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Stimulation of Neurogenesis and Synaptogenesis by Bilobalide and Quercetin via Common Final Pathway in Hippocampal Neurons

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Abstract. Loss of synapses has been correlated with dementia in Alzheimer's disease (AD) as an early event during the disease progression. Hence, synaptogenesis and neurogenesis in adulthood could serve as a therapeutic target for the prevention and treatment of AD. Recently, we have demonstrated enhanced hippocampal neurogenesis by oral administration of *Ginkgo biloba* extract (EGb 761) to a mouse model of AD. This study aims to identify the constituents that contribute to EGb 761-induced neurogenesis. Among the constituents tested, bilobalide and quercetin significantly increased cell proliferation in the hippocampal neurons in a dose-dependent manner. Bilobalide and quercetin also enhanced phosphorylation of cyclic-AMP Response Element Binding Protein (CREB) in these cells, and elevated the levels of pCREB and, brain-derived neurotrophic factor in mice brain. Immunofluorescence staining of synaptic markers shows remarkable dendritic processes in hippocampal neurons treated with either quercetin or bilobalide. Furthermore, both constituents restored amyloid-β oligomers (also known as ADDL)-induced synaptic loss and phosphorylation of CREB. The present findings suggest that enhanced neurogenesis and synaptogenesis by bilobalide and quercetin may share a common final signaling pathway mediated by phosphorylation of CREB. Despite a recent report showing that EGb 761 was insufficient in prevent dementia, its constituents still warrant future investigation.

Keywords: Amyloid-beta derived diffusible ligands (ADDL), bilobalide, CREB, neurogenesis, quercetin, synaptogenesis

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder of old age leading to death in about eight years. This disease is believed to initiate from loss of synapses and the subsequent loss of neuronal cells in the hippocampus and cortex, as synaptic loss is best correlated with early events of AD [1–3]. Accordingly, hippocampal synaptogenesis represents a promising target for the discovery of novel therapies for the treatment of AD [4]. Studies have demonstrated that enriched environment [5], physical activities [6,7], and selective serotonin reuptake inhibitors (SSRIs) stimulated hippocampal neurogenesis in adult rats [8,9]. We have shown that oral administration of the *Ginkgo biloba* extract (EGb 761) enhanced adult hippocampal neu-

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rogenesis in a mouse model of AD [10] which may explain cognitive improvement by EGb 761 in these mice [11] and antidepressant activity of EGb 761 in behavioral models [12,13]. However, a recent report demonstrated that EGb 761 failed to prevent dementia in a large clinical trial Ginkgo Evaluation of Memory [14].

EGb 761 is a standardized ginkgo biloba leaf extract containing 24% flavonoids and 6% terpenoids. Substantial experimental evidence indicates that the constituents of EGb 761 have neuroprotective and neuromodulatory effects in various models [15,16]. Quercetin is a component of flavonoids in EGb 761, which is thought to attribute to the antioxidative properties. Among the terpenoid fraction, ginkgolides A, B, C, J, and M, and bilobalide [17], ginkgolide B is a potent antagonist of platelet-activating-factor receptor [18]. Ginkgolide J protects against Aβ-induced abnormal hippocampal long-term potentiation (LTP) and neuronal cell death [19].

A significant player mediating synaptic plasticity is pro-survival transcription factor CREB, the cyclic-AMP response-element-binding protein. It is responsible for a variety of gene expression essential for neuronal activities and synaptic functions related to learning and memory [20,21]. Various receptors and protein kinase pathways converge to regulate CREB activity, including NMDA receptors, serotonin receptors, and kinases such as cAMP-dependent protein kinase PKA, calmodulin dependent kinase CaMKII, and MAP kinases [22] that are required for LTP [23]. We recently demonstrated that defective phosphorylation of CREB (pCREB) was associated with amyloid-β (Aβ) oligomers in hippocampal dentate gyrus of a transgenic mouse model of AD and EGb 761 restored the levels of CREB phosphorylation in these mice [10]. Furthermore, its constituents re-established a reduced pCREB in Aβ-expressing neuroblastoma cells in a dose-dependent manner [24].

Soluble Aβ oligomers (also known as amyloid-β derived diffusible ligands, or ADDLs) have been reported to be accumulated in the brain of postmortem AD patients [25], to impair hippocampal LTP *in vitro* [26–28], and to be associated with synaptic loss [29,30] and inhibition of insulin receptors (IRs) function by binding to the receptors [31]. ADDLs at physiological concentration also selectively disrupted the activation of the downstream kinases ERK/MAPK, CaMKII and PI3 kinase that are required for hippocampal LTP [23] and that contribute to loss of synaptic plasticity as well as suppress spine maintenance during development of AD [32,33].

The neurogenic potential of EGb 761 [10], its ability to reduce Aβ-induced toxicity *in vivo* [34] and rescue impaired LTP induced by Aβ and aging in hippocampal slices [19,35], prompted us to hypothesize that its neuroprotective activity is due to its constituents that modulate neurogenesis and synaptogenesis in the brain. This study aims to test the hypothesis by identifying the constituents of EGb 761 that regulate cell proliferation and synaptic density in hippocampal neurons. We demonstrate that bilobalide and quercetin significantly enhance hippocampal neuronal proliferation and synaptogenesis and protect against Aβ oligomer-induced synaptic loss in these neurons. Possible signaling pathways mediating these actions are discussed.

