

# **Posttranslational Nitro-Glycative Modifications of Albumin in Alzheimer's Disease: Implications in Cytotoxicity and Amyloid $\beta$ -Peptide Aggregation**

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**Abstract.** Glycation and nitrotyrosination are pathological posttranslational modifications that make proteins prone to losing their physiological properties. Since both modifications are increased in Alzheimer's disease (AD) due to amyloid  $\beta$ -peptide ( $A\beta$ ) accumulation, we have studied their effect on albumin, the most abundant protein in cerebrospinal fluid and blood. Brain and plasmatic levels of glycated and nitrated albumin were significantly higher in AD patients than in controls. In vitro turbidometry and electron microscopy analyses demonstrated that glycation and nitrotyrosination promote changes in albumin structure and biochemical properties. Glycated albumin was more resistant to proteolysis and less uptaken by hepatoma cells. Glycated albumin also reduced the osmolarity expected for a solution containing native albumin. Both glycation and nitrotyrosination turned albumin cytotoxic in a cell type-dependent manner for cerebral and vascular cells. Finally, of particular relevance to AD, these modified albumins were significantly less effective in avoiding  $A\beta$  aggregation

than native albumin. In summary, nitrotyrosination and especially glycation alter albumin structural and biochemical properties and these modifications might contribute for the progression of AD.

Keywords: Albumin; Alzheimer's disease; amyloid; glycation; nitrotyrosination; oxidative stress.

## **INTRODUCTION**

Alzheimer's disease (AD) is characterized by amyloid  $\beta$ -peptide ( $A\beta$ ) aggregates forming senile plaques in the brain parenchyma [1] and vascular amyloid deposits in brain vessels [2].  $A\beta$  aggregation produce oligomers and fibrils [3,4] that generate reactive oxygen species (ROS) [5]. These ROS due to their gaseous nature can diffuse into the surrounding tissues to neuron and vessels where its deleterious effects are amplified by the intracellular free radical cascades. In the downstream of the cellular  $A\beta$  effects there is a high production of superoxide anion from mitochondria that reacts with nitric oxide (NO), mainly produced by glial and endothelial cells but also by neurons [6,7,8], to render peroxynitrite ( $ONOO^-$ ) [9]. This peroxynitrite nitrates the tyrosine residues in proteins, a process termed nitrotyrosination, which dramatically affects their function [10,11]. Consistently protein nitrotyrosination has previously been detected in brain proteins in AD patients [12,13,14] and free 3-nitrotyrosine (NT-3) in serum from AD patients [15].

The intracellular effects of  $A\beta$  also includes high methylglyoxal (MG) production from abnormal glycolysis [16]. MG reacts with proteins to glycate them but protein glycation can be also indirectly produced in a pro-oxidant environment by advanced glycation end products through the Maillard reaction, in both cases facilitating the pathological crosslink

between glycated proteins [17]. Consequently high protein glycation has been reported in AD brains [18,19,20].

Albumin is the most abundant protein in both blood and cerebrospinal fluid (CSF). Plasmatic albumin is produced by the liver and a small fraction of plasmatic albumin enters the brain [21] although most of the albumin found there is produced endogenously by the glia [22]. The functions of albumin are the regulation of blood and CSF volume by maintaining the oncotic pressure, and the transport of different molecules like hormones, free fatty acids, ions and some drugs. Albumin can also buffer oxidative damage due to the presence of a free cysteine that is not forming disulfide bridges [23]. Interestingly, albumin has been previously reported to bind A $\beta$  [24] that could be favoring amyloidogenic clearance by the liver. In addition, albumin has a potential role as a molecular chaperone, preventing the misfolding and aggregation of proteins [25] and specifically inhibiting A $\beta$  fibril formation [26].

Albumin has 19 tyrosines susceptible to modification by peroxynitrite [27] as well as a high number of lysines and arginines, which are also quite prone to glycation [28]. Therefore albumin can be affected by the increased nitro-glycative stress present in AD. This study analyses the albumin modifications caused by nitro-glycative stress and their effects in its functions, its impact on different cell types present at both sides of the blood brain barrier (BBB) and the effect in the inhibition of A $\beta$  aggregation.

## **MATERIALS AND METHODS**

### *Cell lines*

Human umbilical vein endothelial cell line (HUVEC) was cultured in M-199 medium supplemented with 10% fetal bovine serum (FBS; Sigma), 2 mM of L-Glutamine (Sigma), 100 UI/mL of penicillin and 100  $\mu$ g/mL of streptomycin (Sigma). Human aortic vascular

smooth muscle cell line (HA-VSMC) were cultured with MCDB 131 medium (Invitrogen), supplemented with 5% FBS, 0.5 ng/mL of epidermal growth factor (Sigma), 2 ng/mL of basic fibroblast growth factor (bFGF; Invitrogen), 5  $\mu$ g/mL of insulin (Invitrogen), 2 mM of L-Glutamine and antibiotics. Mouse hippocampal and cortical neurons were isolated from 18-day-old CB1 embryos. Pregnant mice were killed by CO<sub>2</sub> inhalation in accordance with the directives of the Council of the European Communities N. 86/609/CEE. Embryos were rendered hypothermic and decapitated. Hippocampi and cortex were aseptically dissected and trypsinized for 17 min. After centrifugation (1 min; thrice) and mechanical dissociation, cells were seeded in phenol red-free DMEM (Sigma) plus 10% horse serum into 1% poly-L-Lysine coated plates. After 120 min, medium was removed and neurobasal medium was added containing 1% B27 supplement (Gibco BRL) plus antibiotics. On day 3 of culture, neurons were treated with 2  $\mu$ M 1- $\beta$ -D-arabinofuranosylcytosine (AraC; Sigma) for 24 h to reduce the number of proliferating non-neuronal cells. Primary neuronal cultures were used at day 10. Murine astrocytes were isolated from 18-day-old CB1 embryos. Mice were sacrificed as indicated above. Brain were aseptically dissected and trypsinized for 17 min. After centrifugations (5 min; thrice), cells were seeded in DMEM (Sigma) plus 10% FBS. Primary astrocyte cultures were used at day 15. The human hepatocellular carcinoma cell line (HepG2) was cultured in DMEM supplemented with 10% FBS and antibiotics.

#### *Human CSF samples*

A total of 20 CSF samples obtained at the Hospital de la Santa Creu i Sant Pau (Barcelona, Spain) were included in this study. The procedure was approved by the ethics committee of the Hospital de la Santa Creu i Sant Pau. CSF samples were from 10 cognitively normal controls ( $66.9 \pm 2.2$  years) and from 10 AD patients ( $66.6 \pm 2.5$  years) using previously described procedures [29].

### *Human brain samples*

Human brain tissue samples were supplied by the Neurological Tissue Bank (Serveis Científico-Tècnics, Hospital Clínic, Universitat de Barcelona), the Department of Pathology (Hospital del Mar, Barcelona) and the Neuropathology Unit and Brain Bank (Fundación Hospital Alcorcón, Madrid). The procedure was approved by the ethics committee of the Institut Municipal d'Investigacions Mèdiques-Universitat Pompeu Fabra. Brain samples were obtained from the frontal cortex of 13 healthy aged male and female individuals (mean  $\pm$  SEM of  $71\pm 2$  years) and 18 male and female AD patients at stage IV-VI (mean  $\pm$  SEM of  $78\pm 3$  years). The samples were lysated with a cocktail containing NP40 lysis buffer (150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 0.05% aprotinin, 1 mM dithiothreitol) and protease inhibitors (Complete mini-EDTA free) from Roche Diagnostics GmbH. Lysates were mechanically disaggregated using plastic micropestles (Eppendorf) and 1 mL syringes and the obtained brain tissue solution was centrifuged at 12,500 rpm for 10 min. The supernatant (SN) was quantified by the Bicinchoninic Acid assay (BCA; Pierce® BCA Protein assay kit, Thermo Scientific).

### *Human plasma samples*

Plasma samples were obtained from 14 healthy elderly males and females ( $70\pm 1$  years) participating in a population-based survey [30], and from 19 male and female AD patients ( $74\pm 2$  years) diagnosed with mild to moderate AD (NINCDS-ADRDA criterion) and an Mini Mental State Examination score between 20 and 24. All the procedures were approved by the ethics committee of the Institut Municipal d'Investigacions Mèdiques-

Universitat Pompeu Fabra. All individuals, close relatives or legal representatives signed the corresponding informed consent before participation. Plasma samples were stored at -80°C.

#### *Albumin nitrotyrosination and glycation*

Unless indicated otherwise, 1.25 µg/µL of human albumin (Grifols) was incubated with increasing concentrations (100 µM to 50 mM) of the peroxynitrite donor 3-Morpholinosydnonimine hydrochloride (SIN-1; Sigma) and the glycating agent MG (CosmoBio Co., LTD) in a phosphate buffer solution (PBS). The solutions were stirred (300 rpm) for 3 hr at room temperature (RT). After treatment, the albumin was filtrated by centrifugation at 11,000 rpm for 10 min in 30 KDa desalting filters (Vivacon 500 and Ultrafree-MC microcentrifuge filters, Sigma). Protein was recovered by revert spinning at 2,500 rpm for 2.5 min and quantified using the BCA.

