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Improvement in neurogenesis and memory function by administration of *Passiflora incarnata L.* extract applied to sleep disorder in rodent models

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ABSTRACT

Recently, there have been reports that chronic insomnia acts as an insult in the brain, causing memory loss through the production of ROS, inflammation, and, Alzheimer's disease if persistent. Insomnia remains the leading cause of sleep disturbance and as such has serious implications for public health. Patients with Alzheimer's disease are also known to suffer from severe sleep disturbance. Meanwhile, vitexin is a key ingredient in *Passiflora incarnata L* (passion flower, PF) extract, which is known to help with sleep. This medicinal plant has been used as a folk remedy for sedation, anxiety and sleep since centuries ago, but the standardization work has not been done and the extent of the effect has not been clearly demonstrated.

For this reason, we tried to test the possibility that repeated administration of PF could improve the memory by promoting hippocampal neurogenesis at the DBA/2 mice known have inherited sleep disorders, as well as preventive effects of Alzheimer's disease.

Here, we found that vitexin, which is the main bioactive component of ethanol extracts from leaves and fruits (ratio; 8:2) of PF, confirmed the improvement of neurogenesis (DCX) of DBA/2 mice repeated PF oral administration by immunohistochemistry (IHC) and western blot analysis. PF-treated group showed increased the neurotrophic factor (BDNF) in the hippocampus compared with that of vehicle-treated group, but the inflammation markers Iba-1 (microglial marker) and COX-2 were inconsistent between the groups. However, we found COX-2 signal is essential for hippocampal neurogenesis according to the additional IHC experiments using COX-2 inhibitor and pIkappaB have shown. In addition, although prescription sleeping pills have been reported to show significant changes in appetite and metabolic rate from time to time, no changes in the feeding behavior, body weight, metabolic rate and body composition of the animals were observed by administration of PF. Interestingly, we found that short-term oral administration of PF displayed improved memory according to the water maze test. Quantitative analysis of Tau protein, which is a marker of Alzheimer's disease, was performed in the SD rats and DBA/2 mice by repeated PF oral administration and pTau/Tau values were significantly decreased in PF-treated group than vehicle-treated group.

In conclusion, our results suggest that PF lead high hippocampal neurogenesis in the animals even in inherited sleep-disturbed animals. The increased hippocampal neurogenesis functionally enhanced memory and

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learning functions by repeated PF oral administration. These results identify PF as a potential therapy for enhancing memory functions and prevention of Alzheimer's disease through actions on the hippocampus.

1. Introduction

Sound sleep is very important for healthy daily life. However, millions of people worldwide suffer from sleep disorders and are forced to use medications such as benzodiazepines, non-benzodiazepine receptor agonists, and suvorexan. Unfortunately, these can have adverse effects such as dementia and unexpected behavioral issues (Li et al., 2013). In addition, the safety and efficacy of sleep medicines are not completely determined yet (Schroeck et al., 2016). Sleep problems can adversely affect neuronal health, and can expose sufferers to subsequent risks of incident all-cause dementia and Alzheimer's disease (AD)(Hahn et al., 2014). It has been reported that patients with chronic primary insomnia commonly have daytime cognitive impairment, memory consolidation during sleep, and disturbed sleep is negatively associated with performance on memory tasks compared to sound sleepers (Backhaus et al., 2006; Fulda and Schulz, 2001; Joo et al., 2014). Sleep disorders can affect the cerebral neuroenvironment and enhance neuroinflammatory responses, or lead to neurohormonal stress conditions (Backhaus et al., 2006; Choi et al., 2014). Therefore, rapid correction of the sleep disorder is needed to protect brain microenvironment structures and brain function by preventing the brain from accumulating insults caused by chronic insomnia (Joo et al., 2014; Kent and Mistlberger, 2017).

Passion flower (*Passiflora incarnata L*; PF) has been widely known as a medicinal plant in South America and Europe since the 16th century (Miroddi et al., 2013). It has sedative, anxiolytic, and anticonvulsant effects on the central nervous system (CNS)(Akhondzadeh et al., 2001; Jawna-Zboinska et al., 2016; Sarris et al., 2011; Tortoriello and Romero, 1992). However, its primary pharmacological constituents were unknown. Using chromatography we identified the active ingredient in PF extract as vitexin, a chemical with diverse biological activities, including anti-oxidant, anti-cancer, anti-inflammatory, anti-hyperalgesic, and anti-AD properties (Abbasi et al., 2012; Choi et al., 2014; He et al., 2016; Jawna-Zboinska et al., 2016; Malar et al., 2018; Miroddi et al., 2013; Sarris et al., 2011; Soulimani et al., 1997). In addition, vitexin could induce sleep in mice as well (Brown et al., 2008; Guerrero and Medina, 2017). This compound is expected to be more effective and safer for those suffering from chronic insomnia, by inducing easy sleep to maintain brain function. Interestingly, while studying the sleep-inducing efficacy of PF we found that neurogenesis was greatly improved in the hippocampus of DBA/2 mice, which are known to be inherently deficient in sleep function. Recently, there have been many reports suggesting a close relationship between improved neurogenesis and resistance to AD (Choi and Li, 2011; Gagnon et al., 2008; Kent and Mistlberger, 2017; Wang et al., 2014). Since numerous studies have reported that sleeping pills are responsible for animal feeding, exercise, and behavioral changes (Guerrero and Medina, 2017; Hermesh et al., 2001; Park and Shin, 2016; Schroeck et al., 2016), the most important considerations in this study are whether the administration of PF minimizes physiological and behavioral changes, and whether improvements to cognitive and memory functions are significant or not. Thus, we wanted to see if the administration of PF could improve brain function safely and without behavioral changes such as orexigenic stimulus and/or degradation of activities. Therefore, in this study, behavioral, physiological and metabolic changes following PF administration, were measured. Moreover, PF was administered to DBA/2 mice as well, and Morris water maze test was performed to determine whether memory capacity was improved by PF administration.

In this study, we determined whether oral administration of PF extracts could enhance neurogenesis at the hippocampus without any behavioral changes. Further, we investigated the possibility of PF extract providing resistance to AD by comparing differences in expression of pTAU/TAU at the hippocampus in experimental and control groups of SD rats. (The research flow is shown in supplement 1)

2. Materials and methods

2.1. Experimental design

2.1.1. Preparation of passion flower (Passiflora incarnata L.) extract and validation

Passiflora incarnata L.(PF) was cut and dried. It contained 80% by weight of leaves and 20% by weight of fruits. Air-dried passion flower leaves and fruits were ground, mixed with 60% ethyl alcohol, heated to 80 °C for 2 h, and filtered. Passion flower leaves and fruit extracts were concentrated and spray dried. Vitexins present in PF extracts were analyzed and quantitated using HPLC-UV. PF extract powder was dissolved in 25 mL of methanol (40 mg/mL). After filtration through a 0.45 nm Millipore membrane filter, aliquots of 20 mL were directly injected into an Agilent HPLC system equipped with Agilent 1260 binary pump, Agilent 1100 auto sampler, and Agilent 1200 diode array detector (Agilent Technologies, Palo Alto, CA, USA). HPLC separation of vitexin was performed using a Shim-pack VP-ODSC18 column (250 × 4.6 mm i.d., 5 μm) and Shim-pack GVP-ODSC18 guard column (10 mm × 4.6 mm i.d., 5 μm). The isocratic mobile phase consisting of tetrahydro-furan/acetonitrile/0.05 phosphoric acid solution (20:3:77, v/v/v) was delivered at a flow-rate of 1.0 mL/min. Prior to use, the mobile phase was filtered through 0.45 mm Millipore membrane filters and degassed by sonication in an ultrasonic bath. Detection wavelength was set at 360 nm and the column temperature was maintained at 25 °C.

2.1.2. Experimental rodent animals and PF oral administration

2.1.2.1. Mice. Male ICR male mice are commonly used experimental animals (Jo et al., 2018; Kim et al., 2019), because they have no special disease, pathogen, and genetic defects. DBA/2 mice have been used as a useful model of insomnia in several previous studies (El Helou et al., 2013; Tinarelli et al., 2014; Toth and Bhargava, 2013). DBA/2 mice inherently have genetic defects in their sleep, thus they experience sleep disturbances. We used them as a preliminary pilot study to identify neurogenic effects and mice were separated into four groups: Sham (ICR mice, Veh-treated only), control (Veh-treated animal), PF 10 mg/kg treated animal, and PF 50 mg/kg treated animal. Male DBA/2 mice were purchased from Samtako (Korea). In addition, a normal Sham group was included using male ICR mice purchased from the same company. Each group of DBA/2 had 5 mice (mean weight of 22 g, range, 19˜24 g, 7-week-old). The Sham group had 5 ICR mice (mean weight of 24 g, range, 22˜26 g, 7-week-old) for use as a positive control. In addition, in a study of water maze analysis to evaluate the memory task function following chronic administration of PF, the DBA/2 mice were separated into two groups, Veh-treated and PF-treated. For the pilot study, distilled water (Veh) or soluble PF extract (10 ml/kg/day and 50 mg/kg/day in the vehicle) was carefully administered daily with an oral sonde to DBA/2 mice. The same amount of Veh was administered orally to DBA/2 (control group) and ICR mice (sham group) for 3 days. These animals were sacrificed on the 4th day after beginning the administration, brains were perfused with 4% paraformaldehyde solution and used following post-fixation with the same fixation. For the water maze study, the animals were administered with 100 mg/kg/day with Veh and PF for the same period with the

following rodent (SD rat) models. For determining whether chronic PF administration created adverse side effects for feeding, activity or metabolic changes, 7-week-old male C57BL/6 mice were purchased from Charles River (Japan, Yokohama) and investigated for total behavioral and metabolic changes. They were separated into control; Veh-treated and PF; PF-treated and monitored with Phenomaster® for the experiments. Mice were housed at room temperature (22 ± 2 °C) with 60% humidity under a 12-h light: dark cycle (light cycle: dark cycle from 07:00 to 19:00). These animals were provided free access to normal chow diet (2018S; Harlan, USA) and water.

