

Effects of *Ginkgo biloba* Extract EGb 761, Donepezil and their Combination on Central Cholinergic Function in Aged Rats

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ABSTRACT - Purpose. *Ginkgo* extract EGb 761 and cholinesterase inhibitors have been shown to be effective in the treatment of dementia patients. In addition to neuroprotective effects, *Ginkgo* extract EGb 761 has been reported to elevate brain levels of certain neurotransmitters such as dopamine, noradrenaline, and acetylcholine. In the present study, we investigated the impact of EGb 761, donepezil and the combination of both drugs on the central cholinergic system in aged rats. Methods. 24 month old rats received EGb 761 (100 mg/kg/day), donepezil (1.5 mg/kg/day), the combination of both drugs or vehicle control by oral gavage for 14 days. We used microdialysis in rat hippocampus to monitor extracellular concentrations of acetylcholine (ACh), choline, glucose and lactate. Brain homogenates were prepared to measure activities of acetylcholinesterase (AChE), choline acetyltransferase (ChAT) and high affinity choline uptake (HACU). Results. While EGb 761 alone had no effect, donepezil and the combination of donepezil and EGb 761 increased basal ACh levels by 2- to 3-fold. Concomitantly, significant reductions of AChE and HACU were measured in both groups. No differences were seen between donepezil and the combination in these parameters. Treatment with EGb 761 decreased extracellular choline release and showed a tendency to moderately elevate ChAT activity. Conclusions. We found that donepezil and EGb 761 do not display a pharmacological interaction when given together. Adding EGb 761 did not modify the effects of donepezil on the hippocampal cholinergic system. Reduced choline levels indicate neuroprotective properties of EGb 761. Therefore, the combination of EGb 761 and donepezil may be beneficial in the treatment of Alzheimer's disease (AD).

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INTRODUCTION

Alzheimer's disease (AD) is the most common adult-onset dementia, and its incidence highly correlates with age. The number of patients is projected to reach 115 million by 2050 (1). Effective treatments are needed to alleviate the great burden of the disease for patients, families and health care systems. According to the "cholinergic hypothesis", impairments in the central cholinergic system, especially in brain areas dealing with memory, learning, and emotional response are of great importance for the severity of clinical symptoms (2). Cholinergic therapy with acetylcholinesterase (AChE) inhibitors is a well-established strategy for symptomatic treatment of AD. Donepezil is a specific and reversible inhibitor of acetylcholinesterase (AChE) which increases acetylcholine (ACh) levels by reducing its breakdown. Donepezil is approved for mild-to-moderate AD in Europe and Japan, and for all AD stages in the USA and several other countries. Clinical studies have repeatedly shown

improvements in cognitive functions, global assessment of change, behavioural symptoms and functional outcome measured by several scores such as the ADAS-cog (3–5).

Ginkgo biloba extract EGb 761 is approved in many European countries for the treatment of mild to moderate AD, as well as for vascular and mixed dementia. Its efficacy and safety has recently been confirmed in three large, independent meta-analyses (6–8). Based on the clinical evidence, the German Institute for Quality and Efficacy in Health Care (IQWiG) and the European Medicines Agency (EMA) concluded that treatment with EGb 761 in a dose of 240 mg/d shows a beneficial effect on activities of daily living and an indication for improvement of cognition and quality of life (9). However, few data are available for the combination of EGb 761 and donepezil. One small randomised clinical trial compared the efficacy

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of EGb 761 alone with that of donepezil alone and in combination with EGb 761 (10). The results revealed similar response rates in all groups with an apparent but not statistical significant tendency in favour of the combination measured by different scores for cognition, behaviour and related diseases. In a recent cohort study, beneficial effects on MMSE but not on ADAS-Cog from the combined therapy compared to cholinesterase inhibitor therapy alone were reported (11). Potential synergistic actions of the two drugs on the central cholinergic system were never investigated. This seemed interesting because EGb 761 has been shown to moderately increase extracellular levels of acetylcholine in the medial prefrontal cortex of freely moving rats (12). In the present study, we used microdialysis to study the impact of orally administered EGb 761, donepezil, and the combination of both on the hippocampal cholinergic system in aged rats. In addition to cholinergic markers, we also monitored extracellular levels of glucose and lactate, two major metabolites reflecting neuronal energy metabolism, and choline as a marker of neuronal death.

METHODS

Animals

Male Sprague-Dawley rats (24 month of age, 54 rats in total) were obtained from the Institute of Pharmacology and Toxicology of the Freie Universität Berlin. The animals were given two weeks to adapt to the new environment before experiments started. Rats were maintained on a 12 h light–dark cycle, at a room temperature of 22.5 ± 2.5 °C and a humidity of 50-70 % with food and water available ad lib. The experimental procedures described here were carried out in accordance with the guidelines as set and approved by the responsible government agency (Regierungspräsidium Darmstadt, Germany).

Treatment

Four groups of rats received either vehicle solution, EGb 761, donepezil, or both drugs for four weeks. The first group (control; "Ctr") received an aqueous 30% sucrose solution (vehicle solution). The second group (EGb 761 group; "EGb") received 100 mg/kg EGb 761 extract suspended in vehicle solution. EGb 761[®], provided by Dr. Willmar Schwabe Co., Karlsruhe, Germany, is a dry extract from Ginkgo biloba leaves adjusted to 22-27% ginkgo flavonoids; 5-7% terpene lactones consisting of ginkgolides A, B, and C and bilobalide; and less than 5 ppm

ginkgolic acids. The extract is stable when kept cold and dry, and solutions were prepared freshly for the present experiments. The third group (donepezil group; "Don") received 1.5 mg/kg donepezil dissolved in the sucrose solution. The fourth group (combination group; "EGb+Don") received 100 mg/kg EGb 761 extract and 1.5 mg/kg donepezil. A dose of 100 mg/kg/d for EGb 761 was chosen because it was reported to affect neurotransmitter levels in the brain in a previous study (12). Major constituents such as ginkgolides and bilobalide are known to enter the brain in relevant concentrations (13,14). The relatively low dose of 1.5 mg/kg/d donepezil was chosen because it is sufficient to decrease AChE activity in the brain (15) but not too high to mask potential effects of EGb 761 on the cholinergic system. Doses were given orally by gavage, once daily in the morning for four weeks. The last doses were given on the days of the experiments, i.e. immediately before the microdialysis experiment started.

