Application of Aurintricarboxylic Acid for the Adherence of Mouse P19 Neurons and Primary Hippocampal Neurons to Noncoated Surface in Serum-Free Culture

Tsung-Yih Lee
Dept. of Biological Science and Technology, National Chiao Tung University, Hsinchu, 30068, Taiwan

Wen-Shin Chen and Yung-An Huang
Institute of Bioinformatics and Systems Biology, National Chiao Tung University, Hsinchu, 30068, Taiwan

Ting-Wei Liu
Dept. of Biological Science and Technology, National Chiao Tung University, Hsinchu, 30068, Taiwan

Eric Hwang
Dept. of Biological Science and Technology, National Chiao Tung University, Hsinchu, 30068, Taiwan

Institute of Bioinformatics and Systems Biology, National Chiao Tung University, Hsinchu, 30068, Taiwan

Institute of Molecular Medicine and Bioengineering, National Chiao Tung University, Hsinchu, 30068, Taiwan

Ching-Ping Tseng
Dept. of Biological Science and Technology, National Chiao Tung University, Hsinchu, 30068, Taiwan

DOI 10.1002/btpr.1638
Published online October 23, 2012 in Wiley Online Library (wileyonlinelibrary.com).

Dissociated primary neuron culture has been the most widely used model systems for neuroscience research. Most of these primary neurons are cultured on adhesion matrix-coated surface to provide a proper environment for cell anchorage under serum-free conditions. In this study, we provide an alternative technique to promote the adhesions of these neurons using aurintricarboxylic acid (ATA), a nonpeptide compound, without surface manipulations. We first demonstrated that ATA could promote Chinese hamster ovary cell attachment and proliferation in serum-free medium in a dosage-dependent manner. We later showed that ATA significantly enhanced the attachment of the retinoic acid differentiated P19 mouse embryonal carcinoma (P19) neurons, with an optimal concentration around 30 μg/mL. A similar result was seen in primary hippocampal neurons, with an optimal ATA concentration around 15 μg/mL. Further morphological assessments revealed that the average neurite length and neuronal polarization were almost identical to that obtained using a conventional method with poly-L-lysine surface. The advantages of using the ATA treatment technique for immunochemical analysis are discussed. © 2012 American Institute of Chemical Engineers *Biotechnol. Prog.*, 28: 1566–1574, 2012
Keywords: aurintricarboxylic acid, serum-free medium, P19 neuron cells, hippocampal neurons, cell attachment reagent

Introduction

Cell attachment and spreading on the extracellular matrix are essential cellular processes for the promotion of cell growth, migration, and invasion.[1,2] These interactions play key roles in a variety of cellular processes, including embryonic development, tumor metastasis, and wound healing.[3–6] Cell culture studies have often used biomaterials to modify the surfaces of a culturing container for cell attachment and growth. Factors involved in cell adherence have been widely studied in cell culture areas. For mammalian cell culture, most attachment-dependent cell lines require an extracellular matrix on the growth substratum. Serum is one of the factors that provide components to facilitate cell attachment and growth.[7,8] However, it produces unclear or undesirable disruptions in cellular responses and is costly. On the other hand, cells often lose their adhesion ability under serum-free conditions, leading to the death of anchorage-dependent cells. Therefore, culture plates are frequently precoated with a suitable matrix before seeding cells, such as poly-L-lysine (PLL), collagen, fibronectin, or laminin. Other commercially available attachment reagents with more complex composition are also used; these include Matrigel (BD Biosciences, Franklin Lakes, NJ), FNC Coating mix® (Biological Research Faculty and Facility, Ijamsville, MD), and ProNectin® F (Protein Polymer Technologies, Inc., San Diego, CA). Most of these complex materials are secreted from other cells or have a specific synthetic peptide

Additional Supporting Information may be found in the online version of this article.

Correspondence concerning this article should be addressed to C.-P. Tseng at cpts@cc.nctu.edu.tw.

containing the sequence from Arg-Gly-Asp.[9] Recently, some new parylene polymers have been used on tissue engineering and biosensor for neuron adhesive growth in culture. These polymers [poly-chloro-para-xylylene (diX C), poly-monoaminomethyl-para-xylylene (diX AM), and poly-monoaldehyde-para-xylylene (diX H)] can be coated on SiO_2 surface in combination with proteins and UV irradiation and have been shown to markedly improve the neuronal cell attachment on micropatterned surfaces.[10–13]

PLL has been widely used in neuron cell culture for cell adhesion. It functions by providing an ionic interaction with cell membrane to promote cell adhesion and growth.[14,15] Pluripotent mouse P19 embryonal carcinoma cells have been used extensively as a model system for in vitro differentiation. These cells can differentiate into a number of distinct lineages, including neurons, skeletal muscle cells, and cardiomyocytes.[16] Normally, the retinoic acid-induced neuronal P19 cells are grown on a culture dish precoated with PLL to support cell attachment. Similarly, primary mouse hippocampal neurons also require PLL-coated or other biomaterial-coated culture surfaces for cell attachment.[15,17] The extra matrix coating steps are time consuming and may increase the risk of contamination.

Aurintricarboxylic acid (ATA), a triphenylmethane derivative, has been used in diverse biological experiments, such as that for nuclease inhibitor,[18] virus reproduction blocker,[19,20] and gene transfection enhancer.[21] Liu et al. suggested that ATA acts as an insulin agonist for Chinese hamster ovary (CHO) cell growth and protein production in a serum-free system.[22] Although the mode of action involved in ATA-induced cell activation remains mostly unclear, ATA had been shown to improve CHO cells proliferation and morphologic change from suspension to attachment in a serum-free culture.[22] However, the application of ATA in other serum-free culturing systems has not been explored.

The purpose of the present study is to test whether ATA could be applied for P19 neuron, and dissociated hippocampal neuron attachment in a serum-free culture system. Presently, we found that ATA could be an alternative agent for these neurons in culture plates without surface manipulations. The optimal concentrations of ATA used in these neuronal cultures and the morphological changes of these neurons compared with that in conventional PLL-coating method are characterized.

