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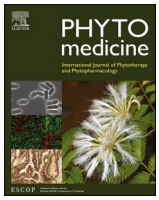

Original Article

Ginkgo biloba extract EGb 761® ameliorates cognitive impairment and alleviates TNFα response in 5xFAD Alzheimer's disease model mice

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ABSTRACT

Background: *Ginkgo biloba* leaf extract EGb 761® has shown clinical efficacy in patients with mild cognitive impairment and dementia. However, the pharmacological action of EGb 761® in Alzheimer's disease (AD) remains unclear and molecular mechanisms targeted in the brain are not completely understood.
Hypothesis/Purpose: We aimed to investigate 1) the potential sex-dependent effects of oral administration of EGb 761® in 5xFAD mice, an AD mouse model, and 2) the underlying microglial subtype responsible for the observed anti-inflammatory effects in the brain.
Methods: Eight-week old 5xFAD and wild type mice received EGb 761®-supplemented diet or control diet for eight weeks. The study investigated changes in cognitive function as well as amyloid plaque load, expression of AD-related genes, and anti-inflammatory effects. Moreover, we used organotypic brain slices for confirmation and assessment of concentration-dependency of the observed EGb 761® effects and performed single cell RNA sequencing on the prefrontal cortex of male mice with focus on microglia.
Results: We demonstrate that EGb 761® treatment improves cognitive function in 5xFAD mice in several behavioral tests. Analysis of the brain tissue from these animals indicated a reduction in amyloid plaque load in the prefrontal cortex (PFC). This brain area was further investigated to assess the molecular changes that occurred following EGb 761® intake. Alterations in the expression of genes related to AD were highly sex-specific with effects on the cholinergic system, the γ-secretase complex, and neuroinflammation. Anti-inflammatory effects of EGb 761® with a particularly pronounced reduction of the TNFα-response could be shown for the PFC but also peripherally in the serum of 5xFAD mice of both sexes. Single-cell RNA sequencing revealed that EGb761® mainly affected disease-associated microglia stage 2 (DAM2), which are thought to have a detrimental role in AD.
Conclusions: EGb 761® shows efficacy in the treatment of cognitive deficits in the 5xFAD mouse model via multimodal activity, including sex-specific and sex-unrelated mechanisms including the normalization of neuroinflammatory parameters.

Abbreviation: 4ABAH, 4-aminobenzoic acid hydrazide; 5xFAD, Mouse line overexpressing human APP and PSEN1; Aβ, Amyloid beta peptide; A2m, Alpha-2-microglobulin; Actb, Beta-actin; AChE, Acetylcholinesterase; AD, Alzheimer's disease; APP, Amyloid precursor protein; B2m, Beta-2-microglobulin; Bace1, beta-secretase 1; BBB, Blood-brain barrier; BSA, Bovine serum albumin; C57BL/6J, Inbred mouse strain; ChAT, Choline acetyltransferase; CSF, Cerebrospinal fluid; DG, Dentate gyrus; DMSO, Dimethyl sulfoxide; DAM, Disease-associated microglia; ELISA, Enzyme-linked immunosorbent assay; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

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Introduction

The prevalence of neurocognitive disorders is increasing with a rapidly aging society, as so far, potentially disease-modifying treatments have shown at best moderate effects (summarized in (Tatulian 2022)). This largely unmet medical need has challenged research to develop more effective treatments. The pathological processes associated with, for example, Alzheimer's disease (AD) are multifactorial, including aggregation of pathological proteins, neuroinflammation, depletion or insufficient synthesis of neurotransmitters and oxidative stress (summarized in (Ferrari and Sorbi 2021)). Therefore, therapeutic approaches that target only a single aspect of the pathology might fall short in providing therapeutic benefit. In contrast to single-target synthetic drugs, medicinal products based on herbal extracts consist of a large number of different molecules with distinct pharmacological activities. Conceptually, a multi-component and poly-pharmacological therapy could present an alternative valuable approach for the treatment of multifactorial diseases.

Indeed, herbal medicinal products based on *Ginkgo biloba* leaf extracts such as EGb 761® are used in therapy of cognitive disorders and have been assessed by the European Medicines Agency to be safe and effective for improving (age-associated) cognitive impairment and quality of life in mild dementia (Agency 2015). Most of the studies on the mechanism of action and clinical efficacy of *Ginkgo* extracts in dementia have been conducted with the special *Ginkgo biloba* leaf extract EGb 761®. EGb 761® is the result of a proprietary extraction process aimed at optimizing the ranges of active constituents while depleting potentially harmful substances in relation to the natural composition in *Ginkgo* leaf (Kulić et al., 2022). Clinical efficacy of EGb 761® has been shown in patients with mild cognitive impairment and mild to moderate dementia in AD (von Gunten et al. 2016). However, the extent of EGb 761®'s protective role in AD remains unclear, and the molecular mechanisms targeted in the brain are not completely understood.

Beneficial effects of EGb 761® have been demonstrated in a number of preclinical studies utilizing mice and rats (Nowak et al. 2021). So far, three studies have investigated the impact of EGb 761® using three different transgenic mouse models of AD: APP/PS1 mice, TgAPP/PS1 mice, and TgCRND8 APP-transgenic mice (Xie et al. 2022). However, in all studies, the effects of EGb 761® were investigated in only one sex. Sex bias has been highlighted as one of the factors contributing to implementation and reproducibility problems in preclinical research. In AD, females are at greater risk to develop the disease, emphasizing that sex should be considered as a biological variable in studies (Podcasy and Epperson 2016).

In our study, we assessed for the first time the therapeutic potential of oral administration of EGb 761® in both sexes of 5xFAD mice, a comparatively aggressive, quickly proceeding mouse model (Oakley et al. 2006). 5xFAD mice exhibit increased Aβ load as early as two months of age, develop plaques by three months of age, and by four months of age, the mice show AD-related behavioral deficits and neuroinflammation (Bilkei-Gorzo 2014). We employed a complementary *in vivo* and *ex vivo* approach including assessment of behavioral changes, amyloid plaque load, expression of AD-related genes, measurement of inflammatory proteins and single-cell RNA sequencing of prefrontal cortex microglia, to identify molecular mechanisms, relevant cell populations and potential sex-dependency of treatment effects of EGb 761®.

Material and methods

Animals

For maintenance, B6SJLTg (APPSwFlLon, PSEN1-M146L-L286 V) 6799Vas/Mmjax (5xFAD) (Oakley et al. 2006)) mice (Jackson Lab, Bar Harbor, ME, USA) were crossbred with C57BL/6J. SAMR1/TaHsd (Envigo, Indianapolis, IN, USA) were used for isolation of macrophages. All animals were reared and maintained on a 12 h light/dark cycle (6 am to 6 pm light on) with free access to food and water. All procedures were performed in accordance with the European Communities Council Directive regarding care and use of animals for experimental procedures and were approved by local authorities (Landesuntersuchungsamt Rheinland-Pfalz; general approval number G-17–035, approval of extension E5-G17–1–035 on 27th May 2020).

EGb 761®-supplemented diets

EGb 761® is a dry extract from *Ginkgo biloba* leaves (DER 35–67:1), extraction solvent: acetone 60 % (w/w). The batch of EGb 761® used in the present study was adjusted to 25.4 % *Ginkgo* flavonoids calculated as *Ginkgo* flavone glycosides (HPLC-UV), and 5.93 % terpene lactones consisting of 3.02 % ginkgolides A, B, C and 2.91 % bilobalide (HPLC-IR). The extract contained < 5 ppm ginkgolic acids (HPLC-UV). A detailed description of the proprietary production process of EGb 761® (Kulić, Lehner et al. 2022) and an analytical characterization of constituents (Kulić, Ritter et al. 2022) have been published recently. Mice were randomly assigned by rolling a dice to two groups treated with a control standard diet (AIN96 G + ssniff Spezialdiäten GmbH, Soest, Germany) or a standard diet supplemented with 750 mg EGb 761® per kg (0.075 %) (produced by ssniff Spezialdiäten GmbH, Soest, Germany on the basis of AIN96 G, EGb 761® provided by Dr. Willmar Schwabe GmbH & Co. KG, Karlsruhe, Germany) for 8 weeks. The concentration of EGb 761® in food pellets was calculated to provide an expected daily dose of approximately 100 mg/kg.

Treatment of mice

At an age of seven weeks, all animals received the control diet for one week. Afterwards, the mice were assigned to a group remaining on the control diet ($n = 7$ for male wild type mice; $n = 8$ for female wild type and male/female 5xFAD mice) or receiving food supplemented with EGb 761® ($n = 7$ for male wild type mice; $n = 8$ for female wild type and male/female 5xFAD mice). Animals were kept on the respective diet for further eight weeks. The behavioral tests were conducted between weeks 13 and 14 before the mice were sacrificed at 16 weeks of age. Body weight and food consumption were recorded weekly throughout the treatment. The experimenters were blinded to the genotype and treatment condition during all behavioral testing. Treatment for the single-cell RNA sequencing experiment was performed as described above using male 5xFAD mice. Three batches of mice, each consisting of two control-diet fed and two EGb 761®-diet fed animals, were conducted independently. The tissue of two animals of each group was pooled for the single-cell preparation to account for biological variability.

