Exercise Rehabilitation and/or Astragaloside Attenuate Amyloid-beta Pathology by Reversing BDNF/TrkB Signaling Deficits and Mitochondrial Dysfunction

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Abstract
We aim to investigate the mechanisms underlying the beneficial effects of exercise rehabilitation (ER) and/or astragaloside (AST) in counteracting amyloid-beta (Aβ) pathology. Aβ oligomers were microinjected into the bilateral ventricles to induce Aβ neuropathology in rats. Neurobehavioral functions were evaluated. Cortical and hippocampal expressions of both BDNF/TrkB and cathepsin D were determined by the western blotting method. The rat primary cultured cortical neurons were incubated with BDNF and/or AST and ANA12 followed by exposure to aggregated Aβ for 24 h. In vivo results showed that ER and/or AST reversed neurobehavioral disorders, downregulation of cortical and hippocampal expression of both BDNF/TrkB and cathepsin D, neural pathology, Aβ accumulation, and altered microglial polarization caused by Aβ. In vitro studies also confirmed that topical application of BDNF and/or AST reversed the Aβ-induced cytotoxicity, apoptosis, mitochondrial distress, and synaptotoxicity and decreased expression of p-TrkB, p-Akt, p-GSK3β, and β-catenin in rat cortical neurons. The beneficial effects of combined ER (or BDNF) and AST therapy in vivo and in vitro were superior to ER (or BDNF) or AST alone. Furthermore, we observed that any gains from ER (or BDNF) and/or AST could be significantly eliminated by ANA-12, a potent BDNF/TrkB antagonist. These results indicate that whereas ER (or BDNF) and/or AST attenuate Aβ pathology by reversing BDNF/TrkB signaling deficits and mitochondrial dysfunction, combining these two potentiates each other’s therapeutic effects. In particular, AST can be an alternative therapy to replace ER.

Keywords Alzheimer’s disease · Exercise rehabilitation · Astragaloside · BDNF · Mitochondrial dysfunction · Amyloid-beta

Abbreviations

Aβ Amyloid-beta
p-Akt Phosphorylated serine/threonine-protein kinase
T-Akt Total form serine/threonine-protein kinase
ANA12 N-[2-[[Hexahydro-2-oxo-1H-azepin-3-yl]amino]carbonyl]phenyl] benzo[b] thiophene-2-carboxamide

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Introduction

Alzheimer’s disease (AD) is the most common form of age-related memory loss. It is characterized by cognitive deficits, β-amyloid (Aβ) deposition, neurodegeneration, neurofibrillary tangle formation, and central neuroinflammation [1]. The exercise provided cognitive benefit in a mouse AD model by elevating hippocampal and cortical levels of brain-derived neurotrophic factor (BDNF). The hippocampus, parietal cortex, temporal cortex, and frontal cortex of AD brains had substantially reduced BDNF levels [2]. Exercise rehabilitation (ER) shares with BDNF therapy the similar beneficial effects on cognition in an AD’s mouse model [1].

The astragaloside (AST), a small-molecule saponin purified from Astragalus membranaceus, also shares with BDNF administration similar beneficial effects in preventing the occurrence of memory loss, synaptotoxicity, oxidative stress, and mitochondrial dysfunction in rat brains caused by Aβ deposition [3–7]. Again, the neurodegeneration mechanisms underlying the beneficial effects of ER and/or AST on AD conditions remain unclear.

It has been promoted that abnormal precursor protein (APP) processing and Aβ production in mitochondria disturbed the axonal transport by impairing mitochondrial function and attenuate BDNF-neurotrophic tyrosine receptor kinase B (TrkB) signaling subsequently [8]. Evidence has accumulated to indicate that Aβ overproduction is a causative event in AD pathogenesis. The present study aimed to investigate whether ER and/or AST attenuate AD pathology via reversing Aβ-induced mitochondrial dysfunction and BDNF/TrkB signaling deficits.

To deal with the question, we used bilateral intracerebroventricular Aβ microinjected [3] to ascertain whether the Aβ-induced neurobehavior deficits, neuropathology, and altered BDNF/TrkB signaling can be affected by ER and/or AST. Additionally, we used cultured primary cortical neurons in vitro model to confirm whether local application of BDNF (to mimic ER effects) and/or AST can inhibit Aβ-induced cytotoxicity, apoptosis, synaptotoxicity, mitochondrial dysfunction, and altered BDNF/TrkB signaling. Furthermore, we would elucidate whether the proposed gains from ER (or BDNF application) and/or AST in reducing Aβ-induced AD condition can be eliminated by inhibition of BDNF/TrkB signaling with ANA-12 [9]. Figure 1 depicts the schematic diagrams showing the in vivo and in vitro experimental designs.

Methods

All authors had access to the study data and reviewed and approved the final manuscript. All in vivo studies represent 20 animals per group. All in vitro studies are from at least three replicate experiments. All animal experiments were designed in accordance with the ARRIVE guidelines [10]. All animal experiments were approved and carried out in accordance with the Institutional Animal Care and Use Committee at the Chi Mei Medical Center, Tainan, Taiwan (approved no. 108120110).

Animals

The laboratory animals were male Wistar rats with a weight of 250–350 g (BioLASCO Taiwan Co., Ltd.). The rats were housed in an air-conditioned animal facility at 26 ± 0.5 °C under a 12-h light–dark cycle and given unlimited access to water and food. All experiments were conducted during the daytime 10:00–18:00.

