

Baicalein Reduces β -Amyloid and Promotes Nonamyloidogenic Amyloid Precursor Protein Processing in an Alzheimer's Disease Transgenic Mouse Model

She-Qing Zhang,^{1,2*} Demian Obregon,² Jared Ehrhart,² Juan Deng,² Jun Tian,² Huayan Hou,² Brian Giunta,³ Darrell Sawmiller,² and Jun Tan^{2*}

¹Department of Neurology, Shanghai Changhai Hospital, Shanghai, China

²Rashid Laboratory for Developmental Neurobiology, Silver Child Development Center, Department of Psychiatry and Behavioral Neurosciences, Morsani College of Medicine, University of South Florida, Tampa, Florida

³Neuroimmunology Laboratory, Department of Psychiatry and Behavioral Neurosciences, Morsani College of Medicine, University of South Florida, Tampa, Florida

Baicalein, a flavonoid isolated from the roots of *Scutellaria baicalensis*, is known to modulate γ -aminobutyric acid (GABA) type A receptors. Given prior reports demonstrating benefits of GABA_A modulation for Alzheimer's disease (AD) treatment, we wished to determine whether this agent might be beneficial for AD. CHO cells engineered to overexpress wild-type amyloid precursor protein (APP), primary culture neuronal cells from AD mice (Tg2576) and AD mice were treated with baicalein. In the cell cultures, baicalein significantly reduced the production of β -amyloid (A β) by increasing APP α -processing. These effects were blocked by the GABA_A antagonist bicuculline. Likewise, AD mice treated daily with i.p. baicalein for 8 weeks showed enhanced APP α -secretase processing, reduced A β production, and reduced AD-like pathology together with improved cognitive performance. Our findings suggest that baicalein promotes nonamyloidogenic processing of APP, thereby reducing A β production and improving cognitive performance, by activating GABA_A receptors. © 2013 Wiley Periodicals, Inc.

Key words: β -amyloid; Alzheimer's disease; flavonoid; baicalein; sAPP α ; GABA

Alzheimer's disease (AD) is a neurodegenerative disease characterized pathologically by senile plaques (SPs) and neurofibrillary tangles (NFTs) and clinically by progressive cognitive deterioration. Over the last 2 decades, the β -amyloid (A β) hypothesis has been the dominant theory for the mechanism of AD pathogenesis (Hardy and Higgins, 1992; Hardy et al., 1998; Hardy and Selkoe, 2002). Amyloid precursor protein (APP) proteolysis is the fundamental process for the production of A β peptides implicated in AD pathology. A β peptides are produced

by the initial action of β -secretase (BACE) cleavage, which creates an A β -containing C-terminal fragment (CTF) known as β -CTF and an N-terminal, soluble APP- β (sAPP β) fragment, which is released extracellularly. Intracellularly, β -CTF is then cleaved by a multi-protein γ -secretase complex that results in generation of the A β peptide and a smaller γ -CTF. Under physiological conditions, A β is constitutively generated at relatively low levels and, hence, most APP is processed by the non-amyloidogenic pathway involving α -secretase. In the nonamyloidogenic pathway, APP is first cleaved at the α -secretase site, which results in the release of N-terminal sAPP α and the generation of α -CTF. Because of the limiting amount of APP in the cell and the failure to saturate the BACE pathway during APP overexpression, it is believed that the above-mentioned amyloidogenic and nonamyloidogenic pathways compete for substrate in the process of APP proteolysis (Hardy and Selkoe, 2002). Therefore, it is often inferred that extracellular elevation of sAPP α can be taken as indirect evidence of activation of α -secretase or inhibition of BACE and the associated amyloidogenic pathway (Rezai-Zadeh et al., 2005).

Contract grant sponsor: NIH/NIA; Contract grant numbers: R01AG032432; R42AG031586 (to J.T.); Contract grant sponsor: STCSM Funds; Contract grant number: 12ZR1438200.

*Correspondence to: Dr. She-Qing Zhang, Department of Neurology, Changhai Hospital, No. 168 Changhai Rd., Shanghai 200433, China. E-mail: sheqingzhang@126.com; or Dr. Jun Tan, Department of Psychiatry, Morsani College of Medicine, University of South Florida, Tampa, FL 33613. E-mail: jtan@health.usf.edu

Received 13 December 2012; Revised 8 February 2013; Accepted 22 March 2013

Published online 17 May 2013 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jnr.23244

Over the past decade, intense focus has been given to investigating the processes of APP proteolysis and A β metabolism as possible targets for AD therapy. One naturally occurring compound is baicalein (5,6,7-trihydroxyflavone), extracted from the root of the Chinese medicinal herb *Scutellaria baicalensis* Georgi (Jianjun and Huiru, 2008). Previous studies have demonstrated the usefulness of baicalein in reducing cognitive deficits in rats induced by chronic cerebral hypoperfusion and improving memory in ibotenic acid-induced rat models (Liu et al., 2007; Heo et al., 2009). Further, Wang et al. (2004) have shown baicalein can reduce β -amyloid peptide-(25–35)-induced amnesia in mice. Heo et al. (2004) reported that baicalein potently reduces A β -induced neurotoxicity in PC12 cells, possibly by a reduction of oxidative stress. Likewise, Zhu et al. (2004) observed that baicalein can inhibit fibrillation of α -synuclein and disaggregate existing fibrils, which raises the possibility of baicalein as a potential treatment for neurodegenerative diseases, such as Parkinson's disease. However, whether baicalein can directly inhibit A β production and ameliorate the subsequent cognitive impairments observed in an animal model of AD has not been determined.

