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Intracellular pH regulates amyloid precursor protein intracellular domain accumulation

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The amyloid precursor protein (APP) metabolism is central to pathogenesis of Alzheimer's disease (AD). Parenchymal amyloid deposits, a neuropathological hallmark of AD, are composed of amyloid-beta peptides (Aβ). Aβ derives from the amyloid precursor protein (APP) by sequential cleavages by beta- and gamma-secretases. Gamma-secretase cleavage releases the APP intracellular domain (AICD), suggested to mediate a nuclear signaling. Physiologically, AICD is seldom detected and thus supposed to be rapidly degraded. The mechanisms responsible of its degradation remain unknown. We used a pharmacological approach and showed that several alkalinizing drugs induce the accumulation of AICD in neuroblastoma SY5Y cell lines stably expressing APP constructs. Moreover, alkalinizing drugs induce AICD accumulation in naive SY5Y, HEK and COS cells. This accumulation is not mediated by the proteasome or metallopeptidases and is not the result of an increased gamma-secretase activity since the gamma-secretase cleavage of Notch1 and N-Cadherin is not affected by alkalinizing drug treatments. Altogether, our data demonstrate for the first time that alkalinizing drugs induce the accumulation of AICD, a mechanism likely mediated by the endosome/lysosome pathway.

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Keywords: Amyloid precursor protein; APP intracellular domain; Alzheimer's disease; Lysosome; Gamma-secretase; Alkalinizing drugs

Abbreviations: APP, amyloid precursor protein; AICD, amyloid precursor protein intracellular domain; Aβ, Amyloid-beta peptide; CTF, carboxy-terminal fragment.

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Introduction

The amyloid precursor protein (APP) metabolism is central to the pathogenesis of Alzheimer's disease (AD). Extracellular amyloid deposits, a neuropathological hallmark of AD, are composed of the aggregation of amyloid-beta peptides (Aβ). Aβ is released from a type I transmembrane amyloid protein precursor (APP) by sequential cleavages. Carboxy-terminal fragments (CTFs) are first generated by alpha- or beta-secretase (for a review, see Suh and Checler, 2002). The alpha-secretase cleaves APP inside Aβ sequence (Buxbaum et al., 1998; Allinson et al., 2003). BACE1, the beta-secretase protease, releases the amino-terminus of Aβ (Vassar et al., 1999). The gamma-secretase further processes APP-CTFs (Sastre et al., 2001; Kim et al., 2003), releasing Aβ from beta-CTF and the APP intracellular domain (AICD or epsilon-CTF) from all APP-CTFs (Gu et al., 2001; Sastre et al., 2001; Weidemann et al., 2002; Andrau et al., 2003; Baulac et al., 2003; Vingtdeux et al., 2005).

Cytosolic domains are released by the gamma-secretase cleavage of several type I transmembrane proteins, such as Notch intracellular domain (NICD) or Cadherins intracellular domains (De Strooper et al., 1999; Marambaud et al., 2003). By similarities to Notch I signaling, AICD associates with nuclear factors Fe65 and Tip60 to form a *trans*-regulatory complex, regulating the expression of KAI1, Nephrelysin or its own expression (Cao and Sudhof, 2001; Baek et al., 2002; Cao and Sudhof, 2004; von Rotz et al., 2004; Pardossi-Piquard et al., 2005). AICD produced physiologically is hardly detected *in vivo* and is rapidly degraded (Cupers et al., 2001). The mechanism responsible for its degradation remains uncertain. AICD is likely degraded by insulin-degrading enzyme (Edbauer et al., 2002; Farris et al., 2003) but not by the proteasome (Cupers et al., 2001). In contrast, the intracellular domain of Notch I (Gupta-Rossi et al., 2001), that of lipoprotein receptor protein (May et al., 2002) or that of

p75NTR (Jung et al., 2003) is processed by the proteasome. The generation of AICD takes place at the plasma membrane and/or in early endosomes (Kaether et al., 2006). In addition, gamma-secretase activity has been localized at the endosomal and lysosomal membrane (Lah and Levey, 2000; Pasternak et al., 2003; Vetrivel et al., 2004). Together, those data suggest that the endosomal/lysosomal pathway is of importance for regulating AICD production and/or degradation.

In the present study, we investigated the role of the endosome/lysosome pathway for the regulation of AICD production using a pharmacological approach. Our results show that treatments of neuroblastoma SY5Y stably expressing APP constructs with several alkalinizing agents result in an accumulation of AICD. We demonstrate that this accumulation did not result from an increased gamma-secretase proteolytic activity. Altogether, our results suggest that AICD accumulation is pH-sensitive and regulated via the endosome/lysosome pathway.

Materials and methods

Antibodies

The APP^{Cter}-C17 is a well-characterized rabbit antibody raised against the last 17 amino acids of the human APP sequence (Sergeant et al., 2002; Casas et al., 2004; Santiard-Baron et al., 2005; Vingtdoux et al., 2005). The mouse monoclonal antibody, 8E5, was a generous gift from Dr. Peter Seubert (Elan Pharmaceuticals). 8E5 is raised against the 444–592 residues of human APP770. The mouse monoclonal antibody, 6E10 (Signet Labs), recognizes an epitope in the first 17 amino acids of the Abeta peptide. The mouse monoclonal antibody 4G8 (Signet Labs) is reactive to amino acid residue 17–24 of Abeta peptide. The rabbit polyclonal anti-Cyclin D antibody was purchased at Euromedex, anti c-Jun was from Cell Signalling (Euromedex). The beta-Tubulin antibody was purchased at SIGMA Immunochemical. Myc-tags were visualized using 9E10 (Santa Cruz Biotechnology). Lamp2 antibody (H4B4) was from Santa Cruz Biotechnology. Secondary antibodies coupled with horseradish peroxidase were from SIGMA.

Cell culture and transfections

The human neuroblastoma cell line SKNSH-SY5Y (SY5Y), HEK and COS cells were maintained in Dulbecco's modified Eagle medium (DMEM, GIBCO BRL) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 mM non-essential amino acids, 50 units/ml penicillin/streptomycin (Invitrogen, France) in a 5% CO₂ humidified incubator at 37°C. APP695 cDNA was subcloned into eukaryotic expression vector pcDNA3 (Invitrogen), allowing for a G418 (Invitrogen) selection of clones. This APP cDNA was transfected into SY5Y cells using the ethyleneimine polymer ExGen 500 (Euromedex) according to the manufacturer's instructions. Cells expressing APP (SY5Y APP^{WT}) were selected by the addition of 200 µg/ml G418 in the cell medium. SY5Y cells stably expressing APP with the Swedish mutation (SY5Y APP^{SW}) were cultured as described previously (Vingtdoux et al., 2005). For transient expression, SY5Y cells were transfected with ΔE Notch (Notch I lacking the extracellular domain) using ExGen 500. Cells were collected 48 h after transfection. Myc-tagged ΔE Notch was obtained from Pr. Raphael Kopan (Washington University, St. Louis, MO, USA).