#### *Albumin immunoprecipitation (IP)*

100 µL of CSF and 200 µL of plasma sample and 400 µg of homogenized brain were used for IP. Samples were pre-incubated for 30 min at 4°C with G protein (GE Healthcare UK Limited) previously washed with PBS. This step is required to avoid unspecific G protein binding. The samples were then centrifuged at 10,000 rpm for 10 min. The SN was incubated overnight (o.n.) with 5 µg of anti-albumin antibody (Ab; Acris Antibodies and Cell Signalling). Following the addition of sepharose-immobilized G protein (GE Healthcare UK limited), samples were shaken for 2 hr at RT. Albumin was precipitated by centrifugation at 10,000 rpm for 10 min and washed trice. 60 µL of loading buffer (x5) were added to the pellet and the mix was boiled for 6 min at 100 °C. The boiled samples were centrifuged at 10,000 rpm for 10 min and 30 µL of this SN was resolved in 8% polyacrylamide gel. Gels were transferred to polyvinylidene fluoride membranes (Immobilon-P transfer membranes)

and the nitrotyrosination and glycation detection was performed as described below. A stripping solution was applied to membranes to wash the previous Abs (three washes with 0.05% Tween- PBS; incubation for 30 min at 80 °C with 0.2 M Glycine at pH 2.5; and three more washes with Tween-PBS). Membranes were then incubated with anti-albumin Ab in a 1:1000 dilution with 0.05% Tween Tris Buffer Saline (TTBS)-5% milk o.n. at 4 °C and they were developed for MG and NT-3 detection as explained below. Band quantification was performed using Quantity One from BioRad and the MG and NT-3 detection was normalized by the amount of albumin in each sample.

#### *Nitrotyrosination and glycation detection by WB*

18.75 µg of native, glycated or nitrated albumin were resolved in 8% polyacrylamide gels. Gels were transferred to polyvinylidene fluoride membranes. Membranes were boiled for 5 min before the glycation study and then blocked with 5% milk-TTBS- solution for 1 hr. Nitration and glycation were detected by incubating the membranes with 1:1000 mouse NT-3 Ab (Cayman and Merck Millipore) and 1:1000 mouse anti-argpyrimidine Ab (CosmoBio Co, LTD) o.n. at 4°C, followed by three washes with TTBS for 5 min. Goat anti-mouse secondary Ab (1:2500, GE Healthcare UK limited) was used at RT for 1 hr. The membranes were washed thrice with TTBS for 5 min and developed with the SuperSignal West Femto Chemiluminescent Substrate (ThermoScientific) in the ChemiDoc MP imaging system (Bio-Rad). Blotting quantification was performed with the ImageJ (NIH) program.

#### *Quantification of A $\beta$ <sub>1-42</sub>, tau and p-tau*

CSF A $\beta$ <sub>1-42</sub>, tau and p-tau were measured by ELISA using commercial enzyme-linked immunosorbent assay kits for A $\beta$ <sub>1-42</sub> (Innotest<sup>TM</sup>  $\beta$ -Amyloid1-42, Innogenetics), Tau (Innotest<sup>TM</sup> hTAU Ag, Innogenetics), P-Tau181 (Innotest<sup>TM</sup> Phospho-Tau181P,



Innogenetics). These assays are based on a solid-phase sandwich ELISA using specific anti- $A\beta_{1-42}$ , anti-tau and anti-p-tau antibodies.

#### *Aggregation assays*

Turbidimetric assays were performed with 10  $\mu\text{g}/\mu\text{L}$  of albumin in PBS (control) and plus 25 mM SIN-1 for nitrative assays or with 25 mM MG for glycative assays. The assays were carried out in Nunc 96-well plates stirred (300 rpm) at RT for up to 24 hr. Absorbances were measured at 405 nm in the Tecan Infinite M200 spectrophotometer.

#### *Osmolarity assays*

30  $\mu\text{g}/\mu\text{L}$  of albumin were incubated with 100  $\mu\text{M}$ , 12.5 mM or 25 mM of SIN-1 or MG and stirred (300 rpm) at RT for 48 hr. Sample osmolarity was measured with an osmometer (Fiske One-ten osmometer).

#### *Atomic force microscopy (AFM) images*

1.25  $\mu\text{g}/\mu\text{L}$  albumin were treated with 25 mM SIN-1 or 25 mM MG for 3 days with continuous stirring (300 rpm) at 37°C. Sample solutions were vortexed for 2 min prior to the deposition of a 20- $\mu\text{l}$  drop on graphite substrates. After an adsorption time of 3 min the sample was washed once with 100  $\mu\text{l}$  of PBS. Measurements were performed on a Multimode 8 atomic force microscope attached to a Nanoscope V electronics unit (Bruker). The imaging mode was Peak Force tapping mode. Triangular-shaped, silicon nitride cantilevers with silicon oxide pyramids were used (SNL-10, nominal spring constant: 0.35 nN/nm, Bruker). Measurements were performed in liquid environment (PBS) at a scan rate of 1Hz.

### *Transmission electron microscopy images*

1.25  $\mu\text{g}/\mu\text{L}$  albumin were treated with 25 mM SIN-1 or MG for 4 weeks with continuous stirring (300 rpm) at RT. Nickel mesh grids were charged with ultraviolet light for 5 min and set on a drop of sample (with 0.625  $\mu\text{g}/\mu\text{L}$  of albumin) for 1 min. They were then washed three times with milliQ water (1 min in total). Finally the grid was set on a drop of 2% uranyl acetate solution for 1 min and dried. Samples were observed with a Jeol 1010 electron microscope.

### *Albumin degradation by trypsin*

Native, glyco- and nitro-Albumin (G-Alb and N-Alb) were incubated with 0.05% Trypsin-EDTA (Gibco BRL, Invitrogen) for 24 hr. A soy trypsin inhibitor (STI; Sigma) was used at 1 mg/mL to stop trypsin activity. Albumin samples were resolved in 8% non denaturing polyacrylamide gels. Protein detection used Coomassie protein staining techniques.

### *Albumin uptake by HepG2 cells*

HepG2 cells were seeded in 6-well plates at a density of  $7.5 \times 10^5$  cells/mL/well. Native albumin, G-Alb and N-Alb at 30  $\mu\text{g}/\mu\text{L}$  were then added. The medium was withdrawn after 24 hr and the albumin concentration was measured by BCA and Bradford protein quantification method (Bio-Rad protein assay).

### *Cell viability assays*

HUVEC cells, HAVSMC cells, primary cultures of murine hippocampal and cortical neurons and astrocytes were seeded in 96-well plates in their respective media at a density of  $10^4$  cells/100 $\mu\text{L}$ /well. Cells were incubated with PBS (controls), untreated, and filtrated

nitrotyrosinated, and glycated albumin at 1  $\mu\text{M}$  (0.068  $\mu\text{g}/\mu\text{L}$ , on the physiological concentrations in CSF). 500  $\mu\text{M}$  albumin (34.5  $\mu\text{g}/\mu\text{L}$ , on the physiological concentration in plasma) and the corresponding amount of nitrotyrosinated and glycated albumin were also tested in HUVEC cells since they are exposed to both brain parenchyma and blood. Cells were treated for 24 hr at 37°C. Cell viability was measured by methylthiazolyldiphenyl-tetrazolium bromide (MTT) reduction. Briefly, 10  $\mu\text{L}$  of MTT (Sigma) stock solution (5 mg/mL) were added and after 2 hr the reaction was stopped with 100  $\mu\text{L}$  of dymethylsulfoxide. MTT was determined in a plate reader spectrophotometer (FLUOstar optima, BMG labtech) at 540 and 650 nm. Control cells were taken as 100%.

#### *A $\beta_{40}$ aggregation assay by ThT*

ThT assay is based on the ThT property to bind  $\beta$  sheet rich structures, such as those amyloid aggregates, and display enhanced fluorescence and a characteristic red shift of its emission spectrum. Albumin, G-Alb and N-Alb were co-incubated with A $\beta_{1-40}$  (Anaspec) and ThT (Sigma-Aldrich) in a black, flat bottom 96-well plate for 21 h. A $\beta_{1-40}$  was co-incubated with albumin and modified albumin in a 1:2 molar ratio (corresponding to 0.125  $\mu\text{g}/\mu\text{L}$  of A $\beta_{1-40}$  and 3.35  $\mu\text{g}/\mu\text{L}$  of the native or modified albumin). The measurements were made each 5 min after orbital shaking mode exciting the ThT at 430 nm and reading the fluorescence emission at 470 nm with a plate reader spectrophotometer (FLUOstar optima, BMG labtech) .

#### *Statistical analysis*

Data are expressed as the mean  $\pm$  SEM of the values from the number of experiments as indicated in the corresponding figures. Data was statistically evaluated using Student's *t*-test and Pearson product-moment correlation coefficient.

## RESULTS

### *Albumin is glycosylated and nitrotyrosinated in brain and plasma samples from AD patients*

The presence of N-Alb and G-Alb in the CSF, plasma and brains of AD patients and aged non-demented controls is shown in Fig. 1. The studies of albumin glycation were performed with an argpyrimidine Ab since argpyrimidine is a main modification in albumin glycosylated by MG [31]. We found similar percentages of albumin glycation in the CSF of AD patients and controls ( $84.5 \pm 12.5$ ) but significantly higher in brain and plasma ( $150.6 \pm 19.3$  and  $120.5 \pm 8.7$  respectively;  $p < 0.05$  in both cases; Fig. 1A). Albumin nitrotyrosination also presented similar values in CSF from controls and AD patients ( $100 \pm 32.9$  and  $62.1 \pm 7.6$  respectively) but significantly higher in brain and plasma from AD patients ( $134.9 \pm 15.1$  and  $141 \pm 12.8$  respectively;  $p < 0.05$  and  $p < 0.01$ ; Fig. 1B).

### *Albumin nitrotyrosination in CSF from AD patients correlates with $A\beta_{1-42}$ levels*

The levels of  $A\beta_{1-42}$ , tau and p-tau were analyzed in CSF samples from cognitively normal subjects and AD patients (Fig. 2, S1 and S2). We found that there was no correlation between the levels of  $A\beta_{1-42}$  and glycosylated or nitrated albumin when total samples or just controls were analyzed (Fig. 2A-D). Interestingly, there was a high negative correlation between  $A\beta_{1-42}$  levels and nitrated albumin in the CSF from AD patients ( $r = -0.97$ ;  $p < 0.005$ ; Fig. 2F). This correlation was not found for glycosylated albumin (Fig. 2E).

