Berberine ameliorates β-amyloid pathology, gliosis, and cognitive impairment in an Alzheimer’s disease transgenic mouse model

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Abstract

The accumulation of β-amyloid (Aβ) peptide derived from abnormal processing of amyloid precursor protein (APP) is a common pathological hallmark of Alzheimer’s disease (AD) brains. In this study, we evaluated the therapeutic effect of berberine (BBR) extracted from Coptis chinensis Franch, a Chinese medicinal herb, on the neuropathology and cognitive impairment in TgCRND8 mice, a well established transgenic mouse model of AD. Two-month-old TgCRND8 mice received a low (25 mg/kg per day) or a high dose of BBR (100 mg/kg per day) by oral gavage until 6 months old. BBR treatment significantly ameliorated learning deficits, long-term spatial memory retention, as well as plaque load compared with vehicle control treatment. In addition, enzyme-linked immunosorbent assay (ELISA) measurement showed that there was a profound reduction in levels of detergent-soluble and -insoluble β-amyloid in brain homogenates of BBR-treated mice. Glycogen synthase kinase (GSK)3, a major kinase involved in APP and tau phosphorylation, was significantly inhibited by BBR treatment. We also found that BBR significantly decreased the levels of C-terminal fragments of APP and the hyperphosphorylation of APP and tau via the Akt/glycogen synthase kinase 3 signaling pathway in N2a mouse neuroblastoma cells stably expressing human Swedish mutant APP\textsubscript{695} (N2a-SwedAPP). Our results suggest that BBR provides neuroprotective effects in TgCRND8 mice through regulating APP processing and that further investigation of the BBR for therapeutic use in treating AD is warranted.

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1. Introduction

Alzheimer’s disease (AD) is a neurodegenerative disease characterized by memory impairment and behavioral disturbances. Extracellular senile plaques and intraneuronal neurofibrillary tangles (NFTs) are the two classic hallmark microscopic pathologies of AD (Selkoe, 2004). Senile plaques comprise a dense core of amyloid-β peptide (Aβ) that is surrounded by dystrophic neuritis (Selkoe, 2004). Aβ deposition in cerebral vessels contributes to cerebral amyloid angiopathy (CAA) in AD (Weller et al., 2008). Aβ, consisting of 39–43 amino acids, is a proteolytic product of a much larger amyloid precursor protein (APP) (Selkoe, 2004). APP is an integral membrane protein processed by the 3 proteases, α-, β-, and γ-secretases to release Aβ, and accumulation of Aβ has been implicated as a causal factor in AD (Wilquet and De Strooper, 2004).

Treatment strategies for AD based on the amyloid hypothesis mainly involve β- and/or γ-secretase inhibitors and anti-Aβ vaccination; however, there are still unresolved
issues with clinical application (Boche et al., 2008; Griffiths et al., 2008; Lukiw, 2008). Therefore, there is a need for alternative drugs. Based on advances in the treatment of AD using herbs, phytotherapy seems to hold promise (Howes and Perry, 2011). One potential phytotherapeutic agent for AD could be berberine (BBR) because BBR has shown its safety and efficiency in human and animals (Kong et al., 2004). BBR is an isoquinoline alkaloid (Fig. 1a) that has been isolated from *Coptis chinensis* Franch. (Huanglian, Chinese goldthread) found in China (Kamath et al., 2009). *Coptis chinensis* has been used in Chinese medicine with a long history of clinical benefits (Cho, 1990). BBR is 1 of the important components of Oren-gedoku-to (Huanglian-Jiedu-Tang) extract, which has been used in clinical therapies for several types of dementia in China and Japan (Yu et al., 2010). Recent reviews have indicated that BBR has a well-documented neuroprotective effect against cerebral ischemia, mental depression, schizophrenia, anxiety, and AD (Ji and Shen, 2011; Kulkarni and Dhir, 2010; Ye et al., 2009). Recently, the anti-AD effects of BBR have been mainly demonstrated through acetyl and butyl cholinesterase inhibition, indoleamine 2, 3-dioxygenase inhibition, amelioration of Aβ1–40-induced cognitive impairments and inhibition of Aβ1–42 fibril formation (Jung et al., 2009; Shi et al., 2011; Yu et al., 2010; Zhu and Qian, 2006). These findings suggested that BBR is a multifunctional compound with neuroprotective properties. It has also been reported that BBR reduces amyloid plaque production in Swedish APP-expressing cells (Asai et al., 2007); however, the in vivo efficacy of BBR on Aβ clearance is not yet validated.

In this study, we report for the first time that chronic administration of BBR reduces Aβ deposits, tau hyperphosphorylation, gliosis, and cognitive impairments in a most
well characterized strain of APP transgenic mice (TgCRND8 mice) (Chishti et al., 2001; Woodhouse et al., 2009).

2. Methods

2.1. Animals and treatment

All animal procedures were approved by the Hong Kong Baptist University Committee on the Use of Human and Animal Subjects in Teaching and Research and by the Committee on the Use of Live Animals for Teaching and Research (CULATR), the University of Hong Kong. TgCRND8 mice expressing human APP695 with the Swedish (K670N/M671L) and Indiana (V717F) mutations under the regulatory control of the PrP gene promoter, heterozygous with respect to the transgene, on a C57BL/6 F3 background (Chishti et al., 2001) were used to breed a colony of experimental animals. When TgCRND8 mice are 3 months of age, Aβ deposits become visible in their cortical brain and hippocampal regions together with astrocytic activation, microglial activation, neuritic dystrophy, inflammation, and behavioral deficits that closely resemble human AD (Chishti et al., 2001).

All animals were housed 4–5 to a cage, of the same sex, and maintained on ad libitum food and water with a 12-hour light/dark cycle in a controlled environment. BBR hydrochloride (98% purity) was purchased from Sigma-Aldrich Company (St. Louis, MO, USA). BBR administration started at 2 months of age and lasted for 4 months up to 6 months of age. Transgenic (Tg) mice were orally administered by gavage once daily with a low dose of BBR (25 mg/kg per day), a high dose of BBR (100 mg/kg per day), or vehicle for 6 months before being killed. The doses of BBR were chosen according to previous studies (Kong et al., 2004; Peng et al., 2007). The body weight, coat characteristics, and in-cage behavior were monitored throughout the study.

