CHAPTER III

MATERIALS AND METHODS

3.1 The functional relationship between iron, glycogen synthase kinase-3β and microglial activation

3.1.1 Reagents

Lipopolysaccharide (LPS; *Escherichia coli* serotype 026-B6), lithium (LiCl), ferric ammonium citrate, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), gelatin and goat anti-rabbit IgG horse radish peroxidase (HRP)-conjugated secondary antibody were purchased from Sigma (St. Louis, MO). All cell culture reagents were obtained from Hyclone (South Logan, UT).

3.1.2 Cell culture

Murine BV2 microglial cells and murine neuroblastoma (NA) cells were kind gifts from Dr. James R Connor (Department of Neurosurgery, The Pennsylvania State University, College of Medicine, Hershey, PA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS), 2 mM L-glutamine, 100μg/ml streptomycin and 100U/ml penicillin at 37°C under humidified 95% O_2 and 5% CO_2. The medium was refreshed twice a week. See appendix A for preparing the medium.

3.1.3 Western blotting analysis

BV2 microglia were first seeded into cell culture flasks and allowed to grow for 24 hour (hr) in growth medium. The medium was removed and replaced with freshly prepared FBS-free medium containing with LPS, iron citrate or LiCl as indicated in the figure. After 1 hr of treatments, the medium was removed and whole cells lysates were prepared in a lysis buffer containing 50 mM Tris, pH 8.0, 150 mM

NaCl, 0.5% Triton, and protease inhibitors. Samples were assayed for protein concentration using the BCA protein assay kit (Pierce, Rockford, IL). Protein (50 μg for each sample) were mixed with sample buffer containing beta-mercaptoethanol, boiled for 10 min, and separated on 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Then, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Pierce, Rockford, IL), blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline with Tween-20 (TBST) solution for 1 hr at room temperature, rinsed with TBST buffered, and incubated overnight at 4°C in primary antibody specific to rabbit anti-phospho-Ser9-GSK-3β or rabbit anti-total-GSK-3β polyclonal antibody or mouse anti beta actin monoclonal antibodies (1:1,000 dilution in TBST) (Cell Signaling Technology, Beverly, MA). Subsequently, the membranes were washed with TBST and incubated in the secondary antibody (1:3,000 dilution in TBST) goat anti-rabbit or goat anti-mouse IgG (H+L), HRP conjugate (Sigma, St. Louis, MO) for 2 hr at room temperature. The blots were developed using SuperSignal West Pico chemiluminescence (Pierce, Rockford, IL) and then exposed on CL-X Posture film (Pierce, Rockford, IL). The band intensity was determined by Scion Image analysis software (Scion, Frederick, MA).

3.1.4 Measurement of NF-κB p65 nuclear translocation

Immunofluorescence assay. BV2 microglia were first seeded in six-chamber slides at a density of $2x10^4$ cells/chamber and allowed to grow for 24 hr in growth medium. The medium was removed and replaced with freshly prepared FBS-free medium containing with LPS, iron citrate or LiCl as indicated in the figure. After 4 hr of treatments, the medium was removed and cells were fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) (pH 7.5) at room temperature. Cells were then washed and permeabilized with 0.1% Triton X-100 for 5 min at room temperature. Permeabilized cells were washed 3 times with PBS and incubated in blocking solution (10% goat serum, 1% bovine serum albumin, 0.5%

Tween 20 in PBS) for 1 hr at room temperature to remove nonspecific binding of the antibody. All subsequent steps were carried out at room temperature. Cells were incubated with a 1:200 dilution of rabbit anti-p65 (Santa Cruz, CA) in 1% goat serum overnight at 4°C. Cells were washed and incubated for 1 hr with Alexa-Fluor 488-conjugated goat anti-rabbit secondary antibody (Invitrogen, Carlsbad, CA) at a 1:1,000 dilution in PBS containing 1% BSA and then for 10 min with DAPI for staining of nuclei. Coverslips were mounted with 50% glycerol and examined with a Leica confocal microscope (Bannockburn, IL).