Implantation of the microdialysis probe

On day 1, rats were anaesthetised with isoflurane (Forene[®], Abbvie, Ludwigshafen, Germany) in concentrations (v/v) of 4% isoflurane in air for induction and 1.5% isoflurane for maintenance of anaesthesia by a vaporisator (Kent Scientific, USA). Self-made, I-shaped, concentric dialysis probes with an exchange length of 3 mm and a molecular cut-off of 10,000 Da were constructed as previously described (16,17). Using a stereotaxic frame (Stoelting, Chicago, USA), the probes were implanted into the right ventral hippocampus using the following coordinates (from bregma): AP: -5.2 mm, L: -5.2 mm, and DV: -7.0 mm (18). Glass ionomer eluting cement (Micron[®] i-Cem, PrevestDenPro, Heidelberg, Germany) was used to fix the probe on the skull. All animals received 2 ml of Ringer-lactate solution i.p. and were allowed to recover overnight.

Microdialysis experiments

Experiments were carried out in freely moving animals on the two days following probe implantation. Microdialysis probes were perfused with artificial cerebrospinal fluid (aCSF) containing 147 mM NaCl, 2.7 mM KCl, 1.2 mM MgCl₂ and 1.2 mM CaCl₂ (all VWR, Darmstadt, Germany) at a perfusion rate of 2 µl/min. Samples were collected in intervals of 15 min, immediately stored on ice and subsequently frozen at -80 °C until analysis. After 90 min perfusion with aCSF, the dialysis fluid was switched to a CSF containing 0.1 µM neostigmine (Sigma Aldrich, Munich, Germany); neostigmine was required to increase

and stabilize ACh levels *in vivo* for the following behavioral experiments. After an additional 90 min, the rats were placed in a novel environment ("open field") and microdialysis was continued (17,19). The testing chamber was a gray plastic container (45x32x20 cm), and each rat was allowed to freely explore the chamber during the time of the experiment. Subsequently, the rats were returned to their home cage, and ACh efflux was monitored for another 90 min. Animals were kept in their home cages overnight, with food and water available *ad lib*. On the second experimental day, baseline values (with neostigmine in the perfusion fluid) were again sampled for 90 min, then the dialysis fluid was switched to aCSF containing 0.1 μ M neostigmine and 1 μ M of scopolamine (Sigma Aldrich, Munich, Germany) while the rats remained in their home cage. After 90 min, the perfusion fluid was switched back, and the rats were monitored for an additional 90 min. After the end of the microdialysis experiments (approx. 8 hours after the last drug treatment), the rats were sacrificed and the left hemispheres were used to prepare homogenates for the measurement of acetylcholinesterase (AChE) activity, choline acetyltransferase (ChAT) activity and high-affinity choline uptake (HACU). The correct location of the microdialysis probes was verified by cutting 1 mm coronal sections of the right hemisphere.

Measurement of acetylcholine, choline, glucose and lactate

Acetylcholine and choline in dialysates were determined by microbore HPLC-ECD using the Eicom HTEC-500 system (Kyoto, Japan) that included degasser, low-speed pump, pre- and separation column, enzyme reactor carrying immobilised AChE and choline oxidase, and electrochemical detector with a platinum electrode operating at 500 mV relative to the Ag/AgCl reference electrode. The system is contained in a temperature-controlled cabinet. ACh was retained on the separation column and cleaved to choline and acetate by AChE; choline was then oxidised to hydrogen peroxide by choline oxidase. Hydrogen peroxide was detected electrochemically. The mobile phase consisted of KHCO₃ 50 mmol/l (Merck, Darmstadt, Germany), EDTA-2Na 134.3 mmol/l (BDH, Poole, UK) and sodium decane-1-sulfonate 1.64 mmol/l (Alfa Aesar, Karlsruhe, Germany) in RotisolV[®] HPLC gradient grade water (Sigma-Aldrich, Munich, Germany), brought to pH 8.4. The flow rate was 150 μ l/min. At an injection volume of 10 μ l, the detection limit of this system was 2 fmol/injection. Data acquisition was performed using EPC-500

PowerChrom[®] software. Glucose and lactate concentrations in the dialysates were analysed by photometry using a CMA 600 microanalyser (CMA Microdialysis, Solna, Sweden).

Acetylcholinesterase activity

Brain homogenates were prepared by weighing the hemispheres and adding a 10-fold volume of isotonic HEPES-sucrose buffer (HEPES sodium salt 10mM, Sucrose 0.32 M, pH 7.4). Homogenization was performed with a tissue grinder (Potter S, B. Braun, Melsungen, Germany) at 1100 rpm and 15 strokes. The homogenates were assessed for AChE activity using the Ellman method (20) with minor modifications (21,22). Briefly, 50 ml of tissue homogenate containing a final volume of 0.5% Triton X-100 were centrifuged for 10 min at 12,000 g and 4°C. 10 μ l of the supernatant was mixed with Ellman buffer and iso-OMPA (final concentration 100 μ M). Acetylthiocholine and dithionitrobenzoic acid (1 mM and 500 μ M final concentrations, respectively) were added before measuring the absorbance at 405 nm using a Victor multilabel plate reader (Perkin Elmer, Bedford, USA). Enzyme activity was calculated using a standard curve prepared with each assay and expressed in relation to protein amount (mU/mg protein). Protein determination was carried out using the Bradford method.

Choline acetyltransferase activity

ChAT was measured by a modification of the Fonnum method (23). Aliquots of brain homogenate (0.5 mg/ml protein) were added to a total volume of 250 μ l reaction mix containing 0.5% Triton X-100, 0.3 M NaCl, 0.02 M EDTA, 0.05 M Na₃PO₄ (pH 7.4), 2 mM choline chloride, 1 mM neostigmine bromide, and 0.5 mCi [³H]acetylcoenzyme A. Following incubation at 37°C for 15 min, the reaction was stopped by addition of 250 μ l of ice-cold phosphate buffer (1 mM, pH 7.4). ACh was extracted with 1 ml 0.5% sodium tetraphenylboron in 85% toluene-15% acetonitrile. After centrifugation, aliquots of 0.8 ml were used for tritium quantification with a scintillation counter (Wallac system 1409, Perkin Elmer). ChAT activity was expressed as nmol of ACh formed per hour per mg of protein (nmol/h/mg). Blank values were obtained by omitting the brain homogenate and subtracted.

