

# Natural Dietary Supplementation of Anthocyanins via PI3K/Akt/Nrf2/HO-1 Pathways Mitigate Oxidative Stress, Neurodegeneration, and Memory Impairment in a Mouse Model of Alzheimer's Disease

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**Abstract** Well-established studies have shown an elevated level of reactive oxygen species (ROS) that induces oxidative stress in the Alzheimer's disease (AD) patient's brain and an animal model of AD. Herein, we investigated the underlying anti-oxidant neuroprotective mechanism of natural dietary supplementation of anthocyanins extracted from Korean black beans in the amyloid precursor protein/presenilin-1 (APP/PS1) mouse model of AD. Both in vivo (APP/PS1 mice) and in vitro (mouse hippocampal HT22 cells) results demonstrated that anthocyanins regulate the phosphorylated-phosphatidylinositol 3-kinase-Akt-glycogen synthase kinase 3 beta (p-PI3K/Akt/GSK3 $\beta$ ) pathways and consequently attenuate amyloid beta oligomer (A $\beta$ O)-induced elevations in ROS level and oxidative stress via stimulating the master endogenous anti-oxidant system of nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase-1 (Nrf2/HO-1) pathways and prevent apoptosis and neurodegeneration by suppressing the apoptotic and neurodegenerative markers such as activation of caspase-3 and PARP-1 expression as well as the TUNEL and Fluoro-Jade B-positive neuronal cells in the APP/PS1 mice. In vitro ApoTox-Glo™ Triplex assay results also showed that anthocyanins act as a potent anti-oxidant neuroprotective agent and reduce A $\beta$ O-induced neurotoxicity in the HT22 cells via PI3K/Akt/Nrf2 signaling. Importantly, anthocyanins improve memory-related pre- and postsynaptic protein markers and memory

functions in the APP/PS1 mice. In conclusion, our data suggested that consumption and supplementation of natural-derived anti-oxidant neuroprotective agent such as anthocyanins may be beneficial and suggest new dietary-supplement strategies for intervention in and prevention of progressive neurodegenerative diseases, such as AD.

**Keywords** Alzheimer's disease (AD) · APP/PS1 · Anthocyanins · Reactive oxygen species (ROS) · Oxidative stress · p-PI3K/Akt/GSK3 $\beta$  pathways · Nrf2/HO-1 pathway · Neurodegeneration · Pre- and postsynaptic protein markers · Memory functions

## Introduction

Alzheimer's disease (AD) is the most common age-related neurodegenerative disorder and is characterized by progressive learning and memory deficits. The neuropathological hallmarks of AD include senile plaques, neurofibrillary tangles, synaptic deficits, and neurodegeneration of the brain. Senile plaques are extracellular accumulations of amyloid beta (A $\beta$ ) peptide, and neurofibrillary tangles are composed of hyperphosphorylated tau protein [1]. A $\beta$  peptides are produced by cleavage of the transmembrane glycoprotein amyloid precursor protein (APP) by the  $\beta$ -secretase and presenilin-dependent  $\gamma$ -secretase enzymes. According to Hardy and Selko and the A $\beta$  cascade hypothesis, A $\beta$  is the major cause of neurotoxic insults in AD; it activates a number of biochemical pathogenic mediators, which lead to AD pathology, including oxidative stress and synaptic dysfunction [2–4]. However, the exact underlying molecular mechanisms associated with dementia remain unknown. Importantly, reactive oxygen species (ROS)-

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induced oxidative stress triggers AD pathology mediators because the brain, as a result of its increase consumption of oxygen and limited anti-oxidant enzymes, is the most sensitive region to oxidative stress [5]. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a key redox-regulated gene that has a critical role against oxidative stress, and the level of Nrf2 in the nuclei is decreased in the neurological disorders such as AD [6]. Recently, accumulating studies have reported that activated nuclear Nrf2 further regulates several endogenous redox-regulated enzymes such as heme oxygenase-1 (HO-1) and glutathione cysteine ligase modulatory subunit (GCLM) via the phosphorylated phosphatidylinositol 3-kinase, phosphorylated Akt, and phosphorylated glycogen synthase kinase 3 beta (p-PI3K/Akt/GSK3 $\beta$ ) pathway. The PI3K/Akt/GSK3 $\beta$  signaling is the most important pathways, having a key and pivotal role in various survival signaling and in other functions. Impaired PI3K/Akt/GSK3 $\beta$  signaling has been investigated in the AD brain and AD mouse models [7–15]. Notably, HO-1 is beneficial in various diseases, and its activation improves learning and memory functions [6, 16]. Recently, it was reported that nuclear translocation of Nrf2 increased the expression of HO-1 [17]. Elevated expression of Nrf2 in both in vitro and in vivo AD models decreased A $\beta$ -induced neurodegeneration and oxidative stress [18]. Similarly, Kanninen et al. revealed that overexpression of hippocampal Nrf2 improves spatial learning and memory in a mouse model of AD [19].

Natural plant-derived polyphenolic flavonoids have beneficial effects on health and possess versatile biological activity, such as anti-oxidant, anti-neuroinflammatory, anti-cancer, and anti-neurodegenerative actions. The development and use of natural substances, particularly polyphenolic flavonoids, may prevent various metabolic disorders, such as diabetes mellitus and obesity; cardiovascular disease; and chronic neurodegenerative disorders, such as AD [20–26]. Previously, we evaluated polyphenolic anthocyanins from Korean black consists of cyanidine-3-glucosides (C3G) (67%), delphinidin-3-glucosides (D3G) (25%), and petunidin-3-glucosides (P3G) (5%), and showed that these compounds exert neuroprotective effects, via attenuation of oxidative stress, against D-galactose- and ethanol-induced neurodegeneration in the adult, neonatal and developing rat brain respectively; and reduce kainic acid-induced excitotoxicity and apoptosis in hippocampal neuronal cells [27–31]. It has been reported that anthocyanins and C3G protect against cerebral ischemia and A $\beta$ -induced mitochondrial degeneration [32, 33]. Dietary flavonoids such as anthocyanins improved spatial working memory in aged and LPS-treated rodents [34, 35]. Recently, we also found that Korean black bean anthocyanins exert neuroprotective effects against A $\beta$ <sub>1–42</sub> in both in vitro and in vivo A $\beta$ <sub>1–42</sub>

models [36, 37]. In the present study, we hypothesized that Korean black bean anthocyanins, a natural potent anti-oxidant neuroprotective agent, would reduce synaptic and memory dysfunction and neurodegeneration by attenuating A $\beta$ -induced ROS-mediated oxidative stress via regulation of the anti-oxidant neuroprotective mechanism of the PI3K/Akt/Nrf2 pathways in the in vitro AD model and APP/PS1 transgenic mouse model of AD.

## Materials and Methods

### Materials

Anthocyanins were extracted from Korean black bean, as previously described [27]. A $\beta$ <sub>1–42</sub> peptide, 2'7'-dichlorodihydrofluorescein diacetate (DCFH-DA), 2-(4-Morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride (LY294002), propidium iodide (PI), 4',6'-diamidino-2-phenylindole (DAPI) and dimethyl sulfoxide (DMSO), diaminobenzidine tetrahydrochloride (DAB) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

### Animals Grouping and Drug Treatments

Male C57BL/6N wild type (WT) mice were purchased from Samtako Bio (Osan, Republic of Korea) and double transgenic B6.Cg-Tg (APP<sup>swe</sup>, PSEN<sup>dE9</sup>)85Dbo/Mmjax (APP/PS1) AD-model mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The double transgenic mice express a chimeric mouse-human amyloid precursor protein bearing Swedish mutation (Mo/HuAPP695<sup>swe</sup>) as well as a mutant human presenilin 1 protein (PS1-dE9) in the brain. The mice were kept in the university animal house under a 12-h/12-h light/dark cycle at 23 °C, 60 ± 10% humidity with food and water ad libitum. The mice when reached to 10 month of age were brought to injection and behavioral room to acclimatize for 1 week.

The animals were grouped as following: (1) WT vehicle (Veh)-treated, (2) APP/PS1 (Veh)-treated, (3) APP/PS1 anthocyanins-treated, and (4) WT anthocyanins-treated.

Anthocyanins were dissolved in DMSO and make the final administered amount in the normal saline. Anthocyanins were intraperitoneally (i.p.) administered at dose of 12 mg/kg/day body weight for 30 days to the APP/PS1 and WT mice. Similarly, the same amount of DMSO and volume of normal saline as a Veh injected to the WT and APP/PS1 mice.

After the treatment and behavioral analysis, the experimental animals were sacrificed for further biochemical and immunohistochemical analysis.

## Behavioral Study

The behavioral study was performed after the completion of the treatment for all the experimental mice using a Morris water maze (MWM) task and Y-maze task.

MWM test was performed with some modification as we described previously [38]. Briefly, each mouse received four training periods per day for six consecutive days. Latency to escape from the water maze (finding the submerged escape platform) was calculated for each trial. On day 7, the probe test was performed by removing the platform and allowing each mouse to swim freely for 60 s. The time that the mice spent swimming in the target quadrant and the number of platform crossings (where the platform was located during hidden platform training) was measured. All data were recorded using video-tracking software (SMART, Panlab Harvard Apparatus, Bioscience Company, USA).

Y-maze test was performed as we described previously [39]. Briefly, each mouse was placed at the center of the apparatus and allowed to move freely through the maze for three 8-min sessions. The series of arm entries was noted. Spontaneous alternation was defined as the successive entry of the mouse into the three arms in overlapping triplet sets. Alternation behavior (%) was calculated as [successive triplet sets (entries into three different arms consecutively)/total number of arm entries-2]  $\times$  100.