NF-κB ELISA assay. BV2 microglia were first seeded in 6-well plates at a density of $5x10^5$cells/well, allowed to grow for 24 hr and treated as indicated in the figure. After 4 hr of treatments, the medium was removed and cells were washed twice with ice-cold PBS, scraped off with a rubber policeman and centrifuged at 1,600 rpm for 10 min at 4°C. To prepare nuclear extracts, cells were resuspended in a buffer containing 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, and 0.2 mM PMSF, followed by vigorous vortex for 15 sec before standing at 4°C for 12 min. Then, samples were centrifuged at 2000 rpm for 3 min. The pelleted nuclei were resuspended in 30 μl buffer containing 20 mM HEPES (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.2 mM PMSF and incubated for 20 min on ice, and nuclear lysates were then centrifuged at 15,000 rpm for 3 min. Supernatants containing the solubilized nuclear proteins were collected and the amount of NF-κB in the nuclear fractions was measured by a NF-κB p65 ACTIVELISA kit (BIOCARTA, Carlsbad, CA) according the manufacturer's instructions.

3.1.5 Measurement of IL-1β, TNF-α, NO and MMP-9 levels in culture supernatants

BV2 microglia ($1x10^5$ cells/well) were first seeded in 24-well plates with DMEM medium supplemented with 5% FBS and incubated for 24 hr. Then, the

medium was removed and replaced with FBS-free medium with or without LPS (1μg/ml), iron citrate or LiCl (10 mM). Twenty-four hours after treatment alone or after combined treatments, cell culture supernatants were collected.

Enzyme-linked immunosorbent assay (ELISA). IL-1β and TNF-α in culture supernatants were measured using ELISA kits from R&D Systems (Minneapolis, MN). According to manufacturer's instructions, a 50 μl of assay diluent was added to each well followed by 50 μl of each sample or standard samples to the antibody coated plates. Plates was covered, incubated at room tem-perature for 2 hr, washed 4 times with wash buffer before adding biotinylated antibody and incubated at room temperature for additional 2 hr. Then, substrate solution was added to each well and incubated for 30 min at room temperature. The enzyme reaction was stopped by adding stop solution into each well. The absorbance at 450 nm was determined using a microplate reader (Bi0-Tek Instruments Inc., Winooski, VT). The concentrations of IL-1β and TNF-α in each sample were then calculated with reference to the standard curves.

Nitric oxide (NO) assay. NO levels in the culture supernatants were determined by measuring nitrite accumulation in the cell culture supernatants using Griess reagent (1% sulfanilamide/0.1% N-(1-naphthyl)-ethylenediamine dinydrochloride/2.5% phosphoric acid) (Invitrogen, Carlsbad, CA). Briefly, 150 ul/well of the nitrite-containing sample were place in 96-well plate, mixed with 20 μl of Griess reagent and 130 μl of deionized water and incubated for 30 min at room temperature. Absorbance values were read at 540 nm using a microplate reader (Bi0-Tek Instruments Inc., Winooski, VT). The concentrations of NO were calculated with reference to a standard curve generated from known concentrations of sodium nitrite.

Gelatin zymography. Secreted MMP-9 was analyzed by gelatin zymography, as previously described (Kaomongkolgit et al., 2008). Briefly, after 24 hr of treatments, the culture supernatants were collected and centrifuged at 1500 x g for 15 min to remove cellular debris. Proteins in the supernatant were concentrated in

centriprep-10 concentrators (Millipore) to approxi-mately 25 fold by centrifugation at 12000 x g. Total protein concentration in each con-centrated sample was determined using the BCA protein assay kit (Pierce, Rockford, IL). Samples (50 µg/lane) were mixed with 2x non-reducing sample buffer and incubated at room temperature for 10 min. Protein samples were separated on 10% SDS-PAGE containing 1 mg/ml gelatin. Then, the gelatin gels were washed three times with 2.5% Triton X-100, incubated in a buffer containing 50 mM Tris, 10 mM CaCl2, 0.15 M NaCl, and 0.02% Briji-35 at 37°C overnight stained with 0.1% Coomassie Brilliant Blue R250 in 7.5% acetic acid and 12.5% methanol for 30 min. Finally, gels were destained in 7.5% acetic acid and 5% methanol until transparent bands were visible on a dark blue background. The relative molecular weights of the clear bands were determined using pre-stained protein markers (Fermentus, Hanover, MD). Quantitative analysis of band density was performed using Scion Image analysis software (Scion, Frederick, MA).