High affinity choline uptake

HACU was determined in synaptosomes following a previously described, slightly modified protocol (16,24,25). To obtain the P2 fraction, brain

homogenates were centrifuged at 1000 g for 10 min and then at 17,000 g for 10 min. The resulting pellets (containing the synaptosomes) were used for HACU determination. Aliquots were incubated at 30°C in the presence of 0.5 μM [^3H]choline (diluted to 0.5 Ci/mmol; Biotrend, Cologne, Germany) in Krebs-Henseleit buffer (KHB; containing NaCl 115 mM, KCl 7.1 mM, CaCl_2 1.2 mM, MgSO_4 1.2 mM, NaHCO_3 25 mM, Na_2HPO_4 1.5 mM, glucose 12.8 mM, and saturated with carbogen adjusting to pH 7.2-7.4). Incubations were done both in the presence and absence of 1 μM hemicholinium-3 (HC-3). Choline uptake was stopped after 5 min by placing the reaction mix on ice and by washing with ice-cold KHB. After three centrifugation (14,000 g, 10 min) and washing (KHB buffer) steps, the pellets were solubilised in 4.5 ml scintillation fluid (IRGA-SAFE PLUS, Perkin Elmer) and 0.5 ml methanol and used for tritium quantification by liquid scintillation counting. The HC-3-sensitive, high-affinity choline uptake was calculated as the difference between uptake in the absence and presence of HC-3 and expressed as dpm/mg protein. Protein determination was carried out using the Bradford method.

DATA ANALYSIS AND STATISTICAL EVALUATION

Concentrations of ACh, choline, metabolites and enzyme activities were expressed as means \pm standard error of the mean (SEM) for the respective group with the number of experiments indicated in figure legends. Metabolites, HACU, AChE and ChAT activity were analyzed by one-way analysis of variance (ANOVA) with Bonferroni's post-tests, or with Student's t-test. ACh and choline time courses as obtained by microdialysis were analysed using two-way analysis of variance (ANOVA) for repeated measures and Bonferroni's post-hoc test using Prism 5 (GraphPad® Software, San Diego, USA). Significance of data was assumed when statements could be made with 95% confidence.

RESULTS

Effects of the different treatments on extracellular levels of ACh

The microdialysis probe has access to the extracellular space; hence, measured values reflect extracellular concentrations. Fig. 1A shows ACh

levels of the four treatment groups obtained from hippocampus under basal conditions, i.e. without neostigmine in the perfusion fluid. 30 minutes after drugs were given by oral gavage, six continuous samples were collected reflecting basal ACh values. Mean ACh levels of all time points were 4.51 ± 0.26 fmol/10 μl for controls, 13.7 ± 0.70 fmol/10 μl for donepezil treatment, 4.73 ± 0.17 fmol/10 μl for EGb 761-treated rats and 10.21 ± 0.20 fmol/10 μl for rats which received both donepezil and EGb 761. Thus, the two groups receiving donepezil had significantly higher ACh levels than the other two groups which did not differ substantially. While the donepezil group had higher ACh levels than controls throughout the 90 min of measurement, the EGb+Don group reached similar ACh levels after 75-90 min.

Preliminary experiments showed that behavioural stimulation such as the "open field" increases ACh levels under basal conditions; however, those values were too variable to render useful results. Therefore, we stabilized ACh levels with the addition of 0.1 μM neostigmine to the perfusion fluid which increased ACh concentrations to approximately 70 to 77 fmol/10 μl in each group (Fig. 1B). ACh levels further increased after placing the rats into a novel environment ("open field"). Maximum increases were 142 ± 17 fmol/10 μl for controls, 144 ± 20 fmol/10 μl for EGb 761-treated rats, 162 ± 20 fmol/10 μl for donepezil-treated rats and 147 ± 23 fmol/10 μl for rats receiving both EGb 761 and donepezil. Thus, while all rats responded with elevated ACh levels, the differences between the groups were not significant. Returning the animals back to their home cages yielded a small initial increase of ACh followed by lower ACh concentrations which returned to basal levels after 90 min in all treatment groups.

On the second day of experiments, basal levels of ACh were similar as towards the end of day 1 (Fig. 1C). Upon infusion of scopolamine, hippocampal ACh levels increased 3 to 4-fold in all groups (Fig. 1C). ACh concentrations reached a maximum after 30 to 45 min infusion with 363 ± 52 fmol/10 μl for controls, 291 ± 61 fmol/10 μl for donepezil-treated rats, 345 ± 41 fmol/10 μl for EGb 761-treated rats and 314 ± 96 fmol/10 μl for rats that received both drugs. ACh concentrations slightly decreased approximately 30 min after the end of the scopolamine infusion but remained elevated for more than 90 min.