## Protein Extraction from Mouse Brain

After behavioral analysis the mice were sacrificed, brains were immediately removed, hippocampus was dissected carefully and the tissue were frozen on dry ice and stored at  $-80^{\circ}\text{C}$ . The hippocampus tissue was homogenized in pro-prep<sup>TM</sup> protein extraction solution according to the provider instruction (iNtRON, Biotechnology, Inc) using homogenizer (DAIHAN, Scientific Co, Ltd., Republic of Korea) having specifications e.g., 230 V, 50/60 Hz; Watts 280 W, Fuse 250 V, T3AL. We calculated the amount of homogenized proteins in pro-prep<sup>TM</sup> protein extraction solution and further some of the amount were separated into the cytosolic and nuclear fractions using a nuclear and cytoplasmic protein extraction kit, according the manufacturer's instruction (catalog # K266-25) Biovision Incorporated, A 95035 USA. The cytosolic and nuclear fractions protein were collected and stored at  $-80^{\circ}\text{C}$ . The remaining protein of the hippocampus homogenate in pro-prep<sup>TM</sup> protein extraction solution were then centrifuged at  $10000\times g$  at  $4^{\circ}\text{C}$  for 25 min (min). The supernatants were collected and stored at  $-80^{\circ}\text{C}$ .

## Western Blot Analysis

The protein concentration from tissue homogenates and cell lysates were measured (BioRad protein assay kit, BioRad

Laboratories, CA, USA). Equal amounts of protein (15–25  $\mu\text{g}$ ) were electrophoresed under same experimental condition using 4–12% Bolt<sup>TM</sup> Mini Gels and MES SDS running buffer  $1\times$  (Novex, Life Technologies, Kiryat Shmona, Israel), broad range prestained protein marker (GangNam stain<sup>TM</sup>, Intrin Biotechnology) was used as molecular size control. The membranes were blocked in 5% (*w/v*) skim milk to reduce non-specific binding and incubated with primary antibodies (detailed information provided in the Table 1) overnight at  $4^{\circ}\text{C}$ . After reaction with a horseradish peroxidase-conjugated secondary antibody, as appropriate. Secondary antibodies HRP-conjugated IgG used were goat-anti-mouse (1: 3000) was purchased from Promega, Madison, WI, USA, goat-anti-rabbit (SC: 2004) (1: 2000–2500), mouse-anti-rabbit (SC: 2354) (1: 2000–2500), mouse-anti-goat (SC: 2354) (1: 4000–5000) were purchased from Santa Cruz Biotechnology, USA. After incubation with secondary antibodies, the membranes were washed three time with 1X TBST. After washing, the proteins were detected using an ECL detection reagent according to the manufacturer's instructions (Amersham Pharmecia Biotech, Uppsala, Sweden). The X-ray films were scanned, and the optical densities of the bands were analyzed through densitometry using the computer-based Sigma Gel program, version 1.0 (SPSS, Chicago, IL, USA).

## A $\beta_{1-42}$ Oligomer (A $\beta$ O) Preparation for In Vitro Experiments

For in vitro experiments, A $\beta$ O was prepared as we described previously [39].

## ROS Assay both in Mouse Hippocampus and In Vitro HT22 Cells

ROS assay was performed to determine the elevated ROS in the hippocampus ( $n = 8$  mice per group) homogenates. The assay was based on the oxidation of DCFH-DA to 2',7'-dichlorofluorescein (DCF) as we described previously [38]. The conversion of DCFH-DA to the DCF was analyzed by spectrofluorimeter with excitation at 484 nm and emission at 530 nm. For background fluorescence (conversion of DCFH-DA to DCF in the absence of homogenate), was run a parallel blanks and used as a standard DCF. We quantified ROS production from a DCF-standard curve and expresses as relative DCF pmol/mg protein.

Further we also analyzed intracellular ROS via DCF fluorescence intensity through confocal microscopy as we described previously [29] with some modification in vitro using mouse hippocampal (HT22) neuronal cells, a kind gift from Prof. Koh (Gyeongsang National University, S. Korea). HT-22 cells ( $2 \times 10^4/\text{ml}$ ) were grown in chamber slides (Thermofisher Scientific 75 Panorama Creek Drive Rochester, NY14625–2385, USA) in Dulbecco's modified

Eagle medium (DMEM) (Gibco by life technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin-streptomycin) at 37 °C in humidified air containing 5% CO<sub>2</sub>. After 70–80% confluence, cells were co-treated with A $\beta$ O (5  $\mu$ M) and anthocyanins (100  $\mu$ g/ml) for 24 h, besides the control group (cells treated with DMSO (0.01%)). After 24 h, cells were washed with PBS, followed by adding 50  $\mu$ M DCFH-DA in DMSO and incubated at 37 °C in humidified air containing 5% CO<sub>2</sub> for 30 min covered by aluminum foil. After this the cells were washed with PBS and fixed in 4% paraformaldehyde. The slides were mounted with DAPI and Prolong Antifade Reagent (Molecular Probe, Eugene, OR, USA) and analyzed by a laser confocal microscopy (Fluoview FV 1000, Olympus, Japan) with excitation and emission wavelengths of 484 and 530 nm, respectively. Fluorescence intensity was measured using image J software and expressed as a relative fluorescence of the samples to control cells.

### Lipid Peroxidation (LPO) Analysis in Mouse Hippocampus

The LPO levels determined in the hippocampus ( $n = 8$  mice per group) homogenates through analyzing the malondialdehyde (MDA) level, a biomarker of LPO, by using the commercial lipid peroxidation kit (catalog # K739-100) from Biovision Incorporated, A 95035 USA. The assay was performed according to the provided protocol.

### Glutathione (GSH) Analysis in Mouse Hippocampus

The GSH levels in the hippocampus ( $n = 8$  mice per group) homogenates were assessed by using the commercially available glutathione Assay kit (BioVision's Catalog #K264-100) according to the provided protocol.

### Tissue Collection and Sample Preparation from Mouse Brain

For the tissue analysis, the mice ( $n = 5$  mice per group) were perfused transcardially with 4% ice-cold paraformaldehyde, and the brains were post-fixed for 72 h in 4% paraformaldehyde and transferred to 20% sucrose for 72 h. The brains were frozen in O.C.T compound (A.O, USA), and 14- $\mu$ m coronal sections were cut using a CM 3050C cryostat (Leica, Germany). The sections were thaw-mounted on probe-on plus charged slides (Fisher, Rockford, IL, USA).

### Immunofluorescence Staining and Confocal Microscopy

For immunofluorescence staining the slides were washed twice for 10 min in 0.01 M PBS, followed by incubation for 1 h in blocking solution containing 2% normal serum

according to the antibody treatment and 0.3% Triton X-100 in PBS. After blocking, the slides were incubated overnight at 4°C in the primary antibodies e.g., p-Akt, HO-1, Nrf-2, SNAP-23, PSD-95, GCLM and 8-Oxoguanine (8-OxoG) (detailed information provided in Table 1) diluted 1:100 in blocking solution. Following incubation with primary antibody, the sections were incubated for 2 h in the secondary tetramethyl rhodamine isothiocyanate (TRITC)/fluorescein isothiocyanate (FITC)-labeled antibodies (1:50) (Santa Cruz Biotechnology). Subsequently, after the incubation of the TRITC/FITC-labeled, sections were incubated overnight in another primary antibody, following incubation with primary antibody, the sections were incubated for 2 h FITC/TRITC-labeled antibody (1:50) (Santa Cruz Biotechnology) under the same conditions. The slides were mounted with DAPI and Prolong Antifade Reagent (Molecular Probe, Eugene, OR, USA). Staining images of the double immunofluorescence and single immunofluorescence were examined using a confocal laser-scanning microscope (Fluoview FV 1000, Olympus, Japan). Three to five original immunofluorescence images per sample were captured from each group and the images were converted to tagged image file format (TIF). The immunofluorescence intensity in the same region of hippocampus/total area of TIF images for all groups was measured using imageJ software. By using the ImageJ software, the TIF image background was optimized according to the threshold intensity and the immunofluorescence intensity was analyzed at the same threshold intensity for all groups and were expressed as the relative integrated density of the samples relative to the control which is normalized as one.

### Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Immunohistochemical Assay

TUNEL immunohistochemical staining was performed using the DNA Fragmentation Detection Kit (Oncogene Research Products, Cambridge, MA, USA). Briefly, sections were two times washed for 5 min each with PBS. After washing the sections were incubated in equilibration buffer and terminal deoxynucleotidyl transferase (TdT) enzyme for minimum of 60 min at room temperature (R.T). The reaction was terminated by incubation in stop buffer for 5 min at R.T. Sections were labeled with streptavidin HRP conjugate and visualized with DAB substrate. TUNEL-positive cells were analyzed using light microscopy. The number of TUNEL-positive nuclei cells is analyzed and counted through image J program.

### Fluoro-Jade B (FJB) Staining

FJB staining was performed according to the provider protocols (Millipore, USA, cat# AG310, lot# 2159662). The slides of the brain tissue were air-dried for at least 4 h. Slides were washed twice for 5 min. Following washing

**Table 1** Primary antibodies information used in the Western blotting (WB) and immunofluorescence (IF) staining

Antibody	Host	Application	Manufacturer	Catalog number	Concentration
p-PI3K	Rabbit	WB	Santa Cruz Biotechnology, USA	SC-2931	1:1000
Total-PI3K	Mouse	WB		SC-376641	1:1000
p-GSK3 $\beta$ (Ser 9)	Goat	WB		SC-37480	1:1000
Total-GSK3 $\beta$	Rabbit	WB		SC-9166	1:1000
Nrf2	Rabbit	WB/IF		SC-722	1:1000/1:100
HO-1	Mouse	WB/IF		SC-136961	1:1000/1:100
Synaptophysin	Rabbit	WB		SC-9116	1:1000
SNAP25	Goat	WB		SC-7538	1:2000
SNAP23	Mouse	WB/IF		SC-374215	1:2000/1:100
PSD95	Mouse	WB/IF		SC-71933	1:2000/1:100
$\beta$ -Actin	Mouse	WB		SC-47778	1:2000
Caspae-3	Mouse	WB		SC-7272	1:2000
PARP-1	Mouse	WB		SC-8007	1:1000
SAP102	Rabbit	WB		3733S	1000
p-Akt	Rabbit	WB/IF	9271S	1:1000/1:100	
Total-Akt	Rabbit	WB	9272S	1:1000	
p-CREB (Ser 133)	Rabbit	WB	9198S	1:1000	
p-GluR1 (Ser 845)	Rabbit	WB	8084S	1:1000	
GCLM	Rabbit	WB/IF	ABCAM, USA	AB81445	1:1000/1:100
8-Oxo-G	Mouse	IF	Millipore, USA	MAB3560	1:100

the slides were immersed in a 1% sodium hydroxide and 80% ethanol solution for 5 min and then in 70% alcohol for 2 min followed by 2 min in distilled water. Next, the slides were transferred to a solution of 0.06% potassium permanganate for 10 min over a slow shaker, rinsed with distilled water and then immersed in a 0.1% acetic acid and 0.01% FJB solution for 20 min. The slides were then rinsed with

distilled water and dried for at least 10 min. The slides were mounted with PI and glass cover slips using DPX non-fluorescent mounting medium. The representative images were captured using a FITC filter on a confocal laser-scanning microscope (FV 1000, Olympus, Japan). The number of FJB-positive cells is analyzed and counted through image J program.