2.2. Morris water maze

After 4 months of drug treatment, mice were assessed for spatial reference memory in the Morris water maze (MWM) (Morris, 1984). The water maze consists of a white circular pool of a diameter of 100 cm and filled with opaque water (22 ± 1 °C). A white plexiglass platform (9 cm diameter and 29 cm height) was submerged in 1 of the pool quadrants. The pool was virtually separated into 4 quadrants and north, west, south, and east positions located at the intersections of the quadrants. The visible platform trials were used to evaluate sensorimotor and/or motivational deficits that could influence performance during the spatial navigation task. Mice underwent visible-platform training for 2 consecutive days (4 trials per day), each time with the platform in a different location; mice were allowed to swim to a flag-mounted platform (length 10 cm) located above the water. Training on the hidden platform water maze task commenced 24 hours after the last visible-platform trial. Hidden-platform training was carried on over 6 consecutive days until each mouse had reached the criterion. During the hidden-platform trial, a set of distal visual cues was used on the black screen around the pool. The hidden platform was submerged 1 cm below the surface of the water in the southwest quadrant of the pool (target quadrant) and invisible to the mice while swimming. Mice were permitted a maximum time of 60 seconds, starting from release in a randomly chosen quadrant to find the hidden platform. On each testing day, animals performed 4 trials separated by a 30-minute interval.

To assess memory retention, a probe trial was performed at the beginning of the 3rd, 5th, and 24th hour after the last training trial. In this trial, the platform was removed from the pool and the mice were permitted to swim freely for 60 seconds to search for the platform. The time spent in the target quadrant was taken to indicate the level of memory retention that had taken place after learning. During each trial, the distance taken to find the hidden platform (path length in cm) and percent time spent in each quadrant of the pool during probe trials of the mouse were recorded using a video-tracking system (EthoVision 2.0, Noldus Information Technology, Leesburg, VA, USA).

2.3. N2a-SwedAPP cell culture and BBR treatment

N2a-SwedAPP cells are mouse neuroblastoma N2a cells stably transfected with human Swedish mutant APP695, and were gifts from Dr. Gopal Thinakaran (University of Chicago, Chicago, IL, USA). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, penicillin, and streptomycin and 200 μg/mL G418 (Invitrogen) (Thinakaran et al., 1996). Cells were cultured either in multiwell dishes or on 20 mm tissue culture plates in DMEM 10% with fetal bovine serum (FBS). Cell cultures were incubated at 37 °C in a humid 5% CO₂/95% air environment. After cells were grown close to confluence, they were treated with/without different concentrations of BBR in DMEM (1% fetal bovine serum) for 24 hours. We first measured whether BBR showed toxicity in the N2a-SwedAPP cells using the 3-(4,5)-dimethylthiazol-2-yl)-3,5-diphenyltetrazoliumromide (MTT) (Sigma-Aldrich) assay as described earlier (Durairajan et al., 2011). The treatment of BBR did not affect cell viability up to 25 μM in N2a-SwedAPP cells (data not shown). For the concentration-dependent treatment, cells were treated with serial dilutions of BBR ranging from 0 to 20 μM for 2 or 12 hours prior to lysis with ice-cold radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton-X, 0.5% sodium deoxycholate, 1 mM EDTA) containing protease inhibitor cocktails (EMD Chemicals, Philadelphia, PA, USA) to find the optimal range of BBR. For the time-dependent application, cells were treated with a 20 μM concentration of BBR and incubated at time points
ranging from 0 to 180 minutes prior to lysis. For immunofluorescent staining, N2a-SwedAPP cells were seeded on glass cover slips at a density of $0.5 \times 10^5$ viable cells per well in a 24-well plate. Cells on cover slips were treated with BBR (20 μM) for 3 hours with or without pretreatment of LY294002 (Cell Signaling Technology, Danvers, MA, USA), a specific inhibitor of Akt upstream kinase phosphatidylinositol 3-kinase (PI3K) (Vlahos et al., 1994) in serum-free DMEM for 1 hour, whereas control cells were incubated with dimethyl sulfoxide (DMSO) alone in DMEM.

2.4. Immunohistochemistry and immunocytochemistry

After the Morris water maze experiment, animals were deeply anesthetized with an intraperitoneal injection of and transcardially perfused with 0.9% saline. The brains were removed and bisected in the midsagittal plane. Half of each brain was frozen on dry ice for Aβ immunohistochemistry. Three coronal sections (per set) from anterior, medial, and posterior hippocampus with a 120-μm interval were made on a cryostat at $-20^\circ C$ at a thickness of 30 μm in each region. Three sets of sections in each region were prepared for analyses of Aβ, ionized calcium binding adapter molecule 1 (Iba-1) (microgliosis) and glial fibrillary acidic protein (GFAP) (astrocytosis). Sections were immunostained using the following antibodies: a biotinylated human amyloid-β monoclonal antibody (4G8; 1:1000; Covance, Princeton, NJ, USA), GFAP polyclonal antibody (1:500; Dako, Carpinteria, CA, USA), and an Iba-1 polyclonal antibody (1:1000; Wako, Osaka, Japan). Immunohistochemical staining was performed according to the manufacturer’s instructions using a Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA, USA) linked with the diaminobenzidine reaction, with the exception that the biotinylated secondary antibody step was excluded for Aβ staining. Isotype control serum or PBS (0.1 mM, pH 7.4) was used instead of primary antibody as a negative control. All images were acquired using a Nikon fluorescence inverted microscope with image acquisition system (Nikon Instruments Inc. Melville, NY, USA). A threshold optical density representing specific immunoreactive signal was established after subtracting the background and nonspecific staining level, and was then held constant throughout the image analysis. To facilitate quantitative measurement of Aβ burden, a region of interest was captured on each section from anterior, medial, and posterior regions (9 sections per mouse). Images were converted to gray scale and thresholded using an unbiased computer-assisted ImageJ software (National Institutes of Health [NIH], Bethesda, MD, USA). Quantification of Aβ immunostaining was performed to detect the proportion of region occupied by Aβ immunoreactivity as described previously (Josephs et al., 2008). Aβ burden expressed as the area of Aβ per the total area of the region of interest. Iba-1 (microgliosis) and GFAP (astrocytosis) burden was also estimated as the area of immunoreactivity expressed as a percentage of full area. For Aβ plaque morphometric analyses, diameters of plaques were measured, and numbers of plaques in 3 categories of diameter (25, 25–50, or >50 μm) were calculated. Assessments were carried out by a single examiner blinded to sample identities.

For immunofluorescent staining, cells were fixed, permeabilized, blocked, and stained with a combination of primary antibodies as described previously (Durairajan et al., 2011; Lee et al., 2003a). To allow combination with the first and second primary antibodies, both raised in the same species, further immunostaining was done following the method reported by Negoescu et al. (1994) with little modifications. Permeabilized cells were incubated with primary antibody specific for phosphorylated APPThr668 (p-APP) (1:100, Cell Signaling Technology) overnight at 4 °C. After incubation of the first primary antibody, cells were rinsed with PBS containing 0.05% Tween 20 (PBST) 3 times. Subsequently, cells were incubated with Alexa Fluor 488-conjugated Fab fragments of goat anti-rabbit immunoglobulin (IgG) (H+L) for 1 hour (1:500; Invitrogen). After washing, cells were incubated with unlabeled Fab fragments of goat anti-rabbit IgG (H+L) for 1 hour (1:100; Invitrogen) to block all possible remaining binding sites of the second primary antibody. The p-APP-labeled cells were incubated with a second rabbit polyclonal antibody raised against Rab5 (endosome marker) (1:100; Cell Signaling Technology) overnight, followed by an Alexa Fluor 594-conjugated Fab fragments of goat anti-rabbit IgG (H+L) (diluted 1:500; Invitrogen), for 1 hour. After several washes with PBS containing 0.05% Tween 20, cells were mounted using an antifading mounting medium.