MATERIALS AND METHODS

Chemicals

The individual constituents of EGb 761, ginkgolide A, B, bilobalide, and quercetin were purchased from Sigma (St Louis, MO). Stock solutions were made with 100% DMSO. The final concentration for DMSO was $< 0.1\%$ in the cell culture media.

Animals

Eighteen days timed-pregnant Sprague-Dawley rats were purchased from Harlan Prague (Indianapolis, IN). Wild type mice (C57B6/BL) were purchased from the Jackson Lab (Bahabro, ME). The Institutional Animal Care and Use Committee (IACUC) of the School of Pharmacy (University of Maryland) approved all animal handling and treatment.

Hippocampal neuronal culture

Hippocampal progenitor cells were prepared from day 18 rat fetuses as previously described [36]. In brief, hippocampi were dissected and then digested with 0.02% trypsin in HBSS (Invitrogen, Grand Island, NY). Dissociated cells were plated on poly-D-lysine-coated 60 mm Falcon Petri dishes at a density of 1×10^5 cells/cm^2 for biochemical study, on poly-D-lysine-coated glass cover slips at a density of 2×10^3 cells/cm^2 for immunostaining or on laminin-coated black clear-bottom 96-well Falcon microplates at a density of 4×10^4 cells per well for 5-bromo-2-deoxyuridine (BrdU) incorporation chemiluminescence ELISA assay. All cells were incubated in Neurobasal medium (In-

vitrogen, Carlsbad, CA) supplemented with 10 U/ml penicillin, 10 μg/ml streptomycin, 0.5 mM glutamine, 25 μM glutamate, and 2% B27 (Invitrogen, Gaithersburg, MD). Cultures were maintained at 37°C in humidified 5% CO_2 conditions for 3, 7, or 21 days (3DIV, 7 DIV or 21DIV) as indicated.

Cell proliferation assay

3 DIV hippocampal cells plated in the 96 wells microplates were treated overnight (12 h) with 3–15 μM of EGb 761 constituents including ginkgolide A, B, bilobalide, or quercetin. Other sets of 3 DIV cells plated in the 96 wells microplates were pretreated for 30 min with pharmacological blockers, namely APV (a competitive NMDA receptor antagonist) [37], mematine (a non-competitive NMDA receptor inhibitor), ketanserin (5-HT2$_A$ antagonist [9]), H-89 (PKA inhibitor), or KN-93 (CaM kinase inhibitor). The pretreatment was followed by overnight treatment with bilobalide and quercetin at concentrations that showed the most potent effect on cell proliferation (bilobalide10 μM, QT7 μM). All final treatments contained 10 μM of BrdU (marker of cell proliferation). Rolipram was used as positive control in the BrdU incorporation assay. Levels of BrdU incorporation were then determined using a BrdU incorporation ELISA kit (Roche, Germany) and the procedure was carried out following manufacturer's instructions. In brief, cells were fixed with Fixodent, incubated with the anti-BrdU-POD antibody, washed, and incubated with the substrate, and then light emission was read using a microplate reader with the chemiluminescence technology (Bio-Tek Instruments Inc., Winooski, VT).

Immunostaining for dendrites formation and synaptic functionality

7 DIV hippocampal cells treated with or without 10 μM bilobalide or 5 μM quercetin (concentrations that significantly increased BrdU incorporation) for 12 h, were co-stained for MAP-2 (marker of dendrite formation) and TAU-1 (marker of axonal formation) or MAP-2 and Egr-1 (Early Growth Response Protein 1, also called zif 268, a marker for functional synapses) as previously described [10]. Cells cultured on glass coverslips were fixed in 4% paraformaldehyde, blocked with 5% serum for 1 h, then incubated with a mixture of primary antibodies against TAU-1 (1:600; Santa Cruz, CA) and MAP-2 (1:1000; Sigma, St Louis, MO) or against MAP-2 and Zif268 (1:800; Santa Cruz, CA), and then overnight incubation at 4°C. This was followed by incubation with a mixture of secondary antibodies anti-Mouse ALEXA FLUOR 488 and anti-Rabbit ALEXA FLUOR 594 (1:500, Invitrogen, Carlsbad, CA) for 2 h. Cover slips were mounted on slides with an antifading agent (Gel/mount; Biomeda, Foster City, CA) and fluorescent signals were detected with a fluorescence microscope (NIKON E-2000).

Immunostaining for the formation of dendritic spines and data analysis

21 DIV hippocampal cells pretreated for 10 min with bilobalide, quercetin, and ginkgolide B at the concentrations of 1, 5, and 10 μM before incubating with ADDLs at 500nM or vehicle overnight (12 h) were immunostained for drebrin (marker of dendritic spines) as previously described [30]. Briefly, 21 DIV cultures were fixed in 2% paraformaldehyde for 20 min followed by an additional 20 min fixation with 4% paraformaldehyde. They were then blocked with 10% serum for 45 min and incubated with antibodies against drebrin (1:500; Stressgen, Victoria, BC, Ca) overnight at 4°C. The next day cells were incubated with mouse ALEXA FLUOR 488 for 90 min. All cover slips were mounted on slides with an antifading agent (Gel/mount; Biomeda, Foster City, CA) and fluorescent signals detected using a fluorescence microscope (NIKON E-2000) or a Leica TCS SP2 laser confocal microscope. Images were analyzed by NIH ImageJ 1.36b and Metamorph v6.3 (Universal Imaging Corporation, Downingtown, PA) software as previously described [30]. Data from two independent experiments were collected.

