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Ginkgo biloba Extract EGb 761 and Its Specific Components Elicit Protective Protein Clearance Through the Autophagy-Lysosomal Pathway in Tau-Transgenic Mice and Cultured Neurons

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Abstract. Alzheimer's disease (AD) is a neurodegenerative disease pathologically characterized by extracellular amyloid-β (Aβ) deposits and intracellular neurofibrillary tangles (NFT) in many brain regions. NFT are primarily composed of hyperphosphorylated tau protein (p-Tau). Aβ and p-Tau are two major pathogenic molecules with tau acting downstream to Aβ to induce neuronal degeneration. In this study, we investigated whether *Ginkgo biloba* extract EGb 761 reduces cerebral p-Tau level and prevents AD pathogenesis. Human P301S tau mutant-transgenic mice were fed with EGb 761, added to the regular diet for 2 or 5 months. We observed that treatment with EGb 761 for 5 months significantly improved the cognitive function of mice, attenuated the loss of synaptophysin and recovered the phosphorylation of CREB in the mouse brain. Treatment with EGb 761 for 5 but not 2 months also decreased p-Tau protein amount and shifted microglial pro-inflammatory to anti-inflammatory activation in the brain. As potential therapeutic mechanisms, we demonstrated that treatment with EGb 761, especially the components of ginkgolide A, bilobalide, and flavonoids, but not with purified ginkgolide B or C, increased autophagic activity and degradation of p-Tau in lysosomes of neurons. Inhibiting ATG5 function or treating cells with Bafilomycin B1 abolished EGb 761-enhanced degradation of p-Tau in cultured neurons. Additionally, we observed that 5- instead of 2-month-treatment with EGb 761 inhibited the activity of p38-MAPK and GSK-3β. Therefore, long-term treatment with *Ginkgo biloba* extract EGb 761, a clinically available and well-tolerated herbal medication, ameliorates AD pathology through mechanisms against multiple AD pathogenic processes.

Keywords: Alzheimer's disease, autophagy, Ginkgo biloba extract, inflammation, tauopathies

INTRODUCTION

Alzheimer's disease (AD), the most common form of dementia in the elderly, is pathologically characterized by intracellular neurofibrillary tangles (NFT)

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and extracellular amyloid-β (Aβ) deposits in the brain parenchyma [1]. NFT are primarily composed of hyperphosphorylated tau protein (p-Tau) [1, 2]. There is evidence showing that oligomeric Aβ induces hyperphosphorylation and aggregation of tau [2, 3], and drives tau pathology expanding from a restricted region around medial temporal cortex to the whole neocortex [4]. Reducing tau expression might prevents Aβ-induced neuronal death [5]. Neuroimaging and pathological experiments have shown that accumulation of tau instead of Aβ is associated with the poorer cognitive performance and brain atrophy [4, 6–8]. In the cerebrospinal fluid of AD patients, the protein level of p-Tau, which are phosphorylated at threonine 181 or 231, and total amount of Tau (t-Tau) proteins are both elevated and even predict the future brain atrophy during AD progression [9–11]. Transgenic mice that overexpress mutant forms of human tau in neurons [6] and knock-in mice in which a single copy of C-terminal tau fragment is expressed under the control of human tau promoter [12] both develop AD-like pathological changes in the brain, such as NFT, synaptic dysfunction, neuronal loss, and microglial activation.

How p-Tau impairs neuronal network is unclear, but might include the following mechanisms: 1) hyperphosphorylation of tau disrupts the normal function of tau in stabilizing the cytoskeleton and regulating axonal transport [13]; 2) tau targets the Src kinase Fyn and mediates the Aβ-induced loss of N-Methyl-D-aspartate receptor at the postsynapse [14]; 3) tau accumulation dephosphorylates cAMP response element binding protein (CREB), and thereby impairs the formation of synaptic connections [15]; and 4) p-Tau even induces oligomeric insulin accumulation and insulin resistance in neurons [16]. Thus, p-Tau is a key pathogenic molecule in AD and reduction of p-Tau might ameliorate the AD progress.

Growing evidence has shown that macroautophagy (hereafter referred to as *autophagy*) might be an efficient mechanism to clear p-Tau. Co-localization of aggregated p-Tau and autophagic vacuoles was observed in AD postmortem brain tissues [17]. p-Tau accumulated in the mouse brain, when the key autophagic component, ATG7, was ablated in the forebrain neurons [18]. On the other hand the following autophagy-enhancing interventions decreased the protein level of cerebral p-Tau in the tau-transgenic mouse brain: 1) treating mice with rapamycin [19], CCI-779 [20], trehalose [21], or miR-132 mimics [22]; 2) activating Nrf2 signaling pathway [23, 24]; or 3) overexpressing transcription factor EB [25, 26]. Recently, we enhanced neuronal autophagy in tau-transgenic mice by activating mild and chronic microglial inflammatory activation and observed that the cerebral p-Tau was decreased and the cognitive deficit was attenuated [27].

We have also observed that a 5-month-long oral administration of *Ginkgo biloba* extract EGb 761 inhibits inflammatory activation in the brain of AD amyloid-β protein precursor (AβPP)-transgenic mice by activating autophagy and facilitating degradation of NACHT, LRR and PYD domains-containing protein 3 (NLRP3)-contained inflammasomes in microglia [28]. We hypothesized that treatment with EGb 761 could also activate neuronal autophagy and increase the p-Tau clearance from the AD brain. To examine whether EGb 761 alleviated the consequences of the p-Tau production and its mechanism, here we treated both tau-transgenic mice and cultured neuronal cells with EGb 761 and examined the effects of autophagy of neuronal cells on AD-related cognitive deficits, tauopathy, and inflammatory activation.

MATERIAL AND METHODS

Animal models

Tau-transgenic mice (B6;C3-Tg(Prnp-MAPT*P301S)PS19Vle/J; Stock No: 008169) overexpressing human tau mutant (P301S) under the direction of mouse prion protein promoter [29] were imported from The Jackson Laboratory in December 2012. The original mice were on a genetic background mixed with C57BL/6J and C3H/HeJ. Before experiments, tau-transgenic male mice were back-crossed with C57BL/6J female mice for two generations to purify their genetic background. Compared to the phenotype displayed by the original mouse strain [29], our mice develop less severe pathology, for example: 1) NFT is not detectable in brain cells before 9 months of age; 2) from January 2013 to July 2018, 201 tau-transgenic mice and 69 wild-type littermates were prepared for experiments (including the current study, but not limited to it). All experiments ended before the mice were 9 months old. There were 23 (11.4%) transgenic mice and 8 (11.6%) wild-type mice which died without clear reasons. χ^2 test shows no difference between the rates of mouse death (χ^2 (1) = 0.000, $p = 0.997$). Our preliminary experiments showed that male and female tau-transgenic mice differed neither in Morris water maze test nor in cerebral load of p-Tau

(see the following methods). Thus, we used both male and female mice in this study to save experimental animals. Animal experiments were performed in accordance with all relevant national rules and were authorized by the local research ethical committee.

Administration of EGb 761 in animals

Tau-transgenic littermate mice were randomly assigned to one of four groups ($n \geq 15$ per group) and treated with EGb 761-supplemented diet according to our previous study [28]: Group 1 consisted of 4-month-old mice treated with a low-flavonoid control diet (C1000, Altromin Spezialfutter GmbH & Co. KG, Lage, Germany) for 5 months. Group 2 consisted of 4-month-old mice treated with C1000 diet supplemented with 600 mg EGb 761 per kg (0.6%) for 5 months. EGb 761® was provided by Dr. Willmar Schwabe Pharmaceuticals, Karlsruhe, Germany. It is a dry extract from *Ginkgo biloba* leaves (35–67:1) with extraction solvent: acetone 60% (w). The extract is adjusted to 22–27% Ginkgo flavonoids calculated as Ginkgo flavone glycosides and 5–7% terpene lactones consisting of 2.8–3.4% ginkgolides A, B, C and 2.6–3.2% bilobalide and contains less than 5 ppm ginkgolic acids. On the basis of ad libitum diet intake measures, the average dose of EGb 761 provided corresponds to 69 mg per kg body weight per day. Group 3 consisted of 7-month-old mice treated with C1000 for 2 months. Group 4 consisted of 7-month-old mice treated with C1000 supplemented with 600 mg EGb 761 per kg for 2 months. Before the intervention period, all mice were adapted to the C1000 diet for 1 week.

Morris water maze

The Morris water maze test was used to assess the cognitive function of tau-transgenic mice after the chronic treatment with EGb 761 or control using our established protocol [27]. Mice were trained 4 times per day for 6 days to find the hidden escape platform with an interval of ≥15 min between each trials. Latency time, path length, and velocity were recorded with Ethovision video tracking equipment and software (Noldus Ethovision, Wageningen, the Netherlands). After the training phase, there was 1 day of rest, and a probe trial on the 8th day.

Tissue collection for histological and biochemical analysis

Animals were euthanized by isoflurane inhalation. Mice were perfused with ice cold PBS through the heart, and the brain was removed and divided. The left hemisphere was fixed in 4% paraformaldehyde (PFA; Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) in PBS and used for immunohistochemistry. A 0.5-μm-thick piece of tissue was sagittally cut from the right hemisphere. The cortex and hippocampus were separated and homogenized in TRIzol (Thermo Fisher Scientific, Darmstadt, Germany) for RNA isolation. The remainder of the right hemisphere was snap frozen in liquid nitrogen and stored at –80°C until biochemical analysis.

Western blotting

Brain tissues were homogenized in 5× volumes of ice-cold lysis buffer (50 mM Tris/HCl [pH = 7.4], 150 mM NaCl, 2 mM EDTA, 50 nM okadaic acid, 5 mM sodium pyrophosphate, 50 mM NaF, 1 mM DTT, 1% Triton X-100, and protease inhibitor cocktail; Roche Applied Science, Mannheim, Germany) followed by centrifugation at 16,000× *g* for 30 min at 4°C. After determination of protein concentrations with Bio-Rad Protein Assay (Bio-Rad Laboratories GmbH, München, Germany), the protein samples were separated through 10% or 12% SDS-PAGE gels. Proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes and incubated overnight at 4°C with the following antibodies: rabbit monoclonal antibodies against LC3B (clone D11), beclin1 (clone D40C5), phosphor-CREB (clone 87G3), CREB (clone 48H2), phospho-glycogen synthase kinase (GSK)-3β (clone D3A4), and GSK-3β (clone 7C10) (all bought from Cell Signaling Technology, Danvers, MA); and rabbit polyclonal antibodies against SQSTM1/p62 (Cat.-No: 5114), phospho-p38-mitogen-activated protein kinase (MAPK) (Thr180/Tyr182) (Cat.-No: 9211), and p38-MAPK (Cat.-No: 9212) (also from Cell Signaling Technology). After thoroughly washing, relevant HRP-conjugated secondary antibodies were used. The detected proteins were visualized via Plus-ECL method (PerkinElmer, Waltham, MA). To quantify proteins of interest, rabbit monoclonal antibody against β-actin (clone 13E5; Cell Signaling Technology) or mouse monoclonal antibody against α-tubulin (clone DM1A; Abcam, Cambridge, United Kingdom) were used to determine the amount of loading proteins. Densitometric analysis of band densities was performed with Image-Pro PLUS software version 6.0.0.260 (Media Cybernetics, Inc., Rockville, MD).