Drug treatment, cytotoxicity and quantification of Abeta

The following drugs were used at the indicated final concentration: *N*-[*N*-(3,5-Difluorophenacetyl)]-L-alanyl-3-(*S*)-amino-1-methyl-5-phenyl-1,3-dihydro-benzo[*e*] (1,4) diazepin-2-one (Compound E, Gamma-secretase inhibitor XVIII) (30 nM), *N*-[*N*-(3,5-Difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-Butyl Ester (DAPT, Gamma-secretase inhibitor IX) (250 nM), MG 132 (3 mM), phosphoramidon (100 mM), bestatin (100 mM), clioquinol (20 mM), bafilomycin A1 (BafA1) (100 nM), concanamycin A (ConA) (100 nM), were purchased at Calbiochem (VWR, International S.A.S.). 1,10-*o*-Phenanthroline (100 mM), NH₄Cl (10 mM) and chloroquine (10 µM) were purchased at SIGMA. Cells were plated into 6-well plates 24 h before drug exposure. After treatment, the medium was collected for dosage of Abeta. Cells were washed with PBS and scraped with a policeman rubber in 70 µl of Laemmli sample buffer containing protease inhibitors (Complete Mini, Roche Molecular Biochemicals). The cell homogenate was sonicated and heat-treated for 5 min at 100°C. Protein concentration was determined using the PlusOne™ 2-D Quant Kit (Amersham Biosciences) and samples were kept at –80°C until used.

Measurement of cell toxicity was performed by the measurement of lactate dehydrogenase release according to the manufacturer's instructions (Cyto Tox 96; Promega). For each experiments, measurements were performed in triplicate.

For quantification of Abeta released in the cell culture medium, the collected medium was spun at 200×g to eliminate the cell debris. Secreted Abeta_{1–40} and Abeta_{1–42} concentrations were determined using the Human Abeta (1–40) Assay Kit (IBL) or the INNOTEST™ beta-Amyloid_(1–42) ELISA Kit (Innogenetics), according to manufacturer's instructions.

In vitro γ-secretase assays

Assays were performed as previously described (Marambaud et al., 2003). Briefly, SY5Y cells were resuspended in 1 ml/100-mm dish of hypotonic buffer (10 mM MOPS, pH 7.0, 10 mM KCl) and homogenized on ice. A post-nuclear supernatant was prepared by centrifugation at 1000×g for 15 min at 4°C. Crude membranes were isolated from the post-nuclear supernatant by centrifugation at 16,000×g for 40 min at 4°C. The membranes were then resuspended in 50 µl of assay buffer (150 mM sodium citrate, pH 6.4, 1× Complete protease inhibitor cocktail, Roche) and incubated at 37°C for 4 h in the presence or absence of gamma-secretase inhibitor (L-685,458, 1 µM). Samples were then analyzed by Western blotting using C32 anti-N-Cadherin antibody (BD Transduction Laboratories).

Immunoprecipitations

After treatments, the media containing the secreted proteins were collected, centrifuged at 3000 rpm and made to 1× Buffer (250 mM NaCl, 50 mM Tris, pH 8.8, 5 mM EDTA, 2.5% Triton X-100, 0.1% SDS). Secreted APPs were isolated from 0.5 ml of conditioned medium. Immunoprecipitation was performed using the mouse monoclonal antibody 8E5 (4 µg) for 2 h at 4°C. Protein-antibody complexes were isolated by incubation with protein A/G Sepharose (PIERCE) for 1 h at 4°C, washed three times, resuspended in 20 µl of sample buffer and boiled before loading.