Behavioral tests

Behavioral tests were performed on a fixed time schedule. Cage labels did not include information regarding the treatment to minimize bias during the experimental procedure.

Pole test

The pole test was conducted as described in (Rödig et al. 2022).

Nesting test

Mice were habituated to paper stripe material (ssniff Spezialdiäten, Soest, Germany) as described previously (Dos Santos Guilherme et al. 2019).

T-Maze

The T-maze was conducted using equipment as described in (Valeri et al. 2021).

Radial arm water maze

The radial arm water maze was performed as previously described with slight modifications (Valeri et al. 2021).

Sacrifice and tissue preparation

Animals were weighed, anesthetized with isoflurane (Piramal, Mumbai, India) in a sealed chamber containing the inhalant, and sacrificed by decapitation. Trunk blood was collected and used 20 min later for serum preparation by two consecutive centrifugation steps. One brain hemisphere of the animals was drop-fixed in 4 % PFA. The second brain hemisphere was immersed in RNA later (Qiagen, Hilden, Germany) and stored at −80 °C until further use. Prefrontal cortex (PFC) samples were divided for the experiments: a total of 21 PFC samples were selected for histology, 18 PFC samples for tissue array, and 18 PFC samples for RT-qPCR.

Immunohistochemistry

Aβ from heterologous overexpression was detected in different brain regions as previously described (Valeri et al. 2021) using the antibody 6E10 (RRID:AB_662,804, Covance, Princeton, NJ, USA). Hemispheres were embedded in paraffin and two slices per mouse were stained and anonymized by a person not involved in the investigation. Microscopic pictures were taken with 10 × 10 magnification (EVOS XL, Life Technologies, Darmstadt, Germany) and staining intensity was assessed by using Aida image analyzer 4.26 software (Raytest, Straubenhardt, Germany).

Western blotting

Western Blotting was performed according to (Valeri et al. 2021). The primary antibodies were as follows: 6E10 to detect human APP. anti-GAPDH (RRID: AB_561,053, Cell Signaling Technology, Danvers, MA, USA), anti-IGF2 (Signalway Antibody, A38493, MD, USA), anti-MPO (SantaCruz, sc-39,010, CA, US). Secondary antibodies were labelled with HRP for chemiluminescent detection, which was performed as described previously.

Expression of AD-relevant genes

The Mouse Alzheimer's Disease QuantiNova LNA PCR Focus Panel (Qiagen, Hilden, Germany) determines the expression of 84 genes important for the onset, development, and progression of AD. RNA was isolated from PFC using the RNeasy lipid tissue mini kit (Qiagen, Hilden, Germany). 100 ng of RNA pool sample from three different animals from the same treatment group were subjected to the panel according to the manufacturer's instructions. Changes in mRNA level were considered a "hit", if the effect size was greater than the mean variation of the five different housekeeping genes.

Expression changes of genes identified as hits and TNFα (Qiagen, Hilden, Germany) were re-evaluated by RT-qPCR. The reaction was performed as described in (Rödig et al. 2022).

Inflammation antibody array

For the simultaneous detection of multiple cytokines, PFC tissue samples were subjected to the Mouse Inflammation Antibody Array (abcam, Cambridge, UK) according to the manufacturer's instruction. PFC specimens were homogenized in cell lysis buffer supplemented with protease inhibitors (Roche, Basel, Switzerland). Then, 250 μg of protein were subjected to the membrane.

Measurement of serum NfL and TNFα

NfL (NF-light™ Serum ELISA kit, UmanDiagnostics, Umea, Sweden) and TNFα levels (TNFα ABTS ELISA kit, Peprotech, Thermo Fisher Scientific, Waltham, MA, USA) in serum were quantified following the manufacturer's instructions.

Assessment of TNFα secretion in macrophages

Primary murine bone marrow macrophages were isolated from the femurs of 3-month-old SAMR1 mice (see Supplementary method section). Cells were incubated for 3 days at 37 °C, 5 % CO_2 and 95 % air humidity. On day 4, cells were treated with LPS (10 μM), $A\beta_{42}$ (2.5 μM; AnaSpec, Fremont California, USA) and co-treated with EGb 761® (1 μg/ml) for 24 h

Preparation of organotypic brain slice culture and treatment

Following brain extraction, the separated hemispheres (without olfactory bulbs and cerebellum) were transferred to phosphate-buffered saline (PBS) and sliced into sections of 200 μm (McIlwain Tissue Chopper, Camden Instruments, Loughborough, UK). The sections were placed into pre-warmed TC-inserts (Sarstedt, Germany) containing 2 ml culture medium (2 % B27, 2 mM l-glutamine, Neurobasal A) and cultured at 37 °C with 5 % CO_2. Following one day of incubation, 50 % of the growth medium was exchanged. On day 3, another medium exchange was conducted, where the medium contained either EGb 761® at concentrations of 1 or 10 μg/ml or the solvent (DMSO) and incubated for 24 h Afterwards, PFC was dissected and weighed. Potassium phosphate buffer with protease inhibitor cocktail (Roche, Basel, Switzerland) was added (4 ml/g tissue) and the tissue was homogenized using a tissue homogenizer and pre-chilled steel beads (Qiagen) at a frequency of 50 Hz for 5 min.

Enzymatic activity assays

The AChE activity measurement was performed as previously described (Stoye et al. 2020) using 1 μl of the supernatant of the sample (diluted 10 ml/ g tissue in potassium phosphate buffer). ChAT activity was quantified following the manufacturer's instructions (Elabscience, TX, USA) with 10 μl of PFC homogenate. For the MPO activity, 0.5 μl homogenate were mixed with 1 μl of 0.43 % H_2O_2 solution (Carl Roth GmbH & Co. KG Karlsruhe, DE) and 44 μl tetramethylbenzidine (TMB) (Sigma Life Science, St. Louis, MO, USA) solution (0.1 mg/ml in 0.1 M sodium acetate buffer (pH 6)). After an incubation of 20 min at 37 °C the reaction was terminated by the addition of 20 μl of hydrochloric acid (HCl) and the optical density (OD) was measured at 450 nm. Specificity of all three assays was proven by using inhibitors or heat-denaturation of the enzyme (Suppl. Figure 1).

Single-cell RNA sequencing: sample preparation and analysis

PFCs were collected and washed with ice-cold phosphate-buffered saline (PBS) supplemented with 5 % (w/v) trehalose. The samples were cut into smaller pieces, placed in C tubes (Miltenyi Biotec, Bergisch Gladbach, Germany) and 1900 μl of dissociation buffer Z, supplemented with 5 % (w/v) trehalose, were added to each C Tube on ice. Enzymo-mechanical dissociation was performed according to the manufacturer (program 37C_ABDK_01) using a gentleMACS Octo dissociator with heaters (Miltenyi Biotec, Bergisch Gladbach, Germany). The C tubes were briefly centrifuged and samples were resuspended and applied to MACS SmartStrainer 70 and 40 μm (Miltenyi Biotec, Bergisch Gladbach, Germany). C tubes were washed with 10 ml ice cold PBS to collect remnant material. Finally, the cell suspension was centrifuged at 300 × *g* for 10 min at 4 °C and the supernatant completely aspirated. Following this, myelin debris removal and red blood cell removal were performed according to the manufacturer's guidelines (Miltenyi Biotec, Bergisch Gladbach, Germany) on the cell suspension.

Single-cell capturing, isolation of mRNA and synthesis of cDNA was

performed using the BD Rhapsody Single-Cell Analysis System (BD Biosciences, Heidelberg, Germany) following the manufacturer's guidelines. Upon cDNA synthesis and exonuclease treatment, whole transcriptome libraries were generated using BD WTA Amplification Kit (BD Biosciences, Heidelberg, Germany). Quantity and size of libraries were assessed by Qubit flex (Invitrogen, Waltham, USA) and Bioanalyzer 2100 (Agilent, Santa Clara, USA). Sequencing (PE150 reads) was performed on a NovaSeq6000 plus (Illumina) at Novogene (Cambridge, UK).

Resulting raw data were preprocessed according to the Illumina standard protocol. Counting and demultiplexing was performed using the BD Rhapsody™ Sequence Analysis Pipeline (v2.0) yielding to around 10,000 called putative cells for each sample with a mean of around 45,000 aligned reads per cell. Pipeline output files were imported in Partek Flow software (Version 1.3) and cells that contained <500 or >4000 detected RNA molecules and a percentage of mitochondrial counts higher than 10 % were removed from subsequent analysis, resulting in 49,623 cells for subsequent analyses. Single-cell counts were normalized by Partek Flows recommended normalization order. For dimensionality reduction PCA was used, followed by Graph-based clustering (using the SmartLocalMoving (SLM) clustering algorithm) and U-map algorithm for visualization. Populations shown in 2D U-map projections were annotated according to ImmGen's (www.immgen.org) data browser. Annotation of cell population was performed by expression of cell-type-specific marker genes based on the UniProt-major data base (Gja1 for astrocytes; Cldn11 for oligodendrocytes; CD11b for microglia; Ccdc153 for endothelial cells; Cldn5 for ependymocytes; and Syt1 for neurons). Separation into DAM-type subpopulations of microglia was performed as described in (Singh et al. 2022).

