Oxidative insults to neurons and synapse are prevented by Aged Garlic Extract (AGE) and S-allyl-L-Cysteine (SAC) treatment in the neuronal culture and APP-Tg mouse model

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Abstract

Alzheimer disease (AD) is one of the most common forms of dementia in the elderly. In AD patients, β-amyloid peptide (Aβ) plaques and neurofibrillary tangles are common features observed in the central nervous system. Aβ deposition results in the production of reactive oxygen species (ROS) leading to the hyperphosphorylation of tau that are associated with neuronal damage. Cholinesterase inhibitors (ChEI) and a partial NMDA receptor antagonist (memantine) have been identified as potential treatment options for AD. However, clinical studies have found that these drugs fail to prevent the disease progression. From ancient times, garlic *(Allium sativum)* has been used to treat several diseases. By "aging" of garlic, some adverse reactions of garlic can be eliminated. Recent findings suggest that "Aged Garlic Extract" (AGE) may be a therapeutic agent for AD due to its antioxidant and Aβ lowering properties. To date, the molecular properties of AGE have been sparsely studied *in vitro* or *in vivo*. The present study tested specific biochemical and molecular effects of AGE in neuronal and AD rodent models. Furthermore, we identified SAC as one of the most active chemicals responsible for the AGE-mediated effect(s). We observed significant neuroprotective and neurorescue properties of AGE and one of its ingredients, S-allyl-L- cysteine (SAC), from ROS (H_2O_2)-mediated insults to neuronal cells. Treatment of AGE and SAC were found to protect neuronal cells when they were independently co-treated with ROS. Furthermore, a novel neuropreservation effect of AGE was detected in that pre-treatment with AGE alone protected ~80% neuronal cells from ROS-mediated damage. AGE was also found to preserve pre-synaptic proteins SNAP25 from ROS-mediated insult. For example, treatment with 2% AGE and SAC (20mg/kg) independently increased (~70%) levels of SNAP25 and synaptophysin in Alzheimer's APP-Tg mice, of which the latter was significantly decreased in AD. Taken together, the neuroprotective, including preservation of pre-synaptic proteins by AGE and SAC can be utilized in future drug development in AD.

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Keywords

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Alzheimer disease (AD), a progressive neurological disorder, is the most common form of dementia in the elderly and also the 7th major cause of death in US alone (A.A 2009). The development of AD is influenced by multiple factors including both genetic and epigenetic (environmental) factors. Environmental factors such as dietary, lifestyle, education and exposure to heavy metals and pestichemicals play major roles in the pathogenesis of sporadic cases of AD (Lahiri *et al.* 2009, Lahiri & Maloney 2010). Neurochemically, AD is characterized by deposition of amyloid β (Aβ)- containing neuritic plaques in the brain parenchyma followed by neuronal loss (Hardy & Selkoe 2002). Additionally, AD is associated with hyperphosphorylated tau, a neuronal microtubule stabilizing protein (Schmidt *et al.* 2001), which also leads to neuronal damage. Aβ, a 40-42 amino acid residue, is a product of transmembrane amyloid precursor protein (APP) that is cleaved by enzymatic actions of β secretase (BACE-1) and γ secretase (Vassar *et al.* 2009). The major species of Aβ, a forty amino acids residue (Aβ1-40), is less pathogenic for the development of AD (Selkoe 2001). However, Aβ with 42 amino acids residue (Aβ1-42) has been shown to fibrillate faster than that of Aβ40, and hence is considered pathognomonic in AD (Walsh & Selkoe 2007). Deposited Aβ in the brain activates microglia, and this interaction between microglia and Aβ generates reactive oxygen species (ROS) and cytochemokines leading to severe neuronal damage (El Khoury *et al.* 1996, Coraci *et al.* 2002). Furthermore, generated ROS is directly involved in lipid peroxidation of the cell membranes (Behl & Sagara 1997). ROS such as hydrogen peroxide (H_2O_2) can also be generated by interaction of Aβ and other metal ions like Cu^{++}, Zn^{++} and Fe^{+++} (Opazo *et al.* 2002).

Apart from a widely used culinary component, garlic *(Allium sativum)* has been utilized to treat a wide variety of diseases, including cancer. The efficacy of garlic has been attributed to its potent antioxidant properties (Durak *et al.* 2004a, Durak *et al.* 2004b, Ichikawa *et al.* 2002). However, the consumption of raw garlic can cause various side effects including anemia, growth retardation and destruction of gut microflora and alteration of serum protein levels (Nakagawa *et al.* 1980, Shashikanth *et al.* 1984). To overcome the adverse effects, aged garlic extract (AGE) has been prepared by soaking sliced garlic in water-ethanol mixture for 20 months, which removes several irritant sulfur containing compounds and also stabilizes some unstable compounds such as allicin (Morihara *et al.* 2006, Borek 2006). Interestingly, the antioxidant property of AGE is greater than those of raw garlic (Park *et al.* 2009, Imai *et al.* 1994). Details of the procedure of making AGE, its quality control and related scientific references can be obtained from Kyolic® [website http://www.kyolic.com/category/truth-about-garlic]

In this study, we have examined the effect(s) of AGE and an active and most abundant ingredient S-allyl-L-cysteine (SAC) in protecting neuronal cells from free radicals (ROS)-mediated damage. We also evaluated AGE and SAC's effect in preserving pre-synaptic proteins in Alzheimer's APP transgenic (Tg) mice, which over produce Aβ peptides. In the cell culture based study, we have observed a significant neuroprotective role of AGE and SAC from ROS (H_2O_2)-mediated insults to the neuronal cells. We also characterized pre-synaptic markers, such as synaptosomal associated protein of 25kDa (SNAP25). SNAP25 is a member of soluble N-ethylmaleimide sensitive factor attachment protein receptors (SNARE), and plays an important role in pre-synaptic vesicle fusion and exocytosis (Sollner *et al.* 1993). Our findings also indicate that AGE treatment preserves SNAP25 in neuronally differentiated PC12 cells when they were co-treated with ROS *versus* cells only treated with

ROS. In the context of ROS mediated damage to the neuronal cells, a novel neuropreservation property of AGE was also observed in this study under different 'challenge conditions'.

For instance, two days pre-treatment with AGE significantly increased cellular viability of differentiated PC12 cells when they were post-treated with ROS alone for 24 hr *versus* the cells not pretreated with AGE but treated with ROS alone for 24 hours. In both experiments, under the first condition, when the neuronal cells were co-treated with the vehicle, AGE and ROS, and under the second condition when the cells were pre-treated with AGE and post-treated with ROS, AGE treatment preserved neuron like morphology, which was completely lost in non-AGE treated (vehicle) but ROS challenged cells. Furthermore, 4 months independent treatment of AGE and SAC to APP-Tg mice yielded an abundant amount of Aβ and significant increase in levels of the pre-synaptic proteins synaptophysin and SNAP25 *versus* untreated APP-Tg mice. In humans, both synaptophysin and SNAP25 are significantly decreased in AD *versus* control cases (Lassmann *et al.* 1992, Greber *et al.* 1999). Furthermore, hippocampal-based memory was found to be impaired in AP-Tg mice (Sadowski *et al.* 2004). Likewise, our findings demonstrate a significant improvement in the hippocampal-based memory as measured by Y-maze spontaneous exploration in SAC treated Tg mice.

Taken together, our findings demonstrate strong evidences that AGE and SAC possess potential neuroprotective and neuropreservative properties against ROS mediated damage, and also preserve pre-synaptic proteins both in cellular and animal models of AD.

Materials and methods

Cell culture

PC12 cells were obtained from American Type Culture Collection (ATCC) and cultured as described previously (Ray & Lahiri 2009). When the cells became 75% confluent, the regular RPMI medium was replaced by low serum (1% FBS) containing RPMI supplemented with 30ng/ml nerve growth factor (NGF, Roche, Indianapolis, IN, USA) to achieve neuronal differentiation. NGF treatment transforms PC12 cell to sympathetic neurons (Ray & Lahiri 2009, Ghosh & Lahiri 1999). Differentiation of PC12 cells with NGF was carried out for 12 days, counted by Trypan blue exclusion method and plated onto a 24 well cell culture plate (Corning, NY, USA) at 150,000 cells/ well in low serum RPMI with NGF. The cells were allowed to settle down for two days prior to the treatment. Drugs were added on 15th day of the differentiation of PC12 cells with NGF.

Drug treatment

AGE (Kyolic™) was manufactured by Wakunaga of America Co. Ltd. (Mission Viejo, CA, USA). Briefly, the raw garlic is aged for 20 months, and the process of aging converts fat soluble sulfur compounds into water soluble compounds, mainly SAC and S-allylmercaptocysteine (Borek 2006). Commercially obtained aqueous aged garlic extract (Kyolic™) contains 300mg/ml of AGE. AGE exerted potential pharmacological effects in cell culture based studies at concentrations between 0.1% to 1.0% *v/v* (Berginc *et al.* 2009, Li *et al.* 2009). We have also tested different doses of AGE and SAC in neuronally differentiated PC12 cells with/without ROS and observed an optimum efficacy of AGE between the dose of 0.2% to 1.0% *v/v* and that for SAC between the dose of 1μM to 3 μM (Supplemental 1A &1B). In our study, we have used 0.3% and 1.0% *v/v* doses of AGE in the cell culture experiments. To evaluate whether SAC produces similar results of AGE or in other word, whether AGE's neuroprotective properties are predominantly due to SAC, neuronal cells were also treated with SAC. SAC was obtained as a gift from Wakunaga of

America Co. Ltd. and was dissolved in sterile PBS. In cell culture experiments, we have used 1μM and 2μM doses of SAC. Apart from our results, previous study revealed potent physiological activities of SAC in cell culture models between the doses of 1-2μM (Kosuge *et al.* 2003).

Co-treatment experimental condition—Prior to start the experiment, 1 ml of AGE liquid was passed through a 0.22μM filter (Millipore Corporation, Billerica, MA, USA) and filtered AGE was mixed with RPMI containing 1% FBS, 1x antibiotic mixture and NGF (30ng/ml). The culture medium from each well of the 24 well plate was aspirated out and either medium alone, 0.3% or 1.0% AGE (dissolved in the medium) was added to the respective wells (n=6 for each treatment group). Both the treatment groups were also co-treated with 200μM of H_2O_2 (Sigma, St. Louis, MO, USA). Differentiated PC12 cells, which were treated with RPMI LSM+ NGF with/ without H_2O_2, were served as 'vehicles with ROS' and 'no ROS vehicle' respectively. In the same experiment, differentiated PC12 cells were also treated with 1μM or 2μM of SAC in the presence of ROS. The duration of the treatment was 48 hours.

Pre-treatment experimental condition—In a separate experiment, 15 days differentiated PC12 cells were treated with 0.3% and 1.0% AGE without the presence of H_2O_2 for 48 hours. After 48 hours, AGE containing medium was gently aspirated out from the wells and RPMI (LSM) + NGF + 200μM H_2O_2 was added to the wells. Post treatment of cells with H_2O_2 was carried out for 24 hours. Cells, which were not pre-treated with AGE, but post-treated with H_2O_2, were served as 'ROS post-treated vehicle'.