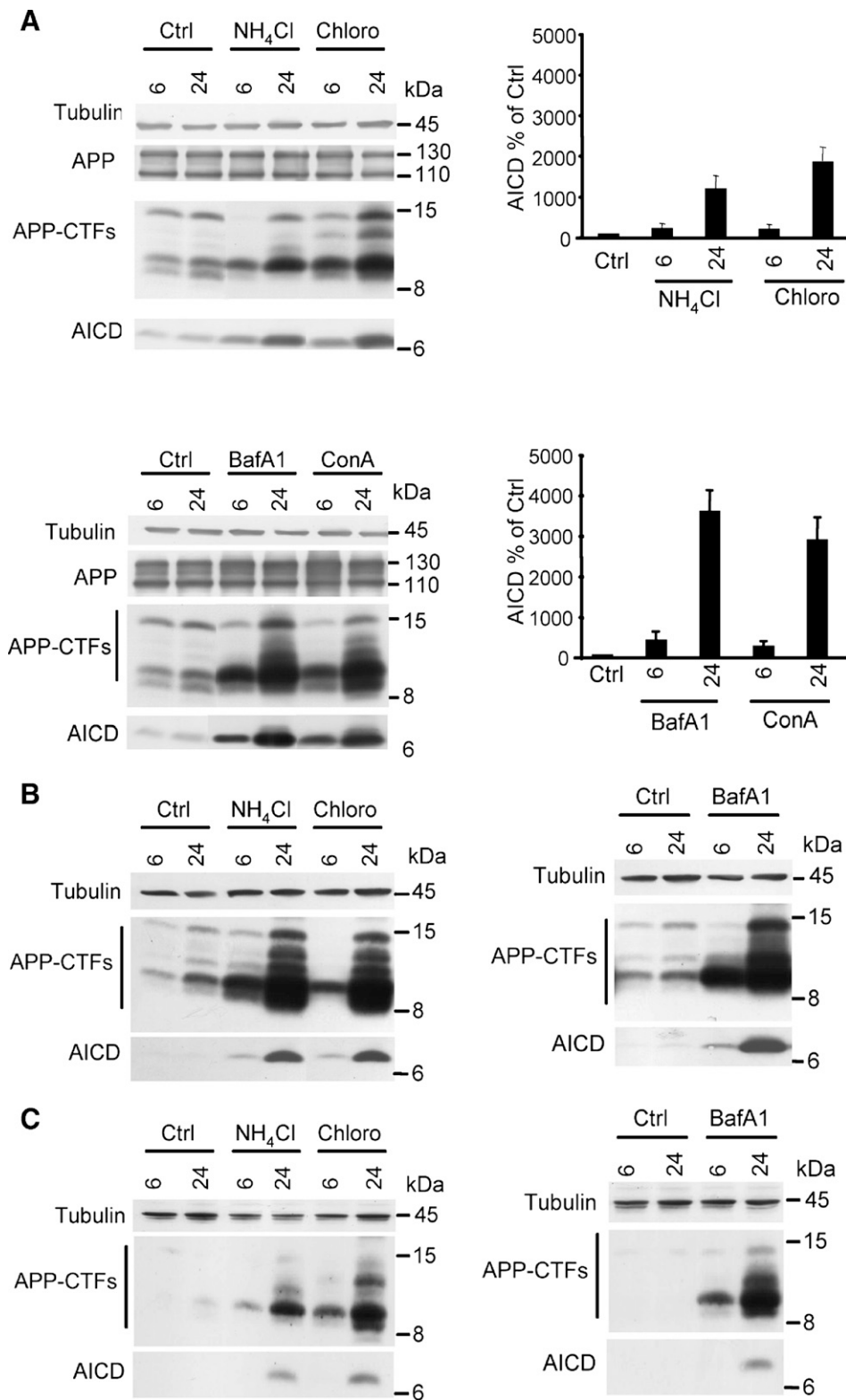


Fig. 1. pH-dependent accumulation of AICD. (A) SY5Y APP^{SW} cells were treated for indicated times with ammonium chloride (NH₄Cl), chloroquine (Chloro), bafilomycin A1 (BafA1) or concanamycin A (ConA). Tubulin, full-length APP, APP-CTFs and AICD were analyzed by Western blot. Percentage of AICD to the control level is determined by semi-quantitative Western blot analysis. Histograms are representative of three individual experiments. SY5Y APP^{WT} (B) or naive SY5Y (C) cells were treated for indicated times with ammonium chloride (NH₄Cl), chloroquine (Chloro) or bafilomycin A1 (BafA1). Tubulin, full-length APP, APP-CTFs and AICD were analyzed by Western blot. Note that, when compared to the control condition, APP-CTFs and AICD labeling is always increased after treatments with alkalinizing drugs.

For secreted A β , immunoprecipitation was performed from 1.5 ml of conditioned medium using the mouse monoclonal antibody 4G8.

Western blotting

Equal quantity of total proteins (20 μ g/lane) was loaded on a 16.5% Tris–tricine or 8–16% Tris–glycine polyacrylamide gel. Tris–tricine SDS–polyacrylamide gel electrophoresis (PAGE) was performed following the procedure of Schagger and von Jagow (1987) with a protean II Xi Cell (Bio-Rad Bioresearch division). Proteins were transferred to nitrocellulose membrane (Hybond, Amersham Biosciences) at 2.5 mA/cm² per gel using the Semidry Novablot Transfert system (Amersham Biosciences), according to the manufacturer's instructions. Proteins were reversibly stained with Ponceau Red to check the quality of protein transfer. Membranes were blocked in 25 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1% Tween-20 (v/v) and 5% (w/v) of skimmed milk for 30 min. Membranes were incubated overnight at 4°C with appropriate dilutions of the primary antibodies and incubated for 1 h at room temperature with secondary antibody. The immunoreactive complexes were revealed using the ECLTM Western Blotting kit and Hyperfilms (Amersham Biosciences). Western blot films were digitized using a UMax scanner (Amersham Biosciences). The scanner was calibrated for optical densities. Image-MASTER 1D ELITE Software (Amersham Biosciences) was used to quantify the signal, and data were collected using Excel Software (Microsoft). Loading variations between Western blot lanes were normalized according to the tubulin signal.

Immunofluorescence (IF)

SY5Y APP^{WT} cells were grown on poly L-Lys-coated glass coverslips. After 24 h of treatment with BafA1, cells were fixed in 0.1 M phosphate buffer (PBS) containing 4% paraformaldehyde for 30 min at room temperature and further permeabilized with 0.25% (v/v) Triton X-100 in PBS. After blocking in 1% (w/v) bovine serum albumin (BSA), fixed materials were incubated for 2 h at room temperature with APP^{Cter-C17} or 8E5 antibody in PBS added with 1% (w/v) BSA and 0.25% Triton X-100. After washing, Alexa Fluor-488 anti-rabbit IgG or Alexa Fluor-568 anti-mouse secondary antibodies (Invitrogen, France) were used. Coverslips were mounted to slides with Vectashield (Vector Laboratories). Images were acquired with a Leica TCS NT laser scanning confocal microscope. All data were analyzed using the Leica TCS image analysis software.

Results

AICD accumulates after long-term intracellular pH modulation

Intracellular pH was raised using ammonium chloride (NH₄Cl) or chloroquine that are weak bases and bafilomycin A1 (BafA1) and concanamycin A (ConA) that are specific inhibitors of vacuolar H⁺-ATPase (Bowman et al., 1988). NH₄Cl has been shown to alkalinize endosomes (Gekle et al., 1995); chloroquine accumulates in lysosomes where it raises the pH (de Duve et al., 1974). BafA1 and ConA were shown to inhibit the acidification of endosomes and lysosomes (Umata et al., 1990; Yoshimori et al., 1991). SY5Y cells stably expressing APP with the Swedish

mutation (SY5Y APP^{SW}) were treated with NH₄Cl, chloroquine, BafA1 or ConA. Full-length APP, APP-CTFs and AICD were detected by Western blot with APP^{Cter-C17} antibody (Figs. 1A, C), directed against the C-terminus of APP (Vingtdoux et al., 2005). No modification of full-length APP expression was observed following drug treatments (Fig. 1A). APP-CTFs were resolved as five bands between 15 and 8 kDa, corresponding to beta-, beta', alpha- and alpha'-CTFs, as previously described, and were referred to as APP-CTFs in the present study (Vingtdoux et al., 2005). The APP cytosolic domain AICD was observed at 6.5 kDa (Fig. 1A). Under NH₄Cl or chloroquine treatment (Fig.

