Journal of Neurochemistry, 2002, **82**, 1480–1489

Pyroglutamate-modified amyloid β-peptides – AβN3(pE) – strongly affect cultured neuron and astrocyte survival

Claudio Russo,* Elisabetta Violani,† Serena Salis,* Valentina Venezia,* Virginia Dolcini,* Gianluca Damonte,‡ Umberto Benatti,‡ Cristina D'Arrigo,§,¶ Eligio Patrone,¶ Pia Carlo* and Gennaro Schettini*

**Pharmacology and Neuroscience National Cancer Research Institute – IST c/o Advanced Biotechnology Center- and Section of Pharmacology, Department of Oncology Biology and Genetics, University of Genova, Italy*

†*Neurological Institute Casimiro Mondino, Pavia, Italy*

‡*Department of Experimental Medicine, Section of Biochemistry, University of Genova, Italy*

§*Division of Experimental Oncology. National Cancer Research Institute-IST, Genova, Italy*

¶*ISMAC Institute for Macromolecular studies, Section of Genova, National Research Council, Italy*

Abstract

N-terminally truncated amyloid-β (Aβ) peptides are present in early and diffuse plaques of individuals with Alzheimer's disease (AD), are overproduced in early onset familial AD and their amount seems to be directly correlated to the severity and the progression of the disease in AD and Down's syndrome (DS). The pyroglutamate-containing isoforms at position 3 [AβN3(pE)–40/42] represent the prominent form among the N-truncated species, and may account for more than 50% of Aβ accumulated in plaques. In this study, we compared the toxic properties, fibrillogenic capabilities, and *in vitro* degradation profile of Aβ1–40, Aβ1–42, AβN3(pE)–40 and AβN3(pE)–42. Our data show that fibre morphology of Aβ peptides is greatly influenced by the C-terminus while toxicity, interaction with cell membranes and degradation are influenced by the N-terminus. AβN3(pE)–40 induced significantly more cell loss than the other species both in neuronal and glial cell cultures. Aggregated AβN3(pE) peptides were heavily distributed on plasma membrane and within the cytoplasm of treated cells. AβN3(pE)–40/42 peptides showed a significant resistance to degradation by cultured astrocytes, while full-length peptides resulted partially degraded. These findings suggest that formation of N-terminally modified peptides may enhance β-amyloid aggregation and toxicity, likely worsening the onset and progression of the disease.

Keywords: Alzheimer's disease, amyloid, apoptosis, astrocytes, neurodegeneration, pyroglutamate.

J. Neurochem. (2002) **82,** 1480–1489.

Alzheimer's disease (AD) is characterized by abnormal accumulation of extracellular amyloidotic plaques closely associated with dystrophic neurones, reactive astrocytes and microglia (Terry and Katzman 1983; Glenner and Wong 1984; Itagaki *et al*. 1989; Funato *et al*. 1998; Selkoe 2001). Amyloid-β (Aβ) peptides are the primary components of senile plaques and are considered to be directly involved in the pathogenesis and progression of AD, an hypothesis supported by genetic studies (Glenner and Wong 1984; Borchelt *et al*. 1996; Lemere *et al*. 1996; Mann and Iwatsubo 1996; Citron *et al*. 1997; Selkoe 2001). Aβ is generated by proteolytic processing of the β-amyloid precursor protein (APP) (Kang *et al*. 1987; Selkoe 1998), which is sequentially cleaved by β-secretase at the N-terminus and by γ-secretase at the C-terminus of Aβ (Haass and Selkoe 1993; Simons *et al*. 1996). In addition to the dominant Aβ peptides starting with L-Asp at the N-terminus (Aβ1–42/40), a great

Received February 26, 2002; revised manuscript received June 13, 2002; accepted June 14, 2002.

Address correspondence and reprint requests to Gennaro Schettini, Department of Pharmacology and Neuroscience, IST c/o CBA, Largo Rosanna Benzi, 10, 16132 Genova, Italy. E-mail: schettini@cba.unige.it

Abbreviations used: AD, Alzheimer's disease; APP, amyloid precursor protein; Aβ, amyloid-β; AβN3(pE), Aβ isoforms beginning with a cyclized glutamate residue at position 3; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethylsulphoxide; LDH, lactate dehydorgenase; MALDI-TOF, matrix-assisted laser desorption/ionization with time of flight; MTT, 3-(4,5-dimethylthiazal-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride.

heterogeneity of N-terminally truncated forms occurs in senile plaques. Such shortened peptides are reported to be more neurotoxic *in vitro* and to aggregate more rapidly than the full-length isoforms (Pike *et al*. 1995). N-truncated peptides are known to be overproduced in early onset familial AD (FAD) subjects (Saido *et al*. 1995; Russo *et al*. 2000), to appear early and to increase with age in Down's syndrome (DS) brains (Russo *et al*. 1997, 2001; Tekirian *et al*. 1998). Finally, their amount reflects the progressive severity of the disease (Russo *et al*. 1997). Additional post-translational processes may further modify the N-terminus by isomerization or racemization of the aspartate at position 1 and 7 and by cyclization of glutamate at residues 3 and 11. Pyroglutamate-containing isoforms at position 3 [AβN3(pE)−40/42] represent the prominent forms – approximately 50% of the total Aβ amount – of the N-truncated species in senile plaques (Mori *et al*. 1992; Saido *et al*. 1995; Russo *et al*. 1997; Tekirian *et al*. 1998; Geddes *et al*. 1999; Harigaya *et al*. 2000) and they are also present in pre-amyloid lesions (Lalowski *et al*. 1996). The accumulation of AβN3(pE) peptides is likely due to the structural modification that enhances aggregation and confers resistance to most amino-peptidases (Saido *et al*. 1995; Tekirian *et al*. 1999). This evidence provides clues for a pivotal role of AβN3(pE) peptides in AD pathogenesis. However, relatively little is known about their neurotoxicity and aggregation properties (He and Barrow 1999; Tekirian *et al*. 1999). Moreover, the action of these isoforms on glial cells and the glial response to these peptides are completely unknown, although activated glia is strictly associated to senile plaques and might actively contribute to the accumulation of amyloid deposits.

In the current study we have investigated the toxicity, aggregation properties and catabolism of Aβ1–42, Aβ1–40, AβN3(pE)−42 and AβN3(pE)−40 peptides in neuronal and glial cells cultures, finding that pyroglutamate modification worsen the toxic properties of Aβ-peptides and inhibit also their degradation by cultured astrocytes.

Materials and methods

Aβ peptides and antibodies

Stock solutions (1 mM) of synthetic peptides Aβ1–42, Aβ1–40 (Quality Controlled Biochemicals, Hopkinton, MA, USA), AβN3(pE)−42 and AβN3(pE)−40 (American Peptide Co., Sunnyvale, CA, USA) were dissolved in dimethylsulfoxide (DMSO) 86%, then diluted in phosphate-buffered saline (PBS; pH 7.4) and maintained at 37°C for 3 days prior to further dilution at the concentration indicated. The final maximal DMSO concentration in cell cultures was about 0.8% when Aβ was added at the maximal concentration tested (10 μM), and therefore the same amount of DMSO was added to the cells used as control cell cultures. The mouse monoclonal antibody 4G8 (Signet Pathology Systems Inc., Dedham, MA, USA) specific to human Aβ17–24 residues was used at a dilution of 1 : 200 for immunoprecipitation and immunofluorescence, 1 : 1000 for immunodetection. Polyclonal anti-N3(pE) antibody specific to pyroglutamate at position 3 and polyclonal anti-N1(D) antibody specific to aspartate at position 1 of Aβ sequence kindly provided by Dr T. C. Saido (Saido *et al*. 1995) were employed for immunodetection at a dilution of 1 : 500.