3.1.6 RNA Isolation

After 6 hr of treatment, total RNA from each condition was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Briefly, the medium in each well was completely removed. The cells were washed once with PBS, pH 7.4. Then, Trizol reagent (300 µl) was added into each well. The homogenates were placed on a low speed rotator for 5 min at room temperature. This is to allow the complete dissociation of nucleoprotein complexes. Next, the homogenates were transferred into steriled 1.5 ml tubes. Then, chloroform (60 µl) was added into each tube followed by vortexing vigorously for 15 sec. The mixture was allowed to stay at room temperature for 5 min before centrifugation at 12,000 g for 15 min at 4°C. RNA in the colorless upper aqueous phase was carefully transferred to a fresh tube. Then, isopropanol (150 µl) was added into each tube. Each sample was stored at room temperature for 10 min and centrifuged at 12,000 g for 10 min at 4°C. RNA will be precipitated and form a white pellet at the bottom of the tube. The

supernatant was carefully removed before adding iced-cold 75% ethanol (300 µl) into each tube to wash RNA pellet for 5 min. Subsequently, the pellet was centrifuged at 10,000 g at 4°C for 5 min. At the end of procedure, the RNA pellets were allowed to air dry briefly. It is important not to let the RNA pellet dry completely as it will greatly decrease its solubility. Dissolve the RNA pellets in RNase free water. The amount of RNA obtained by spectophotometry was calculated using the formular below.

RNA (µg) = Absorbance at 260 nm x 40 x dilution factor

The solution containing RNA sample can be stored at -80°C until use.

3.1.7 Reverse transcription (cDNA synthesis)

A volume of 2 µg total RNA from each sample was added into the reaction mixture containing 10x reaction buffer, 25mM $MgCl_2$, dNTPs, random primer, Ribonuclease inhibitor, AMV Reverse transcriptase and RNase free water. All reagents were purchased from Promega (Promega, Madison, WI). Each sample was kept at room temperature for 10 min incubated at 42°C for 60 min followed by inactivation at 99°C for 5 min. Then, distilled water was added into each sample to a final volume of 100 µl. At this point, each sample can be kept at -20°C until use. See appendix B for preparing the reaction mix.

3.1.8 Polymerase Chain Reaction (PCR)

The PCR reaction was performed in a total volume of 25 µl. A 5 µl of cDNA (as described above) was use as templates for subsequent PCR reaction in the perkin Elmer Thermocycle. PCR reaction was composed of distilled water, PCR buffer, dNTP mix (Promega, Madison, WI), Taq DNA polymerase (Fermentus, Hanover, MD) and primer pairs (see Table 3). To ensure there was no genomic DNA contamination in the RNA samples, the PCR reaction was performed without reverse transcription. As a control reaction, the RNase free water was replaced the RNA samples in the PCR

protocol. Subsequently, amplified PCR products were visualized by gel electrophoresis. Briefly, a 10 µl of each PCR product was mixed with 2 µl of loading buffer, vortexed, spined, and then loaded on to 1.5 % agarose gel. The gel was run at 95 volts until front dye reaches the end of gel. The gel was strained with ethidium bromide (0.5µg/ml). Verification of specific gene was established by their predicted size under ultraviolet (UV) light. Estimate size of PCR products was compared to the DNA marker (100 bp ladder, Fermentus, Hanover, MD). Quantitative analysis of band density was performed using Scion Image analysis software (Scion, Frederick, MA). See appendix B for preparing the reaction mix.

Table 3 Specific primer for iNOS, TNF-α, IL-1β, MMP-9, and GAPDH

Gene	Primer sequences (5′→ 3′)	bp.	PCR Protocols
iNOS	ATC CCG AAA CGC TAC ACT TCC	302	1
	GGC GAA GAA CAA TCC ACA ACT C		
TNF-α	ATC CGA GAT GTG GAA CTG GCA	659	2
	CCG GAC TCC GTG ATG TCT AAG T		
IL-1β	GCT ATG GCA ACT GTC CCT GAA C	200	3
	TGA GTG ACA CTG CCT TCC TGA A		
GAPDH	AAG CTC ACT GGC ATG GCC TTC C	322	4
	TTG GAG GCC ATG TAG GCC ATG AG		
MMP-9	ATC CAG TTT GGT GTC GCG GAG	222	5
	AAT AGG AGC CCT CGA AGG		