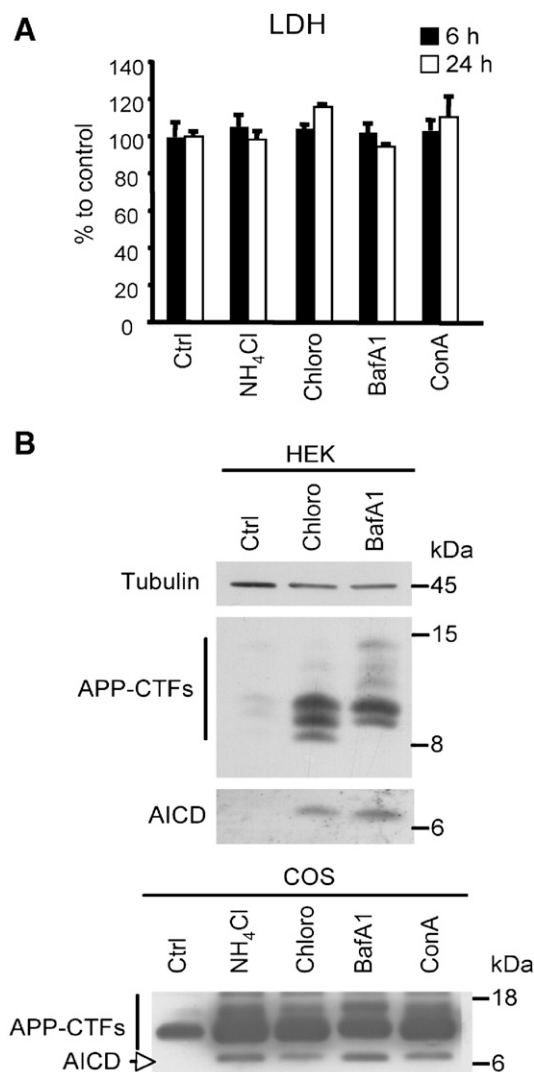


Fig. 2. Alkalinizing drug treatments are not toxic and induce AICD accumulation in naive HEK and COS cells. (A) SY5Y APP^{WT} cells were treated for 6 or 24 h with ammonium chloride (NH₄Cl), chloroquine (Chloro), bafilomycin A1 (BafA1) or concanamycin A (ConA) and ratio of intracellular to secreted lactate dehydrogenase was measured by ELISA. Values are given as the percentage to the control (Ctrl) value. (B) Human embryonic kidney 293 (HEK) or Simian kidney fibroblast-like COS-1 cells (COS) were treated for 24 h with ammonium chloride, chloroquine, bafilomycin A1 and concanamycin A. Tubulin, APP-CTFs and AICD were analyzed by Western blot. Protein extracts from COS cells were resolved on small gels.

1A), we observed an increased of APP-CTFs especially alpha-CTFs. More interestingly, the level of AICD was 10- and 20-fold increased after 24 h of treatment with NH_4Cl and chloroquine, respectively. Under treatments with vacuolar H^+ -ATPase inhibitors BafA1 and ConA (Fig. 1A), the increase of APP-CTFs and AICD was even more marked. AICD level was already 5-fold increased after 6 h of treatment and further increased 30-fold after 24 h. SY5Y stably expressing wild-type APP (SY5Y APP^{WT}) and naive SY5Y cells were also treated with NH_4Cl , chloroquine or BafA1 (Fig. 1B). An increase of APP-CTFs and AICD was observed in both cell types, suggesting that this phenomenon was likely independent of the Swedish mutation or APP expression level. Our results showed that APP-CTFs and AICD levels are increased after a long-term rise of intracellular pH.

Accumulation of AICD is not due to a toxic effect of drug treatments and is observed in several cell lines

The accumulation of AICD could be a consequence of a toxic effect of alkalizing drugs. After 6 or 24 h of drug treatment, we

quantified LDH (Fig. 2A). None of the drugs showed a toxic effect on SY5Y APP-transfected cells, therefore excluding the hypothesis that AICD accumulation was indirectly caused by the toxicity of the drugs used. Alternatively, the accumulation of AICD could be due to the cell line used. As APP-CTFs and AICD were increased in naive SY5Y, HEK and COS naive cells were treated for 24 h with alkalizing drugs (Fig. 2B). Whereas AICD was not detected in control condition, alkalizing drugs induced an accumulation of AICD and APP-CTFs in both HEK and COS cells.

Proteasome or metalloproteinases inhibitors do not reproduce the effect of alkalizing drugs

Alkalizing drugs or inhibitors of the vacuolar H^+ -ATPase could indirectly inhibit other proteases such as the proteasome or metalloproteinases. Several proteasome inhibitors and metalloproteinase inhibitors were used (Fig. 3). SY5Y APP^{SW} cells were treated for 6 h with those drugs and APP, APP-CTFs and AICD were analyzed by Western blot (Fig. 3A). Cyclin D and c-Jun are degraded by the proteasome (Diehl et al., 1997) and

