TABLE OF CONTENT

SUPPLEMENTARY MATERIALS AND METHODS

Cell Viability assay (MTT)

SH-SY5Y-wtAPP or HEK-swAPP cells were seeded in 96-well plates in DMEM/F12 medium supplemented with 0.2% fetal bovine serum (FBS) at a density of 10^4 cells/100μL/well. Cells were incubated for 24 h with increasing concentrations SIN-1. After replacement of the medium with new DMEM/F12 supplemented with 0.2% FBS, cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. Briefly, 11 μL of MTT stock solution (5 mg/mL) were added and after 2 h the reaction was stopped with 120 μL of DMSO. MTT reduction was determined in a plate reader spectrophotometer at 540 and 650 nm. Control cells were taken as 100%.

Human samples

Human brain tissue sections and samples were supplied by Unidad de Neuropatología y Banco de Cerebros (Fundación Hospital Alcorcón), Madrid, Spain. The procedure was approved by the ethics committee of the Institut Municipal d'Investigacions Mèdiques-Universitat Pompeu Fabra, Barcelona, Spain. Samples used in the biochemical studies were from 4 control individuals and 4 AD patients.

Cell-free γ-secretase assays and AICD production

HEK cells were homogenizedin Buffer A (20mM pipes pH7, 140mM KCl, 0.25M sucrose, 5mM EGTA) with complete protease inhibitors (Roche Applied Science) and the microsomal membrane fraction was obtained by ultracentrifugation at 55.000 rpm at 4°C. Cerebral cortex of SOD2 +/- or wt mice, was homogenized in PKM-sucrose buffer (0.5 M sucrose, 100mM Potassium phosphate; 5 mM MgCl2; 3 mM KCl ; pH 6.5) with complete protease inhibitors

(Roche Applied Science) and centrifuged first at 2500 rpm and then twice 10' at 8000 rpm at 4°C. Finally the supernatant was ultracentrifugated at 55.000 rpm at 4°C for 1h to pellet the microsomal fraction.

Cell-free assays were performed as described by Kakuda and colleagues (Kakuda et al, 2006) with some minor modifications. Briefly, microsomal membrane fractions solubilized in Buffer B (50 mM pipes, pH7, 0.25M sucrose, 1mM EGTA) containing 1% CHAPSO were mixed with recombinant APP-C99-3×FLAG substrate (0.5 μM final concentration), 0.0125% phosphatidylethanolamine, 0.1% phosphatidylcholine, and 2.5% DMSO. Reactions were incubated at 37 °C for 4h or 12h for HEK-derived or brain-derived microsomal fractions respectively. Aβ species produced during the reaction were measured by Elisa and the levels were normalized to γ-secretase complex present in the *in vitro* assay, which was estimated from the PS1- CTF levels.

For kinetic studies, previous to solubilization, microsomal fractions were incubated for 24h at 25°C in the presence or the absence of 10 μM of the nitrating reagent SIN-1. After protein extraction with detergent, kinetic experiments were carried out using increasing concentrations of the purified APP-C99-3xFlag as substrate. After 5h of reaction, AICD was determined after SDS-PAGE/western blot by blotting with the primary antibody against FlagM2 (Sigma-Aldrich) at 1:4000 dilution in 1% BSA and Goat anti-mouse antibody labeled with green fluorescence (1:5000) as a secondary antibody. Odyssey was used for fluorescence determination at 800 nm and posterior densitometry. Kinetic constants were estimated by nonlinear curve-fitting using Graphpad Prism 4 software.

Endogenous AICD was produced *in vitro* from membrane preparations of HEK-swAPP cells as described (Guardia-Laguarta et al, 2009). Briefly, cell lysates were resuspended in assay buffer (150 mM sodium citrate pH 6.4 + 1 x protease inhibitors), then treated with vehicle only, or with different concentrations of SIN-1 and incubated at 37 ºC for 2 h. As a negative

control we incubated cells on ice. Cells were centrifuged 30 min at 16,000g, and the supernatant was transferred to a new tube. The samples were electrophoresed in 5–16% Tris-Tricine gels, transferred to 0.2 μm nitrocellulose membranes, and detected by immunoblotting with a rabbit anti-APP C-terminal antibody (Sigma).