The present study shows that baicalein significantly decreased A β production both in Chinese hamster ovary cells expressing a human wild-type APP gene (CHO/APPwt) and in primary neurons from transgenic Tg2576 mice. In concert with these observations, we found that baicalein promotes cleavage of α -CTF and elevates sAPP α . These cleavage events are attenuated with a specific GABA receptor antagonist, bicuculline. As a validation of these findings in vivo, we treated A β -overproducing Tg2576 transgenic mice with baicalein for 8 weeks and found decreased A β levels in the brain associated with promotion of the nonamyloidogenic α -secretase proteolytic pathway. Furthermore, we found the cognitive performance of these mice to be significantly improved compared with untreated mice, suggesting that this natural compound should be tested further for translation into humans.

MATERIALS AND METHODS

Reagents and Antibodies

Baicalein powder (99% purity by high-pressure liquid chromatography) was purchased from Shanghai Jiahe (Shanghai, China). The reagent was initially dissolved in dimethylsulfoxide (DMSO) and then diluted in PBS (pH 7.4) to a final concentration of 0–10 μ M and less than 1% DMSO (de Carvalho et al., 2011). Bicuculline methiodide (BMI) was purchased from Sigma-Aldrich Chemie (Munich, Germany), prepared initially in distilled water and diluted in PBS to a final concentration of 10 μ M (Behrens et al., 2007).

Sterile, azide-free, and low-endotoxin antibodies were used, including anti-C-terminal human sAPP α -specific antibody (2B3; 100 μ g/ml; IBL, Minneapolis, MN), A β _{1–17} antibody (6E10; 1 mg/ml; Covance, Emeryville, CA), APP C-terminal antibody (pAb751/770; 500 μ g/ml; EMD Biosciences, La Jolla,

CA), and β -actin antibody (100 μ g/ml; Sigma-Aldrich). The sAPP α -specific 2B3 antibody was further characterized in our in vitro and in vivo systems, indicating that this antibody recognizes neither A β nor full-length APP.

Cell Culture

CHO cells engineered to express wild-type human APP (CHO/APPwt) were kindly provided by Dr. Stefanie Hahn and Dr. Sascha Weggen, (University of Heinrich Heine, Düsseldorf, Germany). These cells were maintained in Dulbecco's modified Eagle's medium with 10% bovine serum, 1 mM sodium pyruvate, and 100 U/ml penicillin/streptomycin, as described previously (Hahn et al., 2011). Tg2576 mouse-derived neuronal cells were cultured as described previously (Obregon et al., 2006). Briefly, cerebral cortices isolated from 1-day-old Tg2576 mice were mechanically dissociated in trypsin (0.25%) individually after incubation for 15 min at 37°C. Cells were collected after centrifugation at 1,200g, suspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 10% horse serum, uridine (33.6 μ g/ml; Sigma), and fluorodeoxyuridine (13.6 μ g/ml; Sigma) and seeded in 24-well collagen-coated culture plates at 2.5×10^5 cells per well. After reaching confluence (approximately 70–80%), cells were treated with baicalein at 0–10 μ M for 12 hr. In some experiments, cells were treated with baicalein at 5 μ M in the absence or presence of bicuculline at 10 μ M.

Mice

Transgenic mice overexpressing "Swedish" mutant APP (695-aa isoform) under control of the prion promoter (line 2576, Tg2576) were purchased from Taconic Farms (Germantown, NY). For i.p. administration of baicalein, 12 female Tg2576 mice were used; seven mice received baicalein, and five control mice received PBS. Beginning at 6 months of age, these mice were injected i.p. with baicalein (10 mg/kg) or PBS daily for 8 weeks (Rezai-Zadeh et al., 2005). In addition, similarly aged nontransgenic (NT) mice were concurrently given daily i.p. injections of baicalein (10 mg/kg) or PBS for 8 weeks. All mice were sacrificed at 8 months of age for analysis of A β levels and APP processing products in brain homogenates according to previously described methods (Rezai-Zadeh et al., 2005; Zhu et al., 2011). All behavioral testing occurred during the final weeks preceding sacrifice, with treatment being continued. The mice were housed and maintained at the University of South Florida (USF) and all experiments were in compliance with protocols approved by the USF Institutional Animal Care and Use Committee.

Tissue Preparation

Mice were euthanized with isoflurane and transcardially perfused with ice-cold phosphate-buffered saline (PBS). Brains were isolated under sterile conditions on ice, placed in ice-cold lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% v/v Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerolphosphate, 1 mM Na₃VO₄, 1 μ g/ml leupeptin, 1 mM PMSF), sonicated for approximately 3 min, allowed to stand for 15 min at 4°C, and then centrifuged

