FEBS Letters 580 (2006) 6972–6976

Chloroquine-induced endocytic pathway abnormalities: Cellular model of GM1 ganglioside-induced Aβ fibrillogenesis in Alzheimer's disease

Kohei Yuyama[a], Naoki Yamamoto[a,b], Katsuhiko Yanagisawa[a,*]

[a] *Department of Alzheimer's Disease Research, National Institute for Longevity Sciences, National Center for Geriatrics and Gerontology, 36-3 Gengo, Morioka, Obu 474-8522, Japan*
[b] *Japan Society for the Promotion of Sciences (JSPS), Tokyo 102-8472, Japan*

Received 20 October 2006; revised 24 November 2006; accepted 29 November 2006

Available online 5 December 2006

Edited by Jesus Avila

Abstract Endocytic pathway abnormalities were previously observed in brains affected with Alzheimer's disease (AD). To clarify the pathological relevance of these abnormalities to assembly of amyloid β-protein (Aβ), we treated PC12 cells with chloroquine, which potently perturbs membrane trafficking from endosomes to lysosomes. Chloroquine treatment induced accumulation of GM1 ganglioside (GM1) in Rab5-positive enlarged early endosomes and on the cell surface. Notably, an increase in GM1 level on the cell surface was sufficient to induce Aβ assembly. Our results suggest that endocytic pathway abnormalities in AD brain induce GM1 accumulation on the cell surface, leading to amyloid fibril formation in brain.

Keywords: Alzheimer's disease; Amyloid β-protein; GM1 ganglioside; Endosome; Rab5; Chloroquine

1. Introduction

The extracellular deposition of amyloid β-protein (Aβ) is a pathological hallmark of Alzheimer's disease (AD), however, it remains to be clarified how soluble Aβ starts to assemble in the brain. We previously identified a unique Aβ species characterized by its binding to GM1 ganglioside (GM1) in brains exhibiting early pathological changes of AD [1]. On the basis of the molecular characteristics of GM1-bound Aβ (GAβ), we hypothesized that GAβ acts as a seed for Aβ fibrillogenesis in AD brain [1]. Despite accumulating evidence that suggests the pathological implication of GAβ [for review, see Ref. [2]], our hypothesis has been challenged by the simple question of how GAβ is formed in the brain. In this context, we have recently found that GM1 level increases in the membrane microdomains of synaptosomes prepared from aged human apolipoprotein E4 (apoE4)-knock-in mouse brains [3]. This result suggests that GM1 accumulation in neuronal membranes underlies Aβ assembly in AD brain. Importantly, this possibility has recently been supported by studies of AD brains [4,5]. The next issue that must be addressed is how the expression and/or trafficking of GM1 is altered, leading to GM1 accumulation in neuronal membranes in AD brain. Regarding cellular AD pathology, it is noteworthy that endocytic pathway abnormalities, including the enlargement of early endosomes and the upregulation of Rab5 (a small GTPase associated with early endosomes) are observed in neurons of AD brains [6]. In this study, we aimed to clarify the pathological relevance of endocytic pathway abnormalities to Aβ assembly using PC12 cells treated with chloroquine which potently perturbs membrane trafficking from endosomes to lysosomes [7].

*Corresponding author. Fax: +81 562 44 6594.
E-mail address: katuhiko@nils.go.jp (K. Yanagisawa).

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid β-protein; GM1, GM1 ganglioside; GAβ, GM1-bound Aβ; APP, amyloid precursor protein; apoE, apolipoprotein E; CTB, cholera toxin B subunit; HRP, horseradish peroxidase; NGF, nerve growth factor

2. Materials and methods

2.1. Materials

Chloroquine, cholera toxin B subunit (CTB), and horseradish peroxidase (HRP)-conjugated CTB (CTB-HRP) were obtained from Sigma (St. Louis, MO). Alexa Fluor 594-coupled CTB (CTB-AF) was purchased from Invitrogen (Carsbad, CA). Antibodies specific to Rab4, Rab5 and Rab7 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An anti-actin monoclonal antibody, the anti-Aβ monoclonal antibody 6E10 and the anti-amyloid precursor protein monoclonal antibody 22C11 were purchased from Sigma, Signet Laboratories (Dedham, MA) and Chemicon International (Temecula, CA), respectively. Synthetic Aβ1-40 (Aβ) was purchased from Peptide Institute (Osaka, Japan).