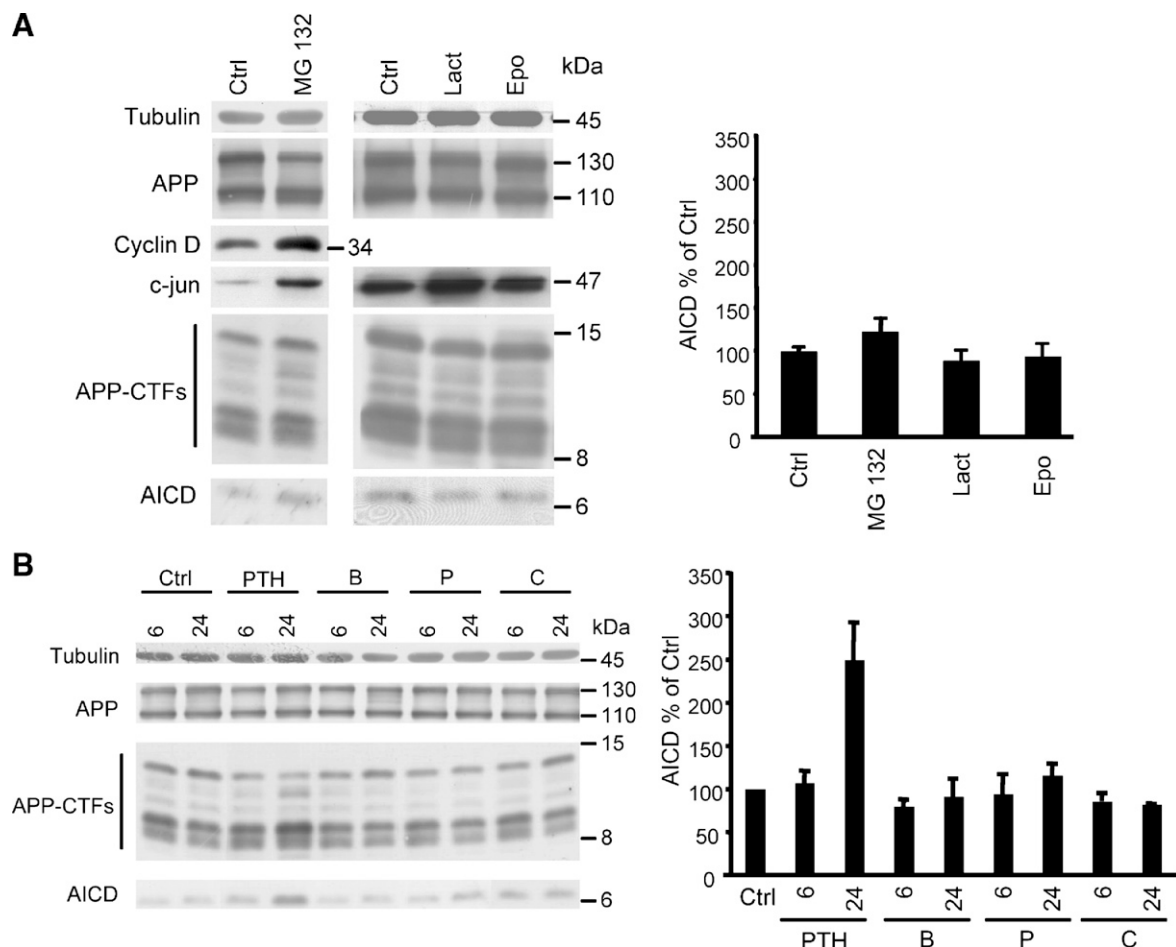


Fig. 3. AICD degradation is not inhibited by proteasome inhibitors or metal chelators. (A) SY5Y APP^{SW} cells were treated with the proteasome inhibitor MG 132, lactacystin (Lact) or epoxomicin (Epo) for 6 h. Western blot of tubulin, full-length APP, APP-CTFs, AICD, Cyclin D and c-jun. (B) SY5Y APP^{SW} cells were treated for the indicated time with various inhibitors: 1,10-phenanthroline (PTH), bestatin (B), phosphoramidon (P), or clioquinol (C). Tubulin, APP full-length, APP-CTFs and AICD were analyzed by Western blot. Histograms in panels A and B are representative of three individual experiments. Values correspond to the percentage of AICD to the control value determined following APP^{CTer}-C17 Western blotting.

were used to assess the repression of the proteasome. Cyclin D and c-Jun were increased in presence of MG 132, lactacystin and epoxomycin (Fig. 3A). None of the proteasome inhibitors modified the amount of APP full-length and APP-CTFs (Fig. 3A). Amount of AICD was slightly increased in the presence of MG 132 (120% to the control level) but remained unchanged with lactacystin and epoxomycin (Fig. 3B). An indirect repression of the proteasome by alkalizing drugs could not explain the accumulation of AICD. Metallopeptidases, such as the insulin-degrading enzyme (IDE), degrade AICD *in vitro* and *in vivo* (Edbauer et al., 2002; Farris et al., 2003). 1,10-Phenanthroline and clioquinol are two metal chelating drugs, bestatin inhibits aminopeptidases and phosphoramidon inhibits Nephrelysin, which degrades Abeta. Among the metallopeptidase inhibitors used, only 1,10-phenanthroline induced an accumulation of AICD (250% to the control level) and a light increase of APP-CTFs (Fig. 3B). Altogether, our results suggest that the increased amount of APP-CTFs and AICD observed with alkalizing agents could not be explained by an indirect inhibition of the proteasome or metallopeptidases as the phenotype was clearly different between alkalizing drugs and those inhibitors.

APP and its metabolites accumulate in large vesicles

To ascertain that alkalizing drugs did not profoundly modify APP cellular localization, we performed a double staining with antibodies against the amino-terminal region and the carboxy-terminus of APP in control and bafilomycin treated cells. Thus, if the routing of either APP or APP-CTFs was altered in treated cells, the localization of one of the two antibodies used would have been modified. When compared to the control condition, the major difference observed was the accumulation of both N- and C-terminus signals in large vesicles (Fig. 4). In an attempt to identify the enlarged vesicles, we performed a co-staining of control or BafA1 treated SY5Y APP^{SW} cells with APP_{Cter}-C17 and Lamp2 antibodies. The latter is a marker of lysosomes (for a review see, Eskelinen et al., 2003). In the control condition, vesicles positive for APP or Lamp2 alone were visualized in the cytoplasm. We observed a co-staining in vesicles with a perinuclear localization (Fig. 4). In BafA1 treated cells, large vesicles were stained both with APP_{Cter}-C17 and Lamp2 antibodies suggesting that those vesicles corresponded to enlarged lysosomes or vesicles that have fused with lysosomes. However, those results were consistent with the accumulation of APP and