Materials and Methods

Chemicals and reagents

ATA, bovine serum albumin (BSA), DNase I, Fluoromount, dimethyl sulfoxide (DMSO), and sodium selenite, insulin, transferrin, ethanolamine (SITE) liquid supplement (containing 2.5 μg/mL insulin, 1.38 μg/mL transferrin, 1.25 ng/mL sodium selenite, and 0.5 μg/mL ethanolamine in Earle's balanced salt solution) were obtained from Sigma (St. Louis, MO). Minimum essential medium (MEM), a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham F12-medium (DMEM/F12), neurobasal medium, Dulbecco's phosphate- buffered saline (PBS), B27 supplement containing vitamin A, insulin, and antioxidant (Cat 17504-044), N-2 supplement containing 500 μg/mL insulin, 10 mg/mL transferrin, 0.63 μg/mL progesterone, 1.61 mg/mL putrescine, and 0.52 μg/mL selenite (Cat 17504-048), 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), Hank's balanced salt solution (HBSS), ethylenediaminetetraacetic acid (EDTA), trypsin-EDTA, trypan blue, and glutamine were from Invitrogen (Grand Island, NY).

Mouse monoclonal antibody-specific against βIII-tubulin (TUJ1) was purchased from Covance (Cat MMS-435P; Berkeley, CA), whereas mouse monoclonal antibody against tau-1 protein (Tau-1) (Cat MAB3420) and rabbit polyclonal antibody against microtubule-associated protein 2 (MAP2) (Cat AB5622) were from Millipore (Billerica, MA). Reagents 4′,6-diamidino-2-phenylindole (DAPI) (Cat D3571), Alexa Fluor 568 phalloidin dye (Cat A12380), Alexa Fluor 488 phalloidin dye (Cat A12379), and Alexa Fluor 568-conjugated goat anti-mouse (Cat A11004) or anti-rabbit (Cat A11011) IgG used as secondary antibodies were purchased from Invitrogen (Grand Island, NY). Dylight 488-conjugated goat anti-mouse (Cat 115-485-003) IgG was from Jackson Immuno Research (West Grove, PA).

CHO cell culture and ATA treatment

CHO-β-Gal-SF cells (BCRC 60378) were obtained from the Bioresource Collection and Research Center of Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were grown in DMEM/F12 supplemented with 1.5 g/L sodium bicarbonate, 2.4 g/L meat peptone, 0.09% (v/v) linoleic-acid BSA, and 0.25% (v/v) SITE as previously described.[23] CHO cells (10^5cells/mL in a 3-mL serum-free medium) were seeded in at least triplicates in each well of a six-well culture plate to examine the effect of ATA on cell adherence. Stock solution of ATA was prepared in DMSO to a concentration of 100 mg/mL. ATA was added to the culture at the beginning of the experiments to make final concentrations from 0 to 100 μg/mL. Treated cells were maintained at 37°C in humidified atmosphere containing 5% CO_2. The cell number was determined using a hemacytometer Hausser Scientific (Horsham, PA), and the viability was judged using a trypan blue exclusion method.

PLL treatment

PLL with molecular weight ranging from 30,000 to 70,000 daltons was dissolved in a boric acid buffer (1.24 g boric acid and 1.9 g sodium tetraborate in 400 mL dd H_2O, pH 8.5) to a final concentration of 0.1 mg/mL, after which it was filter-sterilized by passing through a 0.2-μm syringe-filter. For the coating experiment, 12-mm glass-coverslips were treated overnight with a PLL solution (100 μg/mL) in a 24-well-sterilized culture plate at room temperature. PLL-coated coverslips were then washed twice with sterile water and dried at room temperature before use.

Preparation of retinoic acid-induced neuronal differentiation of P19 cells and ATA treatment

P19 cells were maintained at 37°C in 5% CO_2 in MEM supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 100 μg/mL penicillin/streptomycin, and 10% (v/v) fetal bovine serum. Retinoic acid-mediated P19 differentiation was conducted according to the method previously described.[24] In brief, undifferentiated cells were initially trypsinized and grown in a suspension for seven days in a differentiation medium (MEM supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 5% fetal bovine serum, and 0.5 μM retinoic acid) during which time the cells formed suspended

aggregates. Retinoic acid-induced aggregates were dissociated by trypsinization and plated at a density of 1,000 cells/mm^2 in serum-free neuron maintenance medium (MEM medium plus 2 mM glutamine, 1 mM sodium pyruvate, 10% (v/v) N-2 supplement, and 0.6% (w/v) glucose).

For the ATA treatment, differentiated P19 cells were seeded onto a 24-well plate with coverslips (12 mm in diameter) at the bottom in a serum-free neuron maintenance medium containing various concentrations of ATA and incubated at 37°C in humidified atmosphere containing 5% CO_2. The cultures were terminated by fixing the cells with 3.7% formaldehyde in PBS for further assessments.

Primary hippocampus neuron culture and ATA treatment

Pregnant dams (C57BL/6) from the National Laboratory Animal Center (NLAC, Taipei, Taiwan) were euthanized with CO_2 followed by decapitation on Day 18 (E18), according to the regulations of animal welfare from the National Science Foundation of Taiwan, with the approval of the Animal Study Committee of the National Chiao Tung University. Briefly, E18 embryonic hippocampi were isolated and collected in HBSS containing 10 mM HEPES and 1% penicillin/streptomycin, according to the procedures previously described.[16] Isolated hippocampi were then dissociated at 37°C for 30 min in a digestion medium [0.25% trypsin-EDTA (2.5 g/L trypsin and 0.38 g/L EDTA) with 10 mM HEPES]. The digestion medium was then replaced with a dissection buffer (10 mM HEPES and 50 μg/mL DNase I in HBSS solution) twice by gravity for 3 min. Pelleted cells were triturated, centrifuged at 80 x *g* for 5 min at room temperature and washed with serum-free neuronal maintenance medium (Neurobasal medium plus 0.5 mM L-glutamine and B27 supplement).

Hippocampus cells (300 cells/mm^2) on PLL-coated or uncoated coverslips in a 24-well plate were treated with ATA in a serum-free neuronal maintenance medium, followed by incubation at 37°C in humidified atmosphere containing 5% CO_2. After 4 h, the coverslips were inverted by a pair of forceps. Neurons were then fixed on Day 2 or Day 4 with 3.7% formaldehyde in PBS for further assessments.

