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Astrocytes and microglia but not neurons preferentially generate N-terminally truncated Aβ peptides

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ABSTRACT

The neuropathological hallmarks of Alzheimer's disease include extracellular neuritic plaques and neurofibrillary tangles. The neuritic plaques contain β-amyloid peptides (Aβ peptides) as the major proteinaceous constituent and are surrounded by activated microglia and astrocytes as well as dystrophic neurites. N-terminally truncated forms of Aβ peptides are highly prevalent in neuritic plaques, including Aβ 3-x beginning at Glu eventually modified to pyroglutamate (Aβ N3pE-x), Aβ 2-x, Aβ 4-x, and Aβ 5-x. The precise origin of the different N-terminally modified Aβ peptides currently remains unknown. To assess the contribution of specific cell types to the formation of different N-terminally truncated Aβ peptides, supernatants from serum-free primary cell cultures of chicken neurons, astrocytes, and microglia, as well as human astrocytes, were analyzed by Aβ-ELISA and one- and two-dimensional SDS-urea polyacrylamide gel electrophoresis followed by immunoblot analysis. To evaluate the contribution of β- and γ-secretase to the generation of N-terminally modified Aβ, cultured astrocytes were treated with membrane-anchored "tripartite β-secretase (BACE1) inhibitors" and the γ-secretase inhibitor DAPT. Neurons, astrocytes, and microglia each exhibited cell type-specific patterns of secreted Aβ peptides. Neurons predominantly secreted Aβ peptides that begin at Asp1, whereas those released from astrocytes and microglia included high proportions of N-terminally modified Aβ peptides, presumably including Aβ 2/3-x and 4/5-x. The inhibition of BACE1 reduced the amount of Aβ 1-x in cell culture supernatants but not the amount of Aβ 2-x.

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder in the elderly and is characterized by the neuropathological hallmarks of synaptic and neuronal loss, neuritic plaques, and neurofibrillary tangles (NFT) that consist of hyperphosphorylated tau protein (Glenner and Wong, 1984; Masters et al., 1985; Delacourte and Defossez, 1986; Grundke-Iqbal et al., 1986). The extracellular plaques in AD-brain parenchyma are primarily composed of β-amyloid peptides (Aβ peptides), of which a large proportion is N-terminally truncated. (Glenner and Wong, 1984; Masters et al., 1985; Miller et al., 1993; Saido et al., 1995; Kuo et al., 2001; Sergeant et al., 2003; Guntert et al., 2006; Schieb et al., 2011; Moore et al., 2012; Bayer and Wirths, 2014). In particular, Aβ peptides beginning at Glu3, which is eventually modified to pyroglutamate (Aβ N3pE-x), and Aβ beginning at Phe4 appear to be highly abundant. Other reported N-terminally truncated Aβ-variants include Aβ starting at Ala2 and Arg5. Aβ peptides are ubiquitously generated from the amyloid precursor protein (APP) as products of physiological cellular metabolism and can be detected in cell culture supernatant, cerebrospinal fluid (CSF), and human blood plasma (Haass et al., 1991; Haass and Selkoe, 1993; Wang et al., 1996; Wiltfang et al., 2002; Maler et al., 2007). The pivotal enzyme that exhibits β-secretase activity is BACE1, which is a transmembrane aspartyl protease that cleaves APP between Met671 and Asp672 and between Tyr681 and Glu682 (numbering according to APP770) (Hussain et al., 1999; Vassar et al., 1999; Lee et al., 2003). Recent findings suggest that other enzymes, including glutaminyl cyclase, cathepsin B, meprin, neprilysin, myelin basic protein, plasmin, angiotensin-converting enzyme, and aminopeptidases, may compete with or act in concert with BACE1 to produce several N-terminal variants of Aβ peptides (Howell et al., 1995; Saido, 1998; Van Nostrand and Porter,

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1999; Hu et al., 2001; Takeda et al., 2004; Hook et al., 2005; Cynis et al., 2008; Liao et al., 2009; Sevalle et al., 2009; Bien et al., 2012; Bayer and Wirths, 2014). The subsequent proteolytic cleavage that generates the C-terminus of Aβ is accomplished by γ-secretase, which is a multienzyme complex consisting of presenilin, nicastrin, anterior pharynx defective 1a or 1b, and presenilin enhancer 2 (De Strooper, 2003). Aβ peptides with different C-termini are released from cells because cleavage by γ-secretase can occur at multiple sites of the APP fragment (Struhl and Adachi, 2000; Wiltfang et al., 2002). Variations in the amino acid sequence of Aβ in terms of different N- and C-termini greatly affect their propensity to form oligomers, insoluble protofibrils, fibrils, and, ultimately, Aβ plaques (Thal et al., 2006). In particular, Aβ peptides ending at Ala42 and carrying a truncated N-terminus (e.g., Aβ (pGlu3-42) and Aβ 4-42) appear to be highly prone to aggregation (Bouter et al., 2013). For Aβ N3pE-42 an increased tau-dependent neuronal death has been demonstrated in comparison to Aβ 1-42 alone in cell culture (Nussbaum et al., 2012). Additionally an association between Aβ NpE-x and hyperphosphorylated tau was demonstrated in human brain tissue and the severity of AD neuropathology correlated with frontal NpE-Aβ load, indicating a potential role of this N-terminal truncated Aβ for the initiation and progress of AD (Mandler et al., 2014).

In cell culture supernatant, CSF, and blood plasma, the most abundant Aβ peptide variant is Aβ 1-40 (Wang et al., 1996; Vandermeeren et al., 2001; Esselmann et al., 2004; Maler et al., 2007; Haussmann et al., 2013). CSF predominantly contains Aβ peptides that begin at Asp1; in human blood plasma, a high proportion of N-terminally modified Aβ peptides has been detected (Lewczuk et al., 2004; Maler et al., 2007). In the Aβ plaques associated with AD, Aβ peptides that end at Ala42 (Aβ x-42) are highly prevalent, and the N-terminus exhibits substantial heterogeneity (Sergeant et al., 2003; Rufenacht et al., 2005; Guntert et al., 2006; Thal et al., 2006). Aβ N3pE-x, Aβ N11pE-x, and Aβ 1-x that contain an N-terminal isoaspartate are frequently observed in neuritic plaques (Saido et al., 1996). The percentage of N-terminally truncated Aβ peptides, particularly N3pE-x, Aβ 2-x, Aβ 4-x, and Aβ 5-x, in neuritic plaques appears to increase with disease progression and to be associated with Braak stage of AD-related NFT/neuropil thread (NT) pathology (Braak and Braak, 1991; Braak et al., 2006; Guntert et al., 2006). The Aβ plaques associated with AD are typically composed of a thioflavin S positive, dense-core plaque surrounded by reactive astrocytes that express GFAP, interdigitating microglia, and dystrophic neurites (neuritic plaques) with abnormal tau protein (Selkoe, 2001; Thal et al., 2006). Thioflavin S negative, diffuse plaques can be abundant in brains of non-demented elderly and commonly are nonneuritic and not associated with glial activation. Based on observations from cell culture models and relative BACE1 expression levels, neurons have been suggested to be the major source of Aβ peptides in the brain (LeBlanc et al., 1997; Vassar et al., 1999; Lee et al., 2003). Because of their potential phagocytic activity, microglia and astrocytes were hypothesized to participate in the removal of Aβ plaques (Thal et al., 2006). However, microglia and astrocytes appear to also produce small quantities of Aβ peptides (Haass et al., 1991; LeBlanc et al., 1996, 1997). The contribution of each cell type to the production of different variants of Aβ peptides has not yet been addressed.

To assess whether the heterogeneity of N-terminally modified Aβ peptides may be explained by different cell type-specific profiles of secreted Aβ peptide variants, we established primary chicken cell culture models of neurons, astrocytes, and microglia and examined the Aβ peptide variants released into the cell culture supernatants using ELISA and one- and two-dimensional urea-SDS-PAGE. The chick embryo has previously been described as a suitable model for investigations in the processing of APP. The C-terminal APP domain in chicken is highly homologous to human APP, the amino acid sequence is identical to that in humans, and it expresses BACE-1, BACE-2, presenilin-1, presenilin-2 and nicastrin (Carrodeguas et al., 2005). To investigate potential species differences with respect to the Aβ pattern, the secreted Aβ peptide variants from cultivated human astrocytes were examined. Furthermore, we evaluated the influence of BACE1 and γ-secretase inhibition on the secretion of N-terminally modified Aβ peptide variants.

Material and methods

Isolation, culture, and characterization of primary cells

Fertilized White Leghorn SPF eggs were obtained from Charles River Laboratories (Sulzfeld, Germany). Primary neuronal cultures were derived from the telencephali of 8-day-old chick embryos, and cells were cultivated in serum-free Neurobasal-ATM containing B27 supplement (Gibco, Eggenstein, Germany) at a cell density of $3.75 * 10^5$ cells/cm^2, as previously described (Esselmann et al., 2004). Experiments were performed after the 5th day in vitro (div).

The cells were characterized by morphology and immunocytochemistry using the following primary antibodies: rabbit anti-tau polyclonal antibody (pAb) (1:500; DAKO, Hamburg, Germany), mouse anti-GFAP monoclonal antibody (mAb) (clone GA5, 1:500; DAKO, Hamburg, Germany), mouse anti-fibronectin mAb (clone FN-15, 1:500; Sigma-Aldrich, München, Germany), mouse anti-MOSP mAb (clone 328, 1:500; Chemicon, Temecula, CA, USA), and mouse anti-vimentin mAb (clone Vim 13.2, 1:500; Sigma Aldrich, München, Germany). For immunocytochemistry, cells were fixed with 0.4% w/v paraformaldehyde in PBS at room temperature (RT) for 7 min. Roti®-ImmunoBlock (Carl Roth, Karlsruhe, Germany) containing 0.1% v/v Triton X-100 was used as a blocking agent. Following incubation with primary antibodies, goat anti-mouse and goat anti-rabbit Abs labeled with Alexa Fluor® 488 (1:1000; Invitrogen, Darmstadt, Germany) as well as DAPI were used for visualization under a Leica DM IL HC Bio fluorescence microscope.