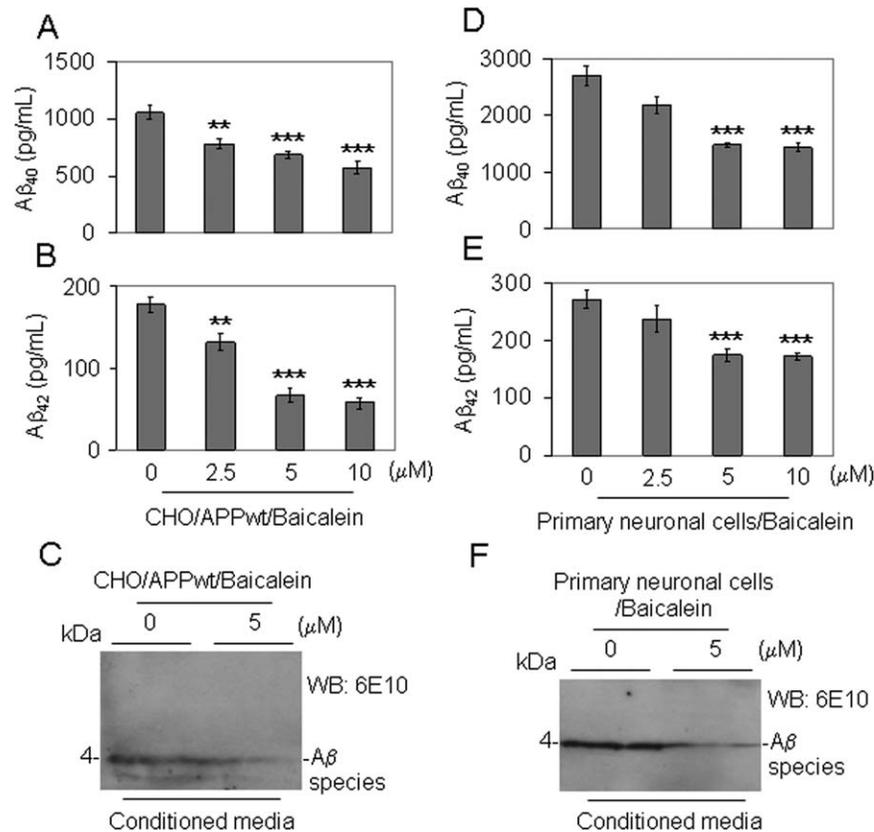


Fig. 1. Baicalein inhibits A β generation in cultured cells. CHO cells expressing wild-type human APP (CHO/APPwt) and primary neuronal cells cultured from brain tissues of 1-day-old Tg2576 mice were treated with baicalein at 0, 2.5, 5, and 10 μ M as indicated for 12 hr, followed by analysis of A $\beta_{40,42}$ peptides secreted in the cell culture media by A β ELISA (A,B,D,E) and IB analysis (C,F). The A β

ELISA results are represented as mean \pm SD of A β_{40} or A β_{42} (pg/ml) in cell culture media after baicalein treatment. These results are representative of three independent experiments, with $n = 3$ for each condition. One-way ANOVA followed by post hoc comparison revealed significant differences between 2.5, 5, or 10 μ M and 0 μ M baicalein treatment in A $\beta_{40,42}$ reduction. ** $P < 0.01$, *** $P < 0.005$.

at 15,000 rpm for 15 min. Supernatants were collected for analyses of soluble A β levels by ELISA and Western blot (WB) analysis, according to methods described previously (Rezai-Zadeh et al., 2005). The pellet was extracted with 5 M guanidine for analysis of insoluble A β as described previously (Zhu et al., 2011).

Western Blot Analyses

Cells were washed with ice-cold PBS three times, lysed with cell lysis buffer (Cell Signaling Technology, Danvers, MA), and stored at -80°C until use. A β species secreted from cells and in brain homogenates were analyzed by A β ELISA and WB (Zhu et al., 2011). Briefly, for WB analysis, 10% bicine/tris gel containing 8 M urea was used for separation of proteins from brain homogenates or CHO conditioned media. Proteins were then transferred to 0.2- μ m-pore-size nitrocellulose membranes (Bio-Rad, Hercules, CA). The membrane was boiled in PBS for 3–5 min before blocking to enhance sensitivity. 2B3 antibody was used for detection of sAPP α , and 6E10 was used for detection of A β . The 16.5% Tris tricine gel was used for detection of APP CTFs using pAB751/770.

Densitometry analysis was performed with a FluorS Multimager with Quantity One software.

ELISA

A $\beta_{40,42}$ species in cell conditioned media and brain homogenates were detected by A $\beta_{40,42}$ ELISA kits (Invitrogen, Carlsbad, CA). The manufacturer's instructions were strictly followed. The A $\beta_{40,42}$ levels are represented as picograms of A β_{40} or A β_{42} per milliliter of conditioned media or as picograms of A β_{40} or A β_{42} per milligram of total cellular protein.

Radial Arm Water Maze

The radial arm water maze (RAWM) test was conducted according to that previously described by Arendash et al. (2001). Briefly, the maze is composed of a 100-cm circular pool with six swim alleys (30.5 cm long and 19 cm wide) radiating from a common circular swim area 40 cm in diameter. An assortment of spatial cues is located on the testing room's walls and ceiling. A submerged escape platform 9 cm in diameter is placed near the end of a different arm for each day of testing,

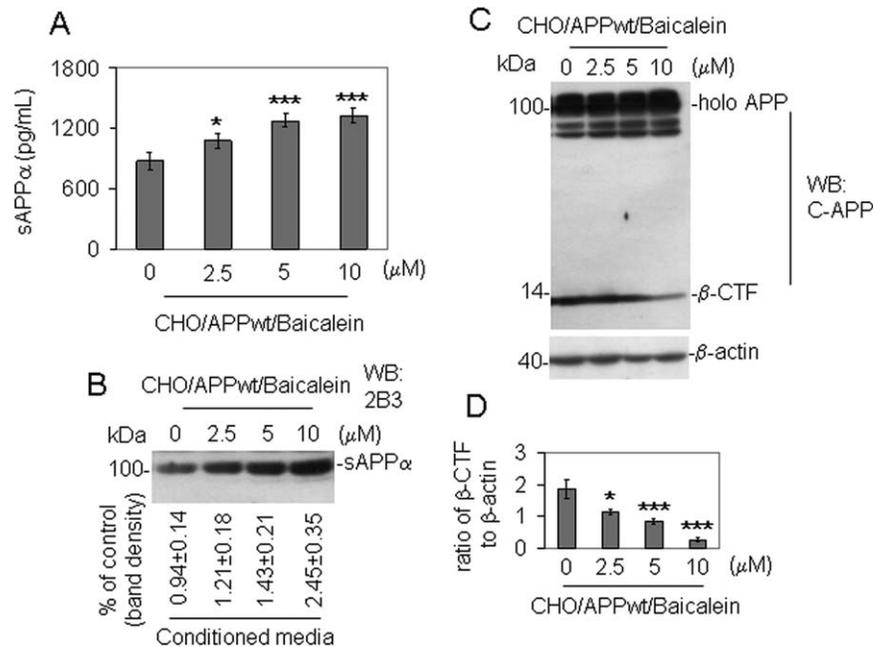


Fig. 2. Baicalein increases nonamyloidogenic APP cleavage in cultured cells. CHO/APPwt cells were treated with baicalein at 0, 2.5, 5, and 10 μM as indicated for 12 hr. Secreted sAPP α in the cell culture media were analyzed by sAPP α ELISA (A) and WB (B), using 2B3 antibody. The sAPP α ELISA results are represented as the mean \pm SD of sAPP α (pg/ml) in the cell culture media after baicalein treatment. Relative band density over vehicle control (1% DMSO in PBS; mean \pm SD) was calculated by densitometry analysis as shown below the WB panel. These results are representative of three independent experiments, with $n = 3$ for each condition. C: Cell lysates were prepared, and APP CTFs were analyzed by WB using a rabbit polyclonal

