CHAPTER III

EXPERIMENTAL

1. Cell line and culture techniques

1.1 Culture of undifferentiated P19 cells

P19 cells were obtained from American Type Culture Collection and routine culturing and induction were performed according to the methods with slight modification (Jones-Villeneuve et al., 1982; Staines et al., 1994). P19 cells were cultured as undifferentiated monolayers at 10^6 cells/ml in 90% α-minimal essential medium (α-MEM) supplemented with 7.5% newborn calf serum (NCS) and 2.5% heat-inactivated fetal bovine serum (FBS) in plastic tissue culture flasks. Cells were grown at 37°C in a 5% CO_2 atmosphere. Cultures were maintained as monolayers in exponential growth.

1.2 Differentiation of P19 cells with retinoic acid

After attaining confluence the cells were subcultured following trypsinization with 0.25 % trypsin solution. Add 2 ml trypsin solution to each T25 flask; swirl and lay the flasks flat making sure the monolayer is coated. Allow the flasks to sit for approximately 1 minute to detach the cells from the surface. Trypsin solution was aspirated, and cells were triturated to single cells with 3 ml α-MEM supplemented with 5% heat-inactivated FBS. Neuronal differentiation was induced by culturing 10^5 cells/ml in a 100X20 mm^2 petri dish containing α-MEM supplemented with 5% heat-inactivated FBS plus 1 μM all-*trans* RA, and allowed to aggregate for 4 days. Cells did not adhere and instead formed free-floating EBs. Media containing fresh RA was replaced every 48 h.

1.3 Plating for neuronal differentiation

EBs were collected by transferring the contents of the dishes to sterile 15 ml conical tubes. Rinse the dishes with 2-3 ml of medium and add to the 15 ml conical

tubes containing the cells. For the EBs sedimentation, the suspension was allowed to stand for 15 min, and the supernatant was carefully removed off the pellet.

To induce extensive morphological differentiation, EBs were exposed to 2 ml trypsin solution for 10 min and centrifuged at 1000 g for 10 min, collected cell pellet. The supernatant was removed and the pellet was gently resuspended in 500 U/ml DNase I in PBS and incubated for 10 min. After incubation, cells were dissociated by trituration and resuspended in MEM containing 10% heat-inactivated FBS, 25 μM L-glutamine. Cells were counted with a hemocytometer, diluted to $5X10^5$ cells/ml, and cells were then distributed in each well of a 24-well dish. In order to discourage non-neuronal cell proliferation, cytosine-β-arabino-furanoside (Ara C) at a final concentration of 20 μM was added to each well to inhibit proliferation of non-neuronal cells at the time of plating (Fukuchi et al., 1992). They were cultured for 7 days prior to experimentation in MEM containing 10% heat-inactivated FBS during which time they elaborated extensive neurites that exhibit characteristics of axons. Under this condition, visual inspection of cultures indicated that > 80% of the cell presence had a morphological phenotype expected for the neuronal cells. The medium was changed every 2 days.

2. Determination of Aβ-induced cytotoxicity

2.1 β-amyloid peptide aggregation

Synthetic $A\beta_{1-42}$ was solubilized in DMSO to a concentration of 1 mM. Aliquots were stored at –20°C until used. At the time of use, dissolve a single aliquot of the store peptide in MEM medium. In vitro formation of synthetic $A\beta_{1-42}$ was induced by incubation of a soluble of $A\beta_{1-42}$ for 3 days at 37°C to form the "aged" state. After manipulation of the aggregation state of the peptide has been achieved, serial dilutions of this stock were made in sterile MEM media immediately before application to the cells. An appropriate final concentration was made into the culture medium (Bozyczko-Coyne et al., 2001).

2.2 Concentration-dependent cytotoxicity of $A\beta_{1-42}$ aggregates

For monitoring concentration-dependent effects of Aβ, cultured NLCs were exposed to $A\beta_{1-42}$ by removing the culture medium and replacing it with fresh medium containing various concentrations of 0.1, 1, 5, and 10 μM. Neuronal cultures were maintained for 24, 48, 72, and 96 h before neuronal survival was assessed visually by phase-contrast microscopy and quantified by measuring (1) the reduction of the tetrazolium salt, XTT, (2) the trypan blue exclusion assay, and (3) the release of the cytoplasmic enzyme, LDH, at various time points.

2.3 Time-dependent study of $A\beta_{1-42}$ aggregates

For monitoring time-dependent effects of Aβ, the seventh day of the culture were exposed to $A\beta_{1-42}$ for 24, 48, 72, and 96 h. The cell viability was determined using the XTT reduction assay, the trypan blue exclusion assay, and the LDH release assay.