Protocol 1 Pre denature at 94°C for 4 min followed by 35 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 2 min with final extension at 72°C for 4 min

Protocol 2 Pre denature at 94°C for 4 min followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 2 min with final extension at 72°C for 4 min

Protocol 3 Pre denature at 94°C for 4 min followed by 32 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 2 min with final extension at 72°C for 4 min

Protocol 4 Pre denature at 94°C for 4 min followed by 35 cycles of 94°C for 1 min, 61°C for 1 min, 72°C for 2 min with final extension at 72°C for 4 min

Protocol 5 Pre denature at 94°C for 4 min followed by 32 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 2 min with final extension at 72°C for 4 min.

3.1.9 Neurotoxicity assay

The effect of conditioned medium from LPS-induced microglial activation with different iron status on the survival of neuronal cells was determined by the MTT reduction assay. In brief, BV2 microglia were seeded in 24-well plates at a density of $1x10^5$ cells/well and allowed to grow for 24 hr in growth medium at 37°C and 5% CO_2. Then, the medium was removed and replaced with freshly prepared FBS-free medium containing with LPS, iron citrate or LiCl as indicated in the figure. Twenty-four hours after incubation, the medium were centrifuged and collected. In a separate set of cultures, neuroblastoma (NA) cells were seed in 96-well plates at a density of $2x10^4$cells/well and allowed to grow for 24 hr in growth medium at 37°C and 5% CO_2. Then, the medium was removed and cells were incubated with the microglial conditioned medium for 24 hr. Finally, a 10 µl of 5 mg/ml MTT reagent was added into each well and incubated at 37°C for 2 hr. The media were gently removed and a 100 µl of DMSO was added to each well. Plates were covered in the dark for 2 hr. The absorbance at 570 nm was determined using a microplate reader (Bi0-Tek Instruments Inc., Winooski, VT).

3.2 The effect of H63D mutation on oxidative stress, mitochondrial function, glycogen synthase kinase-3β activity, apoptosis and Aβ production

3.2.1 Chemicals and antibodies

SH-SY5Y human neuroblastoma cell lines were obtained from American Type Culture Collection (Manassas, VA). Cell culture reagents including DMEM/F12, penicillin/streptomycin/glutamine and geneticin were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Gemini Bio-Products (West Sacramento, CA). Reactive oxygen species (ROS) detection kit was ordered from Calbiochem (CA, USA). Mitochondria activity assay (cytochrome c oxidase activity assay kit) was purchased from BioChain Institute (Hayward, CA). $A\beta_{25-35}$, $A\beta_{35-25}$, $A\beta_{1-42}$, FLAG, β-actin and β-tubulin antibodies were ordered from Sigma Co. (St. Louis, MO). Caspase-9, phospho-GSK-3β (Ser9) and total-GSK-3β antibodies were ordered from Cell Signaling Technology (Beverly, MA). Caspase-8 antibody was ordered from Santa Cruz Biotechnology (Santa Cruz, CA). Bax, cytochrome c, and cytochrome oxidase subunit IV antibodies were ordered from, Upstate (Temecula, CA), Clontech (Mountain View, CA) and Molecular Probes (Eugene, OR) respectively. Secondary anti-rabbit antibody or anti-mouse antibody and ECL detection kits were obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Apoptosis assay kit was purchased from Molecular Probes. Caspase-3 fluorometric assay was ordered from R&D Systems (Minneapolis, MN). Human $A\beta_{1-42}$ colorimetric ELISA kit was purchased from Biosource (Camarillo, CA). Nitrocellulose membrane was ordered from Pall Life Sciences (Pensacola, FL), RIPA buffer and phosphate inhibitor was ordered from Sigma Co. (St. Louis, MO). Cell fractionation buffer was purchased from Clontech (Mountain View, CA). BCA protein assay was ordered from Pierce Chemical (Rockford, IL). All of the other chemicals used were purchased from Sigma Co (St. Louis, MO).