antibody against C-terminal APP (pAb751/770, C-APP). This β -CTF band was further confirmed by the additional WB using 6E10 antibody (data not shown). D: Relative ratio (mean \pm SD) of β -CTF to β -actin was calculated by densitometry analysis. The results are representative of three independent experiments, with $n = 3$ for each condition. One-way ANOVA followed by post hoc comparison revealed significant differences between 2.5, 5, or 10 μM baicalein treatment in both the increased sAPP α and the decreased relative ratio of β -CTF to β -actin. * $P < 0.05$, *** $P < 0.001$, full-length APP, holo APP.

which forces mice to use working memory to perform this task. A semirandom sequence of the remaining five arms is then selected as start arms for each of that day's trials. A different sequence of start arms is selected each day, which never includes that day's goal arm. Four consecutive acquisition trials (T1–T4) give animals the opportunity to learn which arm contains the submerged platform for that day. A retention trial (T5), beginning from the one remaining start arm for that day, is administered 30 min after T4.

For any given trial, animals are placed in the designated start arm facing the common circular swim area. During the following 1-min maximum trial, each time an animal enters an incorrect arm (or fails to select an arm after 20 sec), it is gently pulled back to the start arm for that trial, and an error is charged. For each trial, the number of errors prior to escape onto the goal arm's submerged platform is recorded, as is the latency to escape. If the submerged platform is not located within a given 1-min trial, the mouse is guided to the submerged platform and assigned a latency score of 60 sec. Animals are allowed to remain on the platform for 30 sec before the next trial begins. Mice were tested after 8 weeks of baicalein or PBS treatment. Two animals in the baicalein treatment group were eliminated from the final data analysis because of a consistent lack of arm choices throughout the test period.

Statistical Analysis

All data were normally distributed. In instances of single mean comparisons, Levene's test for equality of variances followed by t -test for independent samples was used to assess significance. In instances of multiple mean comparisons, analysis of variance was used, followed by post hoc comparison using Bonferroni's method. Alpha levels were set at 0.05 for all analyses. The statistical package for the social sciences release 10.0.5 (SPSS Inc., Chicago, IL) was used for all data analysis.

RESULTS

Baicalein Inhibits A $\beta_{40,42}$ Generation From CHO/APPwt Cells and Tg2576 Mouse-Derived Neurons

To examine the effects of baicalein on A β production, we first treated CHO/APPwt cells and primary neurons derived from Tg2576 mice with a wide dose range of baicalein for 12 hr and then measured A β_{40} and A β_{42} generation by ELISA. Baicalein reduced both A β_{40} and A β_{42} generation in CHO/APPwt cells (Fig. 1A–C) and primary Tg2576-derived neurons in a dose-dependent manner (Fig. 1D–F). Baicalein at 10 μM reduced A β_{40} and A β_{42} generation from CHO/APPwt

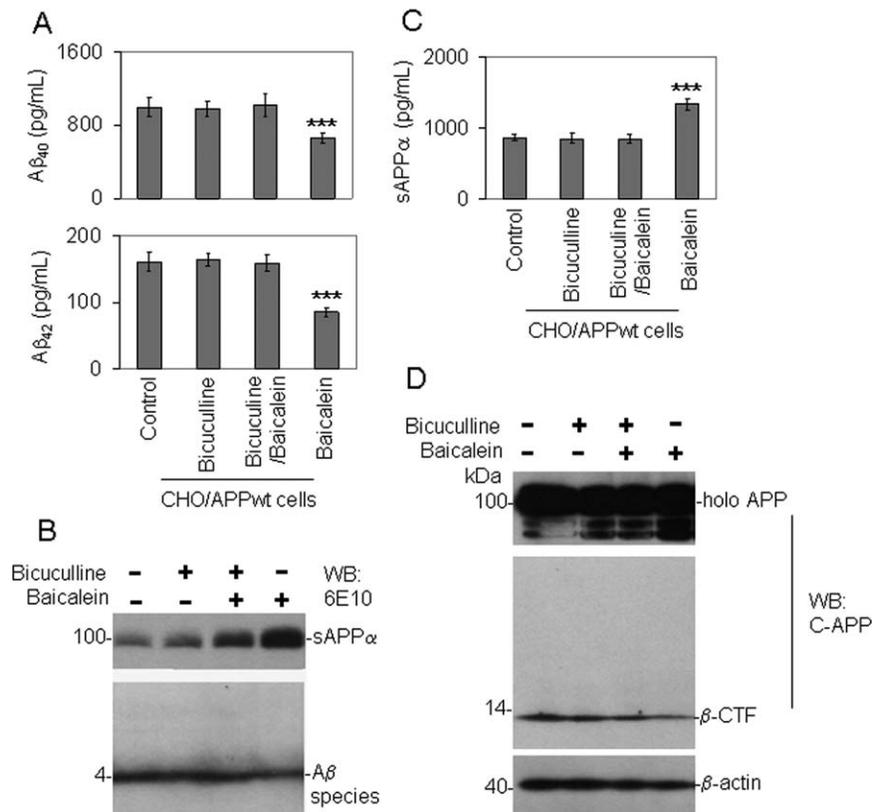


Fig. 3. GABA antagonist blocks baicalein-mediated A β reduction. CHO/APPwt cells were treated with baicalein at 5 μ M in the presence of GABA antagonist (bicuculline, 10 μ M) for 12 hr. Secreted A β _{40,42} peptide (A,B) and sAPP α (B,C) were analyzed in the cell culture media by ELISA and WB analysis using 6E10 antibody. These results are representative of three independent experiments, with