For the identification of microglia, samples were probed with FITC-BS1-B4 lectin (1:50 in PBS/0.02% sodium azide for 30 min at RT; Invitrogen, Darmstadt, Germany), Alexa Fluor® 488-AcLDL (1:250 in cell culture medium for 8 h at 37 °C; Invitrogen, Darmstadt, Germany), Alexa Fluor® 488-heat inactivated-E. Coli (1:5000 in cell culture medium for 8 h at 37 °C; Invitrogen, Darmstadt, Germany), and Fluoresbrite® Yellow Green beads (1:2000 in cell culture medium for 8 h at 37 °C; Polysciences Europe GmbH, Eppelheim, Germany) according to the manufacturer's instructions with the aforementioned modifications. After the 5th div, 60% of the cells in neuronal cultures were tau-positive neurons, and less than 5% of the cells were GFAP-positive astrocytes and latex bead-phagocytosing microglia. Immunostaining using antibodies against vimentin, fibronectin, or MOSP was negative.

For primary cultures of astrocytes and microglia, telencephali of 16-day-old chick embryos were dissected, homogenized, and resuspended in DMEM/10% FBS/1% penicillin/streptomycin. The cells were seeded at a density of 0.01 brains/cm^2. For astroglial cultures, a complete medium exchange was performed every two days. After reaching 80% confluence, the astrocytes were passaged and replated at a cell density of $1.5 * 10^4$ cells/cm^2. After the 12th div, the serum-containing medium was gradually reduced to serum-free DMEM/Ham's F12 containing 10 mM HEPES and G5 supplement (PAA, Cölbe, Germany) or Neurobasal-ATM containing B27 supplement within four days. Experiments were performed after the 16th div. After the 18th div, over 90% of the cells were positive for vimentin and exhibited a flat polygonal shape. The cells were only positive for GFAP after incubation in AM Medium (ScienCell, Carlsbad, CA, USA), indicating a quiescent state of the cultivated astrocytes under the aforementioned culture conditions. Immunostaining with antibodies against fibronectin, tau, or MOSP was negative.

For microglial cultures, a complete medium exchange was performed after the 6th div. After the 12th and 16th div, the culture medium was centrifuged at 500 *g* for 5 min to remove dead cells and was added to the microglial cultures. The cells were maintained in PBS

during centrifugation. After the 21th div, the microglial cells were detached using Accutase (PAA, Cölbe, Germany) and were replated at a cell density of $2.5 * 10^5$ cells/cm^2 in 80% conditioned medium and 20% serum-free microglial medium containing DMEM, 4.5 g/l glucose, 0.2 mg/ml BSA, 5 μg/ml insulin, 5 μg/ml transferrin, and 5 nM sodium selenite. After the 25th div, the serum-containing medium was gradually reduced to serum-free microglial medium within two days. After the 27th div, over 90% of the cells in microglial cultures were phagocytosing cells. Immunostaining with antibodies against tau, GFAP, vimentin, fibronectin, or MOSP was negative. Experiments were performed after the 27th div.

Human fetal astrocytes (ScienCell, Carlsbad, CA, USA) were cultivated according to the manufacturer's instructions. To detect Aβ peptides in supernatants or cell lysates, serum-containing AM Medium (ScienCell, Carlsbad, CA, USA) was gradually changed to DMEM/Ham's F12 (Biochrom, Berlin, Germany) containing G5 supplement (PAA, Germany) and 10 mM HEPES (Biochrom, Berlin, Germany). Experiments were performed between the 5th div and the 32nd div.

Drug treatment and sample preparation

Tripartite compounds and DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester) (Sigma-Aldrich, München, Germany) were dissolved in DMSO at a concentration of 2 mM and 10 mM, respectively, and stored at −20 °C. The tripartite BACE1 inhibitors used in this study, which are designated as "BACE-I53" and "BACE-I89" and correspond to compounds 8e and 8g, respectively, were previously described in detail (Schieb et al., 2010). A complete medium exchange was performed prior to treatment, and medium with drugs diluted in DMSO or medium with DMSO alone was added to the cells, yielding the maximum final concentration of 0.1% v/v DMSO. For the analysis of Aβ peptides in unstimulated cell culture supernatants, a complete medium exchange was performed; neuronal cultures were incubated for 24 h, astrocyte cultures were incubated for 24 h or 48 h, and microglial cultures were incubated for 96 h. Cells treated with tripartite BACE1 inhibitors and DAPT were incubated for 24 h and 48 h, respectively. Subsequently, the cell culture supernatants were centrifuged at 500 *g* for 5 min and stored at −20 °C. Cells were washed with PBS for 5 min and lysed in detergent buffer (50 mM HEPES, 0.037 w/v Complete Mini Protease Inhibitor Cocktail (Roche, Grenzach-Wyhlen, Germany), 150 mM NaCl, 1% v/v NP-40, 0.5% w/v NaDOC, and 0.1% w/v SDS) for 15 min at 4 °C. The lysed cells were centrifuged (13,000 *g*, 5 min, 4 °C), and supernatants were stored at −20 °C. The pellets were resolubilized with shaking in 70% v/v formic acid for 10 min at RT. Formic acid was evaporated in a vacuum concentrator. To evaluate the extracellular degradation of Aβ peptides, conditioned media from chicken astrocytes were incubated at 37 °C and 5% CO_2 for 7 days in polypropylene reaction tubes to minimize the loss of Aβ peptides that results from their adsorption to polystyrene surfaces. Each experiment was repeated at least three times in quadruplicate.

Cell viability assays

Cell viability was assessed after drug treatment using the CytoTox 96® lactate dehydrogenase assay (Promega, Madison, CA, USA) according to the manufacturer's instructions and the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, as previously described (Mosmann, 1983).

Immunoprecipitation of Aβ peptides

For the immunoprecipitation of Aβ peptides, 40 μg/ml 1E8 mAb (nanoTools, Teningen, Germany) or 6E10 mAb (Covance, San Francisco, CA, USA) was covalently coupled to 10 mg/ml magnetic sheep anti-mouse Dynabeads M-280 (Dynal, Hamburg, Germany) according to the manufacturer's instructions.

The quadruplicate samples were pooled and subsequently mixed with five-fold concentrated detergent buffer containing either 20 μl or 25 μl of magnetic beads coupled with 1E8 mAb or 6E10 mAb, respectively, yielding final concentrations of 0.8 μg/ml of immobilized 1E8 mAb and 1 μg/ml of immobilized 6E10 mAb in 50 mM HEPES, 150 mM NaCl, 0.5% v/v Nonidet P-40, 0.25% w/v sodium deoxycholate, and 0.05% w/v SDS. For the immunoprecipitation of pooled cell lysates, the samples were dissolved in detergent buffer yielding final concentrations of 0.8 μg/ml of immobilized 1E8 mAb in 50 mM HEPES, 150 mM NaCl, 1% v/v Nonidet P-40, 0.5% w/v sodium deoxycholate, and 0.1% w/v SDS. Immunoprecipitation was performed under rotation for 15 h at 4 °C. Subsequently, the samples were rinsed three times with PBS/0.1% BSA for 5 min at 4 °C and once with 10 mM Tris-HCl, pH 7.5. For 1D-Aβ-PAGE, rinsed Aβ-coupled magnetic particles were heated to 95 °C in sample buffer (0.36 M Bis–Tris, 0.16 M Bicine, 15% w/v sucrose, 1% w/v SDS, and 0.0075% w/v bromphenol blue). For 2D-Aβ-PAGE, the Aβ peptides were eluted as previously described (Maler et al., 2008).

BCA assay

The concentration of total protein in cell lysates was determined using the BCA assay as previously described (Smith et al., 1985). The absorption at 562 nm was measured using a Benchmark Microplate Reader (Bio-Rad, München, Germany) and was quantified using Microplate Manager 5.1 software (Bio-Rad, München, Germany).

1D-Aβ-IPG-urea-SDS-PAGE

The eluted protein samples and synthetic Aβ peptides (Aβ 1-38/40/42 (Bachem, Bubendorf, Schweiz); Aβ 1-37/39 (Biosynthan, Berlin, Germany); Aβ 2-40/42 and Aβ 3-40/42 (MoBiTec, Rastatt, Germany); Aβ N3pE-40/42 (MoBiTec, Rastatt, Germany); and Aβ 4/5-42(MoBiTec, Rastatt, Germany)) in sample buffer were separated using urea-Bicine/Bis–Tris/Tris/sulfate SDS-PAGE (1D-Aβ-PAGE) as previously described (Klafki et al., 1996; Wiltfang et al., 1997).