3.2.2 Cell transfection and cell culture model

Human neuroblastoma SH-SY5Y cell lines were chosen because they did not express detectable levels of HFE protein or mRNA (Lee *et al.*, 2007). The HFE cDNA was fused to the FLAG octapeptide sequence to more easily detect the HFE protein. H63D cDNA was generated from WT HFE cDNA using a site-directed mutagenesis kit (Stratagene, San Diego, CA) (Wang *et al.*, 2004). The description of the transfection protocol for the SH-SY5Y cell line has been reported previously (Wang *et al.*, 2004). Stable cell lines were obtained following transfection with WT or H63D forms of HFE using the lipid transfection reagent, Lipofectamine (Invitrogen). The clones were confirmed for expression of FLAG/HFE by immunoblotting. Western blot analysis on whole cell homogenates was used to identify differences in protein expression. As a control, we also transfected cells with vector alone. Vector control cells were included in analyses to control for phenotypic changes associated with the cell transfection. Because of the cells transfected with vector alone do not express HFE, the more direct control for study the mutation of HFE, however, is the WT HFE transfected cells. The transfected cells were maintained in DMEM/F12 media supplemented with 10% FBS, 1% penicillin/streptomycin/glutamine, 1x nonessential amino acids, and 1.8g/L sodium bicarbonate and 250 μg/mL geneticin at 37 °C in a 5% CO_2 atmosphere.

3.2.3 Measurement of ROS Generation

The rate of DCFH-DA was measured by a fluorescence microplate reader. 2',7'-dichlorofluorescein diacetate is a sensitive and widely used compound for detection of intracellular oxidant production. Oxidation of DCFH-DA creates highly fluorescent dichlorofluorescein. The DCFH-DA was added to the cell suspension. It diffuses across the cell membrane and is hydrolyzed by intracellular esterases to dichlorofluorescein, which, on oxidation, yields highly fluorescent 2',7'-dichlorofluorescein. The cells were removed from growth media via centrifugation.

Resuspend cells were prewarmed in PBS buffer containing the probe to provide a final working concentration of 10 μM dye. The samples were incubated at 37°C in the dark for 60 min. Fluorescence was measured with the fluorescence microplate reader with excitation of 495 nm and a 525 nm emission filter.

3.2.4 Measurement of Cytochrome c oxidase Activity

Cytochrome c oxidase activity was determined in intact isolated mitochondria using the cytochrome c oxidase assay kit. The colorimetric assay is based on the principle that a decrease in absorbance at 550 nm of the ferrocytochrome c is caused by its oxidation to ferricytochrome c by cytochrome c oxidase. The cytochrome c oxidase assay was performed according the manufacturer's instructions.

3.2.5 Determination of Mitochondrial membrane potential (MMP)

The loss of mitochondrial membrane potential was measured by the JC-1 assay kit. Treated cells with 5 μM DFO for 2 day and untreated cells were removed from growth media via centrifugation. For each condition, 4 x10^5 cells were incubated with JC-1 dye for 30 min. After incubation, the cells were rinsed with PBS. The emission signals at 590 and 527 nm elicited by excitation at 485 nm were measured using a spectrofluorimeter. The ratio of the signal at 590 nm to that at 527 nm (red/green ratio) was calculated.

3.2.6 $A\beta_{1-42}$ Measurements

$A\beta_{1-42}$ levels were determined by ELISA assay for human Aβ (BioSource International, Camarillo, CA). Equal amounts of mitochondrial protein were processed as described in the manufacturer's instructions. Total mitochondrial protein was determined using the BCA (Pierce Chemical, Rockford, IL) assay.

3.2.7 Cell viability assay

$A\beta_{25-35}$, and $A\beta_{35-25}$ were solubilized in sterile water at a concentration of 0.94 mM and were stored frozen at -20°C for at least 1 week prior to use. Cells were plated at a density ($2x10^4$ cells/well) in 96-well flat-bottomed microtiter plates and then cultured for 48 hr. After 48 hr, the cells were exposed to $A\beta_{25-35}$ or $A\beta_{35-25}$ peptides at the concentrations of 10 or 20 μM for 24 hr. After 24 hr treatment, 50 μl of 2 mg/ml MTT (Sigma Co St. Louis, MO) was added to 200 μl of medium present in each well and incubated at 37°C for 2 hr. After the incubation period, an aliquot (220 μl) of the resulting solution was removed from each well followed by the addition of 150 μl dimethyl sulfoxide. After the precipitate in each well was resuspended on a microplate mixer for 10 min, an optical density (OD) reading at 540 nm was measured using a plate reader (Spectramax 340 PC). All results were normalized to OD values measured from an identically conditioned well without cell culture.