2.2. Cell culture

PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 5% fetal bovine serum and 10% horse serum. For nerve growth factor (NGF)-induced differentiation, PC12 cells were seeded on poly-L lysine-coated dishes or chamber slides (Nunc, Roskilde, Denmark) at a density of 2×10^4 cells/cm^2 and cultured in DMEM containing 100 ng/ml NGF (Almone Labs., Jerusalem, Israel). The medium and NGF were replenished every 2 days. After 6 days, the cells were pharmacologically treated with chloroquine and other reagents.

2.3. SDS–PAGE and Western blotting

Cells were lysed in Triton X-100-containing Tris buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, Complete™ protease inhibitor cocktail (Roche Molecular Biochemicals, Penzberg, Germany)]. Cell extracts were centrifuged at $13000 \times g$ for 2 min. Then, the obtained supernatants were solubilized with sodium dodecyl sulfate (SDS) sample buffer. The samples were separated on a 4–20% polyacrylamide gel, and then electrotransferred to nitrocellulose membranes (for GM1 and Aβ) or polyvinylidene difluoride membranes (for other proteins). The blots were probed with CTB-HRP or with primary antibodies followed by HRP-coupled secondary antibodies.

0014-5793/$32.00
doi:10.1016/j.febslet.2006.11.072

2.4. Fluorescence staining of GM1 and endosome-binding proteins

To examine the intracellular distribution of GM1 and the endosome-binding proteins Rab5 and Rab7, PC12 cells, which had been treated with 1 μM chloroquine for 5 days, were fixed with 3% paraformaldehyde/0.5% glutaraldehyde for 30 min at 4 °C, and then permeabilized with 0.01% Triton X-100 for 5 min at 4 °C. After blocking with 5% BSA, the cells were incubated with the anti-Rab5 or anti-Rab7 antibody overnight at 4 °C. Then cells were probed with the 0.1 mg/ml CTB-AF and anti-rabbit IgG-AF594 for 2 h at 4 °C. For detection of cell-surface GM1, the cells were incubated with 0.05 mg/ml CTB-AF in HEPES-buffered DMEM at 4 °C for 5 min and fixed with 4% paraformaldehyde in PBS at room temperature. Stained cells were observed under a microscope equipped with a laser-scanning confocal imaging system, LSM510 (Carl Zeiss, Oberkochen, Germany).

2.5. Evaluation of Aβ fibrillogenesis in cultures and thioflavin-S staining

Cells which had been treated with 1 μM chloroquine for 4 days were incubated with 10 μM seed-free Aβ, which had been prepared as previously reported [8], for 24 h at 37 °C, and centrifuged at 540 000 × *g* for 15 min. The obtained precipitates were subjected Western blotting following solubilization in formic acid. Thioflavin-*S* (ThS) staining was performed according to a method previously reported [9].

3. Results

3.1. Effect of chloroquine treatment on cellular GM1 levels

The GM1 level in the cell lysates markedly increased in chloroquine-dose-dependent and time-dependent manners (Fig. 1A). To investigate the mechanism underlying chloroquine-induced GM1 accumulation, we performed Western blotting of the cell lysates using antibodies specific to amyloid precursor protein (APP), which is endocytosed via a clathrin-dependent pathway [10]. Treatment with 10 μM chloroquine increased GM1 level whereas APP level remained unchanged (Fig. 1B). In contrast, treatment with 100 μM chloroquine decreased GM1 level but markedly increased APP level (Fig. 1B). We treated the cells with ammonium chloride, another weak base similar to chloroquine. Treatment with ammonium chloride also induced GM1 accumulation (Fig. 1C). We monitored cell viability using release assay of lactate dehydrogenase. No significant level of cell death was observed under these conditions (data not shown).