The rats were randomly assigned into eight groups, including (1) sham operation with the sedentary and vehicle-treated group (Sham + Sed + Veh); (2) bilateral intracerebroventricular (i.c.v.) injection of Aβ1–42 with the sedentary and vehicle-treated group (Aβ + Sed + Veh); (3) i.c.v. injection of Aβ1–42 with ER and vehicle-treated group (Aβ + ER + Veh); (4) i.c.v. injection of Aβ1–42 with ER and vehicle-treated group (Aβ + ER + Veh); (5) i.c.v. injection of Aβ1–42 with ER and vehicle-treated group (Aβ + ER + Veh); (6) i.c.v. injection of Aβ1–42 with ER and vehicle-treated group (Aβ + ER + Veh); (7) i.c.v. injection of Aβ1–42 with ER and vehicle-treated group (Aβ + ER + Veh); and (8) i.c.v. injection of Aβ1–42 with ER and vehicle-treated group (Aβ + ER + Veh).

The rats were randomly assigned into eight groups, including (1) sham operation with the sedentary and vehicle-treated group (Sham + Sed + Veh); (2) bilateral intracerebroventricular (i.c.v.) injection of Aβ1–42 with the sedentary and vehicle-treated group (Aβ + Sed + Veh); (3) i.c.v. injection of Aβ1–42 with ER and vehicle-treated group (Aβ + ER + Veh); (4) i.c.v. injection of Aβ1–42 with ER and vehicle-treated group (Aβ + ER + Veh); (5) i.c.v. injection of Aβ1–42 with ER and vehicle-treated group (Aβ + ER + Veh); (6) i.c.v. injection of Aβ1–42 with ER and vehicle-treated group (Aβ + ER + Veh); (7) i.c.v. injection of Aβ1–42 with ER and vehicle-treated group (Aβ + ER + Veh); and (8) i.c.v. injection of Aβ1–42 with ER and vehicle-treated group (Aβ + ER + Veh).
injection of Aβ₁₋₄₂ with ER and AST and ANA12 administration (Aβ+ER+AST+ANA12). The ALZET osmotic pump full of AST was implanted subcutaneously. The ANA12 was injected intraperitoneally every day from day 0 to day 35. The treadmill ER was performed on day 8 post-surgery. The cognitive functions were measured before surgery and every 7 days post-surgery. After the last cognitive evaluation at week 5 post-surgery, the rats were sacrificed.

**Aβ₁₋₄₂ Oligomerization**

Aβ₁₋₄₂ was obtained from Anaspec (Cat.# AS-20276, Fremont, CA, USA). The lyophilized Aβ peptides were prepared by reconstituting the powder in 1% ammonium hydroxide. Subsequently, this stock was dissolved in 1X PBS to obtain a 30-uM working solution and then subjected to oligomerization. For oligomerization, Aβ peptides were incubated at 4°C overnight and then aliquoted to store at –80°C until use. The vehicle solution consists of 1% ammonium hydroxide in 1X PBS.

**ICV Injection of Aβ₁₋₄₂**

Rats were anesthetized with a combination of Zoletil (40 mg/kg; Virbac, Nice, France), xylazine hydrochloride (2 mg/kg; Balanzine, Health-Tech Pharmaceutical Co., Taipei, Taiwan), and atropine sulfate (1 mg/kg; Tai Yu Chemical & Pharmaceutical Co. Ltd., Hsinchu, Taiwan) intraperitoneally (i.p.) and then placed in a standard stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). A middle sagittal incision was made in the scalp and was sterilized using standard procedures. Bilateral holes were drilled in the skull using a dental drill over the lateral ventricles. Rats were bilaterally and intracerebroventricularly (i.c.v., AP: −0.8 mm, ML: ± 1.4 mm, DV: −4.0 mm) injected with 10 μl into each lateral ventricle of Aβ₁₋₄₂ (Cat.# AS-20276, AnaSpec, Inc., Fremont, CA, USA) at a rate of 1 μl/min using Hamilton microsyringe and a minipump (Hamilton, Reno, NV, USA). The syringe was removed 5 min after injection. The sham group received sterile vehicle. After surgery, the scalp was sutured, and sulfamethoxazole was sprinkled on the wound to prevent infection. In addition, penicillin (40,000U) was injected intramuscularly into the gluteus once a day for 3 days.

**Treadmill Exercise Protocol**

On day 8 post-surgery, we trained the rats on a treadmill (model Exer-3/6, Columbus Instruments, Columbus, OH, USA) 5 days a week for 4 weeks. They were acclimatized to run for 15 min at 20 m/min at 0° for 3 days initially, and then they were running for 30 min at 20 m/min at 5°, 30 min at 30 m/min at 5°, 30 min at 30 m/min at 5°, and 30 min at 30 m/min at 5° after 1, 2, 3, and 4 weeks of training, respectively. Rats in the Sham + Veh and Aβ+ Veh groups were placed daily on a stationary treadmill [13].

**Drug Treatment**

Astragaloside (AST, molecular formula: C28H32O17; molecular weight = 640.55, was purchased from Fusol Material Co., Ltd, Tainan, Taiwan) was dissolved in 2 ml of saline with 5% EtOH and administered through an ALZET osmotic minipump (Alzet 2ML4; Alza, Palo Alto, CA, USA) at a dose of 10 mg/kg/day after Aβ₁₋₄₂ injection [3, 14, 15].

The rats received another i.p. injection of vehicle (1% dimethylsulfoxide in physiological saline, 1 ml/kg; Sigma-Aldrich, St. Louis, MO, USA) or N-[2-[(Hexahydro-2-oxo-1H-azepin-3-yl) amino]carbonyl] phenyl benzo [b] thiophene-2-carboxamide (ANA12, a TrkB receptor antagonist, 1 mg/kg/day, i.p.; Selleckchem, Houston, TX, USA), respectively [9].

**Behavioral Tests**

Radial arm maze task, Y-maze task, and rotarod motor coordination test were performed sequentially.

