Neurotoxicity of Alzheimer disease Aβ-peptides is induced by small changes in the Aβ42 to Aβ40 ratio

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Supplemental data

1. Material and methods

Chemicals
Alzheimer’s beta-peptide 1-40 (Cat. A1153) and 1-42 (Cat. A-1163) were from rPeptide (USA). Uranyl acetate was from BDH. Mouse monoclonal (DE2B4) to Aβ was from Abcam (UK) and rabbit polyclonal anti-oligomer antibody (A11) from Biosource (Belgium). The Genetics Company provided specific antibodies against Aβ40 (mouse monoclonal anti-Aβ 40-antibody-peroxidase-conjugate, Clone G2-10) and Aβ42 (mouse monoclonal anti-Aβ 42-antibody-peroxidase-conjugate, Clone G2-13). Mouse monoclonal antibody to synaptophysin was from Sigma-Aldrich (Belgium) and antibody to cleaved caspase-3 from Cell Signaling (Belgium).

Electrospray-Ionization Mass Spectrometry (ESI-MS)
Positive-ion mass spectra were recorded on an orthogonal acceleration quadrupole time-of-flight mass spectrometer (Q-Tof-2, Micromass, Manchester, U.K.) equipped with a standard electrospray probe (Z-spray) and controlled by a dataystem running MassLynx 3.4 (Micromass, Manchester, UK). Samples were dissolved in acetonitrile:water (1:1) containing 1 % acetic acid at a final concentration of 2 µM and infused using a syringe pump with a flow of 5 µL per minute. Cone voltage was set to 30 V, capillary voltage was 3 kV. Spectra were recorded from m/z 600 to m/z 1600. Scan time was set to 4.9 sec with an interscan time of 0.1 sec. At least ten spectra were acquired and averaged. Deconvolution was performed using the MaxEnt algorithm included in the software.

Transmission Electron Microscopy (TEM)
Aliquots (5 µL) of the 50 µM Aβ aggregate preparation incubated for various time intervals, were adsorbed to carbon-coated FormVar film on 400-mesh copper grids (Plano GmbH, Germany) for 1 min. The grids were blotted, washed twice in droplets of Ultrapure water (Merck), and stained with 2 % (wt/vol) uranyl acetate. Samples were studied with a JEOL JEM-2100 microscope at 200 kV.

Atomic force microscopy (AFM)
Atomic force microscopy imaging was performed on a custom built instrument using Si₃N₄ tips (Veeco Instruments, Woodbury NY, USA, type MSCT-AUHW) with a spring constant of 0.5 N/m
and a nominal tip radius of 10 nm. The measurements were made in tapping mode in liquid, with a tapping amplitude of less than 4 nm. The AFM scan settings were optimized to minimum force interaction with the sample. All AFM images have 512x512 pixels. The 50 mM Tris, 1 mM EDTA buffer was 0.02 μm filtered to remove any impurities. Aβ ratios were incubated at 25°C at 70 μM for 1.5h. AFM samples were prepared by placing 4 μL of sample on freshly cleaved mica. After 4 min adsorption time, unbound Aβ was gently washed off with twice 100 μL buffer (10 mM Tris, 1 mM EDTA, pH 7.5). The sample was then mounted on the AFM stage with application of 300 μL extra buffer, a volume sufficient to keep the sample in buffer throughout the experiment. The images are represented in 3D after removal of height discontinuities between subsequent scan lines and compensation for piezo drift using SPIP software (Image Metrology A/S, Lyngby, Denmark).

Time-resolved Western blot and dot blot
Aggregation of Aβ40, Aβ42 and ratios (1:9) and (3:7) (Aβ42: Aβ40) was studied using Western blot analysis. Aggregates were prepared at 100 μM and incubated at 25 °C. At various time points, aliquots of 12.5 μL Aβ were mixed with 12.5 μL 2 x Novex Tricine SDS sample buffer (for SDS-PAGE) or with 4 μL native PAGE sample buffer and 0.5 μL G-250 (for native PAGE) and loaded onto a 10% bis tris gel (SDS-PAGE) or a 3-12 % bis tris gel (native PAGE). The SDS-gel was run at 125 V for 75 min and the native-gel at a fixed voltage of 100V for 120 min, Subsequently the peptides were transferred onto a nitrocellulose membrane (SDS-PAGE) or to PVDF membrane (native PAGE) using a Transfer-Blot Semi-Dry Transfer Cell (Bio-Rad) for 2 h at 25 V at 4 °C using Towbin blot buffer (25 mM Tris, 192 mM Glycine, 20 % MeOH, 0.1 % SDS) (SDS-PAGE) or for 70 min at 0.09 A at 4 °C using NuPAGE transfer buffer (Invitrogen)(native PAGE). For dot blot, 5 μL sample was blotted onto nitrocellulose membrane (Protran BA85, Whatman, Schleicher & Schuell) followed by boiling of the membrane in PBST. Membranes were blocked and incubated with primary antibodies 6E10, and A11 for PAGE and anti-Aβ40 and anti-Aβ42 for dotblots. After blotting with HRP-tagged secondary antibodies the membranes were visualized using an electrochemical luminescence (ECL) system.

Primary hippocampal cultures
Hippocampal neuronal cultures were generated from trypsinized brains obtained from 17-days-old embryos and were maintained in Neurobasal medium (Invitrogen) supplemented with B27 and 12.5 μM L-glutamate After 3 days of culturing, the medium was changed to Neurobasal medium
without glutamate. Cultures were maintained at 37 °C in a humidified 5 % CO₂ atmosphere for 8-10 days prior to further experiments.

