

Pharmacological and biochemical effects of *Ginkgo biloba* extract on learning, memory consolidation and motor activity in old rats

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Effect of administration of the standardized extract of *Ginkgo biloba* leaves (EGb 761) on learning, memory and exploratory behavior was estimated in water maze and hole-board tests. Rats (18-month old) received for three months EGb 761 at doses: 50, 100 and 150 mg/kg b.w. per day. After completion of the behavioral experiment, concentrations of neurotransmitters were estimated in selected brain regions. ANOVA demonstrated significant differences in the content of monoamines and metabolites between the treatment groups compared to the control. The increased level of 5-hydroxytryptamine (5-HT) in the hippocampus and 5-HIAA (5-HT metabolite) in the prefrontal cortex correlated positively with the retention of spatial memory. Positive correlation between platform crossings in SE during the probe trial and neurotransmitter turnover suggest improvement of spatial memory. Long-term administration of *Ginkgo biloba* extract can improve spatial memory and motivation with significant changes in the content and metabolism of monoamines in several brain regions.

Key words: *Ginkgo biloba*, EGb 761, water maze, spatial memory, monoamines

INTRODUCTION

The healing ability of *Ginkgo biloba* has been reported for thousands of years. At present it is one of the most extensively researched medicinal plants in the world, used by medical professionals to aid the treatment of problems typically associated with aging, such as poor circulation, mental confusion and memory loss (Gertz and Kiefer 2004). The most important constituents of the standardized extracts of dried leaves of *Ginkgo biloba* are flavone glycosides (quercetin, kaempferol, isorhamnetin) and terpene lactones (ginkgolides and bilobalide) (O'Reilly 1993, Mahadevan and Park 2008, Xie et al. 2008).

Numerous studies have shown that standardized extract *Ginkgo biloba* has significant influences on neurotransmitter receptors of the central nervous system and can prevent age-related memory impairment (Huang SH et al. 2004, DeFeudis and Drieu 2000, Hadjiivanova and Petkov 2002, Ivic et al. 2003). Various beneficial effects of EGb 761 have been described in vigilance disturbances (Gessner et al. 1985) and memory and cognitive problems associated with ageing and senility (Warburton 1993, Kennedy et al. 2000, Nathan 2000, Keltner et al. 2001, Birks et al. 2002, Le Bars et al. 2002, Le Bars 2003, Muller and Chatterjee 2003, van Dongen et al. 2003, Elsabagh et al. 2005a,b).

While the mechanism underlying the neuroprotective benefits of EGb 761 has remained unclear, the attempts at explanation of its neuroprotective activity proposed its antioxidant properties (Wei et al. 2000,

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Received 29 July 2008, accepted 07 March 2009

Guidetti et al. 2001, Huang P et al. 2004, Zhao 2005), modulation of neurotransmission (Taylor 1986, Davies et al. 2003), inhibition of apoptotic processes (Chen et al. 1999, Smith and Luo 2004, Altiok et al. 2006, Lu et al. 2006) and effects on the energy metabolism of neurons (Karcher et al. 1984, Hoyer et al. 1999, Eckert et al. 2005).

Involvement of serotonergic system on the memory enhancing effects of *Ginkgo biloba* extract has not been precisely assessed. The serotonergic system is thought to be important for emotional states, learning and memory processes. 5-HT neurons are broadly represented in the central nervous system (CNS). 5-HT systems in the hippocampus and amygdala play an important role in regulating emotional behavior and have been widely assumed to be related to anxiety.

Reduced 5-HT turnover is consistently associated with impaired long-term memory functioning and cognitive flexibility (Mössner et al. 2000). In an old rat brain the abnormal morphology of the 5-HT neurones and reduced number of regularly shaped 5-HT fibers are observed (Nishimura et al. 1995). Experimental studies have revealed that manipulation of the serotonin system can produce specific changes in cognitive performance (Schmitt et al. 2006).

Sloley and colleagues (2000) indicate that potential neuroprotective ability of *Ginkgo biloba* is determined by inhibition of monoamine oxidase (MAO)-A and -B in the presence of kaempferol. Current studies confirm that usefulness of *Ginkgo biloba* leaf extract in neurodegenerative disorders is also associated with inhibition of beta amyloid production and aggregation, effect on the amyloid precursor protein (APP), lowering free cholesterol levels and acceleration of acetylcholine release (Bate et al. 2004, Christen 2004, Colciaghi et al. 2004, Lee et al. 2004, Yao et al. 2004).

This study focuses on the influence of EGb 761 long-term pre-treatment on cognition, behavioral functions and alterations in brain neurotransmitters in the rat's aging brain.

METHODS

Subjects

The effect of administration of the standardized extract of *Ginkgo biloba* leaves was analyzed in the modified Morris water maze task in 18 months old male Wistar rats (580–600 g) receiving orally for 3

months the EGb 761 at doses of 50, 100 and 150 mg/kg b.w./day. Daily extract intake was calculated during one week pilot study and the volume of water in which dose of extract should be dissolved (rats drank on the average 48–52 ml of water per day) was established.

The rats were housed individually in plastic cages (26 × 42 cm, 18 cm high) with wood cuttings as bedding, under a 12:12 light: dark schedule with food and tap water or solution of EGb 761 continuously available. Rats received plant extract in drinking water for 3 months and during behavioural experiments. Bottles were protected against spill out.

Animals were divided into four groups and treated as follows: (1) drinking water (Control, $n=8$); (2) *Ginkgo biloba* extract at dose 50 mg/kg b.w. (EGb50, $n=8$); (3) *Ginkgo biloba* extract at dose 100 mg/kg b.w. (EGb100, $n=8$) and (4) *Ginkgo biloba* extract at dose 150 mg/kg b.w. rats (EGb150, $n=8$).