**Radial Arm Maze Task**

The maze comprised 8 arms, extending radially from a central area. Before the training, rats were placed to explore the maze for 5 min and consume food freely. They were trained for 5 days to run to the end of the arms and consumed the baited food. The training trial continued until 5 min has passed. After adaptation, each animal was checked for working and reference memory, in which the same four arms (no. 2, 4, 6, and 8) were baited. All the rats were trained with one trial: (1) before Aβ₁₋₄₂ injection per day for 5 days and (2) after Aβ₁₋₄₂ injection 7, 14, 21, 28, and 35 days. Finally, the number of long-term memory (reference memory) errors (entrance into an arm that never baited) and short-term memory (working memory) errors (repeat entrance into an arm) were counted [16]. Latency was measured as the time elapsed from the beginning to the end of each trial. All measures were averaged across each trial.

**Y Maze Task**

Short-term spatial recognition memory was assessed by analysis of spontaneous alternation in Y-maze, as reported before [16, 17]. In brief, each rat was placed at the end of one arm and allowed to move through the arms for 8 min, and a sequence of arm entries was recorded. Alternation was obtained as successive entries into the three arms on overlapping triplet sets. The maximum number
of possible spontaneous alternations was obtained as the total number of arms entered minus 2, and the alternation was calculated as the ratio of actual to possible alternations \( \times 100 \).

**Rotarod Motor Coordination Test**

Rotarod accelerating test was also performed on each animal to evaluate motor coordination impairment [18]. It was to examine the possible defects in neuromuscular coordination.
that might occur on the Aβ1-42 injection rats. Before the stereotoxic surgery, each rat was placed in a rotarod apparatus and subjected to an accelerating test. The rat was placed on the rotating rod (at the slowest speed, 4 rpm) for 1 min and accelerated to its maximum speed of 30 rpm at 3 min. Each rat that could not hold at the acceleration rod for more than 1 min was excluded from further experiments. For the qualified rats starting from the 2nd day before surgery, the rats were trained per day as described above for 2 days. On days 7, 14, 21, 28, and 35, after surgery, the rotational speed of the rod was then accelerated to its maximum speed of 30 rpm. The length of the time that rats could grasp at the rod was measured. The test score is the average number of seconds that rats could hold onto the rod per trial. 

Histological Analyses

Formalin-fixed brains were embedded in paraffin blocks. Serial sections through the hippocampus (−2.8 mm anterior to bregma to −4.3 mm anterior to bregma) and motor cortex (4.2 mm anterior to bregma to 1.2 mm anterior to bregma) were stained with hematoxylin and eosin for microscopic examination. An examiner who was blinded to the experimental conditions evaluated the extent of neuronal damage for each section. The damage scores were determined by two grading systems. In the first system, Honório et al. [19] determined the damage scores from 0 to 4 in which “0,” “1,” “2,” “3,” and “4” denote no pathological changes, lesions involving 25% of the field, lesions involving 25 to 50%, lesions involving 50 to 75%, and lesions involving 75 to 100%, respectively. In another system, Liu et al. [20] used 0, 1, 2, and 3 which denote normal morphology, minor damage (edema, few pyknotic cells), moderate damage (structural disorganization, edema, moderate pyknotic cells, vacuolization, inflammatory cell infiltration), and intense damage (structural disorganization, edema, intense pyknotic cells, vacuolization, inflammatory cell infiltration), respectively. In our present study, we multiplied these two grading scores to present damage scores.

Thioflavin-S Stain

Amyloid plaques were stained with Thioflavin-S. The deparaffinized and hydrated sections were incubated in 0.25% potassium permanganate solution for 20 min, rinsed in distilled water, and incubated in bleaching solution (2% oxalic acid and 0.9% potassium metabisulfite) for 2 min. After being rinsed in distilled water, the sections were transferred to a blocking solution (1% sodium hydroxide and 0.9% potassium permanganate solution for 20 min, incubated for 5 min in 0.25% acidic ethanol. The sections were washed with 50% ethanol, placed in distilled water, and then covered with a glass cover using a mounting solution [21]. For immunostaining and western blotting assays, brain samples were collected from independent experiments. Actually, the molecular data come from the different animals not used for behavioral tests.

Triple Immunofluorescence Staining

Dissected brains were fixed in 4% phosphate-buffered formalin at 4 °C, and subjected to immunohistochemical staining. The following antibodies were used: Iba1 (1:200; #ab53004, Abcam Inc., Boston, MA, USA), CD68 (1:200, #GTX10042, GeneTex Inc., Irvine, CA, USA), and Neu-N (1:200, MAB377, Merck Millipore, Billerica, MA, USA). Iba1+ and CD68+ cells were visualized using Alexa Fluor 568-conjugated goat anti-rabbit IgG (#A11011, Invitrogen, CA, USA) and Alexa Fluor 488-conjugated goat anti-rabbit IgG (#A11008, Invitrogen), and wavelengths excitation and emission at 578/603 nm and 495/525 nm respectively.
NeuN+ cells were using Alexa Fluor 568–conjugated goat anti-mouse IgG (#A11004, Invitrogen) and excited with 578 nm and observed through 603-nm emission. The sections were finally counterstained with 4', 6-diamidino-2-phenylindole (DAPI, 1:50,000, excitation/emission wavelengths: 358/461; #62,247, Thermo Fisher Scientific Inc., MA, USA) for identification of nucleus.

For degenerative neuron detection, after secondary antibody incubation, the brain tissue slides were immersed in a solution of 5% sodium hydroxide and 100% ethanol for 5 min, followed by immersion in 70% ethanol for 2 min, distilled water for 2 min, and 0.06% potassium permanganate solution for 10 min. The sections were then rinsed in distilled water for 2 min/2 times and placed in a 0.0004% Fluoro-Jade B solution (#AG310, Millipore, MA, USA) made by adding 4 ml of a 0.01% stock solution of Fluoro-Jade B to 96 ml of 0.1% acetic acid. After 20 min in the Fluoro-Jade B staining solution, the stained slides (green fluorescence with an excitation peak at 480 nm and emission peak at 525 nm) were thoroughly washed in distilled water, dehydrated, and coverslipped [22, 23].