2.1.2.2. Rats. Eight-week-old male Sprague-Dawley (SD) rats (Harlan; Madison, WI) weighing 200~225 g were individually housed in a climate-controlled room with a 12:12 light-dark cycle (light cycle: dark cycle from 07:00 to 19:00), and a temperature 22 ± 2 °C and 60% humidity. The animals were provided a normal chow diet (2018S; Harlan, USA). Free access was allowed to water. Food consumption and feeding bouts were measured with a BioDAQ® Food Intake Monitor (Research Diets; New Brunswick, NJ, USA), by the number of times animals physically manipulated the food hopper and removed food. The duration of a bout was defined as a single episode of feeding in which no inter-feeding interval exceeded 15 s. We separated rats into two groups designated as Veh (distilled water in water bottle) and PF extracts treated group (2 mg/ml PF-dissolved in water bottle), and we allowed them to drink freely from the water bottles in their cages. The Soonchunhyang University Institutional Animal Care and Use Committee (IACUC) approved all experiments and procedures (Approval number: SCH16-0037).

2.1.3. Treatment with COX-2 inhibitor

To elucidate the correlation between COX-2 and neuroblast differentiation, the animals were randomly divided into 2 groups; vehicle (a physiological saline)- and COX-2 inhibitor (celecoxib)-treated groups (n = 5 in each group). Vehicle (1 mL/100 g body weight) or the same volume of 20 mg/kg celecoxib (prescription formulation; Pfizer Inc.) were orally administered to rats using the feeding needle every day for 2 weeks, before sacrifice. Afterwards, the brains were removed and immunohistochemistry was performed with goat anti-DCX and mouse anti-phospho-IκBα to show the role of COX-2 on the neurogenesis in the hippocampal dentate gyrus. The quantities were shown as a ratio of cell numbers at the SGZ and numbers of tertiary branches of each neuroblast dendrites.

2.1.4. Cell toxicity by MTT assay

2.1.4.1. Solutions. PF solutions; eight PF solutions with concentrations ranging from 2^2mg/ml to 2^{-5}mg/mL were tested. PF was dissolved in α-MEM media (WELGEN, Gyeongsan-si, Korea) at the maximum soluble capacity of about 2^4mg/ml and filtered with 0.2 μm syringe filter (Corning, Germany). Remaining solutions were prepared by binary dilution of 2^4 mg/mL solution.

2.1.4.2. MTT solution. Thiazolyl blue tetrazolium bromide (Sigma-Aldrich, MO, USA) (10 mg) was dissolved in 2 mL of PBS (pH 7.4) and filtered with 0.2 μm syringe filter. Subsequently the reagent was diluted decuple with α-MEM media, for 0.5 mg/ml final concentration. This yellow-colored solution was stored at 4 °C and kept from light. In brief, bone marrow cells were collected from femurs and tibias of male ICR mice. After cell counting, 2×10^5 cells/200 μL α-MEM media were seeded in each well of a 96-well-plate and incubated at 37 °C with 5% CO_2 for 48 h. Media were suctioned out and 200 μL of PF solutions of various concentrations and α-MEM media without PF (control) were dispensed in each well with duplicates. After incubation for 24 h, PF solutions were completely removed and cells were incubated with 100 μL of MTT solution in the dark for 4 h. Purple precipitates were observed under microscope, 100 μL of dimethyl sulfoxide (DMSO) was dispensed in each well to stop the MTT activity and optical density was measured at 570 nm with Multi SKAN (Thermo Fisher, CA, USA).

2.1.5. Worm culture

The wild-type N2 strain was purchased from the *C.elegans* genetics center (CGC, Minneapolis/St. Paul, USA). The green fluorescent protein (GFP)-expressing strain, CL2070 (dvIs70 [*Phsp-16.2*:: GFP, *rol-6*]) was also purchased from CGC. Worms were cultured on Nematode Growth Media (NGM) agar plates (1.7% agar, 2.5 mg/mL peptone, 25 mM NaCl, 50 mM KH_2PO_4 pH6.0, 5 μg/mL cholesterol, 1 mM $CaCl_2$, 1 mM $MgSO_4$) at 20 °C. *Escherichia coli* OP50 was supplied on NGM plates as a food source. Age-synchronized worms were obtained by permitting five young adult (L4) worms to lay eggs on a fresh NGM plate at 20 °C. After 4 h, all adult worms were removed from the plate and the eggs were maintained at 20 °C for 3 days.

2.1.6. Resistance to oxidative stress

Age-synchronized young adult worms were treated with different dilutions of PF extract (50 mg/L, 100 mg/L, and 500 mg/L) in NGM plates for 24 h. Diluted PF (100 μL of each) was spread on a solid NGM plate (5 mL). Afterwards, 12.5 mg/L of 5-fluoro-2′-deoxyuridiene (Sigma-Aldrich, St. Louis, USA) was added to the NGM plates to prevent internal hatching. Then, the worms were exposed to 2 mM hydrogen peroxide in S-bsal without cholesterol (5.85 g sodium chloride, 1 g potassium phosphate dibasic, and 6 g potassium phosphate monobasic in 1 L sterilized distilled water). The number of dead nematodes was scored after 6 h of exposure to hydrogen peroxide. Three independent repeat experiments were performed and differences among groups were tested and analyzed using one-way ANOVA.

2.1.7. Lifespan assay

Sixty age-synchronized worms were transferred to NGM plates containing different concentrations of PF extract (50 mg/L, 100 mg/L, and 500 mg/L) and 12.5 mg/L 5-fluoro-2′-deoxyuridine. Thereafter, live worms were transferred to a fresh NGM plate every 2–3 days and counted every day until all of the worms were dead. The lifespan of the worms exposed to PF was compared to those of untreated control worms using the log-rank test.

2.1.8. Heat shock stress resistance

Age-synchronized 3-day-old worms were picked from an NGM plate and transferred to a fresh NGM plate containing 500 mg/L PF. After 24 h, the worms were transferred to a 35 °C incubator for 10 h to induce heat shock stress. Then, the worms were transferred back to 20 °C. Survival rates after 24 h at 20 °C were compared between the control and N-acetylcysteine (NAC)-treated worms. We used the standard two-tailed Student's *t*-test for statistical analysis.

2.1.9. Tissue processing

For histology, animals were anesthetized with 1 g/kg urethane (Sigma-Aldrich, St. Louis, MO, USA) and perfused transcardially with 0.1 M PBS (pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate-buffer (pH 7.4). The brains were removed and post-fixed in the same fixative for 12 h at 25 °C. The brain tissues were cryoprotected by overnight infiltration with 30% sucrose. Serial brain sections were cut in the coronal plane at a thickness of 30 μm, using a cryostat (Leica, Wetzlar, Germany), and collected in 6-well plates containing PBS for further processing.

The sections were processed under the same conditions to ensure that the immunohistochemical data were comparable between groups. Tissue sections were selected between -3.00 and -4.08 mm posterior to the Bregma with reference to a rat brain atlas (Paxinos and Watson, 2006) for each animal and between -1.82 and -2.46 mm posterior to the Bregma with reference to a mice brain atlas (Franklin and Paxinos, 2008). Ten sections, 90 μm apart from each other, were sequentially treated with 0.3% hydrogen peroxide (H_2O_2) in PBS for 30 min and 5% normal goat serum in 0.05 M PBS for 30 min at 25 °C.

2.1.10. Immunohistochemistry staining for doublecortin (DCX), Ki67, COX-2 and pIkappaβ

Immunohistochemistry staining was performed under the same conditions for each group in order to examine whether the degree of staining was accurate. Immunohistochemical staining was conducted using protocols described in our previous studies (Yi et al., 2011, 2010). Briefly, sections were sequentially treated with 0.3% hydrogen peroxide (H_2O_2) in PBS for 30 min and 10% normal goat or rabbit serum in 0.05 M PBS for 30 min. They were then incubated with diluted goat anti-DCX antibody (1:500, SantaCruz Biotechnology, CA, USA) and rabbit anti-Ki67 (1:1000, Abcam, Cambridge, UK) overnight at 4 °C and subsequently exposed to biotinylated rabbit anti-goat IgG (diluted 1:200, Vector, Burlingame, CA, USA) and biotinylated goat anti-rabbit IgG (diluted 1:200, Vector, Burlingame, CA, USA), and streptavidin peroxidase complex (diluted 1:200, Vector, Burlingame, CA) for 2 h at 25 °C. The neuroblasts (DCX positive cells) Ki67 positice cells on the subgranular zone (SGZ) were counted and demonstrated with bar graph in the Fig. 2A and B.

Sections first underwent an overnight incubation with rabbit anti − COX-2 antibody (1:200, Cayman, Ann Arbor, MI, USA) or mouse phospho-IκBα (1:500; SantaCruz Biotechnology, Santa Cruz, CA, USA) for 48 h at 4 °C. Thereafter, the sections were treated with biotinylated goat anti-rabbit IgG or anti-mouse IgG and a streptavidin-peroxidase complex (1:200; Vector, Burlingame, CA) for 2 h at 25 °C. Sections were then visualized by staining with 3, 3′-daminobenzidine in 0.1 M Tris − HCl buffer (pH 7.2). Sections were mounted onto gelatin-coated slides with Canada Balsam (Wako, Tokyo, Japan) following dehydration.

2.1.11. Immunohistochemistry staining for microglia (Iba-1)

Freshly prepared brain sections were sequentially treated with 0.3% hydrogen peroxide (H_2O_2) in PBS for 30 min and then with 10% normal goat serum in 0.05 M PBS for 30 min. They were treated with diluted rabbit anti-Iba-1 (1:1,000, Chemicon, Temecula, CA) overnight at 4 °C, and exposed to biotinylated goat anti-rabbit IgG and streptavidin peroxidase complex (1:200, Vector, Burlingame, CA). Sections were visualized using with 3, 3′-daminobenzidine in 0.1 M Tris − HCl buffer (pH 7.2). Sections were mounted onto gelatin-coated slides with Canada Balsam (Wako, Tokyo, Japan) following dehydration.

