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The active role of Ca^{2+} ions in Aβ-mediated membrane damage.

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Supporting Information

Experimental details

Materials. Aβ_{1-40} was purchased from Genscript (Bubendorf, Switzerland) with a purity >98%. Brain total lipid extract (TLBE) was purchased from Avanti Polar lipids Inc. (Alabaster, AL). 4-(2-Hydroxyethyl)-piperazine-1-ethanesulfonic acid sodium salt (HEPES), 6-carboxyfluorescein, Thioflavin T (ThT), Calcium chloride anhydrous ($CaCl_2$), 1,1,1,3,3,3-hexa-fluoro-2-propanol (HFIP) and Fura-2 dye potassium salt were purchased from Sigma-Aldrich (St.Louis, MO) with a purity of 99%. Mouse monoclonal anti Aβ_{1-16} (6E10) was purchased by Covance (Princeton, NJ). Secondary goat anti-mouse labeled with IRDye 800 was purchased by Li-COR Biosciences (Lincoln, NE) .

Preparation of model membranes. In this study, we used large unilamellar vesicles (LUVs) composed of TLBE. Briefly, aliquots of lipid stock solutions in chloroform were dried by using a stream of dry nitrogen gas and evaporated under high vacuum in a round-bottomed flask. Multilamellar vesicles (MLVs), were obtained by hydrating the lipidic film with an appropriate amount of HEPES buffer (10 mM buffer, 100 mM NaCl, pH =7.4) and dispersing by vigorous stirring in a water bath at room temperature. LUVs were obtained by extruding MLVs through polycarbonate filters (pore size = 100 nm, Nuclepore, Pleasanton, CA) mounted in a mini-extruder (Avestin, Ottawa, ON, Canada) fitted with two 0.5 ml Hamilton gastight syringes (Hamilton, Reno, NV). Samples were typically subjected to 23 passes through two filters in tandem.

Aβ_{1-40} preparation. To prevent the presence of any preformed aggregates, Aβ_{1-40} was initially dissolved in HFIP at a concentration of 1 mg/ml and then lyophilized overnight. To be used for the experiments, the lyophilized powder was initially dissolved in NaOH 1 mM to obtain a stock solution with a final concentration of 250 μM. Each stock solution of Aβ_{1-40} was used immediately after preparation.

CD spectra. Circular dychroism spectra were acquired by a JASCO spectrophotometer. Sample were prepared by dissolving Aβ_{1-40} in NaOH 1 mM and then added to HEPES buffer 10 mM, pH 7.4, NaF 0.1 M to obtain a final concentration of 1 μM.

ThT measurements. Kinetics of amyloid formation was measured using the well-known and widely accepted Thioflavin T (ThT) assay. Samples were prepared by adding 1 μL of the 250 μM NaOH peptide stock solution to 100 μL of 10 mM HEPES buffer solution (pH 7.4, 100 mM NaCl, containing 10 μM ThT) and by adding the opportune amount of Ca^{2+} ions. For experiments in

presence of model membrane, samples were prepared by adding 1 μL of the 250 μM NaOH peptide stock solution to 100 μL of 200 μM LUV solution (in 10 mM HEPES buffer pH 7.4, 100 mM NaCl, containing 10 μM ThT) and by adding the opportune amount of Ca^{2+} ions. Experiments were carried out in Corning 96 well non-binding surface plates. Time traces were recorded using a Varioskan (ThermoFisher, Walham, MA) plate reader using a λ_{ecc} of 440 nm and a $\lambda_{em}$of 485 nm at 25 °C, shaking the samples for 10 seconds before each read. To obtain the kinetics parameters relative to the ThT assay reported in table 1 and 2 we fitted the data obtained by three independent measurements with the equation:

$$ThT\ fluorescence = \frac{ThT_{max}}{1 + e^{-k(t - t_{1/2})}}$$

In this equation ThT_{max} is the maximum value of the ThT signal, which is qualitatively correlated with the amount of fiber formed; $t_{1/2}$ is the time to half which is defined as the time the signal needs to reach 50 % of the maximum; *k* is the apparent fiber growth rate.