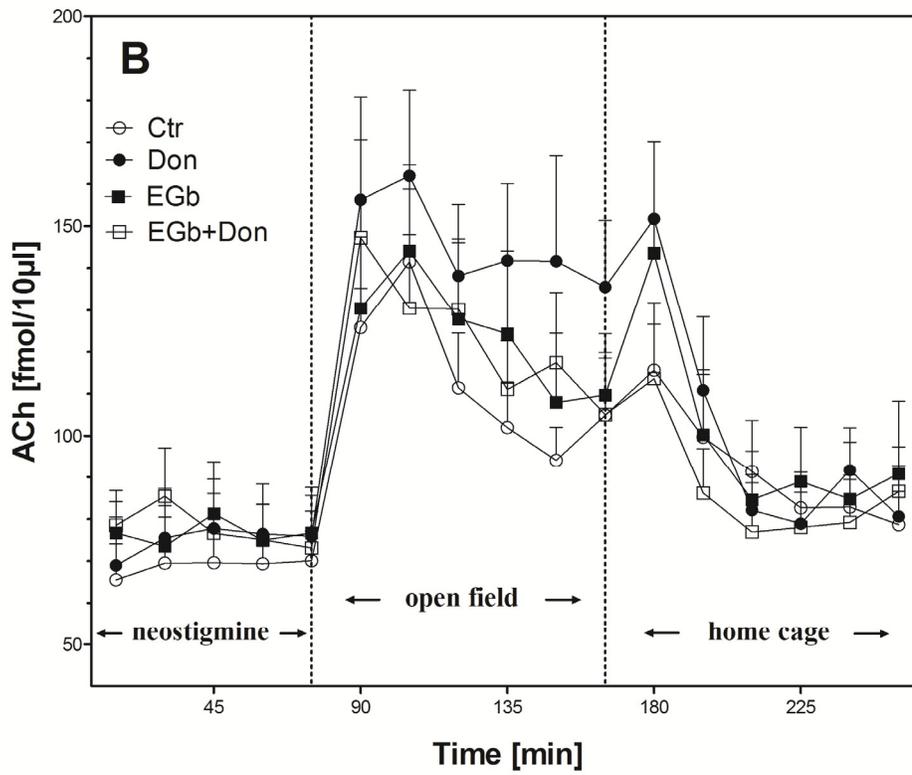
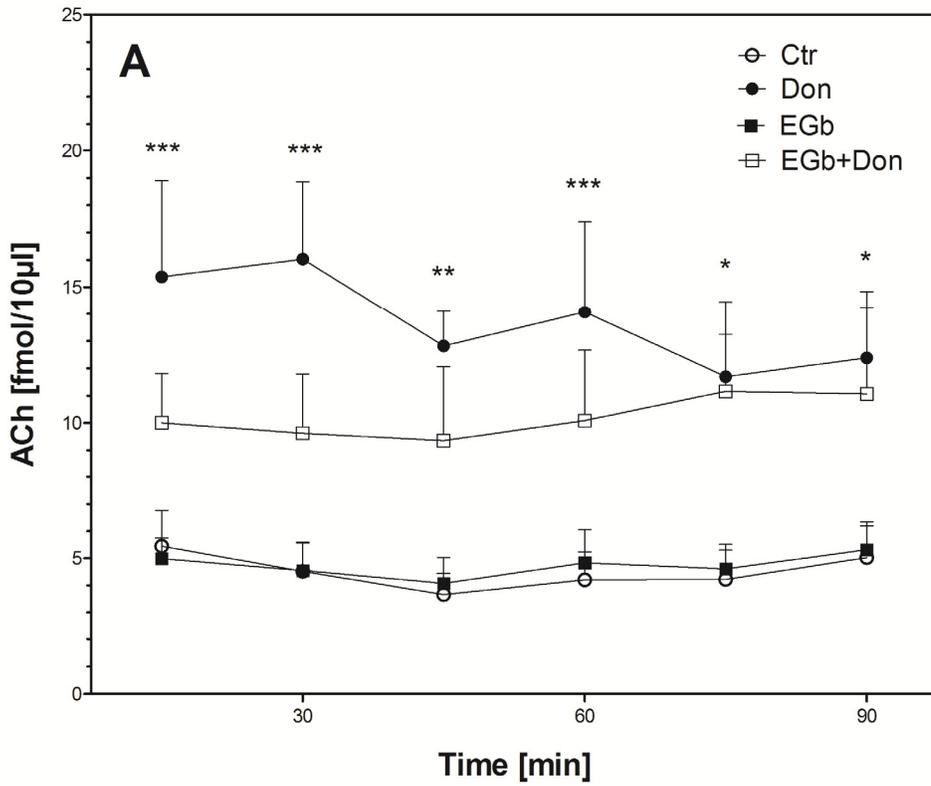


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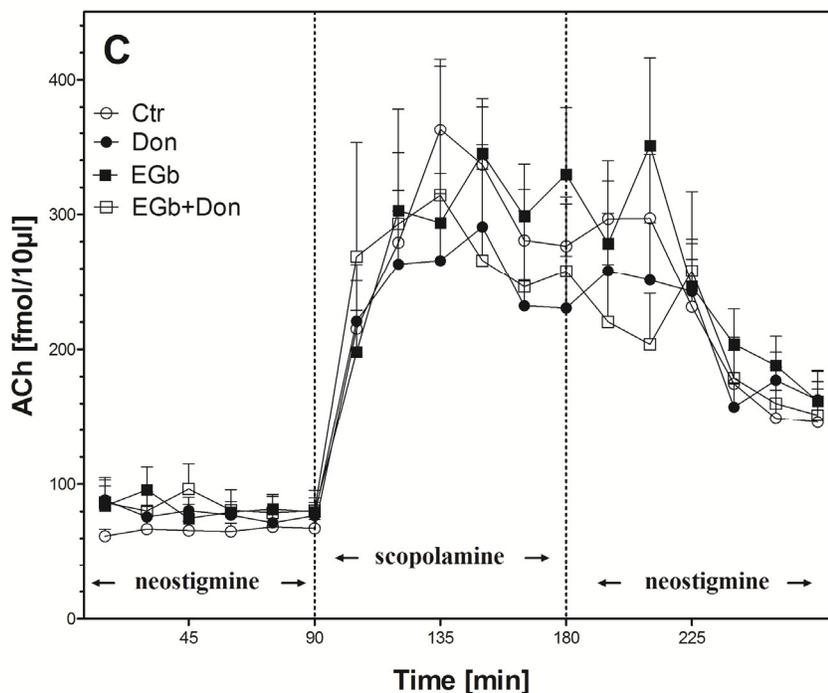