### In Vitro HT22 Cells Culture for Western Blotting

The HT-22 neuronal cells ( $2 \times 10^4$ /ml) were cultured (in 35 mm Petri dish (Nunc A/S, Kamstrupvej 90.P.O.Box 280 DK-4000 Roskilde, Denmark) for western blots in DMEM containing 10% FBS and 1% antibiotics at 37 °C in humidified air containing 5% CO<sub>2</sub>. Nrf2 (SC: 37,049, Santa Cruz Biotechnology, Inc) genes silencing small interference RNA (siRNA) with concentration of 10 μM per transfection for 36 h according to manufacturer protocol. The negative siRNA (Ambion, USA) was used as control. The transfection were performed when culture was 75–80% confluent with lipofectamine™ reagent 2000 (Invitrogen). After 36 h of cells transfection the cells were co-treated with AβO (5 μM), LY294002 (10 μM) and anthocyanins (100 μg/ml) for 24 h. The cell lysates were collected in in pro-prep™ protein extraction solution according to the provider instruction (iNtRON, Biotechnology, Inc). The cell lysates were mixed properly and incubated on ice for at least 30 min to enhance cell lysis. After incubation the cell lysates were centrifuged at 10000×g at 4 °C for 25 min. The supernatants were collected and stored at – 80°C until use for the Western blotting.

### In Vitro ROS Assay in HT-22 Cells with Nrf2 siRNA

The HT-22 neuronal cells were cultured in 75 cm<sup>2</sup> flasks (Thermo scientific, Nunc™ EasYFlask™ 75 cm<sup>2</sup> Nunclon™ Delta surface, thermo fisher scientific A/S, Kamstrupvej 90.P.O.Box 280 DK-4000 Roskilde, Denmark) containing DMEM supplemented with 10% FBS and 1% antibiotics at 37 °C in humidified air containing 5% CO<sub>2</sub>, and the number of HT22 cells were counted using a disposable hemocytometer as described in the above section. The cells ( $2 \times 10^4$ /ml) were further cultured in 96-well plate (Thermofisher Scientific 75 Panorama Creek Drive Rochester, NY14625-2385, USA) containing DMEM supplemented with 10% FBS and 1% antibiotics at 37 °C in humidified air containing 5% CO<sub>2</sub>. After the cells reached 70% confluence, they were transfected with 10 μM Nrf2 siRNA with lipofectamine™ 2000 reagent for 36 h according to the manufacturer's protocol. Negative siRNA was used as a control. After 36 h of transfection, the cells were co-treated with AβO (5 μM), LY294002 (10 μM) and anthocyanins (100 μg/ml) for 24 h. After 24 h of treatment, the cells were exposed to 50 μM DCFH-DA and incubated for 30 min. The relative absorbance of the ROS-positive cells treated with DCFH-DA was measured at 484/530 nm with a multidetector (Promega).

### In Vitro ApoTox-Glo™ Triplex Assay

ApoTox-Glo™ Triplex assay (Promega Corporation, 2800 Woods Hollow Road Madison, WI53711–5399, USA) was

performed to assess viability, cytotoxicity and caspase-<sup>3/7</sup> activation using HT22 cells. HT22 cells ( $2 \times 10^4$ /ml) were cultured in 96-well plate (Thermofisher Scientific 75 Panorama Creek Drive Rochester, NY14625-2385, USA) containing DMEM supplemented with 10% FBS and 1% antibiotics at 37 °C in humidified air containing 5% CO<sub>2</sub>. After 70% confluences the cells were treated with AβO (5 μM) and anthocyanins at different three concentrations of 25 μg/ml, 50 μg/ml and 100 μg/ml for 24 h, besides the control group (cells treated with DMSO (0.01%)). In addition the HT22 cells were transfected with Nrf2 siRNA (m) (SC-37049) and treated with AβO (5 μM), LY294002 (10 μM) and anthocyanins 100 μg/ml for 24 h; the control group subjected to negative siRNA (Ambion, ThermoFisher Scientific) and treated with 0.01% DMSO. The assay was performed according to our previous described protocol [39].

### Statistics

The Western blot bands were scanned and analyzed through densitometry using the Sigma Gel System (SPSS Inc., Chicago, IL). One-way analysis of variance (ANOVA) followed by a two-tailed independent Student's *t* test and Tukey's multiple comparison test were used for comparisons among the treated groups and the control. The ImageJ software was used for the relative immunofluorescence quantitative analysis. The density values of the data were expressed as the means ± SEM of three independent experiments. Behavioral data for escape latency were analyzed using a two-way ANOVA with training days as repeated measurement. For the probe trials, statistical comparisons among groups for the time over quadrants were done using one-way ANOVA. *P* values less than 0.05 were considered to be statistically significant. \*Significantly different from the WT Veh treated; # significantly different from the Veh treated APP/PS1 mice. Similarly, for in vitro represented by bars. \**p* < 0.05, \*\**p* < 0.01 and \*\*\**p* < 0.001; and #*p* < 0.05, ###*p* < 0.01 and ####*p* < 0.001.

### Results

#### Anthocyanins Treatment Upregulated p-PI3K/Akt/GSK3β (Ser9) Signaling, Nuclear Translocation of Nrf2, and Expression of its Target Genes (HO-1 and GCLM) in APP/PS1 Mice and In Vitro AβO-Exposed HT22 Cells

Impairment of the p-PI3K/Akt/GSK3β pathway has been associated with various neurodegenerative disorders [7, 8, 13, 15, 30, 39]. Here, our Western blot results showed reduced p-PI3K, p-Akt (Ser473) and p-GSK3β (Ser9) in the Veh-treated APP/PS1 mice compared with the Veh-treated WT mice.

Anthocyanins treatment (12 mg/kg i.p. for 30 days) significantly upregulated the expression of p-PI3K, p-Akt and p-GSK3 $\beta$  (Ser9) in the APP/PS1 mice compared with the Veh-treated APP/PS1 mice (Fig. 1a). Immunofluorescence results showed decreased immunofluorescence reactivity of p-Akt (Ser473) in the CA1 region of the hippocampi in Veh-treated APP/PS1 mice compared with the Veh-treated WT mice. Anthocyanins treatment significantly increased p-Akt (Ser473) immunofluorescence reactivity in the CA1 region of the hippocampi in APP/PS1 compared with the Veh-treated APP/PS1 group (Fig. 1b).

Recently, several studies reported that Nrf2 is a downstream target of the PI3K/Akt/GSK3 $\beta$  pathway [9–11, 13]. It has been shown that Nrf2 and its target anti-oxidant genes such as HO-1 have a critical role in the mechanism that mitigates oxidative stress and that they activate an anti-oxidant mechanism that might be beneficial in AD and other neurological disorders [40]. Nuclear translocation of Nrf2 increased the expression of its target genes HO-1 and GCLM [19]. Our Western blot results revealed that anthocyanins supplementation significantly increased the nuclear translocation of Nrf2 and decreased the cytoplasmic expression of Nrf2 in the APP/PS1 mice compared with the Veh-treated APP/PS1 mice (Fig. 1c). Consistently, Western blots results indicated that anthocyanins significantly increased the expression of HO-1 and GCLM in the hippocampi of APP/PS1 mice compared with the Veh-treated APP/PS1 mice (Fig. 1c).

Next, we analyzed Nrf2/HO-1 co-localization through double immunofluorescence staining. Figure 1d, e shows decreased expression of Nrf2/HO-1 immunofluorescence reactivity in the hippocampi of Veh-treated APP/PS1 mice compared with that of Veh-treated WT mice. Anthocyanins supplementation (12 mg/kg i.p. for 30 days) significantly increased the immunofluorescence reactivity of Nrf2/HO-1 in the DG and CA1 regions of the hippocampi in the APP/PS1 mice compared with the Veh-treated APP/PS1 mice (Fig. 1d, e). In addition we analyzed the immunofluorescence reactivity of GCLM by using confocal microscopy. The confocal microscopy results reveal that anthocyanins significantly increased the immunofluorescence reactivity of GCLM in the CA1 and CA3 region of the hippocampi of APP/PS1 mice as compared to the Veh-treated APP/PS1 mice (Fig. 1f).