The statistical analysis between tau and p-tau levels and glycosylated and nitrotyrosinated albumin (Fig. S1 and S2) did not show any correlation except for tau levels compared to nitrotyrosinated albumin in AD patients (Fig. S1F), where a positive correlation was obtained ( $r = 0.63$ ;  $p < 0.05$ ).

### *Glycation and nitrotyrosination induce albumin aggregation in vitro*

The ability of albumin to be glycosylated by MG, a glycosylating agent, and nitrated by SIN-1, a peroxynitrite donor, measured by western blot (WB) analysis, is shown in Fig. S3. Albumin glycosylation is augmented with increasing MG concentration up to 7.5 mM whereas higher doses of MG triggered an apparent decrease of albumin glycosylation (Fig. S3A). This effect at concentrations above 7.5 mM MG could be due to cross-linking of glycosylated albumin to form large aggregates unable to enter the polyacrylamide gels and, therefore, not be detected at the molecular weight of monomeric albumin. The glycosylation of albumin at lower  $\mu\text{M}$  concentrations is shown in the inset in Fig. S3A. Albumin nitration however is augmented with increasing doses of SIN-1 up to 12.5 mM when reached a plateau. A SIN-1 concentration of more than 12.5 mM did not produce greater nitration probably due to complete nitration of all the tyrosine at this concentration (Fig. S3B).

Micromolar concentrations of MG and peroxynitrite are found in plasma [32,33], although their tissue concentrations are claimed to be higher. We have carried out the experiments of the present work at 25 mM MG and 12.5 or 25 mM SIN-1 since the production of both harmful compounds is constant in AD and the long turnover of albumin (21 days) [34] makes this protein to be highly exposed to these compounds.

Glycation and nitrotyrosination have been previously reported to induce protein aggregation. Turbidimetric assays consistently showed aggregation of G-Alb and N-Alb compared to untreated albumin (Fig. 3A and B). The aggregation of N-Alb showed higher turbidimetry absorbance values than G-Alb. Not only are the aggregation kinetics between nitrated and glycosylated albumin different, but the appearance of the aggregates differ as well. Structural differences between untreated and glycosylated or nitrotyrosinated albumin aggregates were evident under AFM after 3 days of treatment (Fig. 3C and D). At that time the small

fibrillar forms of the untreated albumin have mostly disappeared in the glycated and nitrotyrosinated albumin, remaining amorphous aggregates bound to the graphite matrix. These amorphous aggregates grow along the time as we found by electron microscopy after 4 weeks of treatment (Fig. 3E and F). G-Alb aggregates showed globular structures different to those present in untreated or nitrated albumin aggregates. The turbidimetry pattern observed for G-Alb could be due to the formation of larger and more expanded aggregates as this would explain the results obtained by WB (Fig. S3) and electron microscopy. In addition, N-Alb aggregates were more condensed than untreated and glycated albumin aggregates

#### *Trypsin digestion and cell uptake of N-Alb and G-Alb*

Albumin turnover, taking approximately 21 days in humans [34] involves its degradation and uptake by liver cells, a process that may be affected by its nitration and glycation, how it was reported for MG-modified bovine serum albumin in proximal tubule epithelial cell [35]. The impairment of albumin digestion and/or uptake will mean a reduction of albumin production by the liver, and consequently it will affect the physiological functions of albumin. To address this point, an evaluation was made of the digestion of modified and native albumin. Untreated, N-Alb and G-Alb samples incubated with trypsin are shown in Fig. 4A. As expected, higher degradation (fragments <50 kD molecular weight) was observed in untreated albumin compared to N-Alb and G-Alb. Digestion of N-Alb produced less low molecular weight fragments. G-Alb was the most resistant to trypsin digestion. Next, an assessment was made of the cellular uptake of modified and native albumin by hepatoma cells (Fig. 4B). In cells incubated with native albumin, G-Alb and N-Alb in a serum-free medium, there was a significant reduction of G-Alb uptake ( $0.41 \pm 0.33 \mu\text{g}/\mu\text{L}$ ;  $p < 0.05$ ) by cells compared to untreated albumin ( $6.89 \pm 3.31 \mu\text{g}/\mu\text{L}$ ) whereas nitrotyrosinated albumin uptake was not affected ( $6.04 \pm 4.03 \mu\text{g}/\mu\text{L}$ ).

### *Albumin glycation reduces osmolarity*

Since nitrotyrosination and glycation produce albumin aggregates, these modifications were studied to determine whether they could also be affecting osmotic pressure by enhancing the binding of ions from the solution. It was previously reported that MG-modified bovine serum albumin had a higher affinity for  $\text{Cu}^{2+}$  and  $\text{Ca}^{2+}$  compared to the native [36]. Measurements were made of the osmolarity of solutions containing the same concentrations of untreated albumin, G-Alb and N-Alb (Fig. 5). Nitrotyrosination of albumin with SIN-1 did not affect osmolarity (Fig. 5B) whereas albumin glycation with increasing concentrations of MG (Fig. 5A) showed significant ( $p < 0.05$ ) reduction in osmolarity compared to control albumin. Since both modifications are coexisting in the same albumin pool, these data support that the pathophysiological effects of albumin modification on osmolarity control would be just due to albumin glycation. This reduction in osmolarity is probably due to trapping of the ions dissolved in the medium.

### *Effect of albumin nitrotyrosination and glycation on cell viability*

Albumin interacts directly with the neurons, endothelium, vascular smooth muscle and glial cells at both sides of the BBB [21,37,38,39]. The effect of albumin glycation and nitration on cell viability was tested using endothelial (HUVEC), vascular smooth muscle (HA-VSMC), primary cultures of murine hippocampal neurons, cortical neurons and astrocytes. The results for cells incubated with the physiological concentration of albumin in plasma (500  $\mu\text{M}$ ) and the albumin concentration range present in CSF (1  $\mu\text{M}$ ), G-Alb, and N-Alb are shown in Fig. 6A and Fig. 6B, respectively. Deleterious effects of G-Alb were observed in muscular cells, hippocampal neurons and cortical neurons ( $p < 0.05$ ) while the toxic effect of N-Alb is on endothelium ( $p < 0.005$ ) and smooth muscle cells ( $p < 0.01$ ).

Astrocytes were the most resistant cells to modified albumins. The controls for the equimolar MG and peroxynitrite are shown in the Fig. S4 and S5 respectively.

#### *Increased A $\beta$ binding to G-Alb and N-Alb*

The results of A $\beta$  binding experiments carried out with soluble and fibrillar A $\beta_{1-40}$  are shown in Fig. 7. The binding assay showed that G-Alb (Fig. 7A) and N-Alb (Fig. 7B) bind more A $\beta_{1-40}$  in soluble form than control albumin ( $p < 0.005$ ;  $p < 0.05$ ). Furthermore, G-Alb (Fig. 7B right panel) binds more A $\beta_{1-40}$  in aggregated form than control albumin ( $p < 0.05$ ).

#### *Albumin glycation and nitration impairs the inhibitory effect of albumin on A $\beta_{40}$ aggregation*

Plasmatic albumin binds A $\beta$  [40] and can inhibit its aggregation to form amyloid fibrils [28]. We have assayed the ability of native, glycosylated and nitrated albumin to inhibit A $\beta_{1-40}$  aggregation. Our results showed that albumin was able to abolish completely A $\beta_{1-40}$  aggregation (Fig. 8A, B; quantified in Fig. S2), as previously reported by [28], but both G-Alb (Fig. 8A) and N-Alb (Fig. 8B) reduced significantly the inhibition of A $\beta_{1-40}$  aggregation ( $p < 0.0001$  for Alb vs. G-Alb;  $p < 0.05$  for Alb vs. N-Alb).

## **DISCUSSION**

High nitro-oxidative stress is associated with the AD brain [5,41,42,43] and this could affect the proteins at both sides of the BBB because of the gaseous nature of ROS, the chain reactions induced by these species in surrounding tissues and the ultrafiltration of proteins from CSF to blood. Albumin is the most abundant protein in both blood and CSF, in addition to its physiological functions, albumin may also play a major role as a scavenger of ROS, favored by its high turnover with a life of 21 days [34]. This process involves its degradation and uptake by liver cells and may be affected by albumin nitration and glycation. However,



this ability might have consequences for albumin when the buffering threshold for oxidative stress is exceeded, as occurs in AD. This study focused on albumin nitrotyrosination and glycation, and how these modifications affect its properties. We found that albumin is significantly glycated and nitrated in brain and plasma from AD patients. Similar results regarding G-Alb have been published by Byun et al. [44]. In AD patients N-Alb from CSF correlates inversely with A $\beta$ <sub>1-42</sub> levels and positively with tau levels. It can be due to the A $\beta$ <sub>1-42</sub> aggregation in the brain parenchyma of AD patients, and the consequent neuronal damage produces tau release. The puzzling lack of differences in N-Alb levels in CSF between controls and AD patients can be the result of the rapid intracerebral aggregation of newly nitrated albumin impairing its ultrafiltration to CSF.

Both nitrotyrosination and glycation make albumin more prone to aggregation. In the case of nitrotyrosination, the interaction of nitrated tyrosines from several molecules of albumin-producing dityrosine bridges could lead to its aggregation [45]. Albumin glycation could also induce aggregation probably due to cross-linking between ketone groups from Schiff bases, the intermediate products after protein glycation [46]. In fact the apparent decrease at concentrations higher than 7.5 mM MG could be due to the cross-linking of glycated albumin forming large aggregates unable to enter the polyacrylamide gels and, therefore, not being detected at the molecular weight of monomeric albumin. Consequently an increased aggregation was obtained in the turbidimetric assay without reaching a plateau. The changes in modified albumin observed under the transmission electron microscopy showed denser aggregates in nitrotyrosinated albumin and more globular aggregates in glycated albumin than those observed in unaltered albumin. These results are consistent with increased albumin aggregation.