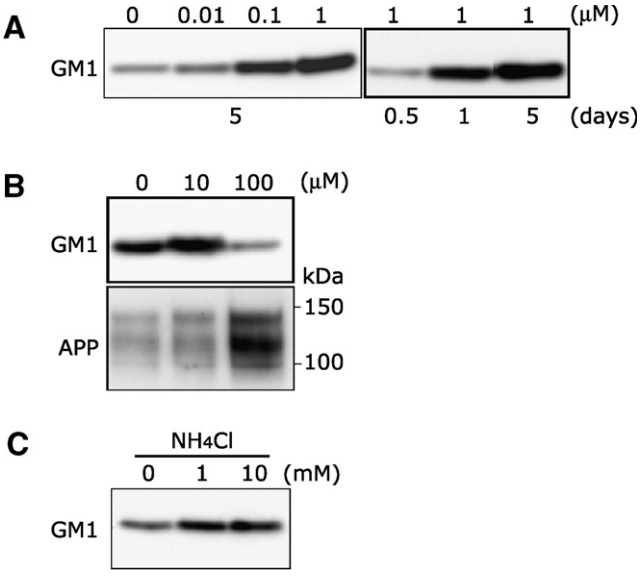

Fig. 1. Effect of chloroquine treatment on GM1 level in PC12 cells. (A) PC12 cells were treated with chloroquine at the indicated concentrations for various durations. The lysates of treated cells were electrophoresed. The blots were reacted with CTB-HRP. (B) PC12 cells were treated with chloroquine at the indicated concentrations for 12 h. GM1 and APP were detected by Western blotting using CTB-HRP and an antibody specific to the N-terminus of APP, respectively. (C) PC12 cells were treated with ammonium chloride at the indicated concentrations for 5 days.

3.2. Rab5 accumulation in chloroquine-treated cells

To examine the effect of chloroquine treatment on endocytic pathway compartments, we performed Western blotting of lysates of the cells, which had been treated with 1 μM chloroquine for 5 days, using antibodies specific to Rab5, Rab7 and Rab4, which are proteins specifically associated with early endosomes, late endosomes and recycling vesicles, respectively. The levels of Rab5 and Rab4 significantly increased following chloroquine treatment (Fig. 2A and B).

3.3. GM1 accumulation in early endosomes and on surface of chloroquine-treated cells

We explored the cellular sites where GM1 accumulation occurred. We treated cells with 1 μM chloroquine for 5 days. In the immunocytochemistry, the level of GM1 stained with CTB-AF following permeabilization pretreatment markedly increased in some organelles and on plasma membranes (Fig. 3A). Double staining with CTB-AF and an antibody

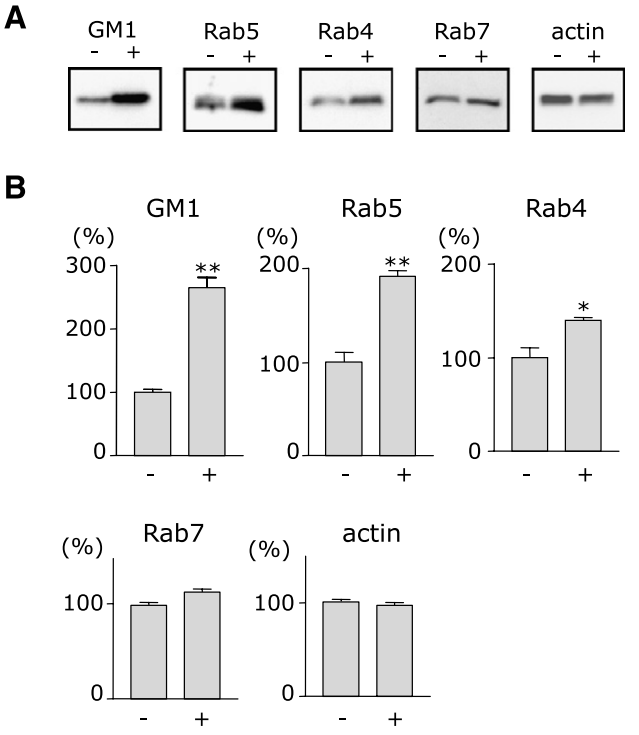

Fig. 2. Effect of chloroquine treatment on the levels of endosome-binding proteins in PC12 cells. PC12 cells were treated with 1 μM chloroquine for 5 days. (A) Cell lysates were subjected to Western blotting using CTB-HRP and specific antibodies. (B) The intensities of the bands were determined by quantitative densitoscanning. Each column represents the average ±1 S.D. of three values as percent of control values obtained from untreated PC12 cells. (−) and (+) indicate without and with chloroquine, respectively. $^{*}P < 0.005$ and $^{**}P < 0.05$ (two-tailed Student's *t*-test).