$n = 3$ for each condition. D: Cell lysates were prepared, and APP CTFs were analyzed by WB using a rabbit polyclonal antibody against C-terminal APP (pAb751/770; C-APP). This β -CTF band was further confirmed by the additional WB using 6E10 antibody (data not shown). Notably, bicuculline markedly abolished baicalein-mediated anti-A β - and α -secretase-promoting effects. *** $P < 0.005$.

cells by 49% and 66% (Fig. 1A,B), respectively, and reduced A β ₄₀ and A β ₄₂ generation from primary Tg2576-derived neurons by 50% and 37% (Fig. 1D,E), respectively, compared with untreated cells. To address this result further, we treated CHO/APPwt cells and Tg2576-derived neurons with 5 μ M baicalein and determined A β ₄₀ and A β ₄₂ secreted into the cell media by WB analysis, using 6E10 antibody. It was noted that A β secretion into the cell media was significantly reduced for both CHO/APPwt and primary Tg2576-derived neurons (Fig. 1C,F).

Baicalein Activates Nonamyloidogenic Processing of APP in CHO/APPwt Cells

To elucidate the mode of action of baicalein on APP cleavage, we examined APP cleavage profiles after treatment of CHO/APPwt cells with baicalein using ELISA and WB analyses. At first, we treated CHO/APPwt cells with different dosages of baicalein for 12 hr and the secretion of sAPP α into the cell culture media was analyzed by ELISA and WB, using 2B3 antibody. In concert with decreased A β generation, baicalein increased

sAPP α in a dose-dependent manner (Fig. 2A,B), suggesting that α -secretase activity was promoted. To confirm this result, we further analyzed the APP C-terminal fragments (CTF) in the cell lysates by WB using a rabbit polyclonal antibody against C-terminal APP (pAb751/770, C-APP). We noted that baicalein treatment significantly reduced the production of β -CTF in the CHO/APPwt cell lysates, indicative of inhibition of the amyloidogenic pathway (Fig. 2C,D). This was confirmed by additional WB for β -CTF using 6E10 antibody (data not shown).

GABA_A Receptor Antagonist Bicuculline Blocks Baicalein-Mediated A β Reduction

A previous study by de Carvalho et al. (2011) has documented that the sedative effect of baicalein is associated with activation of the nonbenzodiazepine binding site at GABA_A receptors. To investigate the mechanism by which baicalein reduces A β production, we treated CHO/APPwt cells with 5 μ M baicalein in the absence or presence of 10 μ M bicuculline, a selective GABA_A receptor antagonist. Secreted A β _{40,42} peptides and sAPP α in the cell culture media were determined by ELISA and WB

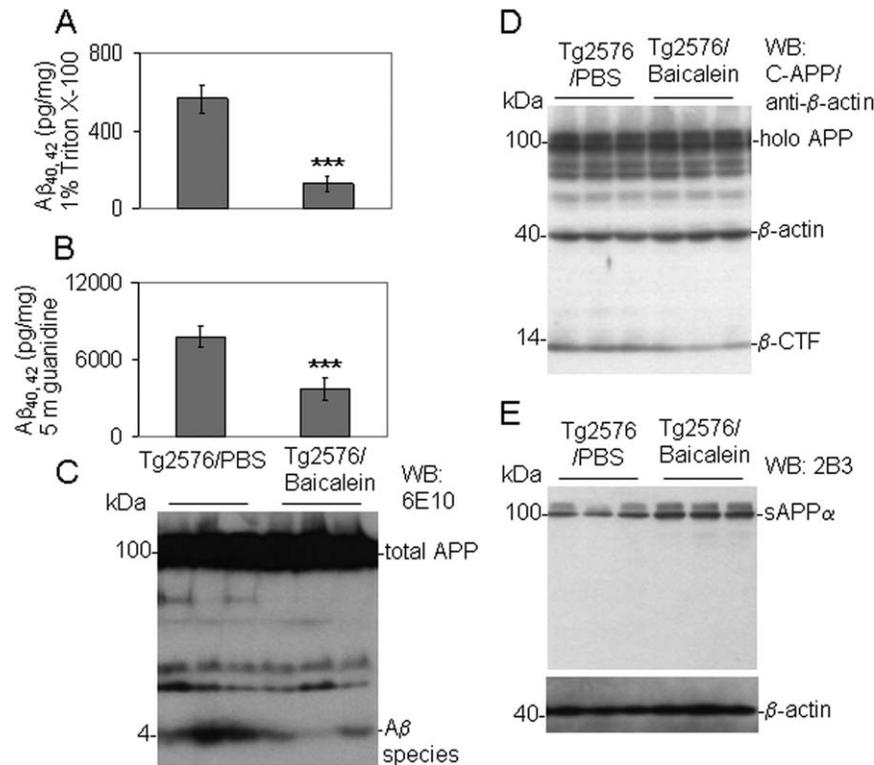


Fig. 4. Baicalein promotes nonamyloidogenic APP processing and reduces cerebral A β levels in Tg2576 mice. Mouse brain homogenates were prepared from Tg2576 mice treated with baicalein or PBS for analysis of detergent-soluble (A) and -insoluble (B) A $\beta_{40,42}$ prepared with 5 M guanidine by ELISA. In addition, soluble A β species were analyzed by WB using 6E10 (C). Data are presented as mean \pm SD of