Membrane leakage experiments. Membrane leakage experiments were performed by measuring the leakage of 6-carboxyfluorescein dye from LUVs. Dye-filled LUVs were prepared by hydrating the dry lipid film with the buffer solution containing 6-carboxyfluorescein (70 mM 6-carboxyfluorescein, pH=7.4) according to the procedure described above. To remove non encapsulated 6-carboxyfluorescein we placed the solution containing LUVs on a Sephadex G50 gel exclusion column (Sigma-Aldrich,St.Louis, MO) and eluted using the buffer solution. The final concentration of lipids was checked by using the Stewart. Membrane damage was quantified by the increase in fluorescence emission intensity of 6-carboxyfluorescein due to its dilution (dequenching) in buffer as a consequence of the membrane leakage.

Fura 2 experiments.

We detected the presence of pores by measuring changes in the 340:380 nm excitation ratio upon binding of Ca^{2+} to the cation-sensitive dye Fura-2 encapsulated within the LUVs. Samples were prepared by first diluting the Fura-2 dye-filled vesicles solution with buffer solution (10 mM Hepes buffer solution, 100mM Fura-2 pentapotassium salt, 100 mM NaCl, pH 7.4; to prevent the formation of solid calcium phosphate, phosphate buffer was not used) to a final concentration of 0.2 mg/ml. Then, Aβ1-40 was added with a final concentration of 10 μM. Fluorescence was measured at 340 and 380 nm with slits set for 10 nm bandwidths to obtain the baseline. After 10 min, Aβ were added to the sample, and changes in the 340:380 ratio were recorded.

Western Blot experiments. Western blotting analysis for aβ aggregation in the presence and absence of Ca^{2+} ions was performed on samples at several time of incubation at 37 °C. Samples were loaded onto 4-12% Bis-Tris gel, (Invitrogen). After separation (160 V), proteins were transferred onto a nitrocellulose membrane. Membranes were blotted at 4°C O/N with the primary antibody mouse monoclonal anti Aβ 1-16 (6E10). Secondary goat anti-mouse labeled with IRDye 800 (1:25.000 Li-COR Biosciences) were used at room temperature for 45 min. Hybridization signals were detected with the Odyssey Infrared Imaging System (LI-COR Biosciences).

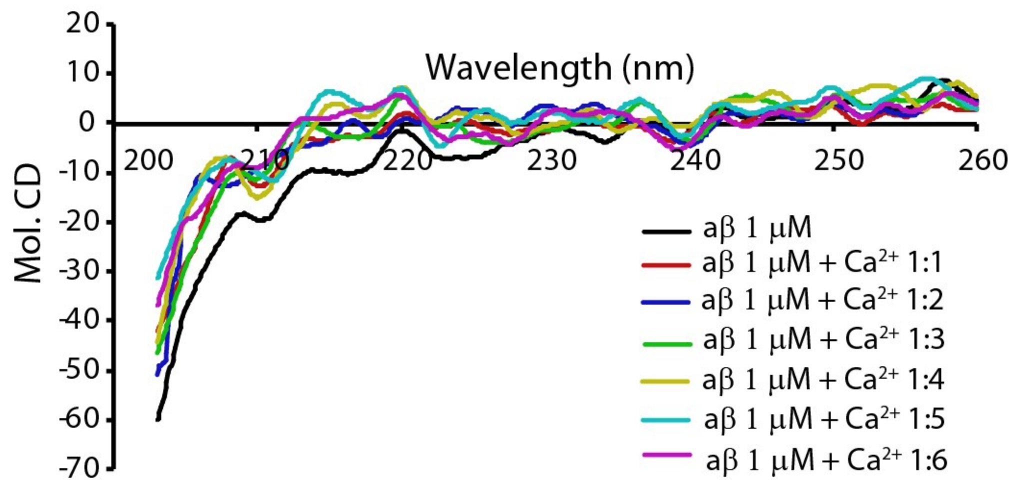

Figure S1. Effect of calcium ions on Aβ_{1-40} secondary structure. Circular dychroism spectra of samples containing 1 mM Aβ1-40 at the indicated concentration of calcium ions. All experiments were conducted at 25°C in HEPES buffer 10 mM, pH 7.4, NaF 0.1 M.

