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Antidepressant natural flavonols modulate BDNF and beta amyloid in neurons and hippocampus of double TgAD mice

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SUMMARY

Increasing evidence suggests that depression may be both a cause and consequence of neurological disorders such as Alzheimer's disease (AD), and that antidepressants could provide an alternative strategy to current AD therapies. Association of side effect and herbal-drug interaction with conventional antidepressant and St. John's wort warrant investigating new antidepressant drugs. Antidepressant effects of *ginkgo biloba* extract (EGb 761) have been demonstrated in animal models of depression and in human volunteers. We report here that ginkgo flavonols quercetin and kaempferol stimulates depression-related signaling pathways involving brain-derived neurotrophic factor BDNF/phosphorylation of cyclic AMP response element binding protein CREB/postsynaptic density proteins PSD95, and reduces amyloid- β peptide (A β) in neurons isolated from double transgenic AD mouse (TgAPPswe/PS1e9). In addition, enhanced BDNF expression and reduction of A β oligomers was confirmed in hippocampus of the double transgenic mice administered with flavonol, which correlates with cognitive improvement behaviors in these mice. The present results suggest that stimulating BDNF and reducing A β toxicity by natural flavonols provide a therapeutic implication for treatment of AD.

Keywords

depression; neuroplasticity; amyloid beta oligomers; natural flavonoids

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INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive impairment of memory and cognition. Increasing clinical and experimental evidence implicates an overlap of pathogenesis between AD and neuropsychiatric depression (Starkstein et al., 2008). Despite controversial results from pharmacotherapy studies of antidepressant treatment in AD, existing clinical data suggest that depression in AD patients responds to antidepressant medication (Raskind 2008), implicating that antidepressants could provide an alternative strategy to current AD therapies.

Like most of the synthetic drugs, the antidepressant drugs are not without side effects. In recent years, St. John's Wort (SJW) and *ginkgo biloba* extract (EGb 761) are often reported as antidepressant and used at therapeutic levels (Blumenthal 2009). Substantial experimental data indicate that EGb 761 has neuroprotective and neuromodulatory effects (Luo 2003) probably due to its two major constituents, flavonoids (24%) and terpenoids (6%) (Smith et al., 2004). In animal studies, EGb 761 improves cognitive function in an age-related memory impairment model (Stoll et al., 1996; Cohen-Salmon et al., 1997), and in a transgenic mouse model of AD (Stackman et al., 2003). It was revealed recently that EGb 761 displays anti-depressive and anti-stress actions in animal behavioral models (Naik et al., 2006; Sakakibara et al., 2006; Kalkunte et al., 2007), and in human volunteers (Hemmeter et al., 2001; Trick et al., 2004). These observations prompted us to test the hypothesis that modulation of depression-related neuroplastic pathways by active constituents of ginkgo extract may explain its anti-depressant action and provide therapeutic impact to depressive disorders in AD.

The flavonoids used in this study were extracted from Ginkgo leaves, primarily composed of quercetin, kaempferol and isorhamnetin, specifically named under a subgroup flavonol (Bedir et al., 2002). Flavonoids extracted from many nature plants is known to exert antidepressant-like effect in animal models of depression (Messaoudi et al., 2008; Zhao et al., 2008; Sanchez-Mateo et al., 2009). Flavonoid from peels of citrus improves memory impairment and A β pathology in a transgenic mouse model of AD (Onozuka, et al., 2008). Flavonoids from green tea and citrus have also been reported to upregulate brain-derived neurotrophic factor BDNF/phosphorylation of cyclic AMP response element binding protein CREB pathway and improve spatial memory (Matsuzaki et al., 2006; Li et al., 2009). Activation of transcription factor CREB and the subsequent induction of plasticity-enhancing genes such as BDNF have been considered as an underlying mechanism for neuroplasticity (Pittenger et al., 2008). Both BDNF and CREB phosphorylation-associated signaling pathway were impaired in animal model of AD and stress (Yamamoto-Sasaki et al., 1999; Lee et al., 2005); and antidepressants rescued both (Duman et al., 2006; Nelson et al., 2007). Our previous studies reported that EGb761 restored impaired phosphorylation of CREB and BDNF expression in A β -expressing neuroblastoma cells and enhances adult hippocampal neurogenesis and phosphorylation of CREB in transgenic mouse model of AD (Tchantchou et al., 2007; Xu et al., 2007). These data suggests that flavonols, the major active constituents of EGb 761, may up-regulate CREB-BDNF pathway that contribute to its antidepressant activity.

In this study, we used primary cortical neurons isolated from double transgenic (TgAPP/PS1) AD mice expressing human mutant amyloid precursor protein APP and presenilin-1 PS1, and employed depression-related behavioral assays in the double transgenic mouse. Our results support the view that stimulating BDNF signaling pathway may serve as a mechanism for antidepressant activity of the flavonols.

METHODS

Material

Flavonols quercetin (4.8% in extract, all based on weight), kaempferol (3.9% in extract) and isohamnetin (0.7% in extract) was analyzed in Dr. Ikhlas Khan's laboratory at National Center for Natural Product Research in Oxford, MS (Bedir et al., 2002). Ginkgo flavonoids extracted was acid hydrolyzed before HPLC analysis. Thus, glycosides had converted to its related aglycon.