All animal testing was carried out according to the European Communities Council Directive of 24 November 1986 (86/609/EEC), after approval of the Ethical Committee for Animal Experiments at Medical University of Warsaw.

EGb 761 treatment

Extract of *Ginkgo biloba* leaves – EGb 761 (INDUSTRIE EGb 761, 5000498 M11359 LA17) was a gift from Beafour Ipsen International, France. It was standardized to bilobalide (2.9%), ginkgolide (3.1%) and flavonoids (24.0%). Solutions of EGb 761 in tap water were prepared fresh daily.

Behavioral tests

Water maze

Water maze paradigm was analyzed for 4 days by training rats using 4 training trials per day. Memory was assessed by a trial given 24 h after the last training. Motor activity and motivation were analyzed in the experiment with visible platform task. The experiment took place during the light portion of the cycle between 08:00 AM and 03:00 PM.

Water escape task, modified from the standard version of the Morris water maze was used (Widytyszkiewicz et al. 1993). The circular pool (1.40 m in diameter and 0.50 m high) was filled with 23°C water (0.30 m) and divided into four quadrants (Northeast – NE,

Northwest – NW, Southeast – SE and Southwest – SW). Rats were trained to locate a transparent hidden plexiglas platform (10 cm × 10 cm, 29 cm high) submerged 1 cm below the water surface. The maze was located in laboratory and was surrounded by several prominent cues to spatial coordinates, including items such as shelves, posters, a video camera, illumination lights and the presence of the researcher and one assistant. All rats were given one session of four trials daily for four consecutive days. For each trial, the rat was placed in the water facing the wall of the pool at one of three equally spaced starting points, excluding the quadrant with the platform. The order in which these starting points were used was determined randomly for each trial and changed each day, but the location of the escape platform remained fixed for all 4 days of the training and was always centered in the SE (Southeast) quadrant. A trial was terminated when the rat reached and entered the platform or when 60 s elapsed. If the animal did not find the platform within this time it was placed on the platform for 15 s before the next trial was initiated. At the end of the session, the rat was wiped in a cloth to dry it, and returned to its cage. The probe trial (memory test) was conducted the day after the last training session (5th day). There was no platform in place during the probe trial when the rats were allowed to swim for 60 s before the end of the session.

The cued task was similar to the acquisition trials except the platform was placed 1 cm above water surface.

Data from the Water maze (latencies to find the platform, distance traveled, number of visits in a target area and the time spent in the goal quadrant were recorded by a VHS image analyzing system (Chromotrack, San Diego Instruments).

Hole-board

Hole-board as a simple method for measuring motor activity and exploration of animals was used. The apparatus consisted of a white box (1 m × 1 m × 0.35 m) with 16 equidistant 3.8 mm-diameter holes in the floor. During 5 days animals were allowed to explore freely the apparatus for 5 min. The behavior of the animals was recorded with a video camera located above the center of the apparatus.

Each rat was placed in the center of the board and total number of head dip (scored if both eyes disappeared into the hole), number of rearings, line crossing

and motor activity were registered. The hole-board was carefully cleaned with a solution of acetic acid and water between the trials.

Biochemistry

After completion of the behavioral experiments, the regional brain concentrations of monoamines and metabolites were estimated in selected brain regions.

Rats were decapitated 24 h after the last behavioral tests, and their brains were rapidly removed and then dissected, according to the method of Glowinski and Iversen (1966), into four regions (prefrontal cortex, hippocampus, hypothalamus and striatum) on an iced plate.

Dissected brain tissue was rapidly weighed, quickly frozen and then stored in a deep freezer (−80°C) for future analysis. To precipitate proteins, tissues were homogenized in ice-cold 0.1 N HClO₄, and centrifuged (13 000 × g for 15 min). The supernatant was filtered (0.2 μm pore size filter; Whatman) and examined for neurotransmitter content. Dopamine (DA; standard substance supplied by RBI), its metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC; RBI); 5-hydroxytryptamine (5-HT; Sigma); 5-hydroxyindolacetic acid (5-HIAA; Sigma); 3,4-dihydroxyphenylethanolamine (NA; Sigma) and homovanilic acid (HVA; Sigma) – were measured using HPLC with electrochemical detection (L-3500A detector; Merck) and a glassy carbon electrode. The electrochemical potential was set at 0.8 V with respect to an Ag/Ag Cl reference electrode. The mobile phase comprised 58 mM sodium phosphate (Sigma), 31 mM citric acid (Sigma), 1 mM octane sulfonic acid (Aldrich), and 27 μM ethylenediaminetetraacetic acid (EDTA; Sigma) in deionised, 18.3 mΩ purified water containing 1% acetonitrile (Merck) and 12% methanol (Merck). Monoamines were separated on a C-18 column (250 mm × 4 mm, reverse phase, Nucleosil, 5 μm particle size; Macherey-Nagel, Germany) and the mobile phase flow rate was maintained at 0.8 ml/min. Sample quantification was achieved by comparison with standard solutions of a known concentration using HPLC software, and area under the peaks was quantified. Data were collected and analyzed by Eurochrom 2000 for Windows (Knauer). Contents of neurotransmitters and metabolites were expressed as ng/g of fresh tissue. Comparison between neurotransmitters and metabolites of the groups was accomplished by one-way analysis of variance, followed by Student's *t*-test.

Statistical analysis

To assess differences during acquisition learning ANOVA with repeated measures (treatment × day × trial) was used. All *post-hoc* tests were performed using Student’s *t*-test to identify any significant differences.

Correlation coefficients between learning performance and the level of monoamines, their metabolites and neurotransmitters turnover in the prefrontal cortex, hippocampus, hypothalamus and striatum were determined using simple linear regression analysis according to Pearson’s *r* correlation. Correlations have been calculated from the results of individual rats. All hypotheses tested used a minimum accepted level of significance of 0.05.