2D-Aβ-IPG-urea-SDS-PAGE

High-resolution two-dimensional electrophoresis of Aβ (2D-Aβ-PAGE) using immobilized pH gradients (IPGs) was performed as previously described (Maler et al., 2007). The samples eluted in rehydration buffer (8.3 M urea, 2% w/v CHAPS, 0.5% v/v pharmalyte pH 3–10, and 20 mM DTT) or synthetic Aβ peptides in rehydration buffer were incubated with IPG DryStrip gels (7 cm, nonlinear pH range of 3–10 or linear pH range of 4–7) over 10 h at 20 °C. Isoelectric focusing was performed using an Ettan IPGphor II IEF System (GE Healthcare, München, Germany). After equilibration of focused IPG strips in equilibration buffer (6 M urea, 0.36 M Bis–Tris, 0.16 M Bicine, 2% w/v SDS, and 20% w/v glycerol) for 10 min with 1% w/v DTT followed by an additional equilibration for 10 min with 4.8% w/v iodoacetamide at RT, the proteins were separated in the second dimension as previously described.

Western blot and immunodetection (2D-IB)

Aβ peptides were detected by immunoblot analysis under semidry conditions (Hoefer Semi-Phor) using a discontinuous buffer system as previously described (Maler et al., 2007). Briefly, the peptides were transferred onto a PVDF membrane (Immobilon, Millipore, Schwalbach, Germany) at 1 mA/cm^2 for 45 min. The PVDF membranes were subsequently boiled for 3 min for optimal antibody detection and blocked for 2 h at RT in Roti®-Block (Carl Roth, Karlsruhe, Germany) for immunostaining using the 1E8 mAb (nanoTools, Teningen, Germany) or the

82E1 mAb (IBL, Hamburg, Germany). For immunostaining with the 6E10 mAb (Covance, San Francisco, CA, USA) or the N3pE mAb (IBL, Hamburg, Germany), membranes were blocked with PBS-T/2% w/v ECL Prime Blocking Reagent (GE Healthcare, München, Germany). The blots were probed with the 1E8 mAb (1:300 in Roti®-Block), the 6E10 mAb (1:1000 in PBS-T/2% ECL Prime Blocking Reagent), the 82E1 mAb (1:1000 in Roti®-Block), or the N3pE pAb (1 μg/ml in PBS-T/2% ECL Prime Blocking Reagent) at 4 °C overnight. The membranes were rinsed three times with PBS/0.075% v/v Tween (PBS-T) and incubated with biotinylated goat anti-mouse IgG (333 ng/ml in PBS-T; Vector Laboratories Ltd., Peterborough, United Kingdom) or with peroxidase-conjugated horse anti-rabbit IgG (Calbiochem, Frankfurt, Germany) for 60 min at RT. Subsequently, the PVDF membranes that were probed with biotinylated goat anti-mouse IgG were rinsed in PBS-T and incubated with streptavidin–horseradish peroxidase complex (1:3000 in PBS-T; Amersham Pharmacia, Germany). Finally, all of the PVDF membranes were rinsed again in PBS-T, and detection was performed using ECL Prime Western Blotting Detection Reagent (GE Healthcare, München, Germany) and a Fluor-S MAX MultiImager (Bio-Rad, München, Germany) according to the manufacturer's instructions. Protein bands and spots detected after 1D-Aβ-PAGE/IB and 2D-Aβ-PAGE/IB analysis were quantified using TotalLab Quant v1.1.4301.25363 (totallab, Jahnsdorf, Germany) as previously described (Maler et al., 2007).

Aβ-ELISA

The concentrations of Aβ 1-42, Aβ 1-40, and Aβ N3pE-40 were studied with commercially available ELISA kits from Innogenetics (Ghent, Belgium) and IBL (Hamburg, Germany), respectively. The dilutions, incubations, and washings steps were performed according to the manufacturer's recommendations and were applied in CSF analyses, but the samples were diluted 1:20 or 1:80 rather than 1:400 for the analysis of Aβ 1-40. The concentrations of Aβ x-42 and Aβ x-40 were analyzed using a multiplex assay (Innogenetics, Ghent, Belgium) in the Luminex platform as previously described (Lewczuk et al., 2010), but the sample dilutions were adjusted for the cell culture supernatants. All of the measurements were performed in duplicate.

Statistical analysis

The data were analyzed using GraphPad Prism version 6.02 (GraphPad Software, San Diego, CA, USA) and SPSS Statistics version 22.0 (IBM, San Jose, CA, USA). Differences between groups were determined using the unpaired t-test, Wilcoxon matched-pair signed-rank test, the Kruskal–Wallis test followed by Dunn's multiple comparisons, one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test, or multivariate analysis of variance (MANOVA) followed by Dunnett's T3 correction as a post-hoc test when a significant effect was observed. All data are expressed as the mean ± standard deviation (SD). Significance levels are indicated as follows: $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$; and ns, not significant.

Results

Astrocytes and microglia produced a higher proportion of N-terminally truncated Aβ than neurons

To estimate the amount of Aβ 1-40 secreted by neurons and astrocytes, Aβ peptides in cell culture supernatants were first assessed using ELISA, and the values were normalized to the amount of total cellular protein as determined using the BCA assay (Fig. 1). The quantity of Aβ 1-40 detected in the supernatants of astrocyte cultures after 24 h of incubation was 1.5 ± 0.3 ng per mg of total cellular protein. In contrast, neuronal cultures released approximately sevenfold more Aβ than astrocytes, i.e., 10.3 ± 2.5 ng of Aβ 1-40 per mg of total cellular protein. The concentrations of Aβ 1-42 and Aβ N3pE-42 were below the limits of detection of the respective ELISA kits. For evaluation of the Aβ42/40 ratios a multiplex immunoassay was employed. Although the total amount of Aβ peptides in cell culture supernatants was significantly different between astrocytes and neurons, the Aβ42/Aβ40 ratios in neuronal and astrocyte cell culture supernatants determined using a multiplex immunoassay did not significantly differ, indicating a similar pattern of γ-secretase cleavage in both cell types. The quantities of Aβ x-40 and Aβ x-42 as assessed by the multiplex immunoassay are shown in the supporting information (Supporting information Fig. S1).

To assess the secretion of additional Aβ peptide variants, including Aβ peptides that possess different N-termini, semiquantitative 1D-Aβ-PAGE followed by immunoblot (IB) analysis (1D-Aβ-PAGE/IB) was performed. 1D-Aβ-PAGE/IB revealed significantly higher proportions of Aβ that comigrated with Aβ 2-40 in the supernatants from astrocytes in comparison to those in the supernatants from neurons and even higher proportions of the presumed Aβ 2-40 in the supernatants of microglia (Fig. 2). Consistent with the ELISA results (see above), the Aβ 1-42/Aβ 1-40 ratios of astrocytes and neurons were not significantly different. Collectively, these results indicate that predominantly N-terminally truncated Aβ peptides may be released in a cell type-specific manner.

The majority of Aβ released by astrocytes or microglia were N-terminally modified

To further characterize the observed Aβ peptides (in addition to an increased resolution and the ability to detect possible additional

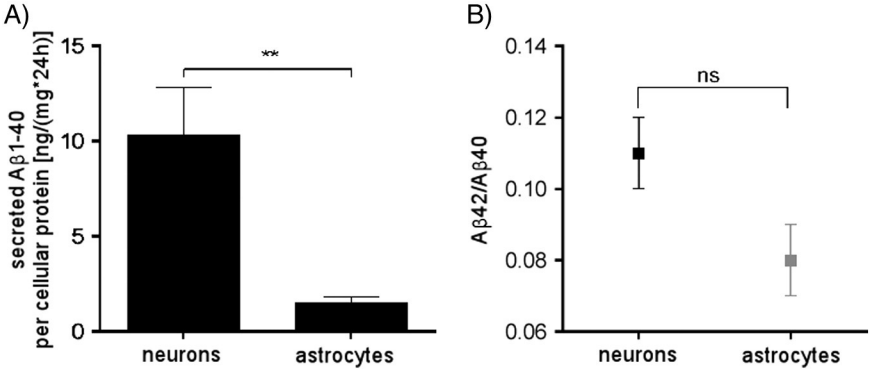

Fig. 1. Quantification of Aβ 1-40 in the supernatants of cultured neurons and astrocytes with ELISA and determination of the Aβ42/Aβ42 ratios with multiplex immunoassay. The amount of Aβ 1-40 secreted by chicken neurons and astrocytes within 24 h was determined using ELISA (unpaired t-test; $^{**}p < 0.01$) (A). Despite the differences in these amounts, the ratios of Aβ x-42/Aβ x-40 that were assessed using a multiplex immunoassay were similar between astrocytes and neurons (Mann–Whitney U test; ns: not significant) (B).