For higher resolution microscopy, images were acquired using an Olympus IX70 Delta Vision microscopy system (Applied Precision, Issaquah, WA, USA) with a 100 × 1.5 numerical aperture oil immersion lens. Images were collected using a Cool SNAP digital camera (Photometrics, Tucson, AZ, USA). Subsequently serial Z-stacks of fluorescence images at 0.2-μm interval were acquired and deconvolved via a controlled iterative algorithm to generate high-resolution images of cells using Softmax Pro 4.8 software (Applied Precision).

2.5. Measurements of Aβ1–40 and Aβ1–42 in brain extracts

To detect differences in the level of Aβ among treatment groups, the levels of Aβ were measured using the 2-step sequential extraction of sodium dodecyl sulfate (SDS) and formic acid (FA) methods as previously described (Kawarabayashi et al., 2001). Briefly, each frozen hemisphere was first homogenized in 2% SDS in water containing protease inhibitors (Complete Mini Protease Inhibitor Cocktail; Roche Diagnostics, Basel, Switzerland) and phos-
photase inhibitor (EMD Chemicals). After sonication, samples were centrifuged at 100,000g at 4 °C for 1 hour to obtain a soluble SDS fraction, which was then stored at −80 °C. The resulting SDS pellets were resuspended in a 70% FA solution and centrifuged at 100,000g at 4 °C for 1 hour. Supernatants were collected and stored at −80 °C until used to measure insoluble Aβ. The 2% SDS extracts were diluted at least 1:300 in 1% bovine serum albumin (BSA) in PBS so that the assays were performed in 0.006% SDS. The FA extract was neutralized by a 1:20 dilution into 1 M Tris, pH 11. The neutralized FA extracts were diluted 1:2 in 1% BSA in PBS. Brain extracts were measured for Aβ (1–40) and Aβ (1–42) using ELISA as detailed in Durairajan et al. (2011). Absorbance values at 450 nm were measured in duplicate wells, and the average of the signal from the 2 wells was considered to represent the Aβ concentration for the sample.

2.6. Western blot analyses

The brain SDS fraction as described above was used to detect the full-length (Fl)-APP, APP C-terminal fragments (CTFs: CTF-β and CTF-α), p-APP, p-CTFs, β-site APP cleaving enzyme (BACE-1), a disintegrin and metalloproteinase domain-containing protein-10 (ADAM-10), neprilysin, insulin-degrading enzyme (IDE), glycogen synthase cleaving enzyme (BACE-1), a disintegrin and metallopro-

Supernatants were collected and stored at 4 °C for 1 hour. The brain SDS fraction as described above was used to detect the full-length (Fl)-APP, APP C-terminal fragments (CTFs: CTF-β and CTF-α), p-APP, p-CTFs, β-site APP cleaving enzyme (BACE-1), a disintegrin and metalloproteinase domain-containing protein-10 (ADAM-10), neprilysin, insulin-degrading enzyme (IDE), glycogen synthase kinase (GSK)3α/β, p-cyclin-dependent kinase 5 (p-CDK5), p-stress-activated protein kinase/Jun-amino-terminal kinase (p-JNK), phospho-tau epitopes, and β-actin in Western blot analysis. For cell homogenates preparation, cells were washed with PBS and solubilized in ice-cold RIPA buffer. Equal amounts of protein (30 µg) were subjected to 10% and 15% SDS polyacrylamide gels, transferred onto polyvinylidene fluoride (PVDF) membranes (GE Healthcare, Piscataway, NJ, USA) at 300 mA for 2 hours, and then blocked with 5% fat-free milk in TRIS-buffered saline and 0.1% Tween 20 for 2 hours at room temperature. The membrane was then incubated with primary antibodies overnight at 4 °C. The antibodies used in Western blots were β-actin (mouse, 1:5000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), p-APP (rabbit, 1:1000; Cell Signaling Technology), BACE-1 (rabbit, 1:1000; Abcam, Cambridge, MA, USA), ADAM-10 (rabbit, 1:2000; EMD Chemicals), IDE (rabbit, 1:1000, Abcam), neprilysin (rabbit, 1:1000; Millipore, Temecula, CA, USA), Akt (rabbit, 1:1000; Cell Signaling Technology), p-Akt (rabbit, 1:1000; Cell Signaling Technology), GSK3α/βSer21/9 (rabbit, 1:1000; Cell Signaling Technology), pGSK3α/βSer21/9 (rabbit, 1:1000; Cell Signaling Technology), Cdk5[Y15] (rabbit, 1:1000; Abcam), JNK (Thr183/Tyr185) (rabbit, 1:1000; Cell Signaling Technology), the C-terminal anti-APP antibody CT15 for full-length APP and CTFβ and CTFα (rabbit, 1:2000; and 3 mouse monoclonal antibodies against phosphorylated tau: AT8 (recognizes phosphorylated tau at Ser-202 and Thr-205, 1:1000; Pierce, Rockford, IL, USA)), AT180 (recognizes phosphorylated tau at Thr-231 and Ser-235, 1:1000; Pierce) and paired helical filament (PHF-1) (recognizes -phosphorylated tau at Ser-394 and Ser 404, 1:2000); and 1 mouse monoclonal against dephosphorylated tau: Tau-1 (recognizes the tau when serines 195, 198, 199, and 202 are dephosphorylated, 1:2000). Mouse monoclonal Tau-5 used to recognize total tau (mouse 1:2000). Blots were washed in and incubated with corresponding horse-radish peroxidase (HRP)-conjugated secondary antibody. Secondary antibody was HRP-conjugated IgG anti-mouse (goat anti-mouse, 1:5000; Invitrogen) or anti-rabbit (goat anti-rabbit, 1:10,000; Invitrogen) as needed. Supersignal chemiluminescent substrate (Pierce) was used to visualize HRP activity on Fuji films (GE Healthcare). The omission of primary antibody resulted in negative staining.

Immunoblots were quantified using ImageJ software (National Institutes of Health), with optical density (OD) measures adjusted for individual loading control optical density levels.