Immunocytochemistry

Immunocytochemical analysis was done according to published procedures.[17] Briefly, fixed neurons (differentiated P19 and hippocampus cells) on coverslips were treated with permeabilizing buffer (0.25% Triton X-100 in PBS) for 5 min at room temperature and washed with PBS three times. Cells were incubated with blocking buffer (10% BSA in PBS) for 1 h at room temperature to prevent nonspecific binding. For samples grown for 2 days, cellular components F-actin was stained with fluorescent-labeled phalloidin dyes in which Alexa Fluor 488 (1:100) was used for hippocampal cells, whereas Alexa Fluor 568 (1:100) was used for P19 cells. βIII-Tubulin of hippocampal cells was first treated with primary TUJ1 antibody (1:4,000) and then stained with a secondary antibody (goat anti-mouse) conjugated with Alexa Fluor 568. For P19 cells, βIII-Tubulin was stained with a secondary antibody (goat anti-mouse) conjugated with Dylight 488. For hippocampal cells grown for 4 days, Tau-1 (marker for axon) and MAP2 (marker for dendrite) were treated with mouse anti-Tau-1 monoclonal (1:2,000) and rabbit anti-MAP2 polyclonal (1:2,000), followed by staining with a secondary antibody (goat anti-mouse) conjugated with Alexa Fluor 488 or (goat anti-rabbit) conjugated with Alexa Fluor 568. Cell nuclei were stained with DAPI dye (5 μg/mL, 1:1000 dilution). Stained samples were incubated for 1 h at 37°C without light exposure and washed with PBS. The coverslips were then mounted with Fluoromount on slides and dried overnight at room temperature in the dark. Nonspecific binding controls were evaluated using normal nonimmune mouse or rabbit serum to replace a primary antibody, but nonspecific binding was not identified.

Image acquisition and analysis

Both phase contrast and fluorescence images were captured using an Olympus IX 71 inverted microscope equipped with a ThorLabs white light-emitting diode (LED) or CoolLED fluorescent light source and a Hamamatsu ORCA-R2 camera. All images were saved in TIF format. The phase contrast images were taken using a phase contrast objective lens (20 $\times$ 0.4 NA). A plan apochromatic objective lens (20 $\times$ 0.75 NA) was used to collect the fluorescent images. For neurite length assessment, the data were calculated from at least five images for each experimental condition based on the neurite length equal to or greater than two times the soma diameter. The selected images were analyzed by the NeurphologyJ software for hippocampal neuron neurite length measurement.[25] Nuclei stained by DAPI were used to determine the percentage of attached cells.

Statistical analyses

Data acquired from image analysis (described above) were evaluated using a GraphPad Prism software (5.01 version) on PC Windows. For neurite length at each condition, 5–11 images with an average of 30 cells/image were analyzed (expressed as means $\pm$ standard deviation) and statistically compared by analysis of variance (ANOVA) for multiple comparisons. Significance difference was accepted at $P < 0.05$. All data points for CHO or neuronal cell attachment (as affected by ATA) were determined by an average of at least three replicates of the samples (total 12 randomized images of each condition with an average of 60 cells/image) before ANOVA.

Results and Discussion

Promotion of CHO cell adhesion by ATA in serum-free culture

One of the characteristics of CHO cells in a serum-free culture is that they form an unattached sphere with aggregated appearance in the medium. Therefore, cells are difficult to establish for immunocytochemical analyses when needed. Previously, Liu et al. reported that ATA (30 μg/mL) exerts insulin-like growth stimulating effects on CHO cells under serum-free conditions;[22] however, it has not been used as an attachment agent for culturing cells. In the present study, we expanded the study by Liu et al.[22] by testing whether ATA could promote the adhesion of some neuronal cells. Initially, we chose CHO cells in the preliminary study to evaluate the effect of ATA on CHO cell growth in serum-free media. We are able to show the morphological changes and adherence of CHO cells following the media supplemented with different concentrations of ATA for cells cultured for 3 days (see Supporting Information). The major advantage is that we observed the capability of ATA in converting cells from