RESULTS

Water maze results

Acquisition trials (days 1–4)

The results of the acquisition in water maze test (escape latency) are presented in Fig. 1A. In the acquisition days 1–4 the performance of all rats improved with subsequent days of testing (reduction of latency), however no significant differences were noted between experimental groups (Con: 28.46 ± 3.71 s; EGb50: 27.21 ± 3.75 s; EGb100: 27.55 ± 3.46 s; EGb150: 25.33 ± 3.28 s) ($F_{3,28}=0.91, P>0.5$). The result did not show a significant main effect for swimming speed (Con: 0.37 ± 0.010 m/s; EGb50: 0.37 ± 0.009 m/s; EGb100: 0.35 ± 0.010 m/s; EGb150: 0.38 ± 0.010 m/s) ($F_{3,28}=1.53, P>0.02$).

The probe trial (day 5)

As shown in Fig. 1B, in the probe trial on day 5 (memory task), the group treated with the extract of *Ginkgo biloba* at a dose of 150 mg/kg b.w./day showed an increase in crossings for the position of the platform in SE quadrant (9.88 ± 2.27) compared to the control group (4.25 ± 1.53) and animals pre-treated with 50 mg EGb 761 (4.50 ± 0.87). Rats treated with a high dose of EGb 761 swam preferentially in the target SE quadrant, where the platform was previously placed.

The *post-hoc* tests show that EGb100 group spent statistically more time in a target quadrant SE (30.29 ±

3.48 s) compared to the other experimental groups (Con: 21.31 ± 1.25 s; EGb50: 22.37 ± 2.6 s; EGb150: 23.72 ± 1.81 s) ($P<0.05$).

Cued task

Analysis of variance demonstrated significant differences between groups pre-treated with high doses of *Ginkgo biloba* extract compared to the lower dose

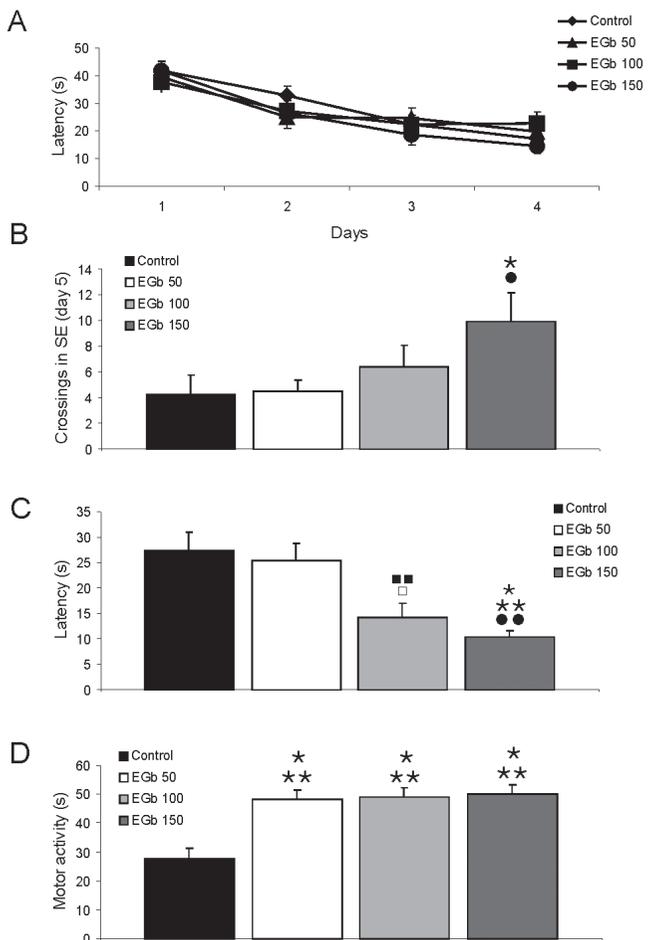


Fig. 1. Effect of chronic pretreatment with standardized extract of *Ginkgo biloba* leaves (EGb 761) on learning and memory of rats in the water maze (A, B, C) and motor activity in the hole-board (D). (A) Mean escape latency (± SEM) during acquisition of the spatial navigation task; (B) spatial probe data from the platform area crossings in the water maze task on day 5 (Trial 17); (C) mean escape latency (± SEM) during cued task. *EGb150 vs. control, $P<0.05$; ***EGb50, EGb100, EGb150 vs. control, $P<0.0005$; • EGb150 vs. EGb50, $P<0.05$; •• EGb150 vs. EGb50, $P<0.005$; □ EGb50 vs. EGb100, $P<0.05$; ■ EGb100 vs. control, $P<0.005$.

(50 mg/kg b.w.) and the control in escape latency in the cued task on day 6 ($F_{3,28}=8.4$, $P<0.00005$). Newman-Keuls analysis of latencies in cued task showed the mean escape latency for the EGb100 (14.20 ± 2.78 s) and EGb150 group (10.35 ± 1.21 s) to be significantly smaller than both the EGb50 (25.43 ± 3.31 s) and the vehicle control group (27.32 ± 3.59 s) (Fig. 1C).

The mean swimming speed in the water maze with visible platform for control was significantly greater than EGb 761 pre-treated groups (Con: 0.36 ± 0.02 m/s; EGb150: 0.45 ± 0.03 m/s) ($P<0.05$).

Hole-board results

The effects of EGb 761 on exploratory behavior of rats in the hole-board test are shown in Table I.

In the hole-board test, motor activity was significantly increased in groups that received EGb 761 ($F_{3,28}=10.75$, $P<0.001$) (Fig. 1D) (Table I). In contrast, the mean number of climbs and crossings was not significantly affected by EGb 761 (Table I).

ANOVA demonstrated significant treatment effects on head-dip counts. EGb 761 at dose of 50 mg/kg b.w. significantly increased head-dipping behaviors compared to the control group (Table I).