2.7. Statistical analysis

Behavioral data were analyzed with 2-way analysis of variance (ANOVA) for repeated measures with “treatment” and “day” and their interactions as fixed factors. After ANOVA analysis, post hoc pair-by-pair differences between groups were determined using the Fisher LSD test. Immunohistochemical data were also analyzed using a 1-way and 2-way ANOVA performed with a Fisher’s least significant difference (LSD)-post hoc comparison. Calculations and graphical presentation were performed with the statistical software GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) and Sigmplot (version 10, SPSS, Chicago, IL, USA). Results are presented as mean ± standard error of the mean (SEM).

3. Results

3.1. Regular treatment and levels of BBR in TgCRND8 mouse brain

We found that regular treatment of TgCRND8 mice with BBR orally administered at 25 or 100 mg/kg per day for approximately 4 months, did not significantly influence animal body weight nor did it cause any noteworthy adverse side effects (Supplementary Fig. 1). To determine how oral administration of BBR influences the concentration of BBR in the brain, we used liquid chromatography (LC) and mass spectrometry techniques (MS) to analyze changes in BBR levels in the TgCRND8 brain tissue in a separate experiment. BBR levels in the brain were found to be 0.88 ± 0.27 and 1.67 ± 0.73 µM for low and high doses of BBR respectively after 3-hour oral administration.

3.2. BBR treatment reverses deficits of learning and memory in TgCRND8 mice

At the beginning of the 15th week of BBR treatment, both Tg and wild type (WT) mice were trained for 4 days (4
trials per day) in the MWM with the visible platform so that they could learn where the platform was located. Although distance traveled for BBR-treated TgCRND8 mice on the first training day of the visible water maze tended to be lower than distances for vehicle treated TgCRND8 mice, differences in overall performance were insignificant \((p = 0.055)\) (Fig. 1b). The visible platform tests showed that the BBR group and Tg control group had a similar path length from 2 to 4 days as revealed by post hoc Fisher LSD multiple comparisons tests \((p > 0.05;\) Fig. 1b), suggesting that BBR treatment did not significantly affect motility or vision in the BBR-treated mice. In other words, the BBR treatment did not influence the visible platform learning trend, which was used in our study as control for the non-spatial factors (e.g., sensory-motor performance).

After the visible platform training, both Tg and WT mice were trained for 6 days in the MWM so that they could learn where the hidden platform was located. From the 24 training trials, average measures of total distance to the platform for each 4-trial block were analyzed to determine and distinguish genotype and treatment differences in spatial learning. Vehicle-treated TgCRND8 mice exhibited a longer path distance \([F(1,96) = 108.6; p < 0.001]\) when compared with the vehicle-treated WT mice during all trial sessions (Fig. 1c). Treatment with low and high doses of BBR reduced the path distance in TgCRND8 mice compared with placebo-treated TgCRND8 mice \([F(2,108) = 7.6; p < 0.001]\). Fig. 1c also suggests that the placebo-treated TgCRND8 mice experienced a spatial learning impairment compared with the WT controls and BBR-treated groups. The observed impairment in spatial memory of TgCRND8 mice is consistent with previous study (Chishti et al., 2001).

In post hoc multiple comparisons, all Tg groups showed no significant changes in swimming distance during the first day \((p > 0.05)\). When the vehicle-treated TgCRND8 control group was compared with WT controls, vehicle-treated TgCRND8 mice showed significantly longer swimming distances than WT-control group mice, from the 1st to 6th day \((p < 0.001)\). From days 4–5, TgCRND8 mice treated with 25 and 100 mg/kg per day of BBR showed a reduction in swimming distance as compared with vehicle control group mice, reaching similar values to those of the WT-control group (Fig. 1c).

In MWM behavior testing, we confirmed that both of the 2 different doses of BBR (25 and 100 mg/kg per day) not only significantly enhanced learning skills during the hidden-platform learning trial, but also significantly enhanced spatial memory retention during the probe trial. The spatial bias of the mice for the position of the hidden escape platform is revealed by the duration spent by the mice in the quadrant of the maze that retained the platform throughout the 3 interpolated probe trials (Fig. 1d). Analyses of probe test search ratio data indicated a significant effect of treatment \([F(3,81) = 16.68; p < 0.001]\) and a significant effect of trial \([F(3,81) = 8.17; p < 0.001]\). Post hoc Fisher LSD multiple comparisons tests indicated that the TgCRND8 mice in the BBR 25 mg/kg treatment group significantly increased their spatial bias in all the probe trials (post hoc Fisher’s LSD). Vehicle-treated mice showed lower times in the target quadrant \((p < 0.001)\) and lower numbers of crossings than vehicle-treated WT mice. Both doses of BBR (25 and 100 mg/kg per day) prevented decrease in the time spent in the target quadrant \((p < 0.001)\) of the previous location of the platform (Fig. 1d). These results demonstrate that spatial memory impairment in vehicle-treated TgCRND8 mice is alleviated by prolonged treatment with BBR.

If the animals had spent more time and had swum further in the target quadrant where the platform had previously been placed during the training session, this would have indicated that the animals had acquired spatial memory improvement (Blokland et al., 2004). Therefore, these results suggest that BBR can ameliorate the long-term memory loss in TgCRND8 mice.

### 3.3. BBR mitigates Aβ pathology and Aβ levels in TgCRND8 mice

The observation that BBR treatment could alleviate the learning and memory deficit led us to further examine if there is any correlation in the reduction of Aβ plaque deposition by BBR in TgCRND8 mice. Aβ plaque immunostaining with the 4G8 antibody and thioflavin S (ThioS) staining in TgCRND8 mice disclosed marked Aβ deposits both in the cerebral parenchyma and in cerebral blood vessels at 6 months of age. Examination of amyloid plaque deposition in the brains of TgCRND8 mice revealed that BBR treatment showed significant reductions in the number and area occupied by Aβ deposits in the coronal sections of the cortex and hippocampus (Fig. 2a). Quantification of the immunoreactivity showed significantly lower plaque burden in BBR-treated animals compared with controls. BBR treatment at concentration of 25 mg/kg per day resulted in a 61% \((p < 0.001)\) decrease in plaque burden compared with control animals (Fig. 2b), whereas 100 mg/kg per day of BBR treatment reduced Aβ plaque burden to 43% of untreated control levels \((p < 0.05)\) (Fig. 2b).

