nature* **331**, 528–530.
- Tartakoff A. M. (1983) Perturbation of vesicular traffic with the carboxylic ionophore monensin. *Cell* **32**, 1026–1028.
- van Weert A. W., Dunn K. W., Gueze H. J., Maxfield F. R., and Stoorvogel W. (1995) Transport from late endosomes to lysosomes, but not sorting of integral membrane proteins in endosomes, depends on the vacuolar proton pump. *J. Cell Biol.* **130**, 821–834.
- Weidemann A., Konig G., Bunke D., Fischer P., Salbaum J. M., Masters C. L., and Beyreuther K. (1989) Identification, biogenesis, and localization of precursors of Alzheimer's disease A4 amyloid protein. *Cell* **57**, 115–126.
- Wild-Bode C., Yamazaki T., Capell A., Leimer U., Steiner H., Ihara Y., and Haass C. (1997) Intracellular generation and accumulation of amyloid  $\beta$ -peptide terminating at amino acid 42. *J. Biol. Chem.* **272**, 16085–16088.
- Williamson P. and Schlegel R. A. (1994) Back and forth: the regulation and function of transbilayer phospholipid movement in eukaryotic cells. *Mol. Membr. Biol.* **11**, 199–216.
- Xu H., Greengard P., and Gandy S. (1995) Regulated formation of Golgi secretory vesicles containing Alzheimer  $\beta$ -amyloid precursor protein. *J. Biol. Chem.* **270**, 23243–23245.
- Xu H., Sweeney D., Wang R., Thinakaran G., Lo A. C., Sisodia S. S., Greengard P., and Gandy S. (1997) Generation of Alzheimer  $\beta$ -amyloid protein in the trans-Golgi network in the apparent absence of vesicle formation. *Proc. Natl. Acad. Sci. USA* **94**, 3748–3752.