Western blot quantification of cerebral p-Tau proteins

To quantify p-Tau proteins, the brain tissue was sequentially homogenized in buffers with increasing extraction strengths according to the published protocol [29, 30]. Briefly, brains were homogenized in 4× volumes of ice-cold high-salt reassembly buffer (RAB-HS) (0.1 M MES, 1 mM EGTA, 0.5 mM MgSO4, 0.75 M NaCl, 0.02 M NaF, 1 mM PMSF, and 0.1% protease inhibitor cocktail, Roche Applied Science) by passing through a 24-gauge needle without significant resistance for 10 times and centrifuged at 50,000× *g* for 40 min at 4°C in a Beckman Optima MAX-XP ultracentrifuge (Beckman Coulter GmbH, Krefeld, Germany). The RAB-HS supernatants were collected as RAB-soluble fractions. The pellets were re-suspended in 3× volumes of RIPA (50 mM Tris, pH 8, 150 mM NaCl, 1% NP-40, 5 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS) and centrifuged at 40,000× *g* for 20 min at 4°C. The supernatants were used as RIPA-soluble fraction. In the end, the RIPA-insoluble pellets were further extracted in 0.8 volumes of 70% formic acid (FA) and centrifuged to collect FA-soluble fraction. The protein concentration of all fractions was measured with Bio-Rad Protein Assay. In one SDS-PAGE gel, the same amount of protein was loaded into each well. The protein levels of p-Tau and α-tubulin were detected with western blot as described in the last paragraph using the mouse monoclonal antibody against p-Tau (Thr231) (clone 4C10; Dianova GmbH, Hamburg, Germany) and α-tubulin (clone DM1A).

Western blot detection of lysosomal p-Tau in the brain

The brain cortex and hippocampus were carefully dissected from the chronically EGb 761-treated or control mice. Lysosomes were isolated using our established protocol [31]. The brain tissue was homogenized in 5× volumes of ice cold buffer (HB; 0.25 M sucrose, 10 mM Hepes, 1 mM EDTA [pH = 7.4]) and centrifuged at 800× *g* for 10 min. The supernatant was collected and the pellet was re-suspended in a half volume of HB for a second centrifugation under the same conditions. The pellet was discarded and the two supernatants were combined. The pooled supernatant was incubated for 10 min at 37°C in the presence of 2 mM $CaCl_2$ and then centrifuged at 3,000× *g* for 10 min to remove large mitochondria. The resultant supernatant was centrifuged for 10 min at 18,000× *g* to obtain a pellet. The pellet was re-suspended in 0.5 ml HB and layered on 4 ml of iso-osmotic Percoll (GE Healthcare, München, Germany) at a concentration of 30% (pH 7.4). Under the Percoll layer, 0.5 ml 2.5M sucrose was laid. Centrifugation was performed at 4°C for 40 min at 44,000× *g*. The subsequent gradients were carefully collected from the top with 0.9 ml /fraction. Protein concentrations in different fractions were determined and western blot was performed to quantify the protein amount of p-Tau and lysosomal-associated membrane protein 1 (LAMP-1), with relevant antibodies (mouse monoclonal antibody AT8 and rabbit monoclonal antibody C54H11, respectively). Non-lysosomal markers, calnexin and β-actin were also detected with the rabbit polyclonal antibody (Cat.-No. ab22595, Abcam) and rabbit monoclonal antibody (clone 13E5), respectively, to identify the fraction enriched with only lysosomes.

Immunofluorescence microscopy and analysis

The 4% PFA-fixed left brain hemisphere was dehydrated in PBS containing 30% sucrose, embedded in Tissue-Tek® O.C.T. Compound (Sakura Finetek Europe B.V., AJ Alphen aan den Rijn, the Netherlands) and then frozen in 2-methylbutane on liquid nitrogen. Serial sagittal sections with 30 μm of thickness were cut from the left brain hemisphere with a Cryostat (Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany). After systematic random sampling, every 10th section throughout the entire hippocampus and the cortex were selected for the histological analysis. Antigen retrieval was performed by heating sections in 10 μM citrate buffer to 100°C for 30 min (pH = 6.0). After blocking with 5% goat serum in PBS/0.3% Triton X-100, brain sections were incubated at 4°C overnight with the following primary antibodies: rabbit polyclonal antibody against ionized calcium-binding adapter molecule (Iba)-1 (Wako Chemicals GmbH, Neuss, Germany), rabbit monoclonal antibody against LC3A/B (clone D3U4C; Cell Signaling Technology), and mouse monoclonal antibodies against S100 (clone 4C4.9; Abcam), synaptophysin (clone SY38; Abcam), and p-Tau (clone AT8; Thermo Scientific), in PBS/0.1% Triton X-100 and 1% goat serum. Afterwards, sections were rinsed thoroughly, and incubated for 1 h at room temperature with the corresponding Alexa Fluor 488 or 546-conjugated second antibodies. All images were acquired with a Zeiss AxioImager.Z2

microscope equipped with a Stereo Investigator system (MicroBrightField Bioscience, Williston).

To count Iba1-, S100-, and cells containing LC 3A/B-immunofluorescence positive puncta, the stereological probe Optical Fractionator with $120 \times 120 \times 18$ μm of a dissector and 400×400 μm of a sampling grid was used as we did in the previous study [32]. The estimated coefficient of error was <0.05. As AT8 antibody-immunoreactive neurons appeared not to equally distribute in different regions of the brain, we counted these cells in the whole cortex and hippocampus without using stereological probes.

To quantify the intensity of the immunofluorescent staining of synaptophysin in the CA3 area of hippocampus, 3 areas using a 63× objective were randomly selected and Z-stacks for 40 images with 0.2 μm interval between two neighboring scans were collected. The serial images were processed with deconvolution and Z-projected with maximal intensity. The immunofluorescent intensity of the final image derived from each area was quantified with Image-Pro PLUS and the mean intensity from three areas was averaged as the result of each section.

Quantitative reverse transcription for analysis of gene transcripts

Total RNA was isolated from the brain homogenate in Trizol. First-strand cDNA was synthesized by priming total RNA with hexamer random primers and using Superscript III reverse transcriptase (Thermo Fisher Scientific). For quantification, we used the 7500 Fast real-time PCR system (Thermo Fisher Scientific) to perform real-time quantitative polymerase chain reaction (PCR) with the Taqman gene expression assays of mouse *tumor necrosis factor (tnf)-α*, *interleukin (il)-1β*, *inducible nitric oxide synthase (inos)*, *chemokine (C–C motif) ligand 2 (ccl2)*, *il-10*, *arginase 1*, *chitinase-like 3 (chi3l3)*, *mannose receptor, C type 1 (mrc1)*, and *glyceraldehyde 3-phosphate dehydrogenase (gapdh)* (all from Thermo Fisher Scientific).

Cell culture, autophagic, and apoptotic analysis, and p-Tau detection

SH-SY5Y neuroblastoma cells were obtained from LGC Standards GmbH (Wesel, Germany) and maintained in DMEM supplemented with 10% fetal calf serum (FCS; PAN Biotech, Aidenbach, Germany), in a humidified incubator with 5% CO_2 at 37°C. LC3-GFP-mRFP-transgenic autophagy reporter cell line overexpressing monomeric red fluorescence protein (mRFP), green fluorescence protein (GFP) and LC3 fusion protein serially [33] and SH-SY5Y cell lines overexpressing wild-type (wt) and dominant-negative (DN, with a substitution mutation: K130R) human ATG5 have been established in our previous study [31].

Cortical neurons were isolated from C57BL/6J mouse embryos at E14-16 and cultured in neurobasal medium supplemented with 2% B27 (Thermo Fisher Scientific), 0.25% L-glutamine (Sigma-Aldrich) and 0.1% glutamate (Sigma-Aldrich). Neuronal cells were used 10 days after culture.

To investigate the effects of EGb 761 on neuronal autophagy, SH-SY5Y cells or primary cultured neurons cultured at 7×10^5 cells/well in 6-well plate were treated with EGb 761 at 0, 2.5, 5, 10, 50 and 100 μg/ml for 24 h, or the major components of EGb 761 (ginkgolides A, B, and C, ginkgo flavonoids and bilobalide) at different concentrations as indicated in the results. Thereafter, the cell lysate was prepared for the quantitative western blot analysis of LC3B, SQSTM1/p62, beclin1 and ATG5 as described above for the western blot analysis of brain homogenate. The antibody used against ATG5 was a monoclonal rabbit antibody (clone D5F5U) obtained from Cell Signaling Technology.

For the apoptosis assay, SH-SY5Y cells were treated with EGb 761 at different concentrations as for autophagy assay. As positive controls of apoptosis induction, SH-SY5Y cells were treated with H_2O_2 at 0.1, 0.3, and 0.6 mM. A rabbit monoclonal antibody against cleaved caspase-3 (Asp175) (clone 5A1E, Cell Signaling Technology) was used to detect activated caspase-3.

LC3-GFP-RFP-transgenic SH-SY5Y cells cultured on coverslips in a 24-well plate (BD Bioscience, Heidelberg, Germany) at a density of 1×10^5 cells/well were treated with EGb 761 as described above. The formation of autophagic vacuoles was evaluated under confocal microscopy (see the following section).

To investigate the effects of EGb 761 on p-Tau degradation, native SH-SY5Y cells were incubated with EGb 761 as for autophagy assay. Thereafter, cell lysate was prepared in RIPA buffer supplemented with proteinase and phosphatase inhibitors (2 mM EDTA, 2 mM EGTA, 50 nM okadaic acid, 5 mM sodium pyrophosphate, 2 mM sodium vanadate, 1 mM DTT, 50 mM NaF, and protease inhibitor mixture; Roche Applied Science). The protein levels of p-Tau and t-Tau were detected with quantitative

western blot using mouse monoclonal antibodies against p-Tau (clone AT8) and t-Tau (clone 8F10, Dianova). The rabbit monoclonal antibody against β-actin (clone 13E5) was used to evaluate the loading amount of proteins. To investigate the potential role of autophagy, SH-SY5Y cells were pre-treated with 100 nM Bafilomycin B1 (Sigma-Aldrich) or vehicle for 1 h, and then with EGb 761 at 0, 5, and 10 μg/ml for another 24 h in the presence of Bafilomycin B1 or vehicle. Moreover, SH-SY5Y cells with overexpression of human wt and DN ATG5 were treated with EGb 761 at 0, 5, and 10 μg/ml for 24 h. After

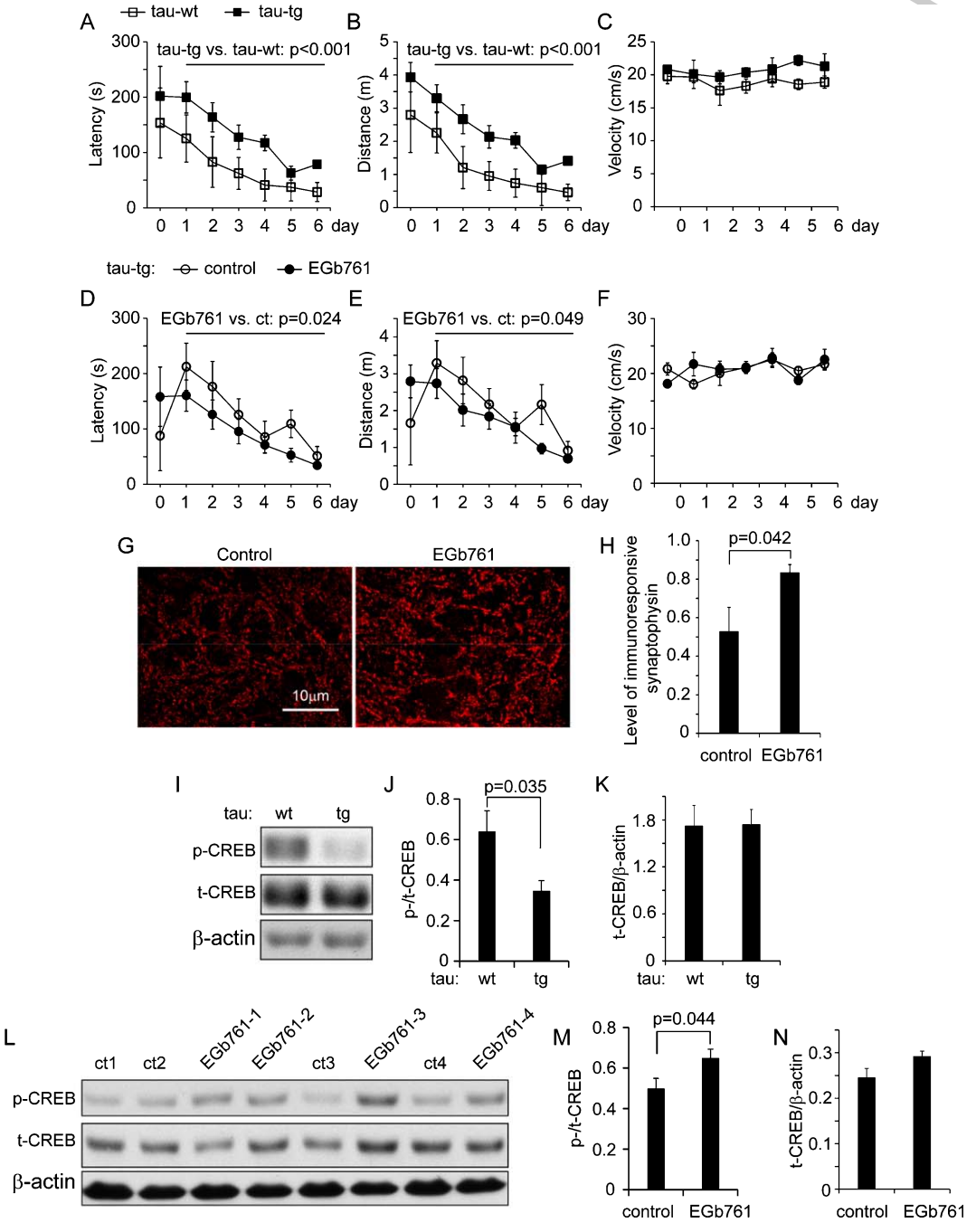

Fig. 1. (*Continued*)

treatments, p-Tau protein and LC3B were detected with quantitative western blot.