BDNF levels were analyzed by BDNF Emax immunoassay system from Promega (Madison, WI). Antibody against amyloid peptide (6E10) was obtained from Covance (Emeryville, CA). Antibody against N-Methyl-D-Aspartate receptors NMDAR1 was obtained from Chemicon International (Temecula, CA). Antibody against PSD 95 was purchased from Cell Signaling (Danvers, MA).

Primary cell culture and treatment

Heterozygous APP^{swe}/PS1 Δ 9 transgenic (TgAPP/PS1) founder mice were purchased from the Jackson lab (Bar Harbor, ME). The pregnant mice were obtained by cross-breeding of female wild-type (WT, B6C3) and male transgenic breeders. The primary neuronal cells were prepared from embryonic day 14 (E14) mice as previously described (Takahashi et al., 2004). Briefly, the cerebral cortex was dissected and incubated with 0.25% trypsin followed with triturating by glass pipettes for dissociation. The dissociated neuronal cells were seeded on the D-polylysine pre-coated 6-well plates at the density of 5×10^5 per well in neurobasal media supplemented with B27 and glutamine. The cells were maintained in a 37 °C incubator with 5% CO₂/95% air for 14 days in vitro (14 DIV). All the embryos were genotyped. 14 DIV cortical neurons were treated with DMSO or the flavonols at 50 µg/ml for 24h. For the dose effect assay, the neurons were incubated with different concentrations of flavonols (12.5, 25, 50 µg/ml) for 24h. To determine glutamate-induced phosphorylation of CREB by flavonol treatment, the neurons were exposed to glutamate 50 µM for 15min after 24 h incubation of flavonols. Pharmacological inhibitors for protein kinase A PKA (H89, 50 µM), Ca²⁺/calmodulin-regulated protein kinase II CaMKII (KN-93, 50 µM), mitogen activated protein kinases MAPK kinase (PD98059, 20 µM) and NMDA receptor antagonist D,L-2- amino-5-phosphonovaleric acid (APV, 50 µM) were used 1h before glutamate exposure.

Western blotting

Cortical neurons or hippocampus were sonicated for 20s in RIPA buffer (100mM Tris-HCl, 100mM NaCl, 1mM EDTA, 1mM EGTA, 1mM NaF, 20mM Na₄P₂O₄, 2mM Na₃VO₄, 1% Triton X-100, 1% glycerol, 0.1% SDS, 0.5% deoxycholate) with cocktail of protease inhibitors and phosphatase inhibitor. The supernatant was collected by centrifugation at 12000g for 15 min at 4°C. Protein (30 µg) from each sample was loaded on 10% SDS-acrylamide gels for electrophoresis and transferred onto 0.22 µm PVDF membrane at 100V for 2 h. After blocking with 5% fat-free milk in PBST, the membrane was incubated with primary antibody overnight at 4°C for BDNF, pCREB detection. For A β detection, the neurons or hippocampus were lysed in lysis buffer (75mM Tris-HCl; 50mM NaCl; 1mM DTT; 5mM EDTA; 1mM EGTA) with protease inhibitor cocktail. Protein (50 µg) from each sample was loaded on 10% tricine gels for electrophoresis. The blots were incubated with corresponding secondary antibodies and developed with enhanced chemiluminescence (ECL) reagents. In some cases, the membrane was placed in stripping buffer (62.5mM Tris-HCl, pH 6.8, 2% SDS, 100mM β -mercaptoethanol) for 30 min at 50°C followed by reprobing with tubulin antibody.

Biotinylation assays

After flavonol treatment, biotinylation of cell surface proteins were performed using a Biotinylation kit (Pierce, Rockford, IL). Cells were rinsed with ice-cold PBS supplemented with 0.1 mM calcium chloride and 1 mM magnesium chloride (PBS-Ca/Mg), cells were then labeled with 1 mg/ml N-hydroxysulfosuccinimidobiotin (NHS-biotin) in PBS-Ca/Mg for 30 min at 4°C with slight agitation. After incubation, biotin was quenched by PBS-Ca/Mg containing 100 mM glycine for 30 min at 4°C. Cells were lysed using RIPA buffer (100mM Tris-HCl, 100mM NaCl, 1mM EDTA, 1mM EGTA, 1mM NaF, 20mM Na4P2O4, 2mM Na3VO4, 1% Triton X-100, 1% glycerol, 0.1% SDS, 0.5% deoxycholate) and a cocktail of protease inhibitors. Lysates were sonicated and centrifuged at 14000g to remove nucleic acids and debris. Half of the lysate fraction was saved for analysis of total protein. The remaining lysate was agitated overnight at 4°C with an equal volume of Ultralink immobilized monomeric avidin beads in order to isolate biotinylated proteins. The lysate-bead mixture was centrifuged, and the supernatant was retrieved (non-biotinylated fraction) and saved for Western blotting analysis. Bead bound protein was eluted into SDS buffer with 2-mercaptoethanol (biotinylated fraction). The lysate and non-biotinylated fractions were all diluted in sample buffer such that the immunoreactivity in the biotinylated and non-biotinylated fractions equaled that observed in the lysate fraction if the yield from extraction is 100%.