Immunoblotting

Standard Western blotting analysis was performed. Primary hippocampus cells (7DIV) from E18 rat and mice brain (4 month old) were homogenized in the lysis buffer containing 10 mM Tris, pH7.4, 100 mM NaCl, 1 mM EDTA,1% Triton X-100, 1% glycerol, 0.1% SDS, 0.5% deoxycholate, protease inhibitor cocktail, and phosphtase inhibitor cocktail (Roche, Pleasanton, CA). Equal amount of proteins (40 μg) were resolved on a 12% SDS-PAGE, transferred and blocked with 5% non-fat dry milk. This was followed by overnight incubation at 4°C with different primary antibodies, which included antibody against phosphorylated CREB (pCREB, Upstate Biotechnology, NY; 1:1000); CREB, BDNF, and tubulin (Santa Cruz, Inc., Santa Cruz, CA; 1:1000). The blots were then incubated with the corresponding secondary antibodies. Immunoreactivities were detected by an enhanced chemilunescence kit (Amersham Biosciences, Piscataway, NJ).

Quantitative and statistical analyses

Data are expressed as the means ± S.D. Statistical comparisons were made using one way ANOVA or paired student t test with a confidence level of 95%. The significance level is set at $p < 0.05$.

RESULTS

Bilobalide and quercetin stimulate proliferation of hippocampal progenitor cells in a dose-dependent manner

To identify the constituents of EGb 761 that contribute to enhanced hippocampal neurogenesis in an aged transgenic mouse model of AD [10] and to determine the mechanism mediating this activity, primary cultures of hippocampal progenitor cells from embryonic day 18 (E18) rats were utilized to assess cell proliferation by quantitative BrdU ELISA. Hippocampal neurons cultured in 96 well plates for 3 days *in vitro* (3 DIV) were treated with or without ginkgolide A, B, bilobalide, and quercetin in the presence of BrdU overnight. Quantitative analysis indicates that only bilobalide and quercetin treatments induced a significant increase in BrdU incorporation in a dose-dependent manner (Fig. 1A). BrdU incorporation in cells treated with QT at concentrations 5, 7, and 10 μM was statistically significant compared with the vehicle-treated controls (Veh), corresponding to respective percent increases of 21 ± 4%, 47 ± 9% and 32 ± 5% ($p < 0.05$ for 5 μM QT treatment, $p < 0.01$ for 7 and 10 μM QT treatments, $n = 6$). Bilobalide treatment displayed a more remarkable change than quercetin (Fig. 1A). At concentrations of 5, 10, and 15 μM, bilobalide significantly increased cell proliferation, corresponding to percent increase of 58 ± 9%, 80 ± 10%, and 55 ± 10% respectively ($n = 6$, $p < 0.01$). Surprisingly, ginkgolides A and B (GA, GB), known to protect against Aβ-induced toxicity [19,34], did not elicit significant increases in hippocampal cell proliferation (Fig. 1A). Rolipram (Rol, 10 μM) was used as a positive control for neurogenesis (Fig. 1A).

Enhanced phosphorylation of CREB by bilobalide and quercetin

Important player implicated in regulation of neurogenesis include activation of a transcription factor CREB by phosphorylation [38] and expression of certain genes such as nerve growth factors BDNF [39]. Previously we observed enhanced phosphorylation of CREB by EGb 761 in the brain of a mouse model of AD [10] and in an Aβ-expressing neuronal cell line [24]. To elucidate whether CREB, an upstream regulator of neurogenesis, mediates the effect of bilobalide and quercetin on cell proliferation, the hippocampal progenitor cells (3 DIV) were treated with or without bilobalide or quercetin at increasing concentrations (1–100 μM) for 12 h. This was followed by Western blotting of CREB, using antibodies specific to phosphorylated and total CREB. Representative blots from two