Cell cultures

Primary cultures of rat hippocampal neurones were obtained from 18- to 20-day-old Sprague–Dawley embryos according to the method of Goslin and Banker (1991) with slight modifications. Briefly, hippocampi were excised, trypsinized and resuspended in neurobasal medium supplemented with 2% B27 (Gibco/Life Technologies, Rockville, MA, USA), 0.5 mM glutamine and antibiotics. Cells were seeded in poly-L-lysine-coated wells and after 8–9 days the cell population was determined to be at least 95% neuronal by immunostaining. Primary cultures of rat cortical type-1 astrocytes were prepared from 2-day-old Sprague–Dawley pups according to the method of Levison and McCarthy (1991). Briefly, cortices were dissected out, trypsinized and cells cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 IU/mL penicillin, 100 μg/mL streptomycin). Astrocyte-enriched cultures were obtained from mixed glial cultures by the shaking off method and were comprised of greater than 95% glial fibrillary acidic protein-positive cells.

Cytotoxicity assay

Cell viability was determined by 3-(4,5-dimethylthiazal-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann 1983). Hippocampal neurones, 8–9 days after seeding 4×10^5 cells/well in 24-well plates, were exposed for 24 h to each Aβ peptide diluted in medium to a final concentration of 0.1, 1 and 10 μM and aged at 37°C for 3 days. Astrocytes were treated identically 3 days after seeding 2.5×10^5 cells/well in 12-well plates. Control cells were exposed to vehicle alone containing the same quantity of DMSO as in the Aβ-treated cells from the stock solution. Neuron and astrocyte viability was further evaluated after a 24-h exposure to 1 μM Aβ peptides by measuring both the release of lactate dehydrogenase (LDH) activity (Cytotoxicity Detection Kit, Roche, Mannheim, Germany) and the cytoplasmic histone-associated-DNA-fragments (Cell Death Detection ELISA Plus kit, Roche, Mannheim, Germany).

Electron microscopy analysis

Diluted aqueous solutions (0.4 μg/μL) of the Aβ peptides were aged at 37°C for 1–5 days and during incubation the aggregation state of each peptide was monitored. Two microliters of each sample were adsorbed onto a carbon-coated 600-mesh copper grid and negatively stained with 3 μL of 2% uranyl acetate. Air-dried specimens were examined and photographed in a Zeiss LEO 900 electron microscope operating at 80 kV.

Confocal microscopy analysis

Cytomorphological analyses were carried out on hippocampal neurones and astrocytes seeded at 5×10^4 cells on glass coverslips and the day after exposed for 24 h to 0.1 μM concentration of each Aβ peptide. Cells, fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton, were incubated with 4G8 antibody together with anti-N3(pE) or anti-N1(D) antibodies for 20 min and then the

secondary antibodies [fluorescein isothiocyanate (FITC)- or rhodamine-conjugated] were applied for 40 min. Samples were analysed on Bio-Rad-MRC 1024 ES confocal microscope (60× objective, argon laser sequential excitation at 468 and 580 nm), 0.2 μm step in z-plane acquisition.

Western blot and mass spectrometry analyses

The proteolytic degradation of Aβ peptides was investigated in astrocytes. Cells were seeded at 8×10^5 cells/well in 6-well plates and after 5 days exposed in serum-free conditions for 24 h to a 1-μM concentration of each Aβ peptide for western blot analysis and mass spectrometry analysis. Aβ species were immunoprecipitated with 4G8 antibody from cell lysates and the antigen–antibody complexes collected by protein A-agarose beads. Electrophoresis, immunoblotting and densitometry were carried out as previously described (Russo *et al*. 1997). Briefly, immunoprecipitated peptides were separated by Tris–tricine gel electrophoresis, blotted to polyvinylidene difluoride (PVDF) membranes, visualized with 4G8 antibody by enhanced chemiluminescence, using 5 ng of Aβ peptides as standard. For MALDI-TOF (matrix-assisted laser desorption/ionization with time of flight)-mass spectrometry the antigen-antibody complexes in the cell lysate were collected by magnetic beads covalently coupled with anti-mouse IgG (Dynal A. S., Oslo, Norway) and then suspended in 10 μL of ddH_2O. Two microliters of the sample were incubated briefly with 2 μL of a matrix prepared dissolving α-cyano-4-hydroxycinnamic acid (Aldrich, Milan, Italy) at 10 mg/mL in 0.05 M HCl and acetonitrile:isopropanol 5 : 1.5 (v/v). One microliter of the incubation mixture together with 1 μL of the matrix solution were placed on the sample plate and allowed to dry. The analysis was performed in linear positive mode on a Kratos Compact MALDI 4 mass spectrometer and a minimum of 100 scans was averaged.

Statistical analysis

Results from MTT assay are expressed as the percentage of viable cells in treated cultures relative to untreated control cultures designated as 100%. For the apoptosis test, data are expressed as the percentage of DNA fragments or oligonucleotides relative to untreated control cultures designated as 100%, while data from LDH assay are expressed as the percentage of the released LDH over the total releasable amount of LDH present in cells. Experiments were repeated at least three times and run in triplicate or quadruplicate. All data are expressed as means ± SEM. Statistical analysis was performed by ANOVA followed by Tukey's *post hoc* test.

Results

The toxicity of aggregated Aβ peptides was examined in rat cultured hippocampal neurones and cortical astrocytes (Fig. 1). Exposure of hippocampal neurones to 0.1 μM AβN3(pE)−40 caused a 23% ($p < 0.001$) decrease in cell viability relative to controls, whereas no significant neurotoxicity was exhibited by the other peptides. At 1 μM AβN3(pE)−40 exerted a 31% ($p < 0.001$) decrease on cell viability, while a toxic effect of 10% ($p < 0.05$) was measured with Aβ1–42 and Aβ1–40 and a 13% ($p < 0.05$) with AβN3(pE)−42. Addition of 10 μM AβN3(pE)−40, Aβ1–42, Aβ1–40 and AβN3(pE)−42 caused a 51, 18, 24 and 19% ($p < 0.001$) cell death, respectively. In comparison to neuronal cells, astrocytes exhibited relatively less susceptibility to the toxic effects of Aβ peptides as evidenced by a general reduced cellular loss. Significant toxicities were observed with AβN3(pE)−40 such that 19% ($p < 0.05$) and 26% ($p < 0.001$) of astrocytes were killed by 0.1 and 1 μM, respectively, whereas the other species were less effective. Treatment of astrocytes with 10 μM AβN3(pE)−40 resulted in a 46% ($p < 0.001$) death while the addition of Aβ1–40 caused a 20% ($p < 0.001$) death; and of AβN3(pE)−42 a 24% ($p < 0.001$), while Aβ1–42 was apparently not toxic to cells. Among Aβ isoforms, AβN3(pE)−40 resulted in the most bioactive species both in neuronal and non-neuronal cultures. Specifically, the peptide induced greater cell loss than Aβ1–42 at any concentration tested ($p < 0.001$). AβN3(pE)−40 caused more degeneration than Aβ1–40 either at 0.1 μM ($p < 0.05$) or at 1–10 μM ($p < 0.001$) and was more effective than AβN3(pE)−42 at 0.1 μM ($p < 0.01$) and at 1–10 μM ($p < 0.001$). AβN3(pE)−40 was the most effective species to cultured astrocytes too, even if at a lower extent than in neurones. At 1 or 10 μM the peptide induced a