The data suggest that these stable aggregates of modified albumin are not easily broken down, and therefore additional, accessory proteins may be required for their

degradation [47,48]. G-Alb was the most resistant to trypsin digestion, and this is consistent with previous reports showing that protein glycation reduces their normal degradation [49,50]. Besides, as proteolysis of large aggregates is more difficult, modified albumin will be circulating for longer periods of time, affecting its turnover and reducing its protective role. Another consequence of albumin modification affects maintenance of osmotic pressure. Compared to native albumin, the presence of glycated, but not nitrotyrosinated albumin, significantly decreases the osmolarity of the solution it is dissolved in. The osmolarity is one of the most-well regulated physiological mechanism [51] and changes around 10% in the osmolarity might have deleterious consequences, especially for small brain vessels where it will favor plasma extravasation [52] and a lack in the homeostasis of the surrounding tissue.

We also found that G-Alb decrease the viability of vascular myocytes and neuronal cells. N-Alb affects mainly endothelium and smooth muscle cells, while astrocytes seem to better tolerate the presence of modified albumin. This difference may indicate that a particular albumin modification will mainly affect a particular set of cell types. Since both nitrative and glycative modifications are additive *in vivo*, their deleterious effects would affect to all the surrounding cells in brain. Another interesting observation is that albumin may act as a buffer for the nitro-oxidative and glycative stress since the presence of albumin reduced the toxic effect of equimolar concentrations of free SIN-1 and MG (Fig. S4 and S5). The reduction in cell viability could be due to a lack of the nutritive properties of albumin [53] or other mechanisms derived from its aggregated structure. Interestingly, nitrating and glycating agents (Fig. S4 and S5) are always more toxic to cells than the corresponding concentration of modified albumin, reinforcing the idea that albumin protects against these reactive species by buffering them [54]. Altogether, modified albumin could not buffer more pro-oxidant challenges in brain, and consequently, also could fail in protecting the brain against nitro-oxidative stress.

On the other hand, 95% of the plasmatic A $\beta$  is bound to albumin [24,40] allowing the hepatic clearance of A $\beta$ -albumin complex. We have found that the modified albumin binds more soluble and aggregated A $\beta$  than the native albumin. Albumin is known to inhibit A $\beta$  aggregation [55]. It traps A $\beta$  likely by hydrophobic interactions since it has hydrophobic pockets [56]. We speculate that glycation and nitration are decreasing the albumin inhibitory effect on A $\beta$  aggregation due to the higher amount of A $\beta$  bound after the modification of its structure. A similar mechanism has been proposed for the prions, which bind A $\beta$  monomers and oligomers depending on its conformational estate [57, 58]. Once the load of A $\beta$  is overcome, the high amount of monomers can interact to fibrillate. An excessive binding to A $\beta$ , together with a reduced enzymatic proteolysis of modified albumin, which is related with an increased half-life, could switch a protective buffering mechanism to a pathophysiological event. Thus, modified albumin bound to A $\beta$  could act as an aggregation seed. In fact, our findings related to the impaired ability of N-Alb and G-Alb to inhibit A $\beta_{40}$  fibrillation point towards that direction. This reduction in the inhibitory effect of albumin on A $\beta$  fibrillation would be especially relevant in brain parenchyma where A $\beta$  aggregation is critical in AD onset and progression.

In conclusion, nitrotyrosination and glycation change the physiological properties of albumin. These modifications can mainly affect to cells at both sides of the BBB and impair the inhibition of A $\beta$  fibril formation normally associated with the native albumin. Since albumin is significantly glycosylated and nitrotyrosinated in AD brain, these posttranslational modifications of albumin would contribute to AD etiopathology.

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## ABBREVIATIONS

AD, Alzheimer's disease; A $\beta$ , Amyloid  $\beta$ -peptide; Ab, antibody; BCA, Bicinchoninic Acid; BBB, Blood Brain Barrier; CSF, cerebral spinal fluid; FBS, fetal bovine serum; G-Alb, glycated albumin; HA-VSMC, human aortic vascular smooth muscle cell line; HepG2, human hepatocellular carcinoma cell line; SH-SY5Y, human neuroblastoma cell line; HUVEC, human umbilical vein endothelial cell line; IP, immunoprecipitation; LRP-1, low-density lipoprotein receptor-related protein 1; MG, methylglyoxal; MTT, Methylthiazolyldiphenyl-tetrazolium bromide; SIN-1, peroxy nitrite donor; BV2, murine microglial cell line; NT-3, nitrotyrosine; o.n., overnight; N-Alb, nitrotyrosinated albumin; ONOO $\cdot$ , peroxy nitrite; PBS, phosphate buffered saline; ROS, Reactive Oxidative Species; RT, room temperature; SN, supernatant; ThT, Thioflavine T; TTBS, Tween-tris buffer saline; WB, western blot.

## FIGURE LEGENDS

Fig. 1. G-Alb and N-Alb in human samples from AD patients. Albumin was immunoprecipitated from CSF (10 controls; 10 AD for G-Alb and 5 for N-Alb), plasma (18 controls and 19 AD) and brain samples (13 controls and 18 AD). Glycation and nitrotyrosination (upper panels), and albumin (lower panels) were analyzed by WB. Glycation (A) and nitrotyrosination (B) were quantified and normalized by the albumin levels. Data are mean  $\pm$  SEM of the number of experiments. \* $p < 0.05$ ; \*\* $p < 0.01$  vs albumin from controls.

Fig. 2. Correlation analysis between  $A\beta_{1-42}$  and G-Alb and N-Alb in CSF samples. Correlation analysis of total samples (10 controls plus 10 AD patients for G-Alb; 10 controls plus 5 AD patients for N-Alb) (A,B); Controls (10 controls) (C,D); and, AD patients (10 AD patients for G-Alb; 5 AD patients for N-Alb) are shown.

Fig. 3. Effect of glycation and nitrotyrosination in albumin aggregation along the time. Turbidimetric assays for G-Alb (A) and N-Alb (B) compared to native albumin. Data are the mean  $\pm$  SEM of 3 independent experiments. AFM images for G-Alb (C) and N-Alb (D) obtained after 3 days of incubation with MG and SIN-1 compared to native albumin. Transmission electron microscopy images for G-Alb (E) and N-Alb (F) obtained after 3 weeks of incubation with MG and SIN-1 compared to native albumin.

Fig. 4. Trypsin digestion and uptake of N-Alb and G-Alb into HepG2 cells. Native albumin, N-Alb and G-Alb were incubated with trypsin at different times. The pattern of degradation

was detected by Coomassie staining. A representative experiment from 4 independent experiments is shown (A). Native albumin, G-Alb and N-Alb (B) uptake in HepG2 cells was studied after incubation for 24 hr. Data are the mean  $\pm$  SEM of 5 independent experiments. \*  $p < 0.05$  vs. native albumin.

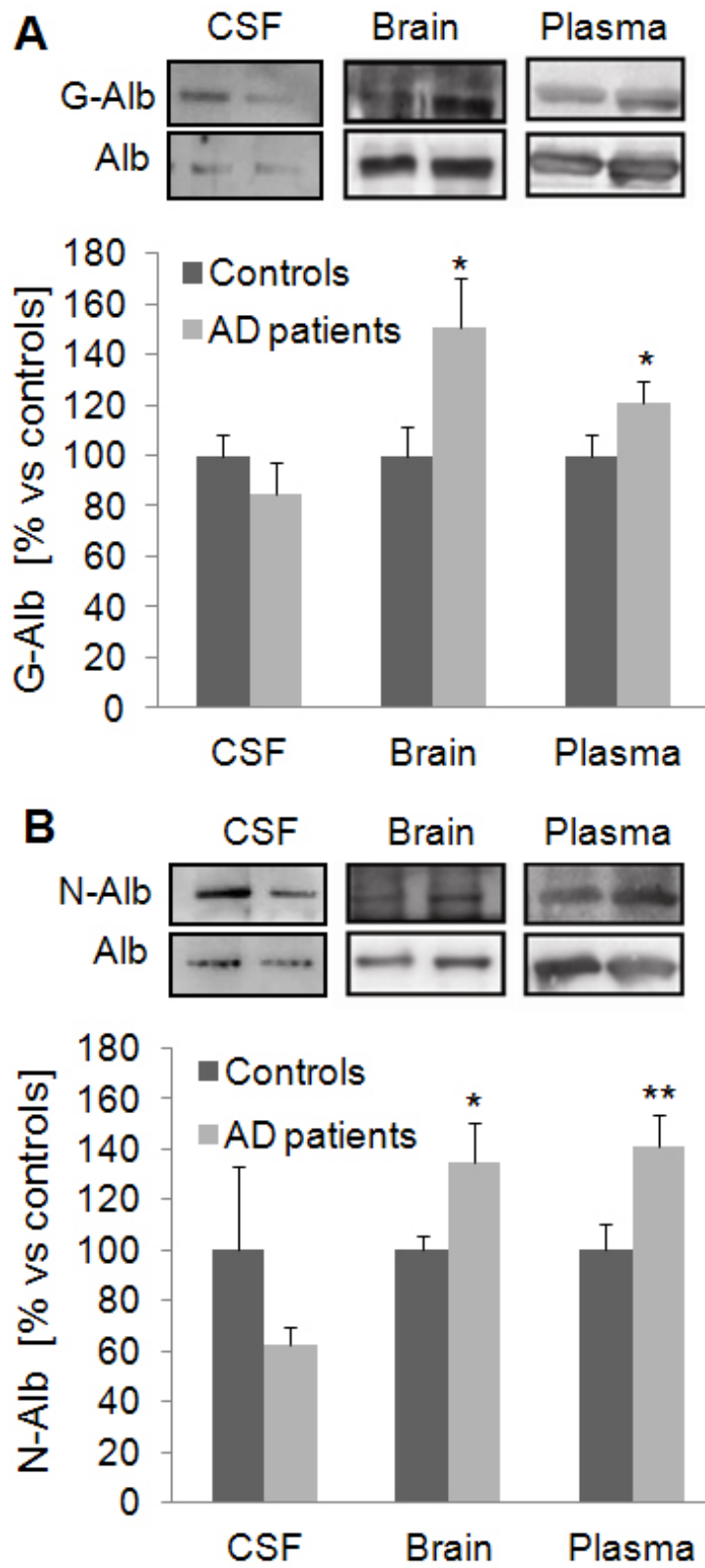
Fig. 5. Effect of albumin modification in osmolarity. The effect of glycation (A) and nitrotyrosination (B) of albumin on the osmolarity of solutions was studied *in vitro*. Native albumin was treated with increasing concentrations of the glycating (A) or nitrating agents (B). Data are the mean  $\pm$  SEM of 3-6 independent experiments. \*  $p < 0.05$  vs. untreated native albumin.