Before the primary antibody incubation, the brain slides were treated with proteinase K (20 µg/ml) for 15 min at room temperature for neuronal apoptosis detection. Subsequently, equilibrium buffer was applied for 10 s, and the brain slides were immersed and incubated for 1 h in working strength terminal deoxynucleotidyl transferase (TdT) enzyme solution at 37 °C. Following incubation in stop/wash buffer for 10 min to terminate the reaction, brain slides were incubated for 30 min in working strength anti-digoxigenin conjugate at room temperature in the dark to visualize the DNA fragments. Proteinase K, equilibrium buffer, and stop/wash buffer were all included in the terminal deoxyribonucleotide transferase–mediated dUTP nick end labeling (TUNEL) assay kit (excitation/emission wavelengths: 480/520 nm; #630,108, Takara Bio USA, Inc., CA, USA) [24]. TUNEL-positive neurons with condensed nuclei were identified as dead neurons.

After a final wash with PBS, slides were mounted in glycerol gelatin mounting medium (#GG1-15 ML, Sigma-Aldrich, St. Louis, MO, USA) and viewed using an upright fluorescence microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). A digital camera linked to a computer running Axioscope version 4 (Carl Zeiss) was used to capture images. A pathologist counted the percentage of Fluoro-Jade+ NeuN/DAPI and TUNEL+ NeuN/DAPI double-labeled cells in 6 fields per section in cortex and hippocampus (×400 magnification).

**Western Blot Assay**

Western blotting was performed using the standard method. The total proteins, obtained from cell cultures or brain tissues, were extracted by the modified RIPA buffer (50 mM Tris–HCl, pH7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA) containing protease and phosphatase inhibitors (Sigma, St Louis, Mo, USA). Protein concentrations were quantified by the Bradford method (Bio-Rad, Hercules, CA, USA). For blot analysis, 10 µg of proteins per condition was boiled for 10 min in loading buffer, separated on SDS-PAGE, and transferred to polyvinylidene difluoride membrane (Pall Corporation, East Hills, NY, USA) using a wet transfer system (Bio-Rad, Hercules, CA, USA). The membranes were blocked in 5% non-fat milk in PBS containing 0.05% Tween-20 (TBS-T) for 1 h at room temperature. The membranes were hybridized with Cathespin D (#MA517236, Thermo Fisher Scientific Inc.), BDNF (#ab108319, Cell Signaling Technology, Inc., Beverly, MA, USA), TrkB (#4603, Cell Signaling), Phospho-TrkB (#ABN1381, Millipore), Phospho-Akt (phosphoresidue serine 473, #4060, Cell Signaling), total Akt (#9272, Cell Signaling), Phospho-GSK-3β (phosphoresidue serine 9, #9336, Cell Signaling), total GSK-3β (#9315, Cell Signaling), β-catenin (#9562, Cell Signaling), and β-actin (#SC-47778, Santa Cruz) antibodies overnight at 4 °C. After washing with TBS-T, the membranes were continuously incubated with appropriate secondary antibodies coupled to horseradish peroxidase (Cell Signaling Technology, Inc.) for 1 h at room temperature. The blots were developed in the ECL Western detection reagents (PerkinElmer, Waltham, MA, USA) and exposed to Hyperfilm ECL (GE Healthcare, Piscataway, NJ, USA). Protein bands were scanned and quantified using ImageMaster TotalLab image analysis software (GE healthcare).

**Aβ-Induced Primary Cortical Neuron Injury Model**

Neuronal cultures were prepared from the cortex of embryonic day 18 rat embryos. In brief, embryos were removed and their brain cortices dissected. The neurons were obtained by digestion of papain (2 mg/ml for 30 min at 30 °C, Millipore, Billerica, MA, USA) followed by trituration with a 1-ml pipette. The cells were plated onto poly-L-lysine (Trevigen, Gaithersburg, MD, USA)–coated 6-well plates or dishes and maintained in neurobasal media supplemented with 2% B27 and 0.5 mM GlutaMax (Invitrogen, Carlsbad, CA, USA) at 37 °C in a humidified 5% CO2 incubator. Culture media were exchanged every 4 days, and cells were grown for 8 days in vitro (DIV) before Aβ25–35 treatment. Aβ25–35 was purchased from Kelowna International Scientific Inc. (Taipei, Taiwan) and dissolved in water with vortex. The solution was allowed to aggregate for 6 days at 37 °C before use. BDNF protein obtained from GenScript (Piscataway, NJ, USA) was dissolved (100 µg/ml) in distilled water. The Chinese herb Astragaloside (AST) was made up as a 100 mg/ml stock in DMSO. ANA12 (TrkB selective antagonist) was purchased from Selleckchem (Houston, TX, USA).
USA) and dissolved in DMSO. To induce cell cytotoxicity, the cortical neurons were exposed to 60 μM Aβ25–35 for 24 h. BDNF, AST, and ANA12 were administered at 400 ng/ml, 400 μg/ml, and 35 μM, respectively.

**MTT Assay for Cell Viability**

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Invitrogen, CA, USA) assay. After treatment, cells were treated with the MTT solution (final concentration, 5 μg/ml) for 2 h. The dark blue formazan crystals that formed in intact cells were solubilized with lysis buffer (20% sodium dodecylsulfate in 50% aqueous N, N-dimethylformamide). The absorbance of the sample was read at 540–595 nm with a MultiSkan GO microplate reader (Thermo Fisher Scientific Inc.). Results were expressed as the percentage (%) of MTT reduction, assuming that the absorbance of control cells was 100%.