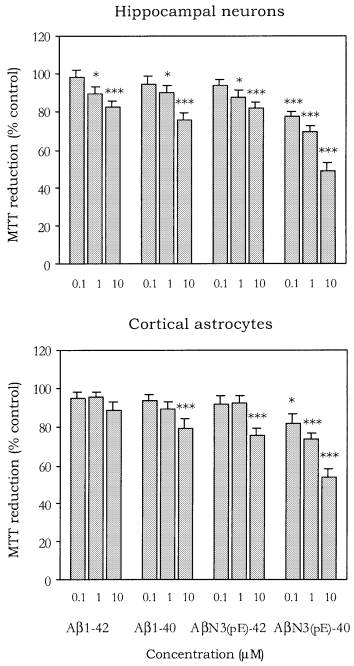

Fig. 1 Cytotoxicity of Aβ1–42, Aβ1–40, AβN3(pE)−42 and AβN3(pE)−40 peptides in rat cultured hippocampal neurones and cortical astrocytes. Cultures were exposed for 24 h to Aβ peptides and cells viability was determined by MTT assay. Data represent mean ± SEM cell loss from at least three independent determinations with 2–3 replicates. Statistical significance was evaluated by ANOVA followed by Tukey's post-hoc test: $*p < 0.05$, $***p < 0.001$ versus control values designed as 100%.

Table 1 Effects of Aβ peptides on apoptosis and LDH release from rat cultured hippocampal neurones and cortical astrocytes

	Neurones		Astrocytes	
	DNA fragmentation (% of control)	LDH release (% of control)	DNA fragmentation (% of control)	LDH release (% of control)
Aβ1-42	115 ± 4**	97 ± 5	105 ± 6	100 ± 5
Aβ1-40	118 ± 4**	89 ± 4	108 ± 6	117 ± 5*
AβN3(pE)-42	117 ± 4**	95 ± 4	100 ± 6	132 ± 5***
AβN3(pE)-40	144 ± 5***	96 ± 5	106 ± 6	140 ± 6***

Oligonucleosome amount and LDH release were assessed in the same cultures exposed for 24 h to 1 μM Aβ peptides. Data represented mean ± SEM from three separate determinations with four samples and are normalised to the effect of vehicle. Statistical significance was evaluated by ANOVA followed by Tukey's post hoc test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. control values designed as 100%.

greater damage than Aβ1–42 ($p < 0.001$), AβN3(pE)−42 ($p < 0.01$) and Aβ1–40 ($p < 0.01$), whereas at 0.1 μM the differences were not statistically significant. Finally, no significance was found comparing Aβ1–42 toxicity with Aβ1–40 or AβN3(pE)−42 toxicities as well as comparing Aβ1–40 with AβN3(pE)−42, either in hippocampal neurones or in astrocytes.

The concentration of 1 μM was chosen to characterize the mechanisms involved in Aβ-mediated cell death. Hippocampal neurones challenged with Aβ1–42, Aβ1–40, AβN3(pE)−42 and AβN3(pE)−40 underwent apoptotic cell death exhibiting the characteristic DNA fragmentation (Table 1). Addition of Aβ1–42, Aβ1–40 and AβN3(pE)−42 increased by 15, 18 and 17% ($p < 0.01$) oligonucleosome formation relative to controls, respectively, and addition of AβN3(pE)−40 by 44% ($p < 0.001$). DNA fragmentation induced by AβN3(pE)−40 was markedly greater than that generated by other species (vs. Aβ1–40, Aβ1–42 and AβN3(pE)−42, $p < 0.001$). Under the same conditions, no nuclear fragmentation was detected in astrocyte cultures. Similar results were obtained after Bis-Benzimide (Hoechst 33258) staining of Aβ-treated cells to detect nuclear fragmentation as well as by morphological examination. Evaluation of LDH leakage into the culture medium, which reflects lysis of damaged cells, showed basically no LDH release by hippocampal neurones (Table 1). On the contrary, in astrocyte cultures, LDH amount increased by 17% ($p < 0.05$) following Aβ1–40 exposure, by 32% ($p < 0.001$) after AβN3(pE)−42 treatment and by 40% ($p < 0.001$) after AβN3(pE)−40, as compared to controls, whereas no effect was observed in Aβ1–42-treated astrocytes. These data suggest that LDH measurement in cultured astrocytes is more sensitive in detecting cell death than MTT reduction assay, likely in force of the delayed mitochondrial involvement in Aβ-treated astrocytes in comparison to apoptotic events as shown in neurones.

Because Aβ neurotoxicity is related to the aggregation state of peptides (Pike *et al*. 1995; Seilheimer *et al*. 1997; Walsh *et al*. 1999), negatively stained samples of each Aβ peptide were analysed by transmission electron microscopy in order to characterize the fibre formation process. As shown in Fig. 2, full-length peptides were completely self-assembled after 3 days of incubation (Figs 2a and b), displaying morphological features quite similar to those previously reported by other groups (Seilheimer *et al*. 1997; Walsh *et al*. 1997). AβN3(pE)−42 (Fig. 2c) aggregated into fibres characterized by constant diameter (6–15 nm) and pitch (105–120 nm) along the axis, consistent with the typical morphology of mature fibres. Frequently, fibres self-associated laterally and assumed a double- or multistranded appearance maintaining the periodic modulation of the width. AβN3(pE)−40 isoform (Fig. 2d) exhibited a specific structural feature polymerizing in small globular particles (5–30 nm in diameter) ordinarily self-organized in fibres with rough surface that assembled into irregular bundles by lateral aggregation. Granular chains became the dominant peptide organization and were invariably observed together with globular particles, most likely oligomers which were present even at prolonged incubation time. As previously reported globular structures have been visualized in full-length samples as well (Seilheimer *et al*. 1997), in this case, however, they were detectable only at the early stage of fibrillogenesis as seed-like particles became undetectable after 3 days of incubation. Aβ1–40 curly fibres, when analysed at higher magnitude (Fig. 2e), revealed that, as for AβN3(pE)−40 (Fig. 2f), are formed by short chains of globular seed-like aligned aggregates. In spite of the marked heterogeneity in fibre morphology, Aβ1–42 and AβN3(pE)−42 filaments grew to the mature fibre type, forming at the end of the aggregation process a dense meshwork of long fibrils, whereas Aβ1–40 and AβN3(pE)−40 assembled more slowly to generate short curly fibres. Moreover, AβN3(pE)−40 did not show any tendency to aggregate into mature fibres, and maintained a globular appearance. To verify if morphological differences between peptides would influence their interaction with cultured cells and their toxic activity, we examined by confocal microscopy with optical sectioning hippocampal neurones and astrocytes exposed to Aβ, in order to explore the localization and the interaction of the peptides with