Further, we sought to determine the underlying neuroprotective anti-oxidant mechanism of anthocyanins, investigating whether anthocyanins regulate the PI3K/Akt/GSK3 $\beta$  pathway and whether they activate the key endogenous anti-oxidant Nrf2 and its target genes. We observed reduced expression of p-PI3K/Akt/GSK3 $\beta$  (Ser 9) as well the Nrf2 and its targets genes HO-1 and GCLM in the A $\beta$ O (5  $\mu$ M)-exposed HT22 cells, which was reversed by anthocyanins (100  $\mu$ g/ml) and significantly increased in its expression. However, anthocyanins were not able to activate and increase the expression of p-PI3K/Akt/GSK3 $\beta$  (Ser 9) and Nrf2/HO-1/GCLM in the

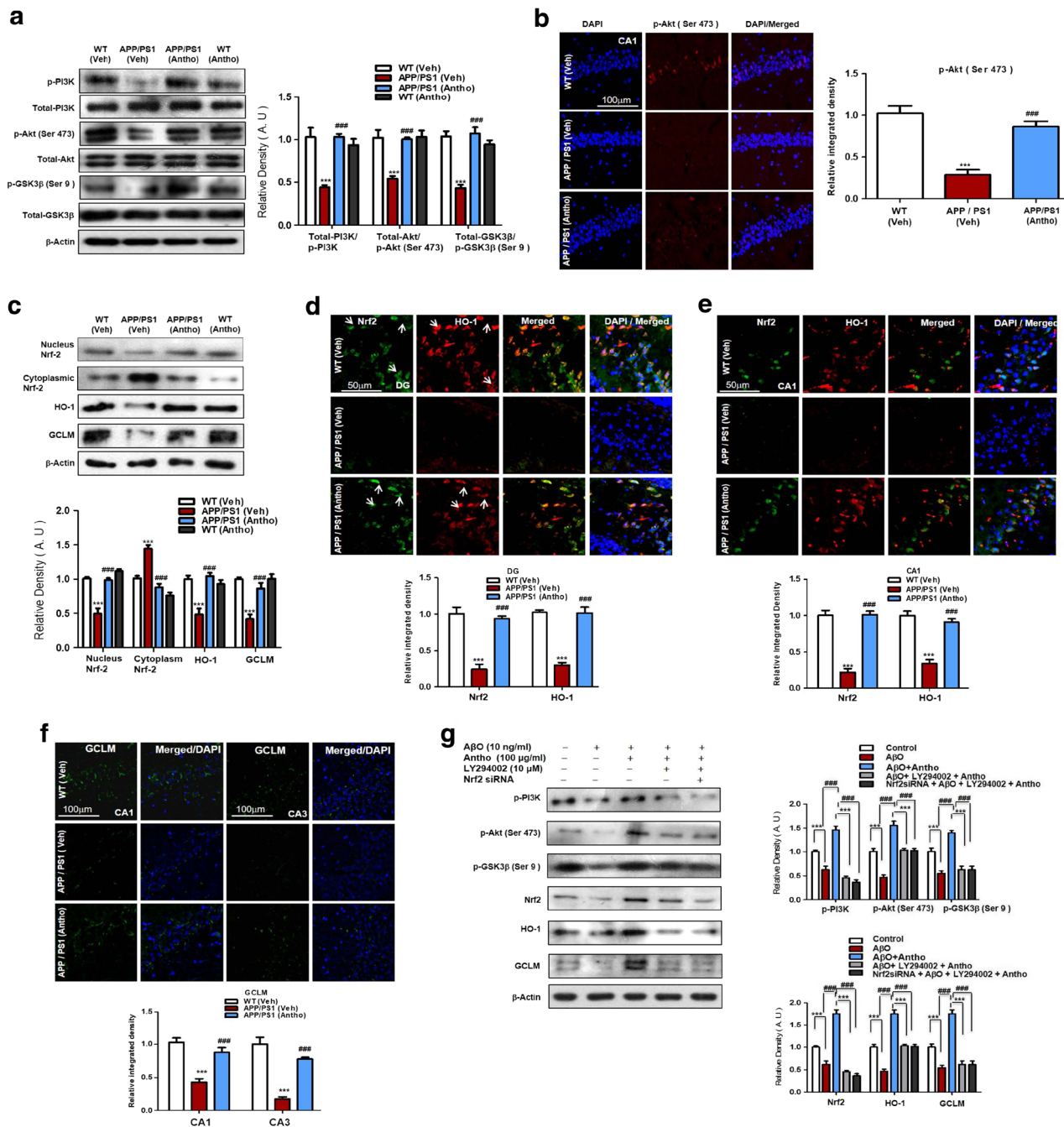
A $\beta$ O-exposed HT22 cells when they were exposed to a PI3K inhibitor (LY294002 (10  $\mu$ M)) and Nrf2 siRNA (Fig. 1g). These results indicated that anthocyanins regulated PI3K/Akt/GSK3 $\beta$  pathway and activated the endogenous anti-oxidant Nrf2/HO-1 pathway which might be associated with the anti-oxidant neuroprotective activity of anthocyanins.

### Anthocyanins Treatment Mitigated ROS and Oxidative Stress in APP/PS1 Mice and In Vitro A $\beta$ O-Exposed HT22 Cells

Further, to assess the potent anti-oxidant activity of natural anthocyanins in the APP/PS1 mice, we conducted ROS assay, LPO assay and GSH assay. The ROS assay results showed that ROS levels [based on the oxidation of DCFH-DA to DCF] significantly increased in the APP/PS1 mice compared with the Veh-treated WT mice. Anthocyanins supplementation (12 mg/kg i.p. for 30 days) overcame ROS activation and significantly reduced DCF levels in the APP/PS1 mice compared with those of Veh-treated APP/PS1 mice (Fig. 2a).

An increased level of MDA has been observed in the transgenic mice [41]. Similarly, we also observed an elevated level of MDA in the Veh-treated APP/PS1 mice compared with the Veh-treated WT mice. Anthocyanins supplementation prevents elevated MDA levels and significantly reduced the MDA levels of the APP/PS1 mice compared with those of Veh-treated APP/PS1 mice (Fig. 2b). Next, we analyzed the GSH levels using a commercial GSH kit. The results revealed that anthocyanins significantly increased GSH levels in the brain homogenates of APP/PS1 mice compared with those of the Veh-treated APP/PS1 mice (Fig. 2c).

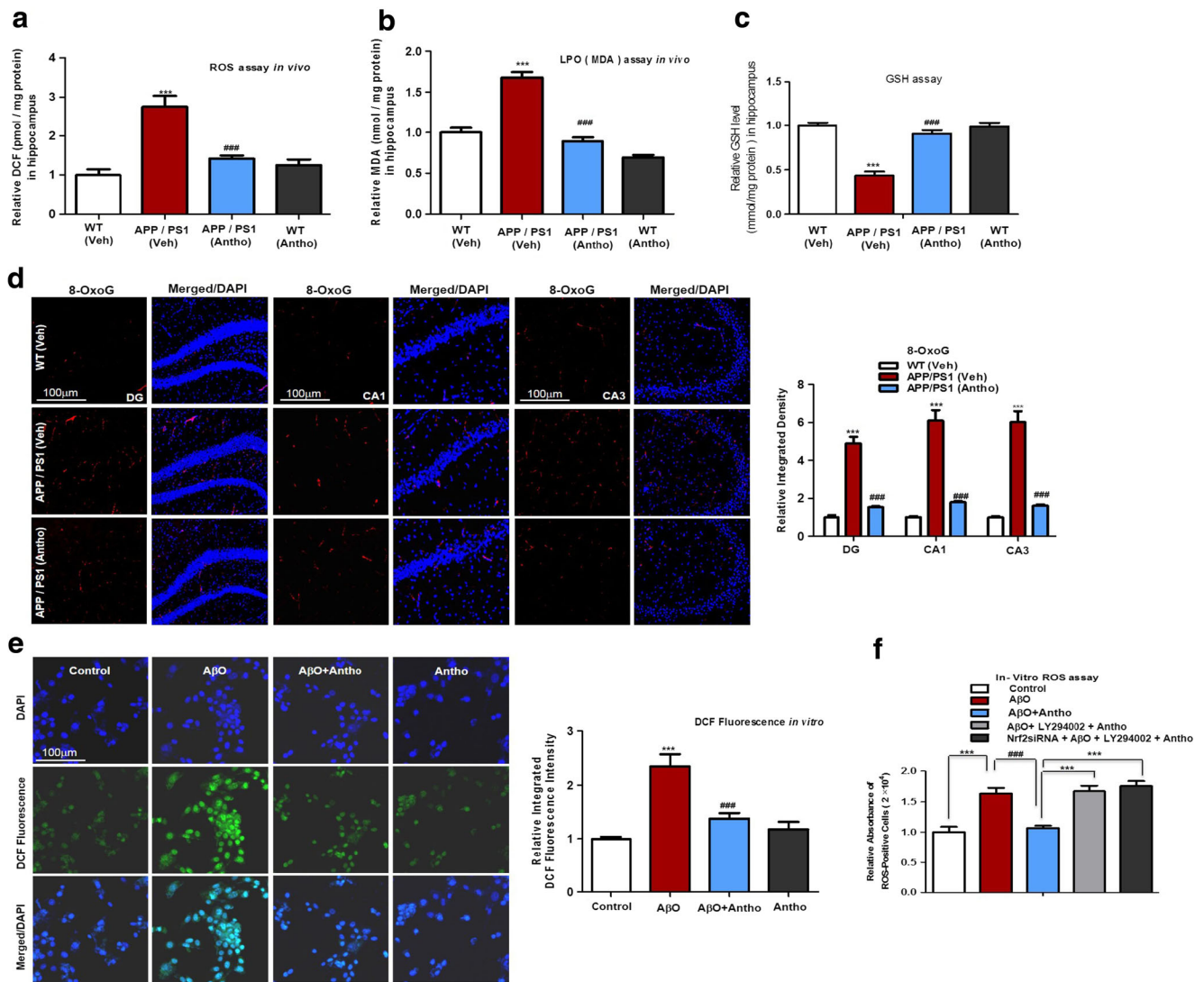
The 8-OxoG is a key biomarker of ROS-induced oxidative stress, increased in the AD brain and the APP<sup>swe</sup>/PS1 $\Delta$ E9 transgenic mouse model of AD [42]. To assess ROS-induced oxidative stress, we analyzed the oxidative stress marker 8-OxoG through immunofluorescence staining. The results showed increased expression of 8-OxoG in the Veh-treated APP/PS1 mice compared with the Veh-treated WT mice. Anthocyanins supplementation overcame oxidative stress and significantly reduced the expression of 8-OxoG in the APP/PS1 mice compared with the Veh-treated APP/PS1 mice (Fig. 2d). Furthermore, we found in vitro that 100  $\mu$ g/ml anthocyanins significantly reduced the elevated ROS production, as indicated by decreased DCF fluorescence intensity, in the A $\beta$ O (5  $\mu$ M)-exposed HT22 cells (Fig. 2e). Additionally, the in vitro ROS assay results manifested that anthocyanins (100  $\mu$ g/ml) significantly mitigated the ROS-positive cells in the A $\beta$ O-(5  $\mu$ M)-exposed HT22 cells. Interestingly, when the HT22 cells were treated with a LY294002 (10  $\mu$ M) and subjected to Nrf2 siRNA, anthocyanins were unable to prevent the formation and accumulation of ROS-positive cells in the A $\beta$ O-exposed HT22 cells (Fig. 2f).