Rescue of SOD2 knockdown in primary neurons with uric acid

Freshly obtained rat hippocampal neurons were transfected in suspension using nucleofection (Amaxa) without or with a shRNA-EGFP construct containing the iRNA sequence 5'-CCACATATGTGTAAGCATA-3' against the ratSod2 mRNA. Transfected neurons were seeded onto poly-L-Lysine coated plates. After 120 minutes, medium was replaced by MEM containing B27 supplement (MEM-B27) alone, or containing 100 μM uric acid.

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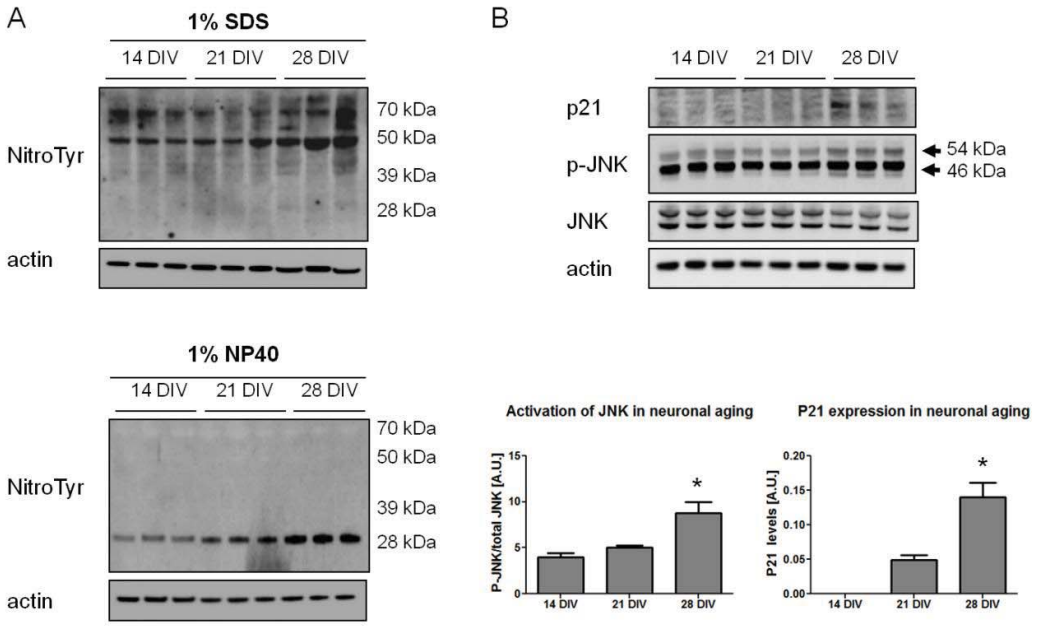
Kakuda N, Funamoto S, Yagishita S, Takami M, Osawa S, Dohmae N, Ihara Y (2006) Equimolar production of amyloid beta-protein and amyloid precursor protein intracellular domain from beta-carboxyl-terminal fragment by gamma-secretase J Biol Chem. 281; 14776-86.

Uemura K, Lill CM, Li X, Peters JA, Ivanov A, Fan Z, DeStrooper B, Bacskai BJ, Hyman BT, Berezovska O (2009) Allosteric modulation of PS1/gamma-secretase conformation correlates with amyloid β(42/40) ratio. PLoS One 4, e7893.