Figure 1. Extracellular concentrations of ACh in rat hippocampus sampled by microdialysis. Different groups of rats received treatments for 4 weeks (once per day) until immediately before start of microdialysis: control ("Ctr"; N=14); donepezil group ("Don") (1.5 mg/kg p.o.; N=12); EGb 761 group ("EGb") (100 mg/kg p.o.; N=14); combination group ("EGb+Don") (100 mg/kg and 1.5 mg/kg p.o.; N=14). Data is presented as means \pm SEM and is given as absolute values, not corrected for recovery. (A) Basal conditions without neostigmine. Statistical significance was calculated by two-way ANOVA for repeated measures (GraphPad Prism[®]). Ctr vs. Don: $F_{1,75} = 22.1$, $p < 0.001$; Ctr vs. EGb: $F_{1,85} = 0.02$, $p = 0.88$; Ctr vs. EGb+Don, $F_{1,80} = 5.74$, $p = 0.03$; EGb vs. EGb+Don: $F_{1,75} = 5.43$, $p = 0.03$; Don vs. EGb+Don: $F_{1,65} = 1.36$, $p = 0.26$. Bonferroni post test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs. Ctr. (B) Behavioral activation on day 1. Perfusion with aCSF containing 0.1 μ M neostigmine. 0 to 75 min basal values, 75 to 165 min behavioral activation (exposure to novel environment, "open field"), 165 to 255 min home cage. Statistical analysis (two-way ANOVA for repeated measures, GraphPad Prism[®]): $F_{3,943} = 0.48$; $p = 0.69$. (C) Pharmacological activation on day 2. Perfusion with aCSF containing 0.1 μ M neostigmine (0-90 min), 90 to 180 min perfusion with aCSF containing 0.1 μ M neostigmine plus 1 μ M scopolamine, 180 to 270 min perfusion with aCSF containing 0.1 μ M neostigmine. Statistical analysis (two-way ANOVA for repeated measures; GraphPad Prism[®]): $F_{3,680} = 0.18$; $p = 0.91$.

Cholinergic enzymes and transporters

Cholinergic parameters were measured in brain homogenates of the left hemisphere. *Ex vivo*- data of the AChE activity (Fig. 2A) reflect the rate of breakdown of acetylcholine. Donepezil and EGb 761 plus donepezil significantly decreased AChE activity compared to control, whereas EGb 761 treatment alone did not affect AChE. Fig. 2B shows the effect of the treatments on ChAT activity, the cytosolic enzyme which catalyzes ACh synthesis. No differences could be seen between groups when tested by one-way ANOVA. However, ChAT activity in the EGb 761-treated group (35 ± 1.2 nmol ACh/h/mg) seemed to be higher compared to control (32 ± 1.2 nmol ACh/h/mg; $p=0.09$). High affinity choline uptake (HACU; Fig. 2C), measured *ex vivo*, is a parameter

for the *in vivo*-turnover of ACh prior to sacrifice (23). While there is an indication of donepezil reducing HACU ($p=0.07$), treatment with the combination of donepezil plus EGb 761 resulted in a significant decrease of HACU activity. In contrast, HACU was not altered by EGb 761 treatment alone.

Effects of drug treatments on extracellular levels of choline

Extracellular choline derives from breakdown of ACh, but also from the hydrolysis of choline-containing phospholipids such as phosphatidylcholine (PC); PC is hydrolyzed by phospholipase A₂ and D, e.g. upon cellular damage (26).