2.1.12. Western blot

Hippocampus and hypothalamic chunk were removed from the skull of SD rats and homogenized in lysis buffer (iNtRon Biotechnology, Seoul, Korea). Protein concentrations were determined with a BCA kit (iNtRon Biotechnology). Total proteins (20 μg per sample) were loaded into each lane of 12% SDS-PAGE, electrophoresed, and transferred to PVDF membranes (Bio-Rad Laboratories, CA, USA). Following transfer, membranes were blocked with TBST [100 mM Tris − HCl (pH 7.6), 0.8% NaCl and 0.1% Tween-20] containing 10% skim milk (BD Biosciences, CA, USA). These membranes were incubated with the following primary antibodies: goat anti-DCX (Doublecortin, 1:200, SantaCruz Biotechnology, TX, USA), rabbit anti-glucocorticoid receptor (GR) (1:1,000, SantaCruz Biotechnology, TX, USA), rabbit anti − COX-2 (1:1000, Cayman chemical, MI, USA), rabbit anti-CRF (1:200, ABBIOTEC, SD, USA), rabbit anti-melatonin receptor 1A (Mela R1A) (1:1,000, abcam), rabbit anti-parvalbumin(1:3,000, swant, Switzerland), rabbit anti-c-Fos (1:500, SantaCruz Biotechnology, TX, USA), rabbit anti-BDNF(1:3000, Proteintech, USA), rabbit anti-Iba1(1:2,000, Wako, Osaka, Japan), mouse anti-tau (1:500, Calbiochem), rabbit anti-phospho-tau (1:1,000, Cell Signaling Technology, MA, USA) and rabbit anti-GAPDH (glyceraldehyde 3-phosphate dehydrogenase, 1:5,000, Cell Signaling Technology, MA, USA) at 4 °C overnight. After further washing, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Vector, CA, USA). Immunoreactive signals were detected through enhanced chemiluminescence (Abclon, Seoul, Korea) and recorded with MicroChemi 4.2 system.

2.1.13. Monitoring of behavioral and metabolic changes by indirect calorimetry

Metabolic performance parameters (energy intake and energy expenditure) were studied by using Phenomaster®, an automated combined indirect calorimetry system (TSE System GmBH, Bad Homburg, Germany). Before the experiment, mice were acclimated for 2 days in a metabolic chamber with food and water, and subsequently oxygen consumption (VO_2), carbon dioxide production (VCO_2) and food consumption were measured for 3 days. Room temperature for all metabolic studies was maintained at 22 °C with a 12-hour-light/dark cycle. The respiratory exchange rate (RER; VCO_2/VO_2) were calculated by using standard in-house software. Body composition (lean tissue, fat, and fluid in live mice on a bench-top platform) was measured following measurements of the animals' phenotyping with Mini-spec LF50 (Bruker Biospins, The Woodlands, TX). This work was technically supported by the Korea Mouse Phenotyping Project (2013M3A9D5072550) of the Ministry of Science, ICT and Future Planning through the National Research Foundation, Republic of Korea.

2.1.14. Morris water maze analysis

We evaluated the ability of hippocampal-dependent learning, including acquisition of spatial memory and long-term memory by PF administration to DBA/2 mice. Vehicle (Veh; n = 5) and PF-treated groups (PF; n = 5) were tested based on the modified Morris water maze test established by Bromely-Brits et al. (Bromley-Brits et al., 2011). In brief, the test consists of three steps day 1 for Visible platform, day 2 to day 4 for Hidden platform, and day 5 for Probe trial. For each day and each mouse, an average of four trials gives a single path length and escape latency for each test subject. For day 5, we simply collected the path length, escape latency, and time spent in the platform quadrant for each mouse. We compared the learning curves for days 2–4 and analyzed by one way-ANOVA. For day 6, comparing the percentage of time spent in the previously learned platform quadrant, and a higher percentage of time spent in the platform quadrant was interpreted as a higher level of memory retention.

2.1.15. Data analysis

To ensure objectivity, all measurements were performed under blinded conditions by two observers per experiment under identical conditions. For quantitation of immunoreactivity, the extent of the staining was measured using 5 sections per animal. The images of DCX, GR and Iba-1 immunoreactive structures were taken using a BX53 light microscope (Olympus, Japan) equipped with a digital camera (DP71, Olympus) connected to a personal computer and a monitor. The intensity of the bands generated during western blotting was evaluated based on the optical density measured by transforming mean gray levels using the formula: optical density = log (256/mean gray level) with the NIH Image 1.59 software.

The data shown represents the experimental mean ± standard error (SE) for each experimental group. Differences between means were analyzed with one-way ANOVA where appropriate or Student's *t*-test. Post hoc analyses were performed with Fisher's least significant difference or Newman-Keul tests where appropriate. Statistical analyses were performed using JMP9 (SAS Institute, USA) and Prism7 (GraphPad Software, San Diego, CA, USA). P values (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$, ****, $P < 0.0001$) were considered statistically significant.

3. Results

3.1. LC/MS/MS analysis

Chromatograms showed the retention time of isoorientin, orientin, vitexin and isovitexin were 8.660, 8.984, 10.263 and 10.578 min,

respectively (Fig. 1D). In addition, chromatograms showed the retention time of isoorientin, orientin, vitexin and isovitexin were 8.669, 9.003, 10.268 and 10.582 min in PF extract, respectively (Fig. 1E). The MS full scan spectra for all analysis showed protonated precursor [M−H]- ions at the positive mode in PF extract. As shown, vitexin is the main substance in PF extract according to these results.

3.2. Enhanced DCX- and Ki67-positive cells expressions in the DBA/2 mice by short-term (3 days) administration of PF extract

DCX-positive neuroblasts and Ki67-positive cells (proliferating cells) were highly expressed in the DBA/2 mice administered with PF extract (10 mg/kg and 50 mg/kg) (Fig. 2A and B). Interestingly, DBA/2 with only vehicle treatment showed very low expression of DCX-positive cell, and the dendrite branches were very poor at the subgranular zone (SGZ) in the dentate gyrus. While high in the PF treated mice, DCX-positive neuroblasts were not as highly expressed or as well developed as in the positive-control (ICR/Veh, Sham) at the SGZ (right enlarged images; Fig. 2A). Ki67-positve cells (proliferating cells) were highly expressed on the SGZ in the dentate gyrus in the DBA/2 mice administered with PF extract (Fig. 2B). The DCX- and Ki67-positive cells on the PF-treated groups were significantly increased than those of Veh-treated group. The neuroblast (DCX) in hippocampus, and Glucocorticoid receptor (GR), which is known as a marker for HPA axis regulation, in hippocampus and in hypothalamus of DBA/2 PF-treated (50 mg/kg) mice were measured compared with those of DBA/ Veh-treated mice (Fig. 2C). Neuroblast and GR protein expressions at the PF-treated mice were significantly different with those at Veh-treated DBA/2 mice (Fig. 2C).

3.3. Cell toxicity

Optical density proportionally correlates with cell viability. Compared to the control group, PF extracts were diluted from 4 mg/ml to 3.125×10^{-2}mg/ml by binary dilution method. Data shows, there was no toxicity to cells at any concentration tested. Instead, they significantly enhanced cell proliferation (****, $P < 0.0001$) and showed increased cell viability (Supplement 2A).

3.4. Increased resistance to induced oxidative stress by PF extract in C. elegans

To examine the effect of PF extract on the response to oxidative stress, we monitored the survival of worms under oxidative stress conditions (6 h exposure with H_2O_2) with or without PF. Exposure to PF (50 mg/L, 100 mg/L and 500 mg/L) seemed to facilitate increased survival under oxidative stress compared to untreated controls (53.32 ± 10.17, 56.63 ± 20.28 and 65.56 ± 5.56 versus 42.22 ± 6.19, respectively)(Supplement 2B), but statistical significance was only achieved at a concentration of 500 mg/L ($P = 0.0485$).

3.5. Lifespan extension with PF extract treatment

We examined whether PF extract could affect *C. elegans* lifespan. PF supplementation significantly extended both the mean and the maximum lifespans of *C. elegans* (Supplement 2C). Among three different PF concentrations analyzed, the lifespan-extending effects of 500 mg/L was greater than that of 50 mg/L and 100 mg/L. The mean and the maximum lifespan values of untreated control worms were 17.6 days and 27 days, respectively. The mean lifespan was extended up to 24.2 days with 500 mg/L PF-treatment, and maximum lifespan increased by 10 days (Table 1.). The effect of PF on the longevity calculated using the mean lifespan of untreated and 500 mg/L PF-treated worms was 19.8% ($P < 0.001$, Table 1.). An independent repeat experiment also showed a significant lifespan-extending effect following supplementation with 500 mg/L PF (Table 1.). These findings indicate that the most effective PF concentration for resistance to oxidative stress and longevity in