**Fig. 1** Anthocyanins treatment upregulated p-PI3K/Akt/GSK3β (Ser9) signaling, nuclear translocation of Nrf2, and expression of its target genes (HO-1 and GCLM) in APP/PS1 mice and in vitro AβO-exposed HT22 cells. **a** The Western blot results for p-PI3K/Total-PI3K, p-Akt (Ser473)/Total Akt and p-GSK3β (Ser9)/Total GSK3β in the hippocampus of the experimental mice. The bands were quantified using Sigma Gel software, and the differences are represented by a histogram. β-Actin was used as a loading control. Data are expressed as the means ± SEM for the indicated proteins (*n* = 8 mice/group), *n* = 3 experiments. **b** A representative image of the immunofluorescence reactivity of p-Akt (Ser473) (red, TRITC; blue, DAPI) in the CA1 region of the hippocampus. *N* = 5 mice/group, *n* = 3 experiments. Magnification ×10. Scale bar = 100 μm. **c** The Western blot results of nuclear/cytosolic Nrf2 expression using Nrf2, HO-1 and GCLM in the hippocampus of the experimental mice. The bands were quantified using Sigma Gel software, and the differences are represented by a histogram. β-Actin was used as a loading

control. Data are expressed as the means ± SEM for the indicated proteins (*n* = 8 mice/group). **d, e** Images of co-localized immunofluorescence reactivity of Nrf2 (green, FITC; blue, DAPI) and HO-1 (red, TRITC; blue, DAPI) in the DG and CA1 regions of the hippocampus. *N* = 5 mice/group. Magnification ×40. Scale bar = 50 μm. **f** A representative image of the immunofluorescence reactivity of GCLM (green, FITC; blue, DAPI) in the CA1 and CA3 region of the hippocampus. *N* = 5 mice/group, *n* = 3 experiments. Magnification ×10. Scale bar = 100 μm. **g** The Western blot results for p-PI3K, p-Akt (Ser473), p-GSK3β (Ser9), Nrf2, HO-1 and GCLM in the HT22 cells subjected to Nrf2 siRNA and exposed to anthocyanins (100 μg/ml), AβO (5 μM) and LY294002 (10 μM). The bands were quantified using Sigma Gel software, and the differences are represented by a histogram. Data are expressed as the means ± SEM for the indicated proteins. The presented data are relative to the control. Significance = \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, #*P* < 0.05, ###*P* < 0.01, ####*P* < 0.001





**Fig. 2** Anthocyanins treatment mitigated ROS and oxidative stress in APP/PS1 mice and *in vitro* A $\beta$ O-exposed HT22 cells. **a** A representative histogram of ROS levels in the hippocampus homogenates of the experimental mice. The graphs were expressed as the means  $\pm$  SEM ( $n = 8$  mice/group) and the number of experiments = 3. The presented data are relative to the control. **b** A representative histogram of MDA levels in the hippocampus homogenates of experimental mice. The graphs were expressed as the means  $\pm$  SEM ( $n = 8$  mice/group) and the number of experiments = 3. The presented data are relative to the control. **c** A representative histogram of GSH levels in the hippocampus homogenates of the experimental mice. The graphs were expressed as the means  $\pm$  SEM ( $n = 8$  mice/group) and the number of experiments = 3. The presented data are relative to the control. **d** Image of immunofluorescence staining

of 8-oxoG (red, TRITC; blue, DAPI) in the CA1, DG, and CA3 regions of the hippocampus.  $N = 5$  mice/group. Magnification  $\times 10$ . Scale bar = 100  $\mu$ m. **e** Images of the DCF immunofluorescence intensity (green, FITC; blue, DAPI) in the HT22 cells exposed to A $\beta$ O (5  $\mu$ M) and anthocyanins (100  $\mu$ g/ml) for 24 h.  $N = 3$  experiments. Magnification  $\times 10$ . Scale bar = 100  $\mu$ m. The presented data are relative to the control. **f** A representative histogram of the relative absorbance of ROS-positive HT22 cells that were subjected to Nrf2 siRNA and treated with A $\beta$ O (5  $\mu$ M) and anthocyanins (100  $\mu$ g/ml) for 24 h. The number of experiments = 3. The data are expressed as the mean  $\pm$  SEM. The presented data are relative to the control. Significance = \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , # $P < 0.05$ , ### $P < 0.01$ , #### $P < 0.001$

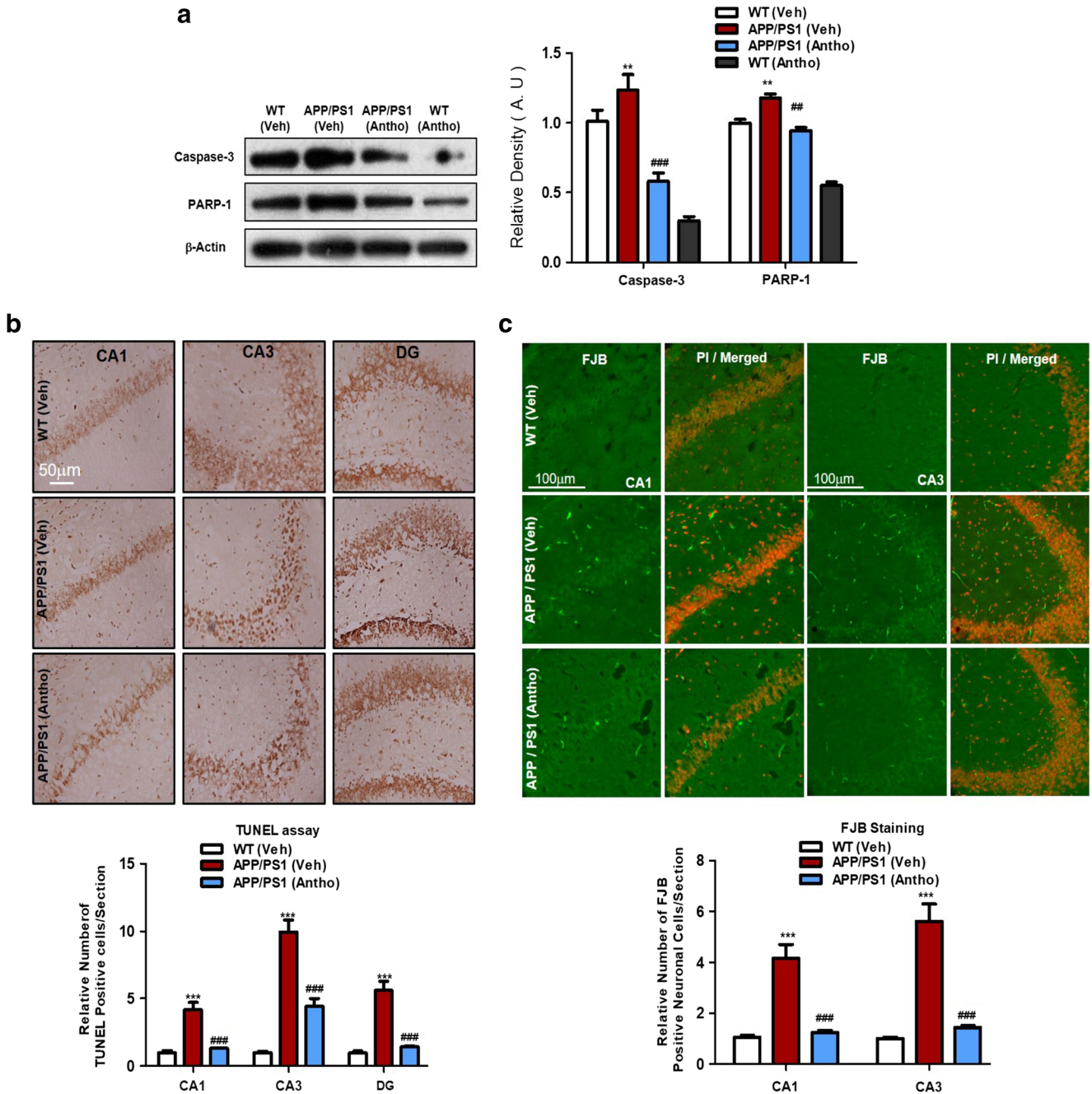
### Anthocyanins via PI3K/Akt/Nrf2 Pathways Prevented Neurodegeneration in APP/PS1 Mice and *In Vitro* A $\beta$ O-Exposed HT22 Cells

Recently, we reported that Korean black bean anthocyanins exert neuroprotective effects against A $\beta$ <sub>1–42</sub> in both *in vitro* and *in vivo* A $\beta$ <sub>1–42</sub> models [36, 37]. Therefore, we assessed the apoptotic and neurodegenerative markers such as activated

caspase-3 and activated poly (ADP-ribose) polymerase 1 (PARP-1) expression level; and TUNEL and FJB positive neuronal cells in the APP/PS1 mice. Our western blot analysis indicated increases in activated caspase-3 and PARP-1 in the Veh-treated APP/PS1 mice compared with the Veh-treated WT mice. Anthocyanin (12 mg/kg *i.p.* for 30 days) supplementation significantly downregulated activated caspase-3 and activated PARP-1 levels in the APP/PS1 mice compared

with Veh-treated APP/PS1 (Fig. 3a). The immunohistochemical TUNEL results revealed that TUNEL-positive neuronal cells were significantly increased in the hippocampi of Veh-treated APP/PS1 mice compared with the Veh-treated WT group. Anthocyanins supplementation significantly reduced the number of these TUNEL-positive neuronal cells in the

CA3, CA1 and DG regions of hippocampi, showing that anthocyanins reduced neurodegeneration in the APP/PS1 mice compared with the Veh-treated APP/PS1 group (Fig. 3b). Similarly, the number of FJB-positive neuronal cells was significantly increased in the hippocampi of Veh-treated APP/PS1 mice compared with the Veh-treated WT group.