Confocal laser scanning microscopy

To determine EGb 761-induced neuronal autophagy, the neuronal autophagic reporter cells were fixed with 4% PFA after treatment with EGb 761 as described in the last paragraph, and examined using a Zeiss LSM 510 Meta Confocal Microscope (Göttingen, Germany). From each treated reporter cells, more than 15 areas using a 40× objective were randomly chosen and >600 cells were counted. The density of puncta with pure red or green (with weak red) fluorescence in each cell was calculated as the total number of puncta divided by the total number of cells. The experiments were repeated independently 3 times.

To investigate the relationship between p-Tau and autophagic vacuoles, brain sections (see above) were incubated at 4°C, overnight with the rabbit polyclonal antibody against SQSTM1/p62 (Cat.-No: 5114, Cell Signaling Technology) and then with Alexa488-conjugated goat anti-rabbit IgG. After washing, the brain tissue was further incubated with AT8 antibody (Thermo Scientific) overnight and then Cy3-conjugated goat anti-mouse IgG (both second antibodies were bought from Thermo Fisher Scientific). Whether p-Tau co-localizes with autophagic vacuoles was analyzed by confocal microscopy.

Statistical analysis

Data were obtained from at least three independent experiments and presented as the mean ± SEM. Two-way ANOVA was used to analyze the results of water maze test with latency, distance, and velocity as dependent variables and tau-transgenic expression, treatments with EGb 761 and training days as fixed factors. One-way ANOVA followed by Bonferroni, Tukey, or Games-Howell *post hoc* test (dependent on the result of Levene's test to determine the equality of variances) was used to examine the effects of treatments (as factors) with EGb 761 or its major components at more than two different concentrations on the levels of autophagic proteins and tau proteins (as dependent variables). The means between two groups of values were compared with 2-tailed unpaired Student *t*-test. All statistical analyses were performed with SPSS version 19.0 for Windows (IBM, New York, NY). Statistical significance was set at $p < 0.05$.

RESULTS

Treatment with Ginkgo biloba extract EGb 761 attenuates both AD-related cognitive deficits and synaptic impairment in tau-transgenic mice

To test anti-AD effects of EGb 761, we fed 4 and 7-month-old tau-transgenic littermate mice with EGb 761-supplemented or control diets for 5 and 2 months, respectively. During feeding experiments, all mice (40/48 [male/female] mice) did not display any gross physical or behavioral abnormalities. No mice died. Interestingly, the body weight (25.15 ± 0.31 g) of mice receiving EGb 761 for 5 months was significantly lower than that (26.42 ± 0.29 g) of mice fed with control diets (*t* test, t (31) = 2.632, $p = 0.013$; $n \geq 15$ per group), which suggests that treatments

Fig. 1. Long-term treatment with EGb 761 improves AD-related symptoms and neuronal plasticity in tau-transgenic mice. In the Morris water maze test, 9-month-old tau-transgenic (tg) spent significantly more time and traveled longer distance to reach the platform than their wild-type (wt) littermate mice (A and B; two-way ANOVA comparing tau-tg and tau-wt mice during the training phase from day 1 to day 6, F (1, 288) = 26.514, $p < 0.001$ for latency and F (1, 288) = 31.805, $p < 0.001$ for distance; $n = 36$ and 14 for tau-tg and tau-wt mice, respectively). Treatments with EGb 761, added to the regular diet for 5 months, significantly reduced the traveling time and distance of tau-transgenic mice in the training phase, when the tau-tg mice receiving standard diets were compared (D and E; two-way ANOVA comparing tau-transgenic mice receiving EGb 761-supplemented and control (ct) diets from day 1 to day 6, F (1, 129) = 5.193, $p = 0.024$ for latency, and F (1, 129) = 3.947, $p = 0.049$ for distance; $n = 12$ per group). The swimming speed did not differ between tau-tg and tau-wt mice, EGb 761-treated and control tau-tg mice and for each mouse at different time points (C and F; two-way ANOVA, $p > 0.05$). Brain sections derived from these EGb 761-treated and non-treated littermate tau-transgenic mice were stained with immunofluorescence-conjugated anti-synaptophysin antibodies (G). As shown by the intensity of fluorescence in the CA3 area of the hippocampus, the protein levels of synaptophysin were significantly increased by EGb 761 treatment (H; *t* test, t (10) = 2.335, $p = 0.042$; $n = 6$ per group). Moreover, phosphorylated and total CREB in the brain homogenate were quantified with western blots. The ratio of phosphorylated CREB (p-CREB) to total CREB (t-CREB) was significantly decreased in 9-month-old tau-tg mice compared with their littermate tau-wt mice (I - K; *t* test, t (14) = –2.341, $p = 0.035$; $n = 9$ and 7 for wt and tg groups, respectively). Interestingly, the ratio of p-CREB/t-CREB was significantly higher in EGb 761-treated 9-month-old tau-tg mice than in the littermate control tau-tg mice receiving control diets (L and M; *t* test, t (16) = 2.184, $p = 0.044$; $n = 8$ and 10 for EGb 761-treated and control groups, respectively). Moreover, treatments with EGb 761 appeared to increase the expression of total CREB protein (L and N; *t* test, t (16.925) = 2.039, $p = 0.057$).

with EGb 761 might inhibit the gain of body weight during growth.

Thereafter, we used the Morris water maze to examine the spatial learning ability of 9-month-old tau-transgenic mice. We observed that tau-transgenic mice spent significantly longer time and swam longer distance than their wild-type littermate controls to reach the target platform during the 6-day acquisition phase, which is the same as we observed in a previous study [27] (Fig. 1A, B; two-way ANOVA showing the difference between tau-transgenic and wild-type littermates, $p < 0.001$ for both latency and distance). When tau-transgenic littermate mice that had received either EGb 761-supplemented or standard diets for 5 months were compared, we observed that the tau mice receiving EGb 761 diets required significantly less time, and traveled significantly shorter distances before reaching the escape platform (Fig. 1D, E; two-way ANOVA showing effects of EGb 761, $p < 0.05$ for latency and distance, respectively). The swimming speed was not different between tau-transgenic and wild-type mice, between tau-transgenic mice fed with and without EGb 761-supplemented diets, and between different training dates for the same animal (Fig. 1C, F; two-way ANOVA, $p > 0.05$). In the probe trial, 48 h after the end of the acquisition phase with the removal of escape platform, tau-transgenic mice crossed the region for original platform with less frequency than their tau-wild-type littermate mice (7.68 ± 0.83 and 14.14 ± 0.90 for tau-transgenic and wild-type mice, respectively; t test, t (29) = –5.266, $p < 0.001$; $n \geq 14$ per group). However, treatment with EGb 761 failed to increase the visiting frequency of tau-transgenic mice (7.88 ± 1.52 and 8.67 ± 0.68 for EGb 761-treated and control mice, respectively; t test, t (15) = 0.625, $p = 0.625$; $n \geq 8$ per group). We also performed Morris water maze test for 9-month-old tau-transgenic mice that had received EGb 761-supplemented or standard diets for 2 months. These two groups of mice differed neither in the acquisition phase, nor in the probe trial (data not shown).

Synaptophysin is a major synaptic vesicle protein. We have observed that its protein level in CA3 region of hippocampus is reduced in tau-transgenic mice [27], which might represent the synaptic impairment in the AD mice. In this study, we observed that the structure of CA3 area appeared to be similar between 9-month-old tau-transgenic mice with and without 5-month treatments of EGb 761 (Fig. 1G); however, treatment with EGb 761 for 5 months significantly increased the immunoreactivity for synaptophysin (Fig. 1H; t test, $p < 0.05$).

Overexpression of human tau was reported to dephosphorylate CREB in association with impaired synaptic plasticity and cognitive deficits in mice [15]. Our result showed that the phosphorylation level of CREB in our 9-month-old tau-transgenic mice was significantly lower than that in their wild-type littermate mice (Fig. 1I, J; t test, $p < 0.05$) supported the previous observation. Interestingly, the reduction of phosphorylated CREB in tau-transgenic mice was recovered by EGb 761 treatment, as the ratio of phospho-CREB/total CREB was higher in the brain homogenate derived from 9-month-old tau-transgenic mice receiving EGb 761-supplemented diets for 5 months than that from the control tau mice receiving standard diets for the same time (Fig. 1L, M; t test, $p < 0.05$). Moreover, we observed that EGb 761 not only affected the phosphorylation of CREB, but also tended to up-regulate the protein expression of CREB (Fig. 1N; t test, $p = 0.057$).

Treatment with Ginkgo biloba extract EGb 761 decreases the protein level of phosphorylated tau in tau-transgenic mouse brain

As p-Tau is one of the major pathogenic molecules in AD [1], we continued to examine whether the chronic treatment of EGb 761 reduced this neurotoxic protein in the tau-transgenic mouse brain. Brains were collected from 9-month-old tau-transgenic mice, which had been fed with and without EGb 761-supplemented diets for 5 months. When brain sections were immunologically stained with AT8 antibody against p-Tau (phosphorylation at Ser202/Thr205), we observed that the AT8-immunoreactive neurons in EGb 761-treated tau mice were significantly less than those in the littermate mice which were fed with control diets (Fig. 2A, B; EGb 761-treated versus control, 117.00 ± 14.52 cells/mm^2 versus 62.00 ± 11.94 cells/mm^2, t test, $p < 0.05$). We also sequentially extracted protein samples from the brain with increasing extraction strengths: RAB, RIPA, and FA using our established protocol [27]. The three fractions were then analyzed by western blotting with antibodies against human p-Tau (phosphorylation at Thr231) (Fig. 2C-E). We observed that the protein amount of p-Tau shown