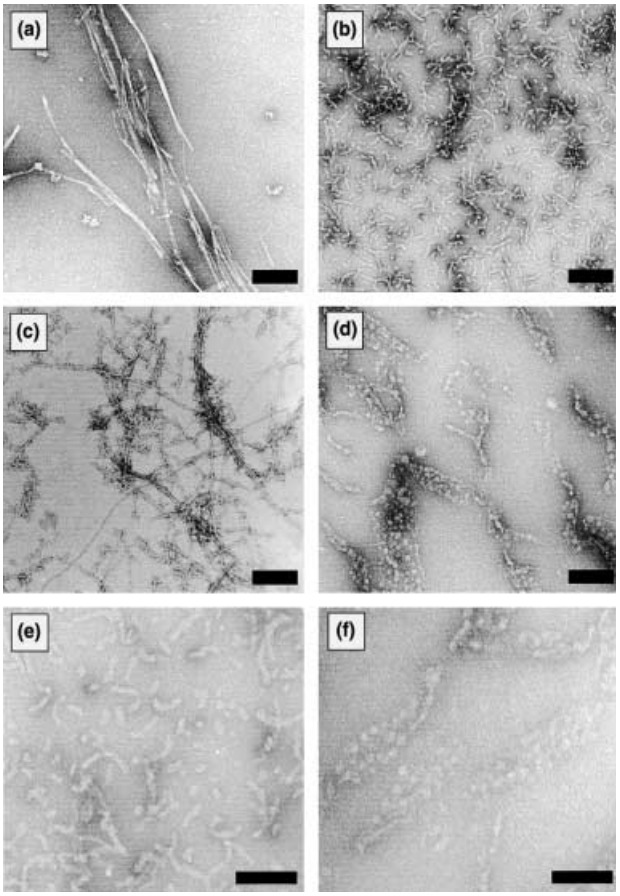

Fig. 2 Electron micrographs of fibres developed by each Aβ peptide after 3-day *in vitro* ageing. Aβ1–42 and AβN3(pE)–42 (a and c) form aggregates showing the characteristic feature of long mature fibre with constant diameter, axial periodicity, and eventually lateral association. Aβ1–40 (b) display morphological features with short curly fibres formed by short chains of globular oligomers like AβN3(pE)–40 (d) which polymerizes into small globular particles (5–30 nm in diameter) that partially self-organize in rough bundles by lateral aggregation. Higher magnification of Aβ1–40 (e) and AβN3(pE)–40 (f) shows the similar appearance of the short curly chains formed by these peptides and the persistent presence of globular particles. Bars corresponds to 150 nm (a–d) and to 100 nm (e and f).

plasma membranes. Figure 3 shows four z-plane sections of AβN3(pE)–40-treated neurones (Figs 3a–c). An intense dotted Aβ labeling after staining with anti-N3(pE) antibody (specific for human N-terminal pyroglutamate, red signal in Fig. 3b) was present throughout the cell. Immunoreactive Aβ granules were variable in size and were located on the cell surface and in the cytoplasm as well as on spines and dendrites (arrows). The staining with the 4G8 antibody (Fig. 3a) in the same sections, co-localized with Aβ aggregates, although in Triton X-100-treated cells 4G8 detected an aspecific signal from the entire cell (likely due to APP and APP's fragments) which, however, in this case, contributed to defining the cell morphology. A line scan (Figs 3d and f) in a Y-plane throughout an Aβ aggregate identified in the correspondent orthogonal plane (Figs 3c and e, white arrow) confirmed that the peptide stuck to membranes and threaded into the intracellular space likely piercing and disrupting the lipid bilayer, both in neurones (Fig. 3c and d) and astrocytes (Fig. 3e and f). The intensity of interaction is in some cases very severe as shown in Fig. 3(g and h; astrocytes) and Fig. 3(I–k; neurones), were cultured cells were treated with AβN3(pE)–40 (green signal). Full-length species and AβN3(pE)–42 behaved similarly to AβN3(pE)–40 peptide, the only difference being a lower level of cell-associated Aβ-immunoreactive granules, suggesting a strong physical interaction between pyroglutamyl-Aβ and cultured cells.

To investigate whether the proteolytic processing of the different Aβ peptides could account in part for the higher glial toxicity of pyroglutamate-modified Aβ with respect to the corresponding full-length isoforms, cell lysates from astrocytes treated with 1 μM of each peptide were subjected to western blot analysis (Figs 4a and b). Synthetic full-length Aβ and AβN3(pE)–peptides migrate, in normal conditions, as two distinct bands with the apparent molecular weight of 4.5 and 4.3 kDa (Fig. 4a), respectively, as previously shown (Russo *et al*. 1997). Cell lysates from Aβ-treated astrocytes were immunoprecipitated with the antibody 4G8 specific for all Aβ isoforms. After sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting, Aβ peptides were detected with the same antibody showing the presence of two bands with apparent molecular masses of 4.5 kDa and 4.3 kDa in astrocytes exposed to Aβ1–40 or Aβ1–42 (Fig. 4b, lanes 1 and 2), suggesting that full-length peptides may be partially digested by cultured astrocytes. Only one band of about 4.3 kDa (Fig. 4b, lanes 3 and 4) was detected in AβN3(pE)–40- and AβN3(pE)–42-treated astrocytes indicating that these species remain uncleaved, or at least that their degradation products were under the detection limit of our method. As example, mass spectrometry analysis (Fig. 4c) provided further evidence that astrocytes exposed to 1 μM AβN3(pE)–40 failed to degrade the peptide (Fig. 4c, upper panel) as only one peak of 4125.1 mass units was observed, while Aβ1–42-treated cells (lower panel) yielded five minor peaks besides the major one at 4515.1 corresponding to Aβ1–42 itself.

Discussion

The abnormal extracellular accumulation of amyloid deposits observed in AD brains is thought to be a central event in the AD pathogenesis (Selkoe 2001). Amyloid deposits consist predominantly of heterogeneous Aβ peptides with different N- and C-termini. Recent data (Saido 1998;