Harvesting of the treated or untreated cells

After the period of treatment, 500μl of the conditioned medium was taken out from each well and stored at -20°C for LDH assay. The remaining conditioned medium from each well was aspirated out, and cells in each wells were rinsed with ice cold 1x DPBS. Cells were lysed by addition of 100μl of M-PER buffer (Pierce) supplemented with protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Cell lysates (CL) were collected and 30μl of CL sample from each of the treated and untreated well were used to determine cell viability (CTG assay) just after lysing the cells, and the remaining CL samples were stored at -20°C freezer for the Western immunoblotting analyses.

Cell viability (CTG) assay

An equal volume (30μl) of the cell lysate samples were taken into each well of a white 96-well plate (Corning), and 30μl of the Cell Titer Glo reagent (CTG, Promega) was added to each well. CTG measures cellular viability by quantifying mitochondrial ATP concentration in the CL, which correlates with the cell number in the lysate (Ray *et al.* 2009). CTG assay measurement was performed in 'Varitas' microplate luminometer (Turner Biosystems, Sunnyvale, CA, USA).

Cellular toxicity (LDH) assay

Cell membrane damage leading to cellular death was measured by actual elevation of lactate dehydrogenase (LDH) in the medium sample. For this, an equal volume of CM samples (15μM) from each treatment group was used to determine cellular toxicity by LDH assay as mentioned previously (Alley *et al.* 2009). To determine the exact amount of LDH present in the CM, a standard curve with known amounts of rabbit muscle LDH was also performed (not shown).

Treatment of transgenic mice with AGE and SAC

Tg2576 transgenic mice modeling familial Alzheimer's disease harbor Human APP695 double Swedish mutation (KM 670/671 NL), originally produced by Karen Ashe Hsiao (Hsiao *et al.* 1996), bred on B6SJLF1 background. These mice are now commercially available from Taconic Farms (Taconic Farms, Inc.). Tg2576 mice begin to accumulate Aβ–40/42 peptides beginning ~5+ months of age developing Aβ plaques, oxidative lipid and glycoxidative damages by 9+ months of age, concomitant with cognitive deficits.

Tg2576 original transgenic breeder males obtained from Taconic Farms (German Town, NY), were crossed with the background strain (C57BL/6J x SJL) females obtained from Jackson Labs (Bar Harbor, ME) to produce Tg2576 progeny. At the end of weaning, transgenic (Tgs/Tg2576) and non-transgenic littermates (Lts) were identified by polymerase chain reaction (PCR) amplification of tail genomic DNA using specific primer pairs as established(Chauhan & Siegel 2003b) (Chauhan & Siegel 2003a).

Mice are reported to consume 150 g of food/kg body weight (Jacoby & Fox 1999). Therefore, a diet containing 2% AGE (2 g AGE/100 g Chow), an optimized dose in our laboratory (Chauhan 2003) will result in the intake of 3 g of AGE per mouse. Since 1 g of AGE is known to contain 1mg of SAC, one mouse will consume 3mg of SAC per day, which will translate into 20 mg SAC/kg diet. Based on this best known estimation, concentration of SAC was determined to be 20 mg/kg.

Special rodent diets containing 2% aged garlic extract (Kyolic) and 20 mg SAC/kg (Sigma–Aldrich) were commercially prepared (Research Diets, Inc., New Brunswick, NJ), in which the test ingredients (AGE or SAC) were incorporated into an AIN-76A based standard purified diet [kcal% of proteins (20.8), carbohydrates (67.7), fat (11.5), casein (8.0), corn starch (60.0) and vitamin mix (4.0)]. A control isocaloric diet delivering equivalent amounts of protein, fat and carbohydrates and containing equivalent calories per gram weight also was commercially prepared (Research Diets, Inc., New Brunswick, NJ). Each batch of special diet was prepared in sufficient quantity (50 kg/batch/diet) that would last for the whole experiment in order to avoid batch-variability. 10-month-old transgenic mice or transgenic littermate controls were fed with respective special diet(s) for 4 months. All animal experiments including transgenic breeding, dietary interventions, behavioral studies and brain tissue preparation, were performed in accordance with the National Institute of Health Guide for the Ethics, Care and Use of Laboratory Animals, and as approved by the local institutional Bioethics, Animal Care and Use Committee at the University of Illinois at Chicago and Jesse Brown VA Medical Center.

At the end of treatment, 14 month old mice were evaluated for behavior and then euthanized with ketamine (100mg/kg, i.p.) and xylazine (10mg/kg, i.p.), and perfused with normal sterile saline. Brains were dissected out and cut into two longitudinal bilateral halves. One half of the brain was utilized for biochemical studies and the other half was fixed in 4% buffered paraformaldehyde. Each half of the brain contained all brain regions including cortex, hippocampus, and cerebellum.

Spontaneous Exploratory Working Memory Test

Spontaneous alterations and exploratory behavior in Y maze is a hippocampal-based task that measures working memory. Spontaneous alteration and exploratory deficits were evaluated using Y maze. Y maze apparatus consisted of dark grey acrylic with 3 arms, each of 21 cms long, 4 cms wide and 40 cms tall, placed at an angle of 33°C with a central triangular entry zone. Each animal was subjected to a single trial of 5 min duration for evaluating spontaneous alterations and exploration by introducing the animal in the central entry zone and allowed to explore freely in all 3 arms. Frequent exploration in all 3 arms and

less time spent in each arm between the alterations indicated normal exploratory behavior. By contrast, restricted arm entries and reduced explorations with more time spent in each arm indicated deficits in spontaneous exploration. Total number of arm entries and alterations between the arms were registered with the use of CCD camera and Accuscan software. Individual values were averaged to obtain a group mean, and group means were subjected to statistical analysis to derive significance.

Behavioral testing was performed only once at the end of treatment period i.e. on the last day of the 16th week of treatment period.

Preparing mice brain lysate—At the end of the treatment brains were dissected out, washed with sterile saline, weighed and immediately frozen in dry ice. Frozen brains were homogenized in Tris-HCl buffer, pH 7.6 [140 mM NaCl, 3 mM KCl, 25 mM Tris-HCl (pH 7.6), 5 mM EDTA, 2mM Phenanthroline, 0.5% SDS, EDTA-free protease inhibitor cocktail (Roche)] and centrifuged at 30,000 RPM (100,000g) in the ultracentrifuge (SW 55 Ti rotor) to obtain TBS soluble fraction of the brain. This fraction was subjected to protein estimation (Bradford assay), and a defined volume of supernatant containing a fixed amount of protein was analyzed for Western immunoblotting.

Western immunoblotting

Protein content of CL samples was measured by Bradford protein assay as described previously (Bradford 1976). Equal proteins from CL and brain denatured lysates were loaded in two separate 10% Bis-Tris 'Criterion' polyacrylamide gels (BioRad) and separated at 180 V for 1.2 hours. Proteins were electrophoretically transferred onto a PVDF membrane (BioRad) using the chilled transfer buffer (25mM Tris, 200mM Glycine and 20% Methanol) at 30V for 6 hours at 4°C. After transfer, the membrane was blocked in 5% nonfat milk in Tris buffered saline, (pH 7.4) containing 0.05% Tween-20 (TBST) at room temperature (RT) for 1 hr. Afterwards, the blot was probed with monoclonal anti-SNAP25 antibody (Clone: SP14; Millipore). The antibody was diluted to 1:3000 in 5% Milk dissolved in TBST and probed overnight at 4°C with gentle rocking. The membrane was washed three times with TBST, and subsequently probed with the secondary antibody, which was HRP-conjugated anti mouse goat IgG (Pierce, Rockford, IL, USA) for 1 hr at RT followed by three times wash with TBST. Detection of protein band signals was achieved by adding chemoluminescent buffer (GE, Buckinghamshire, UK) to the blot, which was immediately photographed using GE chemoluminescent detection film. A specific strip of the membrane was also probed with monoclonal anti-β actin antibody (1:100,000 dilutions, Sigma) for normalization purpose, and β-actin band signals were obtained in the same way as mentioned above.

Immunocytochemistry of differentiated PC12 cells after treatment

In addition to studying the effects of AGE at the molecular level, we also examined the effects at the cellular level. For this, PC12 cells from three wells from each treatment groups were fixed by adding 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) dissolved in phosphate buffer (pH 7.4). Paraformaldehyde fixed cells were washed , permeabilized with 0.12% Triton X-100 (Sigma) and blocked with 10% normal horse serum (Sigma) dissolved in DPBS, followed by incubation with the primary antibody [monoclonal anti α-tubulin (Sigma) diluted 1: 50,000 in 1% horse serum]. After overnight incubation, the cells were washed thoroughly with DPBS and incubated in biotinylated horse anti-mouse secondary antibody (Vector laboratories, Burlingame CA, USA) at 1: 250 dilutions for one hr. After washing, the cells were incubated with a mixture of fluorescein (DTAF) conjugated streptavidin (Jackson Immunoresearch Laboratories, PA, USA) at 1: 300 dilutions for one hr. For nuclear staining, 4', 6-diamidino-2-phenylindole (DAPI, Sigma, diluted to 1.5μg/ml

in water) was added to the wells and examined under Leica DMIL HC inverted fluorescent microscope (Leica Microsystems GmbH, Wetzler, Germany) using the following sets of filters: for FITC, excitation of 480-520 nm, band pass of 505 nm and emission of 535-585 nm and for DAPI excitation of 350-400 nm, band pass of 400nm and emission of 460-510 nm. Combination of these filters resulted in minimum crosstalk between the signals from individual fluorochromes and minimized the 'bleed-through' between any of these three signals. Images were captures with a SPOT RT-SE digital camera (Diagnostic Instruments, Sterling Heights MI, USA) and superimposed with the SPOT basic software (Diagnostic Instruments). The cells stained with α-tubulin exhibited green fluorescence and nuclei stained with DAPI appeared as blue under their respective filter sets.

Quantification of Western immunoblot bands—Densitometric analyses of Western immunoblot bands were performed by using 'Image J' software (NIH) using gel 'analysis tool', which measures 'integrated pixel density' across the region of interest.

Statistical analyses—Statistical analyses were carried out using "GraphPad Prism; 4.0" (GraphPad Software Inc.; La Jolla, CA, USA). All data are presented as mean± SD. ANOVA for more than two groups and 'T test' analyses for less than three groups were carried out. For ANOVA, post-hoc comparisons were assessed after performing Bonferroni correction. 'P values' less than 0.05 were considered significant.

Results

AGE treatment protects neuronally differentiated PC12 cells from ROS mediated damage

For the neuroprotection experiment, three different assays were employed. First, the CTG assay indicated that H_2O_2 treatment alone caused a significant decrease (~80%) in the viability of differentiated PC12 cells compared with 'no ROS vehicle'. We observed a significant increase in the viability of H_2O_2 treated neuronal PC12 cells when they were co-treated with either 0.3% or 1.0% of AGE (Fig.1A). The increase in the cell viability was approximate 60% and 70% as compared to 'vehicle with ROS' with 0.3% and 1.0% AGE co-treatment, respectively. Second, the LDH assay also revealed approximately 80% increase in the secreted LDH in 'vehicle with ROS' *versus* 'no ROS vehicle' indicating severe membrane damage and toxicity caused by 200μM H_2O_2. Interestingly, both 0.3% and 1.0% AGE independently prevented the efflux of secreted LDH in the CM of H_2O_2-treated PC12 cells. Indeed, the co-treatment of AGE restored the membrane integrity of the H_2O_2 treated cells as reflected from the similar low level of LDH release in both the AGE treated and 'no-ROS vehicle'(Fig.1B). Third, cellular morphology studies by immunocytochemistry revealed a substantial loss of cell numbers and neurites in 'vehicle with ROS' group *versus* 'no ROS vehicle'. Both 0.3% and 1.0% AGE with ROS co-treatment prevented cell death and preserved intercellular connections between cells, which is similar to the 'no ROS vehicle' group (Fig. 1C). Thus these biochemical and morphological techniques demonstrate potent neuroprotection properties of AGE against ROS mediated damage.