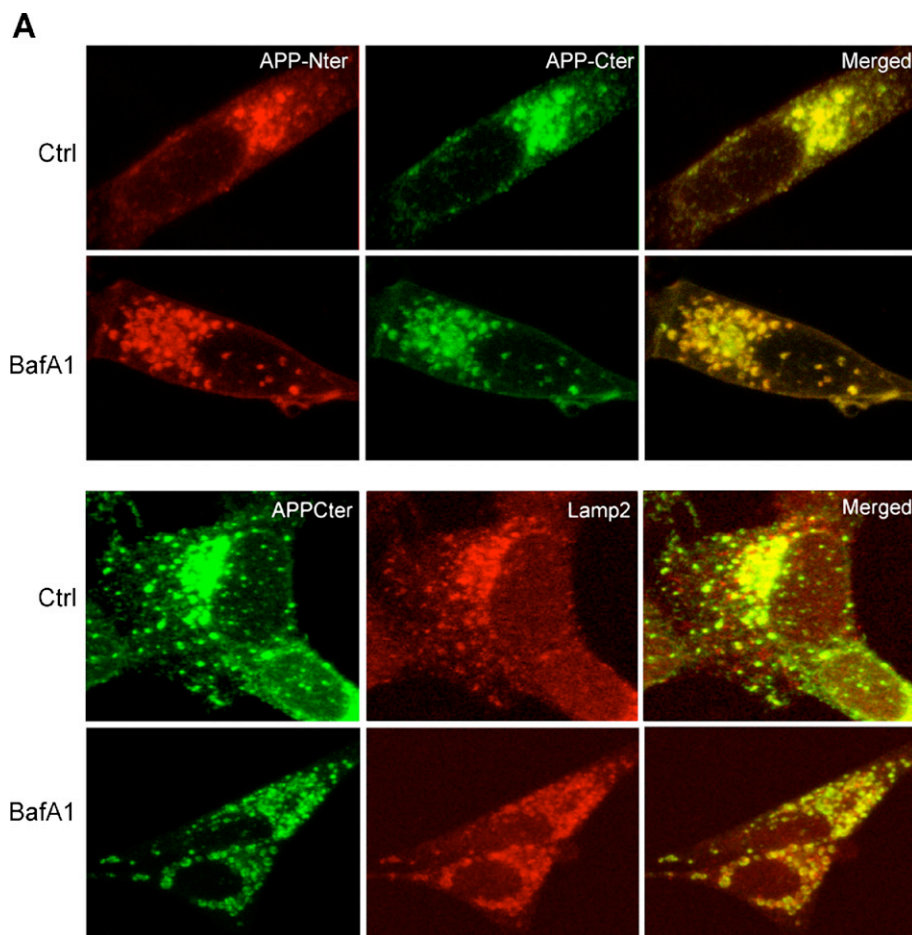


Fig. 4. Alkalizing drugs induce the accumulation APP and APP derivatives in large cellular vesicles which are Lamp2 positive SY5Y APP^{SW} cells were treated or not (Ctrl) with bafilomycin A1 (Baf1) for 24 h, fixed and stained with 8E5 (Nter, Red) or APP_{Cter}-C17 (Cter, Green), upper panel. In the lower, SY5Y APP^{SW} cells were stained with APP_{Cter}-C17 (Green) and Lamp2 (Red). Images are representatives of the stack of 8 confocal sections.

its derivatives in acidic compartments. Alkalinizing drug treatments therefore resulted in the accumulation of APP, APP-CTFs and likely AICD in engulfed vesicles which partly overlap with Lamp2 positive vesicles.

Accumulation of AICD in the presence of alkalinizing drugs necessitates a functional gamma-secretase

To address the function of the gamma-secretase in this process, SY5Y APP^{SW} cells were co-treated with DAPT, a gamma-secretase inhibitor, and ammonium chloride or chloroquine (Fig. 5). When compared to ammonium chloride or chloroquine treatments alone, co-treatment with DAPT almost completely abolished the production of AICD whereas the amount APP-CTFs was similar to that observed with DAPT alone (Fig. 5). As previously established, none of the gamma-secretase used, including DAPT, completely abolished the production of AICD or Abeta release in our assays (Vingtdeux et al., 2005). However, our results showed that the accumulation of AICD necessitated a fully functional gamma-secretase activity.

Alkalinizing drugs do not modify the gamma-secretase cleavage of ΔE Notch and N-Cadherin

We next analyzed whether the accumulation of AICD could result or not from an enhanced gamma-secretase activity. If so, alkalinizing drug treatments would modify the intramembrane proteolysis of other type I transmembrane proteins that are processed by the gamma-secretase. ΔE Notch is processed by gamma-secretase to generate Notch intracellular domain (NICD) (De Strooper et al., 1999). SY5Y cells were transiently transfected with myc-tagged ΔE Notch. After 24 h of transfection, cells were treated for 24 h with the alkalinizing drugs. ΔE Notch and NICD were detected by Western blotting with a myc antibody. Compound E (CompE) which inhibits the gamma-secretase abolished NICD production (Fig. 6A) and was shown previously to abolish AICD release (Vingtdeux et al., 2005). NICD production was unchanged in the presence of alkalinizing drugs demonstrating that the gamma-secretase activity at the epsilon-cleavage site was unaffected by alkalinizing drugs (Fig. 6A). We then assessed the effect of BafA1 on the production of the

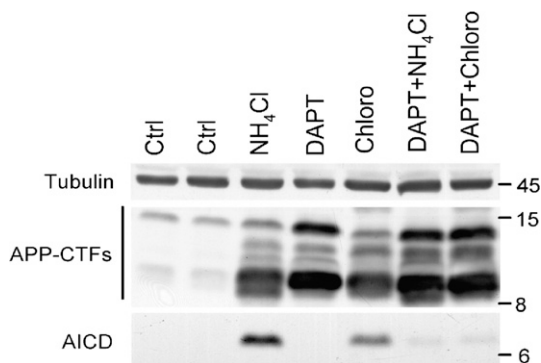


Fig. 5. Accumulation of AICD necessitates a functional gamma-secretase activity. SY5Y APP^{SW} cells were treated with NH₄Cl, DAPT, chloroquine or co-treated with DAPT and NH₄Cl or DAPT and chloroquine for 24 h. Tubulin, APP-CTFs and AICD were analyzed by Western blot.

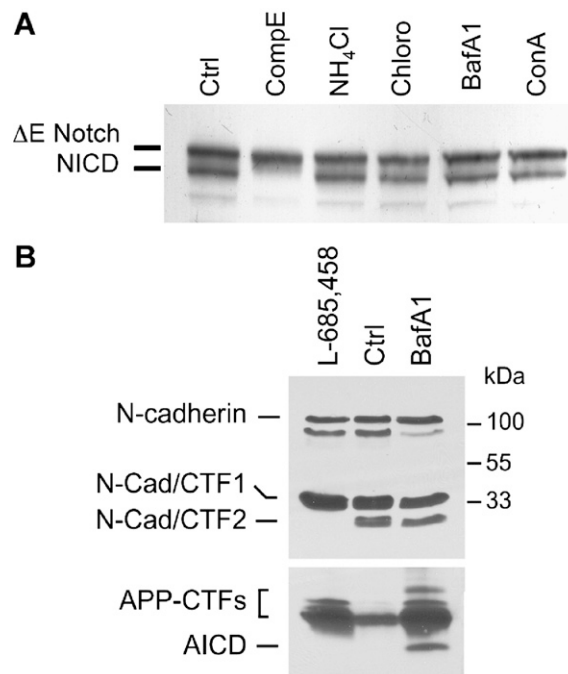


Fig. 6. Alkalinizing drugs do not affect the gamma-secretase cleavage of ΔE Notch nor that of N-Cadherin. (A) SY5Y cells transfected with Myc-tagged NotchΔE were treated for 24 h with Compound E (CompE), ammonium chloride (NH₄Cl), chloroquine (Chloro), bafilomycin A1 (BafA1) or concanamycin A (ConA). ΔE Notch and NICD were analyzed by Western blot. (B) APP-transfected SY5Y cells were treated for 24 h with the γ-secretase inhibitor L-685,458 (1 μM) or with bafilomycin A1 (BafA1, 100 nM). Membrane preparations (see Materials and methods) were then incubated *in vitro* at 37°C for 4 h. N-Cadherin full-length and N-Cadherin-derived C-terminal fragments (N-Cad/CTF1 and N-Cad/CTF2) were analyzed by Western blot (upper panel). APP-CTFs and AICD levels were analyzed in the corresponding total extracts by Western blot using an anti-APP CTF antibody (lower panel).