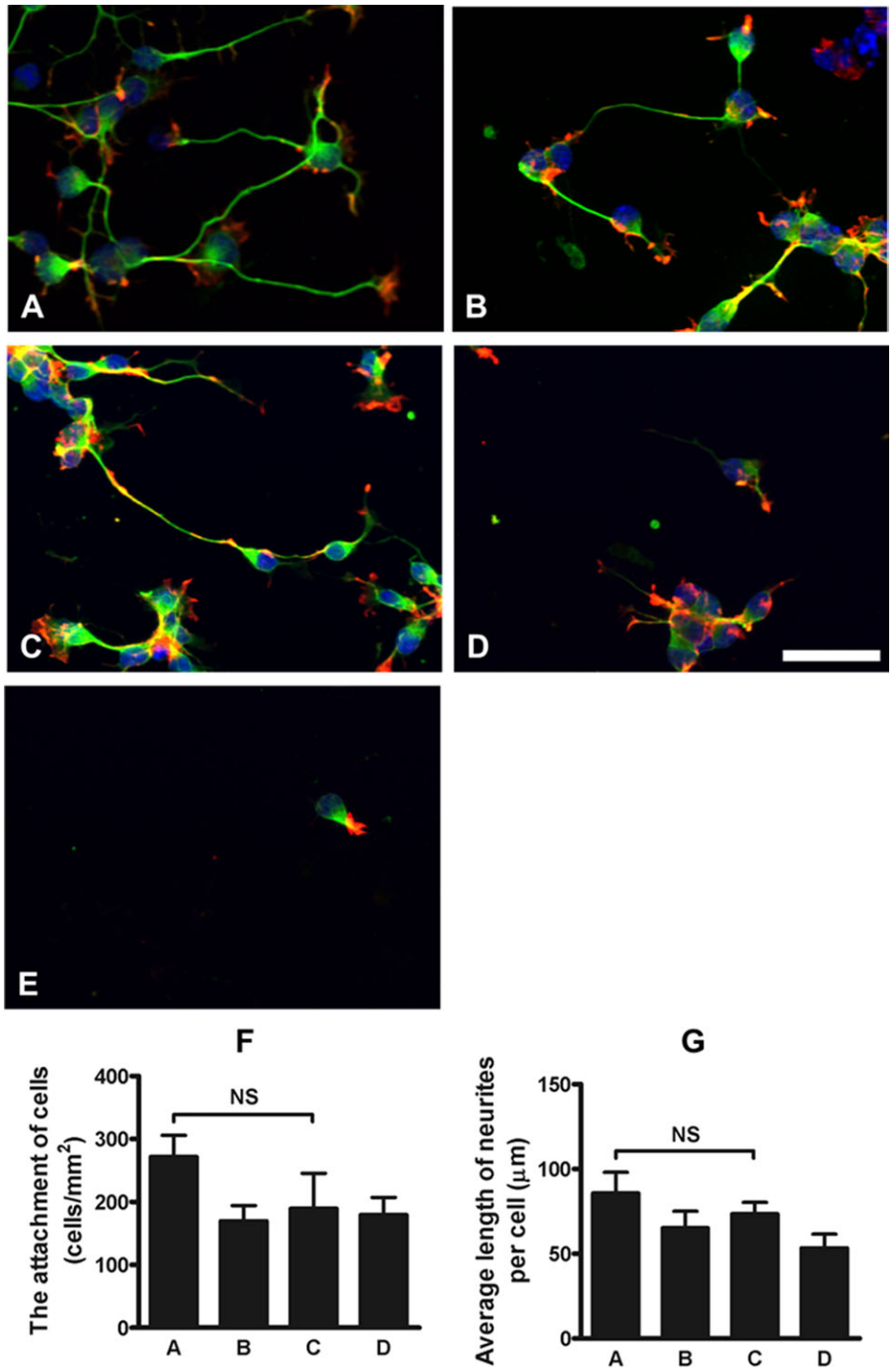

Figure 1. Effect of ATA on the attachment and morphology of mouse P19 neurons.

Differentiated P19 neurons were cultured for 2DIV in serum-free medium. Cells in coverslips were fixed and immunofluorescence staining to visualize neurites (in green). Growth cones were observed in red. Cell nuclei were stained in blue. A: Glass coverslips pretreated with PLL for coating. B–D: Coverslips treated without PLL, but cultured in medium containing 15 μg/mL (B), 30 μg/mL (C), and 50 μg/mL ATA (D). Negative control: non-PLL-treated coverslip cultured in medium containing 0.1% DMSO only (E). F: Number of cells attached per mm^2 (mean of five areas randomly selected). G: Length of neurites. NS: not significant with $P > 0.05$. Scale bar = 50 μm.

suspended aggregation to an almost evenly attached form. The addition of ATA (15 μg/mL to 30 μg/mL) not only increases the total CHO proliferation containing both attached and suspended cells (see Supporting Information) but also enhances the cell attachment up to ~60% of total proliferated cells (see Supporting Information). At ATA concentration $\geq$50 μg/mL, the total attached and unattached cells were attenuated, which may be due to the toxicity of the concentration to the cells. In general, ATA is a compound with low toxicity in vivo. The 50% lethal dosage (LD_{50}) is reported to be 0.34 g/kg or ~425 μg/mL body-volume in mice.[26] In this study, the final optimal concentrations of ATA used in a culture medium were ~10-fold less than the LD_{50} in vivo. Although the mechanism involved in cell toxicity is not readily known, high ATA concentration is likely to reduce the number of cell attachments.