**Synaptic Density**

The cells were fixed with 4% paraformaldehyde in PBS, permeabilized, and blocked with 0.3% Triton-X 100 plus 5% normal bovine serum in PBS. The cultures were then incubated with combinations of the primary antibodies against the following targets: microtubule-associated protein 2 (MAP-2; a neuronal marker; #SC-74421, Santa Cruz) and synaptophysin (an integral membrane protein localized to synaptic vesicles;#MA5-14,532, Thermo Fisher Scientific Inc.) and diluted in 5% BSA overnight at 4 °C. Then, cortical neurons were incubated with the following isotype-specific secondary antibodies for 1 h at room temperature: Alexa Fluor 568–conjugated goat anti-mouse IgG (excitation/emission wavelengths: 578/603) or Alexa Fluor 488–conjugated goat anti-rabbit (excitation/emission wavelengths: 495/525). The neurons were also stained with DAPI (#62,247, Thermo Fisher Scientific Inc.) and diluted in 5% BSA overnight at 4 °C. Then, cortical neurons were incubated with the following isotype-specific secondary antibodies for 1 h at room temperature: Alexa Fluor 568–conjugated goat anti-mouse IgG (excitation/emission wavelengths: 578/603) or Alexa Fluor 488–conjugated goat anti-rabbit (excitation/emission wavelengths: 495/525). The neurons were also stained with DAPI (#62,247, Thermo Fisher Scientific Inc.) as a nuclear marker. Their digital images were captured with a 20× objective (N.A. 0.75) by a fluorescence microscope system (Carl Zeiss Microscopy GmbH, Jena, Germany) with Zen Software (Carl Zeiss). Synaptic density was measured as a synaptophysin-positive area. At least 10 fields obtained from different experiments conducted in triplicate from 3 cultures were captured and measured for each condition. Values obtained for each field (0.15 mm²) were pooled to obtain mean values for each culture.

**Mitochondrial Membrane Potential Analysis**

After culturing, JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide; BD Mitoscreen kit, San Jose, CA, USA) was added to a final concentration of 2.5 μM, and cells were shaken in the dark at 37 °C for 15 min. Following incubation, 400 μl staining buffer was added to each sample and analyzed on the Novocyte flow cytometry (ACEA Biosciences, CA, USA). A total of 10,000 events were measured using FL-1 channel (525 nm), and results were expressed as a percentage.

**Sub-G1 Phase Analysis**

At the end of the treatment period, the cells were harvested, washed with PBS, and fixed in cold 70% ethanol at −20 °C overnight. The fixed cells were washed with PBS and treated with propidium iodide (PI) staining solution containing 10 μg/ml PI (Thermo Fisher Scientific Inc.), 100 μg/ml RNase (Thermo Fisher Scientific Inc.), and 0.1% Triton X-100 (Sigma-Aldrich) for 30 min at 37 °C. Finally, the samples were analyzed in the Novocyte flow cytometer using NovoExpress software to analyze sub-G1 distribution.

**Evaluation of DNA Fragmentation**

DNA was extracted by the use of a commercially available Wizard genomic DNA purification kit (Promega, Madison, WI, USA) following the manufacturer’s instructions. Neuron cell was lysed in Nuclei lysis solution, and then all RNAs were digested with RNase A solution for 30 min at 37 °C water bath. Protein precipitation solution was added, vortexed vigorously, and kept on ice for 5 min. After a short centrifugation, the supernatant was transferred to a new microtube containing 600 ul isopropanol, mixed by inversion, and centrifuged. The supernatant was removed, and the pellet was washed in 600 μl 70% ethanol. Finally, the pellet was air-dried and then rehydrated with hydration solution, and the DNA concentration was determined. Two micrograms of extracted DNA was loaded to 1.8% agarose gel electrophoresis in Tris–borate-EDTA buffer system, stained with ethidium bromide, and visualized through a Quantum CX5 UV-transilluminator (Vilber Lourmat, Torcy, France) and photographed to determine the DNA concentration.

**Statistics**

The person charged with functional outcome measurements was the only one that was blinded to treatments among those working on animals (single-blind). She used cage and animal codes to recognize individuals and to report repeated measurements on data collection forms. Statistical analyses were performed using GraphPad Prism 7.01 (GraphPad Software Inc., CA, USA). Parameters such as histological scores and the immunofluorescence staining data with non-normal distribution were analyzed by the Kruskal–Wallis (KW) test with Dunn’s post-hoc test. One-way analysis of variance (ANOVA) with Tukey’s post-hoc test was used to analyze the flow cytometry (mitochondrial membrane...
potential and sub-G1 phase analysis), western blotting, and synaptic density data. We performed two-way ANOVA with Tukey’s multiple comparisons tests to analyze behavioral performance. All data were expressed as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM). P values <0.05 were considered statistically significant.

Results

ANA-12 Can Ameliorate the Beneficial Effects of ER and/or AST on Aβ-Induced Cognitive and Motor Deficits in Rats

Thirty-five days following Aβ injections, rats displayed deficits in working memory (e.g., decreased % of alternations in Y-maze test, [two-way ANOVA with time and group as factors, time difference $F(5,390) = 36.61, P < 0.0001$; group difference $F(7,390) = 80.45, P < 0.0001$; time-group interaction $F(35,390) = 7.686, P < 0.0001$; Fig. 2a), spatial reference memory (e.g., increased latency time [two-way ANOVA with time and group as factors, time difference $F(5,390) = 9.679, P < 0.0001$; group difference $F(7,390) = 17.44, P < 0.0001$; time-group interaction $F(35,390) = 8.594, P < 0.0001$; Fig. 2b, c, and d), and motor performances (e.g., decreased latency time [two-way ANOVA with time and group as factors, time difference $F(5,390) = 19.8, P < 0.0001$; group difference $F(7,390) = 15.05, P < 0.0001$; time-group interaction $F(35,390) = 1.814, P = 0.0039$] in the radial-maze test; Fig. 2b, c, and d), and motor performances (e.g., decreased latency time [two-way ANOVA with time and group as factors, time difference $F(5,390) = 33.06, P < 0.0001$; group difference $F(7,390) = 19.8, P < 0.0001$; time-group interaction $F(35,390) = 1.406, P = 0.0670$]