A $\beta_{40,42}$ (pg/mg protein) in brain homogenates. For A and B, a *t*-test revealed a significant between-groups difference for either soluble or insoluble A $\beta_{40,42}$. ****P* < 0.01. Brain homogenates were also prepared for analysis of β -CTF by pAb751/770 antibody (D) and sAPP α by 2B3 antibody (E).

analyses. Bicuculline blocked baicalein-mediated reduction of A β (Fig. 3A,B), enhancement of sAPP α (Fig. 3B,C) and reduction of β -CTF (Fig. 3D). However, neither A β , nor sAPP α , nor β -CTF was altered by bicuculline alone (Fig. 3A–D). GABA_A receptor expression was detected in cell lysates prepared from cultured CHO/APPwt cells by WB analysis using a rabbit antibody against GABA_A receptor (06–868; EMD Millipore, Billerica, MA).

Baicalein Promotes Nonamyloidogenic APP Processing and Reduces A β Levels in Tg2576 Mice

We also investigated whether baicalein treatment could promote nonamyloidogenic APP processing and thereby impact cerebral A β levels in AD transgenic mice. Baicalein (10 mg/kg; *n* = 5) based on our previous study (Rezai-Zadeh et al., 2005) or PBS (*n* = 5) was injected i.p. daily for 8 weeks into 6-month-old female Tg2576 mice, and A β levels were determined by ELISA and WB. A β levels were markedly decreased in brain homogenate of Tg2576 mice treated with baicalein compared with those treated with PBS (Fig. 4A–C). As shown, Triton X-100 detergent-soluble A $\beta_{40,42}$ and detergent-insoluble A $\beta_{40,42}$ prepared with guanidine were reduced by 71%, and 53% respectively, compared with PBS-treated mice, as

determined by ELISA (Fig. 4A,B). This baicalein-mediated reduction of soluble A $\beta_{40,42}$ species was confirmed by WB using 6E10 antibody (Fig. 4C). In concert with the A β reduction, baicalein significantly reduced β -CTF (Fig. 4D) and significantly increased sAPP α in brain homogenate of Tg2576 mice (Fig. 4E) compared with PBS controls.

Baicalein Treatment Improves Cognitive Performance of Tg2576 Mice

Inasmuch as baicalein reduced APP amyloidogenic processing in Tg2576 mice, we speculated that baicalein would improve cognitive performance in these mice as well. To test our speculation, we determined working memory in the baicalein- or PBS-treated Tg2576 mice, as well as NT control mice, by the RAWM method. We found that the cognitive performance of the PBS-injected Tg2576 mice was significantly impaired compared with baicalein- or PBS-treated NT control mice in trial 5 (T5) working memory errors (Fig. 5). In addition, baicalein-treated Tg2576 mice performed significantly better than PBS-injected Tg2576 mice but no different from both baicalein- or PBS-treated NT control mice. Most interestingly, baicalein-treated Tg2576 mice and baicalein- or PBS-treated NT control mice, but not PBS-treated

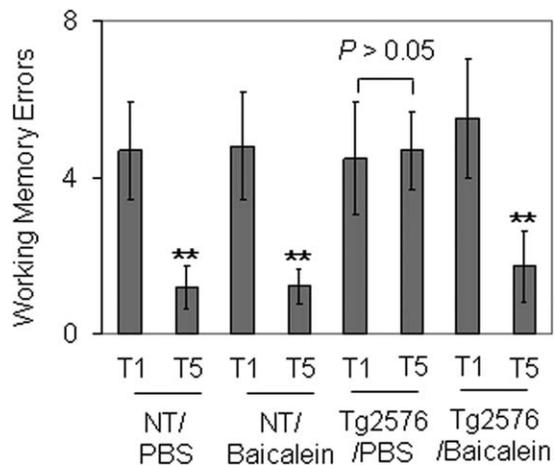


Fig. 5. Baicalein treatment significantly improves cognitive performance of Tg2576 mice. During the final weeks of 8-week treatment, all four groups of mice went to behavioral testing. PBS-injected Tg2576 mice were significantly impaired compared with both PBS- and baicalein-injected NT control mice, as determined by trial 5 (T5) working memory errors. Baicalein-treated Tg2576 mice performed significantly better than PBS-injected Tg2576 mice ($P < 0.05$) and no differently from either PBS- or baicalein-injected NT control mice. Most interestingly, baicalein-treated Tg2576 mice and PBS- or baicalein-injected NT control mice, but not PBS-injected Tg2576 mice, were able to decrease their working memory errors significantly in trial 5 (T5) compared with trial 1 (T1). ** $P < 0.01$. PBS-injected Tg2576 mice made substantially more T5 errors compared with the other three groups.

Tg2576 mice, were able to decrease their working memory errors significantly in T5 compared with T1 (Fig. 5).

DISCUSSION

Since the proposition of the A β hypothesis, many therapeutic studies on AD have focused on clinical trials of potential treatments that can reduce the production of A β ; however, to date, there is no safe and effective agent that can be used clinically (Panza et al., 2010). Our present study demonstrates for the first time that baicalein can directly reduce the production of A β from CHO/APPwt cells in a dose-dependent manner (Fig. 1A–C). This result was seen not only in CHO/APPwt cells but also in primary neuron cells cultured from Tg2576 mice (Fig. 1D–F). This finding was associated with the elevation of sAPP α production in the cell media and reduced β -CTF production in the cell lysate, indicating that baicalein promoted nonamyloidogenic APP processing in these cells (Fig. 2A–D). In concert with these findings, we found that baicalein can significantly reduce the A $\beta_{40,42}$ levels in the brains of an AD mouse model (Tg2576; Fig. 4A–C). These effects were associated with the promotion of sAPP α and reduction of β -CTF, indicating promotion of nonamyloidogenic APP processing (Fig. 4D,E). As was anticipated, the working memory errors tested in RAWM were also decreased after 8 weeks of baicalein treatment (Fig. 5). This indicated that baicalein can improve the learning and spatial memory abilities of Tg2576 mice.