Fig. 6. Effect of G-Alb and N-Alb on cell viability. Vascular cells (HUVEC and HA-VSMC), primary cultures of murine hippocampal and cortical neurons, and primary cultures of murine astrocytes treated with 1  $\mu$ M Alb, G-Alb (A) and N-Alb (B) for 24 hr. 500  $\mu$ M Alb, G-Alb and N-Alb were also tested in HUVEC cells since they are exposed to both brain parenchyma ( $\sim 1$   $\mu$ M albumin) and blood ( $\sim 500$   $\mu$ M albumin). Cell viability was measured by MTT reduction. Data are the mean  $\pm$  SEM of 3-9 independent experiments performed in triplicate. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.005$  vs. treated with native albumin.

Fig. 7. Binding of A $\beta$  to native albumin, G-Alb and N-Alb. Soluble (left panels) and aggregated A $\beta_{40}$  (right panels) were incubated with native albumin, G-Alb (A) and N-Alb (B) for 24 hr. The A $\beta$  binding was measured by an ELISA kit. Data are the mean  $\pm$  SEM of 4 independent experiments. \*  $p < 0.05$  vs. native albumin.

Fig. 8. A $\beta$ <sub>40</sub> aggregation in the presence of native albumin, G-Alb and N-Alb. Synthetic A $\beta$ <sub>40</sub> were incubated with native albumin, G-Alb or N-Alb at a 1:2 molar ratio and ThT for 21 hr. The A $\beta$ <sub>40</sub> fibril aggregation was measured by the excitation of ThT at 430 nm and the fluorescence emission at 470 nm. The kinetic of aggregation along the time is shown in the left panels for G-Alb (A) and N-Alb (B). Quantifications were performed at 21 hr of aggregation and shown in the right panels for G-Alb and N-Alb. Data are the mean  $\pm$  SEM of 3-4 independent experiments. \* p <0.05; \*\* p<0.0001 vs. Alb:A $\beta$ <sub>40</sub>.