SUPPORTING INFORMATION FIGURE S1

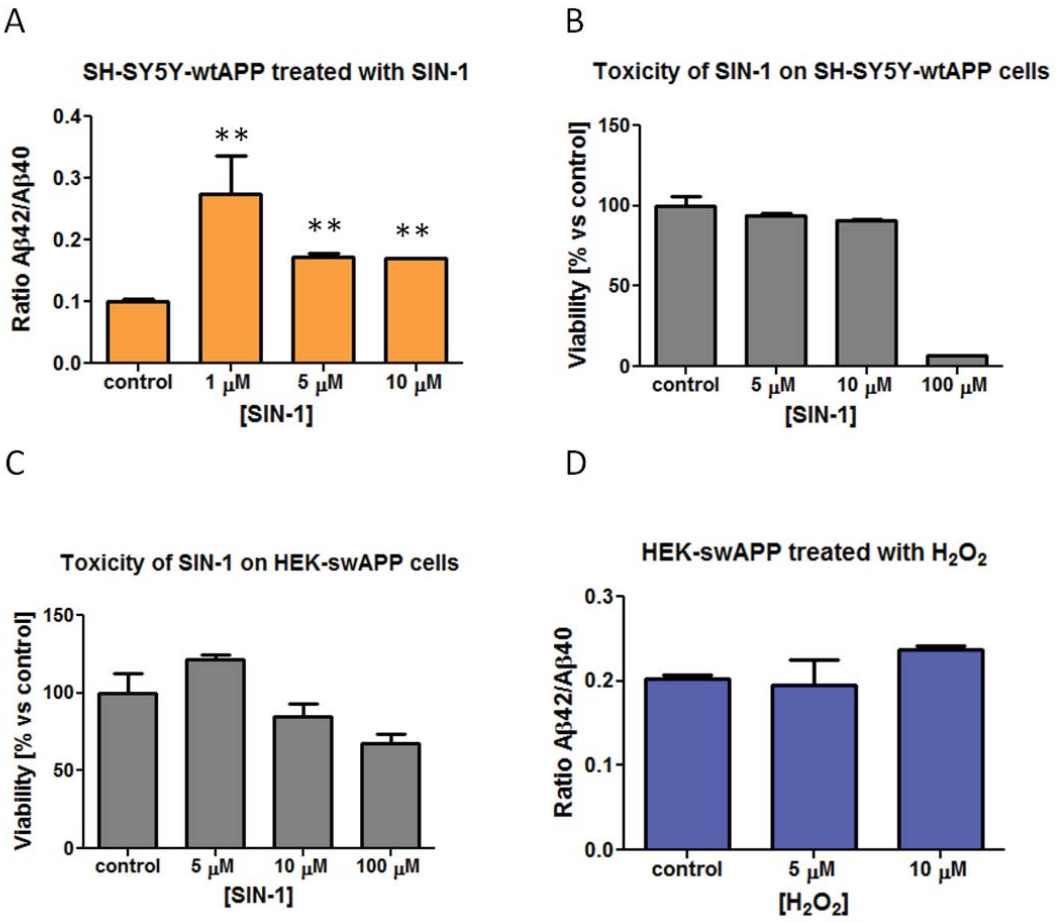


Supporting information Figure S1: Increase of total nitrotyrosination and other age-associated markers during neuronal aging in vitro.

A. Protein from 14, 21 or 28 DIV neuronal lysates was extracted with 1% NP40 and the remaining pelled (resistant proteins) with1 % SDS and run in a 12% Tris-glycine gel. The membrane was probed against a monoclonal anti-nitrotyrosine antibody. Only one band was observed in the NP40 extract but several bands were observed when proteins were solubilized in SDS, showing that nitrated proteins have certain resistance to solubilization. The sum of the signal from both membranes obtained by densitometry and normalized by actin was used to obtain Fig. 3B.

B. Validation of age-associated markers in our system of neuronal aging *in vitro*. After running 20 µg of total protein lysate in a Bis-Tris gel, it can be observed a dramatic increase of the P21 protein levels and phosphorylation of JNK in 28 DIV neurons vs 21 DIV. The data comes from 3 independent aging experiments and is presented in Arbritary Units [A.U.] (*$p<0.05$)..