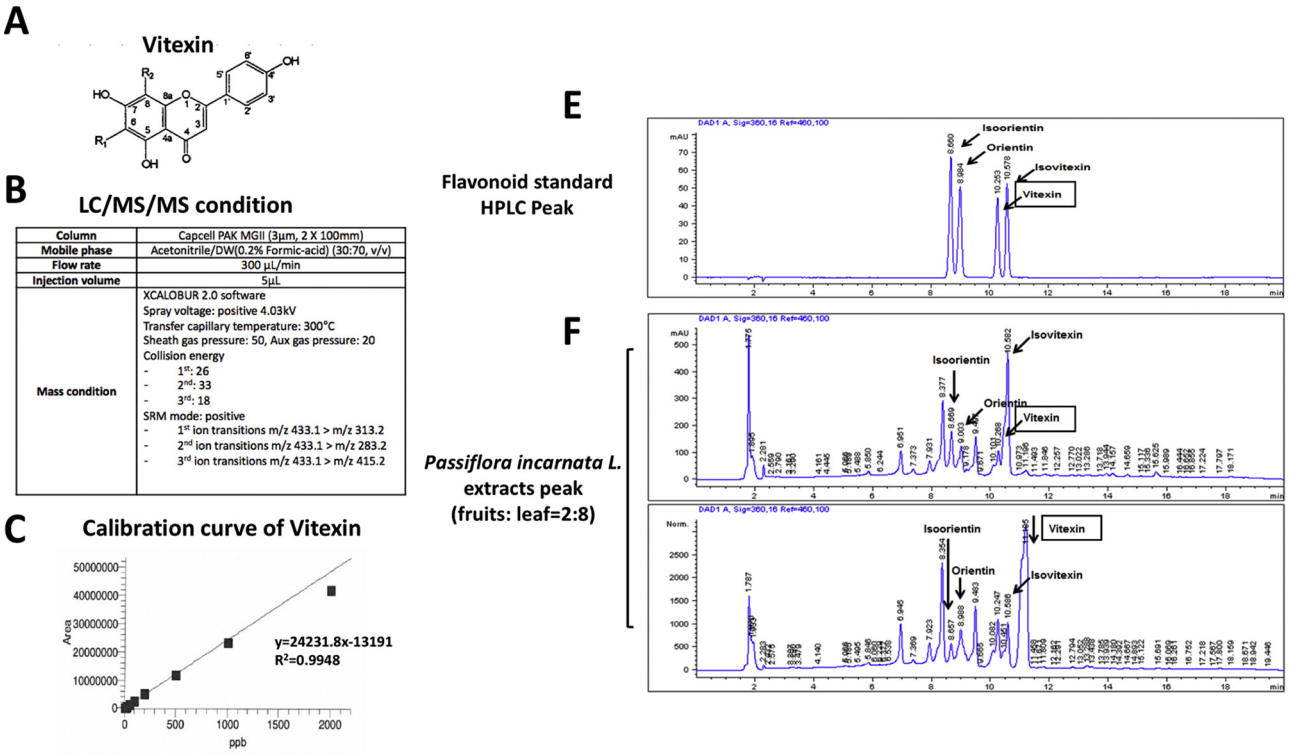

Fig. 1. Vitexin concentration ratio/time and LC/MS/MS chromatogram of *Passiflora incarnata L.* **(A)** Structure of vitexin, **(B)** LC/MS/MS condition, **(C)** calibration curve of vitexin, **(D)** HPLC analysis of isovitexin, isoorientin, orientin and vitexin, **(E)** LC/MS/MS analysis of compounds. Each arrow indicates the mass spectrum of compounds (protonate precursor [M−H]- ions).

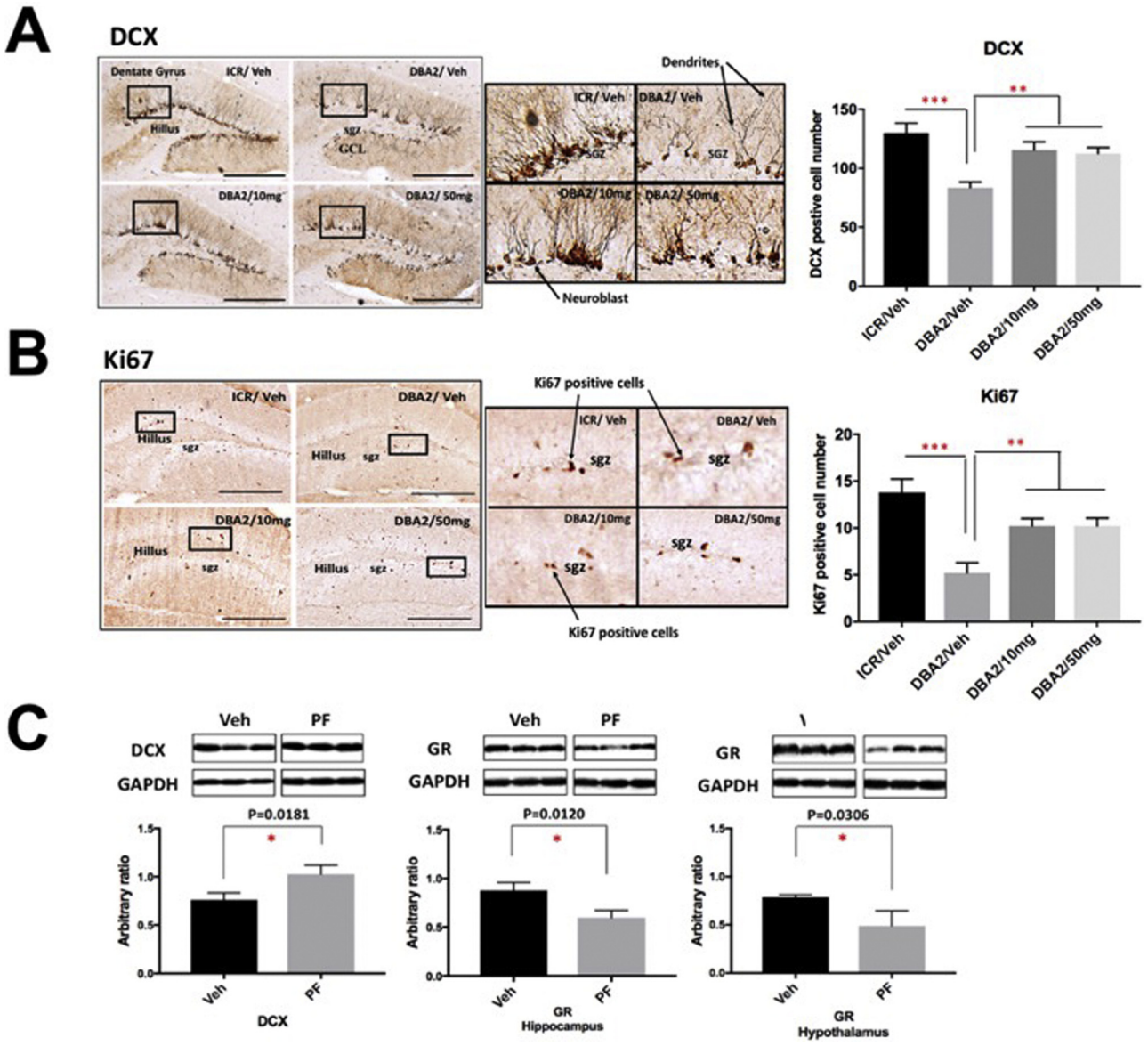

Fig. 2. DCX-positive cell expression in hippocampal dentate gyrus of DBA/2 mice by *Passiflora incarnate L. extract.* **(A)** DCX immunohistochemistry showing positive cell images at the hippocampal dentate gyrus three days after PF oral administration in the DBA2 mouse model (including ICR mice as a positive control for 3 days). The left-side images are overall views of DCX positive cells in the hippocampal dentate gyrus in each group. The right small boxed images are magnified from the black-lined box of the left side images. Neuroblasts were chromogen stained and cell dendrites at the subgranular zone (SGZ) were highly-branched (secondarily and tertiary branching) at the ICR/Veh (Sham), DBA2/10 mg, and DBA2/50 mg groups. However, the DBA2/Veh group showed much lower number of neuroblasts and their branching pattern at the SGZ of dentate gyrus. Scale bar = 100 μm. The DCX positive cell counts were indicated with bar graph, and the significances were demonstrated with (*); **, $P < 0.01$; ***, $P < 0.005$. **(B)** Ki67 immunohistochemistry showing positive cell images at the hippocampal dentate gyrus three days after PF oral administration in the DBA/2 mouse model (including ICR mice as a positive control for 3 days). The left-side images are overall views of Ki67 positive cells in the hippocampal dentate gyrus in each group. The right small boxed images are magnified from the black-lined box of the left side images. Ki67 (proliferating cells) were chromogen stained at the subgranular zone (SGZ). Scale bar = 100 μm. The Ki67 positive cell counts were indicated with bar graph, and the significances were demonstrated with (*); **, $P < 0.01$; ***, $P < 0.005$. **(C)** DCX protein expressions in the hippocampus, GR protein expression in the hippocampus and hypothalamus of PF-treated DBA/2 mice compared to Veh-treated DBA/2 mice were analyzed by arbitrary ratio. They showed significant difference between Veh-treated and PF-treated animals (*, $P < 0.05$). Values are expressed as mean ± SEM.

C.elegans is 500 mg/L.

3.6. Induction of age-related genes by PF extract

Expression of a GFP reporter coupled to the *hsp-16.2* promoter is positively correlated with an organism's lifespan (Rea et al., 2005). This finding suggests that *hsp-16.2* can be used as a predictive molecular marker of *C.elegans* longevity. In this study, we examined whether *hsp-16.2* expression was up-regulated in the long-lived PF-treated (250 mg/L) worms. The *hsp-16.2*::GFP reporter was markedly induced by 250 mg/L PF treatment (Supplement 2D). GFP fluorescence intensity increased from $1.372 \times 10^4 \pm 0.898 \times 10^3$ (mean ± SEM of 20 individual worms) in the control to $3.130 \times 10^4 \pm 1.378 \times 10^3$ in PF-treated worms ($P < 0.0001$) (Supplement 2D). In addition, when the control was set at 100%, the PF-treated (250 mg/L) group was increased by 162.3% (Supplement 2D; $P < 0.0001$).

3.7. Weight and feeding habits of animals upon PF treatment

BioDAQ® real-time food intake monitoring device was used to

Table 1
Lifespan-extending effect of PF extract in *C. elegans*.

		Conc. (mg/L)	Mean lifespan(day)[1)]	Maximum lifespan(day)[2)]	*p*-value[3)]	% increase[4)]
1st	control	0	17.6	27		
Experiment	PF	50	19.8	31	0.025	12.4
		100	21.2	34	0.001	20.8
		500	21.1	33	0.001	19.8
2nd	control	0	21.3	29		
Experiment	PF	50	19.7	30	0.314	−7.2
		100	22.5	34	0.085	5.5
		500	24.2	34	0.012	13.6

[1)] Mean lifespan is the day when 50% of worms were survived.
[2)] Maximum lifespan is the greatest age reached by the last surviving worm.
[3)] *p*-value was calculated using the long-rank test by comparing the survival of control and PF-treated groups.
[4)] % effects were calculated by (*A*-*C*)/*C**100, where *A* is the mean lifespan of *C. elegans* treated with each concentration of PF and *C* is the mean lifespan of control.

observe whether feeding habits were changed by PF administration. Also, no significant difference in body weight was observed after 12 days (Fig. 3A). Although not statistically significant (Fig. 3B), further studies are needed (Fig. 4) because differences in the number of animal accesses to daily food could be a problem in the clinical use of PF extract (104.4 ± 10.79 times and 92.4 ± 5.34 times; Veh versus PF, respectively). However, the total amount of food consumed during the day did not differ between the two groups. This means that the PF-treated animals had a longer stay when they approached the feed, and also had a higher amount of food (309.2 ± 6.33 g and 310.4 ± 9.67 g; Veh versus PF, respectively) (Fig. 3C). PF extract powder was completely dissolved in vehicle (1 mg/mL), and no significant difference was found between Veh and PF for daily water consumption. In addition, since the total drinking water for 12 days did not show any significant difference between the groups (Fig. 3D), PF dosage was maintained within a very constant range (30.5 ± 1.04 mL ~ 34.5 ± 1.19 mL; Veh and 31.5 ± 2.33 mL ~ 36.25 ± 2.32 mL; PF).