3. Treatment of valproic acid and valproyl urea

3.1 Preparation of VPA and VPU

VPA and VPU were dissolved in DMSO at concentrations of 10 mM, 1 mM and 100 μM, and were then added in MEM to an appropriate final concentration. The vehicle-treated cultures received the same amount of DMSO (in medium) that was present in the highest concentration of VPA. The final concentration of DMSO did not exceed 0.01%.

3.2 Concentration dependent and time dependent study of VPA and VPU

For monitoring concentration-dependent effects of VPA and VPU, VPA was applied at the various concentrations of 10, 50, 100, 500 and 1000 μM and VPU was applied at the various concentrations of 10, 50, and 100 μM, for 72 h on the seventh day of the culture. The cell viability was determined using the XTT reduction assay, the trypan blue exclusion assay, and the LDH release assay.

4. Treatment of $A\beta_{1-42}$ and valproic acid or valproyl urea

NLCs were cultured in 24-well culture plates at a density 5 X 10^5 cells/well. The experiments were divided into three groups.

4.1 Co-treatment experiment

NLCs were cultured in MEM with 10% FBS for 7 days. Cells were incubated with or without $A\beta_{1-42}$ at a concentration of 5 μM, together with either VPA at the concentrations of 10, 50, 100, 500, and 1000 μM or VPU at the concentrations of 10, 50, and 100 μM for 72-h period. After the incubation period, cell viability was determined using the XTT reduction assay, the trypan blue exclusion assay, and the LDH release. Trolox at a concentration of 1 mM was used as a positive control to verify system conditions.

Control experiments were performed by incubation with VPA or VPU alone and vehicle control experiments were performed by incubation with DMSO. The final concentration of DMSO per well did not exceed 0.01 %.

4.2 Pre-treatment experiment

NLCs were cultured in MEM with 10% FBS for 7 days. Cells were preincubated with either VPA or VPU at a concentration of 100 μM for 3, 5, and 7days. During treatment periods, fresh control medium or one containing the desired compounds was applied to the cells every 48 h. Following removal of VPA and VPU from well, cells were washed with PBS and then incubated with $A\beta_{1-42}$ at a concentration of 5 μM for 72 h. After the incubation period, cell viability was determined using the XTT reduction assay, the trypan blue exclusion assay, and the LDH release assay. Trolox at a concentration of 1 mM was used as a positive control.

Control experiments were performed by incubation with VPA or VPU alone and vehicle control experiments were performed by incubation with DMSO. The final concentration of DMSO per well did not exceed 0.01 %.

4.3 Post-treatment experiment

NLCs were cultured in MEM with 10% FBS for 7 days and was further pretreated with $A\beta_{1-42}$ at a concentration of 5 μM for 72-h period. After the incubation period, the medium was removed, washed with PBS and replaced with fresh medium lacking $A\beta_{1-42}$ prior to application of VPA or VPU at the concentrations of 50 and 100 μM. The incubation was then continued for 72 h. Cell viability was determined using the XTT reduction assay, the trypan blue exclusion assay, and the LDH release assay. Trolox at a concentration of 1 mM was used as a positive control.

Control experiments were performed by incubation with VPA or VPU alone and vehicle control experiments were performed by incubation with DMSO. The final concentration of DMSO per well did not exceed 0.01 %.

5. Morphology determination

5.1 Photographic method

NLCs were visually analyzed by phase-contrast microscopy using 10-20X magnification on Zeiss inverted microscope to evaluate morphological changes.

5.2 Cresyl violet staining

The staining procedure stains the Nissl substance resulting in a darker stain in some ribosomal RNA, and the acidic RNA-rich ribosomes of the RER. It happens that the secretory activity in neurons is very high, so neurons have vast amounts of RER. Cells were washed twice in PBS for 10 minutes and incubated for 2 minutes in staining solution containing 0.25% cresyl violet, 0.8% glacial acetic acid, and 0.6 mM sodium acetate. Cells were washed 4 times in PBS, then observed using microscope (Fraichard et al., 1995).

6. Determination of cell viability

Viability of neuronal cells was determined using three independent measures: morphology, plasma membrane integrity and metabolic function. Three assays were used to assess the effects of VPA, VPU, and $A\beta_{1-42}$ on NLCs.