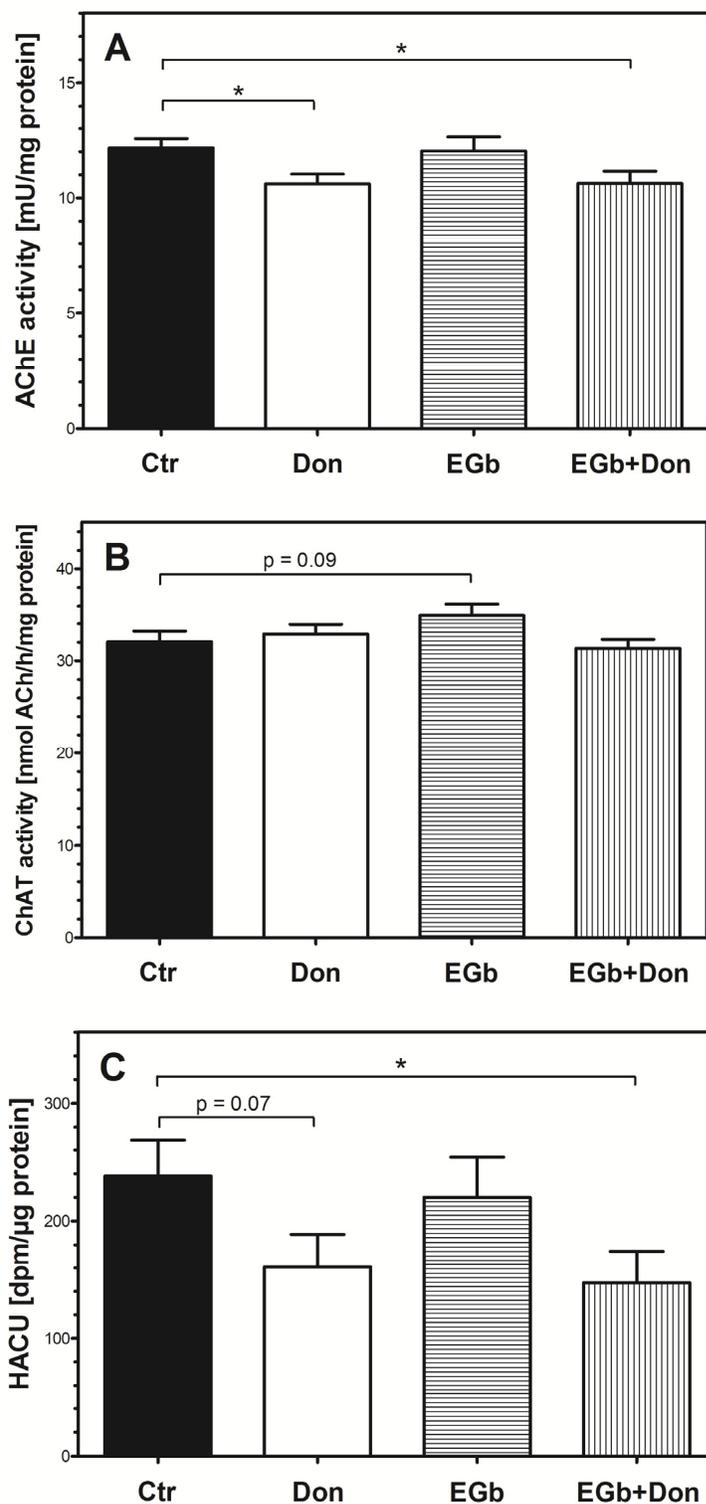


Figure 2. Effect of the drug treatments on the activity of cholinergic enzymes and transporters. (A) Acetylcholinesterase activity and (B) choline acetyltransferase activity were measured in the supernatant of brain homogenates whereas (C) high affinity choline uptake was measured in the P2 fraction. Homogenates and P2 fractions were prepared approximately 8 hours after the last drug administration and stored at -80°C until measurement. Results are expressed as mean \pm SEM with $N=12-14$ each. Statistical analysis (one-way ANOVA with Bonferroni post-test (GraphPad Prism[®]): (A) $F_{3,31} = 2.77$; $p = 0.05$. Student's t-test: Ctr vs. Don, $p=0.02$; Ctr vs. EGb+Don, $p=0.04$. (B) $F_{3,47} = 2.06$; $p=0.12$. Student's t-test: Ctr vs. EGb, $p=0.09$. (C) $F_{3,47} = 2.26$; $p=0.09$. Student's t-test: Ctr vs. EGb+Don, $p=0.04$; Ctr vs. Don, $p=0.07$. *, $p < 0.05$.

In our HPLC assay, choline in microdialysate samples was measured simultaneously with ACh (see Methods). Fig. 3 illustrates changes of choline levels over time in the four treatment groups on day 1 of experiments, i.e. during basal conditions, neostigmine infusion and stimulation with the “open field”. Interestingly, the EGb 761-treated group shows significant lower choline levels than controls and donepezil-treated groups. Mean choline concentrations in dialysates were $0.54 \pm 0.02 \mu\text{M}$ for controls, $0.58 \pm 0.07 \mu\text{M}$ for the donepezil-treated rats, and $0.60 \pm 0.07 \mu\text{M}$ for EGb 761 plus donepezil-treated rats, but only $0.45 \pm 0.03 \mu\text{M}$ for EGb 761-treated rats (Ctr vs. EGb, $p=0.03$).

Extracellular levels of energy metabolites: glucose and lactate

Glucose, the most important source of energy for the brain, is also a precursor of acetyl-CoA, and therefore required for ACh synthesis. In

microdialysates, basal levels of glucose were $0.31 \pm 0.02 \text{ mM}$ for controls, $0.34 \pm 0.04 \text{ mM}$ for donepezil-treated rats, $0.34 \pm 0.04 \text{ mM}$ for EGb 761-treated rats and $0.30 \pm 0.04 \text{ mM}$ for the EGb 761 plus donepezil-treated rats ($p>0.3$); accordingly, the different drug treatments did not have an effect on basal glucose levels (Fig. 4A; empty bars). Glucose levels were unchanged during behavioural or pharmacological stimulation (data not shown).

Fig. 4A (filled bars) shows lactate concentrations under basal conditions. Lactate levels overall were not different between treatment groups. Fig. 4B illustrates, however, that lactate concentrations were elevated when rats were placed in the “open field”. The increases were found to be significant in all treatment groups (paired t-test), but the extent of lactate mobilization was very similar in all groups. Enhanced lactate release could not be seen during pharmacological stimulation with scopolamine (data not shown).

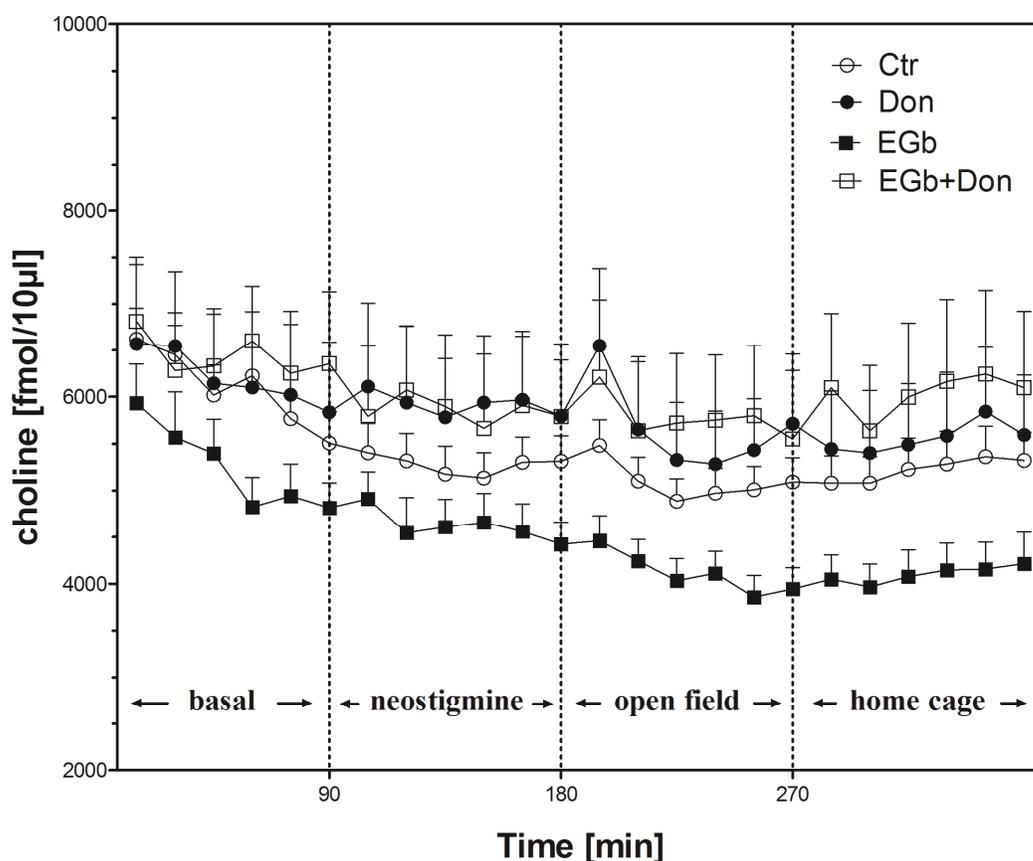


Figure 3. Extracellular concentrations of choline in rat hippocampus on day 1, sampled by microdialysis. Data were obtained using perfusion with aCSF (0-90 min, “basal”), with aCSF containing $0.1 \mu\text{M}$ neostigmine (90-180 min), during behavioural activation (exposure to novel environment, “open field”, from 180-270 min), and after return of rats to the home cage (270 to 360 min). Results are means \pm SEM from the same samples as in Fig. 1A. Statistical analysis (two-way ANOVA for repeated measures, GraphPad Prism[®]): Ctr vs. Don, $F_{1,483} = 0.43$; $p = 0.52$; Ctr vs. EGb, $F_{1,483} = 5.5$; $p = 0.02$; Ctr vs. EGb+Don, $F_{1,506} = 0.82$; $p = 0.37$.