Regional brain monoamines levels

The levels of monoamines and their metabolites in the prefrontal cortex, hippocampus hypothalamus and striatum are summarized in Tables II and III.

ANOVA demonstrated significant differences in the content of monoamines and metabolites in prefrontal cortex, hippocampus, hypothalamus and striatum of

rats between the EGb 761 treatment groups compared to control.

Noradrenaline (NA)

ANOVA demonstrated statistically significant differences between the content of NA in the hypothalamus ($F_{3,28}=3.94$, $P<0.02$). *Post-hoc* comparisons showed a significant increase in the level of NA in the control group compared to the group pre-treated with *Ginkgo biloba* extract at the doses of 100 and 150 mg/kg b.w. ($P<0.05$).

Post-hoc comparisons showed a significant increase in the level of NA in prefrontal cortex in the EGb150 group compared to the control ($P<0.05$). Rats that received EGb 761 at a dose of 100 mg showed higher hippocampal levels of NA compared to the control group ($P<0.02$).

HPLC analysis showed higher content of NA in the striatum of rats from group EGb100 and EGb150 compared to the control ($P<0.05$).

Dopamine (DA)

EGb 761 mg/kg b.w. treatment significantly reduced DA levels in the prefrontal cortex ($F_{3,28}=8.5$, $P<0.0004$) and hypothalamus ($P<0.05$).

Elevated levels of DA after high doses of EGb 761 were observed in hippocampus and striatum ($P<0.05$).

3,4-dihydroxyphenylacetic acid (DOPAC)

EGb 761-treated groups had significantly higher DOPAC levels when compared to the control group in the prefrontal cortex ($F_{3,28}=19.926$, $P<0.0001$), in the hippocampus ($F_{3,28}=3.08$, $P<0.05$) and in the striatum ($P<0.05$).

Table I

Effects of EGb 761 on exploratory behavior of rats in the hole-board test				
Group	Motor activity (s)	Head dips	Climbs	Crossings
Control	27.82 ± 3.26	1.75 ± 0.37	2.00 ± 0.44	7.66 ± 1.02
EGb50	$48.44 \pm 2.97^{***}$	$2.88 \pm 0.42^*$	2.50 ± 0.50	7.38 ± 1.23
EGb100	$48.96 \pm 3.20^{***}$	2.63 ± 0.43	2.75 ± 0.50	7.72 ± 0.85
EGb150	$50.18 \pm 2.96^{***}$	2.47 ± 0.36	2.47 ± 0.47	7.00 ± 0.94

*EGb vs. control, $P<0.05$; ***EGb vs. control, $P<0.0005$

ANOVA showed that the content of DOPAC was significantly lower in the EGb 761 treated rats vs. the control group in the hypothalamus ($F_{3,28}=12.92$, $P<0.0002$).

Homovanilic acid (HVA)

Statistical differences in HVA content observed in the hippocampus ($F_{3,28}=5.56$, $P<0.005$, ANOVA) and the striatum ($P<0.05$, *post-hoc*).

DOPAC/DA ratio

ANOVA demonstrated statistically significant differences between the groups in the DOPAC/DA ratio in the prefrontal cortex ($F_{3,28}=9.85$, $P<0.0002$). Further analysis by means of Student's *t*-test showed that the DOPAC/DA ratio in the cortex was significantly lower

in the control group (Table IV). In the hippocampus, the DOPAC/DA ratio was decreased in the EGb100 group compared to the EGb50 group ($P<0.05$).

HVA/DA ratio

One way ANOVA showed statistically significant differences between the groups in the DOPAC/DA ratio in the hippocampus ($F_{3,28}=3.72$, $P<0.03$). DOPAC/DA ratio was elevated in the control rats compared to the EGb50 and EGb100 group.

5-hydroxytryptamine (5-HT)

Statistically significant differences in 5-HT content between the groups were noted in the hippocampus ($F_{3,28}=5.65$, $P<0.004$, ANOVA), striatum ($F_{3,28}=4.00$,

Table II

Effects of EGb 761 administered daily for over 3 months on brain tissue noradrenaline (NA), dopamine (DA) and serotonin (5-HT) levels in the prefrontal cortex, hippocampus, hypothalamus and striatum

Brain region	Group	Monoamine levels in ng/g wet tissue		
		NA	DA	5-HT
Prefrontal cortex	Control	161.23 ± 16.06	84.16 ± 6.87	582.53 ± 58.76
	EGb50	170.96 ± 9.54	55.45 ± 7.32 ΔΔ	450.44 ± 40.10 Δ
	EGb100	173.10 ± 13.84	46.03 ± 4.13 ■■■	446.54 ± 30.39 ■
	EGb150	209.13 ± 18.85*	53.15 ± 3.84 **	440.94 ± 19.77 *
Hippocampus	Control	128.64 ± 9.80	34.72 ± 2.14	316.32 ± 15.14
	EGb50	144.52 ± 6.76	56.65 ± 19.78	284.72 ± 21.07
	EGb100	125.38 ± 16.76 ■	166.99 ± 62.52 ■□	444.57 ± 57.80 *●●
	EGb150	147.69 ± 16.46	120.28 ± 26.47	445.91 ± 32.27 *
Hypothalamus	Control	76.95 ± 8.55	309.14 ± 41.08	578.27 ± 23.68
	EGb50	60.28 ± 7.16	231.49 ± 15.85	632.51 ± 16.37
	EGb100	51.36 ± 3.2 ■	229.30 ± 15.08	633.95 ± 32.05
	EGb150	51.46 ± 3.52 *	212.59 ± 45.78 *	687.76 ± 55.68 *
Striatum	Control	69.28 ± 6.80	4866.83 ± 479.90	379.03 ± 18.72
	EGb50	100.91 ± 15.41	5372.81 ± 381.06	356.39 ± 19.96
	EGb100	105.83 ± 8.40	5388.66 ± 268.15	437.45 ± 16.95 *●
	EGb150	116.15 ± 16.29 *	6001.2 ± 357.19 *	358.04 ± 19.98 ▲

Data are presented as mean ± SEM levels (ng/g wet tissue). * $P<0.05$, ** $P<0.005$ EGb150 vs. control; ● $P<0.05$, ●● $P<0.005$ EGb50 vs. EGb150; ■ $P<0.05$, ■■■ $P<0.0005$ EGb100 vs. control; □ $P<0.05$ EGb50 vs. EGb100; ▲ $P<0.05$ EGb100 vs. EGb150; Δ $P<0.05$, ΔΔ $P<0.005$ EGb50 vs. control.