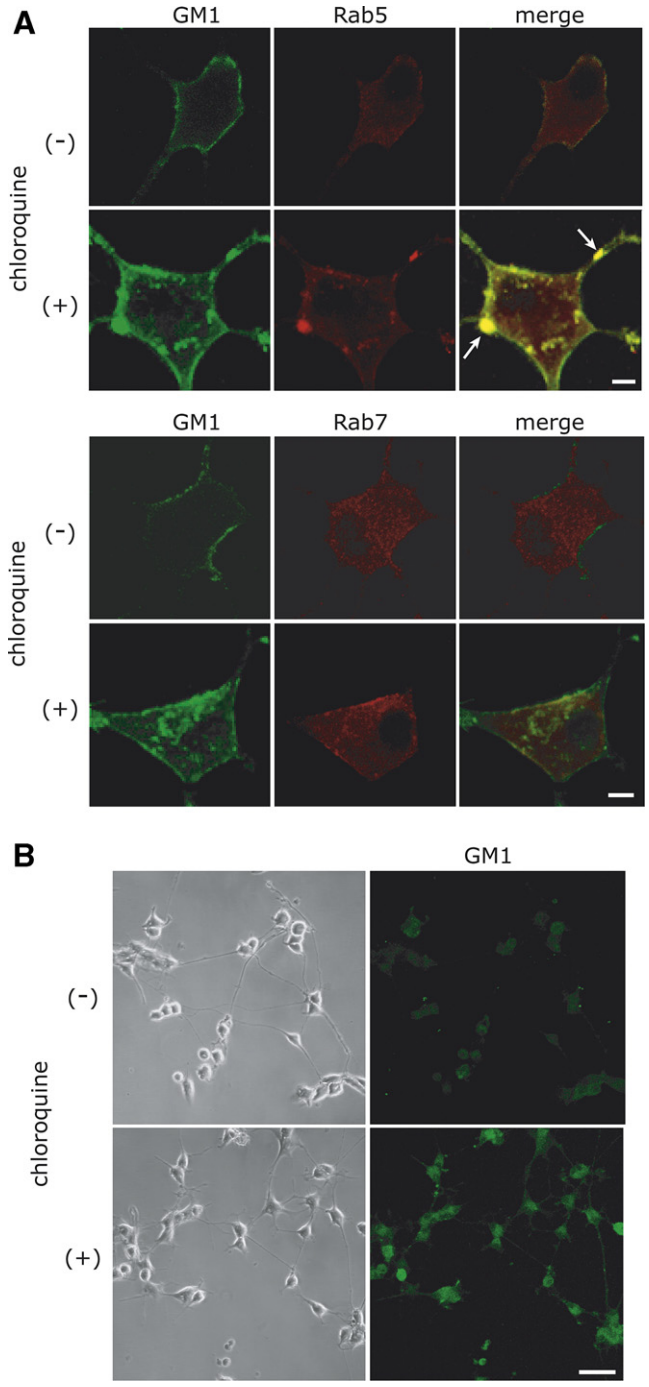

Fig. 3. GM1 accumulation in early endosomes and on surface of chloroquine-treated PC12 cells. PC12 cells were treated with 1 μM chloroquine for 5 days. (A) Images of fluorescence staining of chloroquine-treated or untreated PC12 cells with CTB-AF following permeabilization pretreatment for detecting GM1 (green) and antibodies specific to Rab5 or Rab7 (red). Arrows indicate enlarged vesicles in which GM1 and Rab5 colocalized in chloroquine-treated cells. Bar, 5 μm. (B) PC12 cells, which had been treated with 1 μM chloroquine for 5 days, were incubated with CTB-AF at 4 °C prior to fixation. Bar, 20 μm.

specific to Rab5 revealed that colocalized spots of GM1 and Rab5 were markedly enlarged, which apparently agrees with previously reported enlarged early endosomes in neurons of AD brains [6] (Fig. 3A). We also performed double staining