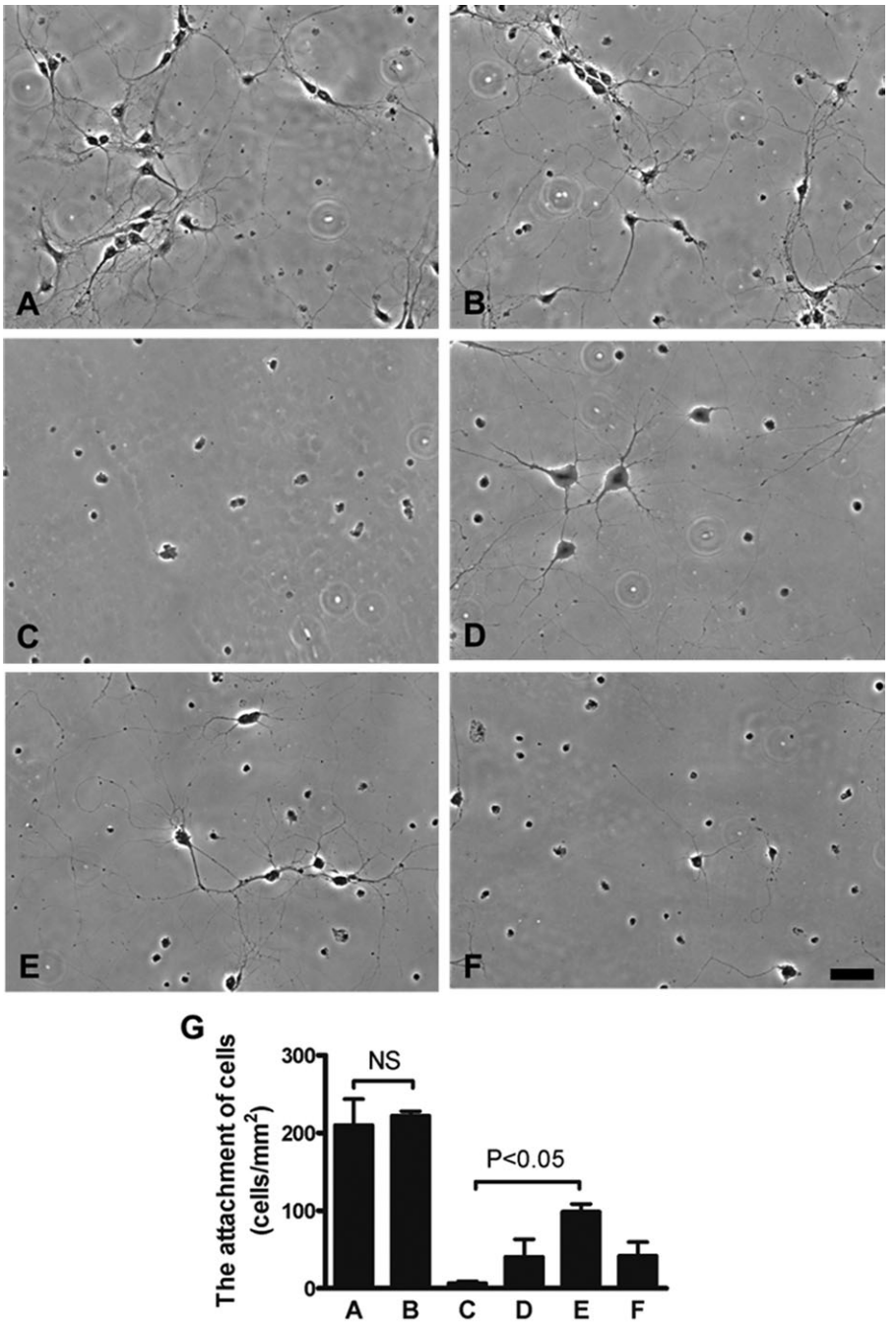

Figure 2. Effect of ATA on the attachment of hippocampal neurons.

Hippocampal neurons grown on coverslips in serum-free medium after 2DIV were shown. A and B: Slips precoated with PLL and grown in medium without (A) or with (B) 0.1% DMSO. C–F: Slips treated without PLL, but cultured in medium containing 0.1% DMSO only (C), 3.0 μg/mL (D), 15 μg/mL (E), and 30 μg/mL ATA (F) (scale bar = 50 μm). G: Number of cells attached per mm^2 (mean of five areas randomly selected). NS: $P > 0.05$.

With respect to the factors that may contribute to ATA-enhanced CHO cell adhesion, we investigated the level changes in some intrinsic factors, such as transcription factor (Snails 1/2), cytoskeletal markers (β-catenin and vimentin), cell-surface proteins (E-cadherin, N-cadherin, occludin, zonula occludens (ZO-1), β1 integrin, and fibronectin), and extracellular matrix protein (laminin β1). Interestingly, we found a significant increase in ZO-1, a gap junction protein, level using a western blot analysis (data not shown). A recent study shows that ZO-1is involved in the attachment of epithelial cells.[22,27] Whether ZO-1 could be responsible for CHO cell adherence remains unknown. Although the detailed mechanism involved in cell adhesion is beyond the scope of the present research, it deserves further study to delineate some possible explanations for our findings.

Application of ATA for the attachment of retinoic acid-induced neuronal P19 cells

The attachment of P19 neurons requires surface modification using PLL or laminin; hence, we next tested whether ATA could enable P19 neuron attachment without surface modification. We maintain the mouse embryonic carcinoma cell line P19 in a standard medium containing 10% FBS

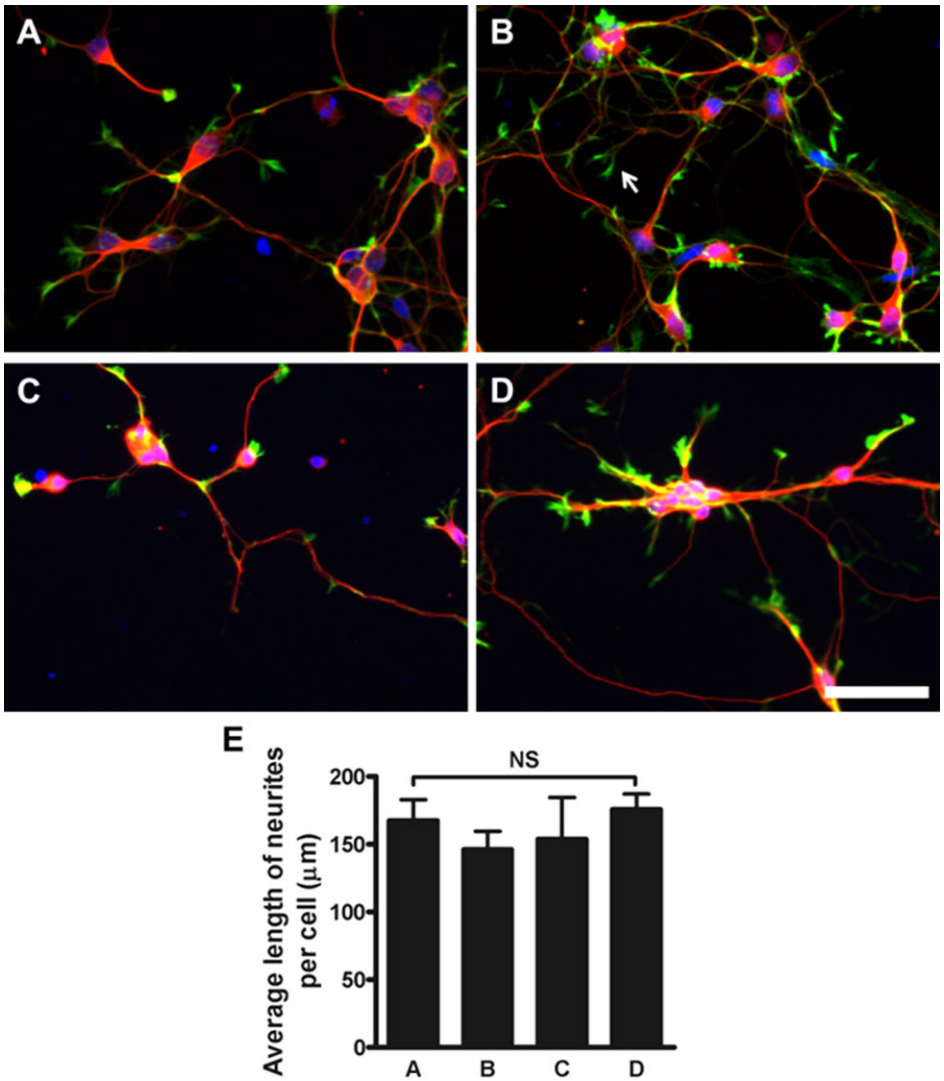

Figure 3. Effect of ATA on the morphology of hippocampal neurons.