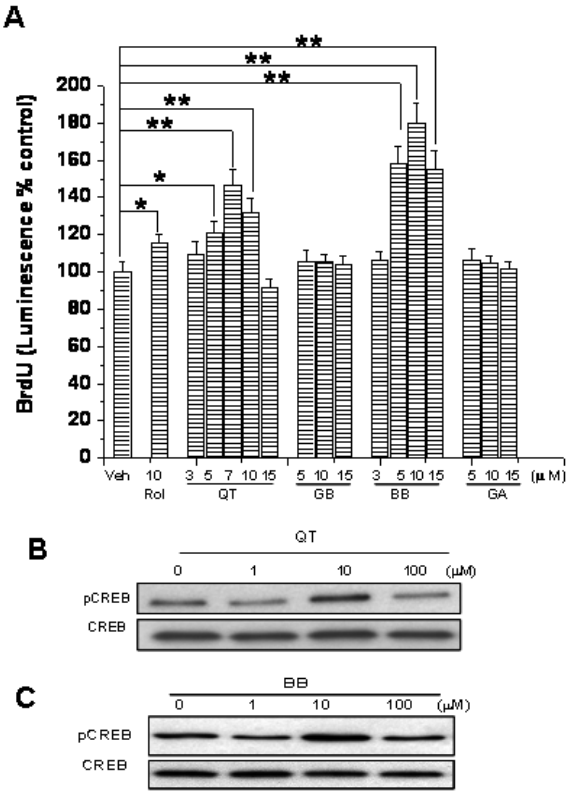

Fig. 1. Bilobalide and quercetin stimulate cell proliferation and phosphorylation of CREB in primary hippocampal cells. **A**) Quantitative cell proliferation determined by chemiluminescence BrdU cell proliferation ELISA. Hippocampi cells from the brain of E18 rats were co-incubated with BrdU (10 μM) and increasing concentrations of quercetin (QT), ginkgolide B (GB), ginkgolide A (GA), or bilobalide (BB) for 12 h. Rolipram (Rol, 10 μM) was used as the control. Quercetin at 5, 7, and 10 μM and bilobalide at 5, 10, 15 μM exhibited statistically significant induction of BrdU incorporation. Data were derived from three independent experiments performed in triplicate and samples were analyzed by one way ANOVA (* $p < 0.05$, ** $p < 0.01$). **B&C**) Representative Western blots of phosphorylated CREB (pCREB) and total CREB in the hippocampal cells (3 DIV) treated with increasing concentrations of QT or BB for 12 h. Blots represent two independent experiments.

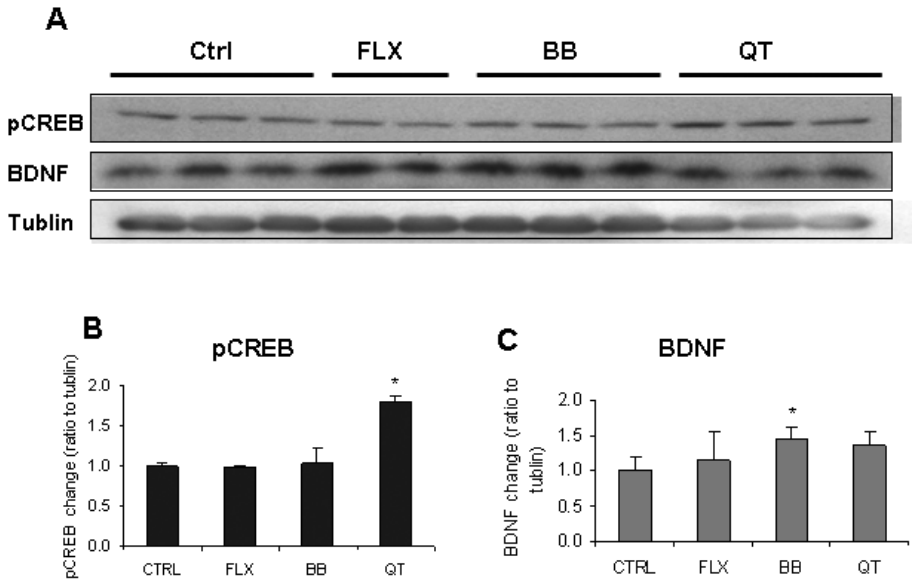

Fig. 2. Representative immunoblots (**A**) and quantitation of the levels of phosphoCREB (**B**) and BDNF (**C**) in homogenates of hippocampi of C57B6 mice (4 month old) treated with quercetin (QT, 10 mg/kg) or bilobalide (BB, 10 mg/kg) or fluoxetin (FLX, 10 mg/kg) for 10 days. Quantification of pCREB and BDNF level were derived from three mice per condition, results are expressed as mean density $\pm$ SE and analyzed by one way ANOVA ($*p < 0.05$).

independent experiments demonstrate that both bilobalide and quercetin remarkably enhanced phosphorylation of CREB (pCREB, Fig. 1B&C) at 10 μM. No notable changes were observed by either constituent on total levels of CREB, suggesting that activation of CREB may mediate stimulation of cell proliferation by bilobalide and quercetin. Stimulation of pCREB by quercetin in hippocampal neurons has been reported in a previous study [40].

Growth factors regulate both cell proliferation and survival, which is considered to be mediated by pCREB [22]. To validate the modulator role of bilobalide and quercetin in this signaling pathway *in vivo*, pCREB and BDNF were probed by immunoblotting of hippocampal tissue from mice (4 month) after inter-peritoneal administration of quercetin (10 mg/kg) and bilobalide (10 mg/kg) for 10 days. Western blots (Fig. 2A) illustrate immunoreactive pCREB and BNDF from three individual mice administrated with bilobalide or quercetin. As a comparison, a known SSRI antidepressant fluoxetine was also administrated to the mice. Data analysis of mean density of the immunoreactivity from three mice brain show significant enhancement of the levels of pCREB in mice treated with quercetin, but not bilobalide (Fig. 2B, CTRL vs. bilobalide, 1.0 $\pm$ 0.03 vs. 1.04 $\pm$ 0.17, $n = 3$, $p = 0.721$; CTRL vs. quercetin, 1.0 $\pm$ 0.03 vs. 1.80 $\pm$ 0.08, $n = 3$, $p = 0.001$). Interestingly, the levels of BDNF (Fig. 2C) were more profoundly enhanced in mice treated with bilobalide but not quercetin (CTRL vs. BB, 1.00 $\pm$ 0.19 vs. 1.44 $\pm$ 0.17, $n = 3$, $p = 0.038$; CTRL vs. QT 1.00 $\pm$ 0.19 vs. 1.34 $\pm$ 0.23, $n = 3$, $P = 0.108$). Fluoxetine did not significantly affect either pCREB or BDNF. These results suggest that bilobalide and quercetin differentially affecting the signaling molecules (pCREB and BDNF) involved in neurogenesis *in vivo*.