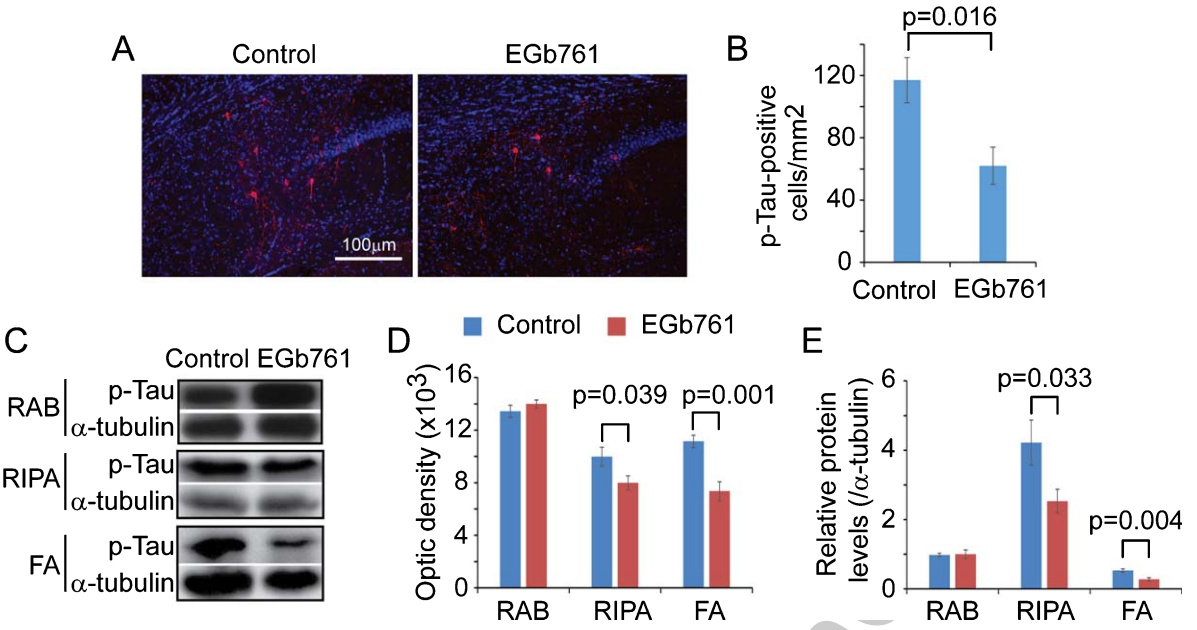

Fig. 2. Long-term treatment with EGb 761 reduces phosphorylated tau protein in the tau-transgenic mouse brain. Four-month-old tau-transgenic mice were treated with or without EGb 761 added to the diet for 5 months. Immunological staining with AT8 antibody against p-Tau showed that AT8-immunoreactive neurons in EGb 761-treated tau mice were significantly fewer than in littermate tau-transgenic mice that received control diets (A and B; *t* test, t (9) = –2.957, $p = 0.016$; $n = 6$ and 5 for EGb 761 treated and control groups, respectively). Tau protein was also extracted using RAB, RIPA, and FA buffers with increasing extraction strengths. Tau and α-tubulin proteins in each fraction of brain homogenate were detected by western blot with the same amount of protein loaded per lane (C). The protein level of p-Tau as shown by direct densitometry or the ratio of optic densities of p-Tau and α-tubulin was significantly lower in both RIPA- and FA-soluble fractions derived from EGb 761-treated tau-transgenic mice than that from control diet-treated littermate transgenic mice (D and E; *t* test, t (15) = –2.266, $p = 0.039$ and t (14.433) = –4.428, $p = 0.001$ for optic density in RIPA and FA fractions; t (15) = –2.369, $p = 0.033$ and t (15) = –3.426, $p = 0.004$ for ratios in RIPA and FA fractions; $n = 10$ and 7 for EGb 761-treated and control groups, respectively). The amount of p-Tau in RAB-soluble fraction was not changed by EGb 761 treatment (D and E; *t* test, t (15) = –1.051 and –1.022, $p = 0.310$ and 0.317, for optic density and ratios, respectively).

with absolute optic density in western blot or relative protein level adjusted by the protein amount of α-tubulin in the same sample, was 20%∼40% lower in the RIPA or FA fraction from EGb 761-treated tau mice, than that from control diets-fed littermate tau-transgenic mice (Fig. 2D, E; *t* test, $p < 0.05$). EGb 761 did not change the p-Tau protein level in RAB fractions (Fig. 2D, E; t test, $p > 0.05$).

Quantitative western blot was also used to detect p-Tau in brains derived from 9-month-old tau-transgenic mice, which had been treated with and without EGb 761 for 2 months. In neither RIPA nor FA fraction, there was a significant difference in the protein level of p-Tau. Optical density of p-Tau from control and EGb 761-treated groups: 12.25 ± 3.44 and 10.19 ± 1.61 in RIPA fraction (*t* test, t (11) = 0.571, $p = 0.579$; $n = 6$ and 7, respectively), and 12.56 ± 1.88 and 11.42 ± 2.06 in FA fraction (*t* test, t (11) = 0.539, $p = 0.601$), respectively.

Treatment with Ginkgo biloba extract EGb 761 reduces pro-inflammatory activation in the tau-transgenic mouse brain

Neurotoxic inflammatory activation contributes to AD pathogenesis [34, 35]. We have recently observed that a long-term treatment with EGb 761 inhibits neuroinflammatory activation by activating microglial autophagy in AβPP-transgenic AD mice [28]. We continued to determine whether treatment with EGb 761 provided a similar anti-inflammatory effect in the tau-transgenic mouse brain. The histological analysis demonstrated that the number of Iba-1-positive cells in the hippocampus was significantly lower ($1.22 \pm 0.10 \times 10^4$ cells /mm^3; *t*-test, $p < 0.05$) in 9-month-old tau-transgenic mice treated with EGb 761 in their diet for 5 months than in littermate tau mice ($1.81 \pm 0.10 \times 10^4$ cells /mm^3) that were given diets without EGb 761 (Fig. 3A, B). However, the number

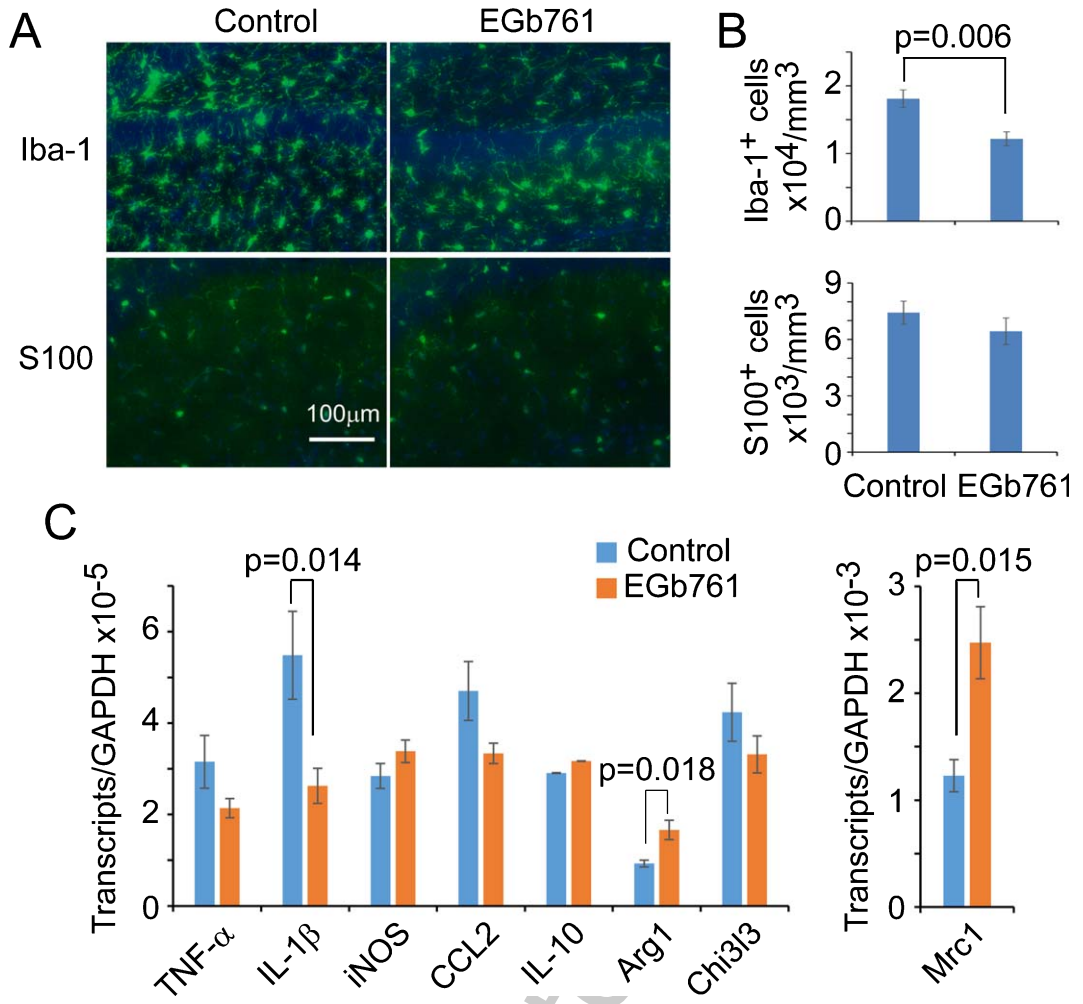

Fig. 3. Long-term treatment with EGb 761 shifts pro-inflammatory to anti-inflammatory activation in the tau-transgenic mouse brain. Four-month-old tau-transgenic mice received EGb 761-supplemented diets or control diets for 5 months. Microglia and astrocytes were immunofluorescently stained with Iba-1 and S100 antibodies, respectively (A; shown in green fluorescence). The number of microglia but not astrocytes in the entire hippocampus was reduced after 5 months of treatment with EGb 761 (B; *t*-test, t (9) = –3.598, $p = 0.006$ for Iba-1 cells, and t (9) = –1.030, $p = 0.330$ for S100 cells; $n = 5$ and 6 for control and EGb 761-treated groups, respectively). Transcription of pro-inflammatory gene *il-1β* as determined by real-time PCR was reduced, whereas, transcription of anti-inflammatory gene markers: *arg1* and *mrc1* was increased in the brains of tau-transgenic mice by 5 months of treatment with EGb 761 (C; *t* test, t (10) = –3.052, 2.816, and 2.948, $p = 0.014$, 0.018, and 0.015, for *il-1β*, *arg1* and *mrc1*, respectively; t $n = 5$ and 7 for control and EGb 761-treated mouse groups).

of S100-positive cells, representing astrocytes, was not changed by the 5-month treatments with EGb 761 (Fig. 3A, B; *t*-test, $p > 0.05$).

We further quantified transcripts of inflammatory genes in the brain. As shown in Fig. 3C, the transcription of pro-inflammatory gene, *il-1*β, was markedly decreased whereas that of anti-inflammatory genes (*arg1* and *mrc1*) was significantly increased by a diet supplemented with EGb 761 for 5 months (*t*-test, $p < 0.05$). However, in 9-month-old tau-transgenic mice that received EGb 761 for only 2 months, the transcription of inflammatory genes in the brain was not affected by EGb 761 (data not shown).

Treatment with Ginkgo biloba extract EGb 761 enhances autophagy in the brain and neuronal cells

We have recently observed that treatment with Ginkgo extract, EGb 761, increases the autophagic activity in the brain and microglia of AβPP-transgenic mice [28]. Indeed, the number of LC3A/B