gamma-secretase-derived intracellular domain of N-Cadherin (N-Cad/CTF2). Using the previously described *in vitro* assay in SY5Y APP^{WT} cells (Marambaud et al., 2003), we show that while N-Cad/CTF2 production is abolished by pretreatment of the cell culture with a gamma-secretase inhibitor L-685,458 (Fig. 5B), N-Cad/CTF2 production was not affected in cells pretreated for 24 h with BafA1 (Fig. 6B). Together our results suggest that the gamma-secretase activity is not modified by long-term alkalinizing drug treatments.

Alkalinizing drugs repressed Abeta secretion

We next quantified the amount of Abeta secreted in the cell medium using ELISA and immunoprecipitation (Fig. 7A). As previously described, secretion of Abeta_{1–40} and Abeta_{1–42} was reduced after alkalinizing drugs treatments (Shoji et al., 1992; Haass et al., 1995; Schrader-Fischer and Paganetti, 1996). NH₄Cl, BafA1 and ConA decreased the amount of secreted Abeta to nearly 70% of control (Fig. 7A). Chloroquine treatment repressed Abeta secretion by 20% (Fig. 7A). The alkalinizing drugs strongly reduce Abeta secretion although they increase APP-CTFs and AICD.

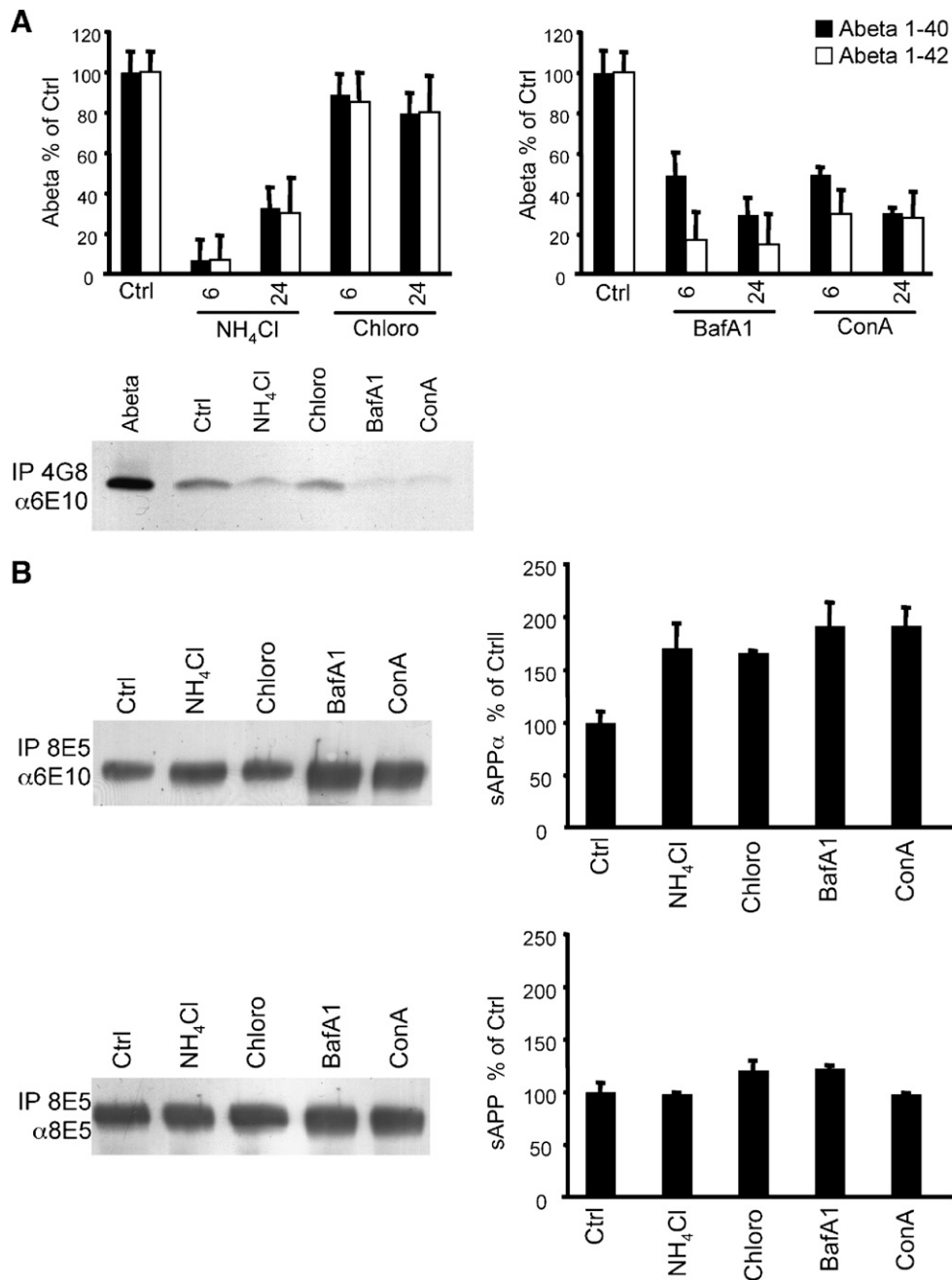


Fig. 7. Alkalizing drugs reduce Abeta secretion and enhance soluble APP alpha production. (A) SY5Y APP^{SW} cells were treated for the indicated time with ammonium chloride (NH₄Cl), chloroquine (Chloro), bafilomycin A1 (BafA1) or concanamycin A (ConA). Secreted Abeta_{1–42} and Abeta_{1–40} are assayed using ELISA. The histograms represent the percentage of Abeta_{1–40} (black bars) and Abeta_{1–42} (white bars) secreted, the value of 100% is given to the control condition. Abeta was immunoprecipitated with 4G8 antibody, from conditioned media of SY5Y APP^{SW} cells treated for 24 h with ammonium chloride (NH₄Cl), chloroquine (Chloro), bafilomycin A1 (BafA1) or concanamycin A (ConA). Abeta was analyzed by Western blot with 6E10 Antibody. (B) sAPP products were immunoprecipitated from conditioned media of SY5Y APP^{SW} cells treated for 24 h with ammonium chloride (NH₄Cl), chloroquine (Chloro), bafilomycin A1 (BafA1) or concanamycin A (ConA). sAPP-alpha was analyzed by Western blot with 6E10 Antibody. sAPP-tot were analyzed by Western blot with 8E5 antibody. Percentages of sAPP-alpha or sAPP-tot to the control level are determined by semi-quantitative Western blot analysis.