To further measure which subsets of Aβ plaques were reduced morphometric analyses of Aβ plaques were performed on whole brain. The 2-way ANOVA revealed both a treatment \([F(2,68) = 17.4; p < 0.001]\) and a size effect \([F(3,68) = 292.6; p < 0.001]\) in the mean number of Aβ plaques. Post hoc multiple analysis revealed that BBR (25 mg/kg)-treated TgCRND8 mice showed a significant reduction in large \((>50)\), medium \((25–50 \mu m)\), small sized \(<25 \mu m)\), and total amyloid plaque subsets; percentage reduction in large, medium, small, and total subsets are 60% \((p < 0.001)\), 41% \((p < 0.05)\), 35% \((p < 0.055)\), and 37% \((p < 0.05)\), respectively (Fig. 2c). However, a 100 mg/kg per day of BBR dose produced only a nonsignificant 21%–28% reduction of amyloid plaque subsets (Fig. 2c).
Moreover, consistent with the indication that BBR treatment reduces the accumulation of total Aβ peptides in the brain, we also found a significant reduction in ThioS-positive fibrillar Aβ in the brains of the same TgCRND8 mice at 25 mg/kg per day (40%; p < 0.05) or 100 mg/kg per day (51%; p < 0.05), relative to age-matched untreated control TgCRND8 mice, as examined stereologically (Supplementary Fig. 2a and c). Values represent group mean ± standard error of the mean (SEM); n = 6–7 mice per group. In (b), *** p < 0.001; ** p < 0.01; * p < 0.05 versus control after 1-way analysis of variance (ANOVA) followed by Fisher LSD post hoc analysis. In (c), *** p < 0.001; * p < 0.05 versus control after 2-way ANOVA followed by Fisher LSD post hoc analysis.

The above findings of reduced 4G8-positive and ThioS Aβ deposits were further corroborated by Aβ ELISA analysis in the opposite hemisphere of the brain. In particular, this assay disclosed that Aβ levels in both SDS-soluble and insoluble formic acid fractions were significantly decreased in BBR-treated TgCRND8 mice (Fig. 3a and b). Soluble Aβ was first extracted with 2% SDS, and the remaining Aβ was pelleted at 100,000 g and extracted with 70% formic acid. In
the SDS-soluble fraction, the diminution of Aβ1–40 was 44% (p = 0.011) and 32% (p = 0.051) by 25 and 100 mg/kg per day, respectively, and there was slight but statistically not significant reduction of Aβ1–42 by BBR treatments (Fig. 3a). One-way ANOVA analyses of both Aβ1–40 and Aβ1–42 in the formic acid fraction showed treatment effects from both low and high concentrations of BBR. In the formic acid extractable fraction, the reductions of Aβ1–40 were 49% (p < 0.01) and 54% (p < 0.01) (Fig. 3b), and the corresponding reductions of Aβ1–42 were 44% (p < 0.01) and 39% (p < 0.05) by 25 and 100 mg/kg per day of BBR, respectively.

3.4. **BBR ameliorates Aβ-associated reactive gliosis and astrocytosis in TgCRND8 mice**

Next, we evaluated microgliosis and astrocytosis in TgCRND8 mice which are elevated phenotypically as a consequence of amyloid deposition. Double immunohistochemical staining with Iba-1/GFAP and ThioS confirmed that only activated microglial cells were in very close association with compact plaques and absent in regions lacking such deposits (data not shown). Iba-1 is expressed in microglia/macrophages, and is upregulated during activation of these cells (Sasaki et al., 2001). The degree of microgliosis as evaluated by Iba-1 load in the 3 brain regions examined was significantly amplified in vehicle-treated TgCRND8 mice relative to WT mice (data not shown), whereas it was significantly reduced to 45% (p < 0.05) and 27% (p > 0.05) in 25 and 100 mg/kg per day of BBR-treated TgCRND8 mice relative to vehicle-treated TgCRND8 mice (Fig. 4a and c). Likewise, the magnitude of astrocytosis as assessed by clusters of GFAP-immunoreactive astrocytes (GFAP burden) was significantly reduced to 54% (p < 0.001) and 28% (p < 0.05) in 25 and 100 mg/kg of BBR-treated TgCRND8 mice, respectively, relative to vehicle-treated TgCRND8 mice (p < 0.001) (Fig. 4b and d).

Activated microglial cells are typically associated with amyloid plaques; thus the decrease in the Iba-1 burden correlates with reduced plaque burden.

3.5. **BBR alters APP processing, APP, and tau phosphorylation probably through inhibition of Akt/GSK3 activities in TgCRND8 mouse brain**

To understand the mechanisms underlying the benefits of BBR for cognitive function and Aβ neuropathology of TgCRND8 mouse, we first examined whether BBR can influence the processing of APP. We found that BBR treatment had no effect on full length APP, BACE-1, and ADAM-10 in brain homogenates (Fig. 5a and b and Supplementary Fig. 3). Moreover BBR did not affect the levels of the Aβ-degrading enzymes neprilysin and IDE (Supplementary Fig. 3). While BBR showed no influence on secretases, BBR treatment (25 mg/kg per day) significantly reduced both CTF-α and -β (Fig. 5a and b). Taken together, these findings suggest that BBR’s Aβ-reducing effects might be related to regulation of APP-CTF expression or intracellular maturation and distribution, rather than modulation of APP processing enzymes or Aβ degradation.

Several studies suggest that APP maturation, subcellular distribution, and generation of Aβ are phosphorylation-dependent (Ando et al., 1999; da Cruz e Silva and da Cruz e Silva, 2003; Iijima et al., 2000). It was also suggested that APP phosphorylation modulates its metabolism, resulting in increased production of CTFs (Lee et al., 2003a). Therefore, we ascertained levels of APP phosphorylation in the brain lysates by immunoblot analysis using an antibody against p-APP-threonine668. This study showed that levels of p-APP and p-CTF were lower in BBR-treated mice compared with the vehicle-treated TgCRND8 mice (Fig. 5a). As shown in Fig. 5a, robust elevation of phosphorylated APP and CTFs were detected in vehicle-treated TgCRND8 mice. In contrast, the APP and CTFs phosphorylation...
were significantly reduced in BBR-treated TgCRND8 mice (Fig. 5a). Quantitative analysis of immunoblots of p-APP and p-CTFs showed 46%–50% decrease in the p-APP and p-CTFs in the low dose group, and 27%–28% decrease in the p-APP and p-CTFs in the high dose group of the BBR-treated TgCRND8 mice compared with the vehicle (Fig. 5b).

Taking into account the inhibitory role of BBR in APP phosphorylation and Aβ accumulation, we sought to determine a possible role of BBR in tau hyperphosphorylation in TgCRND8 mice. It has been shown that amyloid accumulation in TgCRND8 mice is followed by hyperphosphorylation of tau at different sites recognized by PHF-1, AT100, AT8, and CP13 antibodies (Bellucci et al., 2007). To evaluate quantitatively the effects of BBR on tau hyperphosphorylation, we assessed total tau and phosphorylation epitopes on tau, including PHF-1, AT8, and AT180, by Western blot analysis. We observed a robust reduction in the phosphotau recognized by PHF-1, AT8, and AT180 in the brain homogenates of BBR-treated TgCRND8 mice (Fig. 5c). This finding is based on earlier work demonstrating that these sites are phosphorylated by GSK3β (Cho and Johnson, 2003). Quantitative analysis of the Western blot bands of the phosphorylated tau showed 26%, 30%, and 42% decreases at PHF-1, AT8, and AT180 phosphoepitopes, respectively, in the BBR-treated TgCRND8 mice relative to vehicle-treated TgCRND8 mice (Fig. 5d). In addition, Western blotting with a tau-1 antibody, recognizing the dephosphorylated tau at Ser198/Ser199/Ser202, showed a significant increase in tau-1 immunoreactivity in BBR-treated TgCRND8 mice. No significant difference in the total tau levels was observed between the groups (Fig. 5c and d). Both APP and tau hyperphosphorylation were significantly increased in vehicle-treated TgCRND8 mice compared with the WT controls (Supplementary Fig. 4).