AGE treatment restores pre-synaptic proteins in the neuronal cells against ROS mediated insults

Western immunoblotting analysis with cell lysate (CL) showed approximately 60% decrease in the levels of the pre-synaptic protein SNAP25 in 'vehicle with ROS' group *versus* 'no ROS vehicle'. Notably, levels of SNAP25 were significantly increased in CL samples of both 0.3% and 1.0% AGE co-treated with ROS treatment groups *versus* 'vehicle with ROS' (Fig.2).

AGE pre-treatment rescues neuronal cells from ROS mediated insults

To address the 'neurorescue' property of AGE, we have treated neuronally differentiated PC12 cells with 0.3% or 1.0% AGE alone for 48 hours. The dietary supplements were removed and the cells were washed and post-treated with 200μM of H_2O_2 alone for 24 hr. At the end of 24 hr of H_2O_2 treatment, we measured cell viability by CTG and monitored cellular morphology by immunocytochemistry. We have observed ~80% increase in cell viability in 0.3% and 1.0% 'AGE pre-treatment plus ROS only post-treatment' group *versus* 'no AGE pre-treatment plus ROS only post-treatment' group (Fig.3A). Cellular morphology revealed extensive loss and altered morphology with destruction of neurites in the cells of 'no AGE pre-treatment plus ROS only post-treatment' group. Notably, cells in 'AGE pre-treatment plus ROS only post-treatment' group had intact cellular morphology (Fig. 3B), suggesting neurorescue ability of AGE, which overcomes the ROS-mediated insults.

Pre-synaptic proteins (SNAP25 and synaptophysin) are decreased in the brain lysate of APP Tg. *versus* wild type mice

Western immunoblotting analyses of the brain lysate to evaluate the loss of pre-synaptic proteins due to excess Aβ deposition in the untreated APP Tg mice *versus* wild type mice showed a significant difference in levels of pre-synaptic proteins between these two groups of animals. Both levels of SNAP25 and synaptophysin were significantly reduced (~75%) in the brain lysate of Tg mice *versus* those of age matched wild type controls (p<0.0001 and p<0.001 respectively) (Fig. 4A and 4B).

AGE treatment preserves the levels of pre-synaptic proteins (SNAP25 and synaptophysin) in the brain of Tg mice

Western immunoblotting of brain lysate of APP-Tg mice revealed that 4 weeks treatment with a special diet containing 2% AGE significantly increased levels of both SNAP25 and synaptophysin *versus* 4 weeks isocaloric control diet fed Tg mice (p=0.001 and p=0.008 respectively) (Fig. 5A and 5B). Levels of both SNAP25 and synaptophysin were decreased in a time dependent manner in the brain of untreated Tg mice. For example, SNAP25 and synaptophysin levels were decreased approximately by half in the brain lysate of untreated Tg mice at the end of 4-week experiment *versus* untreated Tg mice at the beginning of the experiment (zero week). Interestingly, the treatment of Tg mice with AGE restored or preserved the levels of both SNAP25 and synaptophysin to the level at the beginning of the experiment.

SAC mimics the results of AGE in cell culture and *in vivo*

Since AGE is a mixture of several active and inactive ingredients, we hypothesized that AGE's effects observed in this study are mostly mediated by the active and most dominant constituent SAC. We observed potent neuroprotective property of SAC, when neuronal cells were co-treated with SAC and ROS, which was revealed independently by CTG, LDH and morphology analyses (Fig. 1D, 1E and 1F). Further, 4 months treatment of APP Tg mice with 20mg/ kg of SAC also caused robust increase in levels of SNAP25 and synaptophysin *versus* non-treated APP Tg mice (Fig. 6A and 6B), which is in accordance with the results obtained by AGE treatment to the Tg mice.

Treatment of 2% AGE was found to improve the hippocampal based memory tasks in the APP-Tg mice *versus* untreated APP-Tg mice (Chauhan & Sandoval 2007). SAC treatment data (Fig.7) show that spontaneous exploratory behavior remained unchanged in the littermate controls, as indicated by close-range entries in all arms (24-25 entries) and equal number of average alterations in all arms (31-32 alterations). Compared to littermate controls, the spontaneous exploratory behavior was declined by 1.25-fold reduced number of

entries in all arms in the Tgs fed with isocaloric control diet, and by 1.8-fold reduced average alterations in all arms at ($p<0.0003$, unpaired 2-tailed t-test). Feeding of SAC seemed to restore this deficit almost to a normal level as evidenced by 1.3-fold restored number of entries in all arms, and by 1.8-fold restored average alterations in all arms ($p<0.0001$, unpaired 2-tailed t-test).

Like SAC, we also tested another compound present in garlic, diallyl disulfide (DAD) in cell culture based studies. However, DAD failed to exert any neuro-protection/neurorescue effects against ROS mediated insults (data not shown).

Discussion

Restricting the progression and/or delaying the onset of the disease are the current strategies in AD treatment. Statistical analyses revealed that, if the onset of the disease can be delayed by 2 years, the prevalence of AD would approximately decrease by 2 million after 50 years (Brookmeyer *et al.* 1998). Identifying risk factors and minimizing their effect can potentially be an effective strategy to delay the disease progression associated with AD. For example, it has been proposed that controlling hyperglycemia, obesity and hypertension can delay the onset of AD (Yaffe 2007). In addition, studies have found that moderate physical exercise, engagement in intellectual activity such as reading, increased social interaction and consumption of diet containing fruit and vegetables have beneficial effects in the onset of AD (Angevaren *et al.* 2008, Stern 2006, Gillette Guyonnet *et al.* 2007, Lahiri 2006). Likewise, early diagnosis is another major area of AD research. In addition to molecular areas, recent advancements in brain imaging techniques may lead to the identification of markers well ahead of its onset (Mosconi *et al.* 2010, Nordberg *et al* . 2010). However, treatment options for AD are still limited.

To date, cholinesterase inhibitors (ChEI) such as donepezil, rivastigmine, galantamine and a partial NMDA receptor antagonist such as memantine are the only FDA approved drugs to treat AD (Ray *et al.* 2009) . The main purpose of ChEI is to inhibit enzymatic degradation of the neurotransmitter acetylcholine, resulting in increased amount of acetylcholine in the synaptic terminals(Pepeu & Giovannini 2010). Memantine, which is a partial NMDA receptor antagonist, protects neurons from glutamate induced excitatory damages (Alley et al. 2009). However, none of the FDA approved ChEI or memantine can completely restrict or reverse the disease progression associated with AD. As already mentioned, ROS-mediated damage to the neurons presumably precedes neuropathology associated with AD, suggesting that the intake of anti-oxidants might be beneficial in preventing or treating AD. Previous studies demonstrate that the intake of anti-oxidant rich food has a significant effect in the prevention of cognitive decline (Gillette Guyonnet et al. 2007). For example, it has been observed that higher intake of fruit and vegetables may reduce the risk of dementia (Hughes *et al.* 2009). However, Women Health Study (WHS) found that the oral administration of 600IU vitamin E to 6377 older women did not produce any significant cognitive benefit (Kang *et al.* 2006). Melatonin, which also possesses potential anti-oxidant and free radical scavenging properties, was shown to reduce the production of ROS from Aβ-metal ion complex (Zatta *et al.* 2003), and pre-clinical study did reveal that melatonin treatment reduces Aβ peptide in mice (Lahiri *et al.* 2004). However, convincing evidences for any potential benefit of melatonin in ameliorating symptoms of AD has not yet been generated. Similarly, many other anti-oxidants were tested in pre-clinical and clinical settings to evaluate their effects as potential therapeutic agents in the treatment of AD, but none of them convincingly demonstrated any effect in restricting or reversing the symptoms associated with AD (Cummings & Cole 2002).

Since AD is thought to have a multi-factorial etiology (Lahiri et al. 2003), we hypothesize that an agent that modulates multiple and diverse mechanisms and sites of action would be an effective candidate as a potential drug to restrict the development of AD. A large "Indo-US"epidemiological study revealed approximately 3.7 fold less incidence of AD in Indian elderly when compared to a reference US population (Chandra *et al.* 2001). Environmental factors including dietary habits like consumption of plant derived substances can potentially be responsible for this decreased incidence. In this context, curcumin was shown to have 'pleiotropic' mechanisms and several pre-clinical studies revealed its potential roles in ameliorating neuroinflammation associated with AD (Ray & Lahiri 2009, Frautschy & Cole 2010). However, poor aqueous solubility of curcumin may represent one of the reasons for not achieving its effects in clinical settings (Bisht *et al.* 2007).

Earlier it was shown that AGE prevents H_2O_2 mediated insults to the synaptosomes obtained from young rats (Brunetti *et al.* 2009). In our study, we have observed a direct neuro-protective effect of AGE and SAC, when neuronal cells were co-treated with both AGE or SAC and H_2O_2. Previously, it was documented that AGE and SAC treatment to the same APP-Tg mice studied herein, resulted in a decrease in the brain levels of both Aβ (1-40) and Aβ (1-42) (Chauhan & Sandoval 2007). Spatial learning and spatial memory retention were also improved in those mice that were treated with AGE and SAC. As ROS can be generated by the Aβ-glia or Aβ-metal ions interaction, independent action of AGE or SAC in decreasing Aβ and scavenging ROS represent a potential synergistic effect as a neuro-protective agent for the treatment of AD. In this study, AGE was also found to exert neuro-preserving action *in vitro*, when the neuronal cells were pre-treated with AGE for 48 hours and then challenged with toxic H_2O_2. These findings suggest that AGE's effect of preserving neuronal cells under oxidative stress is just not due to neutralizing ROS but modification of cell's defensive mechanisms against ROS. Our findings also suggest that the neuro-protection property of AGE against oxidative insults can be mediated by the action of SAC present in AGE. Antioxidant property of SAC was previously observed in different experimental models (Ide & Lau 1999, Imai et al. 1994, Maldonado *et al.* 2003). It was also reported that deposition of Aβ peptide in brain parenchyma facilitates cellular apoptosis by inducing caspases (Varma *et al.* 2009). AGE was found to have caspase 3 inhibitory effect, which might slow down the extent of neuronal loss caused by deposition of Aβ peptide (Jackson *et al.* 2002). Furthermore, treatment of AGE to Alzheimer's Tg2576 mice revealed decreased levels of hyperphosphorylation of tau protein in the central nervous system (Chauhan 2006).