3.8. Blood chemistry for sleep induction by PF extract administration

Serum serotonin (5-HT) and melatonin in the animals were 954.0 ± 88.31 ng/mL (Veh) and 1259.0 ± 75.97 ng/mL (PF), and 2.931 ± 0.282 pg/mL (Veh) and 3.841 ± 0.217 pg/mL (PF), respectively. The level of serotonin in PF was 32.0% higher (Fig. 3E; $P < 0.0396$), while melatonin in PF was 31.0% higher (Fig. 3F; $P < 0.0339$).

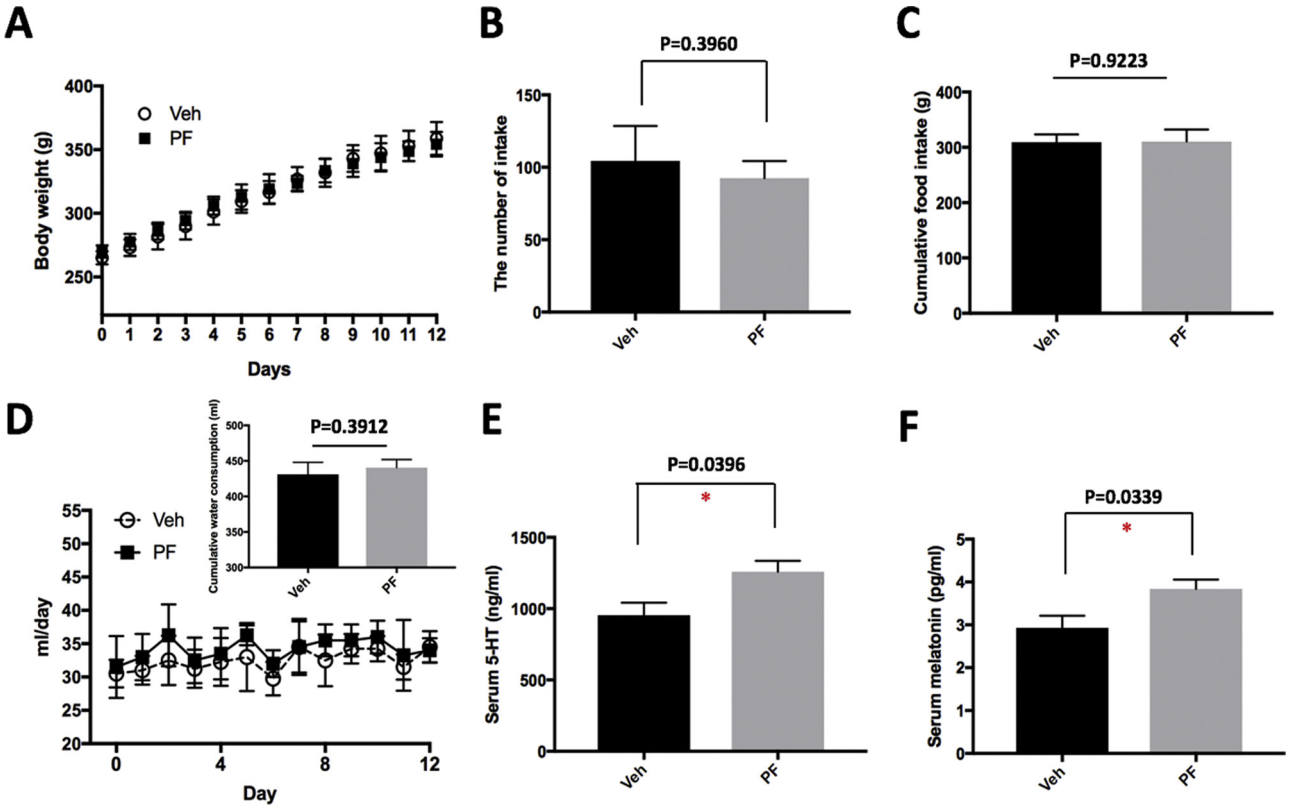

Fig. 3. Physiological data of rats after PF extract oral administration. (A) Body weight changes in control (Veh-) and PF-treated SD rats. **(B, C)** The number of intake (bouting) time and cumulative food intake volumes by BioDAQ® system's running program. According to the records, the number of intake (bouting numbers) seemed reduced in the PF-treated group (PF), however, it was not significantly different from the non-treated group (Con), and cumulative food intake volumes for 12 days were also not significantly different during the experiment. **(D)** Daily water consumption for 12 days was not significantly different. **(E, F)** The serum 5-HT and melatonin levels of PF groups were significantly different from Veh group. Values are expressed as mean ± SEM.

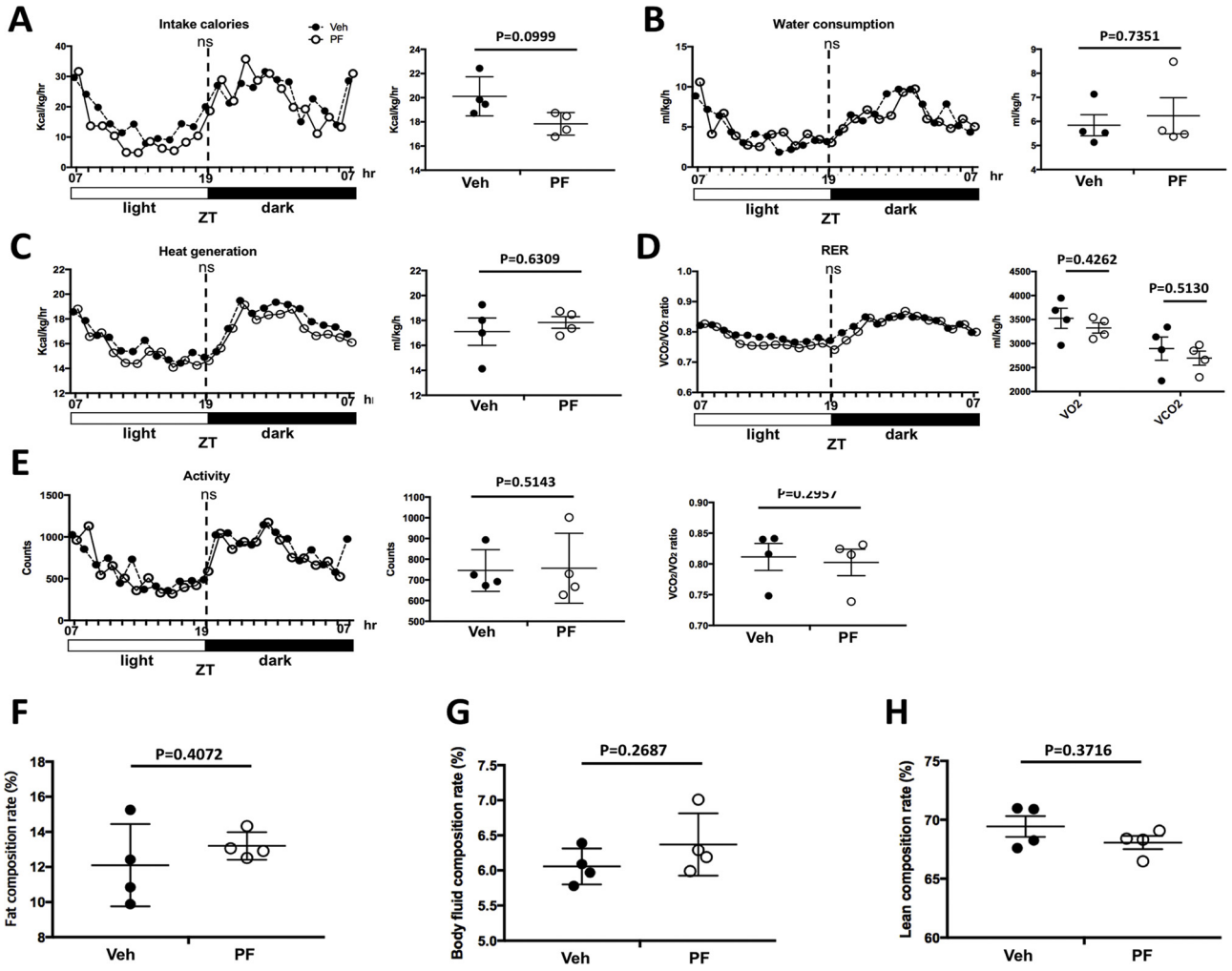

Fig. 4. Automated analysis of significant difference in behavioral, physiological and metabolic parameters by PF administration. In order to eliminate the possibility of behavioral and physiological changes that may be caused by continuous PF ingestion, various parameters were monitored using Phenomaster® equipment. C57BL/6 N mice were used and placed in each phenomaster cage. The results show the mean values of the daily cycle measured over 12 days **(A˜E)**, and lean body mass parameters were measured following Phenomaster® analysis **(F˜H)**. Continuous administration of PF showed no significant differences in mice or in rats compared to the non-treated (Veh) group. ns = non-significance, RER = respiratory exchange rate. Values are expressed as mean ± SEM.

3.9. Automated phenotyping for behavioral, physiological and metabolic data by administration of PF extract

Eight-week old male C57BL/6 N (n = 8; n = 4/group) were acclimated in automated metabolic cages (TSE systems, Germany) for a week before beginning recordings. Mice were continuously recorded for 12 days with following measurements taken every 5 m in. water consumption, food intake, ambulatory activity (in X and Z axes), and gas were calculated according to the manufacturer's guidelines (PhenoMaster Software, TSE systems). The respiratory exchange rate (RER) was estimated by calculating the rate of VCO_2/VO_2 (RER; respiratory exchange rate). Values were adjusted by body weight to the power of 0.75 (kg-0.75) where mentioned. In addition, food intake was determined continuously by integration of weighing sensors fixed at the top of the cage, from which the food containers have been suspended into the sealed cage environment.