Cyclin-dependent kinase 5 (Cdk5), GSK3, and JNK are the 3 key kinases involved in the phosphorylation of APP (Aplin et al., 1996; Iijima et al., 2000; Judge et al., 2011; Muresan and Muresan, 2005). Because BBR reduced the accumulation of CTFs and inhibited the phosphorylation of APP and APP-CTFs, we sought to determine whether BBR plays a role in regulating Cdk5 and/or GSK3. It is known that GSK3 is activated through the phosphorylation at Tyr216 or is inhibited when Ser9 is phosphorylated (Frame and Cohen, 2001). Cdk5 activity is enhanced when Cdk5 is phosphorylated at Y15 and associates with p35, significantly increasing its kinase activity (Patzke et al., 2003; Sato et al., 2008). Thus, we examined the levels of phosphorylated Cdk5, GSK3β, and JNK using specific antibodies. As shown in Fig. 5e, a profound enhancement of inactive form of GSK3β phosphorylated at Ser9 was observed in the brains of the BBR-treated TgCRND8 mice compared with vehicle-treated TgCRND8 controls. Quantitative analysis of the Western blot bands indicated a 50% enhancement in the phosphorylated GSK3β at Ser 21 and 9 in the BBR-treated TgCRND8 mice relative to vehicle-treated TgCRND8 mice (Fig. 5e and f). These data suggest that BBR treatment downregulates GSK3 activity. Immunoblot analysis with antibodies against the phosphorylated Cdk5 and JNK showed no significant differences in...
p-Cdk5 (Y15) and p-stress-activated protein kinase/Jun-N-terminal kinase (Thr183/Tyr185) respectively between the BBR- and vehicle-treated TgCRND8 mice (Supplementary Fig. 3). Because the Akt/GSK3 pathway is found to be dysregulated in AD (Beaulieu et al., 2009; Chung, 2009; Ryder et al., 2004), we determined the levels of inactive GSK3 and active Akt in the TgCRND8 mouse brains. The levels of phosphorylated (p)-Akt in TgCRND8 mice that received BBR was significantly high from that of the vehicle-treated TgCRND8 mice, which showed a significant reduction in p-Akt levels (normalized to total Akt) (Fig. 5e and f). Increased phosphorylation of Ser9 in GSK3β/H9252 indicates decreased activity of GSK3β, whereas p-Akt in Ser473 indicates increased activity of Akt. These results suggest that the level of the active GSK3β is increased in TgCRND8 mouse brains and that BBR treatment increases the activity of protective Akt pathway. The enhanced levels of pGSK3β and p-Akt were not ascribable to an augment in total GSK3α/β or total Akt, because the amount of total GSK3α/β and total Akt was unaltered by BBR treatment.

3.6. BBR alters APP processing, APP and tau phosphorylation through PI3K/Akt/GSK3 pathway in vitro

Given the inhibitory role of BBR in the phosphorylation of GSK3 in TgCRND8 mouse brain, we further evaluated phosphorylation of both GSK3α/β at Ser21 and Ser9 level in N2a-SwedAPP cells following BBR treatment. Because GSK3 activity is inhibited via phosphorylation at specific serine residues (Ser9 for GSK3β and Ser21 for GSK3α) by Akt, we also investigated the effect of BBR on the protein levels of phosphorylated Akt. The amount of phosphorylated GSK3 increased during the first 30 minutes...
of BBR treatment, and remained higher than in the control group at times exceeding 3 hours following BBR treatment (Fig. 6b and d). BBR also increased the phosphorylation of Akt at Ser 473 and GSK3 at Ser21 and Ser9 in a concentration-dependent manner with significant increases achieved at BBR concentrations of 10 and 20 µM, but it did not significantly affect cellular total Akt and GSK3β levels (Fig. 6a and c).

To study the effect of BBR on APP processing and tau phosphorylation in N2a-SwedAPP cells, we first examined the levels of CTFs, p-APP and PHF-1 in cell lysates by Western blot analysis. As shown in Fig. 7a, the level of CTFs from the N2a-SwedAPP cells was reduced by treatment with BBR in a concentration-dependent manner, reaching maximal reduction by 55% of basal level at a concentration of 20 µM. At low concentration of BBR (5 µM), there was a 24% reduction in the level of CTFs but it was not a statistically significant effect. Western blot analysis for p-APP in N2a-SwedAPP cell lysates enabled us to compare APP phosphorylation and its processing. We employed the anti-p-APP-Thr668 antibody, which specifically identifies only this phosphorylated site of the protein, to assess BBR’s inhibitory effect. There was an apparent decrease in the phosphorylated level of APP by BBR treatment in a similarly dose-dependent fashion as demonstrated by Western blot analysis (Fig. 7a). At 20 µM, the level of p-APP was decreased by 75% and at 10 µM by 50% (Fig. 7a and b). It has been shown in AD models that overexpression of Swedish mutant APP (SwedAPP) increases p-tau (Wang et al., 2006). We next tested the effect of BBR on abnormal tau phosphorylation at PHF-1 epitope in N2a-SwedAPP cells. Compared with the control, the level of tau bound to PHF-1 was decreased to 30% and 50% by treatment of 10 and 20 µM concentrations of BBR, respectively (Fig. 7a and b). The data indicate that hyperphosphorylation of tau at Ser-396/404 was increased in N2a-SwedAPP cells and the abnormal modification of tau at this epitope was significantly inhibited by BBR.