Animal treatment

Heterozygous APP^{swe}/PS1^{Δ9} transgenic (TgAPP/PS1) founder mice purchased from the Jackson lab, were maintained by crossbreeding with wild-type (WT, B6C3) breeders and the resultant offspring were genotyped. Eight-month old male TgAPP/PS1 mice and age-matched WT mice (4 in each treatment group) were administered flavonols 50mg/kg or an equivalent amount of vehicle (Carboxymethylcellulose Sodium) once per day by gavage for 4 months. In the last week of flavonol administration, all the mice were trained twice everyday in Morris water maze on five consecutive days. 48h after the last training, mice were sacrificed by decapitation. The right side of hippocampus was used for Western blotting analysis, and the left side was used for immunohistochemical staining analysis. For testing anti-depressant response, flavonol or vehicle was i.p. administered to the mice for 7 days followed by a tail suspension test in the mice. The Institutional Animal care and Use Committee approved all animal handling and treatment.

Immunohistochemistry

The left side of brain was fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) immediately when the brains were removed out for overnight. The brains were transferred to 30% sucrose in 0.1 M PBS until they sank, then frozen in dry ice/isopen-tane and stored at -80°C until sectioning. Each brain was serially sectioned in the coronal plane on a microtome. Sections were cut at an instrument setting of 50 μm. Aβ deposition in the hippocampus were visualized by immunostaining of free floating frozen sections using a mouse monoclonal antibody to 6E10 (1:1000 dilution; Covance) in 1% goat serum and 0.3% Triton X-100 in PBS overnight at 4°C. Sections were washed in PBS and incubated with 1% goat serum and goat anti-mouse biotinylated IgG (1:200, Vector Laboratories, Burlingame, CA) for 1.5 h on a shaker at room temperature, then rinsed and incubated in avidin-biotin complex (Elite kit, Vector Laboratories, Burlingame, CA) for 1.5 h. The visualization reaction was completed with DAB and enhanced by nickel. Sections were mounted on slides and dried overnight, dehydrated in graded ethanols, cleared in xylene and coverslipped with DPX (BDH) mounting medium. Specificity of the immune reactions was ensured by omitting the primary antiserum.

Tail suspension test (TST)

TST was performed according to Steru *et al.* (Steru et al., 1987) with slight modifications. In brief, wild type (wt) and the double transgenic (tg) mice was divided into five groups and treated as follows: one group was inter-peritoneally (i.p) administered fluoxetine (10 mg/kg body weight), two groups were i.p. administered with vehicle (0.1%DMSO in PBS), and two groups were i.p. administered with flavonols (50 mg/kg body weight) for 7 days. Test solutions were injected 30 min prior to the TST. Two stands, each with a clamp located ~ 40 cm from the bench top, were placed at intervals of 30 cm. A mouse was hung 2 cm from the end of its tail on a stand, and recorded with a stopwatch for 6 min. Immobility time was evaluated by observers with no information about the treatment situation of the mice.

Locomotive assays

The locomotor activity was measured by automated activity counter (ENV-510, med associates Inc) in the mice administered with flavonol or vehicle and after recovering for 1 week from the TST. The size of the four-sided chamber is 27×27×20.3 cm. Sixteen evenly spaced I/R beams that are settled around the periphery of the chamber track the movement of the mice for 30 min. One-way ANOVA is applied to determine if there is a difference between different groups followed by post hoc Dunnett's multiple comparison test.

Morris Water Maze

Eight-month old TgAPP/PS1 male mice and age-matched WT mice (6–8 in each treatment group) were oral administered with flavonol (50mg/kg) or equivalent amount of vehicle (Carboxymethylcellulose Sodium) once per day for 4 months. In the last week of the administration, spatial water-task training was performed (Pollak et al., 2005). On five consecutive days mice were trained and tested in a circular black tank (140cm diameter, walls 30cm high) filled to a depth of 25cm with 1% milk (24°C). A submerged platform (15cm diameter, 1.5cm below the surface of the water) was located at a fixed location throughout all the days of training. Mice were placed in the tank with head facing the wall from a fixed position along the side of the tank. Mice were given 120s to find the platform. If a mouse failed to find the platform within 120s, it was guided to the platform by hand and allowed to stay there by 10s. The time needed to find the platform was recorded (latency).

Statistical analysis

Quantification of immunoreactive bands was performed using an Alpha image analysis system. Statistical analysis was carried out using one-way ANOVA by SPSS, the values $p < 0.05$ was considered statistically significant. Graphpad software was used to analyze the statistical difference between the groups in behavioral assays (Tail suspension test, locomotion and Morris water maze). One-way ANOVA was used followed by dunnett's multiple comparison tests. The difference is considered acceptable when $P < 0.05$.