![Figure 2](image-url)

**Fig. 2** Exercise rehabilitation (ER) and/or astragaloside (AST) reversed the motor and cognitive deficits by Aβ, which ANA12 wiped out any gains made from ER and/or AST. On days 7, 14, 21, 28, and 35, the spontaneous alternation in the Y-maze (a), the latency to elapse (b), numbers of reference memory errors (c), and numbers of working memory errors (d) in the radial maze tests, and the latency to fall off the rotarod (e) and the maximum speed reached during the test (f) in the rotarod tests were presented. Data are presented as the means ± SEM (n=8–11). *P < 0.05, compared with Sham + Veh + Veh group; †P < 0.05, compared with Aβ + Veh + Veh group; and ‡P < 0.05, Aβ + AST + Veh group vs. Aβ + AST + ANA12 group or Aβ + ER + Veh group vs. Aβ + ER + ANA12 group or Aβ + BDNF + AST group vs. Aβ + BDNF + AST + ANA12 group.
and decreased RPM counts [two-way ANOVA with time and group as factors, time difference $F(5,390) = 30.62$, $P < 0.0001$; group difference $F(7,390) = 26.06$, $P < 0.0001$; time-group interaction $F(35,390) = 2.247$, $P = 0.0001$] in Rotarod test; Fig. 2e and f). In rats, the Aβ-induced cognitive and motor deficits were significantly reduced by ER and/or AST therapy (Fig. 2). The beneficial effects of ER and/or AST therapy can be significantly attenuated by ANA12 (Fig. 2).

**ANA12 Can Attenuate the Beneficial Effects of ER and/or AST on Aβ-Induced Neuronal Degeneration, Apoptosis, and Aβ Accumulation and Altered Microglia Polarization in Rats**

Rats with intracerebroventriculurally microinjected Aβ displayed neuronal degeneration (as revealed by hematoxylin–eosin staining [Fig. 3a and b] [KW test: $H = 26.43$ in CA1 region, $H = 32.55$ in CA2 region, $H = 38.67$ in CA3 region, $H = 31.81$ in DG region, and $H = 30.33$ in cortex region, all $P < 0.001$], and NeuN with Fluoro-Jade B staining [Fig. 4a and b] [KW test: $H = 29.84$ in CA1, $H = 31.43$ in CA2, $H = 30.04$ in CA3, $H = 28.98$ in DG, and $H = 32.80$ in cortex, all $P < 0.0001$], apoptosis (as revealed by NeuN with TUNEL staining; [Fig. 4a and b] [KW test: $H = 20.04$ and $P = 0.0055$ in CA1, $H = 26.92$ and $P = 0.0003$ in CA2, $H = 32.11$ and $P < 0.0001$ in CA3, $H = 32.86$ and $P < 0.0001$ in DG, $H = 17.78$ and $P = 0.013$ in cortex]), Aβ accumulation (as revealed by thioflavin-S staining; [Fig. 4c and d] [KW test: $H = 20.24$ and $P = 0.0051$ in CA1, $H = 25.09$ and $P = 0.0007$ in CA2, $H = 28.27$ and $P = 0.0002$ in CA3, $H = 17.41$ and $P = 0.015$ in DG, $H = 12.25$ and $P = 0.0925$ in cortex]), and altered microglia polarization in cortical and hippocampal regions of rat brain (as revealed by Iba-1 and CD86 co-positive cells; [Fig. 5a and b] [KW test: $H = 17.21$ and $P = 0.0161$ in CA1, $H = 19.99$ and $P = 0.0056$ in CA2, $H = 18.82$ and $P = 0.0088$ in CA3, $H = 19.45$ and $P = 0.0069$ in DG, $H = 23.81$ and $P < 0.0012$ in cortex]). All of the above-mentioned parameters caused by Aβ injections could be significantly reversed by ER and/or AST therapy (Figs. 3, 4, and 5). Again, any beneficial effects from ER and/or AST could be significantly counteracted by ANA12 therapy (Figs. 3, 4, and 5).

**ANA12 Can Reverse the Beneficial Effects of BDNF (or ER) and/or AST on Aβ-Induced p-TrkB, p-Akt, pGSK3β, and β-catenin Deficits in Cultured Cortical Neurons and Reduced Cortical and Hippocampal Levels of BDNF/TrkB/Cathepsin D In Vivo**

Figure 7c and d showed that BDNF and/or AST therapy inhibited Aβ-induced reduced expression of p-TrkB (one-way ANOVA: $F(7,16) = 111.7$, $P < 0.0001$), p-Akt (Ser473) (one-way ANOVA: $F(7,16) = 39.48$, $P < 0.0001$), p-GSK3β (Ser9) (one-way ANOVA: $F(7,16) = 21.72$, $P < 0.0001$), and β-catenin (one-way ANOVA: $F(7,16) = 27.02$, $P < 0.0001$) in cultured primary cortical neurons, which can be wiped out by ANA12. In vivo studies also showed that the Aβ-induced decreased cortical and hippocampal expression of BDNF (one-way ANOVA: $F(7,40) = 57.21$, $P < 0.0001$ in cortex; $F(7,40) = 15.93$, $P < 0.0001$ in hippocampus), TrkB (one-way ANOVA: $F(7,40) = 72.95$, $P < 0.0001$ in cortex; $F(7,40) = 12.36$, $P < 0.0001$ in hippocampus), and cathepsin D (one-way ANOVA: $F(7,40) = 31.76$, $P < 0.0001$ in cortex; $F(7,40) = 23.02$, $P < 0.0001$ in hippocampus) could be reversed by ER and/or AST (Fig. 7e–g). Again, the beneficial effects of ER and/or AST therapy on Aβ-induced alternations in vivo were all significantly counteracted by ANA12 application.

