MITOCHONDRIAL CHOLESTEROL LOADING EXACERBATES AMYLOID BETA PEPTIDE-INDUCED INFLAMMATION AND NEUROTOXICITY.

Anna Fernández, Laura Llacuna, José C. Fernández-Checa and Anna Colell

SUPPLEMENTAL INFORMATION

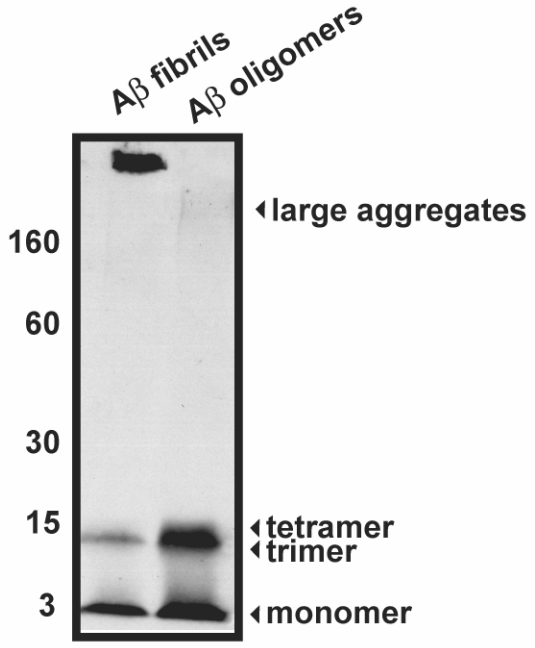

Figure S1. Aggregation state of Aβ1-42 preparations. Representative western blot analysis showing increased presence of soluble oligomers (trimers and tetramers) in samples incubated under oligomeric-forming conditions compared to fibrillar preparations with large aggregates remaining in the well of the gel.

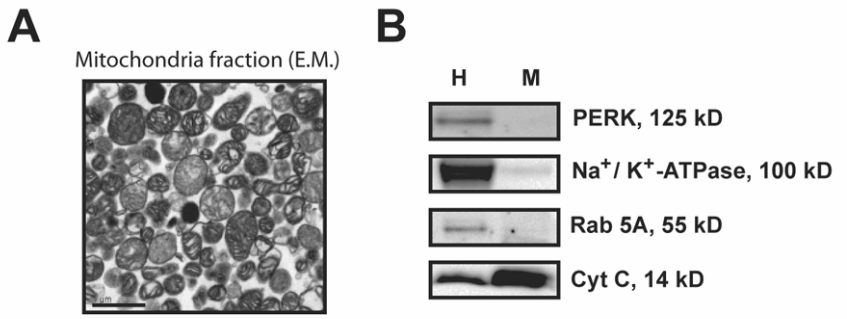

Figure S2. Mitochondria isolated from brain of transgenic SREBP-2 mice. After cellular subfraction, the purity of mitochondria was confirmed (**A**) by electron microscopy (E.M.) and (**B**) by determining in the homogenate (H) or mitochondrial fraction (M) the distribution of specific markers such as PERK (endoplasmic reticulum), Na^+/K^+-ATPase $\alpha 1$ (plasma membrane), Rab 5A (early endosomes), and cytochrome c (mitochondria) using immunoblot analysis. Scale bar: 1 μm.

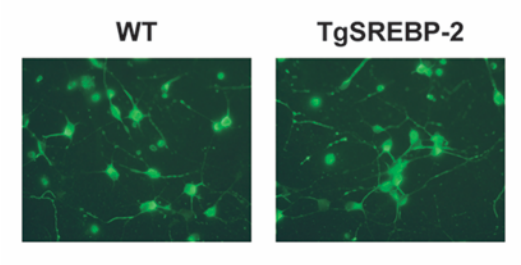

Figure S3. Representative images of neuron-rich cultures from cerebral cortex of wild type (WT) and transgenic SREBP-2 (tgSREBP2) embryonic mice labeled with the neuronal marker microtubule-associated protein 2 (MAP-2) antibody. Original magnification, 400x.