Statistical analyses

Sample size calculation (G*Power, V3.1.9.2, University of Kiel, Germany) was based on previous investigations (Liu et al. 2015) and assuming an effect size of 0.8, type I error $\alpha = 0.05$, and a power of 0.8. Statistical significance for the comparison of two groups was determined by Student's *t*-test, with additional Welch correction if needed. For results with more than two groups, One-way ANOVA was performed with Sidak's post-test, Bonferroni post-test or Fisher's LSD post-hoc test (Graph Pad Prism 6 and 8 (GraphPad Software, Suite, CA, USA)). Data are presented as mean ± SEM. Outliers were detected by performing Grubb's test with a 1 % limit and values were excluded if identified as outliers.

Results

To assess the impact of oral administration of EGb 761®, 5xFAD mice and wild type littermates were kept on a control diet for one week starting at seven weeks of age and afterwards, divided into groups receiving either the control diet or EGb 761®-supplemented feed for further eight weeks (Fig. 1A). The treatment start coincides with onset of AD-like pathology in the respective mouse model. All results from male and female mice were combined when no sex-specific differences were observed. The food consumption was comparable in all groups, showing the acceptance of both diets with a mean daily dosage of 88 mg/kg EGb

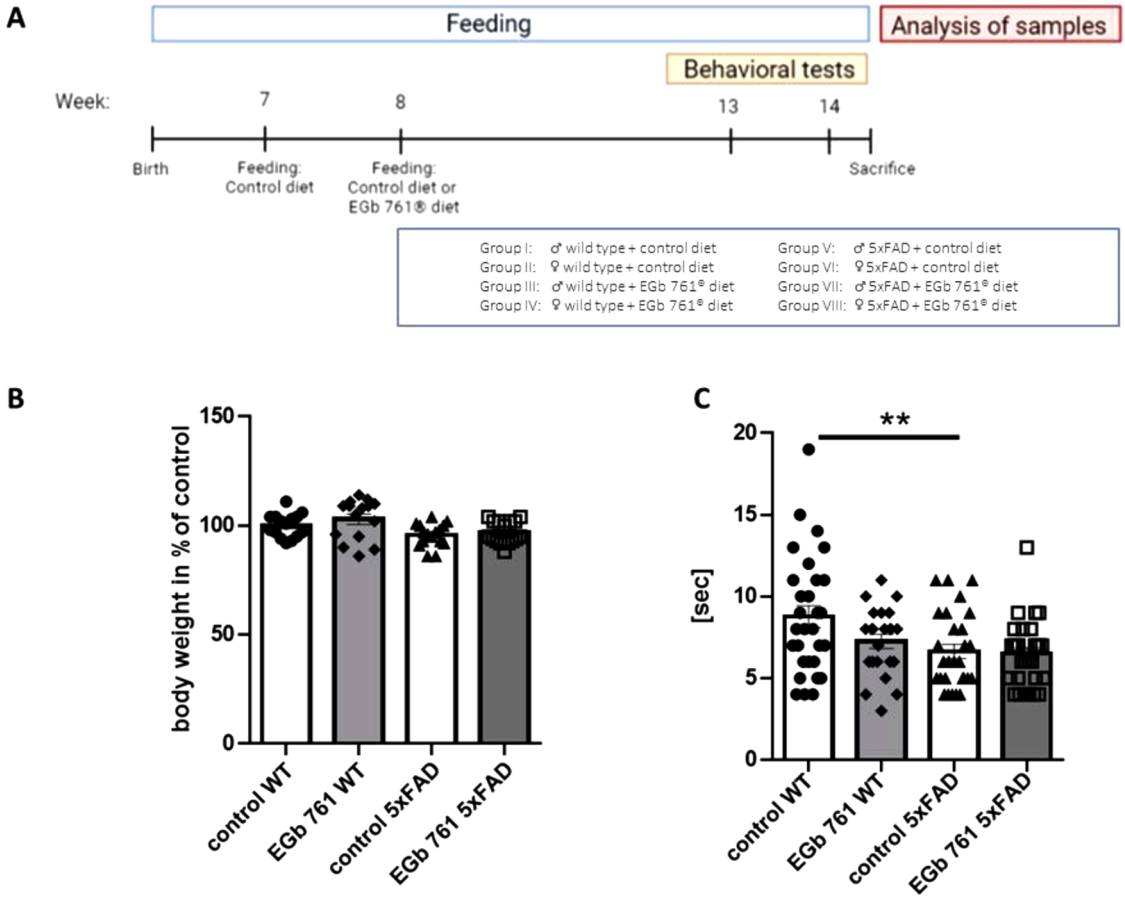


Fig. 1. Experimental procedure and control parameters
EGb 761® treatment improved cognitive function in 5xFAD mice

761® (mean daily dosage per mouse = 2.23 mg, mean body weight at start male = 25 g/ female = 19 g). The EGb 761®-containing diet had no influence on body weight after 8 weeks of treatment (Fig. 1B). Furthermore, EGb 761®-fed mice displayed no locomotive impairment compared to the control-treated group (Fig. 1C), thus, not affecting subsequent analysis of behavior non-specifically. A significant difference was observed only between the wild type and 5xFAD mice treated with the control diet, which could be based on reduced anxiety in 5xFAD mice.

After five weeks of treatment with EGb 761®-containing diet or control diet, mice were subjected to different behavioral tests to investigate cognitive abilities. Nest-building activity is an indicator of an animal's well-being and the integrity of the hippocampus. When assessing the quality of the nests, EGb 761® treatment had no effect on the wild type mice, whereas the decrease in nest building activity observed in 5xFAD mice was counteracted in the transgenic animals receiving EGb 761® (Fig. 2*A*+*B*). The positive effects of EGb 761® on the nest score values were reflected by a significant reduction of material not integrated into the nest as compared to the 5xFAD mice treated with the control diet.

Differences in spatial orientation were assessed in the T-maze test. While wild type mice on both diets showed a comparable percentage of correct alternations of approximately 60 % (control diet: 64.72 % ± 5.52 %, EGb 761®: 57.95 % ± 8.03 %). 5xFAD mice on the control diet displayed a significant reduction to about 37.08 % ± 10.14 of correct alternations in the T-maze which was completely abolished in transgenic mice receiving EGb 761® containing food (66.82 % ± 5.92 %, $p < 0.05$ vs. 5xFAD control diet), suggesting a prevention of the transgene-induced impairment in spatial orientation (Fig. 2C).

Mice were tested in a radial-arm water maze for working memory by measuring the capability to avoid arms already used for escape during each trial and for reference memory by assessing the ability to avoid arms without escape platforms, respectively. No difference was observed between control diet- and EGb 761®-treated wild type mice. In contrast, 5xFAD mice on the control diet showed a significant increase in both latency time as well as the number of errors in finding the hidden platform compared to wild type control animals. Both parameters were significantly improved in EGb 761®-treated transgenic animals which showed cognitive functions in these assays that were comparable to the wild type animals (Fig. 2D+E).

In the next step, molecular changes induced by EGb 761® treatment in mice were analysed. Therefore, deposition of Aβ in the brain of transgenic mice was determined by staining with a human-specific antibody (Fig. 3A). No changes in Aβ-peptide depositions were