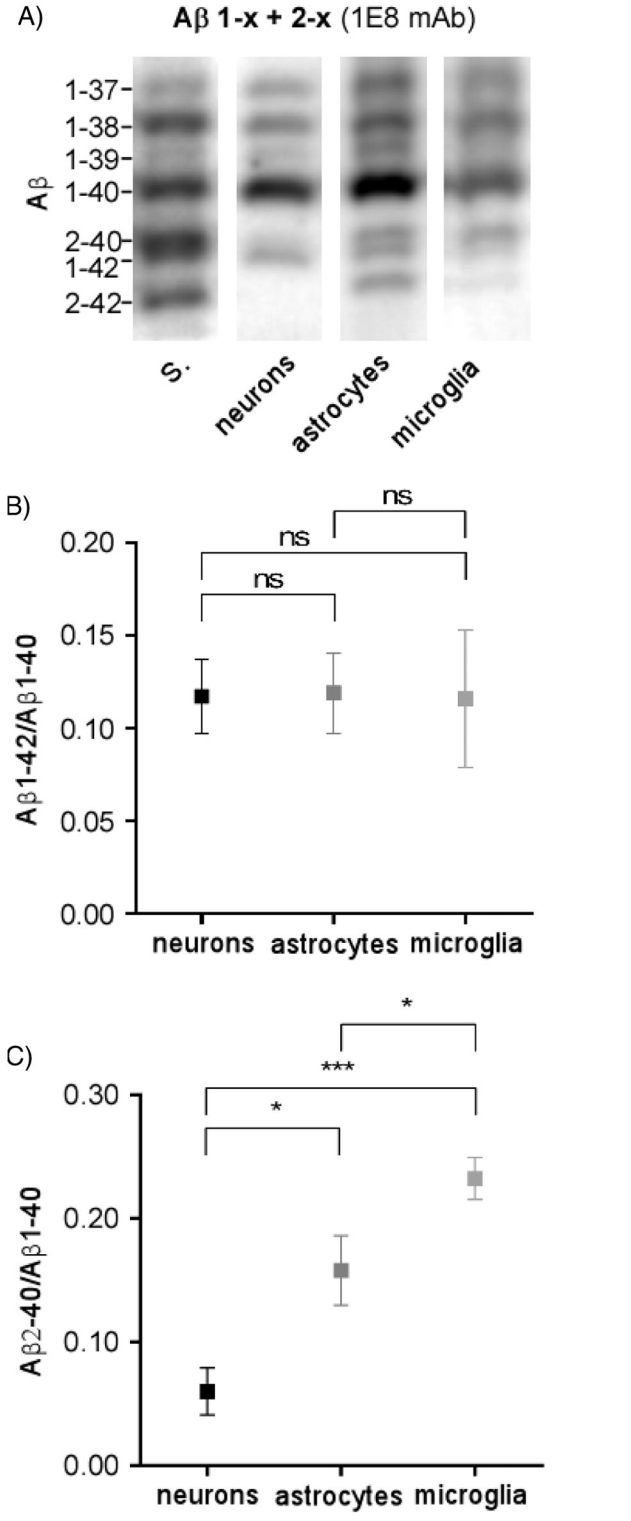

Fig. 2. Quantification of Aβ 1-x and Aβ 2-x in the supernatants of cultured neurons, astrocytes, and microglia using 1D-Aβ-PAGE/IB analysis. Aβ peptides secreted by chicken neurons, astrocytes, and microglia within 24 h, 48 h, and 96 h in 0.8 ml, 1.6 ml, and 2.4 ml of sample volume, respectively, were immunoprecipitated with the 1E8 mAb (0.8 μg/ml). Subsequently, the immunoprecipitated Aβ peptides and a sample of synthetic Aβ peptides (S.) were loaded, separated, and visualized in 1D-Aβ-PAGE/IB using the 1E8 mAb (A). The identities of the loaded synthetic Aβ peptides are indicated on the left. The different Aβ peptide bands were quantified and identified using a dilution series of synthetic Aβ. The most abundant band was Aβ 1-40, independent from the cell type used. The ratio of Aβ 1-42/Aβ 1-40 was not significantly different between the supernatants of neurons and astrocytes (Kruskal–Wallis test followed by Dunn's multiple comparisons; ns: not significant) (B). The ratio of Aβ 2-40/Aβ 1-40 was significantly elevated in samples of astrocytes and microglia in comparison to neurons (Kruskal–Wallis test followed by Dunn's multiple comparisons; ns: not significant; $^{*}p < 0.05$; and $^{***}p < 0.001$) (C). Each experiment was performed at least three times.

charged Aβ variants (i.e., Aβ peptides with different pI values), which indicate N-terminal truncation or elongation), the supernatants of neurons, astrocytes, and microglia were analyzed using 2D-Aβ-PAGE followed by immunoblot (2D-IB) analysis. A panel of different antibodies against the N-terminal region of Aβ was employed to characterize the observed charged Aβ variants. The monoclonal antibody 82E1 is specific for peptides that begin at Asp1 (Aβ 1-x), whereas the 1E8 mAb recognizes the Aβ 1-x and Aβ 2-x. Moreover, the 6E10 mAb detects several N-terminally truncated Aβ peptides that begin at Asp1, Ala2, Glu3, Phe4, or Arg5 (n-x) (Fig. S2 in the Supporting information and Lewczuk et al. (2010)).

The supernatants of astrocytes and microglia exhibited higher proportions of charged Aβ variants with pI values > 5.4 than the supernatants of neurons, and these variants presumably correspond to Aβ 2-x, Aβ 3-x, Aβ 4-x, and Aβ 5-x (Fig. 3).

In 2D-IB analysis probed with the 1E8 mAb, the ratios of Aβ 2-40 exhibiting a pI of 6.4 to Aβ 1-40 (pI of ~5.4) were approximately tenfold higher in the supernatants from astrocytes or microglia than from neurons. In addition to Aβ 2-x and Aβ 3-x, which exhibited pI values of 6.4, astrocytic and microglial supernatants contained additional charged Aβ variants exhibiting pI values of approximately 5.0, 5.5, and 5.7. These variants were minimally detected in neuronal cell culture supernatants (Fig. 3). In 2D-IB analysis probed with the 6E10 mAb, additional Aβ variants were detected at pH 7.0, which presumably corresponded to Aβ 4-x and/or Aβ 5-x. The ratio of Aβ peptides that exhibited pI values of 7.0 to Aβ 1-40 was also more than tenfold elevated in the supernatants of astrocytes compared to neurons (Fig. 3 and Table S1 in the Supporting information).

MANOVA was conducted to determine whether the profiles of secreted charged Aβ variants differed among neurons, astrocytes, and microglia (Fig. 4). The results of this analysis indicated, that each cell exhibited an individual pattern of Aβ variants with different pI values. In general, astrocytes and microglia secreted a significantly higher proportion of Aβ peptides that exhibited a pI greater than 5.4 in comparison to neurons, indicating N-terminal truncations or possibly other modifications.

In contrast to the vast differences in the proportions of N-terminally truncated Aβ peptides among neurons, astrocytes, and microglia, the proportions of Aβ 1-37, Aβ 1-38, Aβ 1-39, and Aβ 1-42 (pI of 5.4) to Aβ 1-40 did not differ and resembled the distribution of the corresponding Aβ peptides in human CSF and blood plasma (Fig. 3 and Table S1 in the Supporting information). In all of the investigated cell culture models, Aβ 1-40 was the most abundant Aβ variant, which exhibited a pI of 5.4, followed by Aβ 1-38 and Aβ 1-42 (Table S1 in the Supporting information).

In cell culture supernatants from astrocytes, but not from neurons or microglia, an Aβ peptide exhibiting a pI of 6.4 was reproducibly detected in 2D-IB analysis using the 82E1 mAb (Fig. 3). This antibody is specific for Aβ peptides that begin at Asp1. Therefore, this particular Aβ variant appears to begin at Asp1 and has presumably acquired an additional positive charge or lost a negative charge due to a non-identified posttranslational modification.

Collectively, approximately more than 80% of all detected Aβ variants that were secreted by neurons began at Asp1, whereas the majority of Aβ peptides released by astrocytes and microglia were N-terminally truncated or otherwise modified, and less than 40% of all detected Aβ peptides were Aβ 1-x in the supernatants of astrocytes (Table S1 in the Supporting information). Aβ N3pE-x was not observed in 2D-IB analysis of the supernatants of astrocytes or neurons. In contrast, synthetic Aβ 3-40, Aβ 3-42, Aβ N3pE-40, and Aβ N3pE-42 (100 pg of each) were equally detected in 1D-IB and 2D-IB analyses probed with the 6E10 mAb (data not shown). Extended incubation times (24 h or 48 h) or the use of a different cell culture medium (serum-free DMEM/F12 containing HEPES and G5 supplement, NbA, or AM) did not influence the pattern of secreted astroglial Aβ peptides when assessed using 2D-Aβ-PAGE/IB analysis probed with the 1E8 mAb. To