It has been proposed that diffuse synaptic loss occurs in AD due to synergistic actions of generated ROS and Aβ oligomers (Selkoe 2002). Postmortem brain analysis of AD patients revealed a reduction in levels of pre-synaptic proteins synaptophysin and growth associated protein 32 (GAP 32) (Masliah et al. 2001). Decreased levels of pre-synaptic proteins that correspond with severity of the disease have also been observed. While the exact mechanism of synaptic damage by Aβ oligomers is still not fully understood, the alteration in NGF signaling pathway and abnormal calcium influxes following activation of NMDA receptors by globular Aβ oligomers play important roles (Yamamoto *et al.* 2007, Shankar *et al.* 2007). Since generalized neuronal and synaptic losses are major consequences as a result of Aβ deposition in the brain interstitium, the protection of neuron as well as synapses remains one of the major therapeutic strategies in AD. We have previously reported that rivastigmine increases the levels of two pre-synaptic proteins SNAP25 and synaptophysin in fetal primary neurons (Bailey & Lahiri), and reasoned that rivastigmine treatment imparts preservation of neurons and synapses. A similar neuro-preservative effect of memantine has also been observed in both primary neurons and differentiated PC12 cells (Bailey, Ray & Lahiri, unpublished data). However, whether these FDA-approved drugs can protect neurons in the presence of oxidative insult is not fully elucidated.

In this study, we have observed a time dependent loss of pre-synaptic proteins, SNAP25 and synaptophysin in the APP-Tg mice brain, consistent with the pathological consequence of gradual deposition of Aβ and subsequent neuro-inflammation. Interestingly, independent treatment of AGE and SAC significantly increased brain levels of SNAP25 and synaptophysin and in fact, restored their levels. We have also observed no significant change in brain levels of SNAP25 and synaptophysin in AGE treated mice sacrificed at the end of 4-week treatment period *versus* untreated Tg mice sacrificed at the beginning of the experiment. SAC (20mg/kg) also significantly increased the hippocampus based memory in APP-Tg mice. These findings demonstrate the potential for AGE and SAC to preserve pre-synaptic proteins and the pathology of the disease. Therefore, preservation of pre-synaptic proteins by AGE and SAC could be preserving neurons from ROS or Aβ mediated damage. Generalized loss of cholinergic neurons in AD results in severe cholinergic deficits. In this context, we have measured ChAT activity in differentiated human SK-N-SH cells as described earlier (Ray *et al.* 2009) and observed preservation of choline acetyltransferase (ChAT) activity by AGE and SAC from ROS mediated damage in differentiated human SK-N-SH cells (data not shown). Consistent with this hypothesis, we measured the levels one of the post synaptic markers PSD-95 in the brain lysate of AGE and SAC treated Tg mice, and observed an increasing trend of PSD-95 in AGE treated Tg mice, albeit not significantly (Ray & Lahiri, unpublished data). However, recent findings revealed that the change in the mRNA levels of PSD-95 in AD brain is region specific. For example, no change in PSD-95 mRNA levels was observed in hippocampus or occipital cortex of AD postmortem brain tissue, while the levels of PSD-95 were significantly decreased in inferior temporal cortex region (Proctor *et al.* 2010). One explanation of this could be a compensatory increase in the levels of post-synaptic proteins in response to severe synaptic loss seen in frontal cortex and hippocampus (Leuba *et al.* 2008). Earlier it was shown that SAC can destabilize Aβ fibrils *in vitro* (Gupta & Rao 2007). Furthermore, Aβ induced neuro-inflammation can also be ameliorated by SAC because of its ability to inhibit the activation of nuclear factor kappa beta (NFκB) (Ide & Lau 2001). Mechanistically, the property of Aβ lowering and consequent preservation of pre-synaptic proteins by AGE could be due to effects of SAC. Thus, this novel property of the garlic compounds may have significant implications in the context of drug development in AD.

In summary, our findings indicate that AGE treatment protects neuronal cells from ROS-mediated oxidative insults, and preserves the levels of pre-synaptic proteins that define pre-synaptic terminal structure. In addition, AGE treatment reverses ROS mediated decline in cholinergic function of the neuronal cells by increasing in levels of neuronal ChAT activity. Furthermore, loss of pre-synaptic proteins because of excessive deposition of Aβ in APP-Tg mice brain can also be prevented by oral AGE treatment. Previous studies demonstrate that AGE supplement can be beneficial in minimizing cardiovascular and diabetic risk factors contributing to AD. Because of the pleiotropic effects of AGE (discussed above), AGE and SAC can be developed as a potential therapeutic agent in the treatment of AD. Ultimately, these findings warrant evaluation of AGE's clinical potency in larger clinical settings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations used

AD	Alzheimer disease
Aβ	amyloid beta protein
AGE	aged garlic extract
APP	amyloid precursor protein
ChAT	choline acetyltransferase
ChEI	cholinesterase inhibitors
LDH	lactate dehydrogenase
LSM	low serum medium
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
PPARγ	peroxisome proliferator-activated receptor γ
PC12	rat pheochromocytoma
RA	retinoic acid
ROS	reactive oxygen species
SAC	S-allyl-L-cysteine
SNAP25	synaptosomal associated protein of 25 kDa
Tg	transgenic

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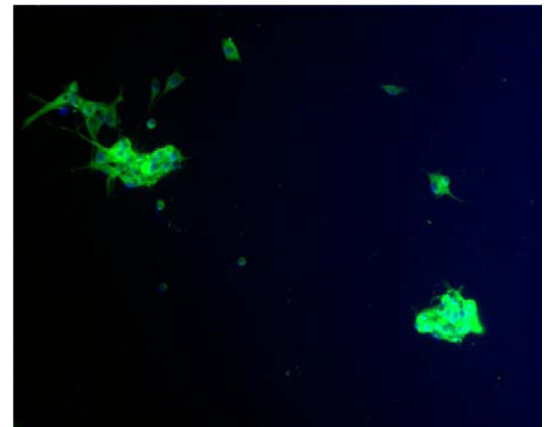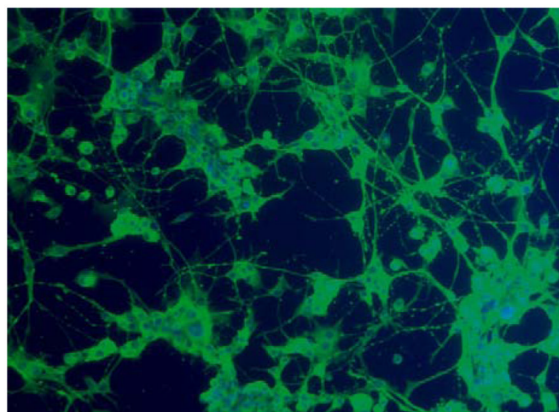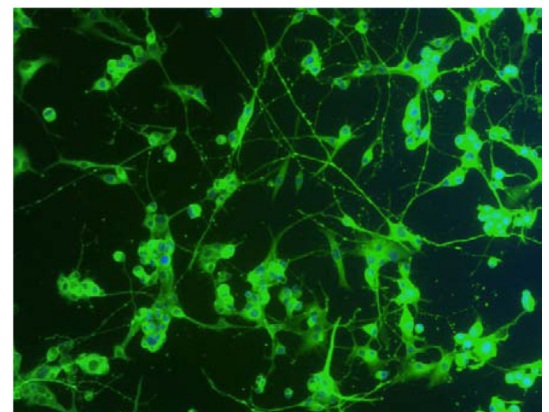
A

Cell viability (CTG) assay

B

Cell toxicity (LDH) assay
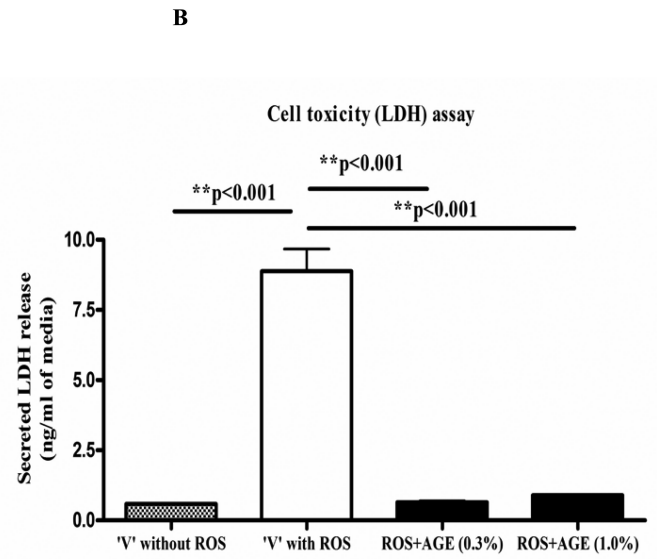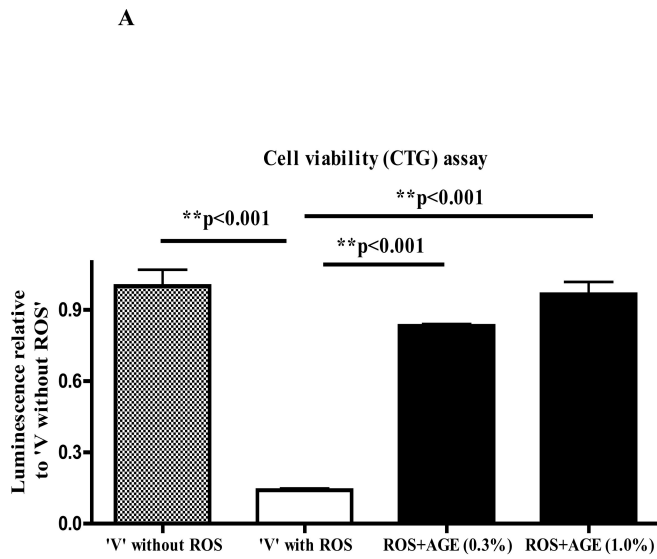
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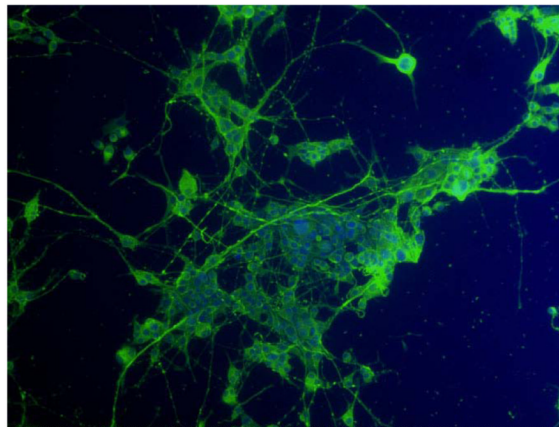
No ROS Vehicle

Vehicle with ROS

ROS + 0.3% AGE

ROS + 1.0% AGE

D

Cell Viability (CTG assay)