Hippocampal neurons were cultured for 2DIV in serum-free medium. Cells on coverslips were fixed and stained to detected neurites (in red). Growth cones were stained with phalloidin dye (in green, arrows). Cell nuclei were observed (in blue). A and B: Glass coverslips pretreated with PLL for coating and cultured without and with 0.1% DMSO, respectively. C and D: Coverslips treated without PLL but cultured in medium containing 3.0 μg/mL (C) or 15 μg/mL ATA (D) (scale bar = 50 μm). E: Average length of neurites per cell (mean of five areas randomly selected). NS: $P > 0.05$.

according to the method previously described.[16] Following proliferation, P19 cells were treated with 0.5-μM retinoic acid in suspension to promote differentiation into neurons.[24] After the dissociation of suspended P19 cell aggregates, differentiated neurons were seeded in a serum-free medium containing various concentrations of ATA.

Figure 1A shows the growth of a differentiated P19 neuron in a conventional PLL-coated coverslip. We demonstrated that ATA promotes P19 cell adhesion to glass coverslips in a serum-free medium without PLL-coating with an optimal concentration around 30 μg/mL judging from immunofluorescence staining for neurite and phalloidin staining for growth cone using the 2-day in vitro (2DIV) culture. Typical morphologic characteristics of the growth cone (in red), neurite (in green), and nucleus (in blue) are shown in Figures 1A–D. Essentially, there was almost no staining in a negative control using non-PLL-coated coverslip (Figure 1E). With ATA dosage $\geq$ 50 μg/mL, the attachment of P19 appears significantly reduced, which is consistent with CHO cells using the same ATA dosage. Since these differentiated P19 cells are widely used in culture today, we propose that ATA may be an alternative reagent for the attachment of stem cell differentiated neurons in a serum-free medium. The average cell number attached and the length of neurites were attenuated in the presence of high ATA concentrations (Figures 1F,G).

Attachment of dissociated mouse hippocampus neurons on culture plate using ATA

Dissociated primary hippocampal neuron culture is one of the most used model systems for neuroscience studies. To this end, pregnant C57BL/6 mice were sacrificed to isolate the embryonic hippocampal neurons. Figures 2A–E show that these hippocampal neurons can attach to the culturing surface at 2DIV in the presence of ATA (3.0–15 μg/mL). However, ATA concentrations greater than 30 μg/mL attenuated the attachment (Figure 2F and G). Notably, the optimal ATA concentration for P19 ($\sim$30 μg/mL) is different from hippocampal neurons (15 μg/mL). We speculate the surface

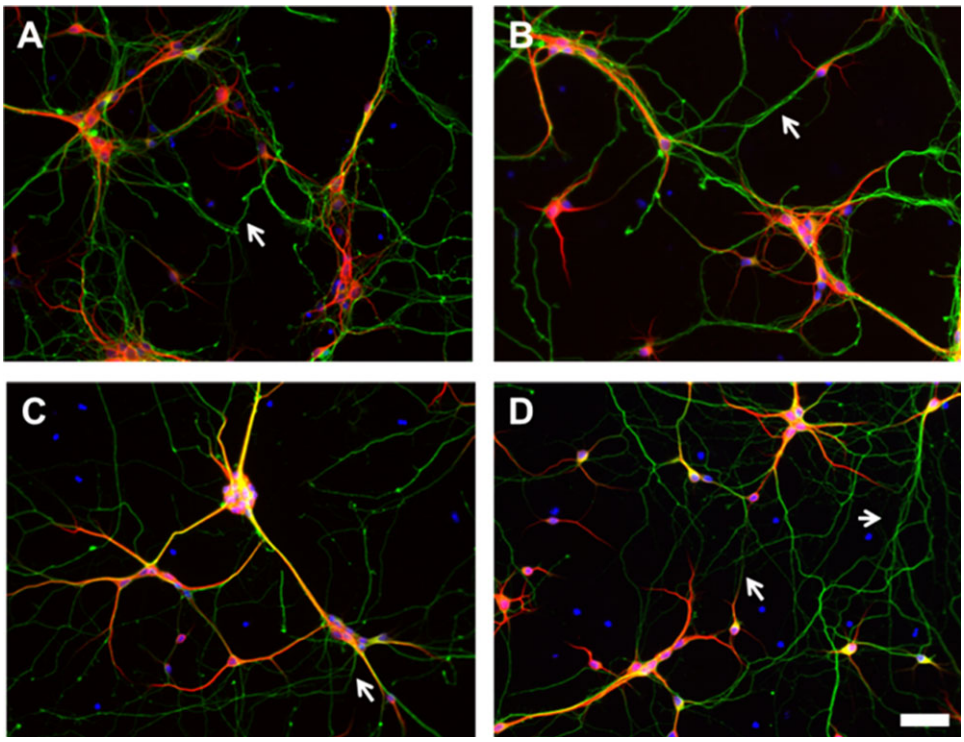

Figure 4. Effect of ATA on the polarization of hippocampal neurons.

Hippocampal neurons cultured for 4DIV in serum-free medium. Cells on coverslips were fixed and stained with axon-specific marker (in green). The dendrites were detected (in red). Cell nuclei were showed (in blue). A and B: Glass coverslips precoated with PLL and cultured without or with 0.1% DMSO, respectively. C and D: Coverslips treated without PLL but cultured in medium containing 3.0 μg/mL (C) or 15 μg/mL ATA (D) (scale bar = 50 μm). Arrows indicate the polarized axon.

molecules that responsible for the cell adherence are regulated differently by the ATA. It appears that the mode of ATA action promoting the adhesion is type specific; P19 cells have a broad range between 15 and 30 μg/mL when compared with that of hippocampal neurons limited at 15 μg/mL. The conventional method using PLL-coated surface, however, showed that the neuron attachment to be approximately twofold better than ATA-treated cells under an optimal condition (15 μ/mL). Furthermore, the addition of ATA to PLL-coated coverslips did not substantiate further adhesion (data not shown), because the attachment between immobilized PLL and neurons is via ionic interaction.[14]

Localization of class III β-tubulin and actin on attached hippocampus neurons on Day 2

Subsequently, we conducted immunofluorescence staining to measure the length of neurite. The essential morphological characterizations (neurite and growth cone) on 2DIV between the two tissue culture techniques (PLL and ATA) were almost identical (Figures 3A–D). Further quantitative analysis using NeurphologyJ software[25] shows that although the average length of neurite over PLL-coated slip is slightly longer than that using ATA, they are not statistically different (Figure 3E).