$P < 0.02$), prefrontal cortex and hypothalamus ($P < 0.05$, *post-hoc*).

Further analysis showed a significant increase in 5-HT content in the striatum between the EGb100 group compared to the other groups ($P < 0.05$), and a lower level of 5-HT in the hippocampus in EGb50 group and control group ($P < 0.001$) compared to the EGb100 and EGb150 group (Table IV).

5-hydroxyindolacetic acid (5-HIAA)

Overall ANOVA showed significant differences between groups in 5-HIAA content in the prefrontal cortex ($F_{3,28} = 9.68$, $P < 0.0002$), hippocampus ($F_{3,28} = 5.65$, $P < 0.004$) and striatum ($F_{3,28} = 4.30$, $P < 0.02$). Low con-

centration of 5-HIAA was observed in the control group.

Post-hoc analysis revealed a significant decrease in the level of 5-HIAA for the control group vs. the EGb150 group in the hypothalamus ($P < 0.05$).

5-HIAA/5-HT ratio

ANOVA demonstrated statistically significant differences in the 5-HIAA/5-HT ratio in the prefrontal cortex between groups ($F_{3,28} = 7.40$, $P < 0.0009$) and in the striatum ($F_{3,28} = 4.24$, $P < 0.02$).

Analysis of differences between groups showed that the 5-HIAA/5-HT ratio in the cortex is significantly higher in the EGb150 group.

Table III

Effects of EGb 761 administered daily for over 3 months on brain tissue metabolite levels: 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxy-indole acetic acid (5-HIAA) in the prefrontal cortex, hippocampus, hypothalamus and striatum

Brain region	Group	Metabolite levels in ng/g wet tissue		
		DOPAC	HVA	5-HIAA
Prefrontal cortex	Control	68.27 ± 6.53	–	148.27 ± 11.50
	EGb50	75.41 ± 5.20	–	162.36 ± 18.04
	EGb100	72.15 ± 3.19	–	198.35 ± 10.90 ■□
	EGb150	151.46 ± 15.46 ▲▲▲***●●●	–	239.32 ± 10.37 ***●●▲
Hypothalamus	Control	177.05 ± 19.40	–	314.16 ± 20.71
	EGb50	109.21 ± 11.47 ΔΔ	–	379.12 ± 21.92
	EGb100	93.39 ± 9.34 ■■■	–	399.20 ± 20.67
	EGb150	72.17 ± 6.39 ***	–	428.45 ± 59.92 *
Hippocampus	Control	45.48 ± 3.77	55.32 ± 7.11	301.9 ± 24.01
	EGb50	58.47 ± 4.57	43.29 ± 4.48	316.40 ± 28.16
	EGb100	96.20 ± 14.57 ■	76.39 ± 8.57	445.07 ± 68.72 ■
	EGb150	105.75 ± 29.10 *	105.52 ± 19.77 *●●	430.38 ± 32.24 *
Striatum	Control	489.63 ± 50.49	260.41 ± 36.78	274.00 ± 15.27
	EGb50	555.01 ± 30.15	366.79 ± 28.80	280.07 ± 12.94
	EGb100	534.77 ± 15.99	355.29 ± 20.56	315.62 ± 16.58
	EGb150	603.82 ± 45.49 *	429.18 ± 69.54 *	344.82 ± 18.43 *●

Data are presented as mean ± SEM levels (ng/g wet tissue). * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$ EGb150 vs. control; ● $P < 0.05$, ●● $P < 0.005$, ●●● $P < 0.0005$ EGb50 vs. EGb150; ■ $P < 0.05$, ■■■ $P < 0.0005$ EGb100 vs. control; □ $P < 0.05$ EGb50 vs. EGb100; ▲ $P < 0.05$, ▲▲▲ $P < 0.0005$, EGb100 vs. EGb150, ΔΔ $P < 0.005$ EGb50 vs. control.

In the striatum the 5-HIAA/5-HT ratio was significantly higher in the EGb100 group vs. the control group (Table IV).

Monoamine levels and spatial memory correlation

The number of crossings over the previous position of the platform during the probe trial was correlated with the levels of monoamines in the prefrontal cortex and hippocampus (Table V).

The accuracy of spatial memory was not reliably correlated with any monoamine, metabolites level or neurotransmitters turnover in the hypothalamus and the striatum ($P>0.05$).

It has been shown that 5-HT concentration in the hippocampus of the EGb150 group positively corre-

lated with results of the probe trial ($r=0.7$; $F_{1,6}=5.76$, $P=0.05$).

Prefrontal cortex 5-HIAA levels correlated positively with the mean annulus crossing of the EGb100 ($r=0.7$; $F_{1,6}=5.85$, $P=0.05$) and EGb150 ($r=0.72$; $F_{1,6}=6.59$, $P=0.04$) group during the probe trial. There was no correlation in the prefrontal cortex between the two measures for the other groups or for the other monoamines and metabolites tested ($P>0.05$).

Monoamine turnover and spatial memory correlation

Further analysis showed positive correlation between 5-HT ratio in the prefrontal cortex and hippocampus with mean annulus crossing during the probe trial (Table VI).

