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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