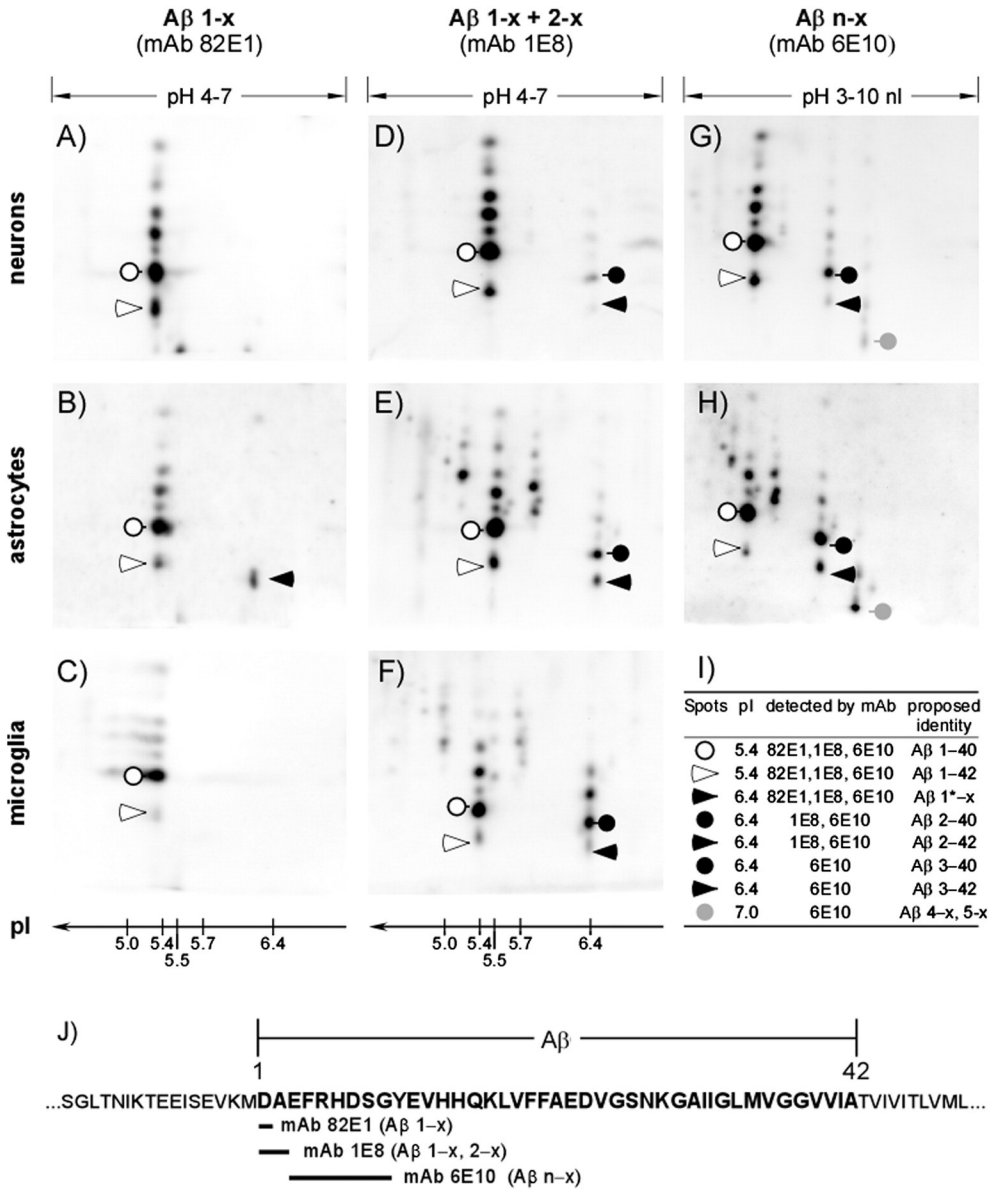

Fig. 3. The pattern of N-terminally modified Aβ variants in the supernatants of cultured chicken neurons, astrocytes, and microglia. Supernatants from serum-free cultures of chicken neurons, astrocytes, and microglia were obtained after 24 h, 48 h, and 96 h, respectively. Aβ from 1.8 ml (A, D), 2.4 ml (B, E, G), and 4.0 ml (C, F, H) sample volumes, respectively, were immunoprecipitated with the 1E8 mAb (0.8 μg/ml; A-F) or the 6E10 mAb (1.0 μg/ml) (G, H). Subsequently, the immunoprecipitated Aβ peptides were dissolved, separated, and visualized by 2D-Aβ-PAGE/IB using IPG strips in a linear pH range of 4–7 (A–F) or a nonlinear pH range of 3–10 (G, H). Representative 2D-Aβ immunoblots (2D-IB) from samples of neurons (A, D, G), astrocytes (B, E, H) and microglia (C, F) that were probed using the Aβ 1-x-specific 82E1 mAb (A-C), the Aβ 1-x- and Aβ 2-x-specific 1E8 mAb (D–F), and the Aβ n-x-specific 6E10 mAb (G, H) are shown. The sequence of Aβ and the epitopes of the different mAbs are shown (J). In 2D-IB analysis using the 82E1 mAb, a series of spots at a pI of 5.4 corresponding to Aβ1-40 and additional Aβ 1-x variants was observed in all examined samples (A–C). An additional single 82E1 mAb-positive spot, which presumably corresponds to a modified Aβ 1-x (identified as Aβ 1*-x), was detected at a pI of 6.4 only in samples obtained from astrocytes (B). In 2D-IB analysis using the 1E8 mAb (D–F), an additional series of spots was observed at pI values of 5.0, 5.5, 5.7, and 6.4. These spots were minimally detected in neuronal samples (D) but were abundant in samples from astrocytes (E) and microglia (F). 2D-IB analysis using the 6E10 mAb revealed an additional series of spots at a pI of 7.0, whereas all of the remaining spots corresponded to those detected in 2D-IB analysis using the 1E8 mAb. The proposed identities of the marked spots and their observed pI values are presented in (I) based on comigration of synthetic Ab peptides (Fig. S2) and mass spectrometry data as previously reported (Schieb et al., 2011). All of the experiments were performed at least three times (nl = nonlinear; and mAb = monoclonal antibody).

investigate the extent of the extracellular degradation of Aβ peptides, conditioned supernatants of astrocytes in the absence or presence of protease inhibitors were incubated for 7 days at 37 °C. The amount of Aβ 1-40 in the conditioned media in the absence of protease inhibitors was reduced to 65 ± 24% in comparison to samples supplemented with protease inhibitors, which indicates a slow degradation of Aβ peptides by secreted extracellular proteases (Fig. S3 in the Supporting information). The relative proportions of the remaining Aβ peptide variants were not different between samples with or without added protease inhibitors.

Cultured human astrocytes secreted a similar pattern of N-terminally truncated Aβ peptides as chicken astrocytes

Because different species may exhibit a different pattern of secreted Aβ peptides, the Aβ profile in the supernatants of cultivated human astrocytes was determined using 2D-Aβ-PAGE/IB analysis (Fig. 5). The relative abundance of the presumed N-terminally truncated Aβ 2/3-x and 4/5-x (pI ~6.4 and ~7.0) in cell culture supernatants of human and chicken astrocytes was very similar. Mann–Whitney U test indicated no significant differences for Aβ with pI 6.4 (Fig. 5). Chicken and human

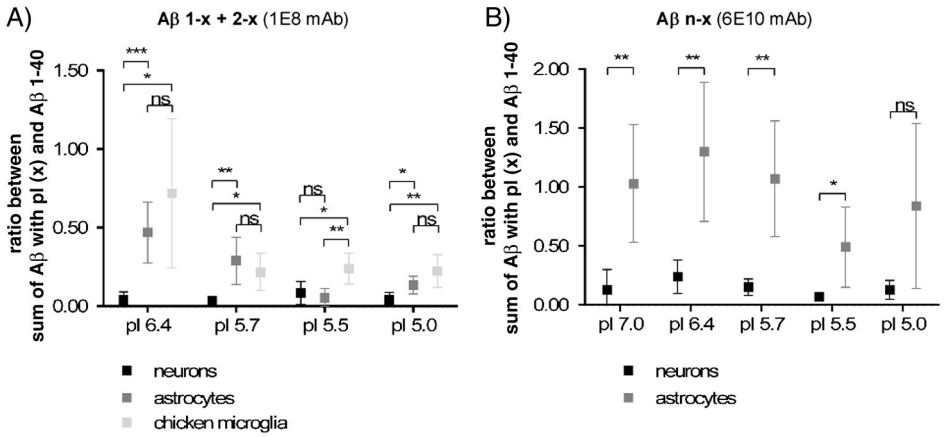

Fig. 4. Comparison of charged Aβ variants in the supernatants of cultured chicken neurons, astrocytes, and microglia by 2D-Aβ-PAGE/IB. Means and standard deviations of the ratios between the sum of all Aβ variants exhibiting an identical pI (i.e., 5.0, 5.5, 5.7, 6.4, or 7.0) and Aβ 1-40 in 2D-Aβ-PAGE/IB are shown. A MANOVA was conducted using cell type (neurons, astrocytes, and microglia) as the independent variable and four ratios of charged Aβ variants (the sum of all Aβ variants exhibiting pI values of 6.4, 5.7, 5.5, or 5.0 to Aβ 1-40) as the dependent variables to determine whether neurons, astrocytes, and microglia were distinguishable by their profile of secreted Aβ peptides that exhibit different pI values. MANOVA, based on data from 2D-Aβ-PAGE/IB analysis using the 1E8 mAb (A), revealed a significant multivariate main effect for cell type (Wilks' $\lambda = 0.092$; $F = 8.600$; $p < 0.001$; and partial eta squared $= 0.696$). Significance levels of post-hoc tests (Dunnett's T3 correction) are shown (A). Astrocytes and microglia secreted a higher proportion of Aβ peptides with pI values of 6.4, 5.0, and 5.7 than neurons. There was no difference between astrocytes and microglia. The relative abundance of Aβ peptides exhibiting a pI of 5.5 from microglia significantly exceeded the relative abundance of these peptides from neurons and astrocytes. MANOVA based on data from 2D-Aβ-PAGE/IB analysis using the 6E10 mAb (B) confirmed that astrocytes and microglia were distinguishable by their profile of secreted Aβ peptides (Wilks' $\lambda = 0.203$; $F = 7.087$; $p < 0.01$; and partial eta squared $= 0.797$). Significance levels of univariate main effects are shown (B) (ns: not significant; $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$). Each experiment was performed at least three times.

astrocytes both secreted additional charged Aβ variants that exhibited pI values of approximately 5.0, 5.5, and 5.7. Consistent with the results from avian cultures, the spot corresponding to Aβ 1-40 that exhibited a pI of 5.4 in 2D-IB analysis of human astrocytes was the most intense followed by spots corresponding to Aβ 1-39 and Aβ 1-42 (Table S2 in the Supporting information).