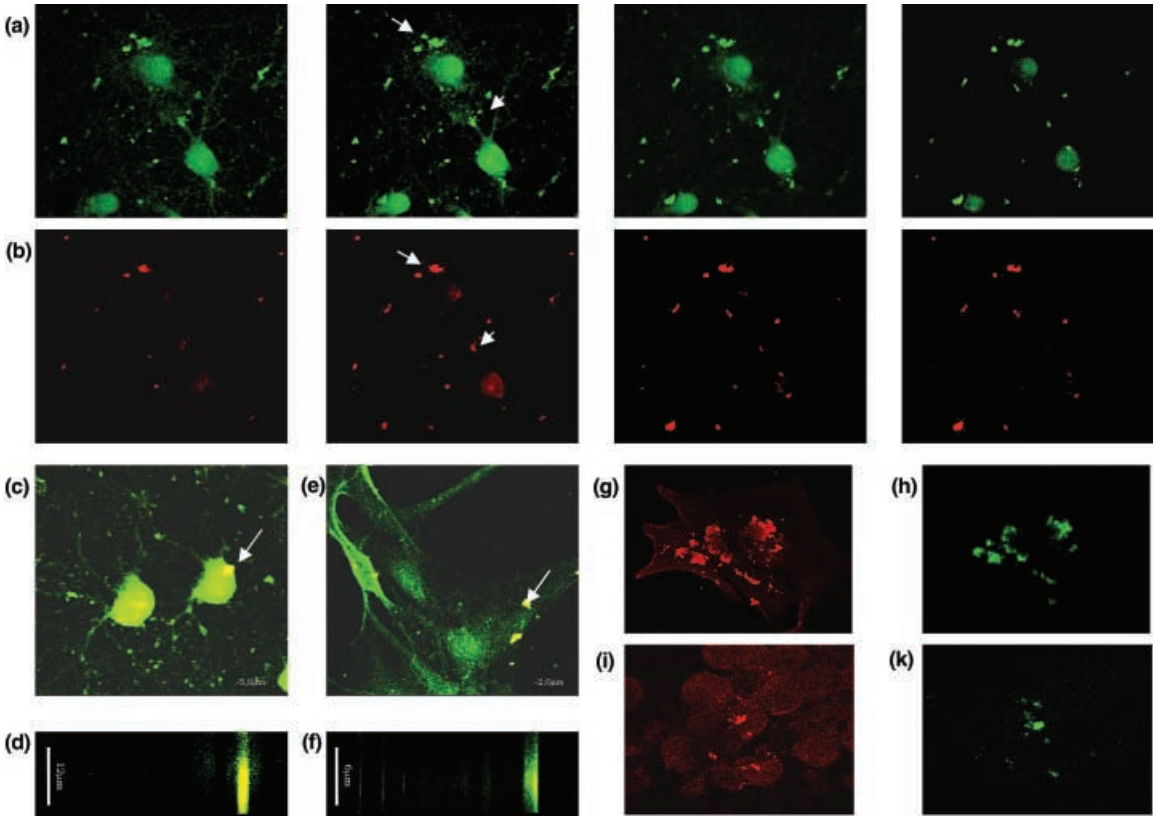

Fig. 3 Double labelling with z-plane optical sectioning of AβN3(pE)–40 immunoreactive aggregates in rat hippocampal neurones (a–d) and cortical astrocytes (e,f). After a 24-h exposure to 0.1 μM AβN3(pE)–40, cultures were immunostained with 4G8 antibody [specific for rat and human Aβ and APP – green signal in (a)] and with anti-N3(pE) antibody [specific for Aβ N-terminal pyroglutamate – red signal in (b)] followed by FITC- and rhodamine-conjugated secondary antibodies, respectively. Serial z-images at 0.2 μm reveal the presence of Aβ aggregates on cell membranes, dendrites and in the cytoplasm, as well as the co-localization of probes (d and e; yellow colour, $r = 0.99$) on AβN3(pE)–40-immunoreactive granules (arrow) which stick to the cell surface or thread into the cytoplasm. This evidence is supported by the orthogonal Y-line scan (d and f) through the Aβ aggregates identified in (c) and (e), which shows the Aβ aggregate penetrating throughout the cell thickness. AβN3(pE)–40 treated astrocytes (g and h) and hippocampal neurones (i–k) are double labelled as above described with inverted secondary antibodies. The images represent sequential acquisitions on the same z-plane, showing the very extensive presence of Aβ aggregates (specifically labelled by the anti-AβN3(pE) modification; green signal) inside the cells.

Harigaya *et al*. 2000) emphasize the role of N-terminally modified Aβ peptides as hydrophobic and amyloidogenic isoforms, probably related to the early deposition of Aβ in plaques. N-truncated Aβ peptides are present at the initial deposition steps in AD and DS (Saido *et al*. 1996; Hosoda *et al*. 1998), their content progressively increases with age in DS subjects (Russo *et al*. 1997) and they are overexpressed in early onset FAD phenotype linked to APP and presenilin 1 gene mutations (Ancolio *et al*. 1999; Russo *et al*. 2000). Among the different N-truncated Aβ species, the pyroglutamyl-modified peptides at residues 3 [N3(pE)] and 11 [N11(pE)] are of particular interest and, specifically, the N3(pE)-modified Aβ peptides are considered the dominant Aβ species in AD plaques (Kuo *et al*. 1997; Harigaya *et al*. 2000). Recently, we found that N3(pE)-modified Aβ peptides do not derive from a progressive proteolysis of full-length peptides but directly from C-terminal fragments of APP processed by β-secretase (Russo *et al*. 2001). Therefore they constitute an independent amyloidogenic entity whose origin may be unrelated to that of full-length species (Paganetti and Staufenbiel 2000). Finally, Aβ peptides present in plaques of transgenic mice overexpressing APP lack N-terminal degradations, post-translational modifications and cross-linkages that are instead abundant in the Aβ deposited in AD (Kalback *et al*. 2002), giving rise to the hypothesis that biochemical diversity might be in part responsible for the neuropathological differences in terms of neuronal loss and glial response between animal models and AD. Despite the amount of information describing pyroglutamate-modified Aβ as molecules likely related to the amyloidogenic process and also to the severity of the disease, little and contrasting information is available about their toxicity and fibrillogenic properties (He and Barrow 1999; Tekirian *et al*. 1999).

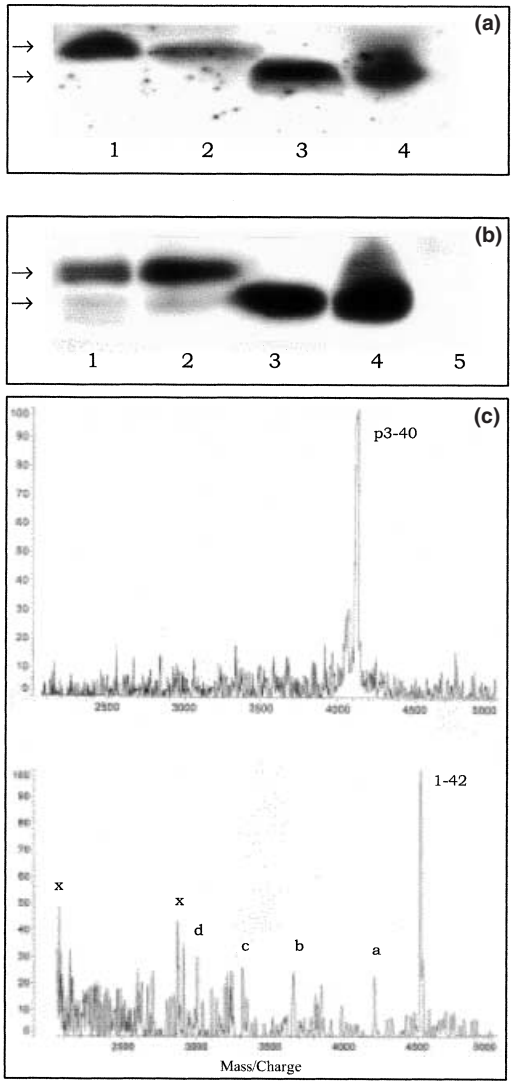

Fig. 4 Aβ proteolytic products recovered from Aβ-treated astrocytes. Astrocytes were exposed for 24 h to 1 μM of each peptide. (a) Electrophoretic migration of unprocessed Aβ1–42, Aβ1–40, AβN3(pE)–42 and AβN3(pE)–40, lanes 1–4, respectively. (b) After immunoprecipitation and immunodetection with 4G8 antibody, lysates of cells treated with Aβ1–42 or Aβ1–40 (lanes 1 and 2) show two bands of about 4.5 and 4.3 kDa indicating a partial cleavage of full-length peptides. A single 4.3 kDa band (lanes 3 and 4) results from AβN3(pE)–42- and AβN3(pE)–40-treated astrocytes, suggesting that these species are resistant to cleavage. Vehicle-treated culture is shown as control (lane 5). (c) Aβ species immunoprecipitated from astrocytes lysates were analysed by MALDI-TOF mass spectrometry. Samples from AβN3(pE)–40-treated astrocytes yield a major peak of 4125.1 m/z corresponding to the peptide itself (upper panel), whereas Aβ1–42-treated cells show peaks corresponding to Aβ fragments besides the major one corresponding to Aβ1–42 itself (lower panel). (a) Aβ4–42, observed 4203.1 m/z, calculated 4198.8 m/z; (b) Aβ1–33, observed 3675.6 m/z, calculated 3674.0 m/z; (c) Aβ10–40 observed 3314.7 m/z, calculated 3314.8 m/z; (d) Aβ4–29, observed 3002.3 m/z, calculated 3004.3 m/z (x) species constantly present but unmatched.