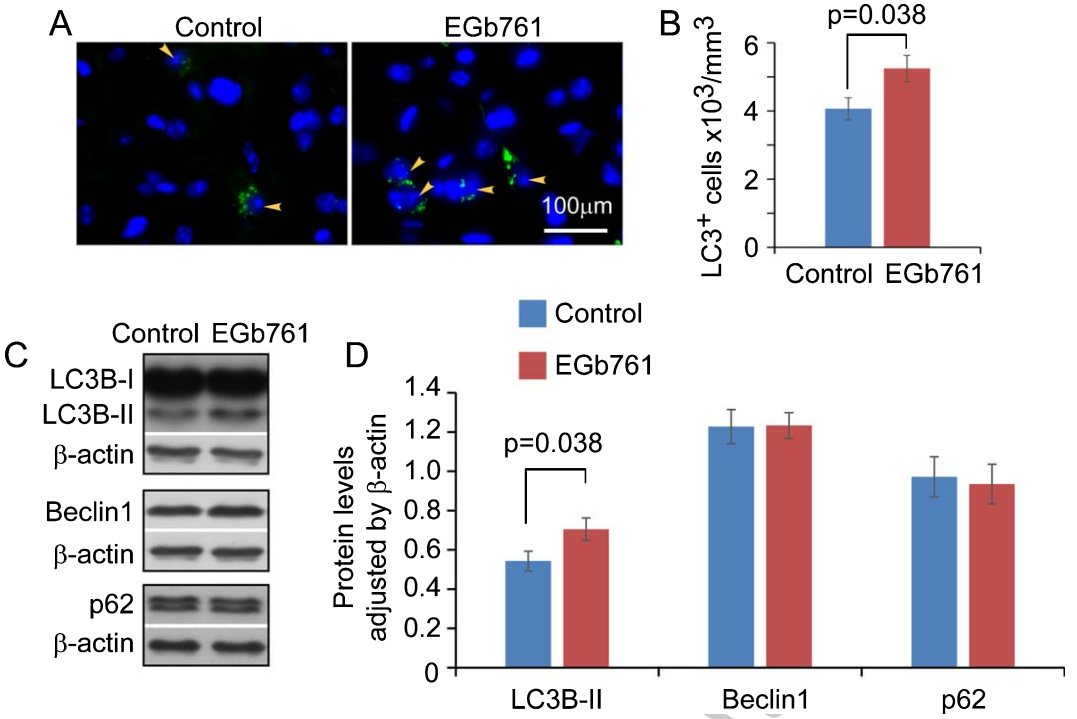

Fig. 4. Long-term treatment with EGb 761 enhances autophagic flux in the tau-transgenic mouse brain. Four-month-old tau-transgenic mice were fed with diets supplemented with or without EGb 761 for 5 months. After immunofluorescent staining of LC3A/B and stereological analysis with Optical Fractionator as a probe, significantly more LC3A/B-immunoresponsive puncta-positive cells were detected in the cortex of EGb 761-treated tau-transgenic mice than in littermate tau mice receiving control diets (A and B; *t* test, t (13) = 2.308, $p = 0.038$; $n = 8$ and 7 for EGb 761-treated and control mice, respectively). Quantitative western blot analysis further showed that the protein levels of LC3B-II (C and D; *t* test, t (36) = 2.150, $p = 0.038$; $n = 19$ per group), but not p62/SQSTM1 (t (22) = 0.684, $p = 0.501$; $n = 12$ per group) and beclin1 (t (12) = –0.050, $p = 0.961$; $n = 7$ per group) were significantly higher in EGb 761-treated tau-transgenic mice than in littermate control mice.

immunoreactive puncta-positive cells in the cortex ($52.47 \pm 3.85 \times 10^3$ cells /mm^3) of 9-month-old tau-transgenic mice receiving 5-month EGb 761-supplemented diets was significantly higher than the number in tau-transgenic littermates ($40.67 \pm 3.22 \times 10^3$ cells /mm^3; Fig. 4A, B; *t* test, $p < 0.05$) receiving 5-month standard diets. Similarly, the protein level of LC3-II, but not p62/SQSTM1 and beclin1, in the brains of tau mice fed with EGb 761-supplemented diet was significantly higher than that in tau mice receiving standard diet (Fig. 4C, D, *t* test, $p < 0.05$).

Moreover, we cultured SH-SY5Y neuronal cells in presence of EGb 761 at different concentrations for 24 h. Treatment with EGb 761 at 5 μg/ml, but not at a lower concentration (2.5 μg/ml), significantly increased the protein level of LC3B-II in the cell lysate (Fig. 5A, B; one-way ANOVA followed by *post hoc* test, $p < 0.05$). Surprisingly, treatment of EGb 761 at increasing concentrations (10 and 50 μg/ml) gradually lost the autophagy-enhancing effects (Fig. 5A, B). We did not observe that treatments with EGb 761 could significantly affect the protein levels of p62 (Fig. 5A, C; one-way ANOVA, $p > 0.05$). We also used a previously established LC3-GFP-mRFP-transgenic autophagy reporter cell line [27]. The mRFP-GFP-LC3 fusion protein showed both GFP and mRFP signals before the fusion of autophagosome with lysosomes; after fusion with lysosomes, the low pH of the lysosome resulted in bleaching of GFP fluorescence, and only the mRFP signal was visible. Similarly, after treatment with EGb 761 at 5 μg/ml, but not at higher concentrations (10, 50, and 100 μg/ml) for 24 h, the neuronal reporter cells showed significant increase in the numbers of both GFP/mRFP puncta and pure mRFP puncta (Fig. 5D-F; one-way ANOVA followed by *post hoc* test, $p < 0.05$). We also treated primary cultured neurons with EGb 761 to verify the results derived from SH-SY5Y cells. As shown in Fig. 5G and H, we observed that treatment of EGb 761 at 5 μg/ml constantly increased the protein level of

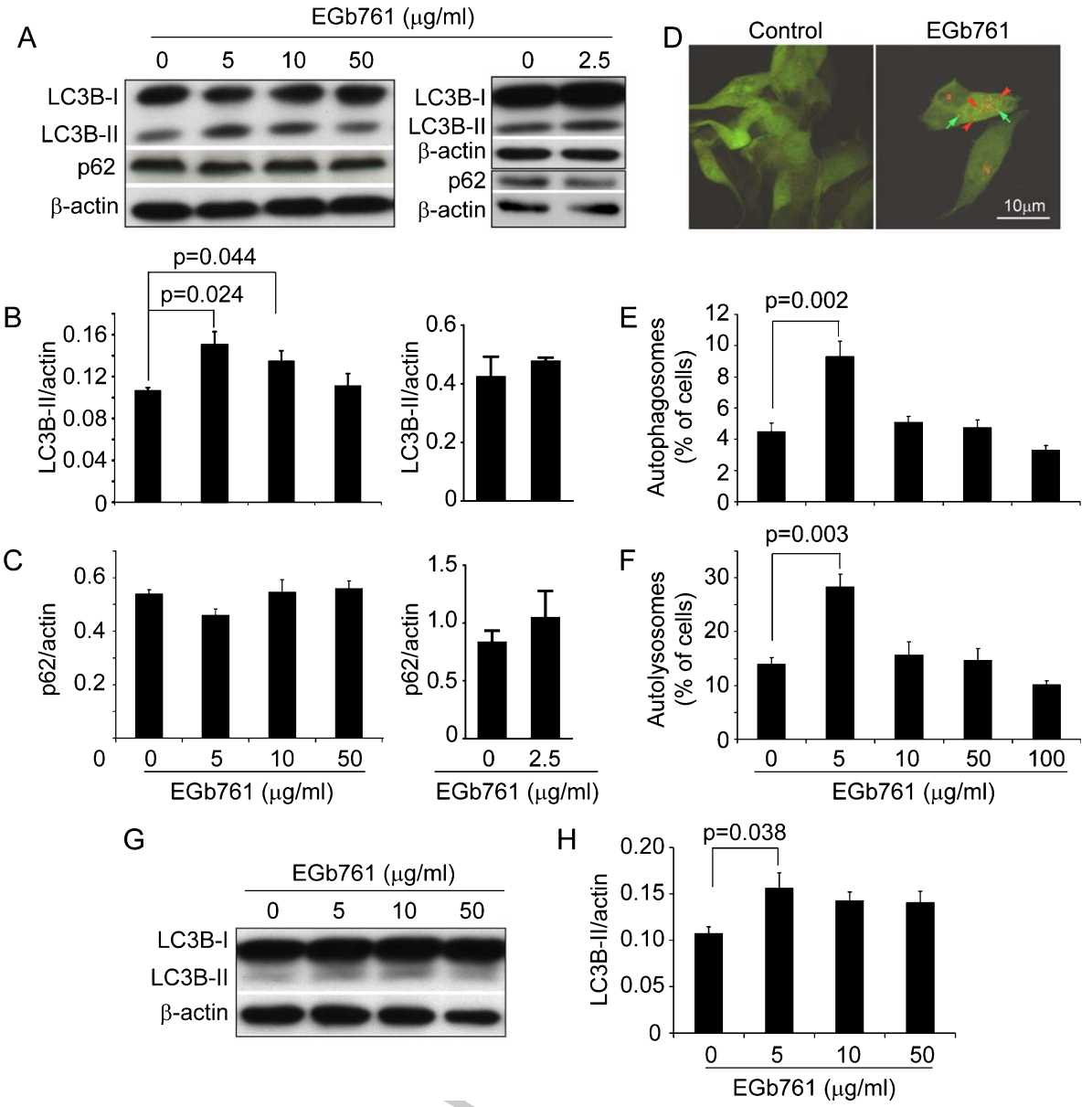

Fig. 5. Treatments with EGb 761 activate autophagy in neuronal cells. SH-SY5Y neuronal cells were treated with EGb 761 at 0, 2.5, 5, 10, and 50 μg/ml for 24 h (A-C). Quantitative western blot was used to detect LC3B and p62/SQSTM1 in the cell lysates. One-way ANOVA shows that EGb 761 treatments regulate the protein levels of LC3B-II but not of p62: $F(3, 32) = 8.957$, $p < 0.001$; $n = 9$ per group, for LC3B-II; and $F(3, 20) = 2.216$, $p = 0.129$; $n = 6$ per group, for p62. A Games-Howell *post hoc* test reveals that treatments with EGb 761 at 5 and 10 μg/ml significantly increase LC3B-II protein levels (B; $p = 0.024$ and $= 0.044$, respectively). Autophagy-reporting cells expressing the fusion protein of LC3-GFP-RFP were treated with EGb 761 in the same way (D-F). Autophagosomes are shown as green puncta (overlap with weak red fluorescence, marked with green arrows) and autolysosomes are shown as red puncta (marked with arrowheads) (D). Similarly, one-way ANOVA shows that EGb 761 treatments enhance autophagic flux: $F(4, 10) = 14.379$ and 12.340, $p < 0.001$ and $= 0.001$, for autophagosomes and autolysosomes, respectively; $n = 3$ per group. Tukey *post hoc* tests reveal that treatments with EGb 761 at 5 μg/ml significantly increase the formation of both autophagosomes and autolysosomes (E and F; $p = 0.002$ and 0.003, respectively). Additionally, we treated primary cultured neurons with EGb 761 at 0, 5, 10, and 50 μg/ml for 24 h and observed similar autophagy-enhancing effects (G and H; One-way ANOVA, $F(3, 16) = 3.263$, $p = 0.049$; $n = 4$ per group). A Tukey *post hoc* test reveals that treatment with EGb 761 at 5 μg/ml significantly increases LC3B-II protein levels ($p = 0.038$).

LC3B-II (one-way ANOVA followed by *post hoc* test, $p < 0.05$). Treatments of EGb 761 at higher concentrations tended to decrease the LC3B-II protein levels (one-way ANOVA followed by *post hoc* test, $p > 0.05$).

In additional experiments, we detected protein levels of ATG5 and beclin1 in EGb 761-treated SH-SY5Y cells. We observed that treatments with EGb 761 increased protein amount of both ATG5 and beclin1 in a dose-dependent manner (Fig. 6A-C; one-way ANOVA followed by *post hoc* test, $p < 0.05$), except that treatment with EGb 761 at 50 μg/ml started to decrease the protein level of beclin1 (Fig. 6C). In order to exclude the potential cytotoxic effects of EGb 761 at high concentrations (e.g., 10 and 50 μg/ml), which might alter autophagic activity, we routinely and carefully examined cells under microscope before and after treatments with EGb 761. We did not observe any significant morphological changes of cells when they were treated with EGb 761 at 0, 2.5, 5, 10, 50, and 100 μg/ml for 24 h. We further detected apoptosis in EGb 761-treated SH-SY5Y cells. H_2O_2 was used as a positive control [36]. We observed that treatments of EGb 761