Table IV

Effects of EGb 761 administered daily for over 3 months on brain tissue neurotransmitter turnover DOPAC/DA, HVA/DA, 5-HIAA/5-HT in the prefrontal cortex, hippocampus, hypothalamus and striatum

Brain region	Group	Neurotransmitter turnover		
		DOPAC/DA	HVA/DA	5-HIAA/5-HT
Prefrontal cortex	Control	0.88 ± 0.14	-	0.27 ± 0.03
	EGb50	1.32 ± 0.26 ΔΔ	-	0.29 ± 0.05Δ
	EGb100	1.44 ± 0.24 ■■	-	0.38 ± 0.07 ■■
	EGb150	3.01 ± 0.45 ***	-	0.55 ± 0.02 **
Hippocampus	Control	1.34 ± 0.13	1.66 ± 0.28	0.95 ± 0.05
	EGb50	1.52 ± 0.28	0.91 ± 0.24 Δ	0.96 ± 0.17
	EGb100	0.82 ± 0.24□	0.57 ± 0.13 ■	1.01 ± 0.07
	EGb150	1.06 ± 0.21	1.19 ± 0.28	1.00 ± 0.10
Hypothalamus	Control	0.59 ± 0.03	-	0.55 ± 0.05
	EGb50	0.40 ± 0.07	-	0.52 ± 0.08
	EGb100	0.38 ± 0.08	-	0.64 ± 0.04
	EGb150	0.56 ± 0.18	-	0.62 ± 0.10
Striatum	Control	0.10 ± 0.007	0.06 ± 0.011	0.73 ± 0.05
	EGb50	0.09 ± 0.014	0.06 ± 0.007	0.80 ± 0.04
	EGb100	0.09 ± 0.014	0.06 ± 0.011	0.64 ± 0.10 ▲
	EGb150	0.10 ± 0.004	0.07 ± 0.011	0.99 ± 0.08 *

Data are presented as mean ± SEM levels (metabolite/neurotransmitter). * $P<0.05$, ** $P<0.005$, *** $P<0.0005$ EGb150 vs. Control; ■ $P<0.05$, ■■ $P<0.005$ EGb100 vs. Control; ▲ $P<0.05$ EGb100 vs. EGb150, Δ $P<0.05$, ΔΔ $P<0.005$ EGb50 vs. control.

DISCUSSION

The present study has shown that EGb 761 improves memory parameters in old rats. This is consistent with earlier preclinical data reporting beneficial effects of *Ginkgo biloba* on memory formation, consolidation and storage (Stoll et al. 1996). Results in humans have been mixed. Part of clinical trials with AD patients has reported significant benefits of *Ginkgo biloba* extract treatment for the cognitive function (Hoerr et al. 2008, Ihl et al. 2008). Results from two epidemiological studies in France showed a positive influence of *Ginkgo*

biloba extract on the onset of Alzheimer's and length of survival. A positive effect on the risk of developing dementia was observed only in those participants who took *Ginkgo biloba* extract regularly (NIH sponsored study). However last study (The Ginkgo Evaluation of Memory – GEM trial), found no significant supporting evidence to say that *Ginkgo biloba* helped to prevent dementia and cognitive decline. *In vivo* and *in vitro* studies have demonstrated that EGb 761 exerts a protective role in neurodegeneration (Bate et al. 2008).

This is of particular interest given that the functional deficits in the central nervous system during

Table V

Correlation between the platform crossings in SE during the probe trial and neurotransmitter levels in the brain of rats of the control group and rats pretreated with EGb 761 (50, 100 and 150 mg/kg b.w.).					
Brain region		Control	EGb50	EGb100	EGb150
Prefrontal cortex	5-HT	$r=0.07; P=0.88$	$r=0.001; P=0.997$	$r=0.24; P=0.58$	$r=0.08; P=0.89$
	5-HIAA	$r=0.65; P=0.08$	$r=0.67; P=0.07$	$r=0.70; P=0.05$	$r=0.72; P=0.04$
	DA	$r=0.21; P=0.61$	$r=0.38; P=0.35$	$r=0.55; P=0.16$	$r=0.15; P=0.72$
	DOPAC	$r=0.67; P=0.70$	$r=0.40; P=0.33$	$r=0.28; P=0.505$	$r=0.60; P=0.11$
	HVA	–	–	–	–
	NA	$r=0.37; P=0.36$	$r=0.25; P=0.55$	$r=0.06; P=0.89$	$r=0.57; P=0.14$
Hippocampus	5-HT	$r=0.65; P=0.08$	$r=0.65; P=0.08$	$r=0.62; P=0.10$	$r=0.70; P=0.05$
	5-HIAA	$r=0.32; P=0.44$	$r=0.24; P=0.57$	$r=0.18; P=0.67$	$r=0.24; P=0.56$
	DA	$r=0.48; P=0.23$	$r=0.43; P=0.29$	$r=0.33; P=0.42$	$r=0.15; P=0.73$
	DOPAC	$r=0.39; P=0.34$	$r=0.55; P=0.16$	$r=0.48; P=0.23$	$r=0.01; P=0.99$
	HVA	$r=0.22; P=0.60$	$r=0.15; P=0.72$	$r=0.37; P=0.37$	$r=0.42; P=0.30$
	NA	$r=0.10; P=0.81$	$r=0.13; P=0.77$	$r=0.098; P=0.82$	$r=0.14; P=0.73$
Hypothalamus	5-HT	$r=0.44; P=0.27$	$r=0.47; P=0.24$	$r=0.40; P=0.33$	$r=0.42; P=0.30$
	5-HIAA	$r=0.51; P=0.20$	$r=0.21; P=0.62$	$r=0.83; P=0.11$	$r=0.35; P=0.40$
	DA	$r=0.42; P=0.36$	$r=0.25; P=0.56$	$r=0.02; P=0.96$	$r=0.51; P=0.19$
	DOPAC	$r=0.61; P=0.11$	$r=0.14; P=0.74$	$r=0.35; P=0.40$	$r=0.44; P=0.27$
	HVA	–	–	–	–
	NA	$r=0.28; P=0.51$	$r=0.67; P=0.07$	$r=0.34; P=0.41$	$r=0.32; P=0.43$
Striatum	5-HT	$r=0.03; P=0.95$	$r=0.11; P=0.79$	$r=0.28; P=0.50$	$r=0.18; P=0.68$
	5-HIAA	$r=0.45; P=0.26$	$r=0.53; P=0.18$	$r=0.12; P=0.78$	$r=0.39; P=0.34$
	DA	$r=0.08; P=0.86$	$r=0.19; P=0.65$	$r=0.34; P=0.42$	$r=0.41; P=0.31$
	DOPAC	$r=0.37; P=0.36$	$r=0.23; P=0.59$	$r=0.43; P=0.28$	$r=0.61; P=0.11$
	HVA	$r=0.80; P=0.17$	$r=0.32; P=0.45$	$r=0.50; P=0.21$	$r=0.47; P=0.24$
	NA	$r=0.04; P=0.92$	$r=0.18; P=0.66$	$r=0.08; P=0.86$	$r=0.41; P=0.31$