Polarization of axon and dendrite of attached hippocampal neurons on Day 4

We further monitored the effect of ATA on the polarization of their axon and dendrite and compared it to the standard method using PLL coating on Day 4. Tau-1 was used as an axonal marker, whereas MAP2 was used as a dendrite marker in immunofluorescence staining. Figure 4 reveals that ATA did not affect the neuronal polarization. It is worth mentioning that although the glass coverslips were used for immunocytochemical staining, the cell attachment can also be achieved in cultures with plastic plates (data not shown). Taken together, our data (Figures 3 and 4) suggest that ATA is a feasible agent for the attachment of hippocampal neurons.

ATA may mimic a hormonal action in stimulating CHO cell proliferation in the absence of growth factor and serum.[22] ATA also acts as an antiapoptotic agent in PC-12 neuron cells[28,29] as well as in sympathetic and hippocampus neurons, to protect these cells from exposure to transient ischemia.[28,30] In some cases, ATA can even stimulate tyrosine phosphorylation cascade in PC-12 cells[31,32] and prevent cell death in numerous cell types, including the cancer cell line.[33,34] However, we believe that usage of this compound to promote neuron attachment in culture technique is interesting. For example, ATA protects hippocampal neurons from glutamate excitotoxicity in vitro,[35] but the detailed mechanism involved is unknown. The advantage of ATA for hippocampal neuron attachment renews interest on the proteomic changes, as affected by ATA.

Possible explanations are available for the neuron attachment and survival in the presence of ATA. First, ATA, as an antiapoptotic agent, could affect the survivability and spread of anchorage-dependent cells on the plate without coating. Second, ATA, as a novel nonpeptide cell attachment chemical, may activate adhesion molecular expression to trigger cell-matrix binding opportunity for these cells, although it remains elusive at present. In some reports, ATA is shown

to induce the activation of a series of membrane glycoproteins not related to cell adhesion.[36] Notably, the treatment of plate surface with ATA (or "precoating") did not produce any cell attachment, including CHO and neurons (data not shown). This result indicates that ATA does not function via modifying culture surface chemistry. Furthermore, it is important to point out that ATA application does not promote attachment for all neurons. For instance, we failed to establish the primary dorsal root ganglion neurons, PC-12 neurons, and primary cortical neurons under similar conditions mentioned above. Interestingly, ATA may be type-specific depending on the surface protein expression in a given neuron type, even if it remains elusive.

In conclusion, we present an alternative method for cell line neuron and primary neuron attachment using ATA. Most importantly, the application of ATA does not affect neurite outgrowth or neuronal polarization. It is possible that this technique can be applied to other neurons or some other cell types in cultures with serum-free medium.

Acknowledgment

The authors gratefully acknowledge Simon J. T. Mao of the National Chiao Tung University for his critical review of this manuscript. We are also thankful to the support by the Center for Bioinformatics Research of Aiming for the Top University Program of the National Chiao Tung University and Ministry of Education, Taiwan, R.O.C.