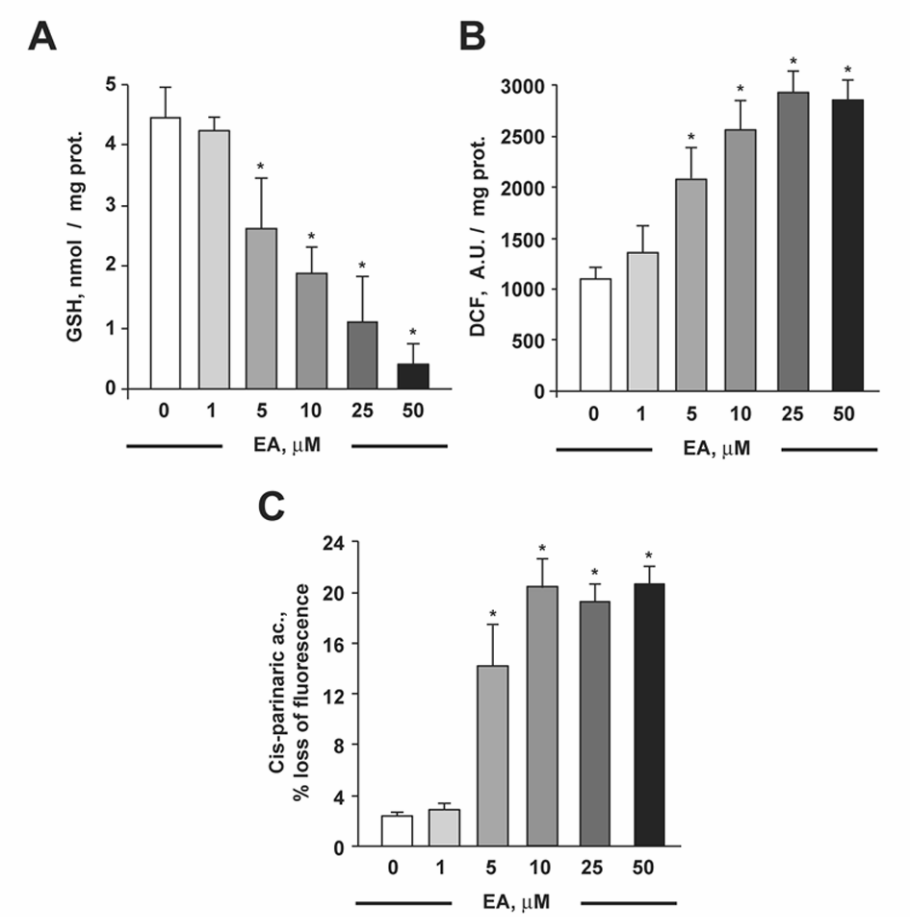

Figure S4. Dose-dependent effect of mitochondrial GSH on ROS and lipid peroxidation induced by Aβ1-42 peptide. Rat brain mitochondria (1 mg/ml) were incubated with increasing concentrations of ethacrynic acid (EA) for 15 min. (**A**) Hydrogen peroxide generation determined by DCF fluorescence from EA-treated mitochondria exposed to Aβ1-42 peptide (5 μM) for 2 h. (A.U.: arbitrary units). (**B**) GSH levels. (**C**) Lipid peroxidation from GSH-depleted mitochondria incubated with 5 μM Aβ1-42 for 2 h and expressed as the percentage loss of cis-parinaric initial fluorescence. **P*<0.05 vs. values at 0 μM. (*n* = 3-4). Values are mean ± S.D.; mean differences were compared by Dunnett's test.