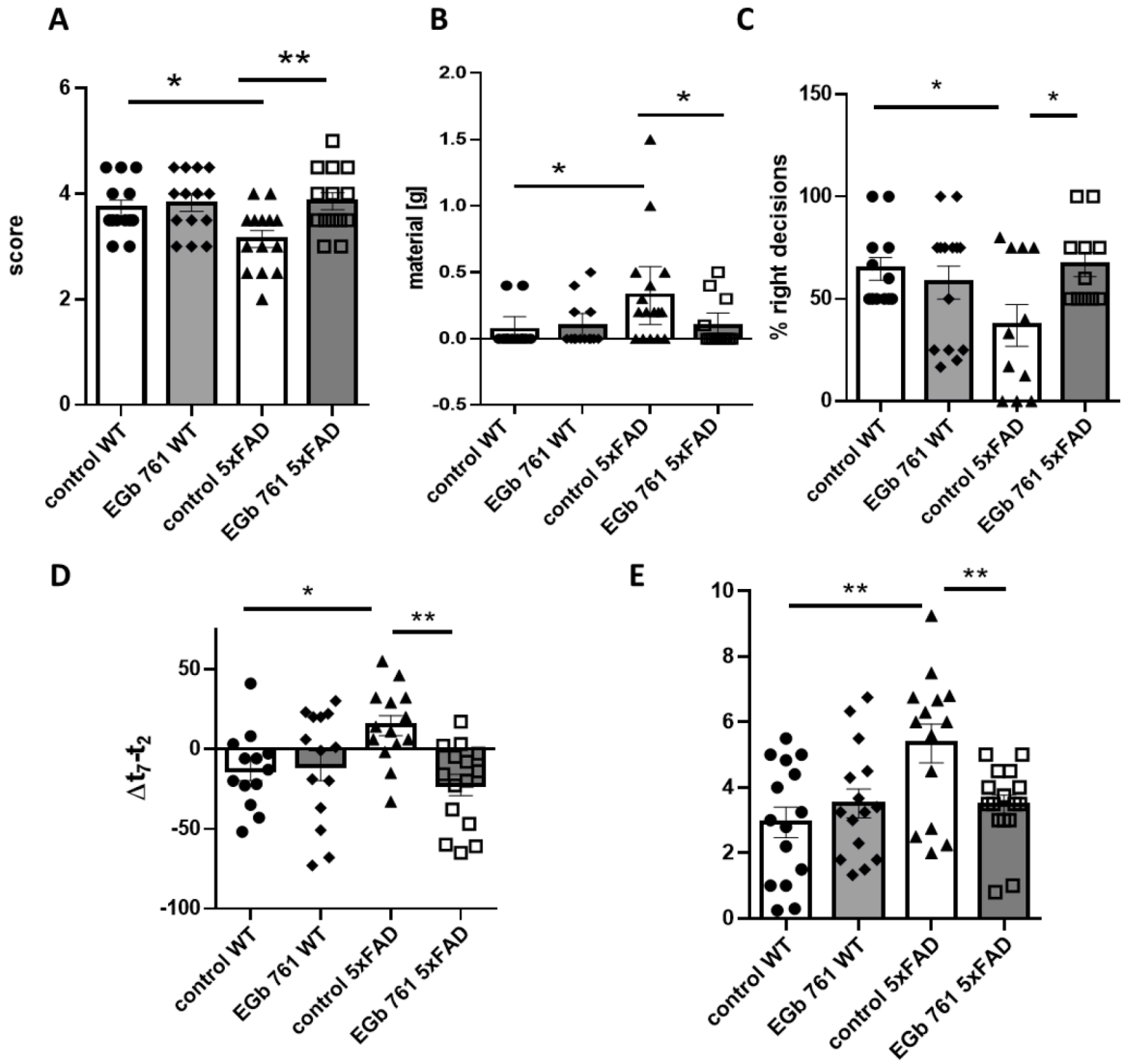


Fig. 2. Cognitive performance of 5xFAD and wild type mice after EGb 761® treatment
Reduction of cerebral amyloid plaques and peripheral NfL in 5xFAD mice after EGb 761® treatment

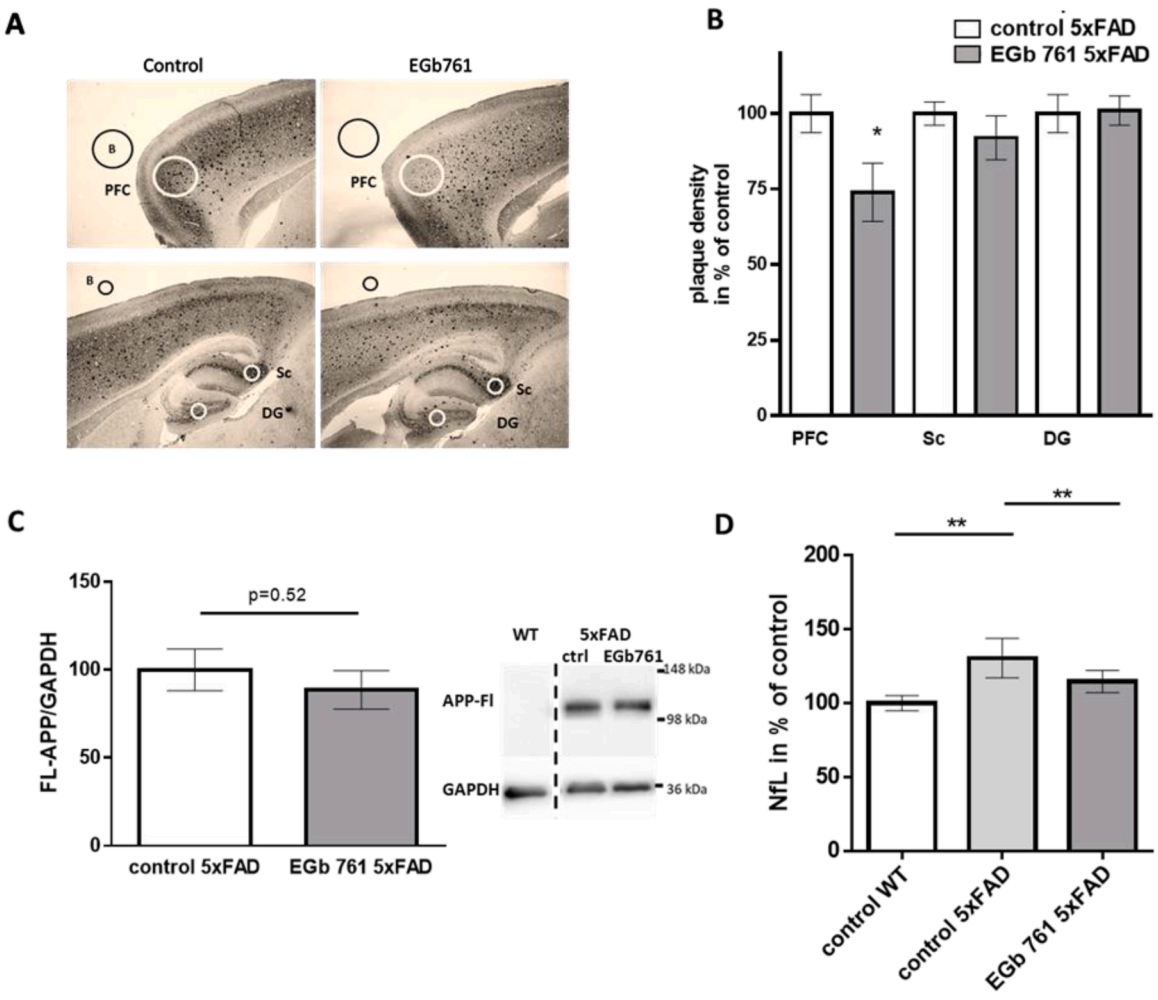


Fig. 3. Influence of EGb 761® on plaque density and NfL levels
Sex-dependent gene expression effect of EGb 761®-treatment in 5xFAD mice.

assessed in the hippocampus (dentate gyrus (DG) and subiculum (*Sc*)). However, a significant reduction in plaque density was observed in the PFC of mice treated with EGb 761® (Fig. 3B). Thus, treatment with EGb 761® elicited changes in the PFC, and therefore the subsequent analyses were focused on this area of the brain.

To exclude the possibility that EGb 761® treatment altered the expression of the heterologous APP, the effect of EGb 761® on APP full-length protein in the PFC was investigated. The results showed that the amount of APP full-length was not affected by the different diets ($p = 0.52$), thus confirming an Aβ-reducing effect of EGb 761® through pathways not relying on regulation of APP expression (Fig. 3C).

Neurofilament-light chain (NfL), has been shown to be significantly increased in the CSF and plasma of AD patients, suggesting that NfL could be a valuable peripheral biomarker for neurodegeneration per se (Gaiottino et al. 2013). In the case of 5xFAD mice, elevated NfL levels were observed in the serum (442.8 pg/ml ± 41.09 pg/ml) as compared to the wild type (342.2 pg/ml ± 18.39 pg/ml). These levels were partially normalized upon EGb 761®-supplemented diet treatment (389.6 pg/ml ± 23.04 pg/ml) (Fig. 3D).