Quercetin and bilobalide promote the dendritic process in hippocampal progenitor neurons

Increased neurogenesis by quercetin and bilobalide is indicative of the ability of these compounds to contribute to the replacement of lost neurons in AD patient. To assess whether quercetin or bilobalide treatments would promote and/or maintain the ability of newly formed neurons to integrate into the neural circuit and connect with others, we examined their effect on dendrite formation and functional spines by co-immune labeling the hippocampal neurons with antibodies against MAP/TAU or MAP/Zif268. MAP-2 and Tau-1 are markers for dendritic or axonal processes, respectively. zif268 (also known as Egr-1, Red) is a marker that indicates functional spines and has been implicated in LTP and memory consolidation [41]. Representative co-immunostaining of the hippocampal

cells with MAP (green), TAU (red), or merge (yellow) are shown in Fig. 5A and 5B. The top three panels are co-staining of vehicle treated hippocampal neuron (Fig. 3A). Remarkably, dendritic arborations were observed in cells treated with either 10 μM bilobalide (middle panels) or 5 μM quercetin (bottom panels), as shown by the intensity of MAP-2 (green) in dendrites and dendritic branches as compared with vehicle- treated controls (Fig. 3A). Consistently, similar dendritic processing stimulated by bilobalide and quercetin can be seen by co-staining with MAP and zif268 (Fig. 3B). The inserts (merged panels) illustrate more functional spines, stained with zif268 (red), in cells treated with both bilobalide and quercetin (Fig. 3B). Quantitative analysis of those immunofluorescent images (Fig. 3C) demonstrate that bilobalide and quercetin treatments induced significant expression of zif268 (Ctrl 100 $\pm$ 3.2% vs. bilobalide 113 $\pm$ 4%, $n = 10$, $p = 0.01$); Ctrl 100 $\pm$ 3.2 % vs. quercetin 142 $\pm$ 5.5%, $n = 8$, $p = 0.001$). These findings indicate that quercetin and bilobalide exhibit their ability to promote the integration of newly formed cells into the neuronal circuitry to assume neuronal functions.

Bilobalide and quercetin significantly prevent synaptic loss induced by ADDL application to hippocampal neurons

Given the deleterious effect of Aβ oligomers (known as ADDLs) on hippocampal neurogenesis in a mouse model of AD [10], spine density in hippocampal neurons in culture [29,30] and the ability of EGb 761 to reduce Aβ toxicity *in vivo* [34], the impact of bilobalide and quercetin on ADDL-induced synaptic impairments was assessed by quantitative immunofluorescent labeling of drebrin, a cytoskeletal marker known to be concentrated within spine heads [30]. Representative confocal microscope images of drebrin immunoreactivity are shown in Fig. 4A&B. Relative drebrin immunofluorescence was quantified and expressed as intensity per unite neuron area (Fig. 4C), which was measured on images including full neurons as well as on images depicting dendritic branches (digitally zoomed). Treatment of the 21DIV hippocampal cells with ADDLs (500 nM) for 24 h significantly induced loss of drebrin expression (37.9 $\pm$ 6.1% over vehicle (VH) set at 100%, $n = 19$, $p < 0.01$) (Fig. 4A–C) as previously reported [30]. Here we show that ADDL-induced drebrin loss was fully prevented in neurons pretreated with bilobalide (1 μM) (128 $\pm$ 19 % over VH, $n = 9$, $p < 0.001$ compared to ADDL group) or quercetin (5 μM) (114.4 $\pm$ 28.7%, $n = 10$, $p < 0.01$) as drebrin levels were comparable to that of vehicle-treated neurons. But treatment with GB (10 μM) for 30 min followed by 24 h ADDL treatment show a rescue of ADDL-induced spine loss that was not statistically significant (71.9 $\pm$ 6.7%, $n = 10$, $p > 0.05$, Fig. 4 A–C). Other concentrations of bilobalide (5 and 10 μM) and quercetin (10 μM) also significantly prevented loss of drebrin, except quercetin at 1 μM did not induce statistically significant improvement (data were not included in the graph for clarity). Prevention of ADDL-induced spine loss by bilobalide and quercetin occurred in the absence of blockage of the ADDL binding to synapses (data not shown) is similarly to what was observed with memantine [29].

ADDL has been recently reported to inhibit insulin receptors and multiple downstream kinases that are necessary for hippocampal LTP [23,31] and some are upstream regulators of CREB. To determine the impact of ADDL to pCREB, 7DIV hippocampal cells were pretreated with ADDL (500 nM) or vehicle peptide for 30 min followed by 24 h treatment with bilobalide or quercetin. Representative Western blots of pCREB from two independent experiments show (Fig. 4D) that both ADDL and vehicle (Veh) treatment alone slightly reduced levels of CREB compared with untreated controls (Ctrl). Co-treatment with quercetin and bilobalide overcame the effect on pCREB by ADDLs (Fig. 4D), suggesting possible involvement of pCREB in ADDL-induced synaptic loss.