Body composition (lean tissue, fat, and fluid in live mice on a benchtop platform) was measured following measurements of the animals' phenotyping with a Mini-spec LF50 (Brucker Biospins, The Woodlands, TX).

Food intakes (Kcal/kg/h) were 20.11 ± 0.808 (Veh) and 17.84 ± 0.465 (PF) (P = 0.0999), water consumption (mL/kg/h) were 5.843 ± 0.441 and 6.235 ± 0.750 (P = 0.7351), Heat generation (Kcal/kg/h) were 17.10 ± 1.093 and 17.84 ± 0.465 (P = 0.6309), Activity (counts) were 745.5 ± 50.45 and 756.3 ± 84.49 (P = 0.5143), VO_2 (ml/kg/h) were 3525.0 ± 208.8 and 3324.0 ± 107.3 (P = 0.4262), VCO_2 (mL/kg/h) were 2894.0 ± 243.2 and 2696.0 ± 147.2 (P = 0.5130), and VCO_2/VO_2 rate were 0.8114 ± 0.0219 and 0.8024 ± 0.215 (P = 0.7802). No significant difference (ns) was observed between the control (Veh) group and PF group when all the data obtained automatically measured at the light:dark cycles (Zeitgeber time; ZT) for 12 days were examined (Fig. 4A-E).

There was no significant difference in body composition between the mice treated with PF and the control (Veh) for 12 days (Fig. 4F-H).

3.10. Protein expressions of neuronal environments in the hippocampus by PF administration

Similar to the results of DBA/2 mice (Fig. 2), DCX (P = 0.008) was significantly increased in rats, and PaV (P = 0.0018), a GABAergic interneuron marker, and BDNF(P = 0.0200), a neuronal plasticity marker, were also significantly increased. Melatonin levels were significantly increased in serum, and Mela R1 (P = 0.0012) was also significantly increased in hippocampus. According to the results between the microglial marker, Iba-1 and COX-2 level, which plays an important

role in the inflammatory process were seemed weird (Fig. 5A). A conflict is found in the tendency to be expressed in Veh- and PF-treated animals. COX-2 expression is increased in the PF-treated group, but Iba-1 expression is decreased in the PF-treated group. To examine how the regulation of the hypothalamic-pituitary-adrenal (HPA) axis is altered by administration of PF, the expression of both the corticotropin-

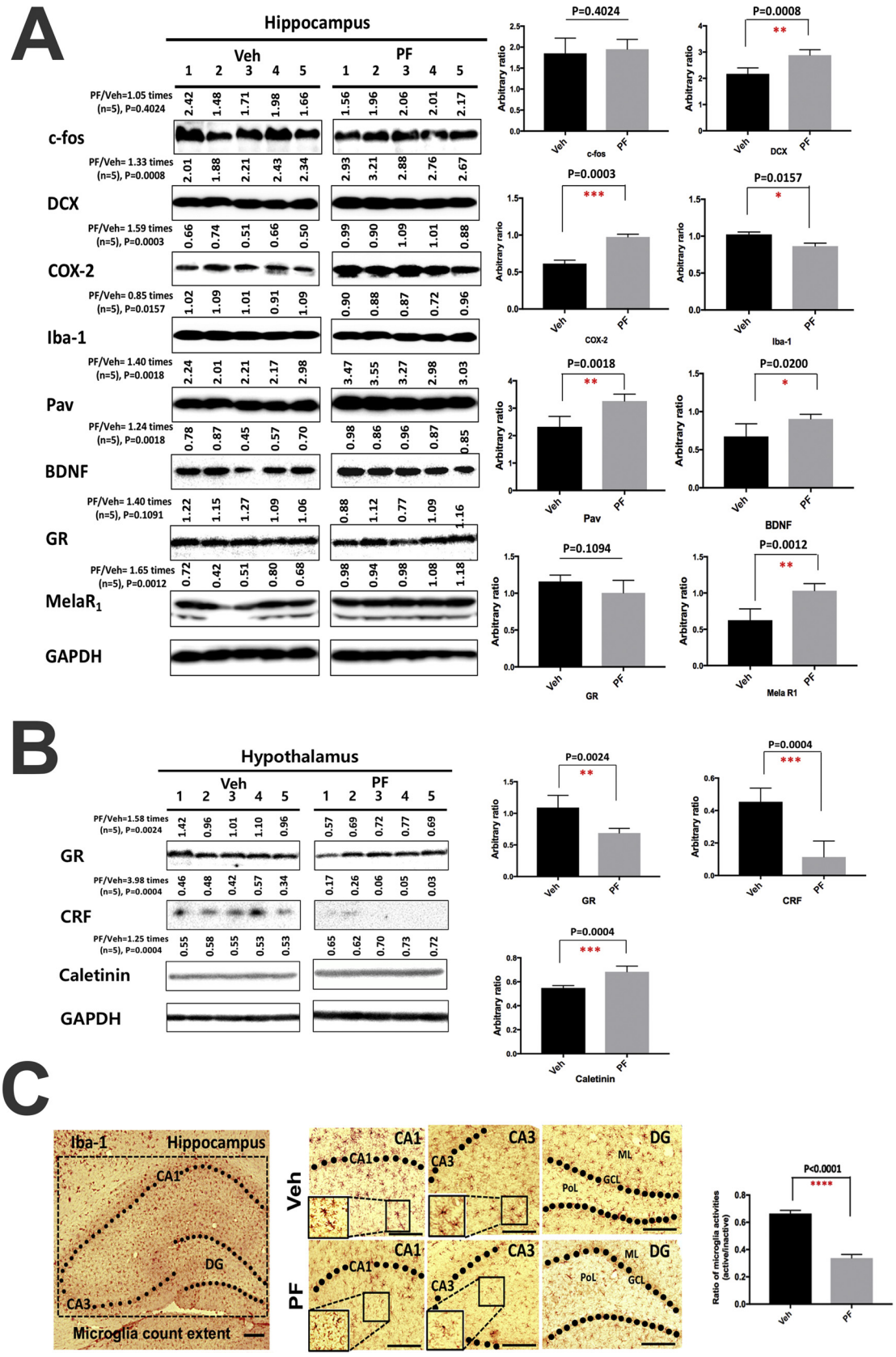

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Fig. 5. Analysis of expression changes of brain environmental improvement markers by PF administration in rats. (A) The expression of DCX, COX-2, Iba-1, PaV, BDNF and Mela R1 were significantly different in the hippocampus upon PF administration, when compared to the non-treated group (Veh). **(B)** The expressions of CRF and GR in the hypothalamus were significantly reduced in the PF group. **(C)** Immunohistochemical staining of Iba-1 in the CA1, CA3 and DG hippocampal areas of Vehicle and PF groups. Microglia in Veh-treated group were found activated and had more round, swollen cell bodies, while microglia in PF-treated group were shown less activated, and thin body. Black lined rectangles have magnified microglia upon different group around hippocampus including CA1, CA3 and dentate gyrus (DG). The black dot lines were demonstrated that CA1˜CA3 and dentate gyrus. The ratio between active and inactive forms were measured and demonstrated with bar graph. DCX = doublecotrin, COX-2 = cyclooxygenase-2, Iba-1 = ionized calcium binding adaptor molecule 1, PaV = parvalbumin, BDNF = brain-derived neurotrophic factor, GR = glucocorticoid receptor, Mela R1 = melatonin receptor 1, The expression rates of each markers were standardized by GAPDH. Values are expressed as mean ± SEM.

releasing factor (CRF) and glucocorticoid receptor (GR) in the hypothalamus was significantly reduced ($P = 0.0004$ and $P = 0.0024$, respectively; Fig.5B).

3.11. Immunohistochemistry for Iba-1

Microglial marker Iba-1 was stained by immunohistochemistry for measurements of microglial activity. As shown in Fig. 5C, Microglia in Veh-treated group were found activated and had more round, swollen cell bodies, while microglia in PF-treated group were shown less activated, and thin body. The ratio was demonstrated with bar graph in the Fig. 5C. The difference between the vehicle- and the PF-treated group was significant (****, $P < 0.001$)

3.12. The role of COX-2 in the hippocampal neurogenesis

According to the results of Fig. 5A, the expression level of microglial marker Iba-1 was low at the PF-treated group, however, the expression of inflammation marker COX-2 at the PF-treated group showed higher than that of Veh-treated group, though it was not significant. The results were seemed to be conflict. However, if COX-inhibitor treated to normal rats, it showed significant lower neurogenesis level than Veh-treated mice (Fig.6A). Cell numbers ratio on the SGZ were calculated and the dendrite numbers tertiary or more branched were counted (Fig. 6B).

3.13. Morris water maze test for memory and learning abilities by PF administration in DBA/2 mice

We have used the Morris water maze test to examine the effect on AD resistance by chronic PF administration in DBA/2 mice. On day 1 (visible platform trials), there is no difference between Veh- and PF-groups in latency (Fig. 7A) and distance to target (Fig.7B) indicating that both of the groups have similar motor and visual capabilities. From this we assume that mice are able to see the flagged-platform and the cues in surrounding environment, and can swim acceptably. For days 2–4 (days 1–3 of hidden platform trials) the example shows a difference in the latency to target (Fig. 7A) and distance to target (Fig.7B) between the groups, suggesting PF treatment performed significantly better than non-treatment (Veh) at day 4. However, the probe trial results on the last day (day 5) show that the number of times (sec) the mice traveled into the second, where the hidden platform was previously placed, was not significantly greater with PF treatment (Fig. 7C and D).