To evaluate the effect of EGb 761®-treatment on the expression of AD-related genes, a disease specific panel with 84 genes (Qiagen) was analysed using pooled samples. In general, the study showed that certain genes related to AD were regulated differently in 5xFAD mice compared to WT mice. For example, for male mice, *Ache* (acetylcholinesterase) and *Mpo* (myeloperoxidase) displayed higher expression levels in 5xFAD, while for female mice, *Igf2* (insulin-like growth factor 2) and *Il-1α* (interleukin-1 alpha) revealed higher mRNA amounts as compared to WT females. Additionally, genotype-dependent downregulation of certain genes was observed, including *Ncst* (nicastrin), and *Snca* (alpha-synuclein) in male 5xFAD mice, as well as *Chat* (choline acetyl-transferase) in female 5xFAD mice. The effects of EGb 761® treatment on the expression of genes related to AD within PFC of 5xFAD mice were highly sex-specific. In males, five genes including *Ache* were down-regulated while nine genes including *Bace1* (β-secretase 1) were upregulated. In females, 12 genes including *Igf2* were downregulated while 10 genes including *Chat* were upregulated (Fig. 4A). Only one gene was equally downregulated in both sexes after EGb 761® administration: *Ncstn*. An example that indicates therapeutic effect of EGb 761® is *Mpo*. *Mpo* was found to be upregulated in male 5xFAD mice compared to wild type mice. However, treatment of 5xFAD with EGb 761® counteracted this effect, bringing it back to wild type level. As the array panel was conducted using pooled RNA samples, which did not allow for analysis of variation, qPCR analyses on samples from individual animals were conducted to confirm the effects for the selected genes with the most pronounced treatment effects (Fig. 4B). The effects of EGb 761® on the cholinergic system were confirmed through qPCR analysis. The increase in *Ache* levels in males and the decrease in *Chat* levels ($p = 0.26$) in female 5xFAD animals on control diet was restored to wild type levels in EGb 761® treated 5xFAD animals, although only *Ache* effects reached statistical significance. 5xFAD animals displayed elevated levels of *Mpo* (male) and *Igf2* (female) as compared to wild type animals, which play a role in regulation of neuroinflammation and metabolism. These levels

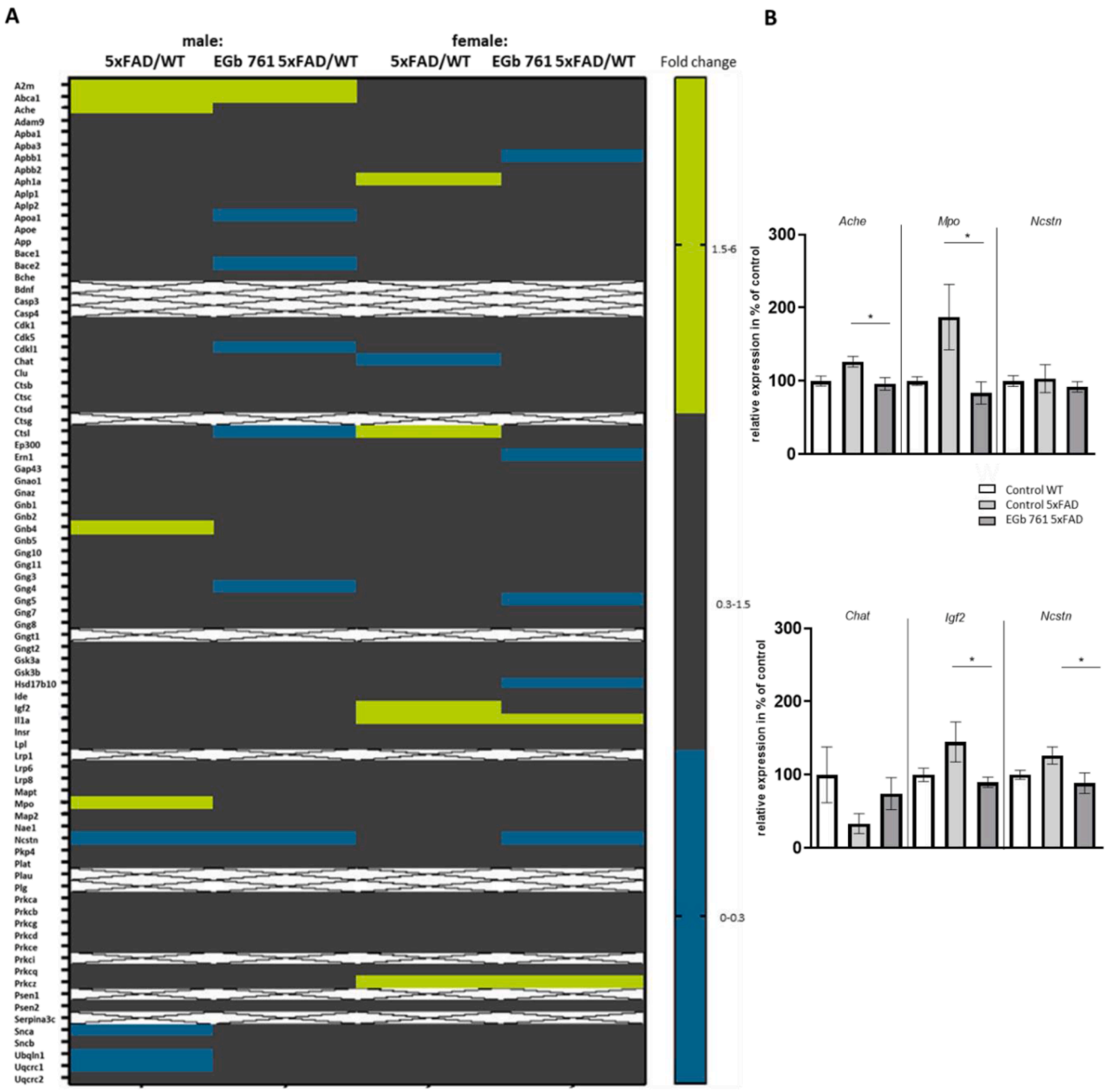


Fig. 4. Impact of EGb 761® on expression of AD-related genes.

were lowered to wild type levels by the treatment with EGb 761® in the transgenic mice, as confirmed by single-animal-based analysis. For *Ncstn*, the elevation observed in the initial pool-sample analysis could only be confirmed for female 5xFAD mice. Here, EGb 761®-treatment successfully restored expression to wild type levels.

Taken together, alterations in the expression of genes related to AD after EGb 761® treatment were highly sex-specific. However, effects on the cholinergic system, γ-secretase complex, and neuroinflammation were still observed in both sexes with pooled samples in the AD-related gene array and confirmed via qPCR.

Organotypic brain slice cultures provide a valuable *ex vivo* model for investigating intricate cellular and molecular processes in the brain. In this study, we used this method to validate our previous findings regarding the sex-specific gene expression effects of EGb 761® treatment in 5xFAD mice with two different concentrations of EGb 761®: 10 μg/ml (equal to the expected plasma concentration reached with the feeding experiment) and a lower concentration of 1 μg/ml were tested (Huang et al. 2014). Brain slices from male and female mice were treated with EGb 761® or solvent for 24 h in culture and compared to solvent-treated wild type animal-derived slices. In male 5xFAD mice, RNA quantitation revealed an elevated expression of *Mpo*, that could be restored by EGb 761® administration via food in the PFC. In the organotypic slice cultures, we observed that MPO protein amount was also increased in slices from 5xFAD mice as compared to wild type animal slices (Fig. 5A, left). The increased amount could be significantly lowered by 1 μg/ml of EGb 761® and further reduced by 10 μg/ml, finally returning to wild type levels. Interestingly, while in living animals no elevation of mRNA for *Mpo* could be found in females, brain slices showed similar protein amount elevation as in males (Fig. 5A, middle). However, therapeutic effect of EGb 761® could be then confirmed in a dose-dependent manner also for slices obtained from female 5xFAD mice. Finally, we tested enzymatic activity of MPO under EGb 761®