DISCUSSION

The recently published negative results from the National Institutes of Health sponsored Ginkgo Evaluation of Memory (GEM) Study were disappointing [14], considering various pre-clinical and epidemiological findings that suggest EGb 761 should have neuroprotective activity. However, as pointed out by Dr. DeKosky, the key author in the *JAMA* paper, that given the importance of midlife risk factors for development of dementia later in life, “prevention might have to start much earlier than at the age of 75”. Prevention is a difficult and relatively new field of research. There are a number of well-controlled studies now – as mentioned in Dr. Schneider’s editorial in *JAMA* – that demonstrate the clinical efficacy of EGb 761 in the symptomatic treatment of dementia, in particular in those patients who suffer from neuropsychiatric symptoms. Therefore, a failed prevention study should not detract from

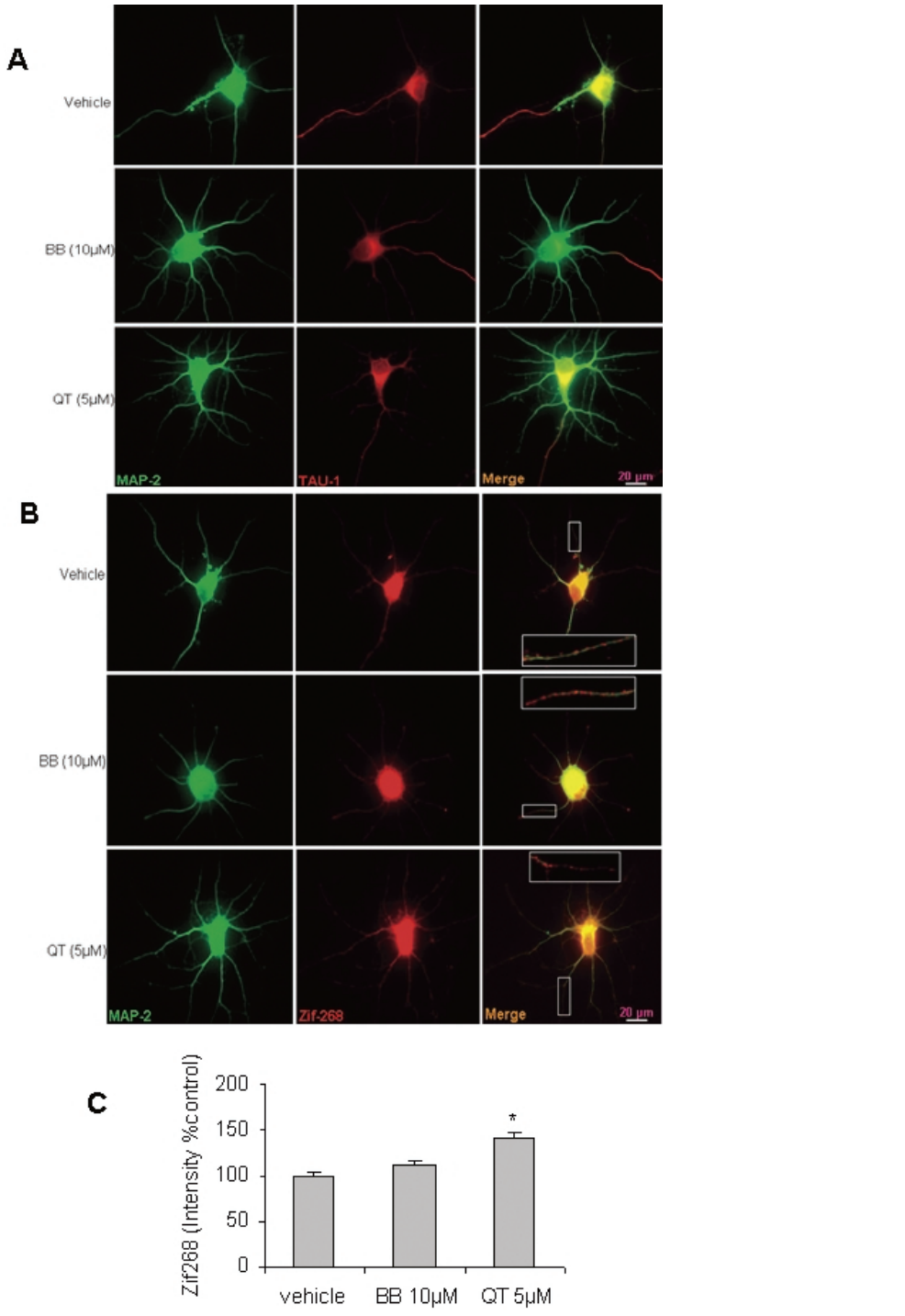

Fig. 3. Co-immunostaining for dendritic processes in neurons treated with or without bilobalide and quercetin. **A**) Representative immunofluorescent images of dendritic processes by co-labeling of the hippocampal neurons with MAP-2 (a dendritic marker, green) and TAU-1 (marker of axonal process, red). The neurons (7 DIV) from E18 rats were treated with bilobalide (BB, 10 μM) or quercetin (QT, 5 μM) for 12 h followed by immunostaining. **B**) Representative immunofluorescent image of functional synapses by co-staining of hippocampal neurons with MAP-2 and zif 268 (marker for functional synapses, red) following treatment with BB (10 μM) or QT (5 μM) for 12 h. Inset: enlarged segment of dendrite co-stained with MAP-2 (green) and zif 268 (red) (Scale bars: 20 μm). **C**) Quantitative analysis of zif268 immunofluorescence density from 8–9 randomly selected images.