E

Cell toxicity (LDH) assay

F

No ROS Vehicle   **Vehicle with ROS**

ROS + 1uM SAC   **ROS + 2uM SAC**
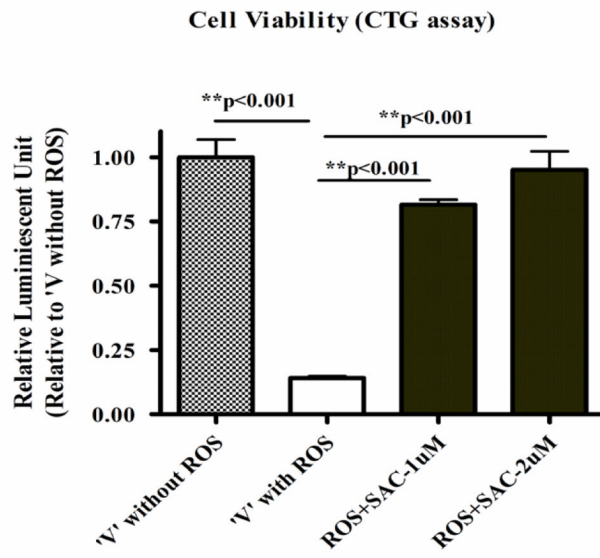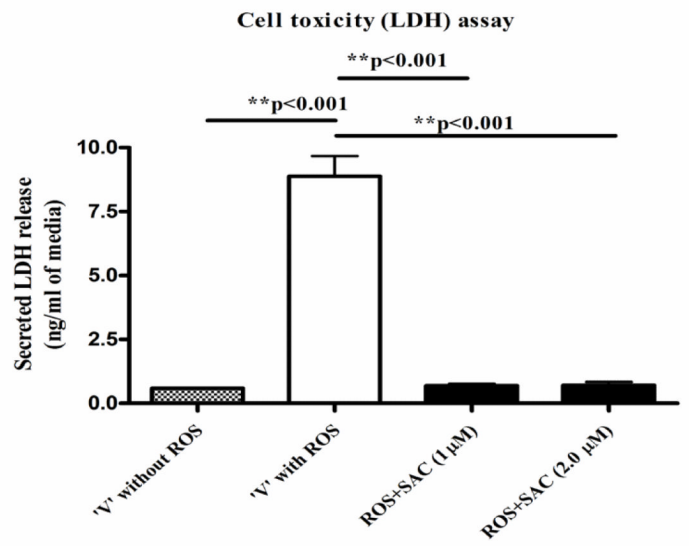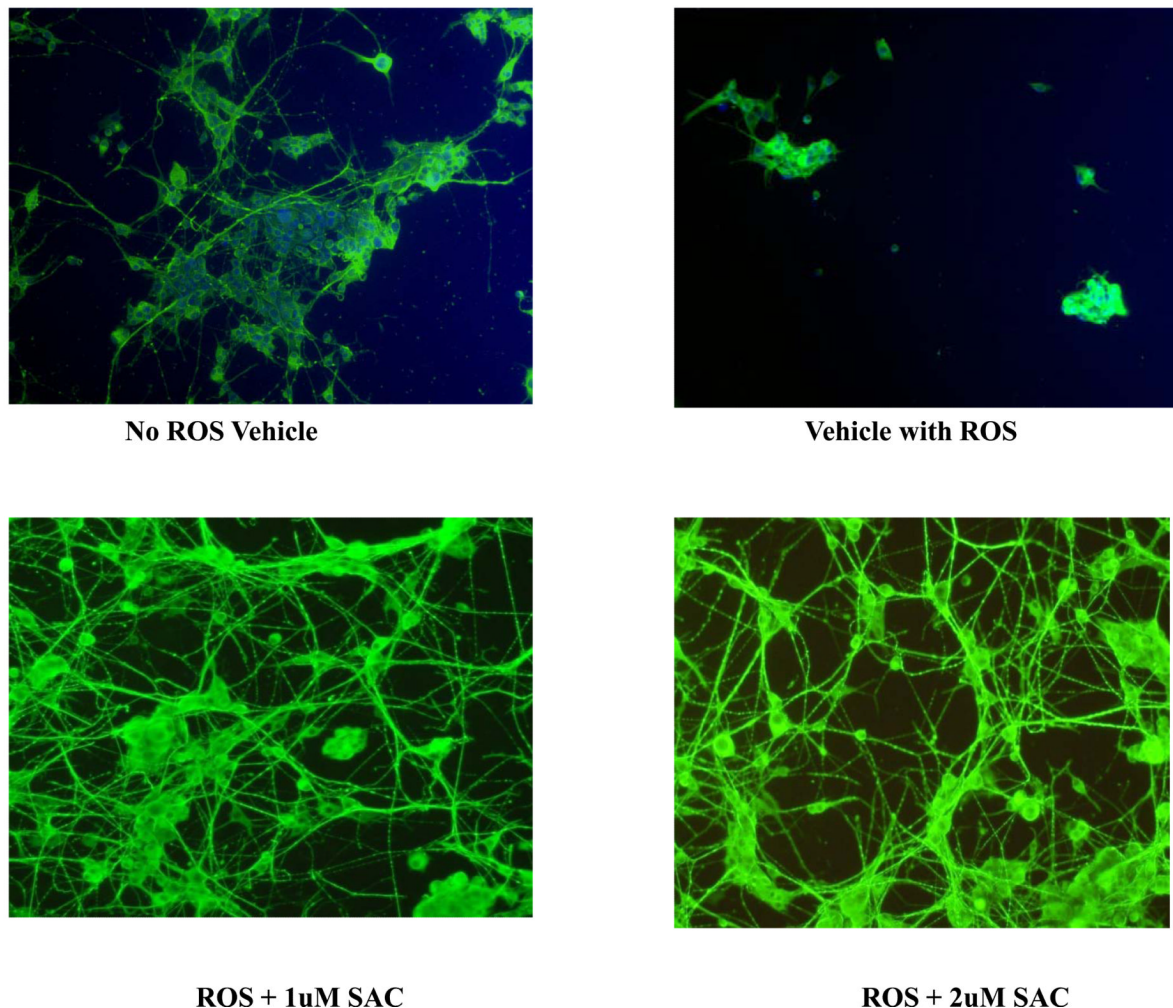
Fig. 1A. Differentiated PC12 cell co-treated with ROS and AGE: Cell viability assay
Differentiated PC12 cells (150,000/well) were co-treated with AGE (0.3% and 1.0%) and
200μM H_2O_2 for 48 hours. After harvest, the viability of cells of different treatment groups
was measured by CTG assay. The numbers in the 'X axis' represent 'relative luminescence
unit' and plotted relative to 'vehicle without ROS'. Results showed a significant loss of
viable cells with ROS (H_2O_2) treatment alone but increase in the viability of cells, which
were co-treated with AGE and H_2O_2 *versus* 'vehicle with ROS'.

Fig. 1B: Differentiated PC12 cell co-treated with ROS and AGE: LDH assay Cellular
toxicity was measured in the previous experiment with equal volume (15μl) of conditioned
media samples. Secreted LDH level was measured and plotted. Results indicate that LDH
release was significantly increased (damage) with ROS (H_2O_2) treatment but lowered in the
AGE and H_2O_2 co-treatment groups *versus* 'vehicle with ROS'. (In both Fig. 2A and 2B, the
error bars represent mean and standard deviation of the experiment performed in triplicate).

Fig. 1C: Differentiated PC12 cell co-treated with ROS and AGE: Cellular morphology
Visualization of the morphology of the cells in the previous treatment group by
immunocytochemistry (ICC) technique using α-tubulin (green) as a cytoskeleton marker,
and DAPI (blue) as nuclear marker. Data revealed a significant loss of cells and altered
cellular morphology in 'vehicle with ROS' group. Notably, AGE co-treatment with H_2O_2
increased the cell number and maintained the intercellular connections between the cells.

Fig. 1D: Differentiated PC12 cell co-treated with ROS and SAC: Cell viability assay
Differentiated PC12 cells (150,000/well) were co-treated with SAC (1μM and 2μM) and 200μM H_2O_2 for 48 hours as mentioned in Fig 1. After treatment, the viability of the cells of the treatment groups was measured by CTG assay Results showed a significant loss of viable cells with ROS (H_2O_2) treatment alone but increase in the viability of cells, which were co-treated with SAC and H_2O_2 *versus* 'vehicle with ROS'.

Fig. 1E: Differentiated PC12 cell co-treated with ROS and SAC: LDH assay Cellular toxicity (LDH assay) was measured with equal volume (15μl) of conditioned media samples. Results indicate that LDH release was significantly increased with ROS (H_2O_2) treatment and lowered in the SAC and H_2O_2 co-treatment groups *versus* 'vehicle with ROS'.

Fig. 1F: Differentiated PC12 cell co-treated with ROS and SAC: Cellular morphology
Visualization of the morphology of the cells after SAC and ROS co-treatment by immunocytochemistry (ICC) technique using α-tubulin (green) as a cellular marker. Data revealed a significant loss of cells and altered cellular morphology in 'vehicle with ROS' group. SAC co-treatment with H_2O_2 increased the cell number and also maintained the intercellular connections between the cells.
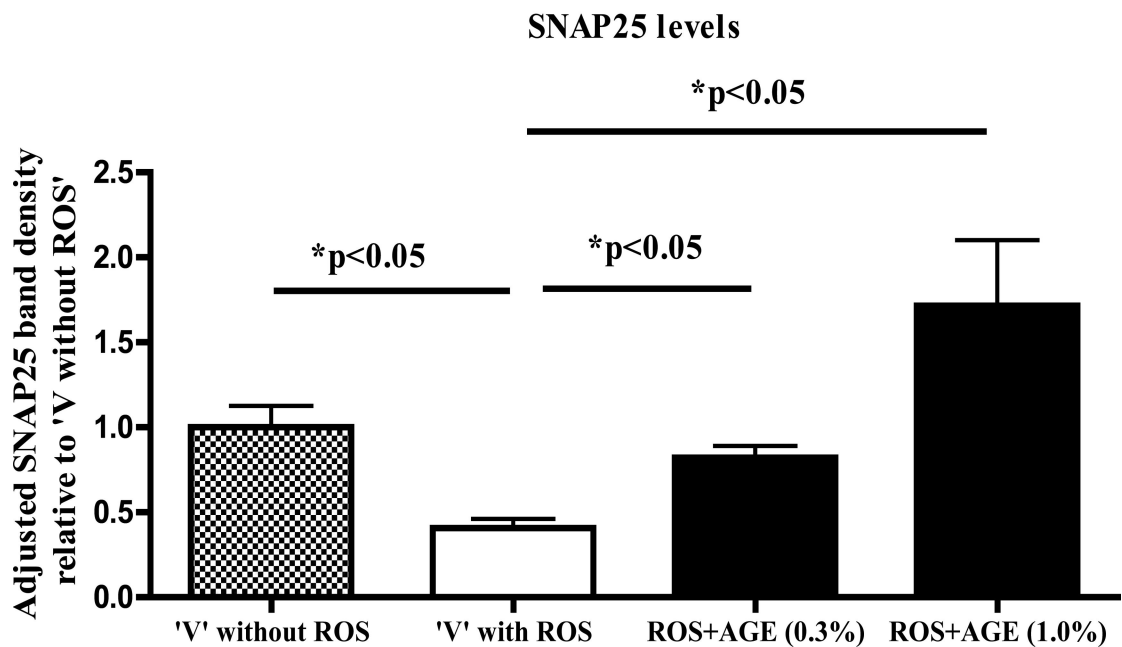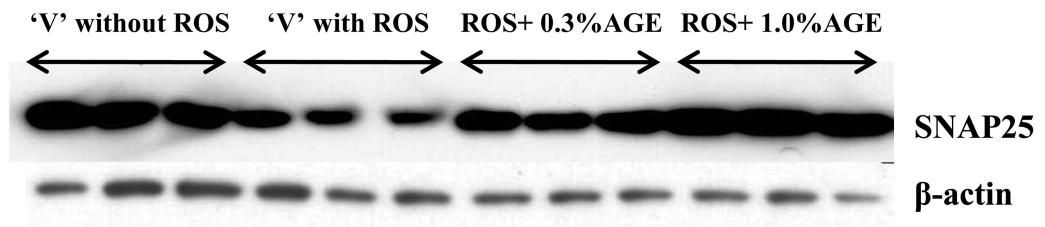
Fig. 2. Differentiated PC12 cell co-treated with ROS and AGE: analysis of pre-synaptic protein SNAP25

Western immunoblot analysis of the pre-synaptic protein SNAP25 from the cell lysate of the experiment mentioned in Fig.1 showed significance increase in levels of SNAP25 in AGE (0.3% and 1.0%) and 200μM H_2O_2 co-treated differentiated PC12 cells *versus* 'vehicle with ROS'. SNAP25 band densities were adjusted with the β-actin band signals and plotted relative to 'vehicle without ROS' with error bars, which represent mean and standard deviation of the experiment performed in triplicate.