Small amounts of Aβ 1-x were detected in cell lysates

To investigate whether the N-terminally truncated Aβ peptides originated from intracellular compartments, detergent-soluble and formic acid-soluble cell fractions were analyzed. In contrast to the findings from the analysis of cell culture supernatants, N-terminally truncated Aβ peptides were not observed in cell lysates of astrocytes and microglia by 2D-IB analysis using the 1E8 mAb (Fig. 6). In all of the detergent-soluble fractions from neurons, astrocytes, and microglia, Aβ 1-x variants that exhibited a pI of 5.4 were nearly exclusively detected. The scarce amount of intracellular Aβ peptides in microglia corresponded to the low concentrations of Aβ peptides in the cell culture supernatants. A comparison of the intracellular and extracellular proportions of Aβ peptides exhibiting a pI of 5.4 indicated an elevated ratio of intracellular Aβ 1-42/Aβ 1-40 in comparison to extracellular Aβ 1-42/Aβ 1-40, particularly in astrocytes. The high variability of Aβ 1-42 could be due to extraction variability of this peptide. The ratios of Aβ 1-39/Aβ 1-40, Aβ 1-38/Aβ 1-40, and Aβ 1-37/Aβ 1-40 were not significantly different between intracellular lysates and extracellular supernatants (Fig. 6 and Table S3 in the Supporting information). Formic acid extractions of detergent-insoluble cell fractions did not yield additional Aβ peptides.

The inhibition of γ-secretase with DAPT reduced the secretion of all N- and C-terminal Aβ variants

To investigate whether the differences between intracellular and extracellular Aβ profiles of astrocytes were related to possible differences in the secretase pathways, the effects of β- and γ-secretase inhibitors were examined. In astroglial cultures treated with the γ-secretase inhibitor DAPT for 48 h, the amount of all of the detected Aβ 1-x and N-terminally modified Aβ was reduced in a dose-dependent fashion as indicated by 1D-IB analysis (Figs. 7A and B). The secretion of the presumed N-terminally truncated Aβ 2-40 and Aβ 1-40 uniformly decreased, indicating that N-terminally modified Aβ peptides and Aβ 1-x both rely on γ-secretase cleavage. In astrocyte cultures, 10 μM DAPT reduced the amount of secreted Aβ 1-40 to 7.1 ± 3.4% in comparison to unstimulated controls. Concentrations below 25 μM DAPT were not cytotoxic according to LDH measurements or the reduction of cell viability, as assessed using the MTT assay (data not shown).

The secretion of N-terminally modified Aβ peptides remained unaffected or increased upon BACE inhibition

Membrane-anchored "tripartite BACE inhibitors" were used to inhibit BACE in astrocyte cultures. The "tripartite structure" consisted of the peptidic BACE inhibitor "GL189" (Tung et al., 2002) that was linked via a 53 Å (BACE-I53) or an 89 Å (BACE-I89) spacer to a dihydrocholesterol anchor (Linning et al., 2012). As expected, the total amount of Aβ peptides released into the cell culture supernatants was substantially reduced in the presence of 25 or 500 nM tripartite BACE inhibitor. In astrocyte cultures treated with BACE-I53, the amount of released Aβ 1-40 significantly decreased to 12.2 ± 5.3% of the controls. Interestingly, neither BACE-I53 nor BACE-I89 had statistically significant effects on the abundance of specific Aβ peptide bands comigrating with Aβ 2-40 compared to controls in 1D-IB (Figs. 7C–E).

Concentrations of 500 nM BACE-I53 were not cytotoxic according to LDH measurements and did not reduce cell viability, as assessed using the MTT assay (data not shown).

Discussion

The present study compares the profiles of Aβ peptide variants of primary neurons, astrocytes, and microglia. Of particular interest was the generation of N-terminally modified Aβ peptides. Specific N-terminally truncated Aβ peptides, such as NpE 3-x and Aβ 2-x, 3-x, 4-x and 5-x are prevalent in neuritic plaques and appear to increase with the Braak stage of AD-related NFT/NT pathology. Their precise origin has not yet been identified unequivocally (Saido et al., 1995; Sergeant et al., 2003; Guntert et al., 2006). Our results demonstrate that, in cell culture supernatants of astrocytes and microglia, N-terminally modified Aβ presumably corresponding to Aβ 2/3 and 4/5-x are highly prevalent and accounted for more than 60% of the

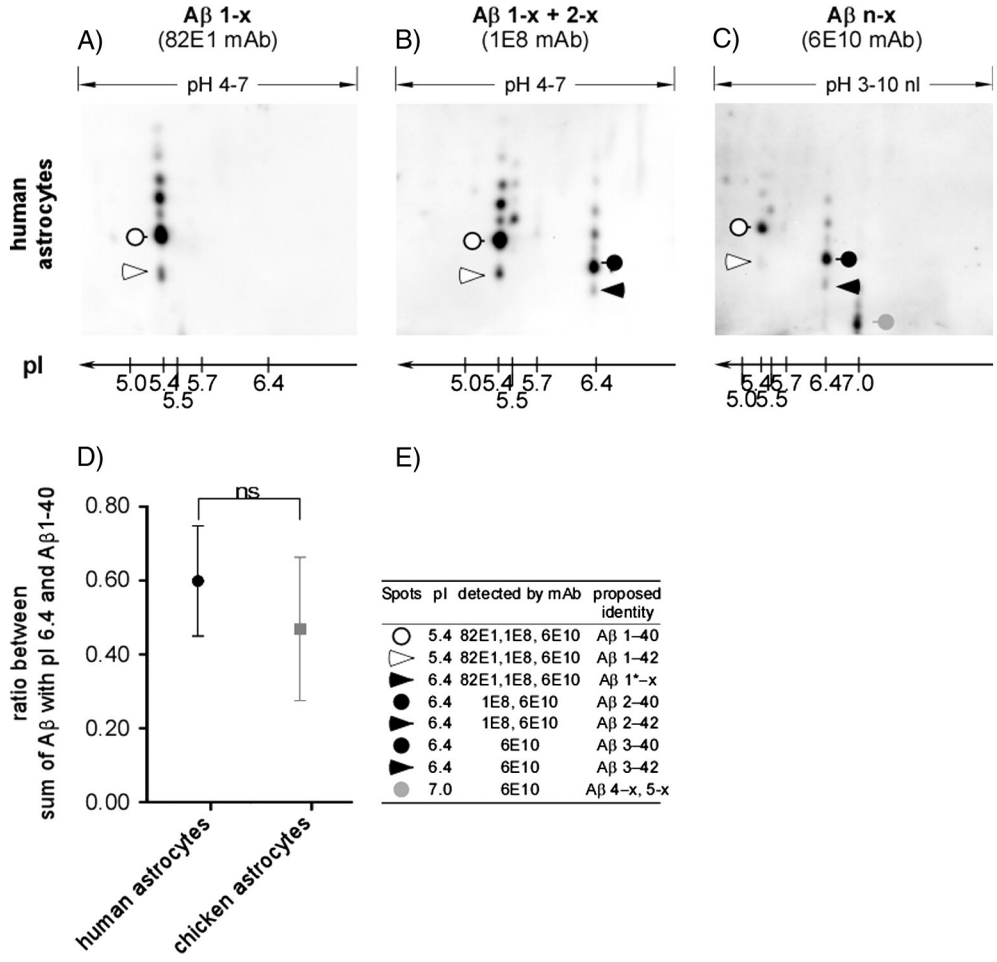

Fig. 5. The pattern of Aβ peptide variants in the supernatants of cultured human astrocytes. Cultured human astrocytes were incubated for 48 h with serum-free cell culture medium. Aβ peptides from a sample volume of 3.2 ml (A, B) and 4 ml (C) were immunoprecipitated with the 1E8 mAb (A, B) or 6E10 mAb (C). 2D-Aβ-PAGE/IB was performed as described in Fig. 3. Representative 2D-IB analyses were probed with the 82E1 mAb (A), 1E8 mAb (B), and 6E10 mAb (C). The distribution of spots in supernatants of human astrocytes corresponded to the distribution of spots in samples of chicken astrocytes at pH values of 5.4, 6.4, and 7.0. The relative intensities of Aβ peptides exhibiting a pI of 6.4 were not significantly different between human and chicken astrocytes in 2D-IB analysis using the 1E8 mAb (Mann–Whitney U test; ns: not significant) (D). The spots with pI values of 5.0, 5.5, and 5.7 were also observed in human astrocytes but exhibited different relative abundance in comparison to chicken astrocytes. Each experiment was performed at least three times. The proposed identities of the marked spots and their observed pI values are presented in (E).

total Aβ peptides. In contrast, neurons predominantly secrete Aβ 1-x and only small amounts of N-terminally modified Aβ peptides. Pyroglutamate-modified Aβ peptides such as NpE x-3 were not detected in the cell culture models used here. The relative abundance patterns of Aβ peptides in supernatants from primary astrocytes from humans were found to be very similar to those from the chick embryo. Importantly, the highly reproducible pattern of secreted Aβ peptides in astrocyte cultures was independent not only of species but also of the incubation period and the cell culture medium. A relatively high proportion of N-terminally modified Aβ peptides was also observed in the supernatant of cultured chicken microglia that closely resembled the pattern of secreted Aβ peptides from untransfected human phagocytes (Maler et al., 2008). The 2D-Aβ-PAGE/IB method employed here allows for a direct comparison of the relative abundances of several Aβ peptide variants differing in charge and electrophoretic mobility in the presence of SDS and urea. However a limitation of this study is that the exact identities of some of the detected peptides remain to be elucidated by mass spectrometry.

To the best of our knowledge, the relative abundance patterns of monomeric Aβ variants secreted by untransfected primary neurons, astrocytes, and microglia have not been published before. Previous studies either compared the amount of total Aβ peptides of these cell types or addressed Aβ variants in cell culture supernatants of transfected cell lines overexpressing wild-type or mutant APP, e.g., N2a/APP695, 7PA2/APP751:V717I, H4/APP695:K670M/N671L, and SH-SY5Y/APP695 (Haass and Selkoe, 1993; LeBlanc et al., 1996; Wang et al., 1996; Vandermeeren et al., 2001; Haussmann et al., 2013; Portelius et al., 2013). The most abundant Aβ peptides identified in these cell models were Aβ variants that begin at Asp1. Additionally, N-terminally truncated Aβ peptides, i.e., Aβ 2-x, Aβ 3-x, Aβ 4-x, and Aβ 5-x, were also detected in small amounts using mass spectrometry or immunoblot analysis (Wang et al., 1996; Haussmann et al., 2013).