Immunocytochemistry
Slides were washed with PBS, fixed with 4 % paraformaldehyde/PBS (PFA) and immunostained with anti-cleaved caspase 3 antibody (1:100, Cell Signalling) or anti-synaptophysin (1:200, Sigma-Aldrich) followed by staining with Alexa-Fluor 488-conjugated GaM secondary antibody (1:200, Invitrogen). For double staining anti-oligomer A11 antibody (1:200) and anti-synaptophysin (1:200, Sigma-Aldrich) were used and subsequently stained with Alexa-Fluor 564-conjugated GaR and Alexa-Fluor 488-conjugated GaM secondary antibodies (1:200, Invitrogen), respectively. Microscopic analysis was performed on a Bio-Rad Confocal microscope. Images were analyzed for their fluorescence intensity using NIH-ImagePro3 software.

Cell-Titer-Blue cell viability assay
Ten µL of Cell-Titer-Blue dye (Promega) was added directly to the growth medium on the cells. After 3 h incubation the fluorescence intensity of the samples was measured at an excitation wavelength of 560 nm and an emission wavelength of 590 nm.

Annexin V/Propidium Iodide (PI) staining
Hippocampal cultures on slides were incubated with annexin V/PI dye mixture from the ApoAlert apoptosis kit (Clontech) for 15 min according to manufacture recommendations. Microscopic analysis was performed using confocal microscope as described above.

Mouse care
Female 3 month old mice of comparable weight were maintained in a specific pathogen-free facility that meets Belgian and European Union requirements for animal care. Mice were group housed in a climate-controlled animal colony with a 12 h light/dark cycle (light on 6:00 A.M.) with access to food and water ad libitum, except during some of the behavioral testing. Adequate measures were taken to minimize pain or discomfort.

Brain intraventricular injections
Animals were locally anesthetized by subcutaneous injection of 5 µL of 2 % Xylocaine. The needle was inserted unilaterally 1 mm to the right of the midline point equidistant from each eye, at an equal distance between the eyes and the ears and perpendicular to the plane of the skull and 6 µg Aβ from an aged (2 h) solution was injected using a Hamilton syringe. The surgery was completed within 8 min. Animals were followed-up for their post-surgery recovery and normally showed unimpaired behavior and motility. 1-1.5 h after the injections the animals were exposed to behavior tests. Different experimental groups of transiently injected animals were used for each behavioral test.

Supplementary figures

Supplemental figure 1: preparation of Aβ ratios.
Deconvoluted electrospray ionization mass spectra of (A) Aβ_{42}:Aβ_{40} (0:10) (B) Aβ_{42}:Aβ_{40} (1:9) (C) Aβ_{42}:Aβ_{40} (3:7), and (D) Aβ_{42}:Aβ_{40} (10:0) at time 0 h. Samples were diluted 100-fold in acetonitrile:distilled water (1:1 v/v) from 100% DMSO and injected directly into the spectrometer. (E) Dot blot confirming the presence of both Aβ_{40} and Aβ_{42} in ratios by employing carboxy-terminus specific antibodies.

Supplemental figure 2: Native PAGE and SDS PAGE of Aβ ratios shows a range of different oligomeric sizes of Aβ present throughout the aggregation process.
Native PAGE (left two panels) and SDS PAGE (right three panels) (10-20% tris-tricine) and subsequent staining by mAb 6E10. Native PAGE shows large oligomers and fibrils (comparable with EM) especially upon longer incubation for synaptotoxic (3:7) and (10:0). SDS PAGE only shows primarily monomeric and some trimeric and tetrameric species indicating that the oligomers and fibrils detected by Native PAGE are constructed from similar building blocks and that the detection of these oligomeric species is dependent on the particular technology used.

Supplemental figure 3: Aβ40 and Aβ42 show direct and non-preferential interaction. (A) Electron Spray Ionization-Mass m/z Spectrum (ESI-MS) of monomeric and dimeric Aβ prepared from a 1:1 Aβ_{1-42}:Aβ_{1-40} ratio upon 100-fold dilution from DMSO in acetonitrile:water (1:1, v/v). The box denotes the spectral location of dimers. Spectral contributions by Aβ_{1-40} or Aβ_{1-42} are denoted by circles or squares respectively. (B) Dimer formation by 1:1 Aβ_{1-42}:Aβ_{1-40} ratio.
Magnification of the ESI-MS ($m/z$) dimer spectrum of Aβ1-42 and Aβ1-40 injected at a 1:1 ratio upon 100-fold dilution from DMSO in acetonitrile:water (1:1, v/v). Data from one of two independent experiments are shown. Spectral contributions by Aβ1-40 or Aβ1-42 are denoted by circles or squares respectively.

**Supplemental figure 4:** Aβ40 and Aβ42 interact equally with each other.

Electrospray Ionization Mass Spectra (ESI-MS) of Aβ42:Aβ40 ratios (A) 1:9 and (B) 3:7 demonstrating that dimer formation between Aβ40 and Aβ42 is random: both heterodimers (Aβ40-Aβ42, m/z 1264.5) and homodimers (Aβ40-Aβ40, m/z 1238 or Aβ42-Aβ42, m/z 1291) are detected. Aβ ratios at ~ 100 µM were diluted 100-fold from DMSO in acetonitrile:water (1:1, v/v) and immediately injected into the ESI-MS for analysis (C). Quantitation of ESI-MS data as in panel A and B of dimers generated with different ratios of Aβ42:Aβ40 indicate that Aβ40 and Aβ42 interact to form dimers in a random manner (calculated/experimental%).
Supplemental Figure 1
Supplemental Figure 2
Supplemental Figure 3
Supplemental Figure 4