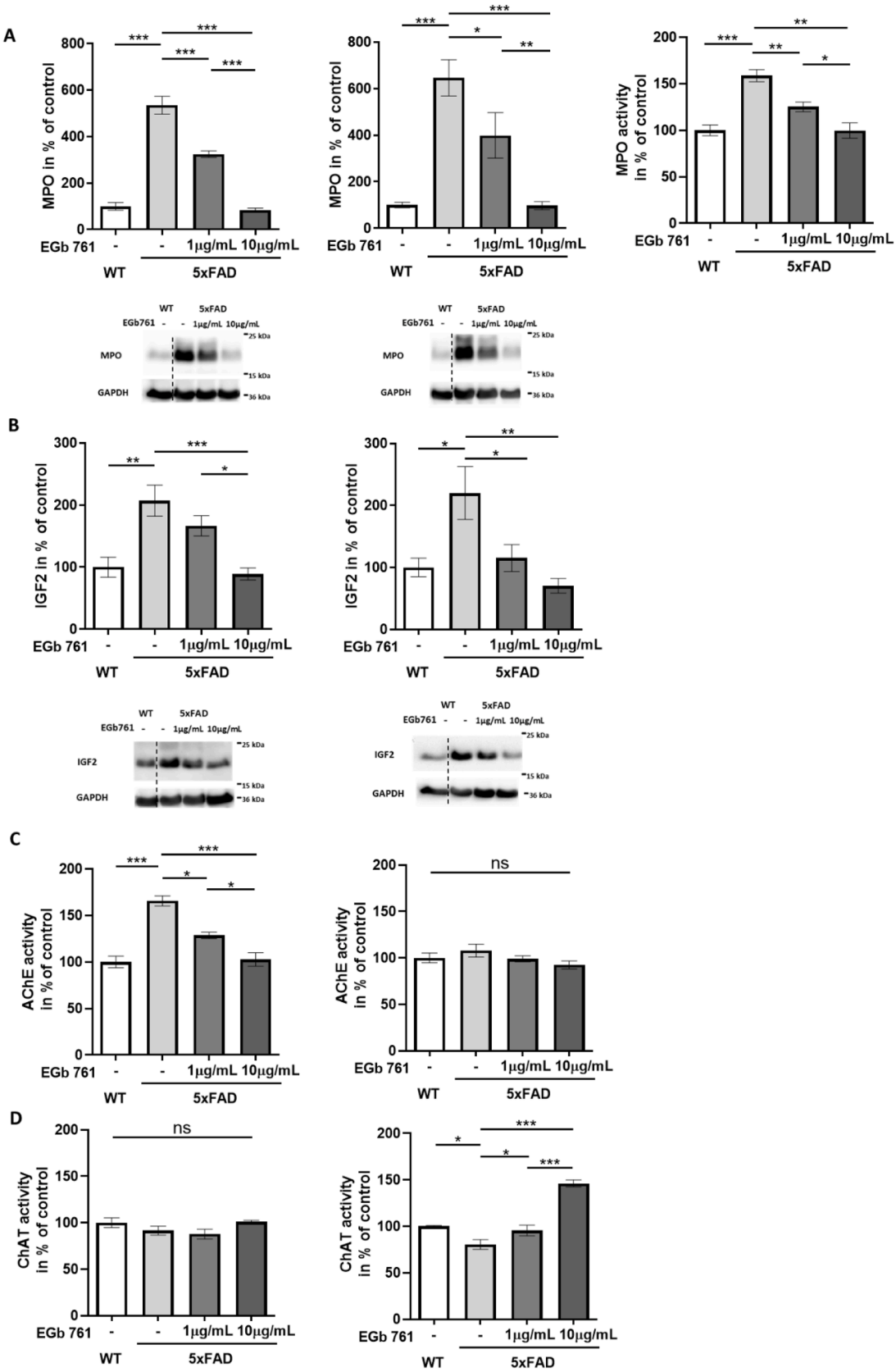


Fig. 5. Sex-dependent response to EGb 761® treatment in organotypic brain slices
Anti-inflammatory effect of EGb 761® in PFC of 5xFAD mice.

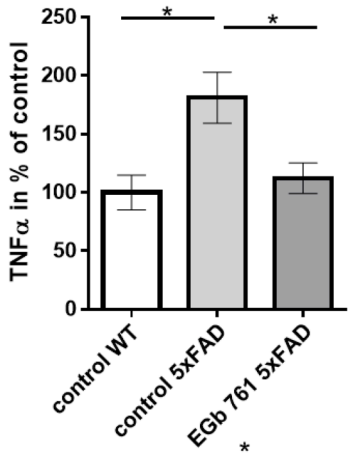
treatment exemplarily in male mice-derived slices and here could also find a dose-dependent reduction, restoring wild type levels (Fig. 5A, right). Subsequently, we assessed IGF2 levels that had been shown to be increased on the mRNA level selectively in female 5xFAD PFC samples and were decreased by EGb 761® supply via diet. Here, we could confirm the finding by *ex vivo* treating organotypic slice cultures of female 5xFAD mice (Fig. 5B, right): IGF2 levels were almost doubled in 5xFAD female-derived slices as compared to wild type and dose-dependently reduced via the pharmacological treatment. Interestingly, for male mice, similar results on the protein level were obtained (Fig. 5B, left). This might indicate that sex-specific effects cannot be fully preserved in the culture model. However, for the selected candidates from the cholinergic system, the slice cultures recapitulated completely our findings on mRNA level from the diet-based experiments: EGb 761® treatment restored the increased activity of the enzyme found in male 5xFAD mice (Fig. 5C, left). As before, this effect was not observed in slices obtained from females, where also no increase in comparison to the wild type was assessed (Fig. 5C, right). Similarly, the significant decrease in ChAT levels observed in female 5xFAD mice compared to wild type mice was observed similarly to the mRNA level. Additionally, a dose-dependent increase in ChAT activity was demonstrated after EGb 761® treatment of the slices (Fig. 5D, right). For males, no changes in ChAT activity, neither due to genotype nor due to administration of the extract could be observed (Fig. 5C, left), which confirms the previous mRNA analyses based on living animals.

Several pathways (cholinergic signaling, inflammation, metabolism) were positively affected in the 5xFAD mice treated with EGb 761®. In the next step, we decided to further analyse the anti-inflammatory potential on the protein levels from single PFC samples by using an antibody array containing 40 immune-relevant analytes (for exemplary images see Suppl. Figure 2A). Overall, the 5xFAD mice exhibited a pro-inflammatory phenotype, with 13 immune-relevant analytes showing increased levels, including IL-7 and TNFα, as compared to wild type mice (Suppl. Figure 2B). In total, out of the 40 analytes examined, EGb 761® treatment of 5xFAD mice restored levels of 26 proteins to wild type level, while nine analytes were regulated deviating from wild type level. The levels of five proteins remained unaffected. Lower levels of proteins, such as interleukin-12-p40/p70 or soluble tumor necrosis factor receptor I (sTNF-RI), were detected in 5xFAD mice compared to wild type mice (Fig. 6A, table shows the cytokines which were changed the most on relative levels upon EGb 761® treatment). Following EGb 761® treatment of the 5xFAD mice, the levels of both proteins increased even above the wild type level. Most prominent treatment effects were observed for TNFα: 5xFAD mice displayed a 50 % increase in TNFα compared to wild type mice, which was completely normalized to wild type levels in EGb 761® treated animals (Fig. 6A). Based on this finding, we decided to additionally analyse the regulation of *Tnfα* mRNA, as this gene was not included in the AD-related gene panel. In both sexes, the same effect was observed: the increased expression of *Tnfα* in 5xFAD mice compared to wild type was reduced by EGb 761® treatment reaching wild type levels (Fig. 6B). Thus, EGb 761® demonstrated an anti-inflammatory effect with particularly pronounced attenuation of the TNFα response in the PFC.

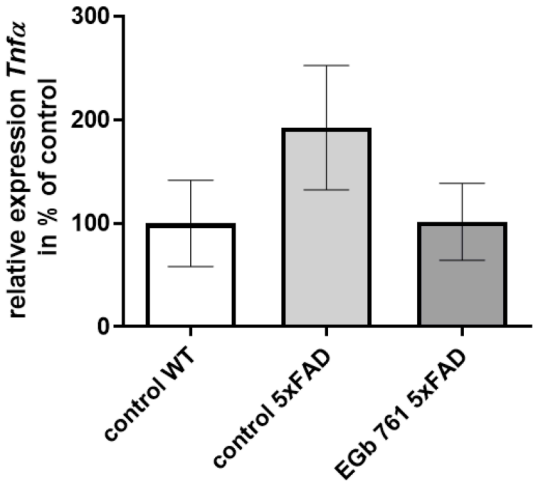
In the next step, we assessed whether the anti-TNFα effects of EGb 761® occurred locally (in PFC) or systemically. The 5xFAD animals receiving control diet displayed significantly increased serum levels of TNFα (130.4 pg/ml ± 4.71 pg/ml) compared to the wild type animals (105.8 pg/ml ± 2.68 pg/ml, $p < 0.05$). Transgenic animals on EGb 761®

A

control WT vs control 5xFAD		control 5xFAD vs Egb 761 5xFAD		effect
protein	*p-value*	*protein*	*p-value*	
TNFα	0.001	TNFα	0.05	neutralizing ↑
Eotaxin	0.01			
IL-12 p40/p70	0.03	IL-12 p40/p70	0.1	
Fas Ligand	0.04			
MIG	0.04	MIG	0.09	neutralizing
GCSF	0.05			
MIP-1α	0.09	MIP-1α	0.07	neutralizing
		RANTES	0.03	↓
		IL-13	0.09	↓
		sTNF R I	0.09	↑



B



C

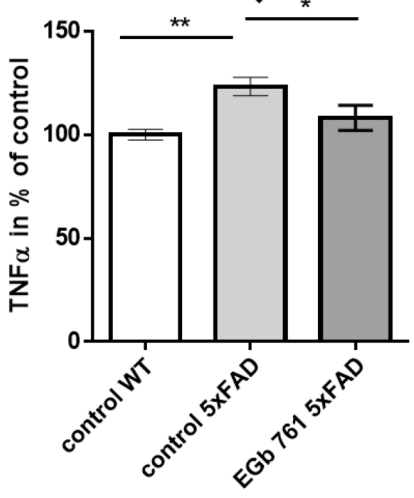


Fig. 6. Anti-inflammatory, TNFα-reducing effect of EGb 761® in 5xFAD mice.

diet showed significantly reduced TNFα levels (114.4 pg/ml ± 6.45 pg/ml, $p < 0.05$) compared to transgenic mice receiving control diet, thus confirming the effects observed in the PFC (Fig. 6C).