**Discussion**

**Brain Aβ Accumulation Causes Neurological Injury via Inducing BDNF/TrkB Signaling Deficits as well as Mitochondrial Dysfunction**

Our in vivo study showed that central injection of Aβ oligomers caused motor and cognitive deficits accompanied by decreased expression of both BDNF/TrkB and cathepsin D and induced neuronal degeneration, apoptosis, Aβ accumulation, and altered microglial polarization in cortical and hippocampal regions of rat brain. Simultaneously,
our in vitro study demonstrated that local application of Aβ caused cytotoxicity, apoptosis, mitochondrial distress, synaptic toxicity, and decreased expression of BDNF/TrkB/p-Akt/p-GSK3β/β-catenin in cultured cortical neurons. These observations suggest that brain accumulation of Aβ oligomers induces BDNF/TrkB signaling deficits and mitochondrial dysfunction, resulting in neurodegeneration and neurobehavioral disorders in rats.

**ER and/or AST Therapy Attenuate Neurological Injury via Restoration of Brain BDNF/TrkB Signaling and Mitochondrial Function in Aβ-Treated Rats**

Our in vivo results showed that ER and/or AST might attenuate neurobehavioral disorders and neurodegeneration by restoring the cortical and hippocampal expression levels of BDNF/TrkB and cathepsin D in Aβ-microinjected rats. Our in vitro results also showed that topical of BDNF and/or AST might attenuate neurodegeneration via restoring the levels of BDNF/TrkB/p-Akt/p-GSK3β/β-catenin expression in Aβ-treated rat cortical neurons. For cellular events, in most of the parameters studied in the present study, the beneficial effects of combined ER (or BDNF) and AST application were superior to ER (or BDNF) or AST alone. However, for neurobehavior disorders, no additive or synergistic effects were observed in the present results. The most striking finding is that all the beneficial effects exerted by ER and/or AST in vivo and BDNF and/or AST in vitro were significantly eliminated by ANA12, a potent BDNF/TrkB antagonist therapy. Thus, it can be derived from these observations that whereas ER or AST therapy alone may improve neurodegeneration and neurobehavioral disorders in AD rats via preserving both cerebral BDNF/TrkB/cathepsin D and BDNF/TrkB/p-Akt/p-GSK3β/β-catenin signaling pathways. The treadmill exercise protocol used in the present study seems quite severe. Forced exercise could be pondered as stressful rather than therapeutic for certain individuals. However, the same extent of exercise protocol has been found to be beneficial in treating Alzheimer’s disease (present results), stroke [25], spinal cord injury [26], and traumatic brain injury [27] in rats.

**BDNF/TrkB/Cathepsin D Signaling Deficits in AD Pathology**

In people with AD [28–31] and in rodent models [32, 33], reduced levels of BDNF and its receptors, TrkB, are related to degeneration of hippocampal and cortical neurons. The increasing of BDNF in the brain with viruses [34, 35] or transplanted cells [36–38] results in marked neuroprotective effects in several animal models of AD. In our results, ER and/or AST application might attenuate neurobehavior disorders and neurodegeneration in Aβ-microinjected rats by preserving neuronal networks’ BDNF-mediated functional integrity [39].

Cathepsin D is essential for lysosomal integrity to degrade misfolded/damaged/oxidized proteins, including Aβ and tau, to prevent toxic accumulation [40]. Genetic depletion of cathepsin D in mice causes a dramatic accumulation of Aβ in lysosomes [41]. They hypothesize that Aβ may trigger tautopathy by competitive inhibition of cathepsin D–mediated degradation of pathological forms of tau. Our present results showed that ER and/or AST inhibits the accumulation of Aβ and increases cathepsin D expression in the brain and leads to the restoration of normal neurobehavioral function.

**Association Between Microglia Polarization and AD Pathology**

Activated microglia can be polarized towards the pro-inflammatory, tissue-damaging M1 (CD86-positive) phenotype or the anti-inflammatory, tissue-repairing M2 (CD206-positive) phenotype [42]. Our in vivo study showed that in response to central injection of Aβ, M1 (CD86-positive) microglia predominated in both the hippocampal and cortical tissues which can be reversed by ER and/or AST therapy. Again, ANA12 significantly wiped out the beneficial effects from ER and/or AST therapy by reducing the accumulation of M1 microglia in Aβ-microinjected rats.

**BDNF/TrkB/p-Akt/p-GSK3β/β-Catenin Signaling Deficits in Aβ Pathology**

Our in vitro results showed that local application of BDNF and/or AST can attenuate Aβ-induced neurodegeneration and reduced expression of BDNF/TrkB/p-Akt/p-GSK3β/β-catenin in cultured rat cortical neurons. Our results are consistent with many investigations described below. Glycogen synthase kinase 3 beta (GSK3β) has been considered as a putative therapeutic target for promoting functional recovery in the injured or degenerative brain [43].