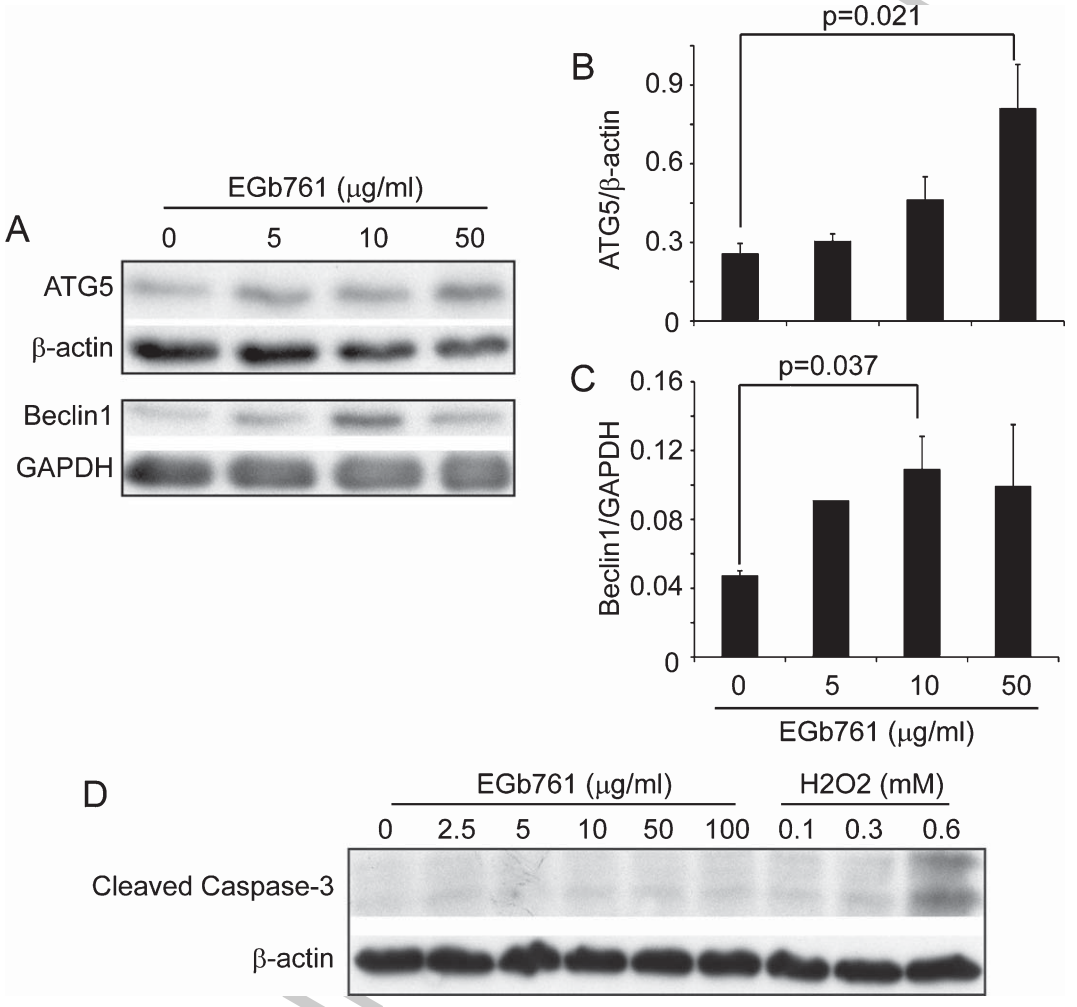

Fig. 6. Treatments with EGb 761 increase autophagy-associated protein levels without inducing apoptosis in neuronal cells. SH-SY5Y neuronal cells were treated with EGb 761 at 0, 5, 10, and 50 μg/ml for 24 h. The expression of autophagy-associated proteins ATG5 and beclin 1 were detected with quantitative western blot (A-C). One-way ANOVA shows that EGb 761 treatments up-regulates protein levels of ATG5 and beclin1: $F(3, 12) = 8.964$ and 3.537, $p = 0.002$ and 0.048, for ATG5 and beclin1, respectively ($n = 4$ per group). Tukey *post hoc* tests reveal that treatment with EGb 761 at 50 μg/ml significantly increases the protein level of ATG5 (B; $p = 0.003$), whereas, treatment with EGb 761 at 10 μg/ml markedly elevates the protein level of beclin 1 (C; $p = 0.037$). Moreover, we detected protein levels of cleaved caspase-3 in EGb 761-treated SH-SY5Y cells and cells treated with H_2O_2 as a positive control of apoptosis induction. EGb 761 at different tested concentrations (0 – 100 μg/ml) did not, whereas 0.6 mM H_2O_2 did cleave caspase-3 (D, one typical experiment from three independent experiments).

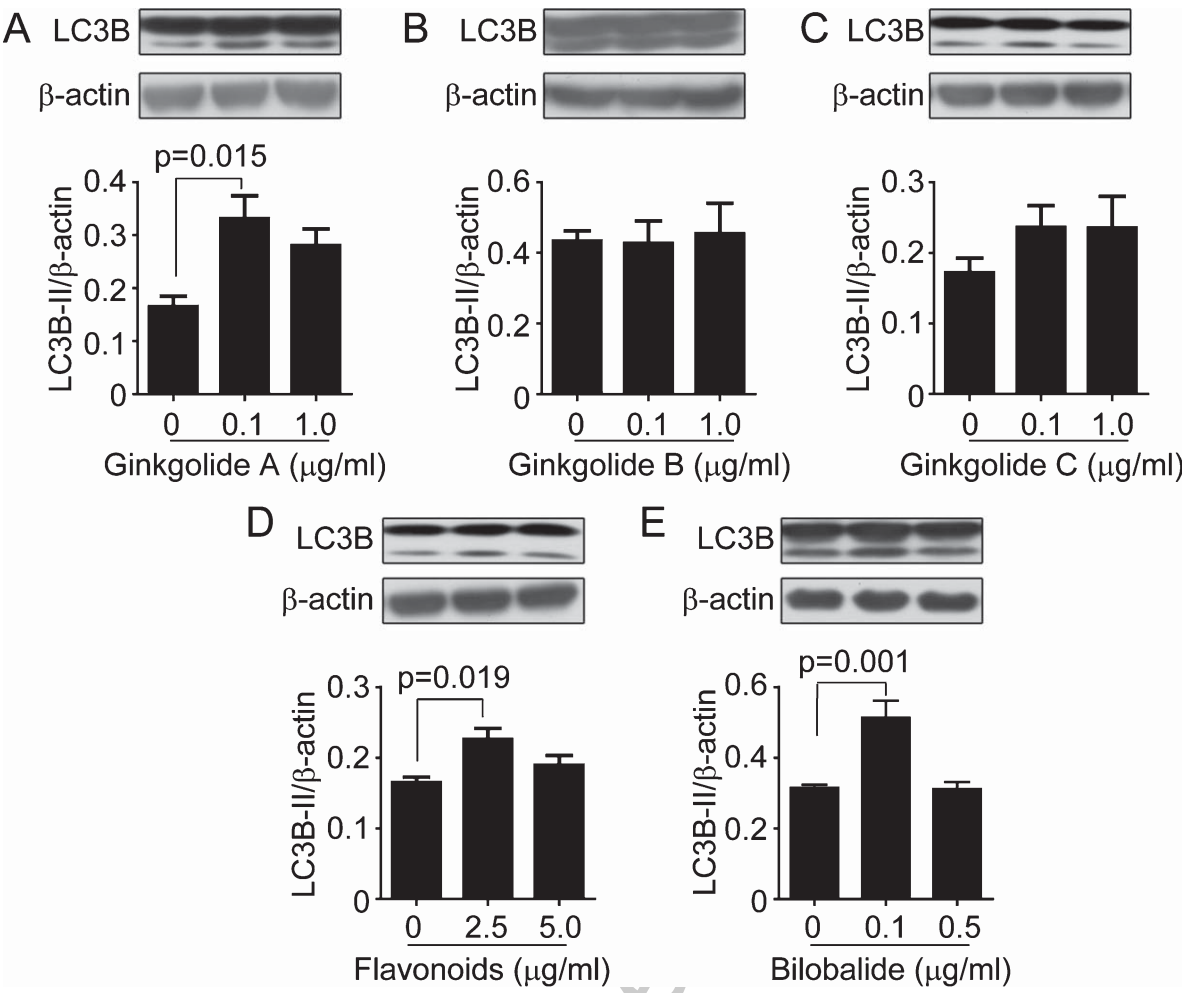

Fig. 7. The major components of EGb 761 differently activate autophagy in neuronal cells. SH-SY5Y cells were treated with the major components of EGb 761 (ginkgolides A, B, and C, flavonoids and bilobalide) at different concentrations for 24 h. Protein levels of LC3B in the cell lysate was detected with quantitative western blot. One-way ANOVA shows effects of ginkgolides A, flavonoids and bilobalide, but not ginkgolides B and C, on the neuronal autophaic activity: $F(2, 9) = 6.694$, 0.050, 1.199, and 5.917, $p = 0.017$, 0.951, 0.345, and 0.023, for ginkgolide A, B, and C, and flavonoids, respectively; $F(2, 15) = 14.145$, $p < 0.001$, for bilobalide. Tukey *post hoc* tests reveal that treatments with ginkgolide A at 0.1 μg/ml ($p = 0.015$), and flavonoids at 2.5 μg/ml ($p = 0.019$) significantly elevate the protein levels of LC3B-II. A Games-Howell *post hoc* test reveals that treatment with bilobalide at 0.1 μg/ml significantly increases LC3B-II protein levels ($p = 0.001$). Treatment with EGb 761 components at higher concentrations lost the autophagy-enhancing effects. $n = 4$ per group for ginkgolides A, B, and C, and flavonoids, and $n = 6$ per group for bilobalide.

at any concentrations tested did not activate caspase-3, whereas administration of H_2O_2 at 0.6 mM did induce the cleavage of caspase-3 into 17- and 12-aminoacid fragments (Fig. 6D).

Treatment with major components of EGb 761 differently enhances autophagy in neuronal cells

After observing that treatments with EGb 761 at higher concentrations always decreased autophagic activity in neurons, we hypothesized that some components in EGb 761 might inhibit neuronal autophagy, especially when they were administered at high doses. Thus, we treated cultured SH-SY5Y cells with different major components of EGb 761: ginkgolides A, B, and C, flavonoids, and bilobalide, at different concentrations for 24 h. As shown in Fig. 7, treatment with ginkgolide A or bilobalide at 0.1 μg/ml strongly elevated the protein level of LC3B-II (the ratio of LC3B-II/β-actin from the basal level to the level after activation: for ginkgolide A, $0.165 \pm 0.020 \longrightarrow 0.331 \pm 0.086$; and for bilobalide, $0.315 \pm 0.009 \longrightarrow 0.513 \pm 0.049$; one-way ANOVA followed by *post hoc* test for each component,

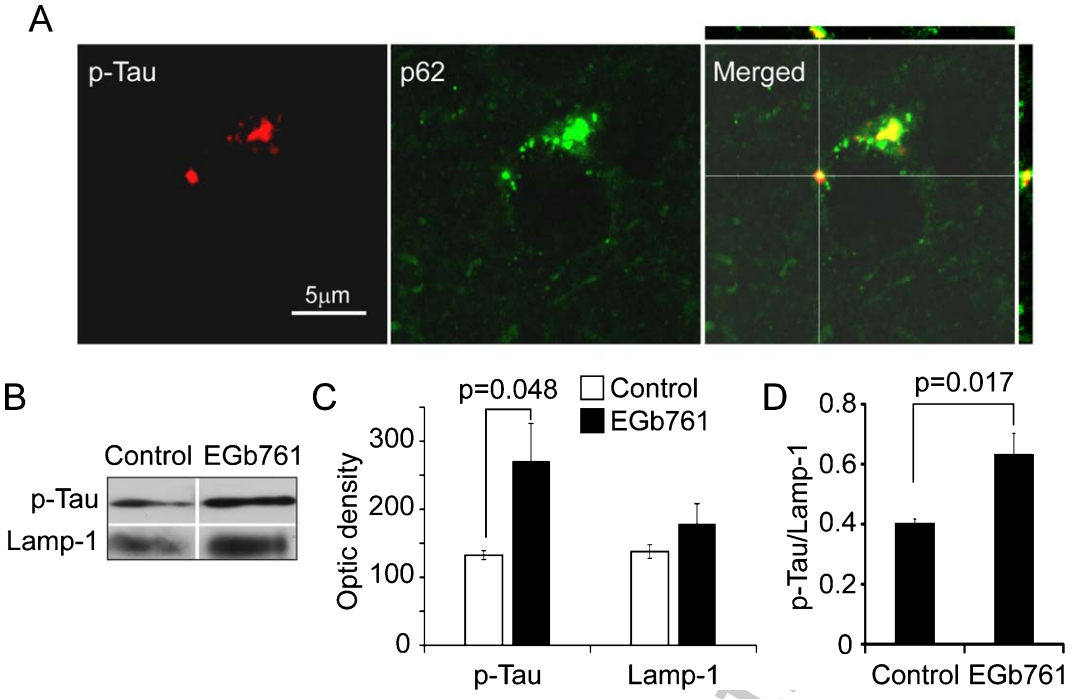

Fig. 8. Long-term treatment with EGb 761 facilitates the transport of phosphorylated tau protein into lysosomes. Four-month-old tau-transgenic mice were fed with a diet either containing or not containing EGb 761 for 5 months. The brain was collected for confocal microscopy analysis of the relationship between p-Tau and autophagic vacuoles. p-Tau was stained with red fluorescence-conjugated AT8 antibody and autophagic vacuoles were visualized by staining p62/SQSTM1 with green fluorescence-conjugated antibodies. Co-localization of p-Tau and p62/SQSTM1 could be observed with yellow fluorescence, superimposing fluorescent images of p-Tau and p62/SQSTM1, in individual cells (A). Moreover, the lysosomes-enriched brain homogenate fraction (B) was isolated by Percoll gradient centrifugation. The protein level of LAMP-1 and p-Tau protein was quantified with western blot. There is significantly more p-Tau protein ($t(6) = -2.485$, $p = 0.048$) or higher ratio of p-Tau/LAMP-1 ($t(6) = -3.291$, $p = 0.017$) in lysosomes isolated from EGb 761-treated mice than in lysosomes from littermate control tau mice (C and D; t test; $n = 4$ per group).