The results of the present study suggest that N-terminal deletion and cyclization contribute to increasing the toxicity of Aβ species. AβN3(pE)–40 peptide resulted in more toxicity than the homologous Aβ1–40, and AβN3(pE)–42 resulted in more toxicity than Aβ1–42 both to cultured neurones and astrocytes. AβN3(pE)-modified peptides significantly affected neuronal and astrocyte viability, and AβN3(pE)–40 resulted in the most toxic species even at the lowest concentration tested. The significant levels of nuclear fragmentation in hippocampal neurones and the consistent amount of LDH leakage in astrocytes suggest two different patterns of Aβ-induced cell death, apoptosis in neurones and necrosis in astrocytes. AβN3(pE)–40 caused the highest DNA fragmentation in cultured neurones and the greatest LDH release in astrocytes, suggesting that the pyroglutamyl-modified peptides may share similar degenerative mechanisms with full-length species, although with higher toxicity. Heterogeneous results are present in the literature about the toxic properties of Aβ peptides, likely reflecting variations in the purity of peptides, ageing conditions, aggregation status, solvents and tests used (Mattson *et al*. 1992; Pike *et al*. 1995; Seilheimer *et al*. 1997; Walsh *et al*. 1997). For example, previous reports on pyroglutamyl-Aβ presented contrasting conformational data by circular dichroism studies: He and Barrow (1999) described a potent effect of the pyroglutamyl modification in inducing a rapid increase in β-sheet content than that detected in the full-length forms; while in the study by Tekirian *et al*. (1999) no significant differences were detected in β-sheet content between full-length and pyroglutamyl-Aβ. Also, Tekirian *et al*. (1999) described similar toxicities between AβN3(pE)-peptides and full-length Aβ, at least in hippocampal neurones, while our data instead suggest a significant effect of the pyroglutamyl-modification in enhancing cellular toxicity in neuronal and in glial cells. A partial explanation for these discrepancies might be due to different Aβ preparations, different experimental settings and different ultrastructures, which mainly depend on solvents and ageing times utilized, as shown in our negatively stained fibres.

Because a great deal of work suggests that Aβ toxicity increases with the enhanced aggregation state (Roher *et al*. 1996; Hartley *et al*. 1999) we sought to determine the level of *in vitro* assembling for each Aβ peptide. After 72 h ageing, the mature fibres of Aβ1–42 and those of the homologous AβN3(pE)–42 aggregated in elongated individual strands forming a dense meshwork of long fibrils with a periodical restriction of diameter along the axis (Figs 2a and c). In the same condition, Aβ1–40 (Fig. 2b) assembled in short and curly fibres as previously reported (Walsh *et al*. 1997), formed by short chains of globular aggregates (Figs 2b and e). AβN3(pE)–40 (Figs 2d and f) also assumed a morphology very similar to that of Aβ1–40 characterized by globular particles often aggregated in dense bundled structures with an irregular surface. Similar particles have

been previously described as monomeric and dimeric Aβ1–40/42 and have been associated with enhanced levels of cytotoxicity probably due to an easier diffusion in comparison to elongated fibrils (Roher *et al*. 1996; Lambert *et al*. 1998). These structures may represent an early step of fibre formation which can last for a more prolonged period in comparison to the Aβx-42 isoforms. Indeed, notwithstanding the different morphologies, the initial steps of Aβ peptides polymerization apparently proceeded by formation of small globular-like structures, which could act as early seeds (Seilheimer *et al*. 1997). Because Aβ1–42 and AβN3(pE)−42 reached rapidly the 'mature' condition forming a dense meshwork of fibres, while Aβ1–40 and AβN3(pE)−40 achieved this condition slowly, it can be speculated that, if the N-terminus influences the toxic properties of Aβ peptides, the C-terminus strongly affects the fibre profile and the aggregation behaviour, and we hypothesize that similar fibres may exert similar toxic properties. Between the AβN3(pE)-modified peptides, the isoform ending at Val40 results in the most toxic species and, considering that both pyroglutamyl and Aβ show similar resistance to degradation by astrocytes, our hypothesis is that this is due mainly to the AβN3(pE)−40 tendency to retain a more diffusible and eventually toxic morphology as short oligomeric particles (Lambert *et al*. 1998).

Immunocytochemistry and confocal analysis provide evidence for a close Aβ–membrane interaction demonstrating that Aβ aggregates could adhere to the cell surface and stick into the cytoplasm. The consequent structural changes in the lipid bilayer may have functional consequences directly or through signal transduction disturbances. For instance, the interaction of Aβ peptides with the membrane could lead to uncontrolled Ca^{2+}-free influx (Mattson *et al*. 1992; Arispe *et al*. 1993; Scorziello *et al*. 1996; Lin *et al*. 2001), which in turn activates cell death pathways involving lipid peroxidation and free radical generation. Alternatively, Aβ peptides could directly activate specific cell surface channel receptors (Ekinci *et al*. 1999) or even bind APP itself leading to apoptotic cell death (Lorenzo *et al*. 2000). We observed a general trend in the peptides tested to interact massively with plasma membrane, to adsorb diffusely to cell soma, dendritic spines, as well as cytoplasmic processes without an apparent order or hierarchy. By comparing the surface distribution of AβN3(pE)−40, AβN3(pE)−42 and full-length aggregates, no apparent preferential localization was found, as peptides were ubiquitously and randomly distributed; nevertheless, AβN3(pE)−40 showed higher levels of immunoreactivity when compared with the other species. Therefore, although apparently similar, the severe interaction between Aβ peptides and cell membranes results in different phenotypic changes as shown by MTT test, LDH release and pro-apoptotic tendency, and we hypothesize that these changes are in part due to intrinsic properties of each Aβ aggregates (fibre morphology, resistance to proteolytic cleavage, hydrophobicity, etc.), but also to specific features of each cell type.

Previous reports (Saido 1998) indicated that AβN3(pE) isoforms are more resistant than full-length peptides to the proteolytic cleavage because of their N-terminal cyclized glutamate, which requires a specific aminopeptidase (Saido *et al*. 1995; Takaki *et al*. 2000), to be processed and digested. As a consequence, in absence of a specific degradative machinery, these modified peptides may exert toxic effects for prolonged times. Evidence for a decreased catabolism of AβN3(pE)-modified peptides compared to that of full-length peptides is found here in Aβ-treated astrocytes. *In vivo* the relative resistance to degradation of N-modified isoforms may constitute a seed for the aggregation of all Aβ species and exert a cumulative toxic effect on both neurones and astrocytes contributing to cellular death, even if their action may be partially blocked by Aβ scavengers, like apolipoprotein E or apolipoprotein J (Russo *et al*. 1998; Munson *et al*. 2000). The presence of activated astrocytes and microglia in the core of senile plaques as well as the occurrence of cytoplasmic Aβ-immunoreactivity in glial cells surrounding amyloid deposits (Funato *et al*. 1998) have been widely interpreted as an active reaction of astrocytes versus Aβ production. This defensive activation may be exacerbated by AβN3(pE) production which contributes to neuronal injury directly or indirectly by impairing neuronal homeostasis.

Recent data show that N-truncated and modified Aβ are absent in plaques of transgenic mice overexpressing APP (Kalback *et al*. 2002), suggesting that these species might be important in sustaining the neurotoxic insult and the cascade of events that characterize AD and are less pronounced or absent in the transgenic models. Also, in the light of recent data on the reciprocal pathophysiological role of Aβ and tau, it would be interesting to evaluate the effect of AβN3(pE) peptides on the status of tau protein in those transgenic mice in which has been shown that Aβ1–42 fibrils can accelerate neurofibrillary tangles formation (Gotz *et al*. 2001).

In conclusion, our data demonstrate that AβN3(pE) isoforms are resistant to proteolysis, aggregate extensively and are more toxic than the homologous full-length isoforms to cultured rat neurones and astrocytes. Because AβN3(pE)-modified peptides are the most abundant species in sporadic and familial AD plaques, we hypothesize that these fragments may impair glial functions and exacerbate neuronal damage, likely worsening the progression of the disease.