*Fig. 3* ER and/or AST reversed the hippocampal and cortical neuron damage and degeneration by Aβ, which can be eliminated by ANA12 in rats. Histology of hippocampus and cortex from different groups of rats using H & E staining (a and b) and NeuN + Fluoro-Jade B + DAPI staining (c and d). Representative photographs and quantification of different stainings were presented. Data are presented as the means ± SEM (n=5–8). *P < 0.05, compared with Sham + Veh + Veh group; †P < 0.05, compared with Aβ + Veh + Veh group; ‡P < 0.05, Aβ + AST + Veh group vs. Aβ + AST + ANA12 group or Aβ + ER + Veh group vs. Aβ + ER + ANA12 group or Aβ + BDNF + AST group vs. Aβ + BDNF + AST + ANA12 group.
For example, suppression of GSK3β activity is required for axon formation and elongation, whereas an enhancement of GSK3β activity is related to mitochondrial dysfunction, DNA fragmentation, and cell death [44]. Increased GSK3β activity or activation of GSK3β is linked to the pathogenesis of AD through Aβ and tau accumulation and mitochondrial distress [45]. Akt has been shown to phosphorylate at serine residue 9 (Ser9) and inactivate GSK3β, leading to stabilized and active β-catenin [43]. The Ser9 phosphorylation results in switching GSK3β from baseline activity to inactive status [43]. Local inactivation of GSK3β would maintain axonal growth and synaptic plasticity [46]. Previous studies established that BDNF activation of TrkB via the phosphoinositide-3-kinase (PI3K)/Akt signaling pathway attenuates the effects of chemotherapy in neuroblastoma cells [47]. Akt (Ser473) and GSK3β (Ser9) phosphorylation was accelerated in the injured cortex and involved neuronal survival after brain injury [48]. Moreover, neuroprotection of β-catenin against brain injury was partly mediated by enhanced and persistent activation of the Akt/GSK3β signaling pathway [48].

Association Between Mitochondrial Dysfunction and AD Pathology

The mitochondrial dysfunction cascade hypothesis holds that amyloid precursor proteins and Aβ accumulation in...
the mitochondrial membrane can evoke mitochondrial dysfunction and may initiate the AD pathology subsequently [49]. Our present results showed that topical application of Aβ-induced mitochondrial dysfunction as evidenced by increased mitochondrial membrane permeability and consequent loss of electrochemical potential (e.g., increased percentage of JC-1 fluorescence positive cell numbers in the cultured rat cortical neurons). The Aβ-induced

Fig. 6 Local application of BDNF and/or AST reversed the dysmorphic changes, decreased cell viability, apoptosis, and mitochondrial distress by Aβ in cortical neurons which could be eliminated by ANA12. BDNF and/or AST inhibited Aβ-induced morphological changes (a), decreased cell viability (b), increased apoptosis (sub-G1 phase) (c, d), DNA fragmentation (e), increased percentage of JC-1 green fluorescence positive cells number (indicates mitochondrial distress) in cultured primary cortical neurons (f, g). Data are presented as the means ± SEM of three independent experiments. *P < 0.05, compared with Control + Veh group; **P < 0.05, compared with Aβ + Veh group; #P < 0.05, Aβ + BDNF vs. Aβ + BDNF + ANA12 or Aβ + AST vs. Aβ + AST + ANA12 or Aβ + BDNF + AST vs. Aβ + BDNF + AST + ANA12. &P < 0.05, Aβ + BDNF vs. Aβ + BDNF + AST or Aβ + AST vs. Aβ + BDNF + AST
Mitochondrial dysfunction can be ameliorated by topical application of BDNF and/or AST. Mitochondrial dysfunction induces BDNF axonal transport deficits [8] and synaptic dysfunction, and neuronal damage [45]. Thus, it can be derived that ER and/or AST may ameliorate neurodegeneration and neurobehavioral disorders in Aβ-treated rats via restoration of mitochondrial function.
Association Between ANA12 and AD Pathology

Our results showed that ANA12 significantly wiped out the beneficial effects from ER and/or AST therapy in Aβ-microinjected rats or in cultured rat cortical neurons. These results indicate that antagonism of cortical and hippocampal BDNF/TrkB signaling with ANA12 significantly alleviates the beneficial effects from ER and/or AST in AD pathology. Our present findings are consistent with a recent investigation showing that ANA12 alleviates the beneficial effects from overexpression of hippocampal BDNF in post-stroke cognitive deficits [50].

Conclusions

In summary, as depicted in Fig. 8, intracerebroventricularly microinjected Aβ in rats might cause neuroinflammation (by M1 or CD86+ microgliosis) as well as neurobehavioral deficits (by Aβ accumulation, neurodegeneration, and neuronal apoptosis) via suppressing cortical and hippocampal levels of BDNF/TrkB/cathepsin D. In vitro studies further confirmed that topical application of Aβ might induce cortical neuronal death via inducing BDNF/TrkB/p-Akt/p-GSK3β/β-catenin signaling deficits as well as mitochondrial dysfunction. Exercise rehabilitation (or topical application of BDNF) and/or AST therapy might attenuate Aβ-induced neurodegeneration as well as neurobehavioral disorders via restoring the cortical and hippocampal levels of BDNF/
TrkB signaling and mitochondrial function. Antagonism of BDNF/TrkB with ANA12 wiped out the beneficial effects exerted by ER (or BDNF) and/or AST. Our data indicate that whereas ER and/or AST attenuate Aβ neuropathology by reversing the brain BDNF/TrkB signaling deficits and mitochondrial dysfunction induced by Aβ accumulation. AST can be an alternative therapy to replace ER for certain individuals.

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**Availability of Data and Materials** The authors confirm that the data supporting the findings of this study are all available within the article.

**Declarations**

**Ethics Approval and Consent to Participate** All animal experiments were approved and carried out in accordance with the Institutional Animal Care and Use Committee at the Chi Mei Medical Center, Tainan, Taiwan (approved no. 108120110).

**Consent for Publication** Not applicable.

**Conflicts of Interest** The authors declare that they have no competing interests.

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