Figure 1



**Figure 2**

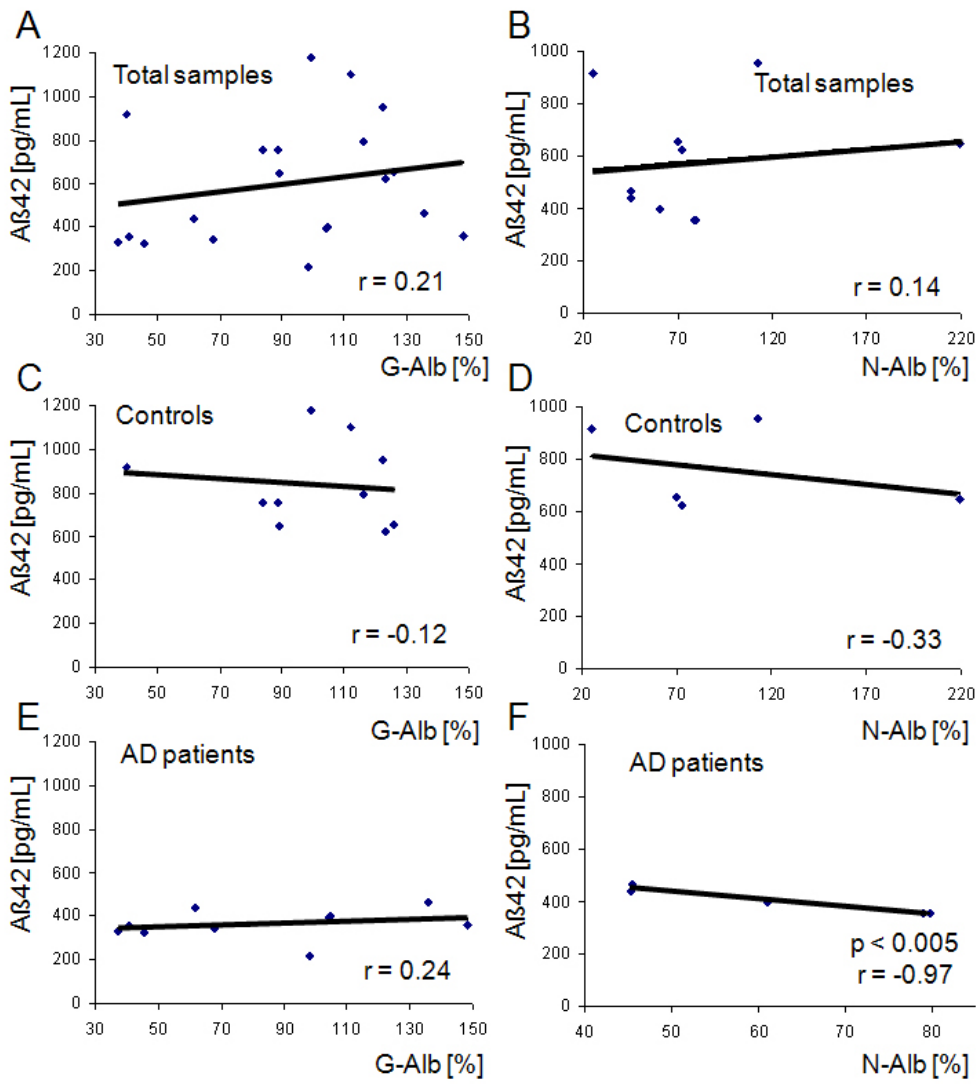




Figure 3

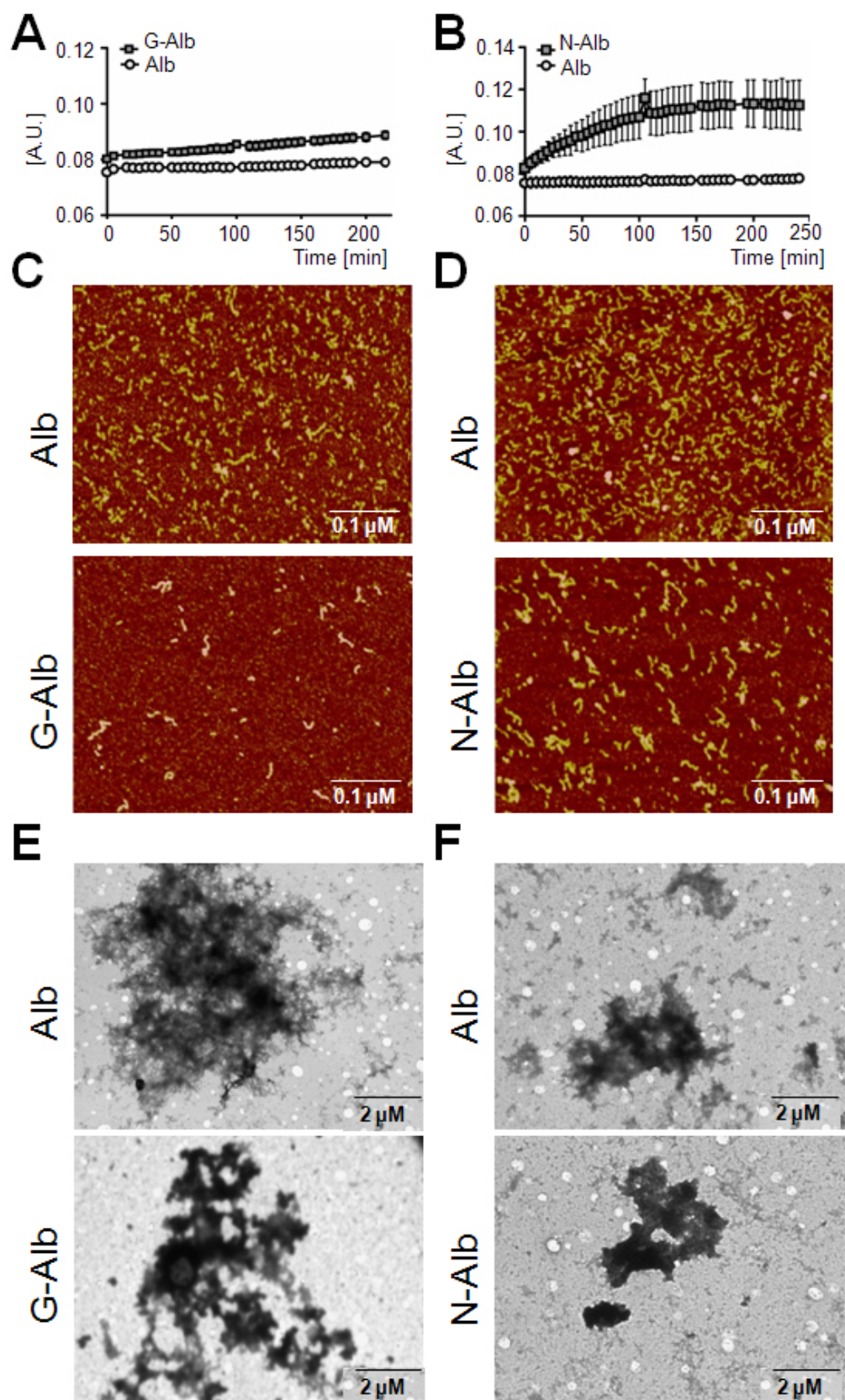


Figure 4

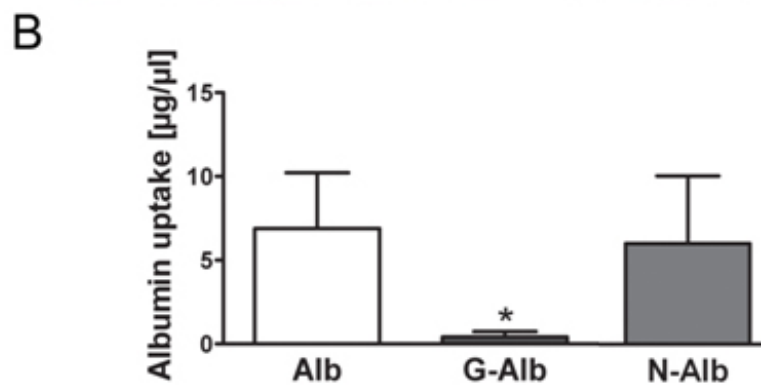
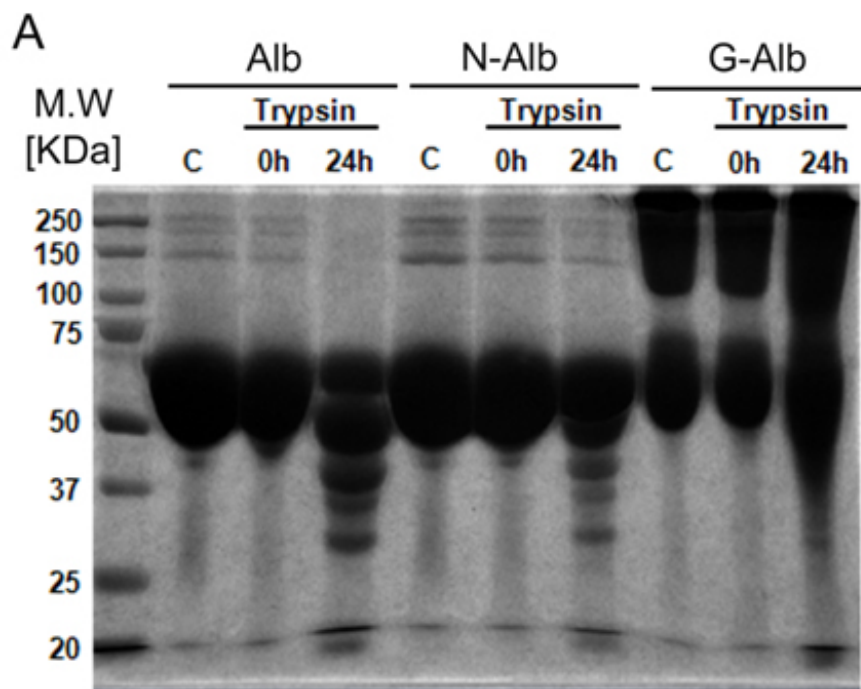
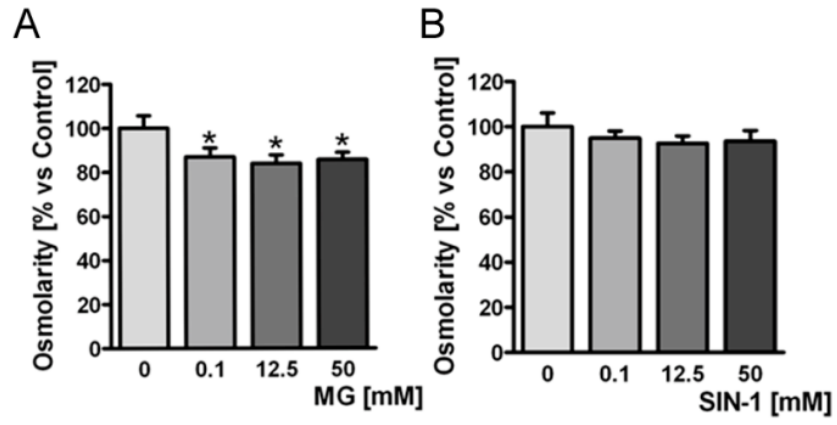
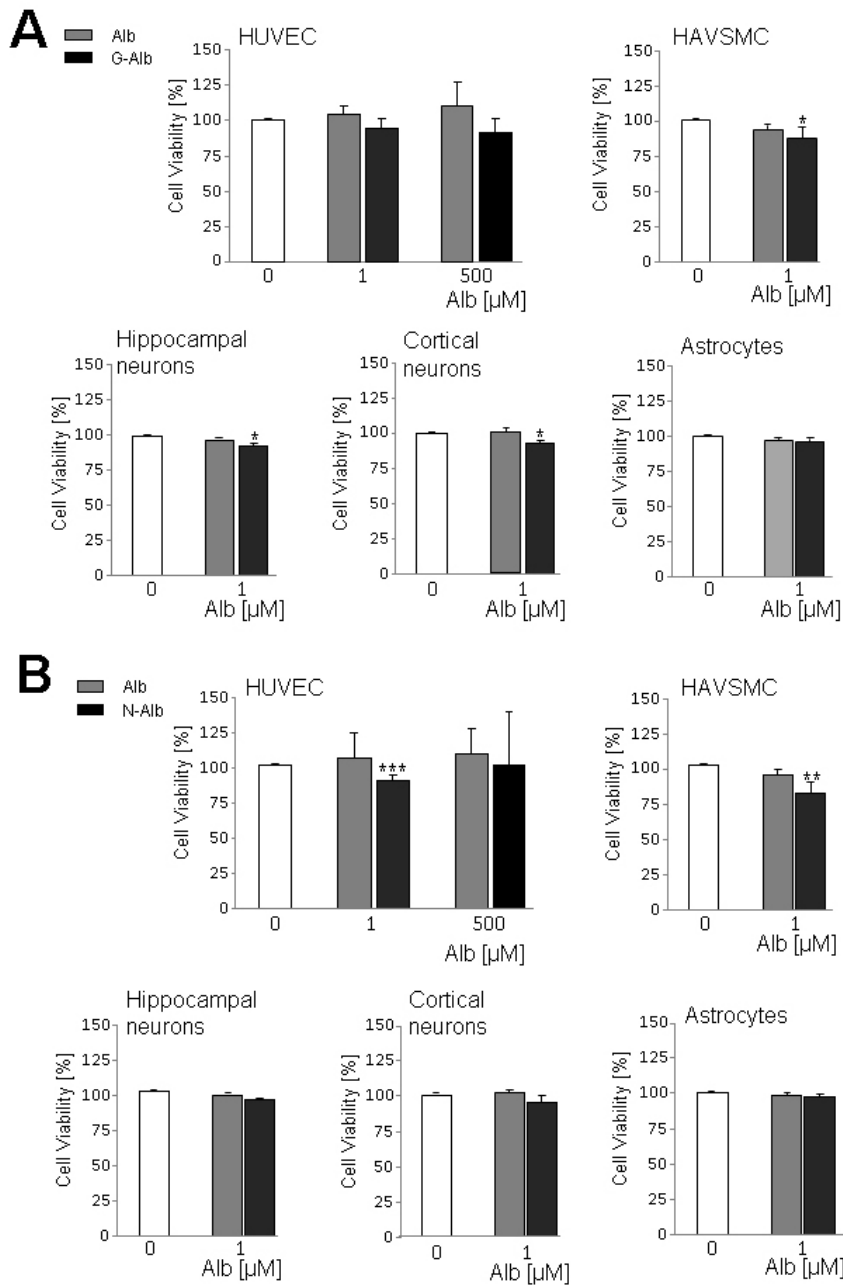