(*r*) Pearson's correlation coefficient. Significant correlations are presented in bold lettering.

Table VI

Correlation between the platform crossings in SE during the probe trial and neurotransmitter turnover in the brain of rats of the control group and rats pretreated with EGb 761 (50, 100 and 150 mg/kg b.w.)

Brain region		Control	EGb50	EGb100	EGb150
Prefrontal cortex	5-HIAA/5-HT	$r=0.71$; $P=0.05$	$r=0.73$; $P=0.04$	$r=0.29$; $P=0.49$	$r=0.74$; $P=0.03$
	DOPAC/DA	$r=0.09$; $P=0.83$	$r=0.03$; $P=0.94$	$r=0.43$; $P=0.29$	$r=0.61$; $P=0.11$
	HVA/DA	–	–	–	–
Hippocampus	5-HIAA/5-HT	$r=0.34$; $P=0.41$	$r=0.79$; $P=0.02$	$r=0.31$; $P=0.46$	$r=0.49$; $P=0.22$
	DOPAC/DA	$r=0.07$; $P=0.87$	$r=0.22$; $P=0.59$	$r=0.69$; $P=0.056$	$r=0.05$; $P=0.91$
	HVA/DA	$r=0.41$; $P=0.31$	$r=0.52$; $P=0.18$	$r=0.47$; $P=0.91$	$r=0.25$; $P=0.55$
Hypothalamus	5-HIAA/5-HT	$r=0.63$; $P=0.65$	$r=0.17$; $P=0.69$	$r=0.32$; $P=0.45$	$r=0.63$; $P=0.10$
	DOPAC/DA	$r=0.37$; $P=0.37$	$r=0.55$; $P=0.16$	$r=0.09$; $P=0.84$	$r=0.63$; $P=0.09$
	HVA/DA	–	–	–	–
Striatum	5-HIAA/5-HT	$r=0.22$; $P=0.59$	$r=0.55$; $P=0.16$	$r=0.23$; $P=0.63$	$r=0.47$; $P=0.25$
	DOPAC/DA	$r=0.31$; $P=0.46$	$r=0.61$; $P=0.11$	$r=0.44$; $P=0.32$	$r=0.61$; $P=0.11$
	HVA/DA	$r=0.47$; $P=0.24$	$r=0.26$; $P=0.53$	$r=0.23$; $P=0.63$	$r=0.32$; $P=0.44$

(*r*) Pearson's correlation coefficient. Significant correlations are presented in bold lettering.

aging may be a result of decrease in the number of neurons, a decrease in the rate of metabolism of neurotransmitters or a change in receptor density.

The results obtained in our experiments indicate that long-term EGb 761 treatment can block age-dependent decline in spatial cognition. Long-term administration of *Ginkgo biloba* extract improved consolidation of spatial learning, motor performance and reversed the learning deficits exhibited in aged rats. The relevance of neurochemical effects of *Ginkgo biloba* to its influence on learning and memory is not clear yet. Precise mechanisms underlying neuroprotective effects of EGb 761 have yet to be established, but they have been reported by multiple antioxidant and rheological properties, improvement of neurotransmission and tissue metabolism (Abdel-Kader et al. 2007, Kampkötter et al. 2007). Loss of receptor function is an important factor in aging mechanisms.

In the present study we have found that EGb 761 significantly modified 5-HT concentration and 5-HT turnover ratio in the prefrontal cortex and hippocampus, structures critically involved in spatial memory and behavioral flexibility. Thus, it seems likely that the

effect of EGb 761 on spatial navigation in rats may be a consequence of an enhanced 5-HT neurotransmission in brain structures. Our finding supports the proposal by Winter and Timineri (1999) that selective 5-HT_{1A} antagonism by *Ginkgo biloba* is due to an enhancement of serotonergic neurotransmission and provides basis for the direct effect on memory and learning. *In vitro* and *ex vivo* *Ginkgo biloba* increases uptake of 5-hydroxytryptamine in a synaptosomal fraction of mice cerebral cortex (Ramassamy et al. 1992).

The serotonergic system is thought to be important for emotional states, learning and memory processes. Many of the drugs used to treat neurological disorders have potent 5-HT receptor actions and are involved in a variety of functions. 5-HT neurons are broadly represented in the central nervous system (CNS). 5-HT systems in the hippocampus and amygdala play an important role in regulating emotional behavior and have been widely assumed to be related to anxiety. Molodtsova (2006) suggests that serotonin transmission is related to emotional mechanisms of memory.