These findings are very important in evaluating the potential use of baicalein in AD treatment as sAPP α is a known neurotrophic and neuroprotective factor. Previous studies have indicated that sAPP α can stimulate long-term potentiation, which is critical for learning and memory, and can also promote the outgrowth of neurites, synaptogenesis, and synaptic plasticity (Turner et al., 2003; Ring et al., 2007). sAPP α confers antiapoptotic and neuroprotective effects against excitotoxic and oxidative insults and prevents A β -induced neurodegeneration in vitro and in vivo (Mattson et al., 1993). Moreover, we have demonstrated that sAPP α can reduce β -secretase activity and A β generation (Obregon et al., 2012). A β oligomerization is very important in the formation of senile plaques (Roychoudhuri et al., 2009; Yankner and Lu, 2009), so sAPP α should also ameliorate these pathological changes in AD. Recently, Lu et al. (2011) found that baicalein can inhibit A β fibrillation and oligomerization, disaggregate preformed A β fibrils, and prevent A β fibril-induced toxicity in PC12 cells. These results undoubtedly provide several explanations for the beneficial effect of baicalein on AD models.

Our present study also demonstrates that baicalein decreases the production of A β and promotes nonamyloidogenic APP processing by activation of GABA $_A$ receptor signaling. As shown in Figure 3A,B, baicalein-mediated reduction of A β was counteracted by cotreatment with the GABA $_A$ receptor antagonist bicuculline. In concert with this finding, baicalein-mediated elevation of sAPP α , and reduction of β -CTF was also inhibited by bicuculline (Fig. 3B–D). GABA is known to be the most important inhibitory neurotransmitter in the CNS, playing a key role in long-term potentiation (Dawson et al., 2006). Previous studies have indicated that activation of GABA $_A$ receptor signaling elicits neuroprotection against A β -mediated toxicity (Gu et al., 2003; Lee et al., 2005). In addition, elevated GABA signaling appears to have a protective effect on neurogenesis. Tao et al. (2008) found that baicalein was the most potent inhibitor of GABA transaminase, which is the key enzyme metabolizing GABA to its inactive metabolite. Marcade and colleagues have demonstrated a direct relationship among GABA $_A$ receptor signaling, α -secretase, and sAPP α , which can directly inhibit the β -secretase activity (Marcade et al., 2008; Obregon et al., 2012).

Taken together, our present study indicates that baicalein can directly reduce A β production and promote nonamyloidogenic pathway in vitro and in vivo, thereby improving the learning and spatial memory performance of Tg2576 mice, effects associated with GABA $_A$ receptor signaling. Future clinical trials using this compound for treatment of AD are warranted, especially in light of recent vaccine trial failures (Liu et al., 2012).

ACKNOWLEDGMENTS

We thank Dr. Stefanie Hahn and Dr. Sascha Weggen (University of Heinrich Heine, Düsseldorf, Germany) for kindly providing us with CHO cells engineered to express wild-type human APP (CHO/APPwt).