**Fig. 3** Anthocyanins prevented neurodegeneration in APP/PS1 mice. **a** Western blot analysis of mouse hippocampi using antibodies against activated caspase-3 and PARP-1. The bands were quantified using Sigma Gel software, and the differences are represented by a histogram. The density values are expressed in arbitrary units (A.U.) as the means ± SEM for the indicated hippocampal proteins ( $n = 8$  mice/group),  $n = 3$  experiments. **b** The TUNEL-positive neuronal cells are shown ( $n = 5$

animals/group),  $n = 3$  experiments. Magnification  $\times 20$ . Scale bar = 50  $\mu\text{m}$ . **c** Representative images of FJB staining (green, FITC; red, PI). ( $N = 5$  animals/group),  $n = 3$  experiments. Magnification  $\times 10$ . Scale bar = 100  $\mu\text{m}$ . The presented data are relative to the control. Significance = \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , # $P < 0.05$ , ### $P < 0.01$ , #### $P < 0.001$

Anthocyanins significantly reduced neurodegeneration in the CA1 and CA3 region of the hippocampi, which showed decreased numbers of FJB-positive neurons in APP/PS1 mice compared with the Veh-treated APP/PS1 group (Fig. 3c).

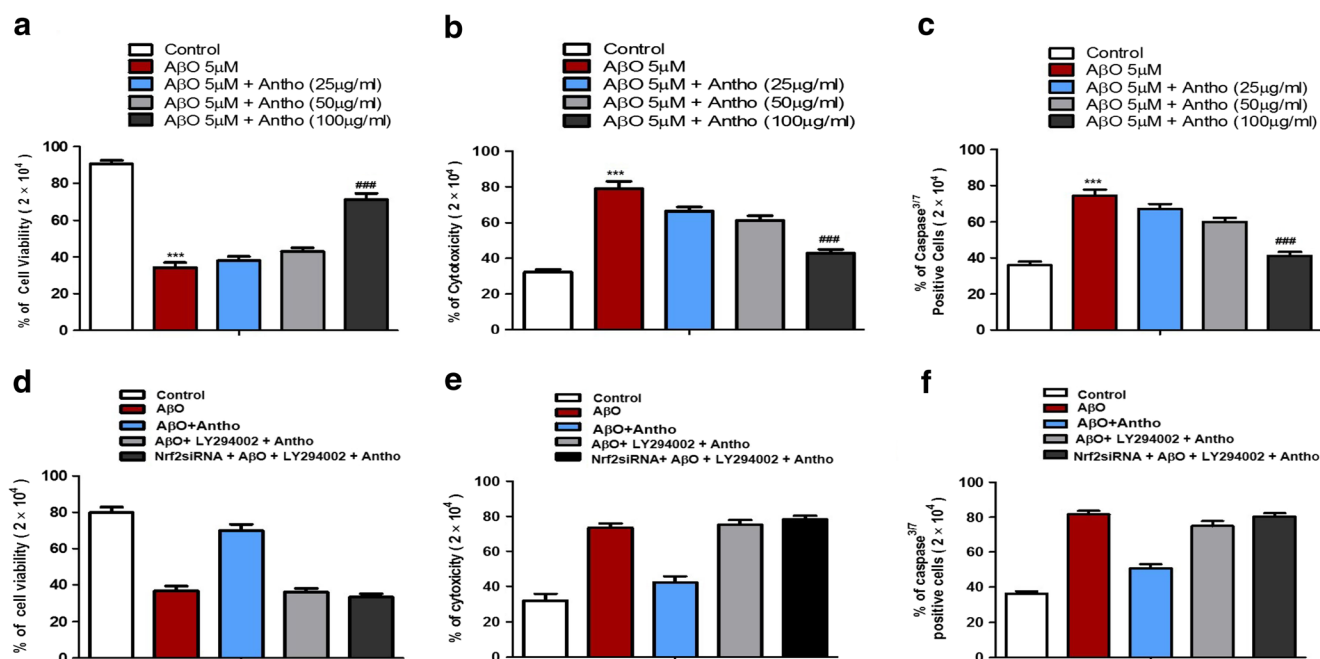
Further, here again we sought to determine in vitro whether anthocyanins ameliorate A $\beta$ O-induced neurotoxicity. The results of an ApoTox-Glo™ Triplex assay in neuronal HT22 cells showed that cell viability was reduced, whereas the cytotoxicity and activation of caspase<sup>3/7</sup> increased, after treatment with A $\beta$ O (5  $\mu$ M) compared with the control [cells exposed to DMSO (0.01%)]. Anthocyanins treatment at three different concentrations (25, 50, and 100  $\mu$ g/ml) reduced the effects of A $\beta$ O (5  $\mu$ M); however, the 100  $\mu$ g/ml dose significantly reduced the effects of A $\beta$ O (5  $\mu$ M), increasing cell viability and decreasing cytotoxicity and caspase<sup>3/7</sup> activation (Fig. 4a–c). Furthermore, to assess the role of the cytoprotective and anti-oxidant PI3K/Akt/Nrf2 pathways in A $\beta$ O-induced neurotoxicity, we performed an ApoTox Glo™ Triplex assay in neuronal HT22 cells by using a PI3K inhibitor (LY294002 (10  $\mu$ M)) and Nrf2 siRNA. Figure 4d–f demonstrates that 100  $\mu$ g/ml anthocyanins reversed the effect of A $\beta$ O, significantly increased cell viability and reduced cytotoxicity and caspase<sup>3/7</sup> activation in the A $\beta$ O-exposed HT22 cells. Nevertheless, when a PI3K inhibitor (LY294002 (10  $\mu$ M)) and Nrf2 siRNA were applied, the anthocyanins were ineffective against A $\beta$ O-induced neurotoxicity and could not increase cell viability and reduce cytotoxicity and caspase<sup>3/7</sup> activation in the HT22 cells (Fig. 4d–f). These

in vitro results suggest that anthocyanins act as a potent neuroprotective anti-oxidant and reduced A $\beta$ O-induced neurotoxicity in a PI3K/Akt/Nrf2-dependent manner.

### Anthocyanins Improved Pre- and Postsynaptic Protein Markers in APP/PS1 Transgenic AD Mice

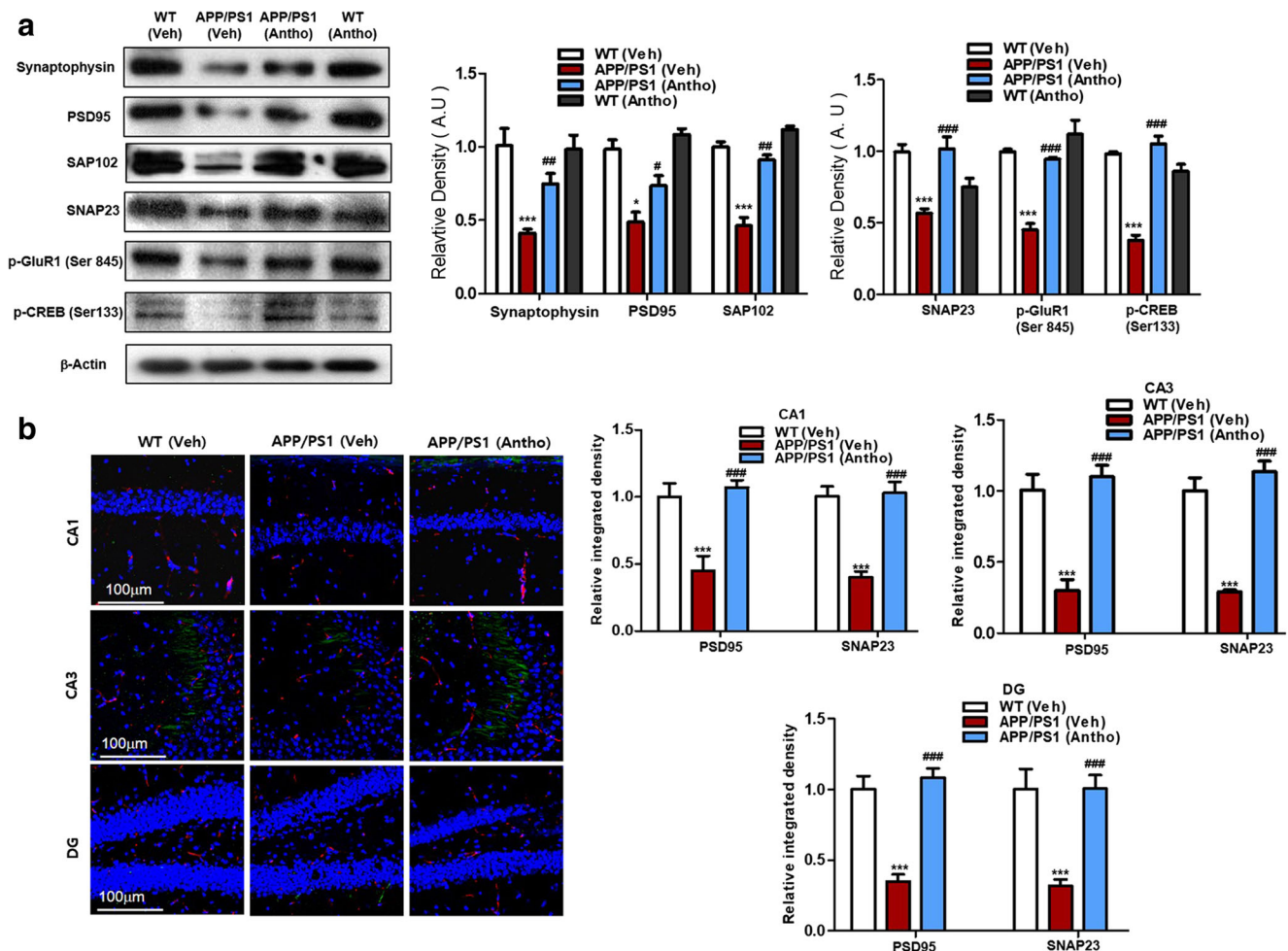
After the antioxidant and neuroprotective activity of anthocyanins in the APP/PS1 mice, we determined the memory-associated protein markers in the APP/PS1 mice. Western blot analysis revealed that memory-associated presynaptic [synaptophysin, synaptosomal associated protein 25 (SNAP-25)] and postsynaptic protein markers [postsynaptic density protein 95 (PSD95) and synaptosomal-associated protein 102 (SAP-102)] were decreased in the hippocampi of Veh-treated APP/PS1 mice compared with the Veh-treated WT mice. Anthocyanins supplementation (12 mg/kg i.p. for 30 days) significantly increased the levels of synaptophysin, SNAP-25, PSD95, and SAP-102 in the hippocampi of APP/PS1 mice (Fig. 5a).