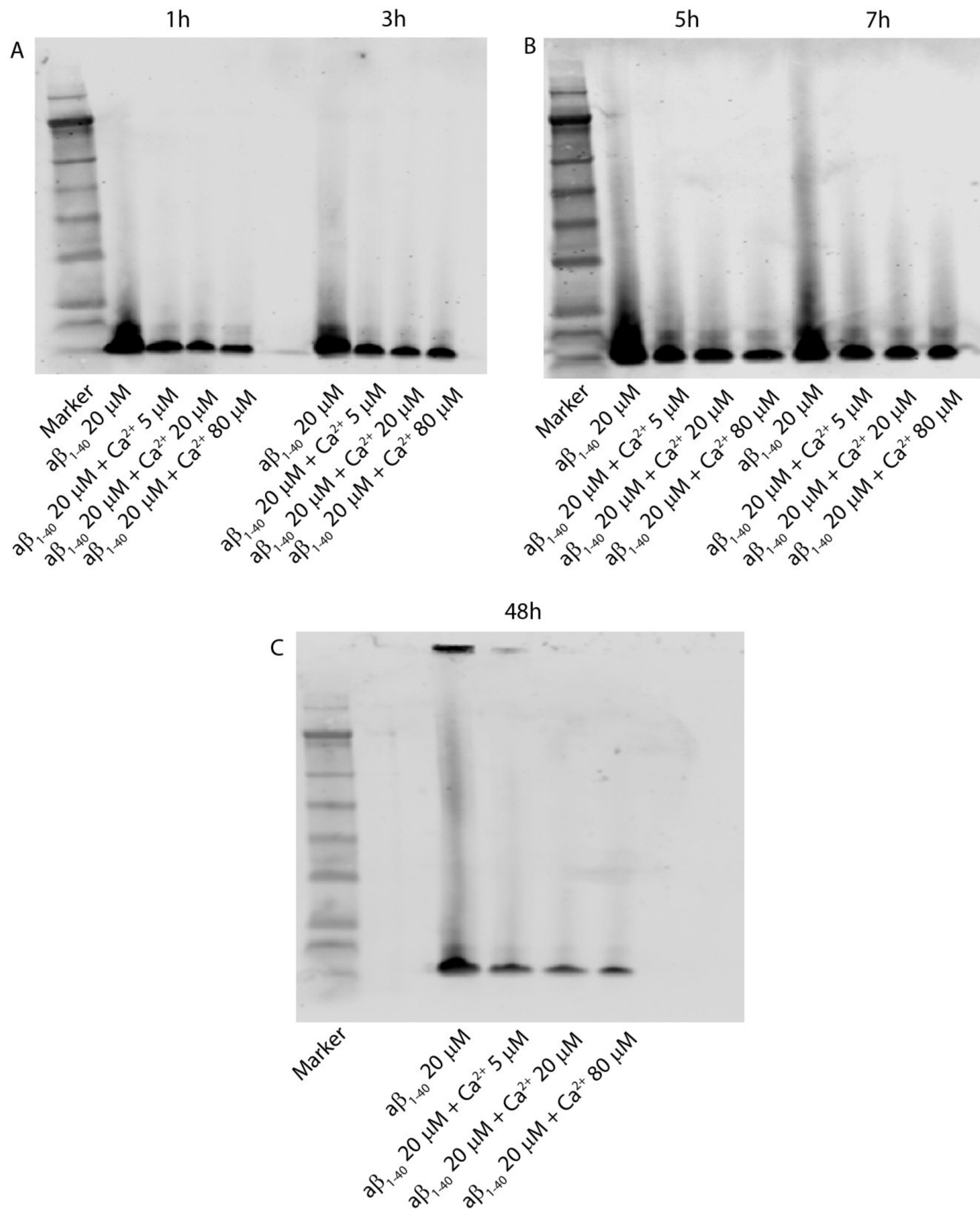

Figure S2. Western Blot analysis of Aβ_{1-40} in the presence of increasing amount of Ca^{2+} ions. Panel A) Western Blot analysis for sample containing Aβ_{1-40} 20 μM and the indicated amount of Ca^{2+} ions after 1h and 3h incubation at 37°C; Panel B) after 5h and 7h incubation at 37°C; Panel C) after 48h incubation at 37°C.