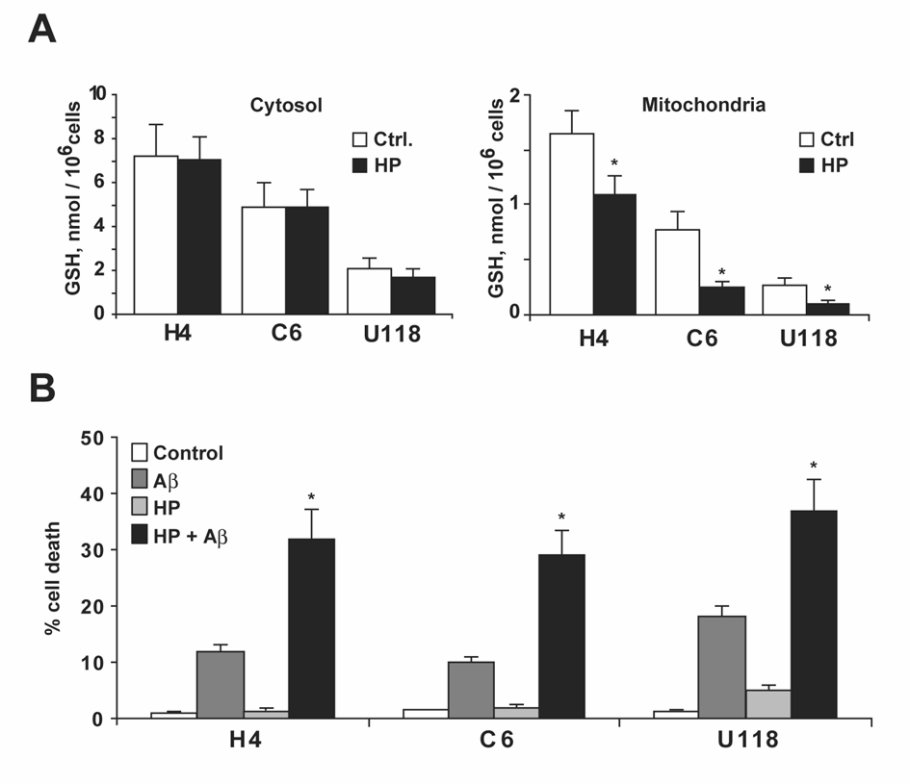

Figure S5. Selective depletion of mitochondrial GSH in rat (C6) and human (H4 and U118) glioma cell lines enhances the cytotoxic effect of Aβ peptide. (**A**) GSH levels in cytosol and mitochondria from H4, C6 and U118 glial cells. Cells were exposed to (S)-3-Hydroxy-4-pentenoate (HP, 5mM) for 10 min and after 2 washes were fractionated to obtain cytosol and mitochondria. **P*<0.05 vs. control cells (*n* = 3). (**B**) Cell death determined by PI staining after exposure of HP-treated cells to Aβ1-42 (5 μM) for 24 h. **P*<0.05 vs. Aβ-treated cells (*n* = 3). Values are mean ± S.D.; mean differences were compared by unpaired Student's t test.