6.1 Assessment of mitochondrial function by XTT reduction activity

The XTT assays, a measure of mitochondrial integrity, were performed in 24-well microtiter plates. This method is based on a conversion of the tetrazolium salt, XTT, a yellow substrate, to produce a dark orange, water soluble formazan by dehydrogenase enzyme in viable, but not dead cells. The viability of cells was determined by adding XTT to the cell cultures to reach a final concentration of 1 mg/ml XTT and 25 μM PMS. Incubation was continued for 4 h, and the colorimetric determination of XTT formazan product, which was soluble in aqueous solution, was made at 450 nm. The 100 μl aliquots of formazan solution was transferred to 96-well plates and using a microplate reader with corresponding filter. The absorbance of samples was then measured against the background control, which contained the same volume of culture medium and XTT solution as used in the experiments (Roehm et al., 1991). Data were presented as a percentage of the value obtained from cells incubated in medium with 0.01% DMSO. Validation of the use of XTT for assessment of cell viability was determined by correlating results obtained using XTT with visual cell count with hemocytometer using trypan blue exclusion. XTT reduction assay was expressed as percentage of control $\pm$ S.E.M from at least three independent experiments.

6.2 Assessment of neuronal survival by trypan blue exclusion

Neuronal survival of 7-day-old neuronal cultures was evaluated by the trypan blue exclusion assay. Cytotoxicity was quantified by examining cultures under inverted light microscopy. After experiment, cells were rinsed 3 times with 1 ml of PBS (pH 7.4). A solution of 0.2% trypan blue was added to cells and incubated for 5 min. Five different microscopic areas were counted for uptake of trypan blue. Dead neurons with a damaged cell membrane, internalize the dye while competent neurons do not. The

viability of the culture was calculated as the percentage of the ratio of the number of unstained cells (viable cells) to the total number of cells counted (viable cells plus nonviable cells). In each experiment, over on two wells were counted at random to obtain mean ± S.E.M of the cell viability. For trypan blue exclusion method, a minimum of 300 cells/well were count (Yan et al., 1999).

6.3 Assessment of plasma membrane integrity by LDH release

Cytotoxicity cells were evaluated by the release of LDH from dead cells. After cell exposed to VPA, VPU, and $A\beta_{1-42}$ in 24 well-plates, LDH activity in cell culture supernatant was measured by the enzymatic test by CytoTox 96 Non-Radioactive Cytotoxicity Assay (LDH assay, Promega) according the manufacture's description. Briefly, cells were harvested and centrifuged at 14,000 g at 4°C for 5 min. Fifty µl of supernatants were transferred to a 96-well microtiter, and the activity of LDH was determined using LDH kit, followed by the addition of 50 µl substrate mix solution. After a 30 min incubation, the reaction was terminated by the addition of 50 µl stop solution (0.5 M acetic acid) On the other hand, cell monolayers were treated with lysing solution (1% tritonX-100) for 45 min at room temperature to lyse the cell membranes, and lysate was collected. LDH activity was measured in both the supernatant and the cell lysate fraction. Optical densities were measured by microplate reader with a 510-nm filter. The amount of the LDH release was expressed as a percentage of the value obtained in comparative wells where cells were 100% lysed by 1% triton X-100. The percentage of LDH value was calculated as the portion of LDH from both supernatant and cell lysate.

7. Determination of cellular acetylcholinesterase activity

EBs were allowed to settle for 15 min in conical tubes, washed twice with 15 ml of cold PBS, and collected by centrifugation at 1000 rpm for 3 min. Cells generally were solubilized in 1% triton X-100 in PBS and briefly sonicated. Insoluble material was removed by centrifugation at 12,000 rpm for 10 min. Aliquots of supernatants were used for enzyme assays (Coleman and Taylor, 1996). For NLCs, monolayers were washed twice with 5 ml of cold PBS and removed from the plates by scraping.

AChE activity was determined by modified Ellman procedure performed in microtiter plates (Ellman et al., 1961). Per 96-well cavity, 1 mM acetylthiocholine iodide, 0.3 mM DTNB solution, 0.1 mM iso-OMPA (specific inhibitor for BchE) and 100 mM sodium phosphate (pH 7.0) were pipetted to 100 µl final volume. After 15 min incubation at room temperature, the reaction was started by adding 100 µl of cell homogenate. The increase in absorption was measured in an ELISA reader at 412 nm. Protein determination was determined according to the method of Lowry. Bovine serum albumin (BSA) was used as a standard.

8. Statistical analysis

All data presented was representative of at least three independent experiments. Individual experiments were performed in 24-well plate containing appropriate negative (media blank and vehicle) control. Within each experiment, data from an individual well was treated as a single data point. The results represented the mean ± S.E.M from three independent experiments. Statistical analysis was conducted by one-way analysis of variance (ANOVA) and post-test comparison performed using LDS multiple comparison methods. Unless otherwise noted, a level of statistical significance was considered $p<0.05$. Error bars indicated the mean ± S.E.M from the indicated number of experiments. Data were analyzed with Excel 2000 (Microsoft Corp.) and SPSS version 10.0.