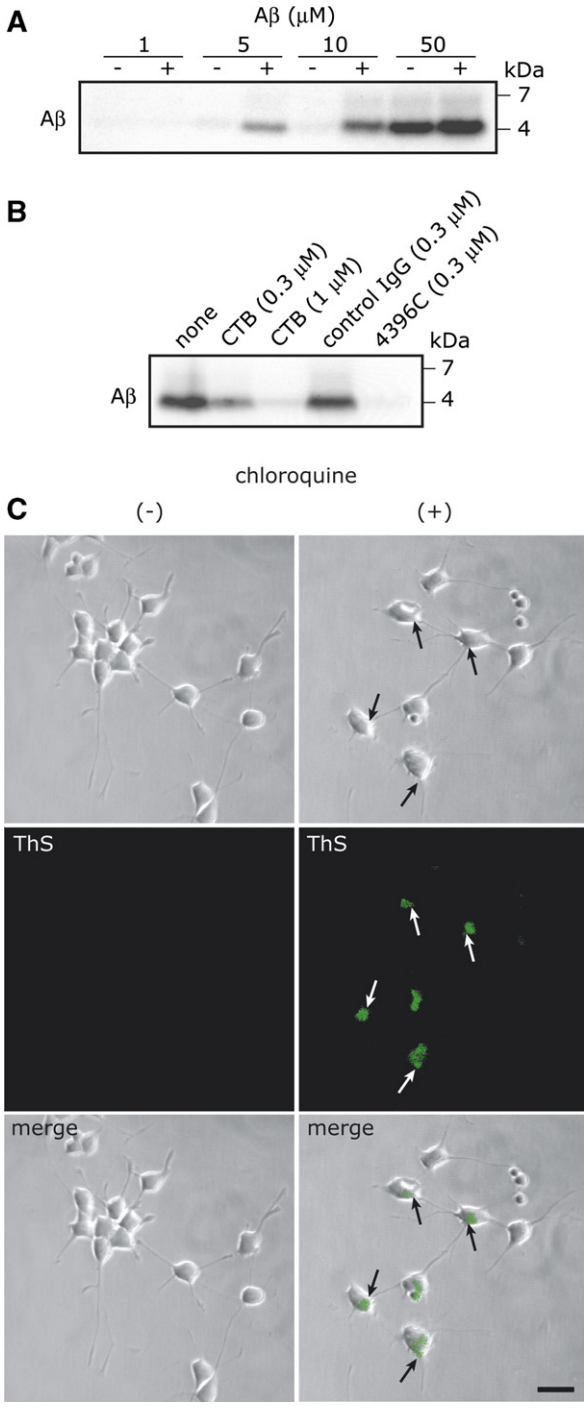

Fig. 4. Accelerated amyloid fibril formation from soluble Aβ in cloroquine-treated PC12 cell culture. (A) Insoluble Aβ formed in the PC12 cell culture, which were incubated with soluble Aβ at 1–50 μM for 24 h following treatment with (+) or without (−) 1 μM chloroquine for 4 days, was subjected to Western blot analysis following solubilization in formic acid. The blot was reacted with a monoclonal antibody against Aβ. (B) Suppression of amyloid fibril formation in the culture by cotreatment with CTB and 4396C, an antibody specific to GAβ [8]. PC12 cells were incubated with Aβ at 10 μM for 24 h with or without CTB or 4396C at the indicated concentrations after prior treatment with 1 μM chloroquine for 4 days. Insoluble Aβ formed in the cultures was detected as indicated in (A). (C) Amyloid fibrils formed in the PC12 cell culture, which was incubated with Aβ at 10 μM for 2 h after pretreatment with 1 μM chloroquine for 4 days, were visualized by ThS staining. Arrows indicate ThS-positive spots. Bar, 20 μm.

with CTB-AF and an antibody specific to Rab7; however, no colocalized spot was observed (Fig. 3A). To confirm GM1 accumulation on the surface of cells, we also incubated the cells with CTB-AF prior to fixation at 4 °C to prevent the internalization of CTB-AF from the cell surface. Compared with untreated cells, chloroquine-treated cells showed a marked increase in the level of CTB-AF bound to their surface (Fig. 3B).

3.4. Accelerated Aβ fibrillogenesis in chloroquine-treated cell cultures

We examined whether accumulated GM1 on the surface of PC12 cells can potently induce the assembly of soluble exogenous Aβ. We performed Western blotting of precipitates of the cultured cells, which had been treated with 1 μM chloroquine for 4 days, following incubation with Aβ for 24 h at final concentrations of 1–50 μM. The level of insoluble, assembled Aβ significantly increased in the cultures treated with chloroquine compared with that in nontreated cultures (Fig. 4A). Importantly, the level of insoluble Aβ significantly decreased in the presence of CTB or 4396C, an antibody specific to GAβ (Fig. 4B). Consistent with the result of Western blotting, the significant level of ThS fluorescence intensity was observed only on the surface of the cells, which had been treated with chloroquine (Fig. 4C).