RESULTS

1. Flavonols dose-dependently stimulate levels of BDNF in neurons derived from Tg APP/PS1 mouse embryo and in hippocampus of the Tg mice

Previously, we have demonstrated differential effect of ginkgolides on pCREB and BDNF expression in a A β -expressing cell line (Xu et al., 2007). To investigate whether ginkgo flavonols affect on BDNF expression, primary neurons (E14) isolated from the brains of wild type mice (B6C3) or double transgenic mice (TgAPP/PS1) embryos were exposed to flavonols or vehicle (DMSO, 0.01%) for 24h, followed by immunoblotting. The concentration of flavonols (50 μ g/ml) was determined based on previous studies conducted in a neuroblastoma cell line and was the maximal non-toxic concentration determined by cell viability assay (data

not shown). Quantitative analysis of the immunoblots (Fig. 1A) shows that BDNF expression was significantly diminished in Tg APP/PS1 neurons (WT Ctrl: 100%±5% vs. Tg Ctrl: 54%±13%, $p=0.004$); flavonols at 50µg/ml significantly restored BDNF expression as compared to the vehicle control in Tg APP/PS1 primary neurons (Tg Ctrl: 54%±13% vs. Tg+flavon: 79%±5%, $p=0.029$). Flavonol at 50µg/ml also increased BDNF expression in WT primary neurons, however it was not statistically significant (WT Ctrl: 100%±5% vs. WT+flavon: 117%±8%, $p=0.055$). Next, the Tg APP/PS1 neurons were treated with flavonols at concentrations ranging from 12.5 to 50µg/ml for 24h, the result of immunoblotting shows that flavonols dose-dependently increased BDNF expression (Fig. 1B).

It is well known that activation of transcription factor CREB and the subsequent induction of BDNF serve as one of the most critical molecular mechanism of neuroplasticity (Soule et al., 2006). As an effector gene, BDNF expression is induced by CREB activation (Johannessen et al., 2004). Through activation of high-affinity cell surface receptor (trkB), BDNF signaling at synapses enhances long-term potentiation (LTP) and promotes the outgrowth of dendrites (Mattson et al., 2004). Since expression of BDNF is regulated by pCREB, we further investigated the effect of flavonols on phosphorylation of CREB. Primary neurons isolated from the brain of wild type mice (B6C3) or transgenic mice (TgAPP/PS1) embryos were treated with flavonols (50µg/ml) for 24h, followed by immunoblotting. Basal levels of pCREB were slightly lower in Tg APP/PS1 neurons as compared to WT neurons (Fig. 1C), and treatment with 50µg/ml flavonola did not affect levels of pCREB as compared to vehicle controls in both the Wt and Tg neurons (Fig. 1C).

To confirm the effect of flavonols on BDNF expression and CREB phosphorylation *in vivo* in an animal model of AD, flavonols (50mg/kg) was orally administrated to TgAPP/PS1 mice at age of 8 month for 4 months. Consistent with our findings of BDNF *in vitro*, oral administration of flavonols significantly increased levels of BDNF in the hippocampus of Tg APP/PS1 mice (BDNF: Tg: 55%±9% vs. Tg+flavon: 78%±6%, $p=0.020$). Mice administrated with flavonols did not shown significant enhanced levels of phosphorylated CREB in the hippocampus (pCREB: Tg: 77%±5% vs. Tg+flavon: 85%±3%, $p=0.077$). Similar as observed in the neurons, this might be due to the nature of CREB activation by phosphorylation, i.e., its basal level remains in a dynamic equilibrium without extracellular stimulus.

2. Flavonols enhance glutamate-evoked pCREB in neurons derived from Tg APP/PS1 mouse, which is associated with activation of NMDA receptors and PSD95 in isolated neurons

Given that basal, dynamic levels of pCREB may mask the changes induced by Aβ and/or flavonols treatment in the neurons derived from the Tg mice, we further examined the effect of flavonols on glutamate-evoked phosphorylation of CREB. Glutamate is the major excitatory neurotransmitter in the hippocampus and mediates the neuronal response through CREB activation, eventually leading to LTP formation and synapse strengthening (Malgari et al., 1992). Brief glutamate stimuli have been used to investigate the biochemical changes underlying synaptic plasticity on cultured hippocampal neurons (Vitolo et al., 2002; Matsuzaki et al., 2006). Therefore, we extended our investigations by measuring pCREB levels in response to a glutamate stimulus. After 24 h incubation with flavonols or vehicle, both WT and Tg neurons were treated with glutamate at 50µM for 15min, followed by immunoblotting. Treatment with glutamate robustly increased pCREB level by 5 folds versus basal pCREB level in WT neurons, whereas in Tg neurons, treatment of glutamate increased pCREB level by 2.5 folds versus basal pCREB level (Fig. 2A). It indicates that glutamate-induced CREB phosphorylation was significantly impaired in Tg neurons. Fig. 2B shows that glutamate-evoked pCREB was significantly restored in flavonol-treated Tg neurons (Tg Ctrl: 42%±8% vs. Tg+flavon: 75%±3%, $p=0.013$), whereas flavonols had no effect on glutamate-evoked pCREB in the wild type mice (WT Ctrl: 100%±4% vs. WT+flavon: 101%±2%, $p=0.718$).