PI3K has a regulatory role in the trafficking of many proteins including APP (PI3K inhibitors increase intracellular APP-CTFs) (Petanceska and Gandy, 1999;
Shineman et al., 2009). Because BBR showed a dose- and time-dependent increase in p-Akt and inactive pGSK3α/β with decrease in p-APP, CTFs, and PHF-1 (Fig. 6a and b), we further determined whether pretreatment of PI3K inhibitor (LY294002) in N2a-SwedAPP cells abolished the BBR-induced reduction of p-APP, CTFs, and PHF-1. To determine the CTFs, p-APP and PHF-1 reducing effect of BBR, N2a-SwedAPP cells were cotreated with 20 µM of BBR together with the PI3K inhibitor, LY294002, used at 20 µM. As expected, BBR-induced reduction of p-APP, CTFs and PHF-1 were reversed by the cotreatment of LY294002 (Fig. 7c and d). To further analyze the BBR effect, we investigated p-APP in N2a-SwedAPP cells by immunofluorescence. We found that p-APP-positive vesicles could be labeled by the endosome markers Rab5 (Fig. 7e [A1–A4]) as determined earlier (Lee et al., 2003a). After 3-hour exposure to 20 µM BBR, p-APP staining was markedly reduced in the endosomal compartments (Fig. 7e [B1–B4]). BBR-induced reduction of p-APP was significantly inhibited by LY294002 in endosomal compartments of N2a-SwedAPP cells (Fig. 7e [C1–C4]). This confirmed the BBR mediated p-APP reduction seen with Western blots (Fig. 7c and d). These data showed that the reduction of p-APP staining inside endosomal compartments is due to BBR’s inhibitory effect on p-APP. Altogether, these results indicate that PI3K/Akt/GSK3 is involved in the BBR-induced reduction of p-APP, CTFs, and PHF-1.
4. Discussion

Although BBR is a well known neuroprotective agent (Ji and Shen, 2011; Kulkarni and Dhir, 2010; Ye et al., 2009), the actual therapeutic role of BBR in AD pathology has not yet been evaluated. BBR has been shown to inhibit the production of Aβ in H4-SwedAPP cells (Asai et al., 2007); however, its effect on Aβ accumulation in vivo has not been documented. The data from this study represent the first evidence that regular administration of BBR can prevent the age-related cognitive impairments and Aβ accumulation observed in TgCRND8 mice with an early-onset AD-like pathology (Chishti et al., 2001). Several lines of our evidence suggest that BBR has beneficial effects in AD. First, BBR readily passed through the blood-brain barrier, and its presence in brain tissue of treated mice was definitively determined by liquid chromatography-mass spectrometry. Second, dose-specific effects on brain Aβ levels, cognitive deficits, amyloid neuropathology, and accelerated gliosis were found in the TgCRND8 mouse model of Alzheimer’s disease. Third, BBR significantly reduced the Aβ and CTFs, probably by downregulating the phosphorylation of APP and of CTFs via the activation of the PI3K/Akt/GSK3 pathway.

Both low and high doses of BBR significantly reduced the cognitive impairment characteristic of AD, both in the conventional reference memory MWM task and memory retention task (probe trial) of the mice (Fig. 1c and d). These results are consistent with the amelioration of hippocampal-dependent memory function (McDonald and White, 1994). The cognitive benefits of long-term BBR administration did not involve significant side effects on sensorimotor function, as evidenced by visible platform training (Fig. 1b).

Notably, BBR-induced decreases in memory deficits are accomplished not only by a significant reduction in the amyloid burden but also by an evident plaque fragmentation in the brains of transgenic mice. In particular, Aβ plaques of all 3 size subsets (<25, 25–50, and >50 μm) were significantly decreased in the brains of BBR-treated TgCRND8 mice (Fig. 2c). The magnitude of this decrease in the number of plaques is similar to the decrease found in brains of mice treated with arundic acid (Mori et al., 2006). The 43%–63% reduction in amyloid burden observed in this study is also comparable with that found in studies of mice treated with curcumin or epigallocatechin 3-gallate (ECGC) (Lim et al., 2001; Rezaizadeh et al., 2005). The present study also includes the important finding that BBR administration can reduce vascular amyloids as well as parenchymal amyloids (Supplementary Fig. 2a and b). Apart from neuronal Aβ deposition, previous studies have shown that the pattern of vascular amyloid deposition in leptomeningeal and small cortical blood vessels observed in TgCRND8 mice mirrors that typically found in human age-related CAA (Chishti et al., 2001; Hawkes and McLaurin, 2009). BBR treatment had significant effects in reducing ThioS-positive vascular amyloids (Supplementary Fig. 2b and d), and might reduce CAA through reduction of Aβ production. Effects reported in the present study are similar to the reported effects of nicotine administration where percentage of vessels with Aβ was significantly reduced (Hellström-Lindahl et al., 2004). Because regular administration of BBR reduces ThioS-positive plaques and insoluble peptides, its effect may be on amyloid fibril formation. We found there was a mild inhibition of Aβ1–42 fibril formation with an IC₅₀ (the half maximal inhibitory concentration) value of 23.5 μM in vitro (data not shown). It has also been recently demonstrated that BBR at a concentration of 20 μM showed 36.3% inhibition of Aβ1–42 fibril formation in vitro (Shi et al., 2011). However, the effective concentration of BBR used in the in vitro studies, approximately 20 μM, were 23 times less than those found in the brain. Therefore, the exact molecular mechanism of BBR-mediated reduction of Aβ accumulation needs further study.

The strong association of reactive astrocytes and activated microglia with amyloid deposition contributes to the progressive course of AD because of amplification of a large range of proinflammatory molecules that mediate, in part, the neuronal loss detected in AD (Akiyama et al., 2000). We found that treating TgCRND8 mice with BBR resulted in a 45% reduction in microgliosis and a 54% decrease in astrocytosis (Fig. 4a and b).

A similar marked decrease in activated microgla (57%) was also observed in ibuprofen-treated animals (Yan et al., 2003). Our results showing that BBR significantly reduced amyloid deposits along with amyloid plaque-associated reactive microgliosis and astrocytosis are in full agreement with the above view. However, this does not essentially entail a unidirectional relationship between the 2 events. The observed reduction in gliosis by BBR is more likely to be linked to its inhibition of Aβ. However, BBR appears to suppress neuroinflammatory responses independent of Aβ (Jeong et al., 2009; Lu et al., 2010). Therefore, the putative action of BBR needs to be determined in relation to the mechanisms underlying both gliosis and amyloidosis. Nonetheless, because neuroinflammation is a risk factor for neurodegenerative disease, the anti-inflammatory effect of BBR in the TgCRND8 mice supports the evidence for its therapeutic potential for AD.