However, the relative proportions of the N-terminally truncated Aβ peptides that are released from transfected cells are difficult to compare with the present results because the overexpression of APP may affect the subcellular distribution and metabolism of APP. Furthermore the cell lines may have lost specific physiological functions during their immortalization. A limitation of all these and similar studies, including our present one, is that the pattern of Aβ peptides secreted from cultured cells does not necessarily reflect the generation of Aβ peptides in the aged human brain especially under disease conditions. The contribution of individual cells to the formation of N-terminal truncated Aβ in vivo remains to be addressed in further studies. The secretion of N-terminal truncated Aβ by neurons might outweigh the contribution of glial cells just because of the larger amount of total Ab secreted by

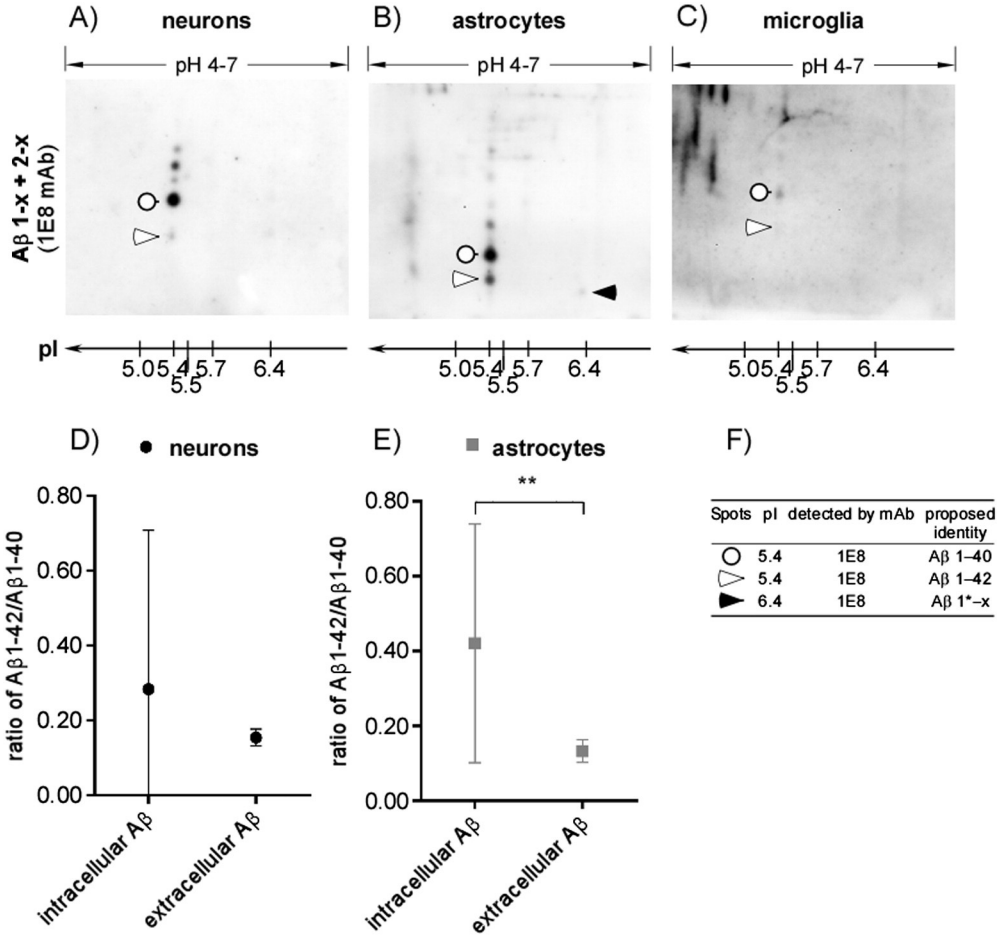

Fig. 6. The pattern of Aβ variants in lysed cell fractions of cultured neurons, astrocytes, and microglia. Detergent-soluble cell fractions of neurons (A), astrocytes (B), and microglia (C) were immunoprecipitated using the 1E8 mAb and analyzed by 2D-Aβ-PAGE/IB. Representative 2D-IB are shown, that were probed with the 1E8 mAb. Only spots with a pI of 5.4, which presumably corresponded to Aβ 1-x, were detected in samples of neurons (A) and microglia (C). In contrast, cell lysates of astrocytes contained a very faint additional peptide exhibiting a pI of 6.4, which shows the same electrophoretic properties as Aβ 1*-x that was observed in the cell culture supernatants of astrocytes. Other N-terminally modified Aβ peptides were not detected in 2D-Aβ-PAGE/IB analysis of cell lysates probed with the 1E8 mAb. MANOVA was conducted to determine whether the ratios of the intracellular Aβ peptides, e.g., Aβ 1-42/Aβ 1-40, Aβ 1-39/Aβ 1-40, Aβ 1-38/Aβ 1-40, and Aβ 1-37/Aβ 1-40, were different from the corresponding ratios of extracellular Aβ peptides in the supernatants. A significant multivariate main effect was detected in astrocytes (MANOVA; Wilks' $\lambda = 0.107$; $F = 16.731$; $p < 0.001$; and partial eta squared $= 0.893$) but not in neurons (MANOVA; Wilks' $\lambda = 0.671$; $F = 0.367$; ns; and partial eta squared $= 0.329$). Significant univariate main effects indicated that the ratio of Aβ 1-42/Aβ 1-40 was significantly higher in intracellular lysates than in extracellular supernatants of astrocytes (**$p < 0.01$) (E). For the remaining ratios of intracellular Aβ peptides, no significant univariate main effects were observed. Each experiment was performed at least three times. The proposed identities of the marked spots and their observed pI values are presented in (F).

neurons. On the other hand it should be considered that the total number of glial cells has been reported to be higher than the number of neurons in the human cerebral cortex (Herculano-Houzel, 2014).

Secreted extracellular proteases appear to have only minimal impact on the Aβ profiles in cell culture supernatants. Only ~35% of Aβ 1-40 in conditioned cell culture media was found to be degraded within seven days at 37 °C. Additionally, the ratios of the remaining N-terminally modified Aβ peptides, presumably corresponding to Ab 2-x, to total Aβ peptides were not significantly altered after this incubation period, indicating that exopeptidases present in the cell culture supernatant did not mediate the N-terminal truncation of these Aβ peptides. Consistent with these results, a lack of degradation of Aβ peptides was reported upon the addition of synthetic Aβ peptides to the medium of N2a/APP695 cells (Wang et al., 1996). The fragments of Aβ peptides cleaved by extracellular proteases were shown to be primarily generated by cleavage near the middle or near the C-terminus of Aβ (Wang et al., 1996). Some studies suggested that Aβ-degrading enzymes were released into the cell culture medium, but the majority of Aβ-degrading enzymes appear to be associated with the plasma membrane or intracellular (Qiu et al., 1998; Saido and Iwata, 2006; Pacheco-Quinto and Eckman, 2013).

Collectively, cultured microglia and astrocytes produce substantially less total Aβ than neurons but a relatively higher proportion of specific N-terminally truncated Aβ variants. These findings may be relevant for the development and progression of the amyloid pathology observed in AD brains. This hypothesis is consistent with histological examinations that have demonstrated increased numbers of reactive astrocytes near Aβ plaques that contain N-terminally truncated Aβ peptides (Thal et al., 2006). Furthermore there are implications from a transgenic mouse model (PS1ΔE9flox mice), that cells other than excitatory neurons can give rise age-dependently to amyloid plaques (Veeraraghavalu et al., 2014). On the other hand in APP/PS1KI mice, in which human APP 751 carrying the London (V717I) and the Swedish (K670N/M671L) mutation was under the control of the neuronal thy-1 promoter, N-terminal truncated Aβ peptides were detected corresponding to the age of mice, which suggests that under these circumstances neurons may be the major source for N-terminal truncated Aβ (Casas et al., 2004). Yet, a glial origin of the of N-terminal truncated Aβ in this model cannot be fully excluded, as long-term primary cultures and cultures using tissue from older animals demonstrate that thy-l does appear on some astrocytes later in development (Pruss, 1979).