To reveal the specific downstream pathway by which flavonols stimulate glutamate-evoked levels of pCREB, various pharmacological inhibitors were used to inhibit PKA (H89, 50 μ M), CaMKII (KN-93, 50 μ M) or MAPK kinase (PD98059, 20 μ M) and to block NMDA receptors (D,L-2- amino-5-phosphonovaleric acid APV, 50 μ M). These inhibitors or antagonist were administered to Tg neurons for 1 h prior to flavonols treatment (for 1 h) and pre-incubation of glutamate (for 15 min). The cells were then subjected to immunoblotting. We assume that if a specific molecule upstream of CREB activation is involved, then the enhanced pCREB by flavonol would be blocked in neurons treated with inhibitor of this molecule. As shown in Fig. 2C, flavonols 50 μ g/ml significantly increased glutamate-evoked pCREB (Ctrl: 100% $\pm$ 7% vs. flavon: 190% $\pm$ 46%, $p=0.029$), and this enhancement was only significantly blocked by NMDA receptor antagonist APV (H89: 177% $\pm$ 15% vs. flavon: 190% $\pm$ 46%, $p=0.658$; KN93: 162% $\pm$ 8% vs. flavon: 190% $\pm$ 46%, $p=0.350$; PD98059: 167% $\pm$ 13% vs. flavon: 190% $\pm$ 46%, $p=0.443$; APV: 108% $\pm$ 8% vs. flavon: 190% $\pm$ 46%, $p=0.038$), implicating that the effect of flavonols is associated with NMDA receptors activation rather than some cytosolic kinases PKA, CaMKII, and MAPK.

To further determine the consequence of NMDA receptor activation, we investigated the effect of flavonols on NR1, the essential subunit of NMDA receptor and PSD95 expression in Tg neurons. Since NR1 and PSD95 primarily located at postsynaptic density, we performed immunoblotting on cell surface protein isolated by biotinylation assay. Tg neurons were incubated with flavonols 50 μ g/ml or vehicle (0.01% DMSO) for 24h, followed by immunoblotting. Figure 2D shows that the levels of NR1 and PSD95 were reduced in Tg neurons as compared with WT neurons (NR1: WT 100% $\pm$ 5% vs. Tg 65% $\pm$ 12%, $p=0.009$; PSD95: Tg: 100% $\pm$ 3% vs. 73% $\pm$ 6%, $p=0.003$). It also shows that flavonols partially restored the levels of cell surface NR1 and PSD95 in Tg neurons (NR1: Tg 65% $\pm$ 12% vs. Tg+flavon: 88% $\pm$ 8%, $p=0.049$; PSD95: Tg: 73% $\pm$ 13% vs. Tg+flavon: 87% $\pm$ 6%, $p=0.048$). Flavonols at 50 μ g/ml had no effect on total NR1 and PSD95 expression (data not shown).

3. Flavonols reduced soluble A β in neurons derived from Tg APP/PS1 mouse embryo and A β load in hippocampus of the Tg mice

The reduced levels of BDNF and glutamate-evoked pCREB in Tg neurons and hippocampus of the transgenic mice (Fig. 1 and 2) implicates that A β may play a role in BDNF/pCREB-mediated, depression-related, signaling pathway. However, flavonols restore reduced levels of BDNF and pCREB in Tg neurons rather than in WT neurons (Fig. 1A and 2B). To delineate the relationship between A β toxicity and flavonols *in vivo*, we further examined the levels of intracellular and extracellular A β in Tg neurons treated with flavonols. Fig. 3B shows that treatment with flavonols at 50 μ g/ml significantly decreased both intracellular and medium A β levels (Intracellular A β , Tg Ctrl: 100% $\pm$ 5% vs. Tg+flavon 50 μ g/ml: 73% $\pm$ 9%, $p=0.0092$; medium A β , Tg Ctrl: 100% $\pm$ 7% vs. Tg+flavon 50 μ g/ml: 74% $\pm$ 11%, $p=0.023$). Flavonols also significantly decreased levels of low molecular A β (~7kD) in hippocampus of the transgenic mice (Fig. 3B, Tg Ctrl 100% $\pm$ 4% vs. Tg+flavon, 69% $\pm$ 19%, $p=0.047$). To confirm and visualize the changes of A β in hippocampus, immunohistochemical staining for A β was performed in the brain of those mice, using anti-A β antibody (6E10). Representative images of brain from three animals in each group showed that in the transgenic mice (8 month olds) administered with flavonols for 4 mon, A β deposition and plaques formation in hippocampus were remarkably reduced compared with that of the vehicle control mice (Fig. 3C), further support an involvement of A β toxicity in the actions of the flavonols.

4. Flavonols exhibit antidepressant-like activity in wild type mice and restore impaired cognitive functions in Tg mice

The anti-depressant activities of EGb 761 in animal models of depression and in clinical trials have been reported from several independent studies (Hemmeter et al., 2001; Trick et al.,

2004; Naik et al., 2006; Sakakibara et al., 2006; Kalkunte et al., 2007). To determine this activity of the flavonols in the double transgenic mouse, tail suspension test (TST) was conducted in 12-month old transgenic mice and their wild type littermate treated with ginkgo flavonols. The dosage of 50mg/kg was chosen for previous studies have reported that at this dose *ginkgo biloba* extract exhibited anti-depressant activity in rodent (Sakakibara et al., 2006). For this test, flavonols was administered by i.p. for 7 days to be consistent with the treatment with fluoxetine which is served as a positive control. As shown in Fig. 4A, administration of flavnols at dosage of 50 mg/kg for 7 days significantly reduced the immobility time in wild type animals (WT: Ctrl 138±16s, falvonol 96±19s, n=7–8, p=0.005). As a positive control, immobility time in the TST mice was also significantly reduced after acute i.p. treatment with SSRI antidepressant drug fluoxetine (10mg/kg) (WT: control 138±16s, fluoxetine 71±16s, n=4–8, p=0.001). However, the changes of immobile time were not statistically significant in the double transgenic mice treated with flavonols (Tg: control 122 ±37s; flavonol 93±19s, n=4–6, p=0.21). The decrease in immobility by flavonols was comparable to that of the fluoxetine group.