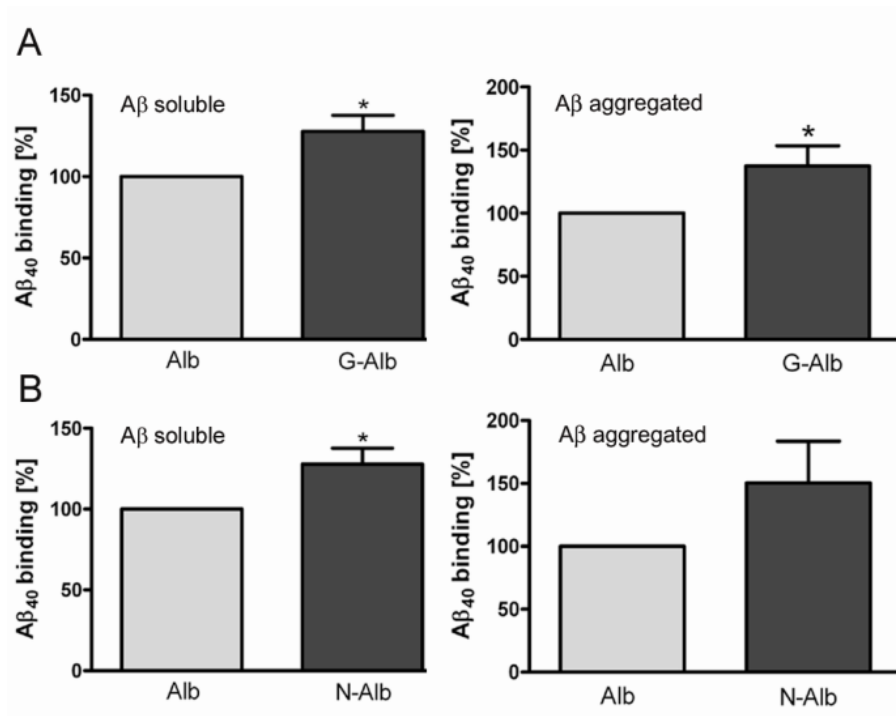
Figure 5



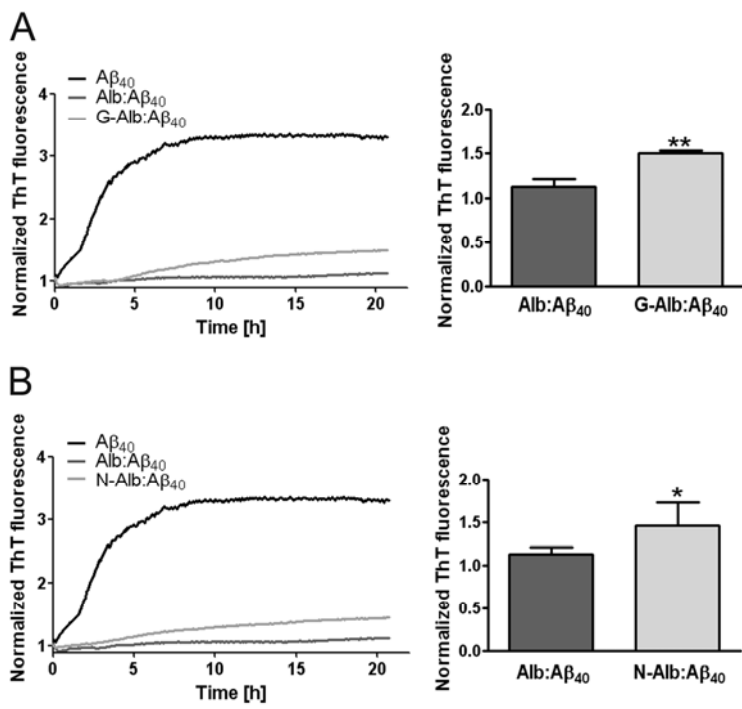
**Figure 6**



**Figure 7**



**Figure 8**



## Supplementary Figures

### Supplementary Legends

Fig. S1. Correlation analysis between tau and G-Alb and N-Alb in CSF samples. Correlation analysis of total samples (10 controls plus 10 AD patients for G-Alb; 10 controls plus 5 AD patients for N-Alb) (A,B); Controls (10 controls) (C,D); and, AD patients (10 AD patients for G-Alb; 5 AD patients for N-Alb) are shown.

Fig. S2. Correlation analysis between p-tau and G-Alb and N-Alb in CSF samples. Correlation analysis of total samples (10 controls plus 10 AD patients for G-Alb; 10 controls plus 5 AD patients for N-Alb) (A,B); Controls (10 controls) (C,D); and, AD patients (10 AD patients for G-Alb; 5 AD patients for N-Alb) are shown.

Fig. S3. Albumin glycation and nitrotyrosination *in vitro*. Native albumin was incubated with increasing concentrations of MG (a glycating agent) and SIN-1 (a peroxynitrite donor). Glycation (A) and nitrotyrosination (B) were detected by WB. Data are the mean  $\pm$  SEM of 5-11 independent experiments.

Fig. S4. Effect of MG on cell viability. Vascular cells (HUVEC and HA-VSMC), primary cultures of murine hippocampal and cortical neurons, and primary cultures of murine astrocytes treated with MG for 24 hr. Cell viability was measured by MTT reduction. Data are the mean  $\pm$  SEM of 3-9 independent experiments performed in triplicate. \*  $p < 0.05$ ; \*\*  $p < 0.01$  vs. untreated controls.

Fig. S5. Effect of SIN-1 on cell viability. Vascular cells (HUVEC and HA-VSMC), primary cultures of murine hippocampal and cortical neurons, and primary cultures of murine astrocytes treated with SIN-1 for 24 hr. Cell viability was measured by MTT reduction. Data are the mean  $\pm$  SEM of 3-9 independent experiments performed in triplicate. \*  $p < 0.05$ ; \*\*  $p < 0.01$  vs. untreated controls.

Fig. S6. Inhibition of A $\beta$  fibrillation by native albumin. Synthetic A $\beta_{1-40}$  were incubated with albumin at a 1:2 molar ratio and ThT for 21 hr. The A $\beta_{1-40}$  fibril aggregation at 21 hr was measured by the excitation of ThT at 430 nm and the fluorescence emission at 470nm. Data are the mean  $\pm$  SEM of 3 independent experiments. \*  $p < 0.0001$  vs. A $\beta$  alone.