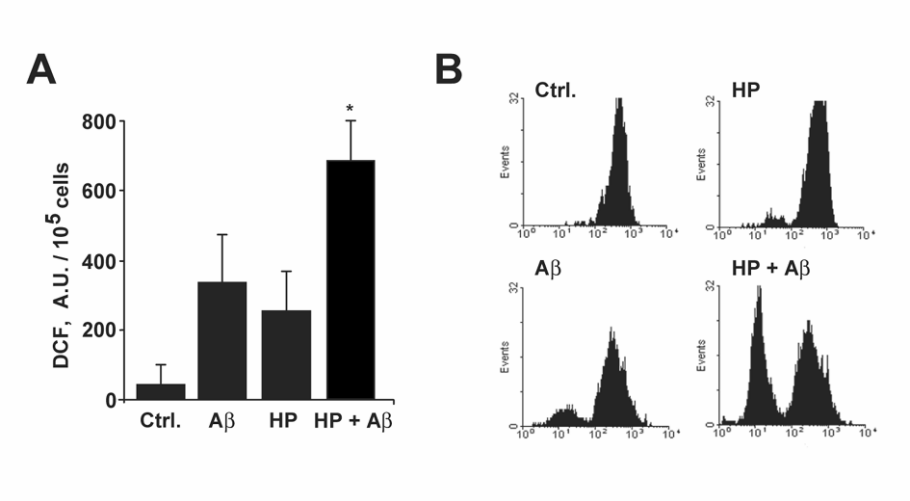

Figure S6. Mitochondrial GSH-depleted SH-SY5Y cells exposed to Aβ1-42 show increased ROS production and loss of mitochondrial membrane potential (ΔΨm). Mitochondrial GSH was selectively depleted by incubation with (S)-3-Hydroxy-4-pentenoate (HP, 5mM) for 10 min and after two washes cells were exposed to Aβ1-42 (5 μM) for 2 h. (**A**) Hydrogen peroxide generation determined by DCF fluorescence. **P*<0.05 (*n* = 3). Values are mean ± S.D.; mean differences were compared by unpaired Student's t test. (**B**) Mitochondrial membrane potential. After Aβ treatment cells were incubated with the fluorescence dye TMRE for 5 min and the percentage of cells with loss of ΔΨm was determined by flow cytometry. Representative analysis of 3 independent experiments is shown.

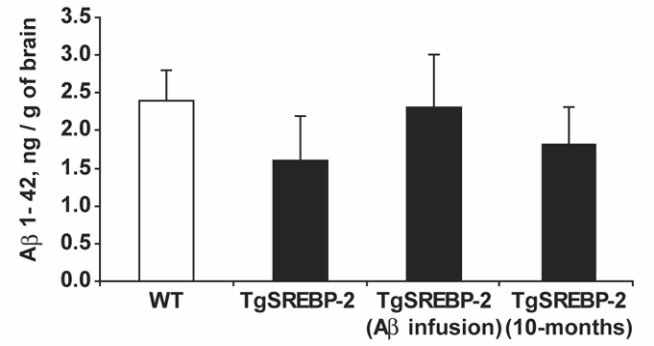

Figure S7. Levels of endogenous Aβ1-42 quantified by ELISA in brain extracts from WT mice, Tg-SREBP-2 mice with or without Aβ-infusion, and 10-months old transgenic mice . Values are mean ± S.D. (n = 4-6).

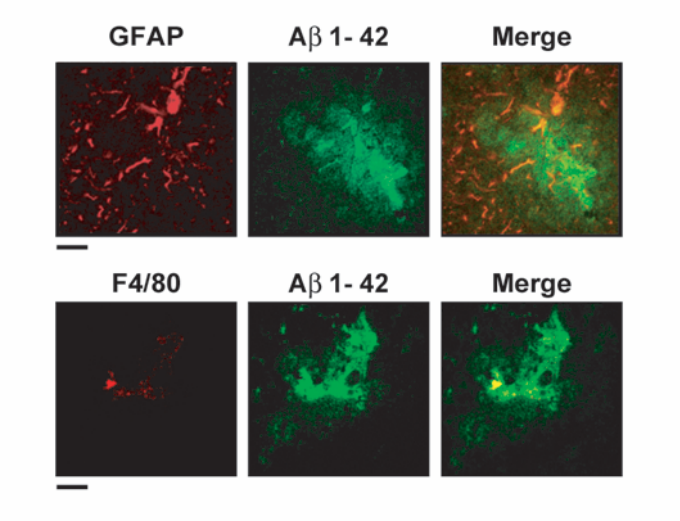

Figure S8. Localization of activated microglia and astrocytes evaluated by confocal microscopy. Representative images of GFAP or F4/80 (red) and Aβ (green) immunofluorescence from SREBP-2 transgenic brain. Merged images provide evidence about the co-localization of glia cells with amyloid deposition (yellow). Scale bar: 20 μm.