To determine if the antidepressant-like action by flavonols is specific, locomotor activities were tested in these mice after a short recovery time (1 day). Neither flavonols (50mg/kg) nor fluoxetine (10mg/kg) administration resulted in any significant changes in locomotor's functions (Fig. 4B) suggesting that reduced immobile time by flavonol is not due to its effect on animal's locomotion.

As an additional measurement and/or a comparison, a cognitive function in Tg mice treated with flavonols was tested for their spatial learning using Morris Water Maze. In the last week of flavonols administration for 4 month (at 50mg/kg daily), the transgenic mice and their wild type littermates were trained in Morris Water Maze. The result illustrates an obvious impairment of spatial memory of TgAPP/PS1 mice as compared to age-matched WT control mice. In TgAPP/PS1 mice administrated with flavonols, significantly improved their impaired spatial learning was observed (Fig. 4C), similar as previously reported observations in Tg2576 mice treated with EGb 761 (Stackman et al., 2003).

DISCUSSION

In this study, we demonstrate stimulation by flavonols on BDNF/CREB-mediated signaling pathway in neurons and in brain of double transgenic mice bearing human mutant APP and PS genes. This stimulus activity correlates with reduced A β burden in neurons and in hippocampus of Tg mice brain, and with restoring cognitive activity in animal behavioral assays.

Previous study in our lab indicated that BDNF expression was impaired in A β expressing neuroblastoma cells (Xu et al., 2007). Here, we found that BDNF expression was prominently decreased in cortical neurons prepared from TgAPP/PS1 transgenic mice as compared to wild type neurons. Flavonols treatment dose-dependently restored BDNF expression in transgenic cortical neurons. However, no significant reduction in basal pCREB level in transgenic neurons as compared to wild type neurons was observed, and flavonols treatment did not affect basal levels of pCREB. This observation may not be surprising, previous studies indicated that A β treatment did not affect basal phosphorylation of CREB in cultured neurons (Snyder et al., 2005) despite decreased pCREB seen in AD patients and animal models of AD. The discrepancy is apparently due to low basal activity of the NMDA receptors, which mask the changes of pCREB in WT and Tg mice, as well as in response to the flavonols treatment. Stimulation of synaptic NMDA receptors by glutamate promotes transcriptional activation through phosphorylation of CREB at Ser133 (Shaywitz et al., 1999), that is proven successfully by our observation that glutamate-evoked pCREB signaling pathway was severely impaired in transgenic neurons. Our result was consistent with previous report that NMDA receptor-

activated phosphorylation of CREB is robustly reduced by A β (Vitolo et al., 2002; Snyder et al., 2005; Matsuzaki et al., 2006). Along the same argument, flavonols treatment significantly restored impaired CREB signaling pathway in transgenic neurons.

In search for cellular mechanisms that mediating flavonol-stimulated phosphorylation of CREB, pharmacological antagonists was applied to the neurons. Our result indicates that among several inhibitors used; only NMDA receptor antagonist APV dramatically blocked flavonol-stimulated phosphorylation of CREB, implicating the effect of flavonols depends on NMDA receptors. Glutamate stimulates CREB phosphorylation via several distinct signaling pathways (Shaywitz et al., 1999), including cAMP/PKA pathway (Chain et al., 1999; Vitolo et al., 2002; Matsuzaki et al., 2006), RAS/MAPK pathway and CaMK pathway (Dineley et al., 2001; Pittenger et al., 2008). The fact that blockage of these pathways had no effect on flavonol-stimulated pCREB suggest that interaction between A β and NMDA receptors might be a primary mechanism for the effect of flavonols. We have reported previously that ERK1/2 but not PKA pathway was involved in EGb761-elevated CREB phosphorylation in neuroblastoma cells (Xu et al., 2007).