Literature Cited

1. Lei T, Hohn HP, Behr R, Denker HW. Influences of extracellular matrix and of conditioned media on differentiation and invasiveness of trophoblast stem cells. *Placenta*. 2007;28:14-21.
2. Cheng X, Lu SH, Cui Y. ECRG2 regulates ECM degradation and uPAR/FPRL1 pathway contributing cell invasion/migration. *Cancer Lett*. 2010;290:87-95.
3. Shay EL, Greer CA, Treloar HB. Dynamic expression patterns of ECM molecules in the developing mouse olfactory pathway. *Dev Dyn*. 2008;237:1837-1850.
4. Skoglund P, Keller R. Integration of planar cell polarity and ECM signaling in elongation of the vertebrate body plan. *Curr Opin Cell Biol*. 2010;22:589-596.
5. Novotny M, Vasilenko T, Varinska L, Smetana K Jr, Szabo P, Sarissky M, Dvorankova B, Mojzis J, Bobrov N, Toporcerova S, Sabol F, Matthews BJ, Gal P. ER-alpha agonist induces conversion of fibroblasts into myofibroblasts, while ER-beta agonist increases ECM production and wound tensile strength of healing skin wounds in ovariectomised rats. *Exp Dermatol*. 2011;20:703-708.
6. Indra I, Beningo KA. An in vitro correlation of metastatic capacity, substrate rigidity, and ECM composition. *J Cell Biochem*. 2011;112:3151-3158.
7. Grainger DW, Pavon-Djavid G, Migonney V, Josefowicz M. Assessment of fibronectin conformation adsorbed to polytetrafluoroethylene surfaces from serum protein mixtures and correlation to support of cell attachment in culture. *J Biomater Sci Polym Ed*. 2003;14:973-988.
8. Sawyer AA, Hennessy KM, Bellis SL. Regulation of mesenchymal stem cell attachment and spreading on hydroxyapatite by RGD peptides and adsorbed serum proteins. *Biomaterials*. 2005;26:1467-1475.
9. Wu SC, Yu JC, Hsu SH, Chen DC. Artificial extracellular matrix proteins contain heparin-binding and RGD-containing domains to improve osteoblast-like cell attachment and growth. *J Biomed Mater Res A*. 2006;79:557-565.
10. Kato YX, Saito I, Takano H, Mabuchi K, Hoshino T. Comparison of neuronal cell adhesiveness of materials in the diX (Parylene) family. *Neurosci Lett*. 2009;464:26-28.
11. Delivopoulos E, Murray AF, MacLeod NK, Curtis JC. Guided growth of neurons and glia using microfabricated patterns of parylene-C on a SiO2 background. *Biomaterials*. 2009;30:2048-2058.
12. Delivopoulos E, Murray AF, Curtis JC. Effects of parylene-C photooxidation on serum-assisted glial and neuronal patterning. *J Biomed Mater Res A*. 2010;94:47-58.
13. Hoshino T, Saito I, Takano H, Samejima K, Mabuchi K, Kato YX. Neurite outgrowth of PC12 cells on diX (parylene) family materials. *Biotechnol Prog*. 2012;28:587-590.
14. Lu H, Guo L, Kawazoe N, Tateishi T, Chen G. Effects of poly(L-lysine), poly(acrylic acid) and poly(ethylene glycol) on the adhesion, proliferation and chondrogenic differentiation of human mesenchymal stem cells. *J Biomater Sci Polym Ed*. 2009;20:577-589.
15. Kaech S, Banker G. Culturing hippocampal neurons. *Nat Protoc*. 2006;1:2406-2415.
16. Bain G, Gottlieb DI. Neural cells derived by in vitro differentiation of P19 and embryonic stem cells. *Perspect Dev Neurobiol*. 1998;5:175-178.
17. Chen WS, Yueh CY, Huang YA, Hwang E. An inverted method for culturing dissociated mouse hippocampal neurons. *Neurosci Res*. 2011;70:118-123.
18. Milanovic M, Radtke S, Peel N, Howell M, Carriere V, Joffre C, Kermorgant S, Parker PJ. Anomalous inhibition of c-Met by the kinesin inhibitor aurintricarboxylic acid. *Int J Cancer*. 2012;130:1060-1070.
19. Hashem AM, Flaman AS, Farnsworth A, Brown EG, Van Domselaar G, He R, Li X. Aurintricarboxylic acid is a potent inhibitor of influenza A and B virus neuraminidases. *PLoS One*. 2009;4:e8350.
20. Hung HC, Chen TC, Fang MY, Yen KJ, Shih SR, Hsu JT, Tseng CP. Inhibition of enterovirus 71 replication and the viral 3D polymerase by aurintricarboxylic acid. *J Antimicrob Chemother*. 2010;65:676-683.
21. Chen YP, Chen IC, Hwang IE, Yuan GF, Liaw LL, Tseng CP. Selection of an effective red-pigment producing Monascus pilosus by efficient transformation with aurintricarboxylic acid. *Biosci Biotechnol Biochem*. 2008;72:3021-3024.
22. Liu C, Chu I, Hwang S. Aurintricarboxylic acid exerts insulin-like growth stimulating effects on Chinese hamster ovary cells under serum-free conditions. *J Biosci Bioeng*. 2001;91:576-580.
23. Liu CH, Chu IM, Hwang SM. Reference recombinant chinese hamster ovary cell lines. *In Vitro Cell Dev Biol Anim*. 2001;37:633-634.
24. Jones-Villeneuve EM, McBurney MW, Rogers KA, Kalnins VI. Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells. *J Cell Biol*. 1982;94:253-262.
25. Ho SY, Chao CY, Huang HL, Chiu TW, Charoenkwan P, Hwang E. NeurphologyJ: an automatic neuronal morphology quantification method and its application in pharmacological discovery. *BMC Bioinformatics*. 2011;12:230.
26. Balzarini J, Mitsuya H, De Clercq E, Broder S. Aurintricarboxylic acid and Evans Blue represent two different classes of anionic compounds which selectively inhibit the cytopathogenicity of human T-cell lymphotropic virus type III/lymphadenopathy-associated virus. *Biochem Biophys Res Commun*. 1986;136:64-71.
27. Maxi H, Thomas von W, Klaus-Dieter W, Nebe JB. Time-dependent effects of low-temperature atmospheric-pressure argon plasma on epithelial cell. *J Phys D: Appl Phys*. 2012;45:025206.
28. Batistatou A, Greene LA. Aurintricarboxylic acid rescues PC12 cells and sympathetic neurons from cell death caused by nerve growth factor deprivation: correlation with suppression of endonuclease activity. *J Cell Biol*. 1991;115:461-471.
29. Cho H, Lee DY, Shrestha S, Shim YS, Kim KC, Kim MK, Lee KH, Won J, Kang JS. Aurintricarboxylic acid translocates across the plasma membrane, inhibits protein tyrosine phosphatase and prevents apoptosis in PC12 cells. *Mol Cells*. 2004;18:46-52.
30. Aronica EM, Gorter JA, Grooms S, Kessler JA, Bennett MV, Zukin RS, Rosenbaum DM. Aurintricarboxylic acid prevents GLUR2 mRNA down-regulation and delayed neurodegeneration

in hippocampal CA1 neurons of gerbil after global ischemia. *Proc Natl Acad Sci USA.* 1998;95:7115-7120.
31. Okada N, Koizumi S. A neuroprotective compound, aurin tricarboxylic acid, stimulates the tyrosine phosphorylation cascade in PC12 cells. *J Biol Chem.* 1995;270:16464-16469.
32. Okada N, Koizumi S. Tyrosine phosphorylation of ErbB4 is stimulated by aurintricarboxylic acid in human neuroblastoma SH-SY5Y cells. *Biochem Biophys Res Commun.* 1997;230:266-269.
33. Beery R, Haimsohn M, Wertheim N, Hemi R, Nir U, Karasik A, Kanety H, Geier A. Activation of the insulin-like growth factor 1 signaling pathway by the antiapoptotic agents aurintricarboxylic acid and evans blue. *Endocrinology.* 2001;142:3098-3107.
34. Haimsohn M, Beery R, Karasik A, Kanety H, Geier A. Aurintricarboxylic acid induces a distinct activation of the IGF-I receptor signaling within MDA-231 cells. *Endocrinology.* 2002;143:837-845.
35. Samples SD, Dubinsky JM. Aurintricarboxylic acid protects hippocampal neurons from glutamate excitotoxicity in vitro. *J Neurochem.* 1993;61:382-385.
36. Kim HK, Kim JE, Park CM, Kim YT, Han KS, Cho HI. Aurintricarboxylic acid upregulates the thrombomodulin expression of endothelial cells and peripheral blood monocytes. *Blood Coagul Fibrinolysis.* 2008;19:489-494.

Manuscript received May 31, 2012, and revision received Aug. 8, 2012.