Acknowledgements

We are grateful to Dr Takaomi C. Saido, Riken Brain Science Institute, Saitama, Japan, for providing generously antibodies anti-N3(pE) and anti-N1(D). This work was supported by Telethon Grant E4411,MIUR PRIN 2000, MISAN IRCCS Progetto Strategico Alzheimer grant and by Angelini Ricerche to GS.

References

Ancolio K., Dumanchin C., Barelli H., Warter J. M., Brice A., Campion D., Frebourg T. and Checler F. (1999) Unusual phenotypic alteration of β amyloid precursor protein (βAPP) maturation by a new Val-715 – > Met βAPP-770 mutation responsible for probable early-onset Alzheimer's disease. *Proc. Natl Acad. Sci. USA* **96**, 4119–4124.

Arispe N., Pollard H. B. and Rojas E. (1993) Giant multilevel cation channels formed by Alzheimer's disease amyloid β-protein [Aβ P-(1–40)] in bilayer membranes. *Proc. Natl Acad. Sci. USA* **15**, 10573–10577.

Borchelt D. R., Thinakaran G., Eckman C. B., Lee M. K., Davenport F., Ratovitsky T., Prada C. M., Kim G., Seekins S., Yager D., Slunt H. H., Wang R., Seeger M., Levey A. I., Gandy S. E., Copeland N. G., Jenkins N. A., Price D. L., Younkin S. G. and Sisodia S. S. (1996) Familial Alzheimer's disease-linked presenilin 1 variants elevate Aβ1–42/1–40 ratio *in vitro* and *in vivo*. *Neuron* **17**, 1005–1013.

Citron M., Westaway D., Xia W., Carlson G., Diehl T., Levesque G., Johnson-Wood K., Lee M., Seubert P., Davis A., Kholodenko D., Motter R., Sherrington R., Perry B., Yao H., Strome R., Lieberburg I., Rommens J., Kim S., Schenk D., Fraser P., St George Hyslop P. and Selkoe D. J. (1997) Mutant presenilins of Alzheimer's disease increase production of 42-residue amyloid β-protein in both transfected cells and transgenic mice. *Nat. Med.* **3**, 67–72.

Ekinci F. J., Malik K. U. and Shea T. B. (1999) Activation of the L voltage-sensitive calcium channel by mitogen-activated protein (MAP) kinase following exposure of neuronal cells to β-amyloid. MAP kinase mediates β-amyloid-induced neurodegeneration. *J. Biol. Chem.* **15**, 30322–30327.

Funato H., Yoshimura M., Yamazaki T., Saido T. C., Ito Y., Yokofujita J., Okeda R. and Ihara Y. (1998) Astrocytes containing amyloid β-protein (Aβ)-positive granules are associated with Aβ40-positive diffuse plaques in the aged human brain. *Am. J. Pathol.* **152**, 983–992.

Geddes J. W., Tekirian T. L. and Mattson M. P. (1999) N-terminus truncated β-amyloid peptides and C-terminus truncated secreted forms of amyloid precursor protein: distinct roles in the pathogenesis of Alzheimer's disease. *Neurobiol. Aging* **20**, 75–79.

Glenner G. G. and Wong C. W. (1984) Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem. Biophys. Res. Commun.* **120**, 885–890.

Goslin K. and Banker G. (1991) Rat hippocampal neurons in low-density culture. In: *Culturing Nerve Cells* (Banker G. and Goslin K., eds), pp. 251–278. MIT Press, Cambridge.

Gotz J., Chen F., van Dorpe J. and Nitsch R. M. (2001) Formation of neurofibrillary tangles in P301l tau transgenic mice induced by Aβ42 fibrils. *Science* **293**, 1491–1495.

Haass C. and Selkoe D. J. (1993) Cellular processing of β-amyloid precursor protein and the genesis of amyloid β-peptide. *Cell* **75**, 1039–1042.

Harigaya Y., Saido T. C., Eckman C. B., Prada C. M., Shoji M. and Younkin S. G. (2000) Amyloid β protein starting pyroglutamate at position 3 is a major component of the amyloid deposits in the Alzheimer's disease brain. *Biochem. Biophys. Res. Commun.* **24**, 422–427.

Hartley D. M., Walsh D. M., Ye C. P., Diehl T., Vasquez S., Vassilev P. M., Teplow D. B. and Selkoe D. J. (1999) Protofibrillar intermediates of amyloid β-protein induce acute electrophysiological changes and progressive neurotoxicity in cortical neurons. *J. Neurosci.* **19**, 8876–8884.

He W. and Barrow C. J. (1999) The Aβ 3-pyroglutamyl peptides found in senile plaque have greater β-sheet forming and aggregation propensities *in vitro* than full-length Aβ. *Biochemistry* **38**, 10871–10877.

Hosoda R., Saido T. C., Otvos L., Jr Arai T., Mann D. M. A., Lee V. M.-Y., Trojanowski J. Q. and Iwatsubo T. (1998) Quantification of modified amyloid β peptides in Alzheimer's disease and Down's syndrome brains. *J. Neuropathol. Exp. Neurol.* **57**, 1089–1095.

Itagaki S., McGeer P. L., Akiyama H., Zhu S. and Selkoe D. (1989) Relationship of microglia and astrocytes to amyloid deposits of Alzheimer's disease. *J. Neuroimmunol.* **24**, 173–182.

Kalback W., Watson M. D., Kokjohn T. A., Kuo Y. M., Weiss N., Luehrs D. C., Lopez J., Brune D., Sisodia S. S., Staufenbiel M., Emmerling M. and Roher A. E. (2002) APP transgenic mice Tg2576 accumulate Abeta peptides that are distinct from the chemically modified and insoluble peptides deposited in Alzheimer's disease senile plaques. *Biochemistry* **41**, 922–928.

Kang J., Lemaire H.-G., Unterbeck A., Salbaum J. M., Masters C.L., Grzeschik K.-H., Multhaup G., Beyreuther K. and Muller-Hill B. (1987) The precursor of Alzheimer's disease amyloid A4-protein resembles a cell-surface receptor. *Nature* **325**, 733–736.

Kuo Y. M., Emmerling M. R., Woods A. S., Cotter R. J. and Roher A. E. (1997) Isolation, chemical characterization, and quantitation of Aβ 3-pyroglutamyl peptide from neuritic plaques and vascular amyloid deposits. *Biochem. Biophys. Res. Commun.* **237**, 188–191.

Lalowski M., Golabek A., Lemere C. A., Selkoe D. J., Wisniewski H. M., Beavis R. C., Frangione B. and Wisniewski T. (1996) The 'non-amyloidogenic' p3 fragment (amyloid β17–42) is a major constituent of Down's syndrome cerebellar pre-amyloid. *J. Biol. Chem.* **271**, 33623–33631.

Lambert M. P., Barlow A. K., Chromy B. A., Edwards C., Freed R., Liosatos M., Morgan T. E., Rozovsky I., Trommer B., Viola K. L., Wals P., Zhang C., Finch C. E., Krafft G. A. and Klein W. L. (1998) Diffusible, nonfibrillar ligands derived from Aβ1–42 are potent central nervous system neurotoxins. *Proc. Natl Acad. Sci. USA* **95**, 6448–6453.