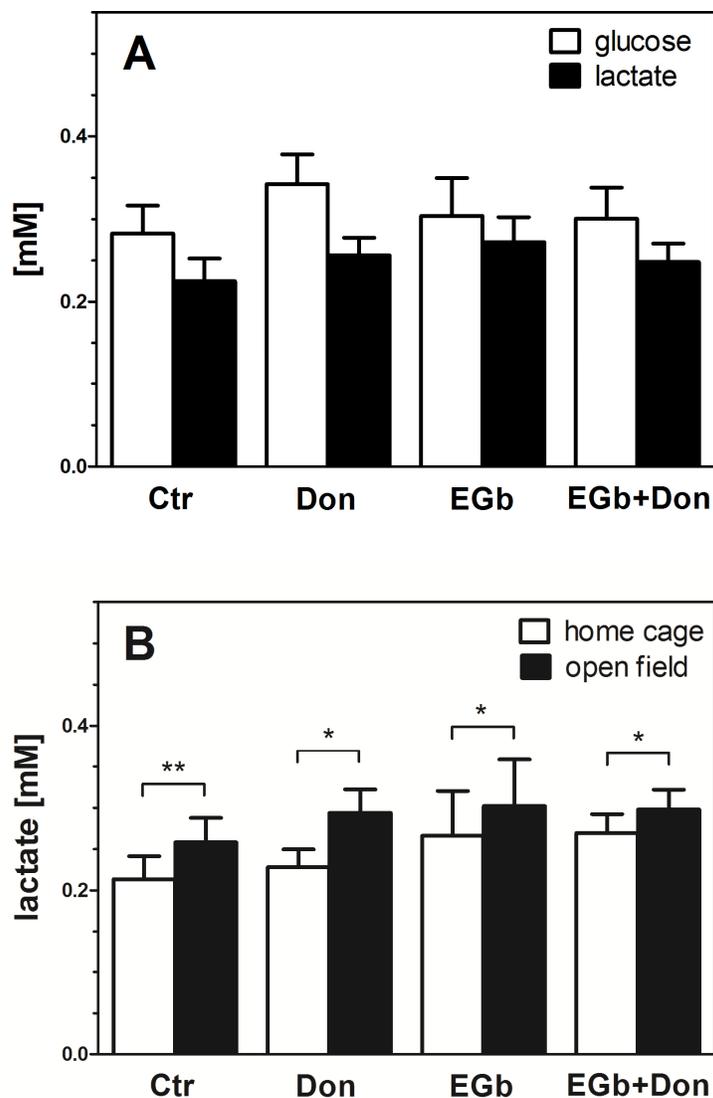


Figure 4. Energy metabolites under basal and stimulated conditions as sampled by microdialysis. (A) Extracellular glucose and lactate concentrations during basal conditions. (B) Effects of behavioural stimulation (“open field”) on lactate levels. Concentrations were calculated as means of the first three samples. (A) Statistical analysis (one-way ANOVA with Bonferroni post-tests, GraphPad Prism®): for glucose, $F_{3,31} = 0.32$; $p = 0.81$; for lactate, $F_{3,30} = 0.56$; $p = 0.64$ (N=8-10 in each group). Student’s t-test: Ctrl lactate vs. EGb lactate, $p = 0.26$ (B) Statistical analysis (paired t-test, GraphPad Prism®): Ctrl, $p < 0.01$; Don, $p = 0.03$; EGb, $p = 0.03$; EGb+Don, $p = 0.04$ (N=8-10 in each group). *, $p < 0.05$; **, $p < 0.01$.

DISCUSSION

The purpose of the present study was to examine the effects of single and combined treatments of donepezil, an AChE inhibitor, and EGb 761, an extract of *Ginkgo biloba*, on the cholinergic system. As outlined in the introduction, both drugs were shown to be effective for senile dementia and are therapeutically used in many countries; however, studies on their potential interaction are

scarce. To increase the translational value of our study, we used moderate doses of the two drugs, applied orally, and we used aged rats as an animal model of the aging human.

The cholinergic system is an important target for senile dementia because its decline correlates with the severity of Alzheimer’s disease (2). Increases of cholinergic transmission, albeit of moderate effectiveness, are the mainstay of current therapy with AChE inhibitors. In the present study,

we applied the microdialysis technique, coupled to a highly sensitive HPLC system, to determine extracellular levels of ACh in the hippocampus. We chose this brain region because the hippocampus has a prominent role in cognitive processes and shows early dysfunction in dementia. Microdialysis is an ideal method to evaluate cholinergic function *in vivo* because sampling of ACh in the extracellular fluid reflects the activity of central cholinergic fibers. This is illustrated by the effect of AChE inhibition with donepezil which resulted in a significant increase of basal ACh concentrations after p.o. administration of the compound (1.5 mg/kg, Fig. 1A). The difference between the treatment groups disappeared when neostigmine was added to the perfusion fluid. Neostigmine was added to increase and stabilize ACh levels *in vivo* for the following experiments, and it has been shown previously that a low concentration of neostigmine (100 nM as in our study) does not impair the cholinergic response to behavioral or pharmacological stimulation (27; Stein, unpublished observations). We cannot exclude, however, that the addition of neostigmine masked a small effect of the treatments on ACh levels.