A $\beta$ -induced synaptic impairment is associated with the reduction of  $\alpha$ -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors (AMPA receptors). Notably, phosphorylation of the AMPA receptor (GluR1) at Ser845 plays a pivotal role in the trafficking of postsynaptic glutamate receptors and is related to spatial working memory [15, 37, 39]. Our immunoblot results showed decreased levels of these post-synaptic markers in the Veh-treated



**Fig. 4** Anthocyanins via PI3K/Akt/Nrf2 pathway prevented A $\beta$ O-induced neurotoxicity in HT22 neuronal cells. **a–c** ApotoxGlo Triplex assays of the cell viability, cytotoxicity and activated caspase<sup>3/7</sup> in HT22 cells treated with A $\beta$ O (5  $\mu$ M) and anthocyanins at three different concentrations (25, 50, and 100  $\mu$ g/ml). **d–f** ApotoxGlo Triplex assays of

the cell viability, cytotoxicity and activated caspase<sup>3/7</sup> in HT22 cells subjected to Nrf2 siRNA and treated with A $\beta$ O (5  $\mu$ M), LY294002 (10  $\mu$ M) and anthocyanins (100  $\mu$ g/ml).  $N = 3$  independent experiments. The data are expressed as the means  $\pm$  SEM. Significance = \*\*\* $P < 0.001$ , ### $P < 0.001$



**Fig. 5** Anthocyanins improved pre- and postsynaptic protein markers in APP/PS1 transgenic mice. **a** The Western blot results for synaptophysin, SNAP25, PSD95, SAP102, SNAP23, p-GluR1 (Ser845) and p-CREB (Ser133) in the hippocampi of the mice. The bands were quantified using Sigma Gel software, and the differences are represented by a histogram. Data are expressed as the means  $\pm$  SEM for the indicated proteins ( $n = 8$

mice/group),  $n = 3$  experiments. **b** Images of co-localized immunofluorescence reactivity of SNAP23 (green, FITC; blue, DAPI) and PSD95 (red, TRITC; blue, DAPI) in the CA1, CA3 and DG regions of the hippocampus.  $N = 5$  mice/group. Magnification  $\times 40$ . Scale bar = 50  $\mu$ m. The presented data are relative to the control. Significance = \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$

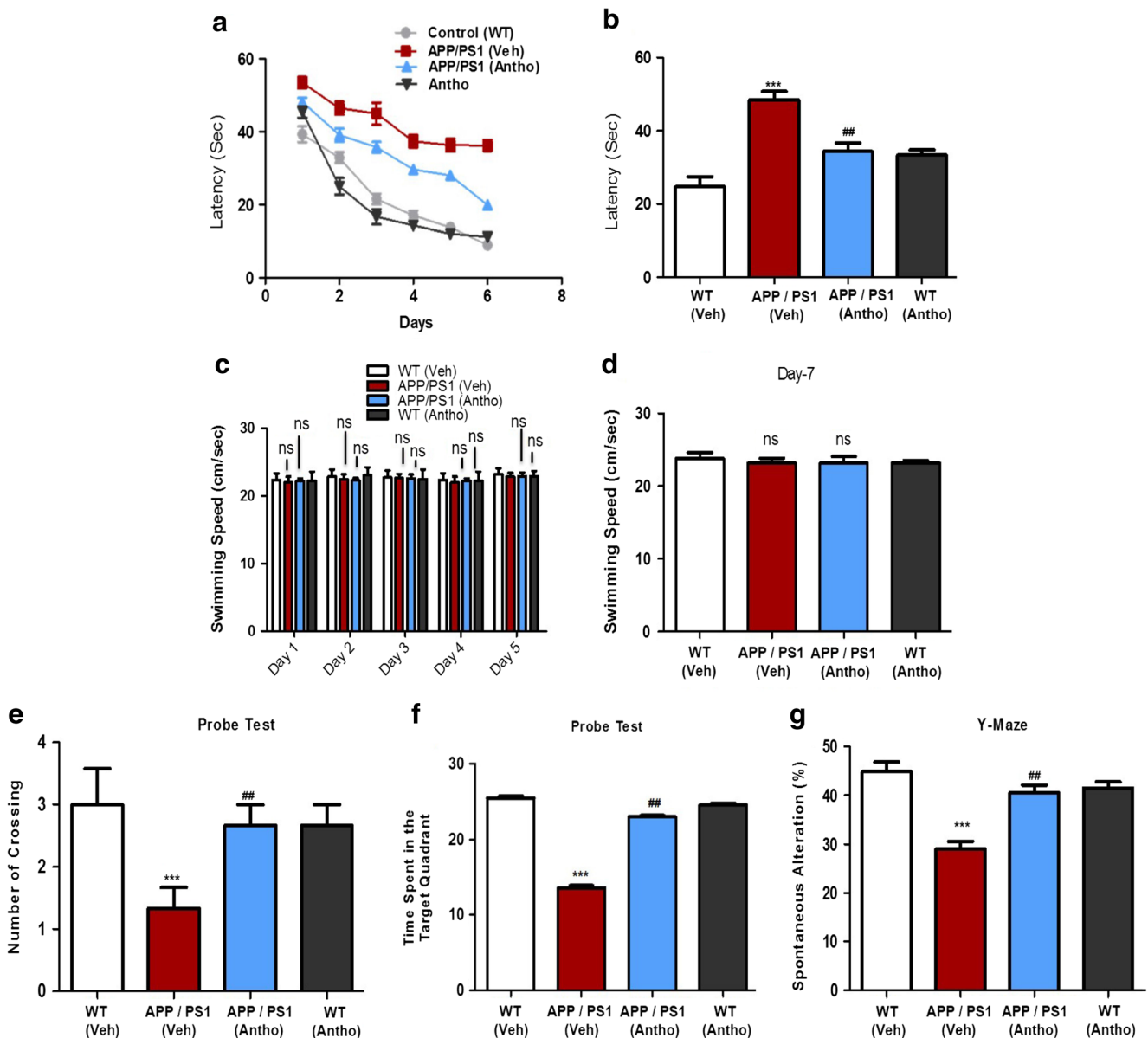
APP/PS1 mice compared with the Veh-treated WT mice. Anthocyanin treatment significantly increased SNAP23 and p-GluR1 (Ser845) in the APP/PS1 mice compared with the Veh-treated APP/PS1 mice (Fig. 5a).

It has been reported that activating phosphorylated cAMP response element binding protein (p-CREB) (Ser133) in the rodent's brain improved spatial working memory [37, 43, 44]. Our black bean anthocyanins supplementation activated p-CREB (Ser133) and significantly increased the level of p-CREB (Ser133) in the APP/PS1 mice compared with the Veh-treated APP/PS1 mice (Fig. 5a).

The immunofluorescence results of PSD95 and SNAP23 revealed that anthocyanins treatment enhanced the immunofluorescence reactivity of PSD95 and SNAP23 in the CA1, CA3 and DG region of hippocampi in the APP/PS1 mice compared with the Veh-treated APP/PS1 mice (Fig. 5b).

### Anthocyanins Improved Memory Functions in APP/PS1 Transgenic AD Mice

The beneficial effects of polyphenolic flavonoid anthocyanins (12 mg/kg i.p. for 30 days) on spatial learning and memory in APP/PS1 mice were investigated using the MWM and Y-maze tasks. MWM training tests were performed for 6 consecutive days, and the latency to escape to the hidden platform and the trajectories to reach the hidden platform were recorded. As shown in Fig. 6a, the mean escape latency was significantly increased in the APP/PS1 mice compared with the WT Veh-treated mice. Anthocyanin (12 mg/kg i.p. for 30 days) treatment significantly improved memory functions and reduced the mean escape latency compared with the Veh-treated APP/PS1 mice. Moreover, we measured the final escape latency on day seven before the probe test. Regarding the



**Fig. 6** Anthocyanins improved memory functions in APP/PS1 transgenic mice. The MWM and the Y-maze test were used to evaluate spatial working memory. Thirteen mice per group were used for the behavioral analysis. **a** Mean escape latency (sec) to the hidden platform during training. **b** Final escape latency on the final day of the probe test. **c**, **d** Histograms represent swimming speed (cm/s) of the mice during their search for the hidden platform during the training sessions and on day 7 before the probe test, respectively. To calculate swimming speed (cm/s),

we used the equation  $v = \text{distance}/\text{time}$ . No significant difference was observed among the swimming speeds of the mouse groups. **e** Number of crossings over the previous platform location during the probe test. **f** Time spent in the target quadrant (where the platform was located during the hidden platform training session) during the probe test. **g** The spontaneous alteration behavior percentage. The graphs express the means  $\pm$  SEM ( $n = 13$  mice/group). Significance = \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , # $P < 0.05$ , ### $P < 0.01$ , #### $P < 0.001$ . ns = no significant

final latency, the Veh-treated WT, anthocyanin-treated APP/PS1 and anthocyanin-treated WT mice took less time than the Veh-treated APP/PS1 mice to reach the hidden platform (Fig. 6b). We also observed the mean swimming speeds during the training days and on day 7 before the probe test. The swimming speed measurements revealed no difference among the Veh-treated WT, anthocyanin-treated WT, Veh-treated APP/PS1 and anthocyanin-treated APP/PS1 mice, indicating

that any differences in ability to reach the hidden platform were unrelated to the motor functions of the mice (Fig. 6c, d).

On day 7, after the escape latency test, we removed the hidden platform and conducted a probe test. The number of crossings over the previous site of the platform was significantly greater in the anthocyanins-treated APP/PS1 mice than in the Veh-treated APP/PS1 mice (Fig. 1e). Additionally, anthocyanin-treated APP/PS1 mice spent more time in the

target quadrant than Veh-treated APP/PS1 mice (Fig. 6f), demonstrating that anthocyanins protected against spatial memory deficits in the APP/PS1 mice.