SUPPORTING INFORMATION FIGURE S2



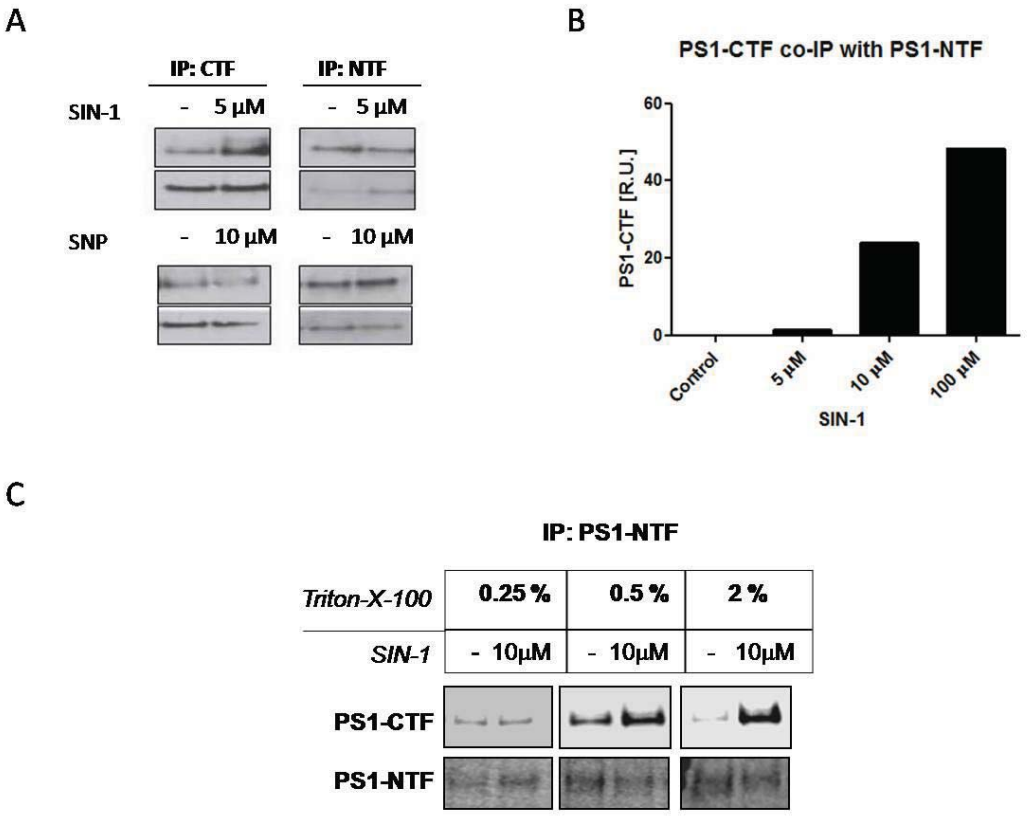
Supporting information Figure S2: Peroxynitrite increases $A\beta_{42}$ in vitro

A. SH-SY5Y-wtAPP cells treated for 24h with increasing concentrations of SIN-1. The $A\beta_{40}$ and $A\beta_{42}$ secreted into the media were determined by Elisa and the ratio calculated. As it is observed, the ratio is highly increased after just 1μM SIN-1 treatment. The experiment was repeated 3 times (*$p<0.05$, *$p<0.01$).

B,C. The toxicity of SIN-1 on SH-SY5Y-wtAPP cells and HEK-swAPP after 24h of exposure was analyzed by at different concentrations of the drug and by MTT viability test. The reduction of MTT was read in a plate reader spectrophotometer at 540 nm for living cells and 650 nm for background. Concentrations of SIN-1 ranging from 1 to 10 μM were chosen to carry out the experiments due to their subtoxic effect (same viability than untreated cells).

D. In order to demonstrate the specific effect of nitrosative stress on the $A\beta_{42}/A\beta_{40}$ ratio,HEK-swAPP cells were exposed for 24h to 5 μM and 10 μM H_2O_2, a general cell oxidant. The $A\beta_{40}$ and $A\beta_{42}$ secreted into the media were determined by Elisa. No differences of the ratio were observed under this experimental conditions.