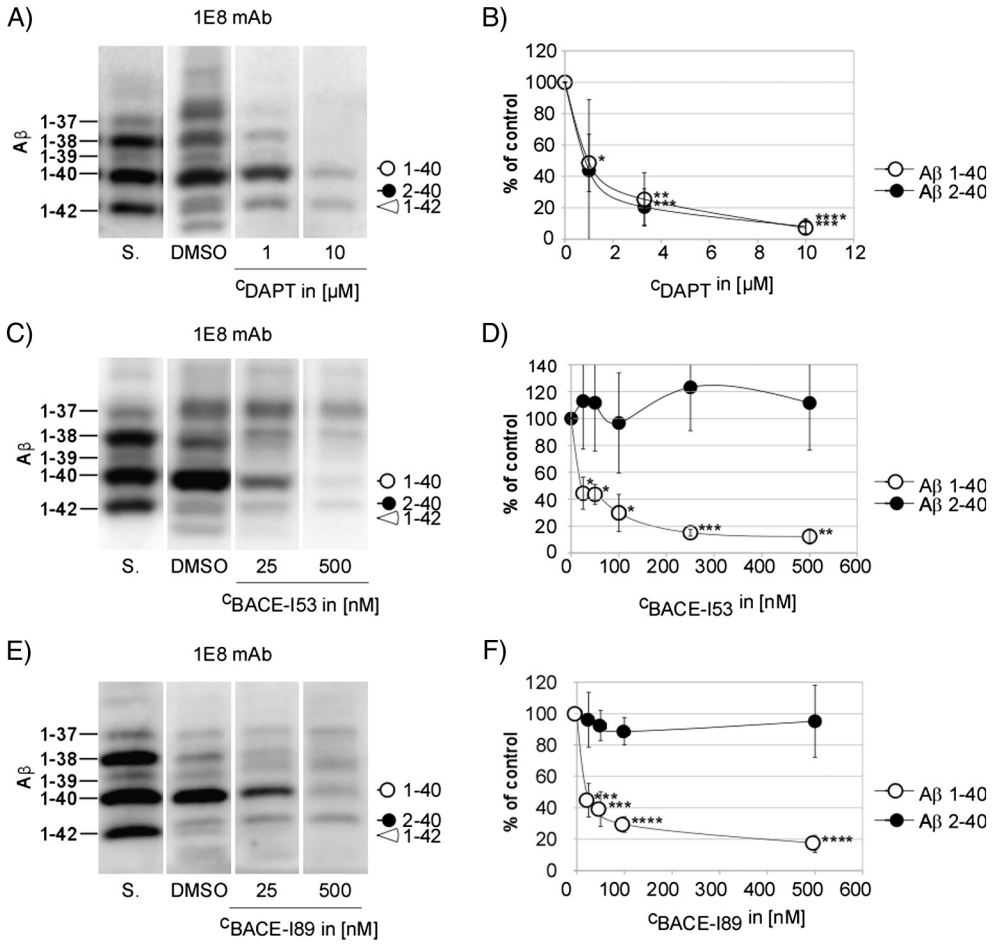

Fig. 7. Effects of the inhibition of γ-secretase and BACE on the pattern of Aβ peptides in the supernatants of cultured astrocytes. Supernatants from cultured chicken astrocytes treated with the γ-secretase inhibitor DAPT or a tripartite BACE inhibitor were analyzed by 1D-PAGE/IB. A) Representative 1D-IB analysis of cell culture supernatants of astrocytes treated with 0.1% DMSO (vehicle alone) and 1 μM, and 10 μM DAPT for 48 h. For comparison, a sample of synthetic Aβ peptides, as indicated on the left of each 1D-IB (S.), was loaded. The blot was probed with the 1E8 mAb. All of the detected Aβ-peptides were substantially reduced after treatment with the γ-secretase inhibitor. B) Dose response curves for selected Aβ-variants indicated statistically significant reductions of Aβ1-40 and Aβ2-40 as compared to vehicle controls (DMSO). The mean relative band intensities and standard deviations are shown. Dose–response experiments were analyzed by one-way ANOVA followed by Dunnet's post hoc test with DMSO treated astrocytes as control group. Significance levels of post hoc tests are shown (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$). C) Representative 1D-IB analysis of cell culture supernatants of astrocytes treated with the tripartite BACE-inhibitor BACE-I53 or vehicle alone (DMSO). While Aβ1-40 was clearly reduced by BACE-I53, specific other Aβ-variants were not. D) Dose response curves for selected Aβ-peptides 1-40 and 2-40 indicated a statistically significant reduction of Aβ1-40 but not of Aβ2-40. Similarly treatment with BACE-I89 reduced Aβ1-40 significantly, while other Aβ-variants were not affected (E, F). All experiments were performed at least three times.

Intracellular N-terminally truncated Aβ peptides, presumably corresponding to Ab 2-x, were not detected in 2D-Aβ-PAGE/IB analysis from primary chicken neurons, astrocytes, and microglia. Only Aβ peptides exhibiting identical electrophoretic properties as extracellular Aβ peptides that begin at Asp1 were detected. The relative abundance of intracellular Aβ 1-42 was slightly elevated in comparison to the relative abundance of the corresponding extracellular peptide. Elevated amounts of intracellular Aβ 1-42, particularly in astrocytes, have been previously reported (Nagele et al., 2003). In comparison to human AD brain extracts, which typically contain higher amounts of Aβ 1-42 than Aβ 1-40 especially, in detergent soluble fractions from astrocytes the amount of Aβ 1-40 exceeded the amount of Aβ 1-42 similarly as observed in cell culture supernatant, human CSF and blood plasma. It appears that in the AD-brain, Aβ42 is preferentially deposited during disease onset and progression reflecting its reported higher propensity to aggregate in vitro (Jarrett et al., 1993; Harper et al., 1997).

The proportions of the remaining intracellular Aβ peptides and their corresponding extracellular Aβ peptides, i.e., Aβ 1-37, Aβ 1-38, and Aβ 1-39, were similar. This result indicates that, in our study, neurons, astrocytes, and microglia did not take up and accumulate the secreted N-terminally truncated Aβ peptides, presumably corresponding to Ab 2-x, to a measurable extent. It has been previously suggested that the intracellular pool of Aβ peptides of cultured SH-SY5Y cells does not consist of phagocytosed Aβ peptides (Pacheco-Quinto and Eckman, 2013). In contrast, astrocytes and microglia appear to be capable of phagocytosing extracellular Aβ peptides of amyloid plaques because in histological preparations, astrocytes in the vicinity of amyloid plaques were positive for Aβ N3pE-x (De Kimpe et al., 2013). Enhanced phagocytosis or generation of intracellular N-terminally truncated Aβ by astrocytes could be triggered by changes of activities and expression levels of enzymes, including glutaminyl cyclase, which was reported to be involved in the formation of Aβ N3pE-x (Cynis et al., 2006; Schilling et al., 2008). In our cell culture model the absence of distinct intracellular N-terminally modified Aβ peptides and the ineffective generation of N-terminally truncated Aβ peptides by secreted extracellular proteases may be due to a different origin and metabolism of Aβ 1-x compared to Aβ 2-x.

Interestingly, in addition to the previously described Aβ 1-x, a peptide that was only observed in astrocyte supernatants and cell lysates was detected using the 82E1 mAb, which is specific for Aβ peptides that begin at Asp1. However, in contrast to the detected Aβ 1-x variant (pI of 5.4), this peptide exhibited a pI of 6.4, indicating that this presumed Aβ 1-x posttranslationally lost an acidic modification or acquired

a basic modification. The exact identity and mechanism of formation of this peptide remain to be elucidated.

The N-terminally modified Aβ peptides, presumably corresponding to Ab 2-x, were secreted by astrocytes in the presence of effective concentrations of membrane-targeted tripartite BACE inhibitors while Aβ 1-x variants were reduced as expected.

BACE plays a pivotal role in the production of Aβ peptides from APP, particularly in primary neurons and cell lines transfected with APP (Vassar et al., 1999; Lee et al., 2003). N-terminally truncated Aβ peptides have been previously suggested to be generated by aminopeptidases following the primary cleavage of APP by BACE (Saido, 1998; Sevalle et al., 2009). In contrast, our results indicate that BACE inhibition specifically reduced the secretion of Aβ 1-x in astrocyte cultures, whereas the amount of Aβ 2-x typically found in astrocytes remained unaltered or was even increased. These findings are consistent with previous reports indicating that treatment with BACE inhibitors reduces the amount of Aβ 1-x in the supernatant from APP-transfected cells, the CSF of dogs, and in neuronal cultures and causes a concomitant increased release of N-terminally truncated Aβ peptides, such as Aβ 5-40 (Schieb et al., 2011; Mattsson et al., 2012; Haussmann et al., 2013). Studies from BACE knockout mice revealed that primary cortical cells still produced small amounts of Aβ peptides despite abolished BACE (Roberds et al., 2001). These findings might indicate the existence of proteases in addition to BACE1 that are presumably responsible for the de novo generation of N-terminally modified Aβ peptides. A potential candidate protease that has been shown to be capable of producing Aβ 2-x is meprin β (Bien et al., 2012). In contrast to BACE1, meprin appears to preferentially cleave APP at the plasma membrane. APP cleavage at the cell surface may explain our observations that Aβ 2-x was not intracellularly detected in cell samples from primary neurons, astrocytes, and microglia. Another candidate for an alternative β-secretase is cathepsin B, which was detected in microglia and astrocytes and exhibits an unspecific cleavage pattern at the N-terminus of the Aβ sequence (Takeda et al., 2004; Hook et al., 2005; Bohme et al., 2008; Sun et al., 2008). In particular, Aβ 5-x appears to be generated by cathepsin B in SH-SY5Y and HEK293 cells (Takeda et al., 2004). Other studies suggested that cathepsin B may contribute to a C-terminal truncation of Aβ 1-42, thereby generating Aβ 1-38 and Aβ 1-33 (Sun et al., 2008).

Conclusion

Collectively, different cell types may genuinely exhibit or modulate the activity and expression of different proteases capable of cleaving APP at or close to the β-secretase site, which may explain the observed cell type specific differences in the patterns of secreted Aβ peptides. Our results indicate that astrocytes and microglia, but not neurons, secrete proportionally high amounts N-terminally modified Aβ peptides, among others Aβ 2-x, and that the release of Aβ 2-x appears to be independent of BACE1 itself. We speculate that the high prevalence of N-terminally modified Aβ peptides, especially Aβ 2-x, in formic acid soluble extracts from AD plaques may originate from glial rather than neuronal cells, thereby providing a novel perspective on the role of glial cells in AD pathology.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nbd.2014.08.031.

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