Next, we performed a Y-maze task to evaluate spatial working memory using spontaneous alternation behavior percentage. The observation showed that the APP/PS1 mice had a lower percentage of alternation than the Veh-treated WT mice, indicating weaker working memory. Anthocyanins treatment significantly increased spontaneous alteration percentage in the APP/PS1 mice compared with the Veh-treated APP/PS1 mice (Fig. 6g), indicating that anthocyanins alleviated short-term memory dysfunction in APP/PS1 AD mice.

## Discussion

The objective of the current study was to investigate the underlying anti-oxidant neuroprotective mechanism of dietary Korean black bean anthocyanins in the APP/PS1 mouse model of AD. Several well-established studies have investigated elevated ROS and oxidative stress in this AD model and it has been shown that in the AD brain, A $\beta$  accumulation induces increased ROS production and oxidative stress, which subsequently leads to neurodegeneration and memory impairment [45–48]. Attenuation of ROS and oxidative stress reduced the memory impairment in the transgenic mouse model of AD [48]. Similarly, the present study noted elevated ROS, enhanced LPO, as determined by increased MDA levels, reduced GSH level and activated immunofluorescence reactivity of 8-OxoG in the APP/PS1 mouse model of AD. Interestingly, anthocyanins supplementation attenuated the elevated ROS levels and oxidative stress in the AD mouse model.

Studies have reported translocation of Nrf2 from the cytosol to the nucleus, where it activates anti-oxidative enzymes, such as HO-1 and NADPH quinone dehydrogenase-1 (NQO1), and produces a notable anti-oxidative response to counteract escalated ROS-induced oxidative stress and protect against oxidative stress in different neurodegenerative models such as ischemia. Moreover, it has been shown that Nrf2 knockout mice possess elevated oxidative stress and ROS levels [49–52]. Recently, Zhang et al. found that activation of Nrf2 and its target anti-oxidant genes is protective against oxidative stress [53]. Both in vitro and in vivo numerous studies have proposed that endogenous anti-oxidant genes such as Nrf2 and HO-1 activation are possibly involved in neuroprotective mechanisms against AD [54, 55]. Notably, it has also been reported that the Nrf2/HO-1 pathway was dysregulated in an AD transgenic mouse model and that overexpression of these Nrf2/HO-1 pathways improves spatial learning in an AD mouse model [19, 40, 41]. Consistently, in our AD model (APP/PS1 mice), the expression levels of Nrf2 and HO-1 were

decreased. Both in vitro and in vivo, we confirmed that anthocyanin treatment reduced the ROS-induced elevation of oxidative stress via the activation of Nrf2 and its target genes such as HO-1 and GCLM, which might be associated with improved spatial working memory in APP/PS1 mice.

PI3K/Akt/GSK-3 $\beta$  signaling is one of the most important pathways, having a key and pivotal role in various signaling and in other functions. Impaired PI3K/Akt/GSK-3 $\beta$  signaling has been investigated in the AD brain and AD mouse models [7, 8, 13–15, 39]. Accumulation of ROS induces oxidative stress that mediates the downregulation of p-PI3K/p-Akt, which triggers the activation of GSK-3 $\beta$ , resulting in Nrf2 translocation from the nucleus to cytosol and producing a low level anti-oxidant activity [56, 57]. ROS accumulation induces oxidative stress that mediates GSK-3 $\beta$  activation, while GSK-3 $\beta$  inhibition is implicated in the attenuation of oxidative stress in hippocampal cell lines [58]. It has been reported that activated GSK-3 $\beta$  induces the impairment and degradation of Nrf2 [59, 60]. Moreover, activation of GSK-3 $\beta$  is prevented by activation of p-GSK-3 $\beta$  (Ser9) via the activation of PI3K/Akt [15]. Kitagishi et al. reviewed the evidence that dietary supplementation of some natural pleiotropic neuroprotective agents, especially anti-oxidants, provides neuroprotection by regulating the PI3K/Akt/GSK-3 $\beta$  pathway [61]. Other studies also reported that nuclear translocation and activation of Nrf2 via upregulation of p-PI3K/p-Akt/GSK3 $\beta$  pathway provides protection against oxidative stress-induced cell injury [61, 62]. Recently, we found that natural anti-oxidant anthocyanins induced neuroprotection against ethanol in the developing rat brain by attenuating oxidative stress through regulation of the PI3K/Akt/GSK-3 $\beta$  pathway [27]. Here, our results also described and elaborated on the anti-oxidant protective mechanism in which chronic anthocyanins treatment regulates the PI3K/Akt/GSK3 $\beta$  signaling cascade and activated the Nrf2/HO-1 pathway and its target anti-oxidant genes, ultimately providing effective anti-oxidant neuroprotection in APP/PS1 mice.

Phenolic and polyphenolic flavonoids act as neuroprotective agents via activation of the PI3K/Akt/GSK3 $\beta$ /Nrf2 pathway, which suppresses the apoptotic caspases and neurodegeneration [63–68]. Previously, we demonstrated that anthocyanins prevents the apoptotic neurodegeneration both in vivo and in vitro studies [69]. Similarly, our Western blot results indicated that anthocyanins administration prevented apoptosis via the suppression of apoptotic markers, e.g., activated caspase-3 and PARP-1 levels, in the hippocampi of APP/PS1 mice. Moreover, the increased numbers of TUNEL- and FJB-positive neuronal cells in the hippocampi of APP/PS1 mice were reduced by anthocyanins treatment, which indicated that polyphenolic flavonoids such as anthocyanins may prevent neurodegeneration via activation of the endogenous Nrf2/HO-1 anti-oxidant

mechanism. Additionally, it has also been determined that anthocyanins dose-dependently (25, 50, 100  $\mu\text{g/ml}$ ) prevented  $\text{A}\beta_{1-42}$ -induced neurotoxicity in vitro in HT22 cells. Furthermore, we exposed HT22 cells to LY294002 (10  $\mu\text{M}$ ) and knocked down Nrf2 with Nrf2 siRNA in the presence of  $\text{A}\beta_{1-42}$ -induced neurotoxicity, against which anthocyanins (100  $\mu\text{g/ml}$ ) were ineffective. This provided further evidence that activation of the endogenous neuroprotective anti-oxidant PI3K/Akt/Nrf2 pathway by anthocyanins has a promising role in the prevention of apoptosis and neurodegeneration in the AD.

In AD synaptic deficit and loss is implicated in the memory and cognitive dysfunction and has been considered a primary target in the AD therapy [70]. Synaptic impairment is associated with the decreased expression of presynaptic proteins such as synaptophysin, and the postsynaptic proteins PSD95 and SAP102 were also decreased in the brains of AD patients and animal models of AD [71]. An  $\text{A}\beta$ -induced deficit of AMPARs (Ser845) has been associated with early synapse and memory dysfunction [15]. P-CREB (Ser133) is an important biochemical marker for memory functions. High expression of p-CREB in the hippocampus increases memory functions and promotes synapse formation. Hippocampus-dependent memory formation is impaired through attenuation of p-CREB [72]. In agreement with previous reported studies, the present study observed a decreased level of pre- (synaptophysin) and postsynaptic proteins (PSD95, SAP102, SNAP23, p-GluR1 at Ser845) as well as p-CREB (Ser133) in the APP/PS1 mouse model of AD. Interestingly, chronic anthocyanins treatment produces beneficial effects and significantly restores these memory-related pre- and postsynaptic markers as well the expression of p-CREB (Ser133) in APP/PS1 mice.

Several studies reported that natural dietary supplements, e.g., phenolic and polyphenolic flavonoids have potent anti-oxidant neuroprotective activities which induce beneficial effects and have been reported to improve learning/memory and cognitive functions in the rodent animal models [63–69, 73–75]. The APP/PS1 mouse is a well-known animal model of memory deficits in the animal AD model [70, 76]. Mounting evidences indicated that PI3K/Akt/GSK3 $\beta$ /Nrf2/HO-1 pathways have a key role in the behavioral performance as well in the learning and memory function of the rodents [75, 77, 78]. Here, we investigated memory impairment via the MWM and Y-maze tasks in 10-month-old male APP/PS1 mice. Notably, in our study, anthocyanins treatment at 12 mg/kg/day i.p. for 30 days improves learning and memory functions as measured by escape latency in the trial session as well as on the final day of the probe test. No significant differences were recorded in the swimming speed among the groups, indicating that spatial learning

and memory functions were not associated with motor function. Anthocyanins also improved the number of platform crossings and the time spent in the target quadrant during the probe test on the final day. Anthocyanins also rescued the spatial working memory deficit, as indicated by increasing spontaneous alteration behavior (%) in the APP/PS1 mice compared with Veh-treated APP/PS1 mice (Fig. 6).

In summary, this study demonstrated the underlying anti-oxidant neuroprotective mechanism of natural dietary Korean black bean anthocyanins in the APP/PS1 mouse model of AD. Our results established that anthocyanins regulate the PI3K/Akt/GSK3 pathway and consequently activate the downstream endogenous anti-oxidant Nrf2/HO-1 pathway and its target genes, reducing the  $\text{A}\beta$ -induced elevation of ROS-mediated oxidative stress and preventing neurodegeneration via a PI3K/Akt/Nrf2-dependent pathway. Importantly, anthocyanins improve memory-related pre- and postsynaptic markers as well cognitive functions in APP/PS1 mice. Taken together, these data suggest that anthocyanins derived from natural sources act as potent anti-oxidant; are a promising potential dietary component and could be beneficial as a dietary supplement for the prevention of age-related neurological disorders, such as AD.

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**Compliance with Ethical Standards** The animal maintenance, treatments, behavioral studies and surgical procedures were carried out in accordance with the animal ethics committee (IACUC) guidelines issued by the Division of Applied Life Sciences, Department of Biology at Gyeongsang National University, South Korea. The experimental methods with animals were carried out in accordance with the approved guidelines (Approval ID: 125) and all experimental protocol were approved by the animal ethics committee (IACUC) of the Division of Applied Life Sciences, Department of Biology at Gyeongsang National University, South Korea.

**Conflict of Interest** The authors declared no conflict of interest.

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