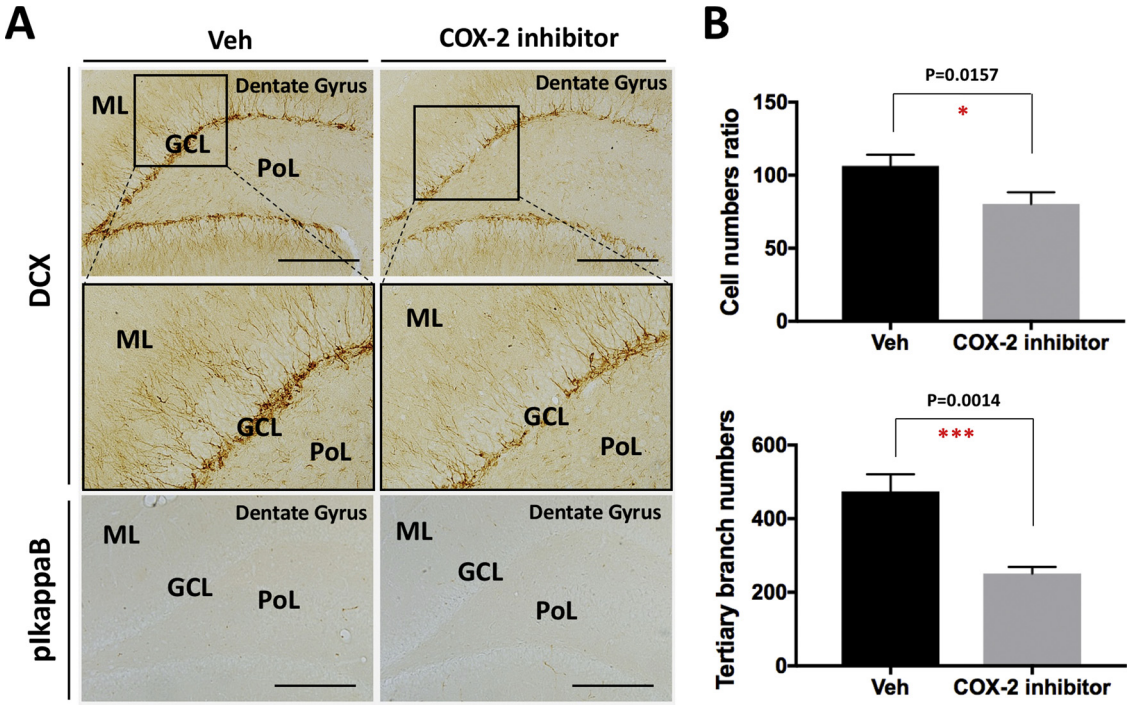

Fig. 6. Immunohistochemistry of DCX expression in the hippocampal dentate gyrus under COX-2 inhibition. (A) The chromogen stained by DCX antibody images are shown in the hippocampal dentate gyrus of SD rats, the neuroblasts were along the SGZ in the dentate gyrus. DCX-positive neuroblasts and their dendrites were well developed in Veh-treated group (Veh), but not in COX-2 inhibitor-treated group. The black lined box images were magnified. The pIkappaB positive cells were not seen in both groups. ML = molecular layer, GCL = granular cell layer, Pol = Polymorphic layer. Scale bar = 100 μm. **(B)** The cell number ratio and tertiary and more branched dendrites from the neuroblasts on the SGZ of dentate gyrus. It showed significant difference between Veh- and COX-2 inhibitor-treated mice (*, $P < 0.05$; ***, $P < 0.005$).

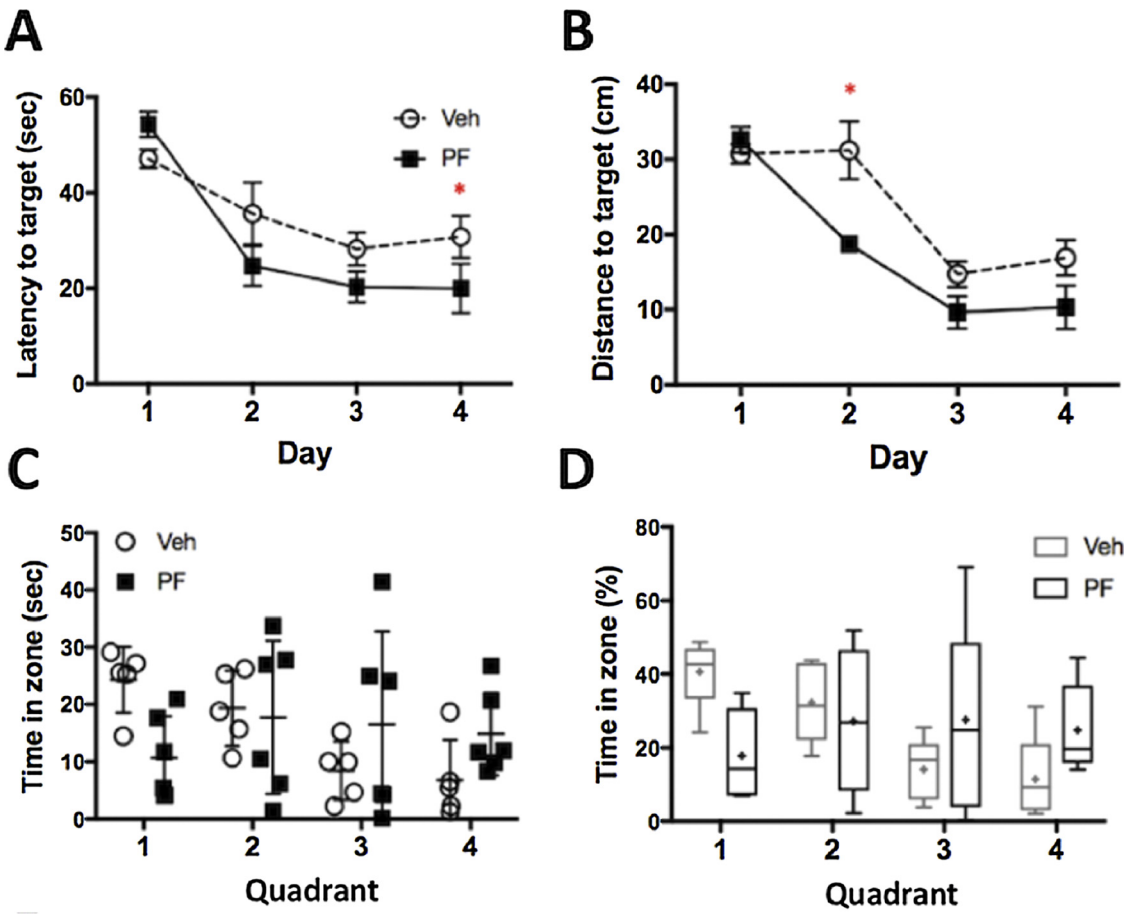

Fig. 7. Enhanced memory function in PF extract administered DBA/2 mice. (A) DBA/2 were administered PF extract during the experiment and educated target finding for evaluating their memory function was tested by Morris Water Maze analysis. As the training was repeated, the time required to find the target in the PF-treated group was shorter than that of the non-treated group, and a statistically significant difference was observed on the fourth day. **(B)** DBA/2 PF administered showed that significant short path length at Day 2 (*, $P < 0.05$). **(C, D)** In the probe test, PF-administered mice showed time (C) and percent(%)(D) stayed in Zone for last three days in the Quadrant. There was not any significance at the probe test.

3.14. AD resistance assessment by phosphoTau(pTAU) over TAU protein expression in PF-treated animals

A comparison of protein expression of Tau and phospho-Tau (pTau), as known of Alzheimer's disease, in animals treated with PF is considered to be critically important. According to Fig. 8, in both SD rats and DBA/2 mice treated with PF, the values of pTau/Tau were significantly higher than those of vehicle treated groups ($P < 0.0001$ in the SD rats and DBA/2 models), Tau and pTau expressions between Veh and PF groups were significantly different compared each other, and finally pTau/Tau values also showed significant different between the Vehicle and PF groups in both rat and DBA/2 mice model. This result is indicating that continuous PF administration conferred resistance to Alzheimer's disease. The administration of PF is expected to have an effect on Alzheimer's disease as well as memory improvement as seen in Fig. 8.

4. Discussion

Lack of sound sleep is emerging as an important problem for people particularly in developed countries. As mentioned above, it is known chronic insomnia demonstrates a kind of insult against the micro-environment of the human brain (Fulda and Schulz, 2001; Joo et al., 2014). However, it has been reported that various side effects continuously occur from the use of sleeping pills such as benzodiazepines used to relieve symptoms. Recently, many studies have reported on the relationship between insomnia and neurodegenerative disease, and the fact that insomnia can progress to AD is disturbing (Bliwise, 2004; Gagnon et al., 2008; Joo et al., 2014; Kent and Mistlberger, 2017; Malhotra, 2018; Raggi and Ferri, 2010; Schroeck et al., 2016; Trotti and Karroum, 2016). However, since many sleep remedies can have side effects such as health and feeding behavior problems (Park and Shin, 2016; Piran and Robinson, 2006; Schroeck et al., 2016), successful treatment of insomnia is more complicated than expected (Hermesh et al., 2001). Therefore, there is a pressing need for safe and effective pharmacological sleep induction.

There are many reported subspecies of Passion flower, therefore, various efficacy and safety issues surrounding the therapeutic use of Passion flowers are being illuminated (Toda et al., 2017). It has been reported that PF has also been used for sleep and anxiety disorders (Akhondzadeh et al., 2001; Elsas et al., 2010; Grundmann et al., 2008) and there are reports that no side effects were found when using PF for short periods in clinical studies (Guerrero and Medina, 2017; Miroddi et al., 2013). However, PF phytocomplex contains many main flavonoids as secondary compounds such as chrysin, vitexin, isovitexin, orientin, isoorientin, apigenin and kaempferol (Miroddi et al., 2013). Thus, it is expected that the content of primary flavonoid, which plays a major functional role will be different depending on the extraction method. We established the main valid compound in the PF extracts as a vitexin through analysis with HPLC and LC/MS/MS (Fig. 1) in the present study. Many studies report that vitexin has the ability to induce sleepiness, is anti-diabetic, anti-inflammatory, and effective for sleep improvement (Abbasi et al., 2012; Choi et al., 2014; He et al., 2016; Malar et al., 2018).