A

Pre-treatment with AGE and post-treatment alone with ROS (Cell viability)
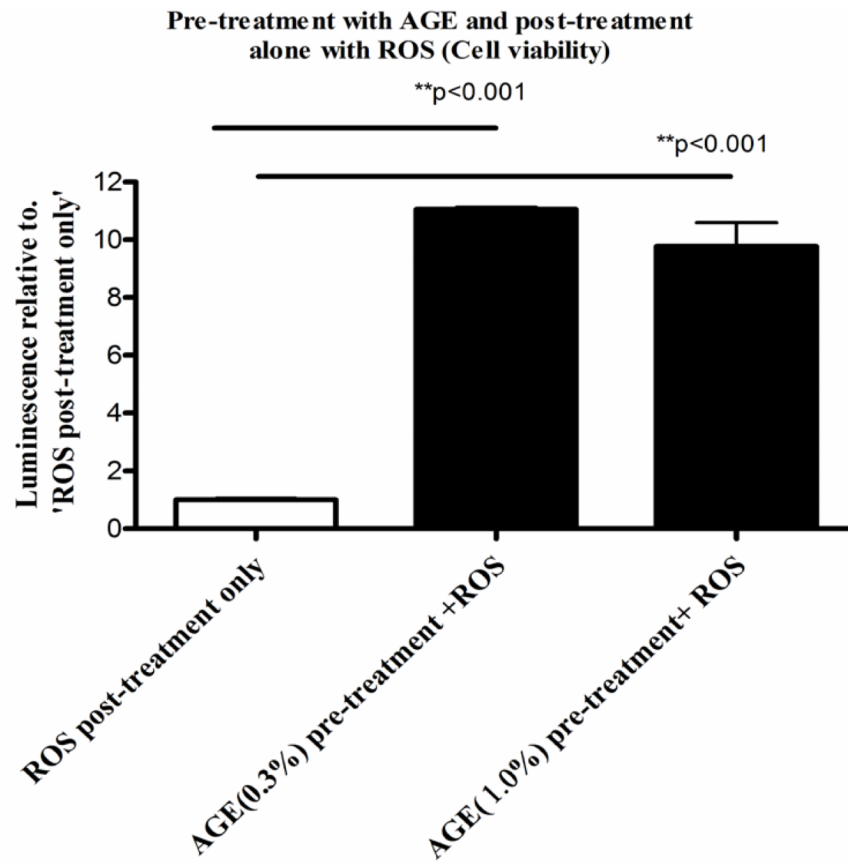
Pre-treatment (48Hrs)	None	AGE (0.3%)	AGE (1.0%)
After 48 Hrs	Washing off pre-treatment	Washing off pre-treatment	Washing off pre-treatment
Post-treatment (24Hrs)	ROS (200µM)	ROS (200µM)	ROS (200µM)

B
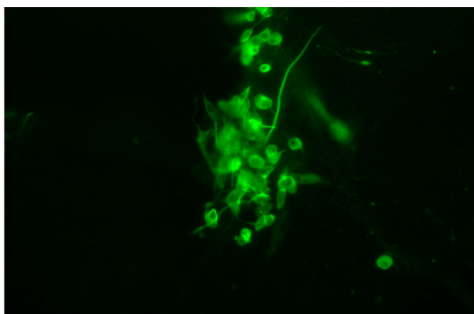
Differentiated PC12 cells with no AGE pre-treatment; post-treated with 200μM ROS for 24 hours
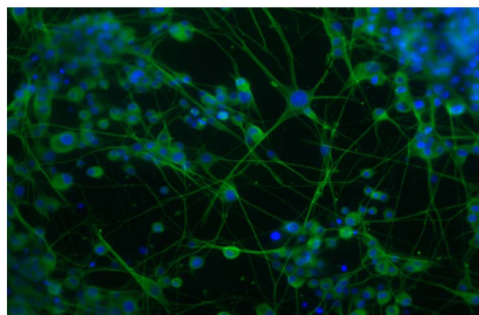
Differentiated PC12 cells pretreated with 0.3% AGE for 48 hours; post-treated with 200μM ROS for 24 hours
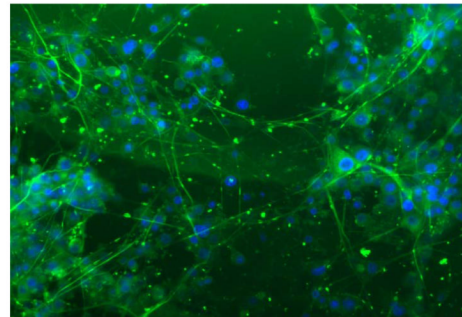
Differentiated PC12 cells pretreated with 1.0% AGE for 48 hours; post-treated with 200μM ROS for 24 hours

Fig. 3A. Differentiated PC12 cell pre-treated with AGE and post-treated with ROS: Cell viability assay

A novel neuro-rescue property of AGE was shown in this CTG data, where differentiated PC12 cells were pre-treated with 0.3% and 1.0% AGE without the presence of H_2O_2 for 48 hours and then treated with 200μM of H_2O_2 alone for 24 hours. Cell viability was almost completely lost by ROS (H_2O_2) without any prior treatment of AGE. Notably, there was a significant increase in cell viability in the AGE pre-treated and H_2O_2 post-treated cells *versus* cells which were only post-treated with H_2O_2.
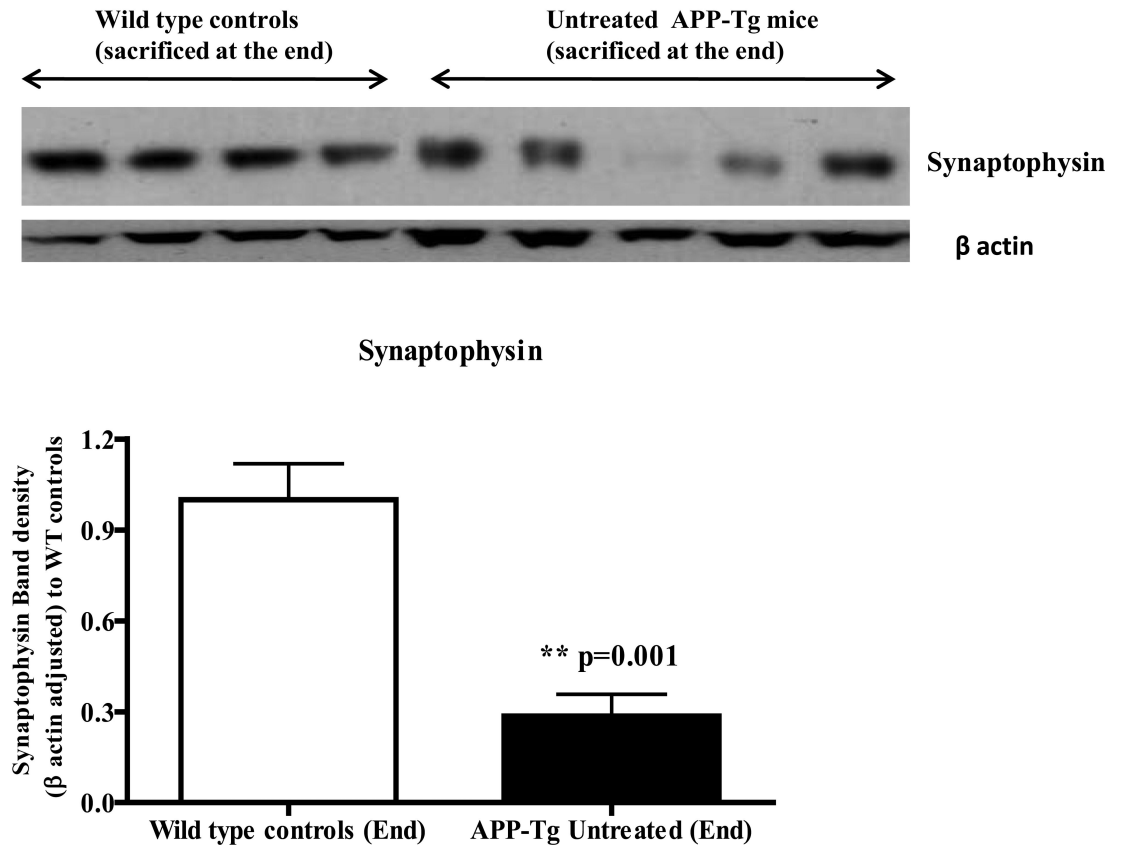
Fig. 3B: Differentiated PC12 cell pre-treated with AGE and post-treated with ROS: Cellular morphology Cellular morphology of the previous experiment was studied by immunocytochemistry using α-tubulin (green) as a cytoskeleton marker and DAPI (blue) as nuclear marker. A gross reduction in cell number with complete loss of neuritic outgrowth was observed in the cells which were not pre-treated with AGE but post-treated with 200μM of H_2O_2 for 24 hours. AGE pre-treatment for 48 hours not only rescues neuronal cells from dying, but also preserves neurite connections even in the presence of ROS (H_2O_2).