There is an evidence for abnormalities in 5-HT neurotransmission in depressive and cognitive disorders.

A loss of serotonergic transmission is also one of the neurological features of dementia (Meneses 1999, Johnson et al. 2008). In Alzheimer's disease (AD) patients, beside typical neurological changes, degeneration of serotonergic neurons and loss of 5-HT and 5-HT transporter is present (Mössner et al. 2000). Stimulation and normalizing the central 5-HT transmission observed in old rats after EGb 761 treatment have specific beneficial effects on spatial memory, cognition and motivation. Earlier investigations conducted on aged rats reported that chronic treatment with *Ginkgo biloba* increased by 33% binding density to 5-HT_{1A} receptors in cerebral cortex membranes (Huguet et al. 1994) and prevented stress-induced desensitization (Bolaños-Jiménez et al. 1995). Significantly elevated serotonin levels in hippocampus after the administration of *Ginkgo biloba* may be a consequence of MAO inhibition (Pardon et al. 2000, Rojas et al. 2004).

In the same mechanism the augmented levels of noradrenaline (NA) and dopamine (DA) are believed to be involved (Wu and Zhu 1999). In our experiment, administration of EGb 761 enhanced levels of the stress-related neurotransmitter NA in the hippocampus, prefrontal cortex and striatum. Previous studies supported this observation and point to a functional association between NA pathways in EGb 761 neuroprotection (Huguet and Tarrade 1992, Brunello et al. 1985, DeFeudis and Drieu 2000). Interestingly, other reports give evidence that EGb 761 improved memory processes and normalized cognitive deficits in rats chronically stressed or treated with corticosterone (Walesiuk et al. 2005, 2006).

Our study also showed a significant increase of DA neurotransmission in the hippocampus and striatum in *Ginkgo biloba* extract-treated rats. Dopaminergic system is involved in the control of locomotion, neuroendocrine secretion and cognition, with critical influence on the working memory in the prefrontal cortex (Gruber et al. 2006). Last evidence suggests that DA plays a very important role not only in motor function but also in cognitive sequence of learning (Shohamy et al. 2005). Evidence from lesion studies, depletion studies and administration of DA receptor agonists and antagonists supports the hypothesis that mesocorticolimbic DA is a neurochemical link between motivational and memory processes (Phillips et al. 2008). Dopamine neuronal loss in the substantia nigra and decrease in the striatal DA levels corresponded

with behavioral deficits of Parkinson's disease. Research in experimental animals suggests that stimulation of DA₁ receptors in the prefrontal cortex can ameliorate cognitive deficits. Ramassamy et al. (1990) have shown prevention of the dopaminergic neurotoxicity induced by MPTP administration with *Ginkgo biloba* ingestion in mice. These data are supported by Wu and Zhu (1999) who confirm that *Ginkgo* effectively protects against MPP⁺ nigrostriatal DA neurotoxicity.

A variety of neurochemical and pharmacological effects have been presented recently for *Ginkgo biloba*. Shif and coworkers (2006) examined effects of low doses of *Ginkgo biloba* extract (10, 20, 40 mg/kg b.w.) on long term reference memory retention in the radial arm maze and water maze. Regardless of the lack of effects of this extract on working memory observed in this study, the researchers suggest that *Ginkgo* may promote learning of spatial information.

Ginkgo biloba extract (50, 100, 200 mg/kg b.w. for 2 months) ameliorated learning and memory deficit induced by aluminum chloride tested in Morris water maze. This effect may be due to inhibition of acetylcholinesterase (AChE) expression in the hippocampus after *Ginkgo biloba* treatment (Gong et al. 2006).

Water maze task and electrophysiological methods were used to study the effects of administration of a standardized extract from *Ginkgo biloba* leaves (60 mg/kg for 30 days) on hippocampal-dependent spatial learning, memory and synaptic plasticity of aged rats. EGb 761-supplemented diet significantly improved spatial learning and memory of aged rats and enhanced magnitude of LTP recorded *in vivo* from the hippocampus CA1 area (Wang et al. 2006). Ability of *Ginkgo biloba* (70 mg/kg for 6 months) to antagonize the age-dependent behavioural impairment exhibited by Tg2576 transgenic mice (model of AD) were confirmed in water maze (Stackman et al. 2003).

Orally administered *Ginkgo biloba* extract can protect the brain against neurotoxicity of intraventricularly infused beta-amyloid and partially reverse the memory deficit through its influence on the cholinergic system (Tang et al. 2002). Improvement of spatial memory deficits induced by scopolamine and diphenhydramine suggests that the mechanism of the *Ginkgo biloba* action is mediated not only by cholinergic transmission but also by the histaminergic system (Yamamoto et al. 2005).

In our experiment, swimming speed was not altered by *Ginkgo biloba* treatment during acquisition trials and probe trial although was increased in the cued task. This finding suggests that EGb 761 administration can improve sensomotor activity and motivation in old rats during water maze performance. Because the swim speed displayed by the rats in acquisition trials and memory test was similar, we supposed that the increase in crossings for the position of the platform could be related to the memory and motivation rather than to the displayed motor activity. On the other hand, procognitive activity may also indicate the recently reported antioxidant effect of *Ginkgo biloba* extract on free radical production. Free radical damage is implicated in several neurodegenerative disorders, including Alzheimer's and Parkinson's disease.

CONCLUSION

In conclusion, old rats treated with high doses of EGb 761 showed considerable memory, cognitive performance and exploratory behavior improvement in a water escape task. Our observations indicate that part of the procognitive properties of *Ginkgo biloba* may be based on 5-HT-dependent learning and memory.

ACKNOWLEDGEMENT

The authors carried out the experiment using EGb 761 supplied by Beafour Ipsen International, France.

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