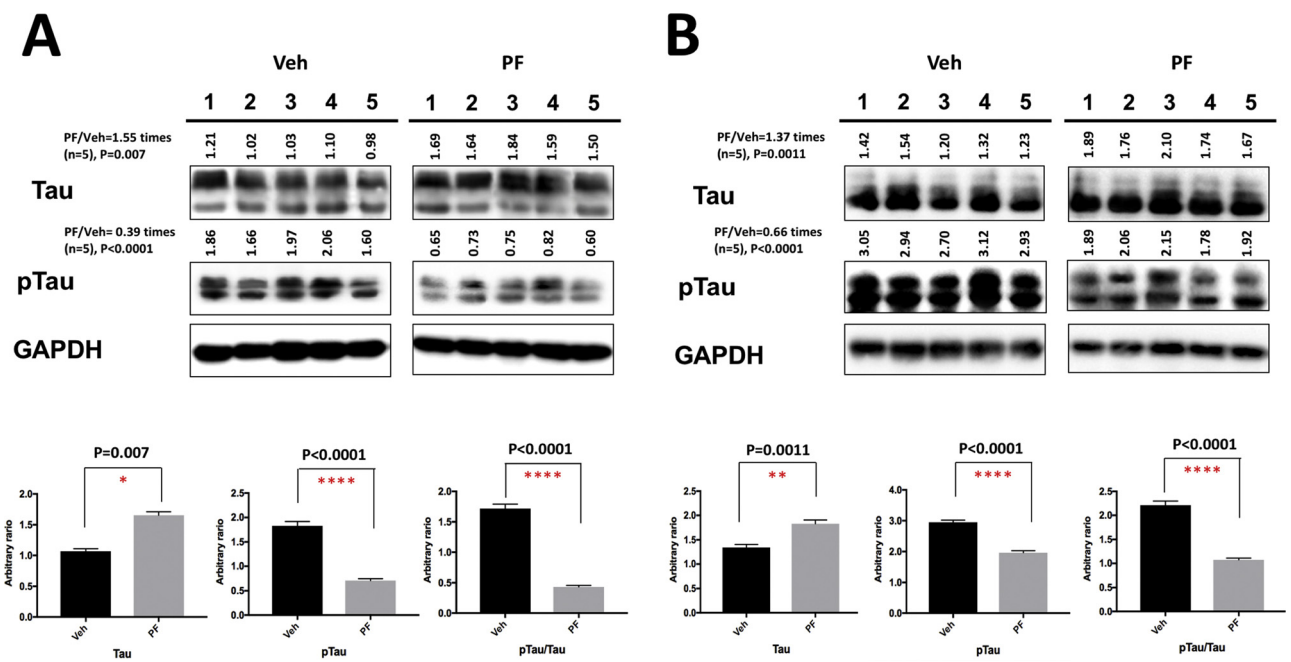

Fig. 8. The results of Tau and pTau in the brain of SD rats treated with PF, parallels the results shown in DBA/2 mice which were also significantly lower for pTau/Tau by Western blot analysis. (A) Tau and pTau protein expressions were measured by Western blot analysis in SD rats. The expression levels were standardized by GAPDH, and PF levels were compared with Veh. Tau expression by PF treatment was significantly higher than that of Veh ($P = 0.007$), and phospho-Tau(pTau) expression of PF was not significantly lower than that of Veh ($P < 0.0001$). The pTau/Tau values were significantly lower in PF group compared to Veh group ($P < 0.0001$). Values are expressed mean ± SEM **(B)** Tau and pTau protein expression measured by Western blot analysis in DBA/2 mice following Morris Water Maze test. Data are from five Veh and six PF. The intensity of the bands was standardized by GAPDH, and compared. Tau expression of PF was not significantly higher than that of Veh ($P = 0.0011$), and pTau expression of PF was significantly lower than that of Veh ($P < 0.0001$). The pTau/Tau values were significantly lower in PF group compared to Veh group ($P < 0.0001$). Values are expressed as mean ± SEM.

As shown in Fig. 2, in the hippocampal dentate gyrus of DBA/2 mice (DBA2/Veh), which had not been subjected to any treatment, neuroblasts and their dendrites responding to DCX was very poor, whereas in the two groups treated with PF, neuroblasts and their dendrites branching is very active. In Sham group, neuroblasts were well developed along SGZ and dendrites were also well developed.

In MTT test using bone marrow cells, there was no significant cytotoxicity at high concentrations of PF extract (< 4 mg/ml) (Supplement 2A). In addition, the survival rate *of C.elegans* was significantly increased by PF treatment at various concentrations followed by 1 mM H_2O_2 treatment (Supplement 2B), and at each concentration of PF, life span curves of *C. elegans* were significantly increased when analyzed by Log-rank (Mantel-Cox) test (Supplement 2C). Protection against oxidative stress and an increased lifespan is also observed in *C. elegans* following treatment with PF at the concentration of 500 mg/L (Supplement 2C), and both the lifespan of *C. elegans* treated with different concentrations of PF (100 mg/L and 500 mg/L) increased significantly (Supplement 2C). Expression of *hsp-16.2*::GFP increased significantly in long-lived worms treated with 500 mg/L PF, indicating the ability of PF to induce longevity-assuring transcriptional alterations (Supplement 2D). Previous reports have found that differential expression of *hsp-16.2*::GFP affects lifespan (Cypser et al., 2013; Rea et al., 2005). According to the data from Supplement 2, it is suggested that dietary supplementation of PF can modulate an organism's response to environmental stressors and aging, potentially through induction of stress-responsive transcriptional marker and genes associated with longevity.

It has been noted that further studies on the safety of PF use are needed (Miroddi et al., 2013). However, no change in body weight was observed in the present study (Fig. 3A). Possibly there was a reduction in food intake count between the groups (Fig. 3B), however, the cumulative intake volumes were not significantly different (Fig. 3C). In the PF-treated group, the number of times feed was accessed may be smaller due to the sedative effect. However, once the feed is approached, it is consumed for a longer period than the non-administered group (Fig. 3B and C), and this characteristic is also doubly confirmed in the mouse experiment using Phenomaster® in Fig. 4. Using BioDAQ® real-time food intake recording system, we could determine the degree of sedation by PF for 12 days, the feeding adverse reaction, and body weight differences compared to the Veh-group. The intake of vehicle-diluted PF for 12 days and vehicle alone was not different, and remained constant for almost 12 days (Fig. 3D). Through PF administration, we found that serum levels of serotonin and melatonin in the serum of the animals were significantly increased compared to those not treated with PF (Fig. 3E and F). These important linked-mechanisms are well introduced in the previous studies (Cubero et al., 2006; Demisch et al., 1987; Paredes et al., 2009). Although melatonin is primarily effective in people with sleep deprivation, it is also known to help sleep disorders in Alzheimer's patients, and animal studies have also shown beneficial effects in reducing beta-amyloid level during sleep (Scullin and Bliwise, 2015; Trotti and Karroum, 2016; Villa et al., 2015). Higher levels of melatonin are also believed to increase sleep quality and increase cognitive ability in Alzheimer's patients (Musiek et al., 2015).

However, the concentration of PF currently in use needs to have evidence that the animals can maintain a similar level of activity when compared to the untreated group, to demonstrate that the low dose used in the present study increases neurogenesis in the hippocampus, and shows anti-AD effects. According to the results, there was no significant difference in the values of each item. However, calorie intake in, the PF administration group was relatively low (Fig. 4A), and the PF administration group in the RER was relatively low in the light-cycle (Fig. 4D). In particular, RER is a comparable indicator of whether an animal has utilized carbohydrates or fatty acids as an energy source. Carbohydrates when it is close to 1, and fatty acids when it is close to 0.7, are used to acquire energy. Although not statistically significant, it

is worth investigating whether energy utilization can be differentiated through PF in the future. Taken together, no bahavioral, physiological, or metabolic changes of significance were caused by PF administration for about 2 weeks through BioDAQ®, Phenomaster®, and Mini-spec equipment, was found using rats or mice.

The results of observing markers related to brain plasticity by PF administration to rats, are shown in Fig. 5. It was confirmed that DCX, PaV, BDNF and Mela-R1 at hippocampi, and CRF and GR were significantly different by administration of PF. Interestingly, although not statistically significant, we found conflicting results with high COX-2 and low Iba-1 in the PF-treated group. However, in our data and previous reports (Baek et al., 2015), we observed a marked decreased in neurogenesis following COX-2 inhibition (Fig. 6), indicating that COX-2 is not necessarily involved in neuroinflammation but is a necessary signal for high neurogenesis. Therefore, PF administration is thought to contribute to increasing brain plasticity by inhibiting the activity of microglia in the brain, without inducing neuroinflammation by COX-2 (Fig. 6). According to the immunohistochemistry for Iba-1 expression, the counts of the Iba-1 positive cells in the hippocampus at the Veh- and PF-groups ('Microglia count extent') were not much different each other, however, more ramified microglia observed at Veh-group than those of PF-group. In particular, microglia had little round and swollen cell bodies and were less activated in PF-group than those of Veh group. These results are consistent with the data of Western blot for Iba-1, and morphologically confirmed that microglial cells are more inactive and stable in the hippocampal area by PF treatment in the animals.

There have been reports that AD progression can be inhibited by inhibiting the activity of microglia (Klein et al., 2016; Silva et al., 2015; Wadhwa et al., 2017). We also found that PF can inhibit neuroinflammation as well as *C.elegans* data in this study.

From prior work, we hypothesized that administration of PF could substantially improve memory in animals and confer some level of resistance to AD. Morris water maze test was used to measure hippocampal-dependent learning, including acquisition of spatial memory and long-term spatial memory following treatment with PF (Bromley-Brits et al., 2011). The results showed that neurocognitive function was significantly increased with PF treatment (Fig. 7). These results indicate that phospho-Tau/Tau was significantly lower in the PF-treated group (Fig. 8), and showed that PF provides resistance to AD with memory improvement.

Before designing genetically-engineered AD animals, we needed to verify the safety and efficacy of PF through normal rodent models. Taken together, all of the above results indicate that administration of PF has both memory and learning enhancement, and provides resistance to AD. These results are due to the regulation of the neuroinflammatory response and by providing strong resistance to environmental stressors. Therefore, based on the results of the present study, we will find the target mechanism of PF through various genetically-engineered mice and achieve therapeutic approaches to effective sleep, memory and degenerative brain diseases.

Ethical statement

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Soonchunhyang University (Approval number: SCH16-0037) in accordance with the Principal of Laboratory Animal Care (NIH publication no. 82-23, revised 1985).

Disclosures

No conflict of interest, financial or otherwise, are declared by the author(s).

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Appendix A. Supplementary data

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