$p < 0.05$). Treatment with flavonoids also significantly increased the amount of LC3B-II protein in the cells, however, it had weaker effects than the treatment with ginkgolide A or bilobalide (Fig. 7D; the ratio of LC3B-II/β-actin from the basal level to the level after activation with flavonoids: $0.165 \pm 0.007 \longrightarrow 0.226 \pm 0.015$; one-way ANOVA followed by *post hoc* test, $p < 0.05$). Surprisingly, it was similar to the direct treatment with EGb 761 that the autophagy-enhancing effects of different EGb 761 components disappeared after the concentrations of components used to treat cells were increased (Fig. 7A, D, E). Ginkgolides B and C did not significantly change the protein levels of LC3B-II (Fig. 7B, C; one-way ANOVA, $p > 0.05$).

Autophagy-lysosome pathway mediates EGb 761-enhanced degradation of phosphorylated tau

After we had observed that a long-term oral treatment with EGb 761 simultaneously reduced cerebral p-Tau load and enhanced autophagy in the brain and neuronal cells, we continued to investigate whether autophagy mediates the degradation of p-Tau. By confocal microscopy, p-Tau was shown to co-localize with autophagosomes or autolysosomes as stained with antibodies against p62/SQSTM1 in individual neurons (Fig. 8A). Then, we used our established protocol to isolate lysosomes from mouse brain [27, 31]. We quantified p-Tau protein levels in the gradient centrifugation fraction, in which LAMP-1 was enriched (Fig. 8B-D) and non-lysosomal proteins, such as calnexin and β-actin were absent (data not shown). We observed significantly more p-Tau protein in lysosomes isolated from EGb 761-treated 9-month-old tau-transgenic mice than in lysosomes from control littermate mice without EGb 761 treatment (Fig. 8C, D; t test, $p < 0.05$).

To examine the role of autophagy in p-Tau degradation, we went on treating SH-SY5Y cells with EGb 761 at different concentrations. We observed that EGb 761 at 5 and 10 μg/ml significantly decreased

protein levels of p-Tau but not total Tau (t-Tau) (Fig. 9A-C; one-way ANOVA followed by *post hoc* test, $p < 0.05$). Then, we treated SH-SY5Y cells with EGb 761 in the presence of a well-known autophagy inhibitor, Bafilomycin B1, at 100 nM. Indeed, co-treatment of Bafilomycin abolished the effect of EGb 761 treatments on the reduction of p-Tau protein in SH-SY5Y cells (Fig. 9D, E; one-way ANOVA followed by *post hoc* test showing effects of EGb 761 treatments on p-Tau reduction in the presence of drug vehicle [$p < 0.05$] but not in the presence of Bafilomycin [$p > 0.05$]). To further test whether autophagy mediates EGb 761 treatments-induced p-Tau degradation, we used recently established SH-SY5Y cell lines overexpressing dominant-negative (DN) and wild-type (wt) human ATG5 [27, 31, 37]. Overexpression of ATG5DN inhibits autophagy of SH-SY5Y cells [31]. We treated ATG5DN- and

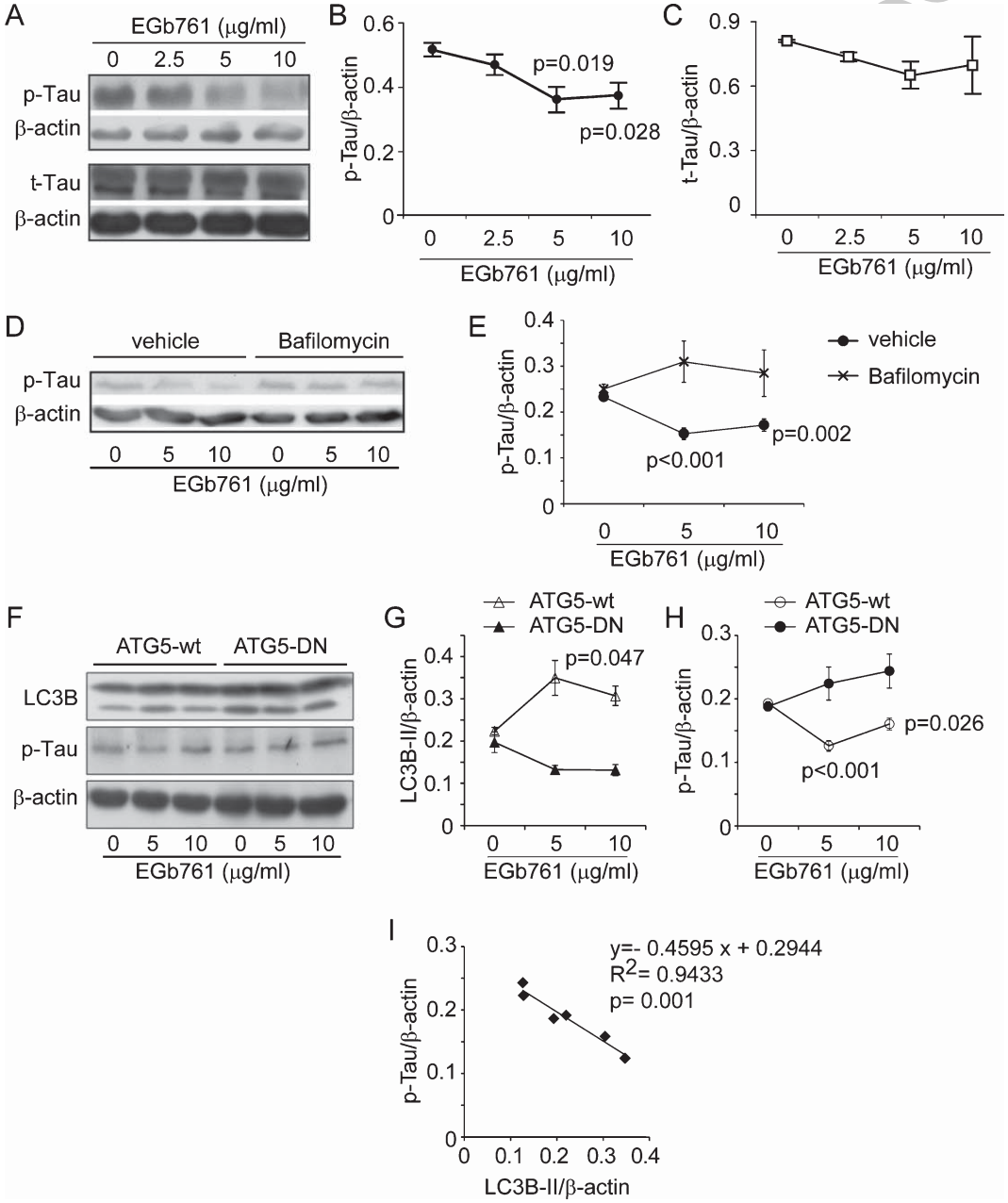

Fig. 9. (*Continued*)

wild-type ATG5-transgenic SH-SY5Y cells with EGb 761 at 0, 5, and 10 μg/ml, and then evaluated p-Tau level in SH-SY5Y cells 24 h after treatments. In wild-type ATG5-transgenic cells, we found that EGb 761 significantly increased LC3B-II protein level (Fig. 9F, G; one-way ANOVA followed by *post hoc* test, $p < 0.05$), but markedly reduced the amount of p-Tau protein especially when cells were treated with 5 μg/ml EGb 761 (Fig. 9H; one-way ANOVA followed by *post hoc* test, $p < 0.05$). In ATG5DN-transgenic cells, neither the protein levels of LC3B-II nor of p-Tau were changed by treatment with EGb 761 (Fig. 9F-H; one-way ANOVA, $p > 0.05$). Interestingly, when Pearson correlation test between all LC3B-II/β-actin and their correlated p-Tau/β-actin was made, we could clearly observe that the increase of LC3B-II protein was closely related to the reduction of p-Tau protein in EGb 761-treated SH-SY5Y cells (Fig. 9I; $p = 0.001$).

Treatment with Ginkgo biloba extract EGb 761 inhibits the activity of p38-MAPK and GSK-3β in tau-transgenic mouse brains

After demonstrating the autophagic degradation of p-Tau, we asked whether EGb 761 also affected p-Tau generation. Western blot was used to quantify the protein amount of phosphorylated and total p38-MAPK and GSK-3β in the 9-month-old tau-transgenic mouse brain. Phosphorylation of GSK3β at Ser9, which was detected in our study, inhibits kinase activity [38]. Compared to the protein level derived from mice without EGb 761 treatment, the phosphorylation of p38-MAPK and GSK-3β (at Ser9) in the brain homogenate from tau-transgenic mice was increased after 2-month treatments with EGb 761; however, after 5-month treatment with EGb 761, the protein level of phosphorylated p38-MAPK turned to be significantly decreased and the protein level of GSK-3β phosphorylated at Ser9 was still elevated (Fig. 10A-C; *t* test, $p < 0.05$). The total protein levels of p38-MAPK and GSK-3β were changed by neither short- nor long-term administration of EGb 761 (Fig. 10A-C; *t* test, $p > 0.05$).

DISCUSSION

AD is the main reason for dementia in the elderly. Currently, there are very few therapeutic options for this disease. In our study, we demonstrated that a long-term (5-month instead of 2-month) oral administration of *Ginkgo biloba* extract EGb 761 reduces the synaptic impairment and improves the cognitive function of tau-transgenic AD mice, which suggests that EGb 761 is a promising natural compound to prevent AD progress, although two recent dementia prevention trials (GEM and GuidAge) failed to prove that 5-6-year treatments with EGb 761 reduced the incidence of dementia in healthy subjects [39, 40].