A

B

Wild type controls
(sacrificed at the end)

Untreated APP-Tg mice
(sacrificed at the end)
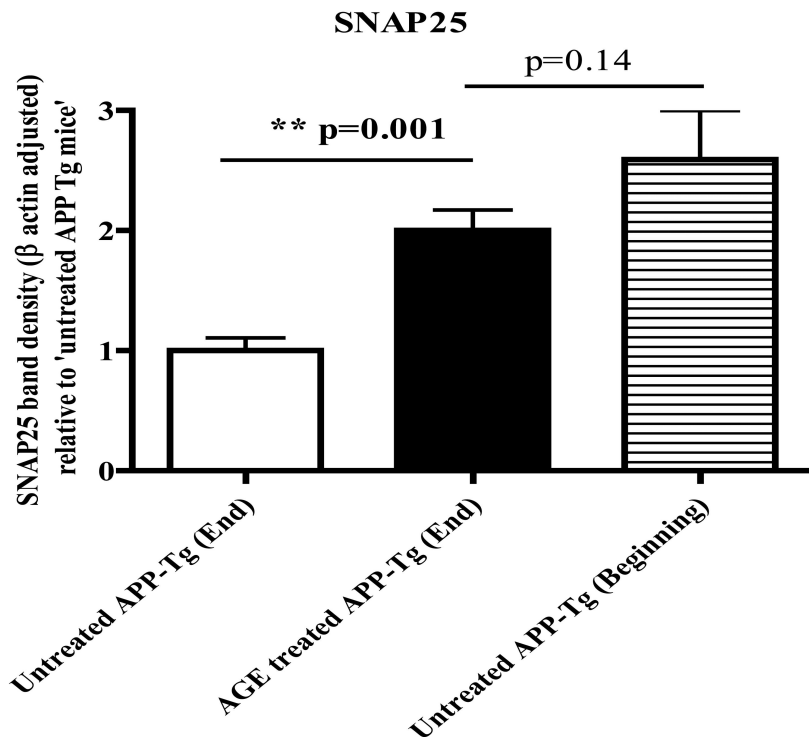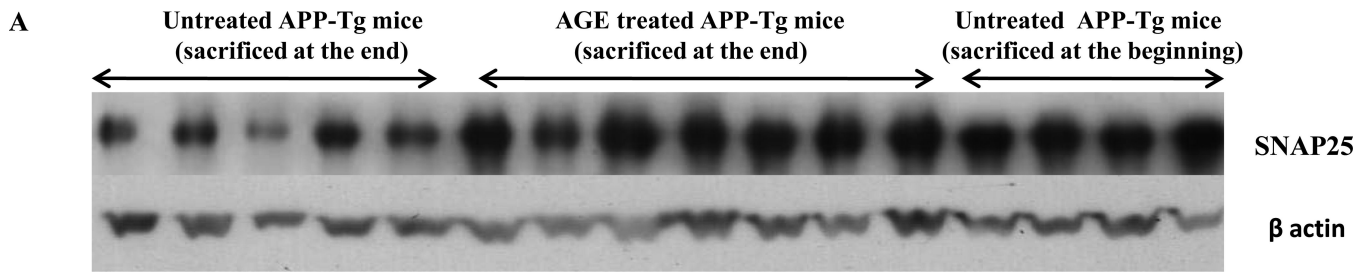

Synaptophysin
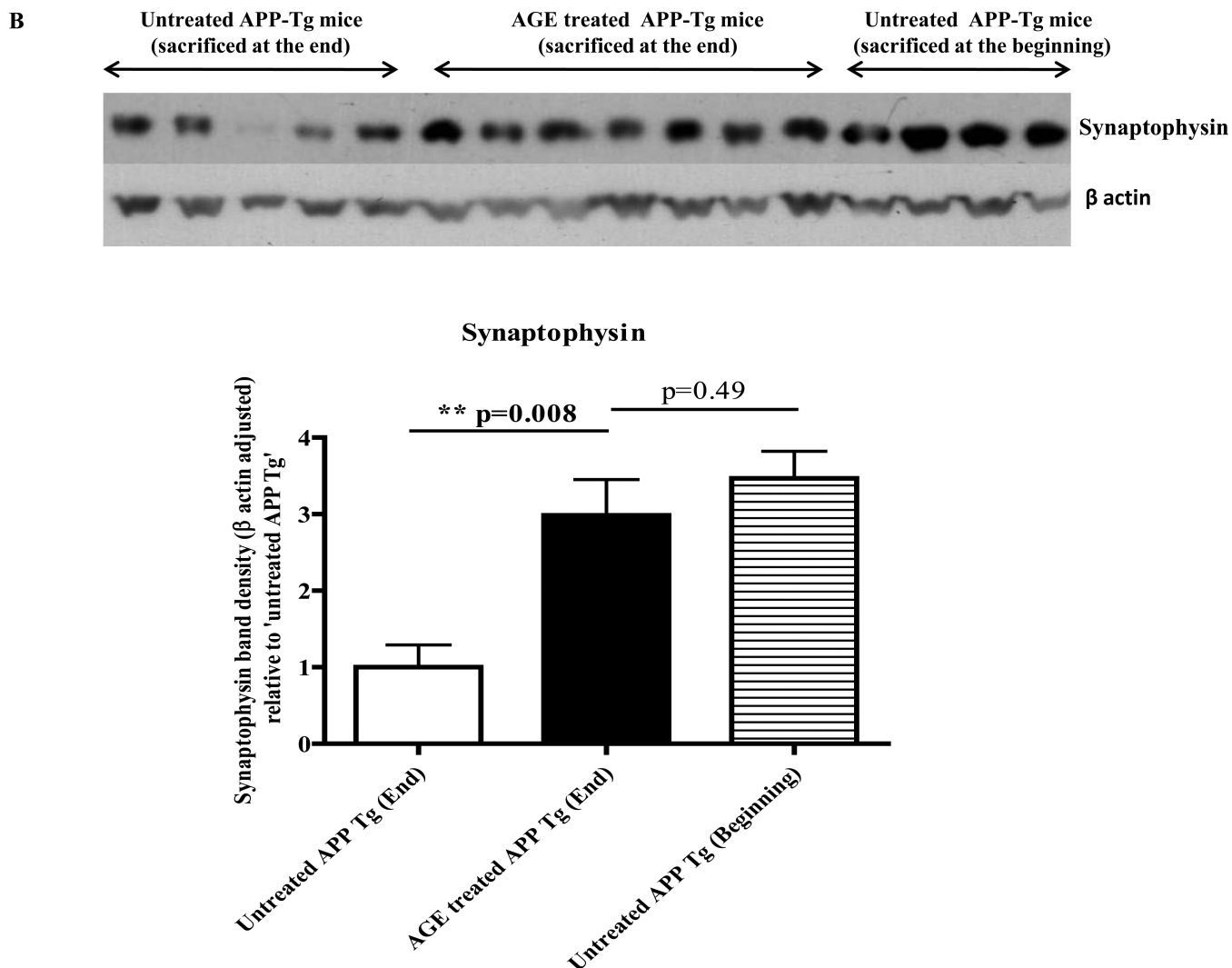
β actin

Synaptophysin
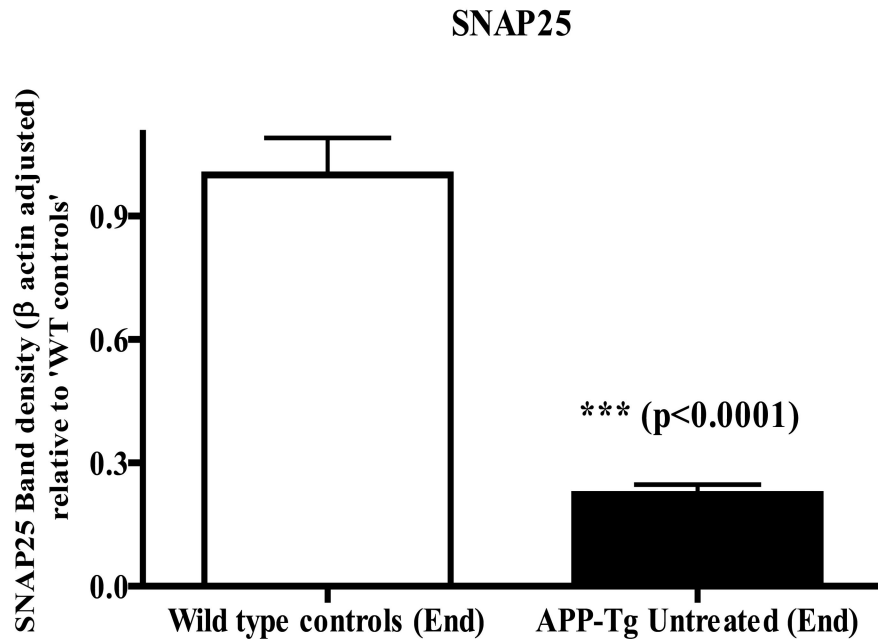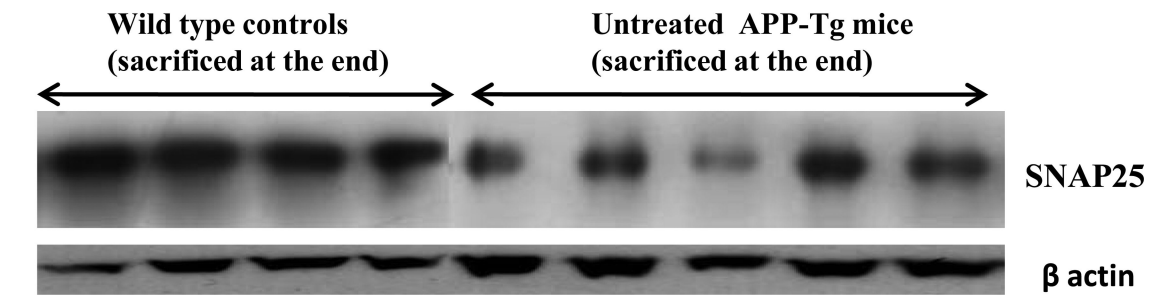
Fig.4A. Levels of SNAP25 in wild type *versus* APP-Tg mice brain lysate
Western immunoblotting with wild type and APP Tg mice brain lysates revealed a significant decrease in brain levels of the pre-synaptic protein SNAP25 in the untreated APP Tg mice *versus* wild type controls of the same age. SNAP25 band densities were adjusted with the corresponding β-actin band densities and plotted relative to 'wild type controls' (n=4 for wild type mice and n=5 for Tg mice).

Fig. 4B: Levels of synaptophysin in wild type *versus* APP-Tg mice brain lysate: A similar significant decrease in the brain levels of another pre-synaptic protein synaptophysin was observed in the untreated Tg mice *versus* wild type controls of the same age. Synaptophysin band densities were adjusted with the corresponding β-actin band densities and plotted relative to 'wild type controls'.