4. Discussion

GM1 molecules on plasma membranes are endocytosed and transported to lysosomes along early and late endosomes, and there, the first hydrolysis catalyzed by β-galactosidase occurs [11,12]. However, little is known about how the endocytosis of GM1 is regulated. In this study, the treatment of PC12 cells with chloroquine at low μM concentrations induced marked GM1 accumulation whereas that at high μM concentration, which is required for the inhibition of lysosomal activity [13], induced the GM1 disappearance.

It remains to be clarified how GM1 disappearance was induced by chloroquine at a high μM concentration; however, possible explanations for GM1 accumulation by a chloroquine at low μM concentrations may be as follows; first, intralysosomal GM1 degradation may be inhibited by chloroquine; second, GM1 trafficking through endocytic pathway may be perturbed by the chloroquine-induced impairment of the acidification of endocytic pathway compartments. Unlike GM1, accumulation of APP, which is degraded in lysosomes [14,15], was only induced by chloroquine at a high μM concentration as previously reported [16]. This result suggests that chloroquine treatment of cells with chloroquine at low concentrations is insufficient to inhibit lysosomal activity. Thus, we favor the second possibility. In this context, it is noteworthy that a recent study revealed the crucial role of endosomal acidification in regulation of endocytic pathway through modulation of the interaction of V-ATPase with Arf6 and ARNO, which are small GTPase molecule and its cognate GDP/GTP exchange factor, respectively [17]. Thus, it is assumed that impaired acidification by chloroquine induced GM1 stagnation in early endosomes and on the cell surface. Taking account of upregulation of Rab4, it is also possible to assume that a population of GM1 molecules stagnated in early endosomes was transported to the cell surface through recycling pathway.

One of the unexpected observation in this study is that GM1 was readily accumulated in early endosomes and on the cell surface without the apparent accumulation of APP, that shall be endocytosed via a clathrin-dependent pathway [10]. Although further studies are needed to conclude, GM1 may be endocytosed and sorted into a population of early endosomes different from those for APP. The following is evidence in favor of this possibility: first, CTB is endocytosed via a clathrin-independent pathway [18]; and second, there are distinct groups of early endosomes with different sorting functions [19].

Overall, our results may imply that endocytic pathway abnormalities in neurons cause GM1 accumulation on the neuronal surface, leading to extraneuronal Aβ fibrillogenesis. A challenge for future studies is to determine how endocytosis and trafficking of GM1 are regulated in neurons and how these are altered in AD brain by risk factors for AD development such as aging and apoE4 expression.

Acknowledgments: This study was supported by a Grant-in-Aid for Scientific Research on Priority Areas-Research on Pathomechanisms of Brain Disorders from the Ministry of Education, Culture, Sports, Science and Technology of Japan (1700220004) and by the Program for the Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO).