SUPPORTING INFORMATION FIGURE S3



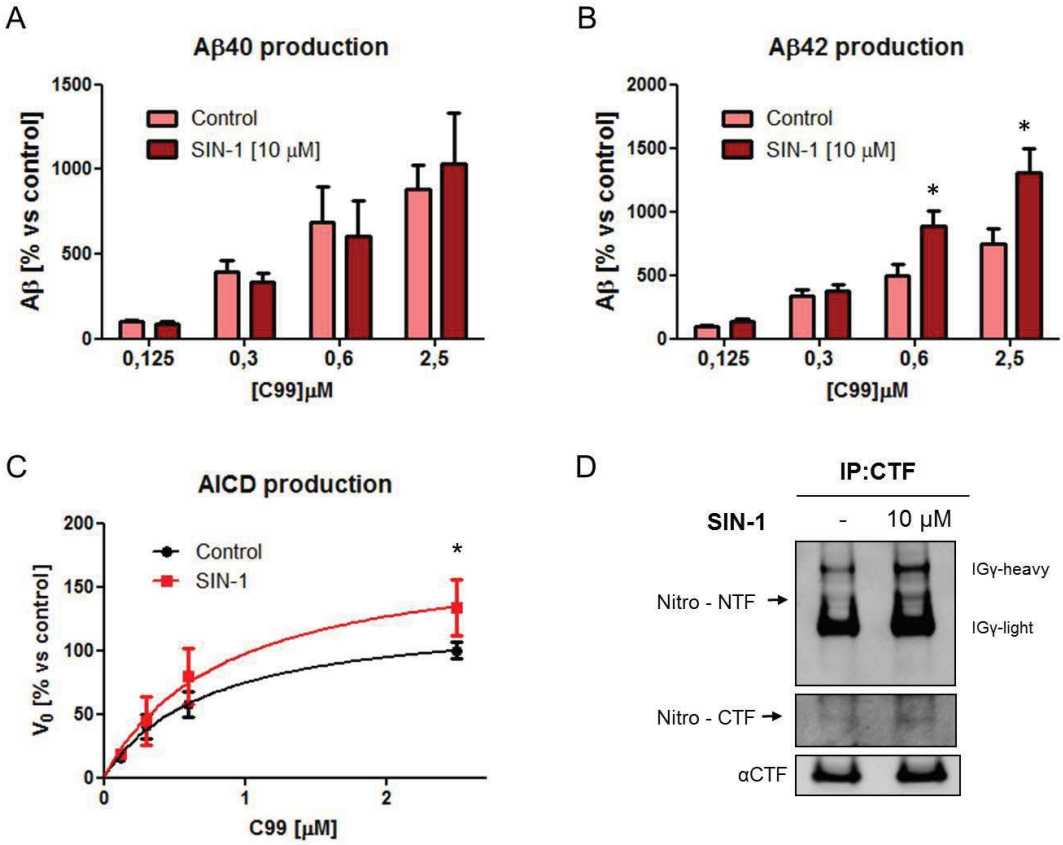
Supporting information Figure S3: Strong co-immunoprecipitation of presenilin fragments induced by nitrosative stress.

A. HEK-swAPP cells were exposed for 24h to SIN-1 before lysis in 0.5% Triton-X-100 and immunoprecipitation of PS1-CTF or PS1-NTF with monoclonal antibodies. The amount of PS1-NTF co-immunoprecipitated together with PS1-CTF and viceversa was determined by Western blot. The same experiment was performed with a donator of nitric oxide (SNP) to rule out the contribution of small amounts of this gas released by SIN-1.

B. Quantification of a western blot by densitometry of the amount of PS1-CTF pulled down when PS1-NTF is immunoprecipitated from 0.5% Triton-X-100 containing lysates from HEK-swAPP cells treated with increasing concentrations of SIN-1.

C. Co-immunoprecipitation of PS1-NTF and PS1-CTF from HEK-swAPP cells treated with or without 10 µM SIN-1 for 24h and using increasing concentrations of triton-X-100 detergent in the immunoprecipitation reaction. PS1-NTF was immunoprecipitated with a specific monoclonal antibody and the amount of PS1-CTF fragment pulled down was detected with a monoclonal antibody.