As a potential therapeutic mechanism, treatments with EGb 761 strongly increase autophagic activity in neurons, which promotes the degradation of

Fig. 9. Inhibition of autophagy abolishes EGb 761 treatment-induced decrease of phosphorylated Tau protein in neuronal cells. SH-SY5Y neuronal cells were treated with EGb 761 at 0, 2.5, 5, and 10 μg/ml for 24 h. Phosphorylated (p-) and total (t-) Tau were detected with quantitative western blot (A-C). One-way ANOVA shows effects of EGb 761 treatments on the protein levels of p-Tau (B; $F(3, 8) = 7.161$, $p = 0.012$; $n = 3$ per group), but not on t-Tau (C; $F(3, 8) = 0.903$, $p = 0.481$; $n = 3$ per group). A Tukey *post hoc* test further reveals that treatments with EGb 761 at 5 and 10 μg/ml significantly decrease the protein levels of p-Tau ($p = 0.019$ and 0.028, respectively). To investigate the role of autophagy in EGb 761-induced p-Tau degradation, we examined p-Tau in EGb 761-treated SH-SY5Y cells in presence of Bafilomycin B1 at 100 nM or drug vehicle for 24 h (D and E). One-way ANOVA shows that treatments with EGb 761 affected p-Tau protein levels in the presence of vehicle (E; $F(2, 36) = 14.942$, $p < 0.001$, $n = 13$ per group). A Games-Howell *post hoc* test reveals that treatments with EGb 761 at both 5 and 10 μg/ml significantly reduce p-Tau proteins (E; $p < 0.001$ and 0.002, respectively). However, in the presence of Bafilomyecin, EGb 761 treatments lost the effects to regulate p-Tau levels (E; $F(2, 21) = 0.566$, $p = 0.576$, $n = 8$ per group). Furthermore, SH-SY5Y cells over-expressing dominant-negative (ATG5-DN) and wild-type (ATG5-wt) human ATG5 were treated with EGb 761 at 0, 5, and 10 μg/ml (F-H). The LC3B-II protein level in ATG5-wt transgenic cells was significantly increased within 24 h after EGb 761 treatments (G; one-way ANOVA: $F(2, 9) = 8.344$, $p = 0.009$; $n = 4$ per group). A Games-Howell *post hoc* test reveals that treatment with EGb 761 at 5 μg/ml significantly increases LC3B-II protein (G; $p = 0.047$). In ATG5-DN cells, treatments with EGb 761 appeared to significantly reduce the LC3B-II protein levels (G; one-way ANOVA: $F(2, 9) = 4.887$, $p = 0.037$; $n = 4$ per group). However, a Tukey *post hoc* test shows that treatments with EGb 761 at 5 and 10 μg/ml did not really affect LC3B-II protein levels (G; $p = 0.059$ and 0.055, respectively). In parallel, the p-Tau protein level in ATG5-wt cells was significantly decreased within 24 h after EGb 761 treatments (H; one-way ANOVA: $F(2, 18) = 20.631$, $p < 0.001$; $n = 7$ per group). A Games-Howell *post hoc* test further reveals that treatments with EGb 761 at 5 and 10 μg/ml both significantly reduce p-Tau proteins (H; $p < 0.001$ and $= 0.026$, respectively). In ATG5-DN cells, p-Tau proteins were slightly increased after the same treatments with EGb 761, whereas, the changes were not statistically significant (H; one-way ANOVA: $F(2, 9) = 1.680$, $p = 0.240$; $n = 4$ per group). After LC3B-II/β-actin and p-Tau/β-actin in ATG5-DN and ATG5-wt cells after treatments with EGb 761 at different concentrations were pooled, the protein amount of LC3B-II was closely correlated with the protein level of p-Tau (I; Pearson correlation test, $p = 0.001$).

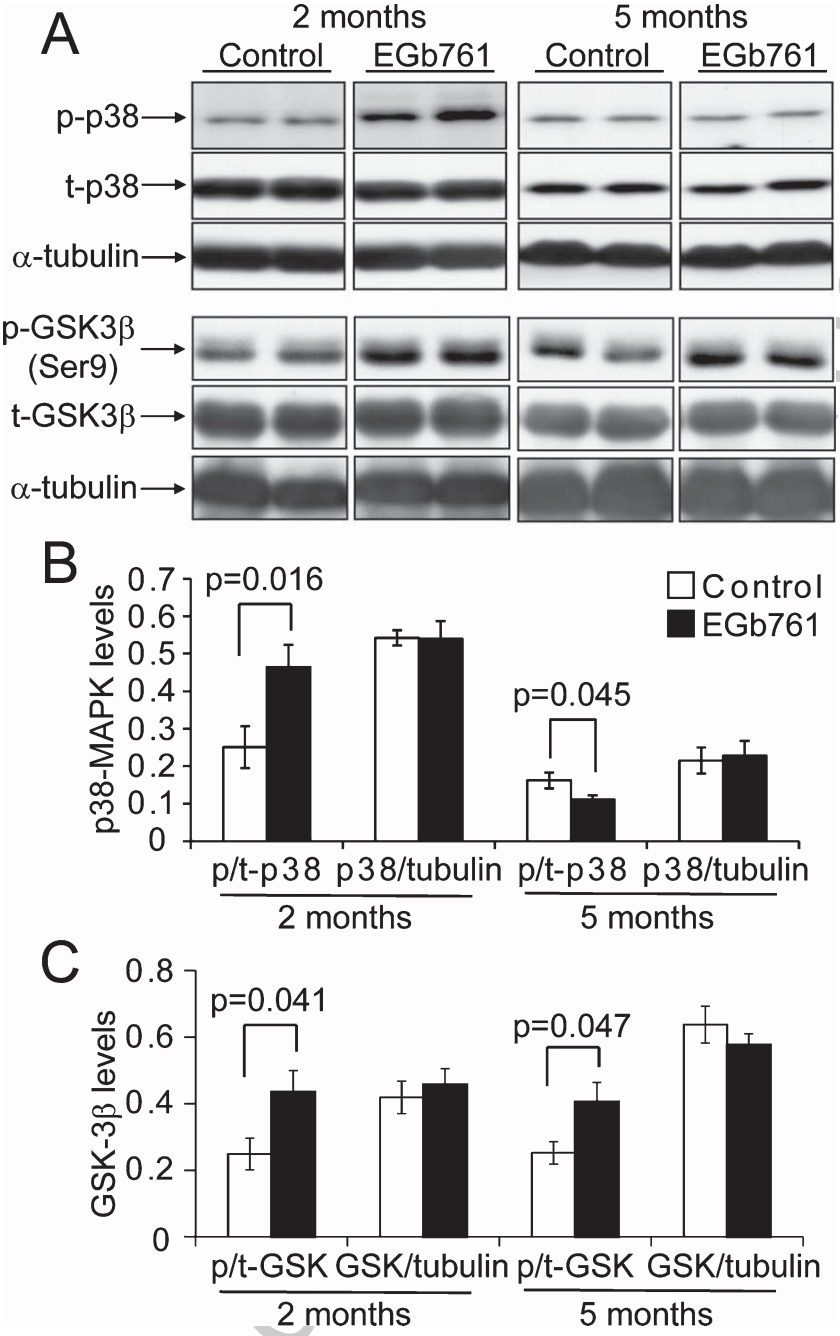

Fig. 10. Long-term treatment with EGb 761 inhibits the activation of p38-MAPK and GSK-3β in tau-transgenic mouse brains. Four-month-old and 7-month-old tau-transgenic mice were treated with or without EGb 761 as a supplement in the diet for 5 and 2 months, respectively. The brain was collected for quantitative western blot analysis. The ratios of phosphorylated (p-) and total protein (t-) of p38-MAPK (p38) and GSK-3β (phosphorylated at Ser9) were both increased after 2-month treatment with EGb 761 compared to control tau mice without EGb 761 treatment (A-C; t test, t (27) = –2.572 and –0.042, $p = 0.016$ and 0.967, for p/t-p38 and p38/tubulin, respectively; $n \geq 13$ per group; t (21) = –2.181 and –0.556, $p = 0.041$ and 0.584, for p/t-GSK and GSK/tubulin, respectively; $n \geq 10$ per group). After 5-month treatment with EGb 761, the ratio of p-/t- p38-MAPK was significantly decreased (B; t test, t (19.334) = 2.148 and –0.257, $p = 0.045$ and 0.801, for p/t-p38 and p38/tubulin, respectively; $n = 14$ per group), whereas the ratio of p-/t-GSK-3β was still elevated compared to that in control mice (C; t test, t (18) = –2.132 and 0.970, $p = 0.047$ and 0.343, for p/t-GSK and GSK/tubulin, respectively; $n \geq 9$ per group).

p-Tau in the tau-transgenic mouse brain. It is evidenced by the following observations: 1) p-Tau co-localizes with autophagic vacuoles in the brain; 2) treatment with EGb 761 facilitates the transportation of p-Tau into lysosomes; 3) treatments with EGb 761 and its major components increase the expression of autophagy-associated proteins, such as ATG5 and beclin1, and enhances neuronal autophagy; and 4) blocking autophagosome formation by inhibiting ATG5 function [41], and interfering with the fusion of autophagosomes and lysosomes by treating cells with Bafilomycin [42], both abolish the effect of EGb 761 to decrease p-Tau level in cultured neuronal cells.

However, how EGb 761 activates neuronal autophagy remains unclear. For example, why treatments with EGb 761 at higher concentrations (e.g., 50 μg/ml) lost the effects to enhance autophagy, even when these treatments still up-regulate the protein levels of ATG5 and beclin1. We hypothesized that there is at least one component among the EGb 761 mixture, which serves inhibitory effects on neuronal autophagy, especially at high concentrations. Thus, we tested different major components of EGb 761 for their autophagy-regulating effects. EGb 761 contains flavonoids and terpene lactones. We observed that flavonoids activate autophagy, which is in accordance with a previous observation that one flavonoid aglycone, kaempferol, activates autophagy and increases the mitochondrial turnover in neuronal cells [43]. To our knowledge, we are the first to report that ginkgolide A or bilobalide activates neuronal autophagy, even with stronger effects than flavonoids; ginkgolide B or C as a single compound does not affect neuronal autophagy. Thus, our observation did not support our hypothesis on the autophagy-inhibiting EGb 761 components; however, it is helpful to modify the recipe of EGb 761 mixture to improve its therapeutic efficacy.

Both Aβ and p-Tau trigger microglial inflammatory activation in the brain [35]. The inflammatory activation, especially release of IL-1β, promotes phosphorylation and aggregation of tau by activating p38α-MAPK in neurons, which also contributes to the spreading of pathological tau in the brain [44–46]. Interestingly, we observed that treatments with EGb 761 reduce the transcription of *il-1*β gene, but increases the transcription of anti-inflammatory genes, such as *arg1* and *mrc1*, in tau-transgenic mouse brains. In our previous study, we observed that treatment of EGb 761 enhances autophagy and subsequent degradation of NLRP3 inflammasome in microglia, which reduces IL-1β secretion [28]. We believe that this mechanism also plays a role in the inhibition of pro-inflammatory activation in tau-transgenic mice, although it needs to be confirmed.

Apart from the autophagy-enhancing effect, EGb 761 treatment must serve other anti-AD effects, for example, substantial experiments have shown that treatments with ginkgolide B protect neurons [47]. CREB is a transcription factor that can regulate the syntheses of synapse- or memory-associated proteins [48, 49]. We observed that EGb 761 treatment attenuated the reduction of phosphorylated CREB in tau-transgenic mouse brains, which corroborates the observation of EGb 761-triggered neuroprotection in AβPP-transgenic mice [50]. Similarly, long-term treatment with EGb 761 inhibits the activation of p38-MAPK and GSK-3β, the two key enzymes generating p-Tau [51], although it cannot be excluded that the inhibition of CREB and p38-MAPK phosphorylation was subject to the reduction of p-Tau protein and inflammatory activation, respectively.

Our study appears to show that a long-term treatment is necessary for EGb 761 to enhance p-Tau degradation and inhibit p-Tau generation in the AD prevention. Similarly, in our previous studies, 5-month but not 2-month treatment with EGb 761 inhibits the inflammatory pathology and prevents the AD pathogenesis in AβPP-transgenic mice [28]. Indeed, in the prevention trials (GEM and GuidAge) based on large numbers of healthy participants [39, 40], when the participants who have received EGb 761 for at least 4 years were analyzed, the long-term use of EGb 761 did reduce the incidence of Alzheimer-type dementia [40].

In summary, we have demonstrated that a long-term oral treatment with EGb 761 improves the cognitive function of tau-transgenic mice, which is associated with the reduction of phosphorylated tau and inhibition of neurotoxic pro-inflammatory activation in the brain. As potential mechanisms, we have observed that EGb 761 treatment not only enhances autophagy and increases the degradation of phosphorylated tau in neurons, but also reduce the generation of phosphorylated tau by inhibiting the activity of p38-MAPK and GSK-3β. Together with our previous observation on anti-AD effects of long-term treatment with EGb 761 in AβPP-transgenic mice [28], our study strongly supports that a long-term treatment with EGb 761 is a promising option to delay AD progression.

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