Figure S1

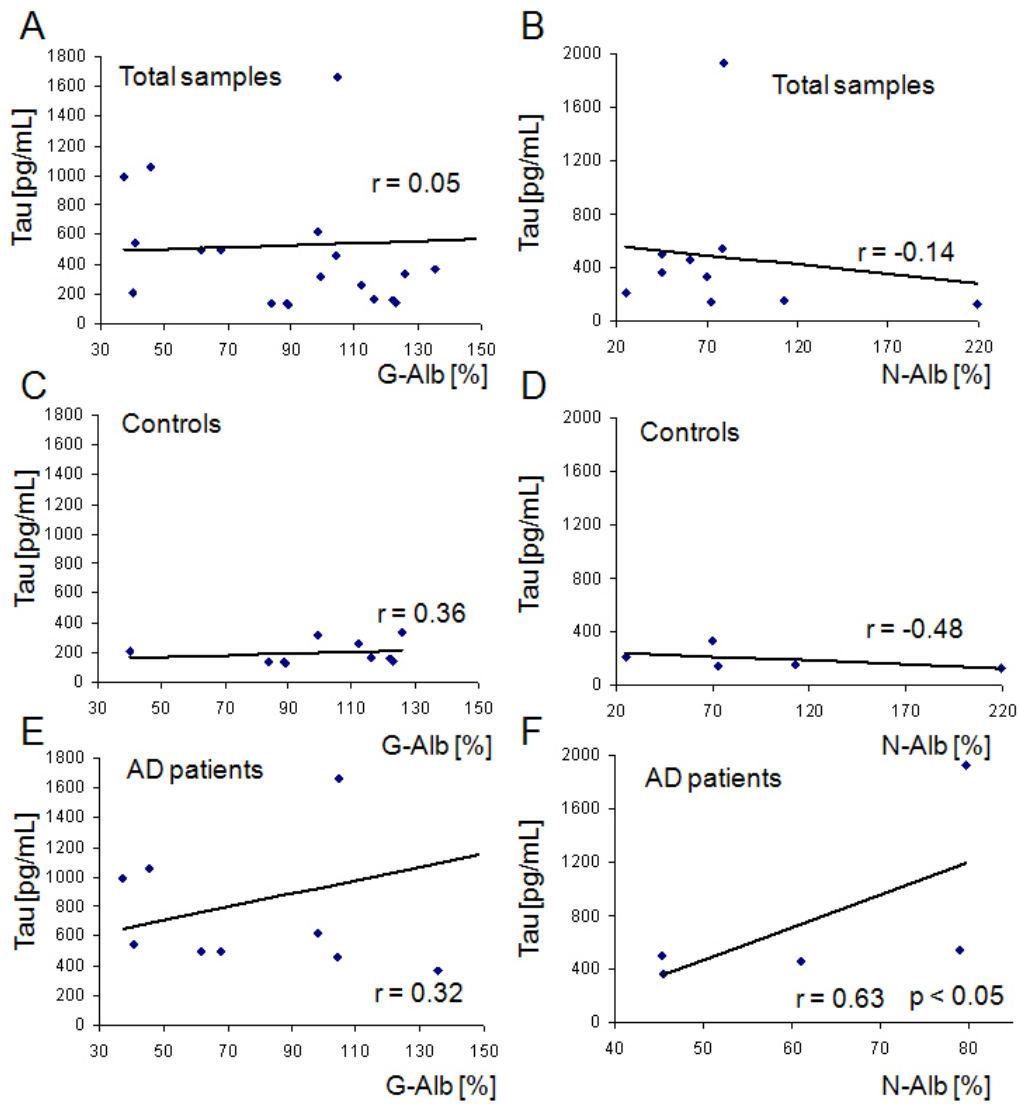


Figure S2

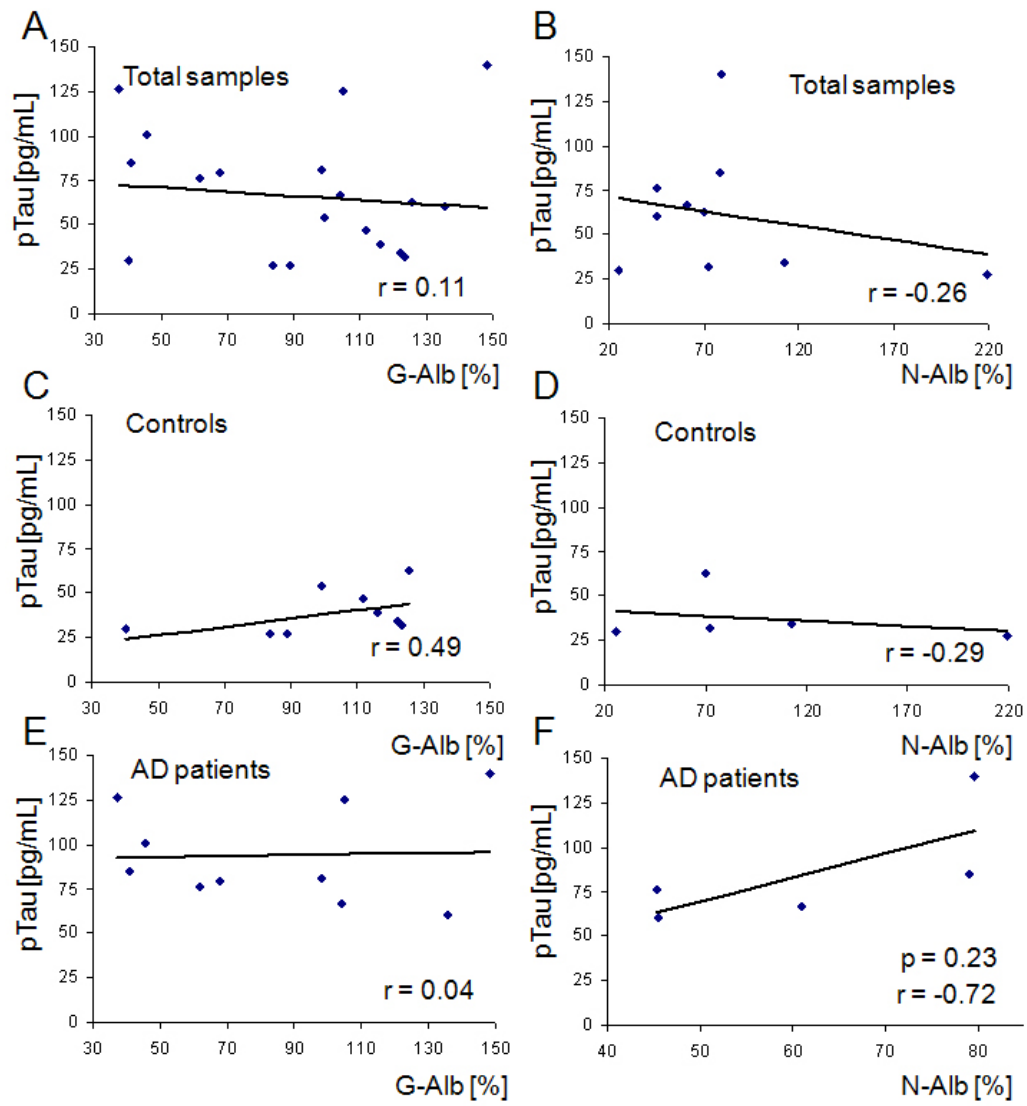
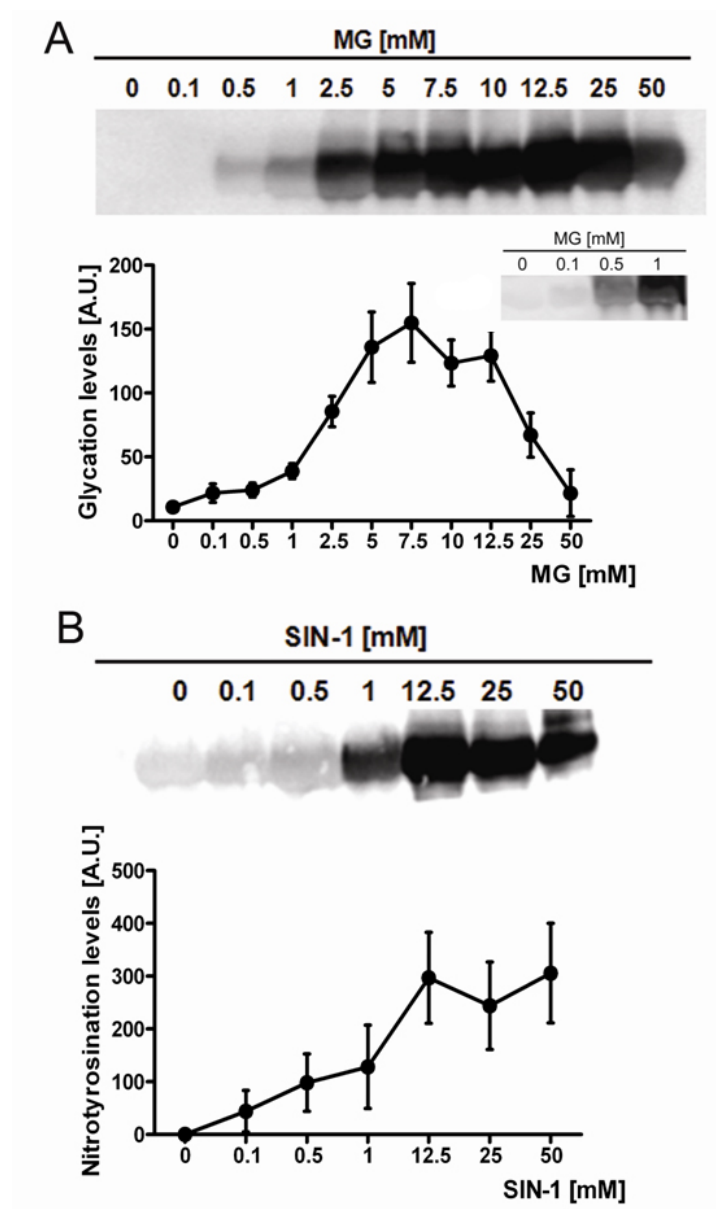
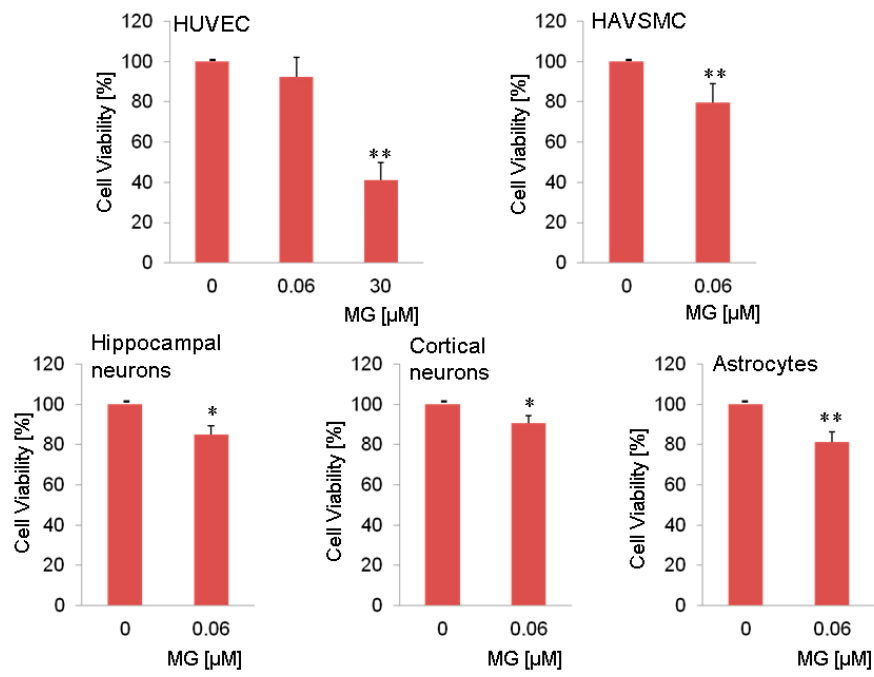


Figure S3



**Figure S4**



**Figure S5**

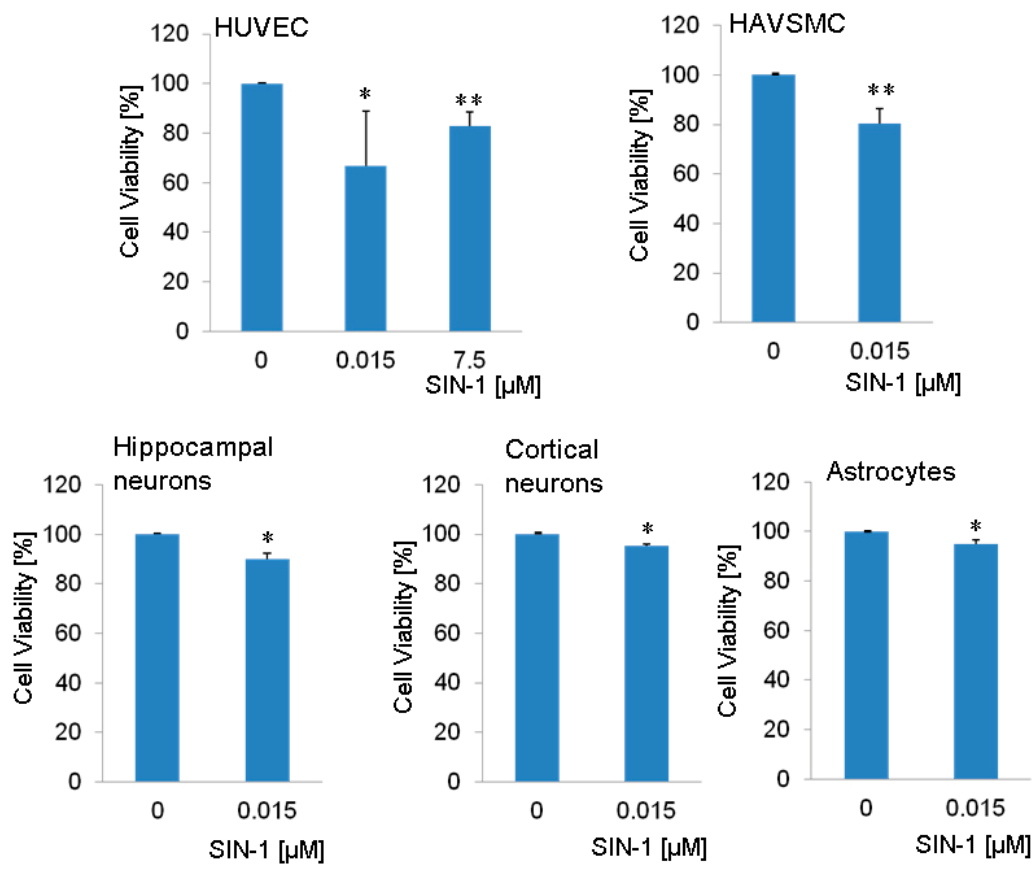


Figure S6