Lemere C. A., Lopera F., Kosik K. S., Lendon C. L., Ossa J., Saido T. C., Yamaguchi H., Ruiz A., Martinez A., Madrigal L., Hincapie L., Arango J. C. L., Anthony D. C., Koo E. H., Goate A. M., Selkoe D. J. and Arago J. C. V. (1996) The E280A presenilin 1 Alzheimer mutation produces increased Aβ42 deposition and severe cerebellar pathology. *Nat. Med.* **2**, 1146–1150.

Levison S. W. and McCarthy K. D. (1991) Astroglia in culture. In: *Culturing Nerve Cells* (Banker G. and Goslin K., eds), pp. 309–336. MIT Press, Cambridge.

Lin H., Bhatia R. and Lal R. (2001) Amyloid beta protein forms ion channels: implications for Alzheimer's disease pathophysiology. *FASEB J.* **15**, 2433–2444.

Lorenzo A., Yuan M. L., Zhang Z. H., Paganetti P. A., Sturchler Pierrat C., Staufenbiel M., Mautino J., Sol Vigo F., Sommer B. and Yankner B. A. (2000) Amyloid β interacts with the amyloid precursor protein: a potential toxic mechanism in Alzheimer's disease. *Nat. Neurosci.* **3**, 460–464.

Mann D. M. and Iwatsubo T. (1996) Diffuse plaques in the cerebellum and corpus striatum in Down's syndrome contain amyloid beta protein (Aβ) only in the form of Aβ42 (43). *Neurodegeneration* **5**, 115–120.

Mattson M. P., Cheng B., Davis D., Bryant K., Lieberburg I. and Rydel R. E. (1992) β-Amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. *J. Neurosci.* **12**, 376–389.

Mori H., Takio K., Ogawara M. and Selkoe D. J. (1992) Mass spectrometry of purified amyloid β protein in Alzheimer's disease. *J. Biol. Chem.* **267**, 17082–17086.

Mosmann T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**, 55–63.

Munson G. W., Roher A. E., Kuo Y. M., Gilligan S. M., Reardon C. A., Getz G. S. and LaDu M. J. (2000) SDS–Stable complex formation between native apolipoprotein E3 and β-amyloid peptides. *Biochemistry* **26**, 16119–16124.

Paganetti P. A. and Staufenbiel M. (2000) Using γ-secretase inhibitors to distinguish the generation of the Aβ peptides terminating at Val-40 and Ala-42. *Alzheimer's Disease.* **32**, 239–248.

Pike C. J., Overman M. J. and Cotman C. W. (1995) Amino-terminal deletions enhance aggregation of β-amyloid peptides *in vitro*. *J. Biol. Chem.* **270**, 23895–23898.

Roher A. E., Chaney M. O., Kuo Y. M., Webster S. D., Stine W. B., Haverkamp L. J., Woods A. S., Cotter R. J., Tuohy J. M., Krafft G. A., Bonnell B. S. and Emmerling M. R. (1996) Morphology and toxicity of Aβ-(1–42) dimer derived from neuritic and vascular amyloid deposits of Alzheimer's disease. *J. Biol. Chem.* **271**, 20631–20635.

Russo C., Angelini G., Dapino D., Piccini A., Piombo G., Schettini G., Chen S., Teller J. K., Zaccheo D., Gambetti P. and Tabaton M. (1998) Opposite roles of apolipoprotein E in normal brains and in Alzheimer's disease. *Proc. Natl Acad. Sci. USA* **95**, 15598–15602.

Russo C., Saido T. C., DeBusk L. M., Tabaton M., Gambetti P. and Teller J. K. (1997) Heterogeneity of water-soluble amyloid β-peptide in Alzheimer's disease and Down's syndrome brains. *FEBS Lett.* **409**, 411–416.

Russo C., Salis S., Dolcini V., Venezia V., Xiang S., Teller J. K. and Schettini G. (2001) Identification of amino-terminally and phosphotyrosine modified carboxy-terminal fragments of the amyloid precursor protein in Alzheimer's disease and Down's syndrome brain. *Neurobiol. Dis*. **8**, 173–180.

Russo C., Schettini G., Saido T. C., Hulette C., Lippa C., Lannfelt L., Ghetti B., Gambetti P., Tabaton M. and Teller J. K. (2000) Presenilin-1 mutations in Alzheimer's disease. *Nature* **405**, 531–532.

Saido T. C. (1998) Alzheimer's disease as proteolytic disorders: anabolism and catabolism of β-amyloid. *Neurobiol. Aging* **19**, S69–S75.

Saido T. C., Iwatsubo T., Mann D. M. A., Shimada H., Ihara Y. and Kawashima S. (1995) Dominant and differential deposition of distinct β-amyloid peptide species, AβN3 (pE), in senile plaques. *Neuron* **14**, 457–466.

Saido T. C., Yamao-Harigaya W., Iwatsubo T. and Kawashima S. (1996) Amino- and carboxyl-terminal heterogeneity of β-amyloid peptides deposited in human brain. *Neurosci. Lett.* **215**, 173–176.

Scorziello A., Meucci O., Florio T., Fattore M., Forloni G., Salmona M. and Schettini G. (1996) β25–35 alters calcium homeostasis and induces neurotoxicity in cerebellar granule cells. *J. Neurochem.* **66**, 1995–2003.

Seilheimer B., Bohrmann B., Bondolfi L., Muller F., Stuber D. and Dobeli H. (1997) The toxicity of the Alzheimer's β-amyloid peptide correlates with a distinct fiber morphology. *J. Struct. Biol.* **119**, 59–71.

Selkoe D. J. (1998) The cell biology of β-amyloid precursor protein and presenilin in Alzheimer's disease. *Trends Cell. Biol.* **8**, 447–453.

Selkoe D. J. (2001) Alzheimer's disease: genes, proteins, and therapy. *Physiol. Rev.* **81**, 741–766.

Simons M., de Strooper B., Multhaup G., Tienari P. J., Dotti C. G. and Beyreuther K. (1996) Amyloidogenic processing of the human amyloid precursor protein in primary cultures of rat hippocampal neurons. *J. Neurosci.* **16**, 899–908.

Takaki Y., Iwata N., Tsubuki S., Taniguchi S., Toyoshima S., Lu B., Gerard N. P., Gerard C., Lee H. J., Shirotani K. and Saido T. C. (2000) Biochemical identification of the neutral endopeptidase family member responsible for the catabolism of amyloid β peptide in the brain. *J. Biochem.* **128**, 897–902.

Tekirian T. L., Saido T. C., Markesbery W. R., Russel M. J., Wekstein D. R., Patel E. and Geddes J. W. (1998) N-terminal heterogeneity of parenchymal and cerebrovascular Aβ deposits. *J. Neuropathol. Exp. Neurol.* **57**, 76–94.

Tekirian T. L., Yang A. Y., Glabe C. and Geddes J. W. (1999) Toxicity of pyroglutaminated amyloid β-peptides 3 (pE)−40 and −42 is similar to that of Aβ1–40 and −42. *J. Neurochem.* **73**, 1584–1589.

Terry R. D. and Katzman R. (1983) Senile dementia of the Alzheimer type. *Ann. Neurol.* **14**, 497–506.

Walsh D. M., Hartley D. M., Kusumoto Y., Fezoui Y., Condron M. M., Lomakin A., Benedek G. B., Selkoe D. J. and Teplow D. B. (1999) Amyloid β-protein fibrillogenesis. Structure and biological activity of protofibrillar intermediates. *J. Biol. Chem.* **274**, 25945–25952.

Walsh D. M., Lomakin A., Benedek G. B., Condron M. M. and Teplow D. B. (1997) Amyloid β-protein fibrillogenesis. Detection of a protofibrillar intermediate. *J. Biol. Chem.* **272**, 22364–22372.