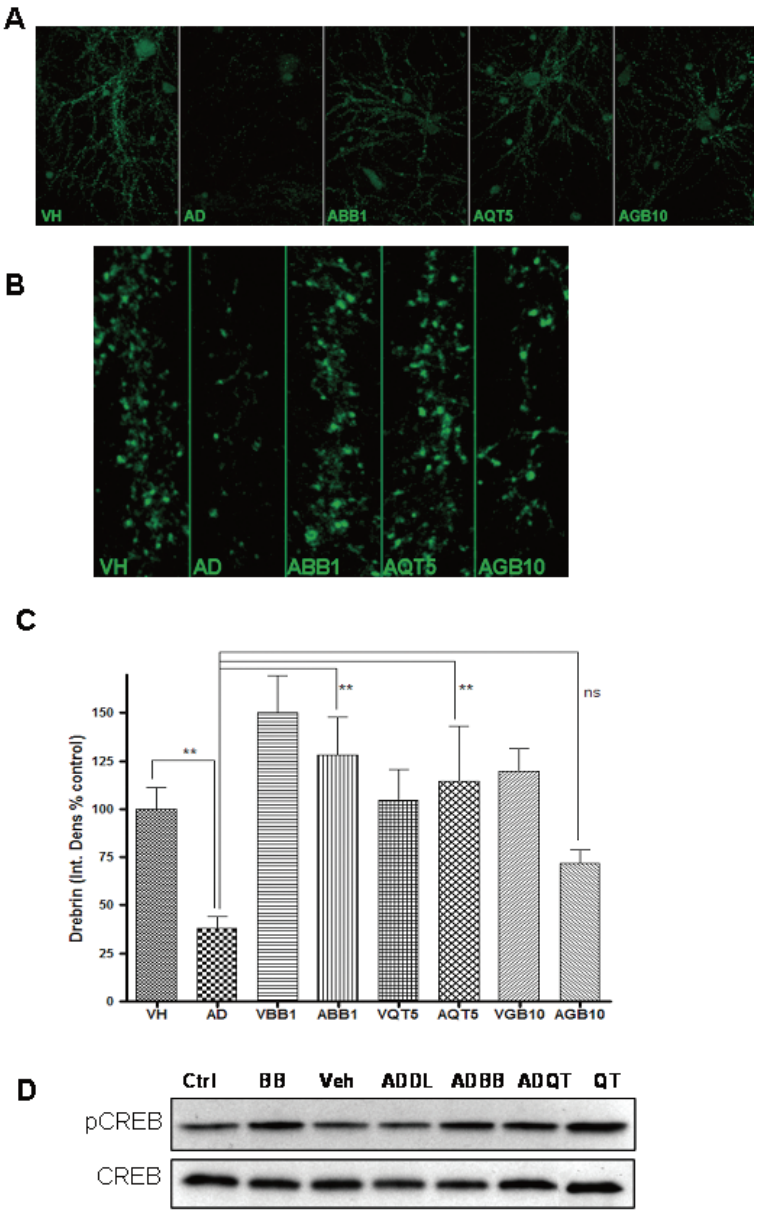

Fig. 4. ADDL-induced synaptic loss. **A**) Drebrin labeling of mature hippocampal cells treated with vehicle or ADDLs (500nM) or Veh/ADDLs+Bilobalide or Quercetin. Bilobalide (1, 5, 10 μM), quercetin (1, 5, 10 μM), or ginkgolide B (1, 5, 10 μM) were added 10 min prior to the 24 h incubation with ADDLs or vehicle, cells were fixed and immunolabeled for drebrin (dendritic spine marker) and ADDLs. Images of ADDL-sensitive neurons were captured on a confocal microscope and quantified with Image J. Data represents the Integrated density for drebrin (% over control). Dendrites of neurons treated with ADDLs for 24 h (AD) exhibit decreased drebrin immunofluorescence when compared with neurons treated with vehicle for 24 h (VH). Neurons treated with the Bilobalide at 1 μM (Fig. 4, ABB1) or quercetin at 5 μM (AQT5) 10 min before 24 h ADDL treatment exhibit drebrin-IR comparable to the VH-treated neurons, demonstrating that BB1 and QT5 have a protective effect on ADDL-induced drebrin loss. Ginkgolide B at 10 μM (AGB10) did not show such protection against ADDL-induced drebrin loss. **B**) At higher magnification, it is noticeable that ABB1 and QT5 exhibit very similar drebrin-IR punctas than controls, while AD and GB10 do not show abundance in drebrin-IR punctas. **C**) Bar graph illustrating the integrated density of drebrin immunofluorescence from confocal image sets as shown in A. Values is normalized to veh-treated cells for 24 h. Differences between Veh ($n = 18$) versus ADDL ($n = 19$) as well as differences between ADDL versus BB+ADDL ($n = 9$) and QT+ADDL ($n = 10$) are significant (**$p < 0.01$) whereas ADDL vs. ADDL+GB ($n = 10$) was not. **D**) Representative Western blots of pCREB from 7DIV hippocampal neurons untreated (Ctrl), pretreated with ADDLs (ADDL, 500nM) or vehicle (Veh) peptide for 30 min followed by 20 min treatment with BB or QT. Data was obtained from two independent experiments.

the efficacy of the drug in manifest dementia. As we known, other attempts to demonstrate preventive action of promising compounds such as cholinesterase inhibitors have failed. In addition, efficacies of EGb 761 in other central nervous system and peripheral nervous system disorders have been well demonstrated. The slight dichotomy observed between the pre-clinical and the clinical studies suggests that clinical pharmacology of EGb 761 needs to be optimized. Thus, further basic research can provide insight by elucidating multitarget mechanisms of the constituents that may have a more favorable pharmacological profile and be more efficacious than EGb 761.