References

[1] Yanagisawa, K., Odaka, A., Suzuki, N. and Ihara, Y. (1995) GM1 ganglioside-bound amyloid β-protein (Aβ): a possible form of preamyloid in Alzheimer's disease. Nat. Med. 1, 1062–1066.
[2] Yanagisawa, K. (2005) GM1 ganglioside and the seeding of amyloid in Alzheimer's disease. Neuroscientist 11, 250–260.
[3] Yamamoto, N., Igbabvoa, U., Shimada, Y., Ohno-Iwashita, Y., Kobayashi, M., Wood, W.G., Fujita, S.C. and Yanagisawa, K. (2004) Accelerated Aβ aggregation in the presence of GM1-ganglioside-accumulated synaptosomes of aged apoE4-knock-in mouse brain. FEBS Lett. 569, 135–139.
[4] Molander-Melin, M., Blennow, K., Bogdanovic, N., Dellheden, B., Mansson, J.E. and Fredman, P. (2005) Structural membrane alterations in Alzheimer brains found to be associated with regional disease development; increased density of gangliosides GM1 and GM2 and loss of cholesterol in detergent-resistant membrane domains. J. Neurochem. 92, 171–182.
[5] Gylys, K.H., Fein, J.A., Yang, F., Miller, C.A. and Cole, G.M. (2007) Increased cholesterol in Aβ-positive nerve terminals from Alzheimer's disease cortex. Neurobiol. Aging 28, 8–17.
[6] Cataldo, A.M., Peterhoff, C.M., Troncoso, J.C., Gomez-Isla, T., Hyman, B.T. and Nixon, R.A. (2000) Endocytic pathway abnormalities precede amyloid β deposition in sporadic Alzheimer's disease and Down syndrome: differential effects of APOE genotype and presenilin mutations. Am. J. Pathol. 157, 277–286.
[7] Lippincott-Schwartz, J. and Fambrough, D.M. (1987) Cycling of the integral membrane glycoprotein, LEP100, between plasma membrane and lysosomes: kinetic and morphological analysis. Cell 49, 669–677.
[8] Hayashi, H., Kimura, N., Yamaguchi, H., Hasegawa, K., Yokoseki, T., Shibata, M., Yamamoto, N., Michikawa, M., Yoshikawa, Y., Terao, K., Matsuzaki, K., Lemere, C.A., Selkoe, D.J., Naiki, H. and Yanagisawa, K. (2004) A seed for Alzheimer amyloid in the brain. J. Neurosci. 24, 4894–4902.
[9] Yamamoto, N., Hirabayashi, Y., Amari, M., Yamaguchi, H., Romanov, G., Van Nostrand, W.E. and Yanagisawa, K. (2005) Assembly of hereditary amyloid β-protein variants in the presence of favorable gangliosides. FEBS Lett. 579, 2185–2190.
[10] Nordstedt, C., Caporaso, G.L., Thyberg, J., Gandy, S.E. and Greengard, P. (1993) Identification of the Alzheimer β/A4 amyloid precursor protein in clathrin-coated vesicles purified from PC12 cells. J. Biol. Chem. 268, 608–612.

[11] Furst, W. and Sandhoff, K. (1992) Activator proteins and topology of lysosomal sphingolipid catabolism. Biochim. Biophys. Acta 1126, 1–16.
[12] Mobius, W., Herzog, V., Sandhoff, K. and Schwarzmann, G. (1999) Gangliosides are transported from the plasma membrane to intralysosomal membranes as revealed by immuno-electron microscopy. Biosci. Rep. 19, 307–316.
[13] Matsuzawa, Y. and Hostetler, K.Y. (1980) Inhibition of lysosomal phospholipase A and phospholipase C by chloroquine and 4,4′-bis(diethylaminoethoxy) alpha, β-diethyldiphenylethane. J. Biol. Chem. 255, 5190–5194.
[14] 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.
[15] Haass, C., Koo, E.H., Mellon, A., Hung, A.Y. and Selkoe, D.J. (1992) Targeting of cell-surface β-amyloid precursor protein to lysosomes: alternative processing into amyloid-bearing fragments. Nature 357, 500–503.
[16] Caporaso, G.L., Gandy, S.E., Buxbaum, J.D. and Greengard, P. (1992) Chloroquine inhibits intracellular degradation but not secretion of Alzheimer β/A4 amyloid precursor protein. Proc. Natl. Acad. Sci. USA 89, 2252–2256.
[17] Hurtado-Lorenzo, A., Skinner, M., El Annan, J., Futai, M., Sun-Wada, G.H., Bourgoin, S., Casanova, J., Wildeman, A., Bechoua, S., Ausiello, D.A., Brown, D. and Marshansky, V. (2006) V-ATPase interacts with ARNO and Arf6 in early endosomes and regulates the protein degradative pathway. Nat. Cell Biol. 8, 124–136.
[18] Kirkham, M., Fujita, A., Chadda, R., Nixon, S.J., Kurzchalia, T.V., Sharma, D.K., Pagano, R.E., Hancock, J.F., Mayor, S. and Parton, R.G. (2005) Ultrastructural identification of uncoated caveolin-independent early endocytic vehicles. J. Cell Biol. 168, 465–476.
[19] Lakadamyali, M., Rust, M.J. and Zhuang, X. (2006) Ligands for clathrin-mediated endocytosis are differentially sorted into distinct populations of early endosomes. Cell 124, 997–1009.