Exploration of a novel environment is known to enhance hippocampal ACh release in mice (16) and rats (28,29), and we used this paradigm to monitor hippocampal cholinergic activity in aged rats. In our hands, all treatment groups responded with an approximately 2-fold increase of ACh release which subsided after return of the animals to the home cage (Fig. 1B). This response was slightly lower than the 3-fold increases observed before with young rats (28,29), likely due to the old age of our animals and the loss of neuronal cells and dendritic spines taking place especially in the hippocampal region of aged rats (30,31). On day 2, increases in ACh levels were induced by pharmacological stimulation, i.e. infusion of scopolamine, which increases ACh release *in vivo* by blockade of inhibitory presynaptic receptors (19). Again, this effect requires partial blockade of AChE by neostigmine to activate the negative feedback mechanism. Importantly, all groups responded with an increase of ACh release approximately 3-fold indicating the functional preservation of cholinergic function. As with behavioral activation, the treated groups responded similarly to scopolamine.

The effect of donepezil was also seen when cholinergic parameters were measured *ex vivo*. As expected, AChE activity was decreased after donepezil. The moderate effect of the drug is explained by the low dose given and by the fact

that approximately 8 hours passed between the last drug administration and sacrifice. An earlier study found a higher extent of AChE inhibition immediately following donepezil administration (15), but the half-life of donepezil in rats is only 3.6 hours (32), and a less prominent inhibition is, therefore, plausible after 8 hours. ChAT activity was unchanged after donepezil, but HACU was decreased. HACU activity is controlled by the presence of choline transporter CHT-1 in the plasma membrane which is internalized depending on the neuronal firing rate (33). The decrease of HACU activity after donepezil is plausible because an increase of ACh by AChE inhibition is expected to decrease ACh turnover due to the above mentioned, presynaptic muscarinic receptors which limit ACh release (19). A similar result was previously observed with galantamine, another AChE inhibitor (34). In contrast to the effects of donepezil on cholinergic parameters, the levels of choline, glucose and lactate were not affected by the drug.

EGB 761 is an extract containing ginkgolides, bilobalide and flavonoids with biological activity. Its mechanism of action in senile dementia is thought to be based on multiple mechanisms including neuroprotective effects (35); for instance, transgenic mice (36) and aged rats (37,38) chronically treated with EGB 761 displayed improved spatial learning, memory and synaptic plasticity. Recent animal studies also suggested an effect of EGB 761 on neurotransmitter levels. A 14-day treatment with EGB 761 (100 or 300 mg/kg, p.o.) increased dopamine and noradrenaline levels in the rat prefrontal cortex (PFC) (39). The present study which tested potential effects of EGB 761 on ACh levels was stimulated by the observation that ACh levels were slightly increased in frontal cortex following the administration of two flavonoids, quercetin glycoside and kaempferol glycoside (10 mg/kg each) (12). However, in the present study oral treatment with 100 mg/kg/day EGB 761 showed no effects on ACh levels in rat hippocampus; neither basal ACh levels, nor stimulated ACh levels were affected by EGB 761 (Fig. 1). The discrepancy to the earlier study (12) may be explained by measurements in another brain region. Moreover, the present study used a moderate dose (100 mg/kg/d p.o.) of extract which contains only about 4.5% of quercetin and kaempferol, respectively.

EGB 761 treatment also did not change AChE and HACU activity, further illustrating that the extract had no direct effect on ACh levels in our study. However, two findings indicated a possible

neuroprotective effect of the extract. First, rats treated with EGb 761 had significantly lower choline levels than the other groups. It is important to note that the extracellular levels of choline are poorly correlated with ACh release and breakdown, a fact that is also obvious in our study because addition of neostigmine or scopolamine, or exposure to the open field did not affect choline levels (Fig. 3). Instead, extracellular levels of choline are known to be dependent on the metabolism of choline-containing phospholipids. Under a variety of pathological conditions including ischemia and excitotoxicity, choline levels increase due to phospholipase A₂ activation and hydrolysis of phosphatidylcholine (26). Ginkgo extract EGb 761 is known from our previous studies to strongly inhibit membrane phosphatidylcholine breakdown and glutamate release and to stabilize mitochondrial functions (40,41,42). Interestingly, the effect of EGb 761 on choline was inhibited by donepezil (Fig. 3), probably because the increase of (excitatory) cholinergic transmission induced by donepezil counteracted the choline-lowering effect of EGb 761.

Measurement of cholinergic enzymes suggested a moderate increase of ChAT activity by EGb 761 treatment. ChAT, the enzyme which is responsible for the synthesis of ACh, has been shown to decrease with progression of dementia (43), and a stabilization of ChAT activity may contribute to improved cognitive function. Although the effects of EGb 761 on ChAT activity did not reach significance, they are in agreement with a neuroprotective and activity-enhancing effect of the extract which was previously described in other studies (35).

While cholinergic parameters responded to drug treatments in this study, the levels of glucose, the major energy source of the brain, was not affected in rat hippocampus. Similarly, lactate levels did not respond to drug treatment. Interestingly, lactate levels increased upon exposure of rats to the open field in all four treatment groups (Fig. 4B). These increased lactate concentrations, earlier thought to reflect ischemic conditions, are now recognized to reflect neuronal activation (44). Astrocyte-derived lactate in the extracellular space is known to increase during physiological stimulation (19) and to support neuronal function, especially during high neuronal activity (44).

In summary, donepezil and EGb 761 are two drugs with different mechanisms of action that are beneficial in senile dementia. The present study tested co-administration of these two drugs in aged

rats in an attempt to elucidate potential interactions. However, while effects were noted on several parameters, we did not detect major and significant interactions between the two drugs. In other words, while EGb 761 has no direct effects on ACh levels, it did also not interfere with the well-known cholinergic properties of donepezil. Donepezil is a well-established drug for the symptomatic treatment of AD. It improves cognition, behavioral symptoms and quality of life by increasing levels of ACh in the brain. Our study shows that EGb 761 has no direct effects on ACh levels but is beneficial by its neuroprotective action. We conclude that EGb 761, while it does not directly affect the central cholinergic system, evidently does not interfere with the cholinergic effects of donepezil. As a consequence, a combination of both drugs might yield additive effects and should be further explored clinically.

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