In these experiments, we revealed that bilobalide and quercetin, two natural constituents, enhanced neurogenesis and promoted synaptogenesis using quantitative BrdU incorporation and immunostaining of synaptogenesis biomarkers. These results suggest that the neurogenic effects of bilobalide and quercetin involve diverse cell signaling molecules and a convergent common final pathway.

The roles and mechanisms of bilobalide in neuroprotection have been understudied compared with its terpenoid ginkgolide partners [17,19,42]. However, studies show that bilobalide (2.3 μM) protects glutamatergic excitotoxicity both *in vitro* and *in vivo* [43–45] by antagonizing GABA receptors [46]. A high dose of bilobalide (100 μM) might exert neuroprotective properties by reducing the release of excitatory neurotransmitters [47]. Quercetin has been shown to produce comparable actions on currents mediated by α4, β2 neuronal nicotinic acetylcholine receptors, serotonin3A receptors and glutamate AMPA/kainate receptors [48,49]. The 5-HT system is particularly interesting because of its interactions with many other neurotransmitters systems [50]. Dysfunctions in the serotonergic system correlate with altered behavioral aspects of AD [51,52].

Our data demonstrated that the dose-response of quercetin on cell proliferation is biphasic, with low concentrations (5–10 μM) promoting, and higher concentration ($>$ 15 μM) inhibiting cell proliferation (Fig. 1A). This biphasic effect of quercetin was also demonstrated by others, which show the phosphorylation of CREB at low dose and cytotoxicity at higher ones [40,53,54]. Furthermore, as a prooxidant [55] and an antioxidant [56], the effects of quercetin on the redox equilibrium of cells may play a decisive role on cell proliferation, viability, and the sustainable neurogenesis [5,22].

Bilobalide and quercetin-stimulated dendrite formation and the expression of zif 268 in hippocampal neural cells (Fig. 3B&C) are considered as possible downstream effectors. These processes could be mediated by phosphorylation of CREB, a transcription factor that is required for the development of dendrites [57] and plays a role in the regulation of zif 268 expression [41]. Although mechanisms of action by quercetin and bilobalide is unclear, the involvement of CREB in the regulation of neurogenesis, dendrite formation, zif 268 expression and the formation of dendritic spines, underscores its importance in neuroplasticity and neuroprotective processes [41,58,59].

The expression of drebrin, a marker of dendritic spines, has been implicated in neuronal interactions and, as a result, promotes the integration of newly formed neurons in the neuronal circuit. Drebrin expression was found to be severely affected by ADDLs in mature hippocampal cells in culture (Fig. 4A&B). These findings are in agreement with those previously reported [29,30], and our recent report that show the effects of Aβ oligomers on adult neurogenesis in a transgenic mouse model of AD [10]. Pretreatment of hippocampal primary cells with bilobalide and quercetin before the addition of ADDLs restored dentritic spines density levels to those observed with vehicle treated controls. Recent research demonstrated that ADDL-induced toxicity in hippocampal neuronal culture by stimulating formation of reactive oxygen species via a mechanism that requires activation of NMDA receptors [60]. Previous reports demonstrate that bilobalide prevents formation of reactive oxygen species induced by xanthine in PC12 cells [61] and protect hippocampal neurons against damage caused by glutamate excitotoxicity, which could contribute to its enhance cell proliferation and spine density. Similarly, the protective effect of quercetin could be attributed to its flavonoid related antioxidant properties. Findings from several studies reported that quercetin prevented peroxide-induced toxicity in glioma C6 cells and cortical neurons via the inhibition of formation of reactive oxygen species [56, 62]. Of additional importance, a recent *in vitro* study indicated that quercetin inhibited Aβ aggregation [63]. The neurogenic effect of bilobalide and quercetin and their ability to enhance synaptic processes, and subsequent promoting integration of newly formed neurons in the neuronal circuit, added to their preventive effect on Aβ toxicity.

The concept of neuronal progenitor cells adding neurons into hippocampal circuitry throughout life has been well accepted in past years [64]. In addition, dentate granule cells generated in developing and adult hippocampus display a remarkably similar afferent con-

nectivity and functionally homogeneous neuronal population [65]. The mechanisms underlying synaptic activity and neurogenesis could overlap [38] or be divergent. Previous studies [19] demonstrated that treatment of hippocampal slices with ginkgolide J and A, but not ginkgolide B and bilobalide rescued impaired long-term potentiation induced by Aβ. The fact that ginkgolide A facilitated LTP, but did not stimulate cell proliferation, suggests that LTP and cell proliferation could be facilitated by different mechanisms. In supporting this notion, it was demonstrated that ginkgolide B affects hippocampal LTP by inhibiting the platelet activating factor receptor [66]. Taken together, the diverse actions of individual natural components on synaptic modification provide a potential combination strategy for more effective therapy in treatment of AD patients.

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