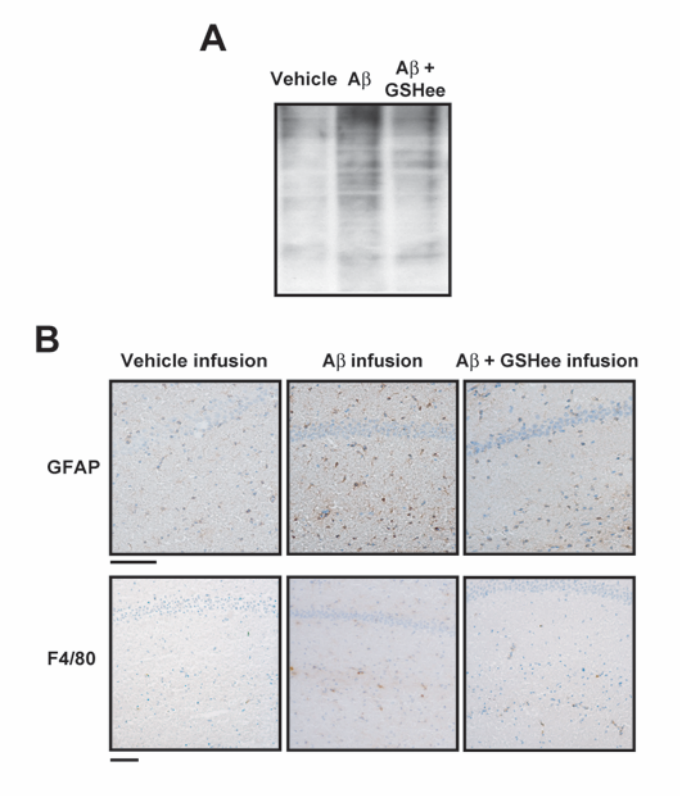

Figure S9. Protective effect of GSH ethyl ester co-infusion in the oxidative stress and neuroinflammation shown by the Aβ-infused SREBP-2 transgenic mice. SREBP-2 transgenic mice were subjected to continuous intracerebroventricular infusion of vehicle or human Aβ1-42 solution (1.2 μg / day) with or without GSH ethyl ester (GSHee) (0.6 mg / day) for 28 days. (n = 6). (**A**) Representative immunoblotting showing presence of carbonyl proteins in TgSREBP-2 brains following vehicle or Aβ infusion with or without GSHee. (**B**) Activation of microglia and astrocytes analyzed by F4/80 and GFAP immunostaining, respectively. Shown are representative photomicrographs of F4/80 and GFAP immunoreactivity as indicated, in hippocampal regions of infused SREBP-2 transgenic mice. Scale bar: 50 μm.

SUPPLEMENTAL METHODS

In vivo GSH depletion. Preweaning rats (14-16 days of age) were given two daily i.p. doses of L-buthionine (S, R)-sulfoximine (BSO) (3 mmol / kg) for 3 days. Controls were injected with an equivalent volume of saline. Isolation of brain mitochondria and GSH measurements were done 2 h after the last BSO injection (Jain et al., 1991).

Measurement of hydrogen peroxide production and lipid peroxidation. Hydrogen peroxide generation was determined spectrofluorometrically using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, 2 μM; Molecular Probes). The fluorescent probe was added in the incubation buffer and at the indicated time fluorescence was recorded at 529 nm for emission and 503 nm for excitation. Lipid peroxidation in mitochondria was measured by quenching of fluorescence of *cis*-parinaric acid (5 μg/ml; Molecular Probes) at 318 nm for excitation and 410 nm for emission. In total brain homogenate lipid peroxidation was assessed by the production of malondialdehyde (MDA) using the thiobarbituric acid (TBA) method. Briefly, 0.4 ml of 0.6% TBA and 1.2 ml of 1% ortophosphoric acid were added to 100 μl of brain homogenate and boiled for 45 minutes. After cooling, 1.6 ml of 1-butanol was added, samples were mixed and centrifuged at 1,200 rpm for 10 min. The supernatant was used to determine concentrations of MDA by measuring the OD at 535 nm. Results were expressed as percentage of untreated controls.