A

Untreated APP-Tg mice
(sacrificed at the end)

AGE treated APP-Tg mice
(sacrificed at the end)

Untreated APP-Tg mice
(sacrificed at the beginning)

SNAP25

β actin

SNAP25
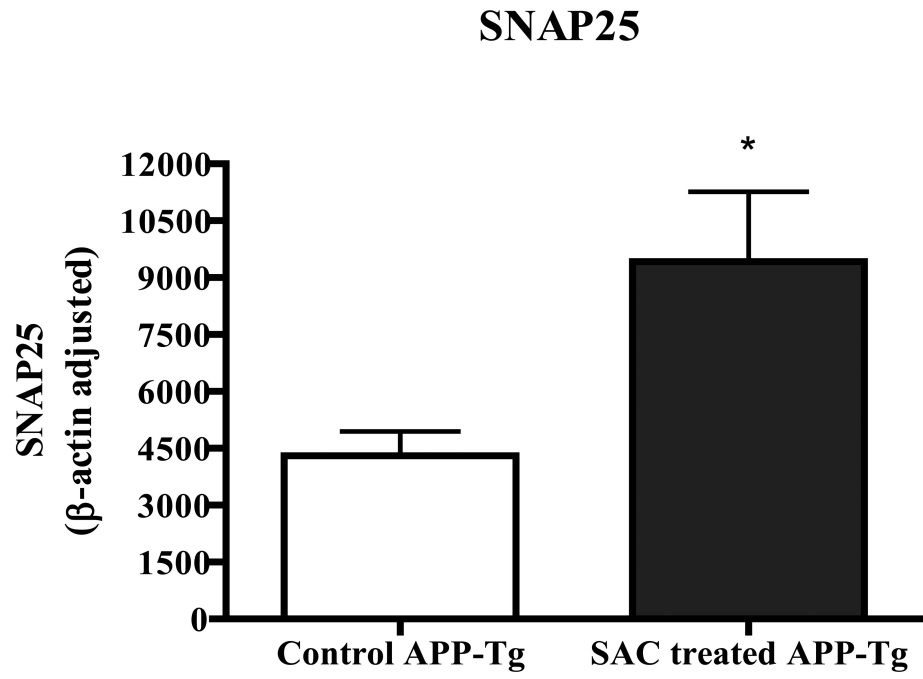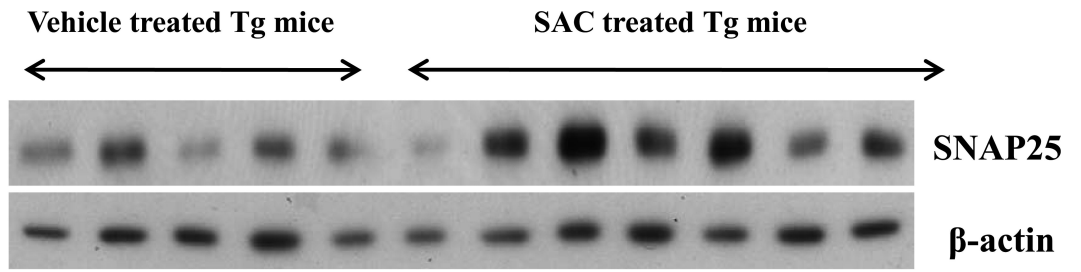
B

Synaptophysin

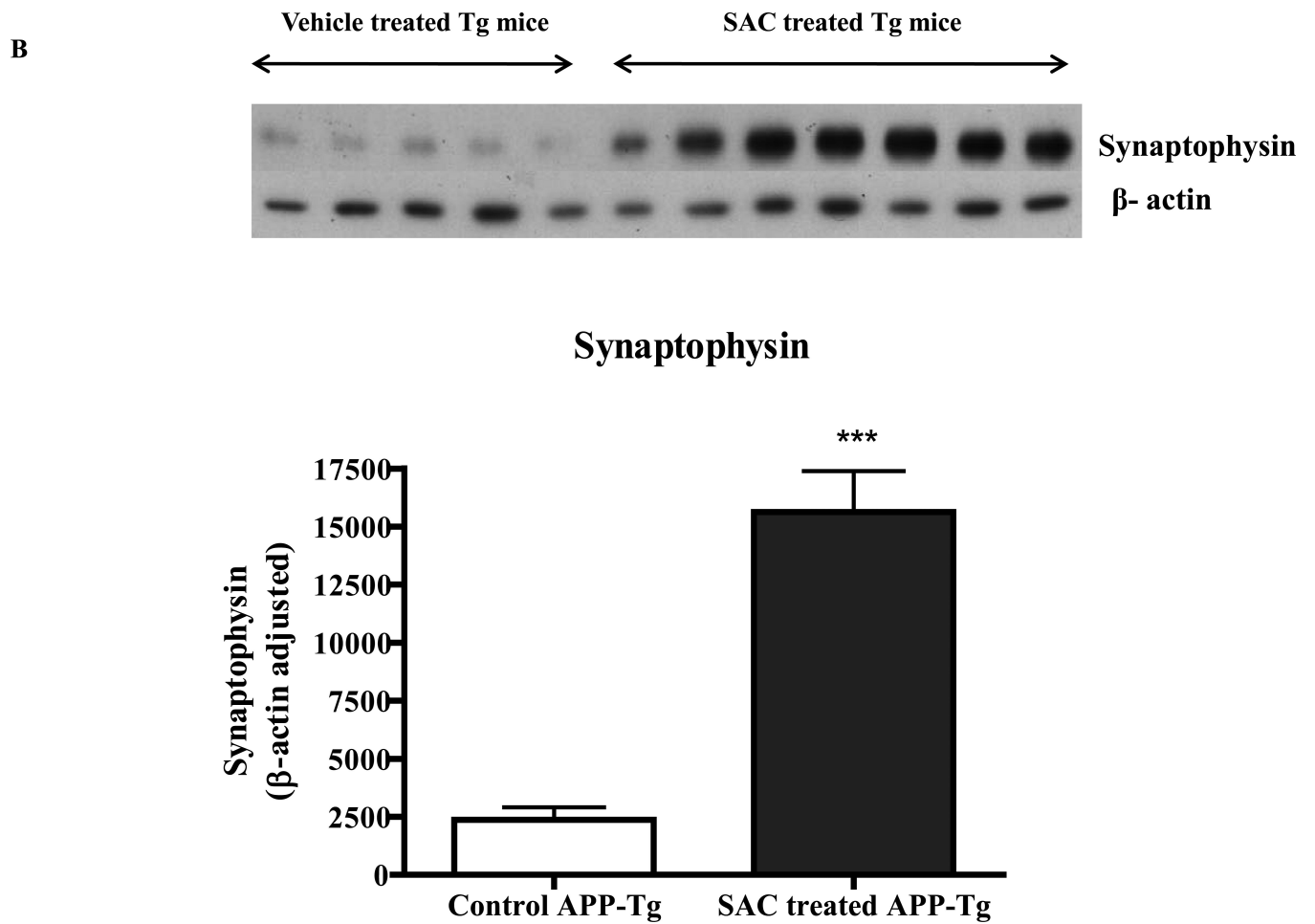**Fig. 5A. Levels of SNAP25 in the brain lysate of AGE treated APP-Tg mice *versus* untreated APP-Tg mice**

Western immunoblotting of the mice brain lysate showed a gradual time dependent decrease in brain levels of SNAP25 in untreated APP Tg mice, which were sacrificed after the 4-week experiment *versus* Tg mice sacrificed at the beginning of the 4-week experiment. Treatment of AGE significantly increases brain levels of SNAP25 *versus* untreated mice. There is no significant difference in brain levels of SNAP25 in AGE treated Tg mice sacrificed at the end of 4-week experiment *versus* untreated Tg mice sacrificed at the beginning of the experiment. Beta actin adjusted SNAP25 band densities were plotted relative to 'untreated APP Tg mice' sacrificed at the end of the experiment. (n=5 for untreated Tg mice; n=7 for AGE treated Tg mice and n=4 for untreated Tg mice sacrificed at the beginning of the 4-week experiment).

Fig. 5B: Levels of synaptophysin in the brain lysate of AGE treated APP-Tg mice *versus* untreated APP-Tg mice: Similar results of SNAP25 were obtained in brain levels of synaptophysin as detected by Western immunoblotting. There is a significant increase in the levels of synaptophysin in AGE treated APP Tg mice *versus* untreated. A time dependent decrease in the levels of synaptophysin was also observed in Tg mice brain and no significant change was detected in the brain levels of synaptophysin between untreated Tg mice sacrificed at the beginning of the 4-week experiment *versus* AGE treated Tg mice

sacrificed at the end of the experiment. Beta actin adjusted synaptophysin band densities were plotted relative to 'untreated APP Tg mice' sacrificed at the end of the experiment. Thus AGE treatment could prevent time-dependent decline of SNAP25 and synaptophysin in APP Tg mice brain.

A

Vehicle treated Tg mice   **SAC treated Tg mice**

SNAP25

β-actin

SNAP25

B

Vehicle treated Tg mice　　　　　　**SAC treated Tg mice**

Synaptophysin

β- actin

Synaptophysin

Fig. 6A and Fig. 6B. Levels of SNAP25 and synaptophysin in the brain lysate of SAC treated APP-Tg mice *versus* untreated APP-Tg mice
Western immunoblot analyses of four months SAC (20mg/kg/day) treated APP-Tg mice brain lysate resulted in significant increase in the levels of pre-synaptic proteins SNAP25 and synaptophysin.
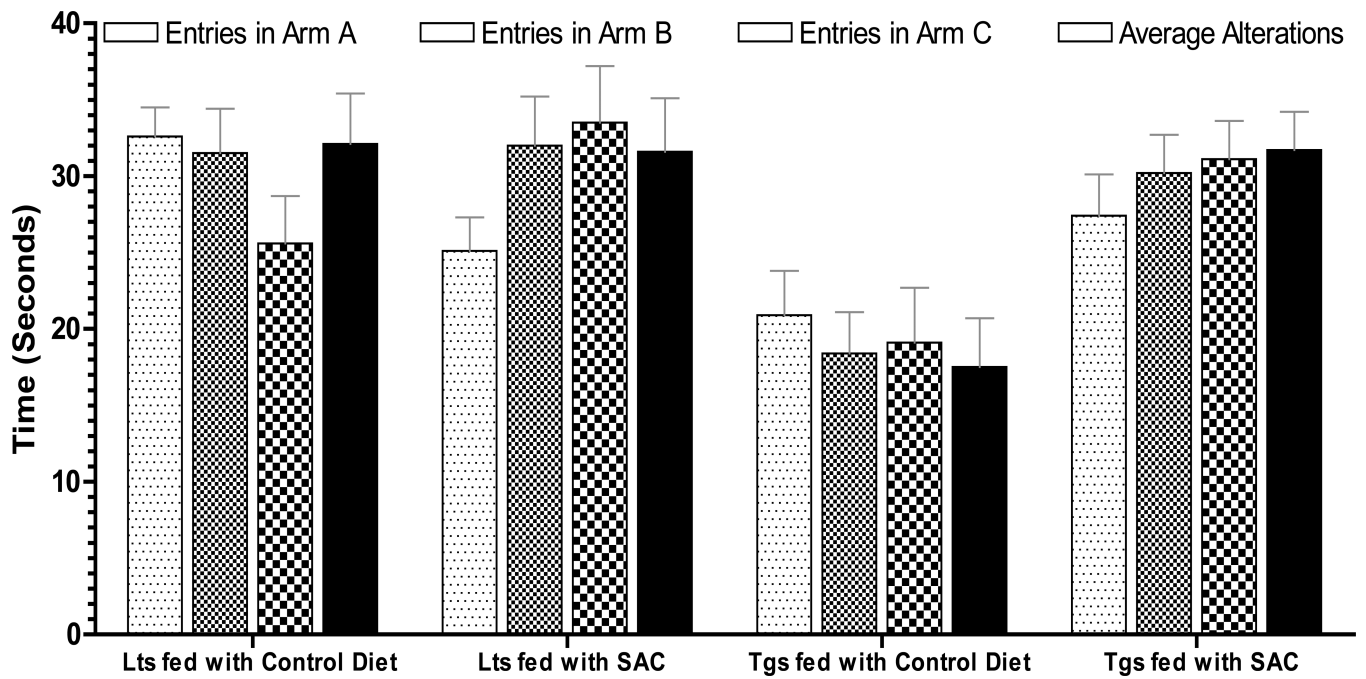
Fig. 7. Effect of SAC treatment in Y maze spontaneous exploration
Hippocampal-based spontaneous alterations and exploration behavior in Y maze after SAC treatment in Tg2576 mice. Note that, impaired exploratory behavior in Tgs fed with isocaloric control diet, and restored spontaneous exploration in Tgs fed with SAC diet.