Previous study indicates that A β treatment promoted endocytosis of NMDA receptors in cultured cortical neurons. A reduction in NMDA receptors at surface and synapse was observed in neuronal cultures prepared from mice bearing APP with familial Swedish mutation, while no change was found in total receptor amounts (Snyder et al., 2005). More recent studies from several laboratories indicated a role of human mutant PS1 in promoting APP trafficking from membrane towards the trans-Golgi network-enriched fractions (Gandy et al., 2007), and also in regulating intracellular trafficking of neurotrophic factor TrkB, which reduce supply of TrkB receptor and presynaptic membrane components at the nerve terminal resulting in compromised motor neuron function (Lazarov et al., 2007). It is possible, that co-expression of mutant PS1 with APP has more impact on intracellular A β and BDNF than cells or mice expressing single mutant APP mutant transgene, due to the role of mutant PS1 in regulating APP (Gandy et al., 2007) and TrkB (Lazarov et al., 2007) trafficking. More over, A β might regulate NMDA trafficking through regulating the binding of NMDA receptor to synaptic scaffolding proteins, such as PSD-95 and AP-2 (Lavezzari et al., 2003). PSD95 is suggested as an anchoring protein for NMDA receptor, PSD95 association with NMDA receptor results in increased number of cell surface receptors (Yamada et al., 1999). In this study, we show that surface-expressed NR1 and PSD95 reduced in transgenic neurons as compared with wild type neurons. Flavonols significantly restored NR1 and PSD95 expression at cell surface, consistent with the result of pharmacological blockade by NMDA receptor antagonist. It indicates that flavonols enhanced BDNF and CREB signaling pathway through restoring NMDA receptor expression at surface. The mechanism of impairment of CREB phosphorylation and BDNF expression in AD remains to be elucidated; however, substantial evidence suggests that A β play an important role. We observed that flavonols partially decreased A β production in transgenic neurons and hippocampus of transgenic mice (Fig. 3), and that flavonols had no effects on CREB/BDNF signaling pathway in wild type neurons. Therefore, it is possible that flavonols restored impaired CREB-BDNF pathway through decreasing A β level.

The effect of flavonols on hippocampal BDNF expression correlates with its enhanced spatial learning in Tg APP/PS1 mice. However, in tail suspension test, short-term administration of flavonols (2 weeks) reduced immobile time in wild type but not transgenic mice, which does not agree with what we observed in cultured neurons isolated from Tg mice. Moreover, the effective concentrations of flavonols required for elevating levels of BDNF is apparently higher than required for generating antidepressant behavioral in vivo by oral administration. For example, 50 μ g/ml applied *in vitro* is roughly equals 50 mg/kg used in the *in vivo* experiments. Taken into consideration of low bioavailability and low CNS penetration via the blood-brain

barrier of flavonols, the actual concentration could be much lower than in the *in vitro* experiments. One possible explanation is that the dose required for detectable change of BDNF is not necessarily reflects the dose required for its effect on antidepressant behavioral. Furthermore, the *in vitro* experiment conducted in isolated neurons is totally different from *in vivo* ones, which consists multiple cell layers, tissues and organs. It takes month for modification of leaning behavioral changes by flavonols to occur, whereas when flavonols were added directly to cells, 24 h treatment was sufficient to affect protein modification and expression of inducible gene products in the cells, a treatment time based on a time course of ginkgo treatment conducted previously in our laboratory in a neuroblastoma cell line (Xu et al, 2007). Another explanation is the complexity within the dTg mice. Studies have demonstrated several non-cognitive behaviors observed in these dTG mice (Pugh et al., 2007), including decreased spontaneous motor activity, disinhibition, increased frequency and duration of feeding and reduced body weight. In male dTg mice, enhanced aggression also observed compared with their wild type littermate. Interestingly, after 10 month old, increased activity was observed in these mice. Similar activities were seen in locomotion assays (Fig. 4B) in the present study. Though not significantly enhance, the dTg mice are more active compared with wt controls, which may explain why antidepressant activity was not seen in flavonol treated dTg mice as measures by immobile time (Fig. 4A).

Supported by previous studies (Stackman et al., 2003), long-term administration of flavonols (4 mon) significantly improved impaired spatial learning in dTg mice (Fig. 4C). Considering the critical role of CREB-BDNF pathway in neuroplasticity of depression and learning, the convergent mechanism of both AD and depression, restoration of BDNF by flavonol treatment is likely to contribute to the improved performance of transgenic mice in both Morris water maze and tail suspension test.

It should be noted that the choice of different administration routes and duration was based on different animal behavioral models. For the MWM and A β deposition, flavonols was administered to 4-month-old mice for 4 month. This long-term treatment model is necessary for restore impaired memory and eliminates A β deposition. Previous study employed oral administration of *ginkgo biloba* for 6 month in TgAPP mice and reported improvement of memory impairment in these mice (Stackman et al., 2003). We used gavage for long-term treatment to ensure the consistent amount of flavonol intake in the animals. On the other hand, for TST, we performed i.p. for 7 days, which was based on our preliminary experiments and other published data showing detectable antidepressant activities at this treatment time. For acute effect, even just 30 min before TST, fluoxetine show significant effect to reduce immobility time. Another reason of chosen this treatment paradigm was to be able to compare the effect of flavonols with that of fluoxetine.

In conclusion, flavon extracted from leaves of ginkgo improves impaired neuroplasticity in transgenic cortical neurons and hippocampus of transgenic mice through upregulating CREB-BDNF signaling pathway, which endue it with potential for the treatment of both AD and depression.

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Figure 1.