We additionally evaluated the anti-TNFα effects of EGb 761® in macrophages derived from bone marrow of 3-month-old SAMR1 mice. In the primary culture, LPS elicited a five-fold elevated TNFα secretion, while $A\beta_{42}$ increased the secretion three-fold after 24 h of treatment. Co-treatment with 1 μg/ml EGb 761® significantly ameliorated the inflammatory response in both cases (Suppl. Figure 3).

As both, male and female mice displayed an anti-inflammatory effect of EGb 761®, we wanted to elucidate if and how microglia cells are affected in the brain of AD model mice. For this purpose, we performed single-cell RNA sequencing after having attributed the dietary regimen (Fig. 1) exemplarily on male 5xFAD mice (Fig. 7). We chose PFC as the region to investigate as this showed a clear reduction in plaques and was also used for TNFα assessment. The efficacy of the treatment was monitored by measuring TNFα and NfL in serum (Suppl. Figure 4). Viability of isolated cells was >80 % for all samples and quality control did not reveal deviating behaviour concerning genotype during cell preparation or any following processing (Suppl. Fig. 5).

Cell types showed a clear distinction in expression patterns and only a minor proportion of cells could not be identified (Fig. 7A). The chosen isolation technique resulted in a high yield of microglia (about 70 %) and a highly comparable composition of cells in either control or EGb 761®-fed mice. Microglia were subsequently clustered following Singh et al. (2022). This revealed similar gene expression patterns for the respective subpopulations (homeostatic, transition, DAM1, and DAM2) as already described for 5xFAD mice (e.g., highest *Axl* (receptor tyrosine kinase) expression in DAM2). When comparing the relative amount of microglial subtypes, an increase of the homeostatic subtype was observed due to the EGb 761®-containing diet, while the DAM1/DAM2 ratio strongly increased due to a reduction in DAM2 (Fig. 7C). Interestingly, the remaining DAM2 cells even showed reduced cellular proliferation and increased homeostatic behaviour within the most strongly upregulated pathways (Fig. 7D), while Rac (Rho GTPase)- and LPS-mediated signalling pathways were decreased (based on the 50 highest expressed genes). To unravel which microglial populations drive reduced TNFα secretion and production, relative expression was investigated (Fig. 7E): here, DAM1 subtypes revealed a significant higher expression than the other subtypes in the 5xFAD PFC, and this was reduced by EGb 761® treatment.

Discussion

In the search for new treatment strategies for AD, natural products have garnered interest with the hope to find a multi-targeted drug.

In our study, treatment of 5xFAD mice with the *Ginkgo biloba* leaf extract EGb 761® over a period of eight weeks, starting at an early pathological stage at the onset of AD-like pathology, resulted in ameliorated cognition disability in both male and female mice as shown by different behavioural tests for learning and spatial orientation. In other AD mouse models, such as male APP/PS1, female Tg2576 mice, and female TgCRND8 APP mice, treatment with EGb 761® significantly improved learning and memory in the Morris water maze test (Stackman et al. 2003; Hou et al. 2010; Wan et al. 2016).

The main reasons for the improved cognitive functions after the use of EGb 761® remained unclear. Pre-clinical studies have proposed different mechanisms of action. However, differences in probable mechanistical pathways could be based on the sex bias of those studies utilizing either only male or only female mice but also on variable duration of treatment ranging from six to eight months.

We could demonstrate in an aggressively progressing AD mouse model that the treatment with EGb 761® resulted in decreased deposition of Aβ-peptides in the PFC after only eight weeks in both male and female mice which corresponds to the minimum recommended human usage time.

In addition to the reduction of amyloid plaques, EGb 761® treatment also exhibited neuronal protection as shown by the decrease in serum NfL levels for both sexes in our study. It was already demonstrated that plasma NfL level increases in several mouse models of different neurodegenerative diseases, including the 5xFAD mouse strain (Loeffler et al. 2020), making it a suitable biomarker and therapy read-out.

In our study, the effects of EGb 761® treatment on gene expression were sex-dependent, such as the restoration of *Ache* decrease in male 5xFAD mice and increase in *Chat* in females. The discovery that acetylcholine plays an important role in memory and learning, coupled with the observation of a cholinergic deficit in the brains of AD patients, led to the cholinergic hypothesis of AD (Bartus et al. 1982). Co-administration of *Ginkgo biloba* extracts and donepezil exerted enhanced pro-cholinergic and antioxidative effects in scopolamine-induced cognitive impairment rats (Zhao et al. 2021). This is consistent with the effects of EGb 761® on the cholinergic system of 5xFAD mice observed in our experiments, which resulted in restoration of AChE decrease in male mice and ChAT increase in female mice.

In addition to its effect on cholinergic signaling, we observed an effect of EGb 761® on γ-secretase complex, as evidenced by a significant downregulation of *Ncstn* in EGb 761®-treated female 5xFAD mice. Ncstn is an integral member of the γ-secretase complex, which is associated with AD due to its role in catalyzing the proteolytic cleavage, that results in the liberation of Aβ-peptide (Haass and Selkoe 1998). Modulation of γ-secretase complex components, such as Ncstn, to reduce γ-secretase activity is suggested as a potential therapeutic approach for AD (Panza et al. 2010; Hur 2022).

In addition to improving cognitive function, EGb 761® has been shown to reduce parameters related to neuro-inflammation in both animal and clinical studies (DeFeudis and Drieu 2000). Our study found that the elevated expression of *Mpo*, a primary mediator of neutrophils oxidative stress response, was reduced after eight weeks of supplementation with EGb 761® in male 5xFAD mice. This indicates an anti-pathological activity as in male 5xFAD mice with MPO deficiency (5xFAD-MPO KO) significantly superior performance in spatial learning and memory and associative learning compared to control was previously shown (Volkman et al. 2019).

To date, the published literature has demonstrated the beneficial effects of EGb 761® in a number of preclinical studies in mice and rats using only one sex with the main focus on males. However, the different treatment responses in males and females show the importance of sex-specific analysis, which is particularly relevant given the higher prevalence of AD in females and underscores the importance of considering sex as a biological variable. Within the last few years, evidence has emerged that besides peripheral immune cells, microglia might play a pivotal role in AD. With the progression of the disease, microglia become hyper-activated within the vicinity of plaques and release cytotoxic mediators such as TNFα (Thu Thuy Nguyen and Endres 2022). Elevated levels of TNFα in the CSF have been associated with higher rates of progression from mild cognitive impairment to AD and also generally have been described for patients with AD (Contreras et al. 2022). We could demonstrate that the increased TNFα mRNA and protein level in the PFC observed in male and female 5xFAD mice as compared to wild type mice, was counteracted by the treatment with EGb 761® in our experimental setting.

The role of peripheral immune cells in AD has been neglected for a long time but is now gaining interest (Prinz and Priller 2017). Patients with AD have shown significantly elevated levels of cytokines in their blood (Su et al. 2016). In our study, we demonstrated that TNFα was increased in 5xFAD mice, and the treatment with EGb 761® normalized TNFα levels not only in the brain but also in the serum of mice. These findings suggest that inhibition of (neuro-) inflammation could contribute substantially to the overall pharmacological treatment effect of EGb 761®. To better understand which cell types are contributing to the anti-inflammatory effect, we performed single cell RNA sequencing with a special emphasis on microglia and identified a strong reduction in