It has been demonstrated that successive extraction of brain homogenates using SDS and FA solubilizes the majority of the total amyloid species from brains of Tg2576 mice (Kawarabayashi et al., 2001). We have noted both low and high doses of BBR significantly reduced both soluble and insoluble Aβ peptides (FA fraction) in the brain of TgCRND8 mice. This reduction in insoluble Aβ peptides is comparable with earlier observed reduction after chronic nicotine administration in Swedish APP mice (Nordberg et al., 2002). The earlier observation that the nicotine treatment reduced the insoluble but not soluble Aβ peptides in the brain of Tg2576 is in accordance with this result. How-
ever, a low dose of BBR reduces SDS-soluble Aβ1–40 (44% reduction); therefore, BBR may also have an effect on potentially neurotoxic soluble Aβ oligomers. Overall, the low dose BBR-induced reduction of total extracted Aβ40 (44%–49%) and Aβ42 (22%–43%) in the brain hemispheres corresponds well with the decrease in plaque number (37%) and plaque load (61%) in other brain hemispheres evaluated by immunostaining, indicating that these biochemical measures are an exact reflection of overall amyloid load (Figs. 2 and 4). BBR treatment prevents spatial memory reference deficits and Aβ pathology in vivo at doses that are equivalent to or lower than the doses prescribed for humans for hypercholesteremia (Kong et al., 2004). In this study we found that the low dosage of BBR showed more significant effect than the high dosage in reducing Aβ pathology and gliosis (Figs. 2 and 4). A similar case has been published, reporting the Aβ-reducing effect of memantine in Tg2576 AD mice (Dong et al., 2008). Thus, it is possible that lower BBR doses may obstruct just enough neuronal activity to reduce amyloid generation, while higher dose obstructs so much neuronal activity that Aβ clearance mechanisms are also reduced.

A possible mechanism of the reducing effect of BBR on Aβ deposition is modulation of APP processing, because the levels of APP-CTFs, the direct precursor of Aβ (De Strooper and Annaert, 2000), were decreased by BBR treatment (Fig. 5a). APP processing can be modulated either by an altered APP expression or by the function of β-secretase (BACE-1). However, we did not notice a significant difference in the protein levels of Fl-APP or BACE-1 between the groups (Fig. 5a). It has been shown that APP phosphorylation at position Thr668 facilitates the accumulation of CTFs and increases Aβ generation (Lee et al., 2003a). Several studies have revealed that APP maturation and targeting for proteolysis requires APP Thr668 phosphorylation (da Cruz e Silva and da Cruz e Silva, 2003; Lee et al., 2003a). Phosphorylated APP undergoes fast anterograde axonal transport to the nerve terminals, where β- and γ-secretase mediated-cleavage occurs, resulting in Aβ release (Kamal et al., 2000; Lazarov et al., 2005; Lee et al., 2005). This indicates that BBR-mediated reduction of APP phosphorylation might subsequently result in a reduction of mature p-APP in the trans-Golgi compartment and a lesser amount of APP directed to the distal axon, which would prevent Aβ production at synaptic terminals. These findings suggest that BBR modulates APP processing through a mechanism beyond regulating the expression of APP and BACE-1. This is consistent with a previous study showing that retinoic acid reduced the Aβ pathology mainly via reducing CTFs, p-APP without influencing the levels of Fl-APP and BACE-1 (Ding et al., 2008). Similarly, indirubin-3-monoxime, a GSK3β inhibitor isolated from the traditional Chinese medicinal herb Indigo naturalis (Qing Dai in Chinese) reduced Aβ accumulation and tau phosphorylation in APP/PS-1 mice without affecting the levels of Fl-APP and BACE-1 (Ding et al., 2010).

Based on the result that BBR treatment decreased the elevation of APP phosphorylation in TgCRND8 mice, we propose that BBR may prevent APP processing by inhibiting its phosphorylation. Among the several protein kinases phosphorylating APP at Thr668 (Aplin et al., 1996; Iijima et al., 2000; Standen et al., 2001), GSK is considered to be a key kinase responsible for APP phosphorylation in neuronal cells (Aplin et al., 1996; Chang et al., 2006; Judge et al., 2011; Ploia et al., 2010). Suppressing GSK3 activity has been demonstrated to decrease the generation and accumulation of Aβ in APP mice (Rockenstein et al., 2007). This coincides with our result showing a concomitant downregulation of GSK activity by BBR treatment in TgCRND8 mice. In TgCRND8 mice and N2a-SwedAPP cells, BBR treatment led to a trend of increased p-Akt and inactive pGSK3α/β protein levels (Figs. 5e and f, and 6). GSK3 activity is inhibited via phosphorylation at specific serine residues (Ser 9 and Ser21) by Akt (Frame and Cohen, 2001). It is also possible that the improved spatial learning can be ascribed to the BBR-induced activation of the Akt pathway because activation of the Akt pathway is essential for the expression of long-term potentiation (Karpova et al., 2006; Sanna et al., 2002). There are several studies indicating that the PI3K/Akt/GSK3β signaling pathway contributes to a number of aspects of AD pathology. In vitro studies suggest that PI3K and Akt protect neurons against Aβ toxicity (Martin et al., 2001). GSK3α also modulates APP processing thereby influencing the production of Aβs (Phiel et al., 2003). Liu et al. (2003) showed that inhibition of PI3K contributes to tau phosphorylation and impairment of spatial memory. GSK3β is strongly involved in tau phosphorylation at various sites including PHF-1, Tau-1, AT8, and AT270 epitopes (Lee et al., 2003b). It has been shown that GSK3β activity increases in cell expressing Swedish APP mutation and in AD presenilin-1 and presenilin-2 mutation lymphoblast cells via a downregulation of both Ser473 Akt and inactive Ser9 phosphorylated GSK3β (Ryder et al., 2004). Although underlying mechanisms of PI3K/Akt/GSK3β signaling in AD are still unexplained they are presently being intensively investigated. We studied the involvement of PI3K/Akt within the complex intracellular signaling pathway involved in the inhibition of APP processing. Pharmacological inhibition of the kinase activity of PI3K not only reversed the BBR-induced reduction of p-APP and PHF-1, but it also blocked the BBR-mediated reduction of CTFs (Fig. 7b). We have been able to detect reduced p-APP staining in the endosomal compartment of N2a-SwedAPP cells in immunohistochemistry (Fig. 7c), suggesting that the phosphorylation state of APP is an important factor due to its association with BACE-1, which mediates the amyloidogenic processing of APP to increase Aβ through increased generation of the CTFs (Lee et al., 2003a). Interference in PI3K/Akt/GSK3 pathway thus rep-
resents a key mechanism through which BBR may decrease AD pathology and improve cognitive function.

In conclusion, our data demonstrate that BBR is able to reduce cerebral Aβ levels, glial activation, and cognitive impairment in the TgCRND8 mouse model. In addition, we found that BBR suppresses both CTFs and p-APPs levels via activating the PI3K/Akt/GSK3 signaling pathway, thereby precluding Aβ generation. Based on the safety and brain bioavailability of BBR, and its ability suppress to Aβ levels, it appears to be a promising drug for the prevention and/or treatment of Alzheimer’s disease. The outcomes from our study will warrant further investigation of BBR-like alkaloids as candidates for Aβ and tau-based therapeutics to modify or delay the onset of Aβ and tau pathology in AD.

Disclosure statement

The authors disclose no conflicts of interest.

All animal procedures were approved by the Hong Kong Baptist University Committee on the Use of Human and Animal Subjects in Teaching and Research and by the Committee on the Use of Live Animals for Teaching and Research (CULATR), the University of Hong Kong.

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Appendix A. Supplementary data


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