Flavonols stimulate levels of BDNF in neurons and hippocampus of double transgenic (TgAPP/PS1) mice. **A.** Levels of BDNF proteins in cortical neurons (14 DIV), derived from wild type (WT) or double transgenic mice (Tg) E14 embryos, incubated for 24 h with vehicle (Ctrl, 0.01% DMSO) or flavonols (flav, 50μg/ml). **B.** Levels of BDNF proteins in Tg neurons treated with increasing concentrations of flavonols (12.5, 25, 50μg/ml). Cell lysates were subjected to standard Western blotting using antibody to BDNF. Immunoreactive bands were analyzed from three independent experiments and expressed as percentage of vehicle control. **C.** Levels of phosphorylated CREB (pCREB) in WT and Tg neurons (14DIV) treated with or without flavonols (50μg/ml) for 24 h. **D.** Levels of BDNF and pCREB in hippocampus of 8-month old

WT or Tg mice brain treated with or without 50 mg/kg flavonols for 4 month (three mice from each group). * $p < 0.05$.

Figure 2.

Flavonols stimulate glutamate-evoked phosphorylation of CREB in neurons derived from TgAPP/PS1 mice. **A.** Levels of phosphorylated CREB in neurons (14 DIV) from E14 embryos of WT or Tg mice treatment with (+) or without (-) glutamate (50μM) for 15min. Cell lysates were subjected to Western blotting using antibody specific to phosphorylated CREB.

Immunoreactive bands were analyzed and expressed as percentage of WT control. **B.** Levels of pCREB in neurons of WT or Tg mice incubated with vehicle (0.01% DMSO) or flavonols (50μg/ml) for 24 h followed by treatment with (+) glutamate (50μM) for 15min. **C.**

Pharmacological blockage of flavonol-enhanced pCREB after glutamate exposure. Neurons were incubated for 24 h with vehicle (DMSO) or 50μg/ml flavonols. 50μM H89, 50μM KN-93,

20 μ M PD98059 and 50 μ M APV were applied 1 h prior to glutamate exposure for 15 min. Cell lysates were subjected to Western blotting to detect pCREB. **D.** Changes of cell surface NMDA receptors NR1 and Postsynaptic proteins PSD95 in WT and Tg neurons (14 DIV). Tg neurons were treated with or without flavonols (flavon) for 24 h. Membrane proteins isolated by biotinylation were subjected to immunoblotting using antibodies to NR1 or PSD95. Immunoreactive bands were analyzed and expressed as percentage of vehicle control. All the results were obtained from three independent measurements and expressed as mean $\pm$ SD. * $p < 0.05$.

A BL: A β in neurons from Tg mice

B BL: A β in Tg mice brain

C Immunostaining of A β (6E10) in Tg mice hippocampus

Figure 3.

Flavonols decrease intracellular and extracellular A β in transgenic neurons and hippocampus of Tg mice brain. **A.** Levels of A β in neurons derived from Tg mice brain (14 DIV) were incubated with vehicle (0.01% DMSO) or flavonols (50 μ g/ml) for 24 h. Cell lysates or cultural medium were subjected to Western blotting using monoclonal antibody 6E10 to detect A β species. Immunoreactive bands (7 kD for intracellular A β blots, the only band at ~ kD for medium A β blots) were analyzed and expressed percentage of vehicle control. **B.** A β levels in hippocampus of TgAPP/PS1 mice. 8-month old TgAPP/PS1 mice and age-matched wild type (WT) mice were oral administered with flavonols 50mg/kg or equivalent amount of vehicle daily for 4 months. The brains were removed and hippocampi were dissected. Equal amount

homogenous protein was probed with antibody 6E10. The changes of immunoreactive A β were analyzed by densitometry and expressed as percentage of vehicle control. Results are obtained from 3 mice and expressed as mean $\pm$ SD. **C.** Immunohistochemical staining of A β in hippocampus of the Tg mice after treatment with flavonols (b and b') for 4 months compared to the control group administered with vehicle (a and a'). (Bar: a & b= 250 μ m, a' & b'=20 μ m). Images represent at least five sections from three mice brains for each group.

A Tail suspension test in mice **B Locomotive assay in mice** **C Morris water maze in mice** **Figure 4.**

Antidepressant-like activity of flavonols in animal behavioral models. **A.** Tail suspension test (TST) in 12-month old male WT and Tg mice. Drugs were administered by intraperitoneal injection daily for 7 days. The results represent mean $\pm$ SEM ($n = 4-8$). The data shows significant difference between WT untreated and flavonols treated hippocampus by ANOVA analysis ($P = 0.0038$). Post hoc Dunnett's test shows significant difference for the treatment in comparison to control. * $P < 0.05$ and ** $P < 0.01$. **B.** Locomotor activity was measured in the same mice tested for TST after one-day recovery. The assay was based on the distance traveled by each mouse in the chamber for 30 min. All results represent the mean $\pm$ SEM ($n = 4-7$). Data shows no significant difference by ANOVA analysis ($P = 0.5940$) between all groups.

Post hoc Dunnett's multiple comparison tests show no significant difference between the Wt control and any other group ($P>0.05$). C. Morris water maze assay in WT and Tg mice orally administrated with or without flavonols (50 mg/kg) for 4 month starting from 8-month old. Mice were trained twice every day during 5 consecutive days. The time needed to find the platform was recorded as latency. Results are expressed as mean $\pm$ SEM. One-way ANOVA is used to analyze the statistical difference between the groups on each day followed by post hoc dunntte's multiple comparison test. On the fifth day, flavonol treated transgenic mice shows significant reduction in the latency time in comparison to vehicle-treated Tg mice (* $P<0.05$).