3.2.8 Preparation of cell lysates

After $A\beta_{25-35}$ treatment (4 hr for caspase-8, Bax and cytochrome c and 9 hr for caspase-9), the cells were washed twice in cold PBS and were then removed by using cell scraper. The cells suspension were centrifuged at 300 x g for 3 min. The cell pellets were resuspended in lysis buffer (1% Triton X-100, 1% phosphates inhibitor, 1% protease inhibitor cocktail and RIPA buffer) and incubated for 10 min. The lysates were cleared by centrifugation at 10,000 x g for 20 min at 4°C. The supernatant was then assayed for active caspase-8 and caspase-9. For evaluation of cytochrome c, Bax and cytochrome c oxidase subunit IV (COX IV) levels, the cells suspension was centrifuged at 300 x g for 3 min and then the cell pellets were resuspended in a fractionation buffer mix, left on ice for 10 min and homogenized by 50 strokes in an ice-cold Dounce homogenizer. The cells extract was centrifuged at 700 x g for 10 min at 4°C, resulting in a pellet containing nuclei and supernatant retaining mitochondria and cytosol. The supernatant was further centrifuged at 10,000 x g for 25 min at 4°C to

collect the pellet enriched-mitochondria fraction. The resulting supernatant containing the cytosolic fraction was collected and the enriched mitochondria pellet was lysed by incubation with fractionation buffer mix. The protein concentrations were determined by BCA protein assay.

3.2.9 Western blot analysis

For western blot analysis, 50 μg of total protein from each sample was performed on 4–20% gradient gels (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes. The membranes were blocked with 5% skim milk and incubated with specific antibodies for anti-FLAG (1:2,000), caspase-8 (1:200), cleaved (activated form) caspase-9 (1:1,000), cytochrome c (1:100), Bax (1:2,000), COX (1:1,000), total GSK-3β (1:1,000), phospho-Ser9-GSK-3β (1:1,000), APP (1:1,000) and β-tubulin (1:2,000). The membranes were washed with Tris buffered saline containing 0.05% Tween-20 (TBST) and processed with an HRP-conjugated anti-rabbit antibody or anti-mouse antibody for ECL detection. The probing for internal control molecules such as cytochrome c oxidase subunit IV (COX) (1:1,000) and actin (1:3,000) was always performed by re-hybridization after stripping the primary antibodies.

3.2.10 Cytofluorometric Determination of Apoptotic Cells by Annexin V/PI (propidium iodide) Staining

HFE cells were exposed to 20 μM $A\beta_{25-35}$ for 24 hr and then labeled with Annexin V/PI. Briefly, $A\beta_{25-35}$-treated and -untreated HFE cells (5×10^5) were washed in PBS and then incubated with 5 μl of 0.2 μg/ml Annexin V-FITC and 1 μl of 100 μg/ml propidium iodide for 15 min at room temperature, prior to flow cytometry analysis. Annexin V^+/PI^- cells were identified as early apoptotic, whereas Annexin V^+/PI^+ cells were classified as late apoptotic cells.

3.2.11 Caspase-3 activity assay.

A caspase-3 fluorometric assay kit was utilized. Briefly, after 8 hr and 12 hr $A\beta_{25-35}$ treatment, the cells were collected by centrifugation at 300 x g for 3 min. The cell pellets were lysed upon addition of the cold lysis buffer (provided by the manufacturer) and incubated on ice for 10 min. The protein content of the cell lysate was estimated using a protein determination assay. Total protein (100 μg) was then mixed with 2x reaction buffer (provided by the manufacturer) and 5 μl of caspase-3 fluorogenic substrate (DEVD-AFC) in a 96-well plate. The reaction was incubated at 37°C for 90 min followed by fluorescence analysis using a fluorescent microplate reader with light excitation at 400 nm and light emission at 505 nm.

3.2.12 Statistical analysis

In comparison more than two groups, statistical significance was analyzed by one-way ANOVA, followed by LSD's test. In comparison of two groups, statistical significance was analyzed by *t*-test. Differences among the means were considered statistically significant when the p value was <0.05. Data were presented as the mean ± SEM.