Increase production of soluble APP-alpha by alkalizing drugs treatments

BACE1 has an optimum proteolytic activity at acidic pH. Alkalizing drugs could repress or abolish BACE1 aspartyl protease activity leading to a decreased beta-CTFs and Abeta

production. Thus, APP that is not processed by beta-secretase could undergo increased alpha-secretase cleavage, resulting in more sAPP-alpha in the conditioned medium. This would lead also to more alpha-CTFs thereafter processed by the gamma-secretase to produce AICD. We analyzed the soluble APP produced in the medium. sAPP were immunoprecipitated with an

antibody against the N-terminus of APP, sAPP were analyzed by Western blot with 6E10 (sAPP-alpha) or 8E5 (sAPP-tot). A two-fold increase of sAPP-alpha was observed after the treatments (Fig. 7B), although the global amount of soluble APP (sAPP-tot) remained equal (Fig. 7B). These results could explain the decrease of Abeta secretion since APP was rather processed by alpha-secretase than by beta-secretase, under alkalizing drugs treatments. In sharp contrast, AICD accumulation could not be explained by these results as the global level of soluble APP remained identical.

Discussion

The discovery of gamma-secretase has pioneered the mechanism of regulated intramembrane proteolysis. Intracellular domains released from several type I transmembrane proteins are essential for signaling pathways such as gene transactivation. For instance, release of NICD is essential for cell fate decisions, N-Cadherin ICD promotes the proteasomal degradation of transcription factor CBP and AICD regulates several gene expression (Jarriault et al., 1995; Marambaud et al., 2003; Cao and Sudhof, 2004). NICD was seldom detected until the degradation pathway was evidenced (Gupta-Rossi et al., 2001). NICD nuclear signaling pathway was therefore established before the polypeptide could be visualized in physiological conditions. In living cells, AICD is ephemeral (Cupers et al., 2001). Knowing the degradation pathway or experimental conditions in which AICD is modulated is therefore essential to unravel its biological function. To address this issue, we studied the production and degradation of AICD physiologically produced from exogenous or endogenous full-length APP. Four different alkalizing agents known to inhibit degradation of proteins by lysosomes induced the accumulation of AICD. We showed previously that AICD could be visualized in SY5Y cells overexpressing APP (Vingtdeux et al., 2005). Presently, we showed that AICD accumulates in three different cell lines after alkalizing drugs treatments, demonstrating that this accumulation is not due to our overexpressing system. Our data further demonstrated that AICD is not degraded by the proteasome (Cupers et al., 2001). AICD generated *in vitro* from membrane extracts was stabilized by EDTA or PTH (Edbauer et al., 2002). Transgenic mice deficient for insulin-degrading enzyme showed a reduced degradation rate of AICD (Farris et al., 2003). We showed that PTH induced an increase of AICD. Clonidine, another metal-chelating compound, inefficiently modified AICD or other APP derivative levels. The effect of PTH is therefore different from that of alkalizing drugs. From the present work, we cannot conclude whether PTH inhibits AICD degradation or enhances its production.

Intracellular accumulation of AICD necessitates a functional gamma-secretase as a co-treatment of SY5Y cells with NH₄Cl or chloroquine and a gamma-secretase inhibitor repressed the production of AICD. An increased gamma-secretase activity by alkalizing drugs was ruled out as those drugs did not modify NICD or N-Cad/CTF2 production. Our results do not support the hypothesis of an increase or even a defective gamma-secretase activity as the gamma-secretase activity was both assessed by an *in vivo* and *in vitro* assay. Therefore, our results show that AICD accumulation is pH-sensitive and not related to increased gamma-secretase activity at the epsilon site. Moreover, a profound modification of APP routing was not evidenced. In contrast,

alkalizing drugs result in the accumulation of APP, APP-CTFs and likely AICD in engulfed vesicles, which were shown to overlap with Lamp2 positive vesicles. Our results therefore suggest that those vesicles rather correspond to acidic compartments or vesicles that fused with lysosomes.

Alkalizing drugs were shown to repress Abeta production (Haass et al., 1993; LeBlanc and Goodyer, 1999; Schrader-Fischer and Paganetti, 1996). We obtained similar results showing that Abeta_{1–42}, Abeta_{1–40} and total Abeta species are strongly reduced. Interestingly, short-term treatments (6 h) with alkalizing drugs diminished the rate of beta-CTF, as previously shown in HEK cells (Schrader-Fischer and Paganetti, 1996). As BACE1 optimal proteolytic activity is at acidic pH (Yan et al., 1999; Sinha et al., 1999), alkalizing drugs may repress its activity. The alpha-secretase further cleaves APP molecules not processed by BACE1, as shown by the increase of soluble APP-alpha. Therefore, the decreased production of Abeta may be explained by the reduced production of beta-CTF. However, after 24 h of treatments we observed an accumulation of beta-CTFs. First, APP-CTFs can be degraded by a lysosome-dependent pathway (Golde et al., 1992; Estus et al., 1992; Haass et al., 1992). Secondly, BACE1 is also degraded following the same pathway (Koh et al., 2005). Together, the long-term accumulation of beta-CTFs may result from the accumulation BACE1 and a lack of beta-CTF degradation by the lysosome. As the gamma-secretase activity is likely unaltered, we propose that AICD accumulates as the result of (1) the release of AICD from alpha-CTFs and (2) a repressed degradation of AICD.

In conclusion, our data highlight the cellular pathway of AICD accumulation. Further work will unravel the mechanisms responsible for this pH-dependent accumulation. Moreover, we demonstrate that Abeta and AICD, which are both released by the gamma-secretase, can be differentially regulated. Molecules mimicking this effect without affecting the lysosome activity could be interesting drugs against Alzheimer's disease since Abeta is reduced whereas the gamma-secretase remains active toward other type I transmembrane proteins.

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