Cell death. The incidence of cell death after Aβ treatment, was assayed either by propidium iodide labelling (0.5 μg / ml) and assessment with a Becton-Dickinson FACScan flow cytometer in FL3 or by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 0.5 mg / ml) conversion monitored at 540nm. Morphological changes in the nuclei of cells undergoing apoptosis were determined by staining with the DNA binding fluorochrome Hoechst 33258 (10 μM, Molecular

Probes) for 10 min. Afterwards, cells were fixed with 3.7% paraformaldehyde and nuclei were visualized using an Olympus IX70 inverted fluorescence microscope. Neuronal damage following the *in vivo* Aβ infusion was analyzed in frozen sections (10 µm) from –1.2 mm through –2.4 mm from Bregma. To detect DNA fragmentation, terminal deoxynucleotidyl transferase-mediated uridine 5′-triphosphate-biotin nick end-labeling (TUNEL assay) was performed according to the manufacturer recommendations (In Situ Cell Death Detection Kit, TUNEL POD, Roche Applied Science). To analyze neuronal degeneration, sections were stained with Fluoro-Jade B (0.0004 %, Chemicon) (Schmued et al., 1997). The tissue was then examined with a Zeiss Axioplan epifluorescent microscope with blue (450-490 nm) excitation light.

Real-time RT-PCR. Total RNA was isolated from brain with the TRIzol reagent (Invitrogen). Real-time PCR was performed using the iScript™ One-Step RT-PCR Kit with SYBR® Green (Bio-Rad) following the manufacturer's protocol. Briefly, 20 ng of total RNA, 600 µmol/l of primers, and 12.5 µl of 2X Reaction Mix were incubated in 25 µl at 50 °C for 10 min and 95 °C for 5 min, followed by 45 cycles of 95 °C for 10 s, 56 °C for 30 s, and 72 ºC for 30 s. Each reaction was run in duplicate and the threshold (C_T) values for each mRNA were subtracted from that of 18S mRNA, averaged and converted from log-linear to linear term. The primers used were: TNF forward 5'-CTGAACTTCGGGGTGATCGGT -3'; TNF reverse 5'-ACGTGGGCTACAGGCTTG TCA -3'; IL-1B forward 5'-GAGCTGAAAGCTCTCCACCTC-3'; IL-1B reverse 5'-CTTTCCTTTGAGGCCCAAGGC-3'; 18S forward 5'-GTAACCCGTTGAACCCCAT T -3'; 18S reverse 5'-CCATCCAATCGGTAGTAGCG -3'; SREBP-2 forward 5'-TATCATTGAGAAGCGGTACCGG-3', reverse 5'-CTGATTTGCCAGCTTCAGCAC C-3'.

Western blot analysis. Samples (20-30 μg of protein / lane) were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Blots were probed with mouse monoclonal anti-Aβ 6E10 (1:2000; Sigma), mouse monoclonal anti-cytochrome c (1:1,000, clone 7H8.2C12, PharMingen), rabbit polyclonal anti-smac/DIABLO (1:1,000, Calbiochem), rabbit polyclonal anti-Rab5A (1:200, Santa Cruz Biotech.), mouse monoclonal anti- Na^+K^+-ATPase α1 (1:200, Santa Cruz Biotech.), rabbit anti-synaptophysin (1:10,000, Abcam), rabbit polyclonal anti-PERK (1:200, Santa Cruz Biotech.), and monoclonal anti-β-actin (1:25,000, Sigma). After 1 h incubation, bound antibodies were visualized using horseradish peroxidase-coupled secondary antibodies and ECL developing kit (Amersham Biosciences). The carbonyl protein levels were analyzed using the Oxyblot^{tm} protein oxidation detection kit (Chemicon) following the manufacturer's instructions. Briefly, the carbonyl groups in the protein side chains were derivatized by reaction with 2,4-dinitrophenylhydrazine (DNPH) and the DNP-derivatized protein samples were analyzed by western blotting.

SUPPLEMENTAL REFERENCES

Jain A, Mårtensson J, Stole E, Auld PA, Meister A (1991) Glutathione deficiency leads to mitochondrial damage in brain. Proc Natl Acad Sci USA 88:1913-1917.

Schmued LC, Albertson C, Slikker W Jr (1997) Fluoro-Jade: a novel fluorochrome for the sensitive and reliable histochemical localization of neuronal degeneration. Brain Res 751: 37-46.