SUPPORTING INFORMATION FIGURE S4



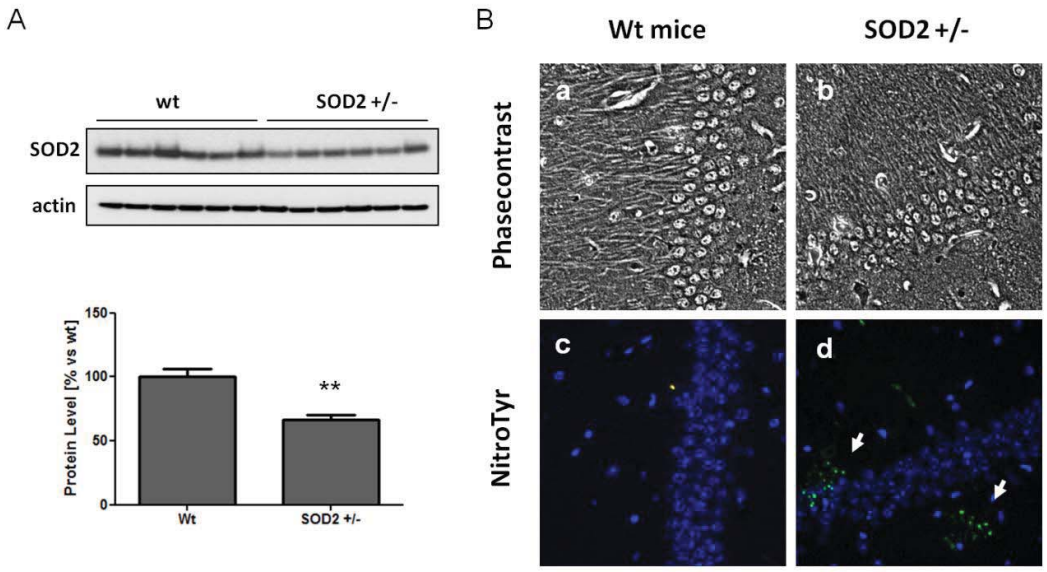
Supporting information Figure S4: study of the kinetic parameters for the nitrated γ-secretase complex.

A, B. $A\beta_{40}$ and $A\beta_{42}$ levels determined by ELISA from cell-free assays using 1%-CHAPSO solubilized microsomal fractions from HEK cells as source of γ-secretase. Previous to solubilization, microsomal fractions were incubated for 24h at 25°C in the presence or the absence of 10 μM of the nitrating reagent SIN-1. The kinetic studies were carried out by giving increasing concentrations of the APP-C99-3xFlag substrate. 3 independent reactions were done per condition. $A\beta_{42}$ levels start to show a significant increase at 0.6 μM of substrate (* $p<0.05$), while $A\beta_{40}$ levels stay unchanged

C. Michaelis-Menten curves for AICD of the reactions carried out with non-nitrated and nitrated γ-secretase. AICD levels were calculated by band densitometry of a 12% SDS-PAGE/western blot and posterior staining with an anti-flagM2 antibody. The initial velocity for each substrate concentration (V_0) was determined by dividing the relative amount of AICD (compared to the AICD levels produced by lowest substrate concentration) by the total time of the reaction (5h). At the maximum substrate concentration there is a significant increase of the AICD production (*$p<0.05$).

D. After the cell-free assays, γ-secretase was pulled down with a monoclonal antibody against PS1-CTF and run in a 4-12% SDS-PAGE gel. After western blot, the membrane was probed against nitration with a monoclonal anti-nitrotyrosine antibody. Presenilin was nitrated in those reactions where γ-secretase was obtained from microsomal fractions exposed to 10 μM SIN-1.

SUPPORTING INFORMATION FIGURE S5



Supporting information Figure S5: decreased SOD2 expression induces general nitrotyrosination.

A. The expression of SOD2 in the cerebral cortex of 18 months wt and SOD +/- mice was determined by Western blot and a monoclonal antibody. Actin was used as a loading control. The quantification by densitometry of the blot shows a 50% reduction of SOD2 in SOD2 +/- mice after normalization by actin (**$p<0.01$).

B. Immunofluorescence staining of the parenchyma of wt (a, b) and SOD2 +/- (c, d) mice shows the presence of widespread nitrotyrosination (green) in knockout mice. Nuclei are stained with DAPI (blue).

TABLE S1

	Control	St Error	SIN-1	St Error
Vmax [%]	**100**	8	**147.4**	20.7
Km [μM]	**0.7293**	0.2	**0.8470**	0.4
Kcat/Vmax [%]	**100**	19.4	**113**	37.6

TABLE S1: kinetic parameters of the in vitro γ-secretase reaction. The Km and the Vmax were calculated from the *in vitro* γ-secretase reactions using a non-modified or a nitrated enzyme. The equation V = (Vmax * [S])/(Km + [S]) was used to calculate Km and Vmax values for the different enzymes, where V was experimental determined using a range of substrate concentrations [S].