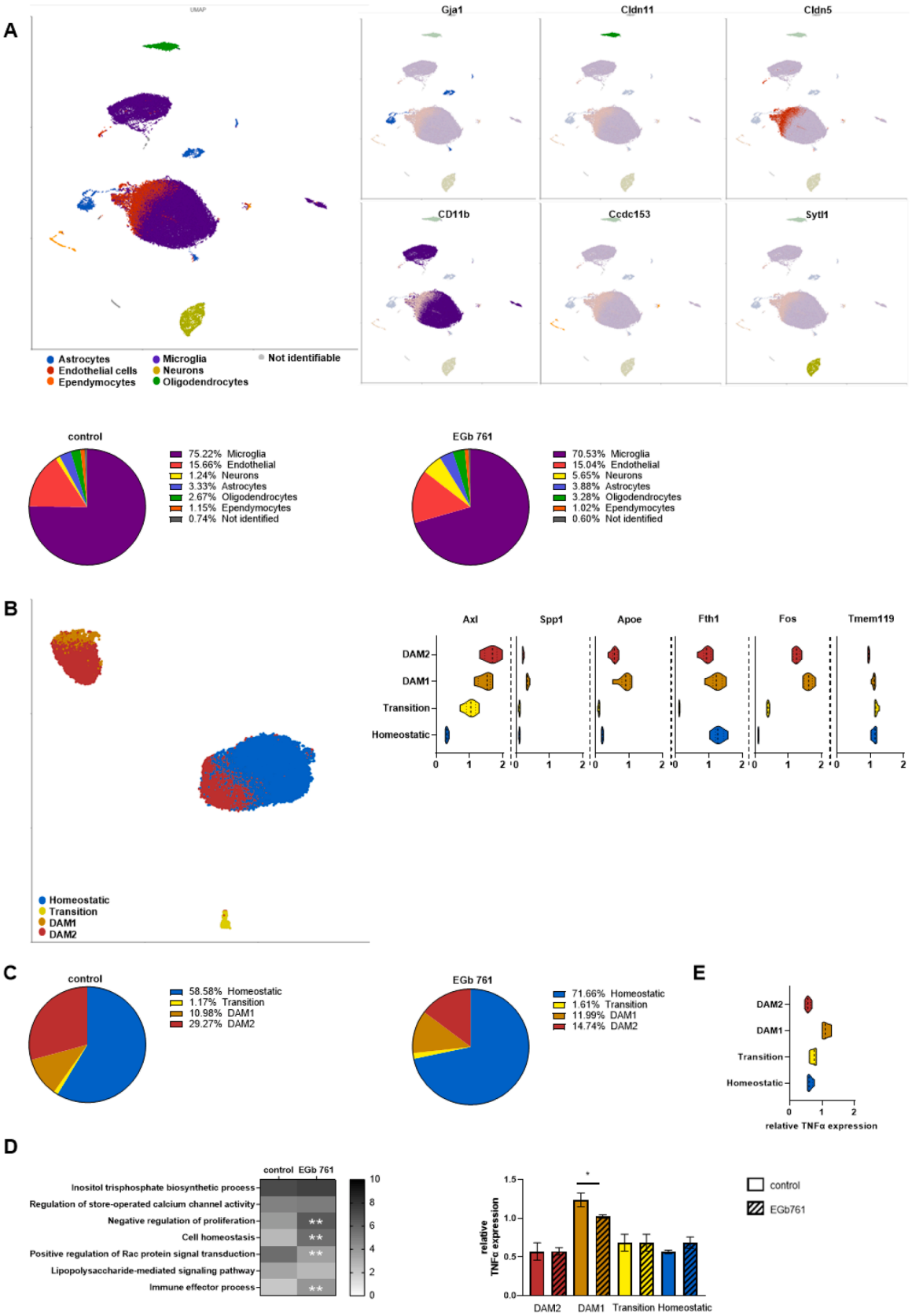


Fig. 7. Effect of EGb 761® on microglial subtypes in 5xFAD mice.

DAM2 microglia and simultaneously an increase in homeostatic microglia.

In wild type mice, microglia are mainly in the homeostatic state. Changes in metabolism might then lead to DAM1 probably via the transition state (Liu et al. 2024), and further result in DAM2. The latter conversion requires triggering receptor expressed on myeloid cells (TREM2) (Nguyen et al., 2020; Zhou et al. 2020). There has been controversy about the function of TREM2 in AD – especially in regard to the stages of pathology. TREM2 deficiency was shown to ameliorate amyloid pathology early, but to exacerbate it at later stages of disease progression (Jay et al. 2017). Moreover, investigation of a microglial BACE-1 knock-out revealed that balancing DAM1/DAM2 ratio towards DAM1 is beneficial concerning pathogenesis (Singh et al. 2022). As 5xFAD mice develop fast disease hallmarks, we assume that the 4 months at the end of our experiments already represent a later stage of disease, thereby defining reduction in DAM2 as a beneficial outcome. Nomenclature of microglia is still fluid and a developing field after decades of a simplified M1/M2 system (Chen and Colonna, 2021; Spurgat and Tang 2022). Therefore, we cannot exclude that the administration of other clustering approaches might not have also been informative for evaluating the effect of EGb 761®. For example, LDAM – lipid droplet accumulating microglia – have been found especially in the aging mouse brain (Marschallinger et al. 2020). In this regard, it is of interest that minocycline seems to exert its anti-inflammatory effect on retinal degradation mainly by suppressing DAM markers, particularly those associated with lipid metabolism (Ozaki et al. 2022), confirming a beneficial outcome of DAM reduction in another pathological setting. We could furthermore show that DAM1 TNFα production was reduced, while the relative amount of this subpopulation remained stable. This confirms a central anti-inflammatory effect of EGb 761® and assigns it to a specialized microglial cell population.

Conclusion

In conclusion, oral EGb 761® treatment for eight weeks improves cognitive function in both male and female 5xFAD mice. This was associated with a reduction of amyloid plaque load in prefrontal cortex and beneficial effects on markers of neurodegeneration, (neuro-) inflammation, cholinergic signalling and microglial overreactivity in the EGb 761® treated animals. Although sex-dependent differences were observed between treatment effects on individual gene expressions, these changes belonged to common mechanisms of action (i.e. reduction of inflammation and modulation of cholinergic signalling) and produced comparable net results on cognitive function in both sexes (Fig. 8).

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Availability of data and materials

All data are provided within the text or within Supplementary Materials.

Studies involving animals

All procedures were performed in accordance with the European Communities Council Directive regarding care and use of animals for experimental procedures and were approved by local authorities (Landesuntersuchungsamt Rheinland-Pfalz; approval number G-17–035).

CRediT authorship contribution statement

Vu Thu Thuy Nguyen: Data curation, Formal analysis, Validation, Visualization, Writing – original draft, Writing – review & editing. **Robert Subirana Slotos:** Investigation, Validation, Visualization. **Malena Dos Santos Guilherme:** Data curation, Formal analysis, Investigation. **Tinh Thi Nguyen:** Investigation, Writing – review & editing. **Sabrina Weisenburger:** Conceptualization, Methodology,

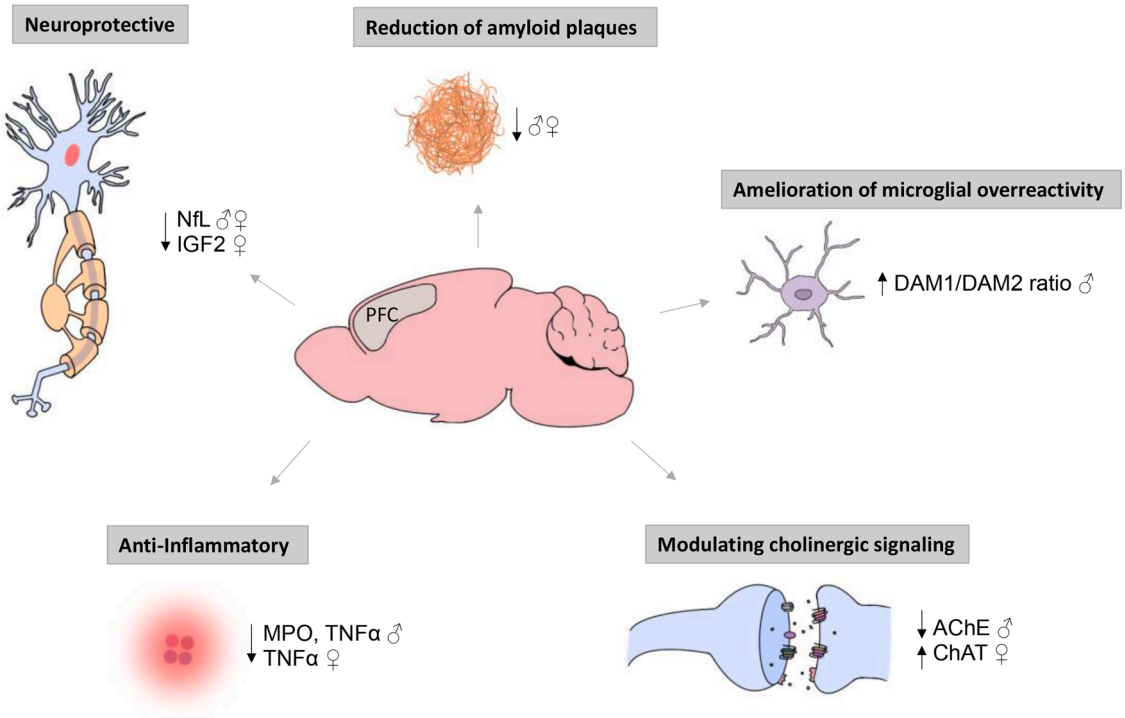

Fig. 8. Schematic of multi-targeted effects of EGb 761®.

Project administration, Validation, Writing – original draft, Writing – review & editing. **Martin D. Lehner:** Conceptualization, Methodology, Project administration, Validation, Writing – original draft, Writing – review & editing. **Kristina Endres:** Conceptualization, Methodology, Project administration, Validation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

S.W. and M.D.L. are employees of Dr. Willmar Schwabe GmbH & Co. KG, a producer of medicinal products containing the special extract from *Ginkgo biloba* leaves (EGb 761®).

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Kristina Endres reports financial support and equipment, drugs, or supplies were provided by Dr Willmar Schwabe GmbH und Co KG Kristina Endres reports financial support was provided by MWG Rhineland-Palatinate. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2024.156327.

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