REFERENCES

- Arendash GW, Gordon M, Diamond DM, Austin LA, Hatcher JM, Jantzen P, DiCarlo G, Wilcock D, Morgan D. 2001. Behavioral assessment of Alzheimer's transgenic mice following long-term Abeta vaccination: task specificity and correlations between Abeta deposition and spatial memory. *DNA Cell Biol* 20:737–744.
- Behrens CJ, van den Boom LP, Heinemann U. 2007. Effects of the GABAA receptor antagonists bicuculline and gabazine on stimulus-induced sharp wave-ripple complexes in adult rat hippocampus in vitro. *Eur J Neurosci* 25:2170–2181.
- Dawson GR, Maubach KA, Collinson N, Cobain M, Everitt BJ, MacLeod AM, Choudhury HI, McDonald LM, Pillai G, Rycroft W, Smith AJ, Sternfeld F, Tattersall FD, Wafford KA, Reynolds DS, Seabrook GR, Atack JR. 2006. An inverse agonist selective for alpha 5 subunit-containing GABAA receptors enhances cognition. *J Pharmacol Exp Ther* 316:1335–1345.
- de Carvalho RS, Duarte FS, de Lima TC. 2011. Involvement of GABAergic non-benzodiazepine sites in the anxiolytic-like and sedative effects of the flavonoid baicalein in mice. *Behav Brain Res* 221:75–82.
- Gu Z, Zhong P, Yan Z. 2003. Activation of muscarinic receptors inhibits beta-amyloid peptide-induced signaling in cortical slices. *J Biol Chem* 278:17546–17556.
- Hahn S, Bruning T, Ness J, Czirr E, Baches S, Gijzen H, Korth C, Pietrzik CU, Bulic B, Weggen S. 2011. Presenilin-1 but not amyloid precursor protein mutations present in mouse models of Alzheimer's disease attenuate the response of cultured cells to gamma-secretase modulators regardless of their potency and structure. *J Neurochem* 116:385–395.
- Hardy JA, Higgins GA. 1992. Alzheimer's disease: the amyloid cascade hypothesis. *Science* 256:184–185.
- Hardy J, Selkoe DJ. 2002. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* 297:353–356.
- Hardy J, Duff K, Hardy KG, Perez-Tur J, Hutton M. 1998. Genetic dissection of Alzheimer's disease and related dementias: amyloid and its relationship to tau. *Nat Neurosci* 1:355–358.
- Heo HJ, Kim DO, Choi SJ, Shin DH, Lee CY. 2004. Potent inhibitory effect of flavonoids in *Scutellaria baicalensis* on amyloid beta protein-induced neurotoxicity. *J Agric Food Chem* 52:4128–4132.
- Heo H, Shin Y, Cho W, Choi Y, Kim H, Kwon YK. 2009. Memory improvement in ibotenic acid induced model rats by extracts of *Scutellaria baicalensis*. *J Ethnopharmacol* 122:20–27.
- Jianjun J, Huiru D. 2008. Preparation of high-purity baicalein from *Scutellaria baicalensis* Georgi. *Nat Prod Res* 22:1410–1412.
- Lee BY, Ban JY, Seong YH. 2005. Chronic stimulation of GABAA receptor with muscimol reduces amyloid beta protein (25–35)-induced neurotoxicity in cultured rat cortical cells. *Neurosci Res* 52:347–356.
- Liu C, Wu JL, Gu J, Xiong Z, Wang F, Wang JZ, Wanu W, Chen JG. 2007. Baicalein improves cognitive deficits induced by chronic cerebral hypoperfusion in rats. *Pharmacol Biochem Behav* 86:423–430.
- Liu YH, Giunta B, Zhou HD, Tan J, Wang YJ. 2012. Immunotherapy for Alzheimer disease—the challenge of adverse effects. *Nat Rev Neurol* 8:465–469.
- Lu JH, Ardah MT, Durairajan SSK, Liu LF, Xie LX, Fong WFD, Hasan MY, Huang JD, El-Agnaf OMA, Li M. 2011. Baicalein inhibits formation of alpha-synuclein oligomers within living cells and prevents A beta peptide fibrillation and oligomerisation. *Chembiochem* 12: 615–624.
- Marcade M, Bourdin J, Loiseau N, Peillon H, Rayer A, Drouin D, Schweighoffer F, Desire L. 2008. Etazolate, a neuroprotective drug linking GABAA receptor pharmacology to amyloid precursor protein processing. *J Neurochem* 106:392–404.
- Mattson MP, Cheng B, Culwell AR, Esch FS, Lieberburg I, Rydel RE. 1993. Evidence for excitoprotective and intraneuronal calcium-regulating roles for secreted forms of the beta-amyloid precursor protein. *Neuron* 10:243–254.
- Obregon DF, Rezaei-Zadeh K, Bai Y, Sun N, Hou H, Ehrhart J, Zeng J, Mori T, Arendash GW, Shytle D, Town T, Tan J. 2006. ADAM10 activation is required for green tea (–)-epigallocatechin-3-gallate-induced alpha-secretase cleavage of amyloid precursor protein. *J Biol Chem* 281:16419–16427.
- Obregon D, Hou HY, Deng J, Giunta B, Tian J, Darlington D, Shahaduzzaman M, Zhu YY, Mori T, Mattson MP, Tan J. 2012. Soluble amyloid precursor protein-alpha modulates beta-secretase activity and amyloid-beta generation. *Nat Commun* 3:777.
- Panza F, Frisardi V, Imbimbo BP, Capurso C, Logroscino G, Sancarlo D, Seripa D, Vendemiale G, Pilotto A, Solfrizzi V. 2010. Review: gamma-secretase inhibitors for the treatment of Alzheimer's disease: the current state. *CNS Neurosci Ther* 16:272–284.
- Rezaei-Zadeh K, Shytle D, Sun N, Mori T, Hou H, Jeannot D, Ehrhart J, Townsend K, Zeng J, Morgan D, Hardy J, Town T, Tan J. 2005. Green tea epigallocatechin-3-gallate (EGCG) modulates amyloid precursor protein cleavage and reduces cerebral amyloidosis in Alzheimer transgenic mice. *J Neurosci* 25:8807–8814.
- Ring S, Weyer SW, Kilian SB, Waldron E, Pietrzik CU, Filippov MA, Hermis J, Buchholz C, Eckman CB, Korte M, Wolfer DP, Muller UC. 2007. The secreted beta-amyloid precursor protein ectodomain APPs alpha is sufficient to rescue the anatomical, behavioral, and electrophysiological abnormalities of APP-deficient mice. *J Neurosci* 27:7817–7826.
- Roychaudhuri R, Yang M, Hoshi MM, Teplow DB. 2009. Amyloid beta-protein assembly and Alzheimer disease. *J Biol Chem* 284:4749–4753.
- Tao YH, Jiang DY, Xu HB, Yang XL. 2008. Inhibitory effect of *Eriogonum breviscapus* extract and its flavonoid components on GABA shunt enzymes. *Phytomedicine* 15:92–97.
- Turner PR, O'Connor K, Tate WP, Abraham WC. 2003. Roles of amyloid precursor protein and its fragments in regulating neural activity, plasticity and memory. *Prog Neurobiol* 70:1–32.
- Wang SY, Wang HH, Chi CW, Chen CF, Liao JF. 2004. Effects of baicalein on beta-amyloid peptide-(25–35)-induced amnesia in mice. *Eur J Pharmacol* 506:55–61.
- Yankner BA, Lu T. 2009. Amyloid beta-protein toxicity and the pathogenesis of Alzheimer disease. *J Biol Chem* 284:4755–4759.
- Zhu M, Rajamani S, Kaylor J, Han S, Zhou F, Fink AL. 2004. The flavonoid baicalein inhibits fibrillation of alpha-synuclein and disaggregates existing fibrils. *J Biol Chem* 279:26846–26857.
- Zhu Y, Hou H, Rezaei-Zadeh K, Giunta B, Ruscin A, Gemma C, Jin J, Dragicevic N, Bradshaw P, Rasool S, Glabe CG, Ehrhart J, Bickford P, Mori T, Obregon D, Town T, Tan J. 2011. CD45 deficiency drives amyloid-beta peptide oligomers and neuronal loss in Alzheimer's disease mice. *J Neurosci* 31:1355–1365.