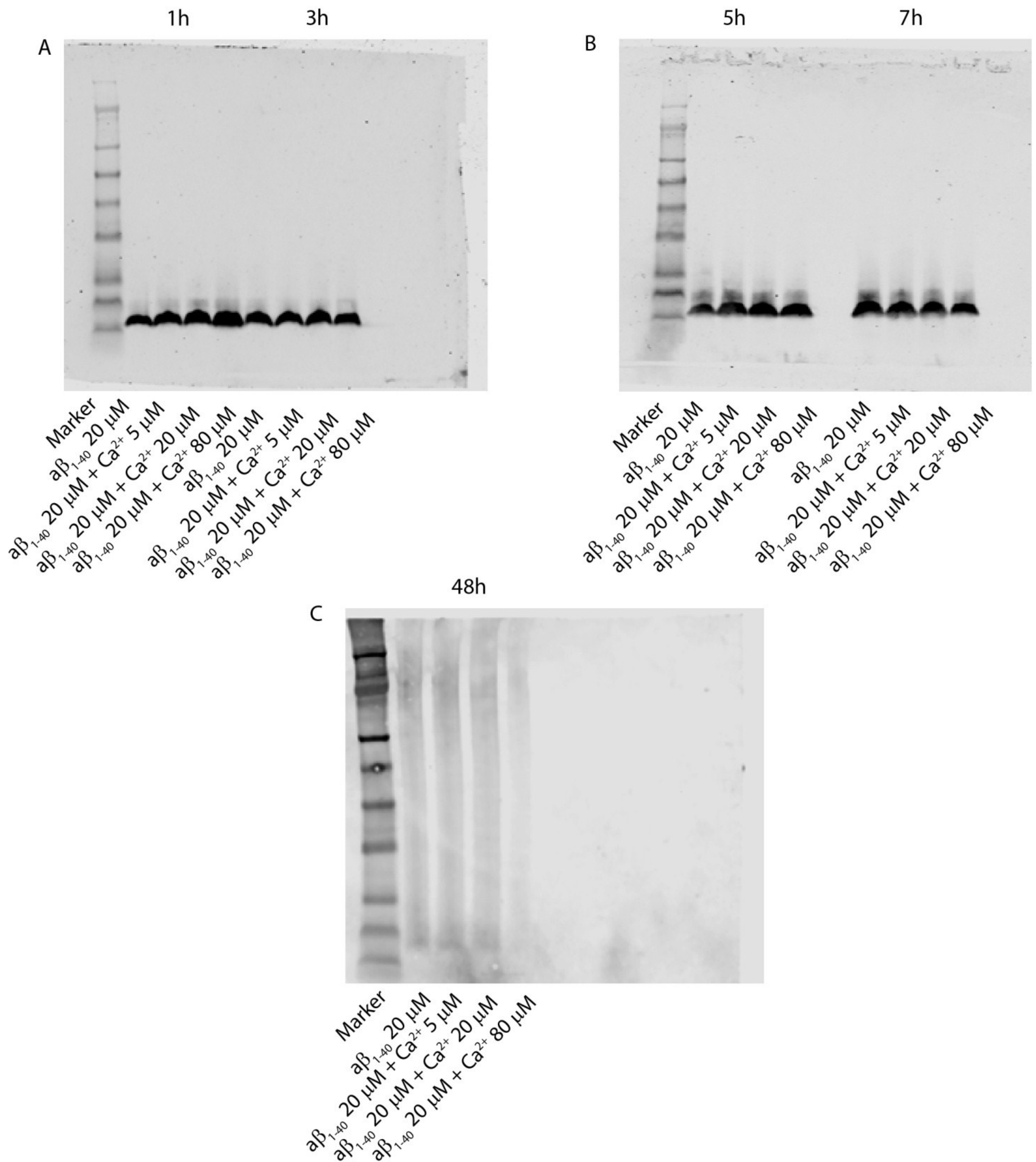

Figure S3. Western Blot analysis of Aβ_{1-40} in the presence LUV TLBE and an increasing amount of Ca^{2+} ions. Panel A) Western Blot analysis for sample containing 200 µM LUV TLBE, Aβ_{1-40} 20 µM and the indicated amount of Ca^{2+} ions after 1h and 3h incubation at 37°C; Panel B) after 5h and 7h incubation at 37°C; Panel C) after 48h incubation at 37°C.