Pharmacological Reports
2005, 57, 176–187
ISSN 1734-1140

Ginkgo biloba extract diminishes stress-induced memory deficits in rats

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Abstract:
Exposure to chronic restraint stress in rats and psychosocial stress in humans has been shown to alter cognitive functions such as learning and memory and has been linked to the pathophysiology of mood and anxiety disorders. Antianxiety or sedative agents used in the management of stress have several disadvantages and undesired effects. Therefore, in this study, we investigated efficacy of a natural medicine, the extract of *Ginkgo biloba* (EGB 761), in prevention and treatment of the post-stress memory dysfunctions. The results showed that chronic restraint stress (2 h for 21 days) or an 'equivalent' dose of exogenous corticosterone (5 mg/kg) impaired nonspatial memory as measured by an object recognition test. In control rats, EGB 761 improved spatial and nonspatial memory in Morris water maze and object recognition tests. Preventive doses of EGB 761 (100 mg/kg) normalized cognitive deficits, seen in rats chronically stressed or treated with corticosterone in object recognition test, and improved memory processes in these rats measured by Morris water maze test. There was no influence of our treatments on locomotor exploratory activity and anxiety measured in open field and elevated 'plus' maze tests, making a contribution of unspecific motor and emotional effects of the used drugs to their performance in the memory tests improbable.

Key words:
Ginkgo biloba, memory, object recognition, Morris water maze, restraint stress

Introduction

Stress, depression, and associated mental problems have become increasingly important [12]. Lack of satisfactory treatments of the cognitive deficits usually accompanying these states presents a constant challenge for psychopharmacological research.

It is well known that glucocorticoid (GK) hormone levels rise in response to stress [7, 9, 31, 33]. Evidence that the exposure to stress (and GKs) causes subsequent impairment of hippocampus-dependent forms of memory in both humans and animals has been repeatedly shown [34].

The hippocampus, a part of the medial temporal lobe necessary for the formation of stable declarative memory in humans [13, 36] and spatial memory in rodents [7, 16] has one of the highest densities of the GK receptors in mammalian brain and participates in the GK-mediated negative feedback of the hypothalamic-pituitary-adrenal (HPA) axis [7, 27, 33, 34]. In the rat hippocampus, corticosterone has been shown to regulate neuronal metabolism, physiological functions, genomic expression, and to alter the cell morphology [5, 32, 34]. Consequently, certain hippocampal functions (such as learning and memory) appear to be susceptible to uncontrollable stress [5, 16].

Stress- or corticosterone-dependent behavioral changes are paralleled by both neurochemical and neuroanatomic alterations.

Chronic exposure to GKs/stress changes hipocampal morphology, with loss of pyramidal neurons, atro-

phy, remodeling of apical dendrites, e.g. in CA1 and CA3 regions [31, 34, 38] *via* NMDA receptors. Corticosterone also alters GABA-mediated inhibitory neurotransmission and causes neurodegeneration produced by the diminished expression of $GABA_A$ receptors (benzodiazepines, positive modulators of $GABA_A$ receptors, can block stress-induced dendritic regression) [38, 51]. Also the role of catecholamines i.e. norepinephrine (NE) and dopamine (DA) in stress is well documented. Stress enhances both NE and serotonin (5-HT) turnover, and noradrenergic system exerts a region-specific tonic inhibitory effect on brain serotonergic activity [6, 30]. $5\text{-}HT_3$ receptor antagonists seem to improve performance in rodents and primates in various cognitive tests [53]. High amounts of corticosterone enhance action of NE *via* β-adrenoreceptors. The increased dopamine turnover in the prefrontal cortex can impair spatial memory performance [20, 46]. Stimulation of various receptor subtypes exerts variable effects on hippocampal cell excitability during chronic corticosterone exposure. DA, GABA, NE and 5-HT were implicated in cognitive processes partly through an interaction with the cholinergic system [4, 44]. Also, histaminergic H_3 receptor antagonists facilitate acquisition of memory, possibly through the modulation of cholinergic mechanisms [4].

The pathogenic effect of GK excess on the neuronal development and in neurological diseases is mainly related to the hippocampal damage [1]. For example, post-traumatic stress disorder and administration of high doses of cortisol in normal humans selectively impair verbal declarative memory without affecting nonverbal (procedural) memory [11, 29, 36]. Similarly, stress and stress hormones have been shown to impair performance in spatial memory tasks [17, 18] and also nonspatial hippocampal memory in rats [5]. *In vitro* and *in vivo* electrophysiological studies indicate that stress and GKs impair hippocampal long-term potentiation (LTP), a putative synaptic mnemonic mechanism in the mammalian brain [18]. Various stressors impair LTP in different regions of the rat brain (e.g. in the CA1 and dentate gyrus) [5, 40].

Antianxiety or hypno-sedative agents are commonly used for the management of stress but their use has several disadvantages [46] and replacing them with safe natural products (e.g. medicinal plants) can be an ideal choice. EGB 761 is one of the candidates [26]. Numerous studies have shown that *Ginkgo biloba* has an antioxidant [24, 39, 49], free radical scavenging [24, 39, 42, 44, 49], neuroprotective [37, 42, 47], and antiplatelet effects [47]. Beneficial action of the plant against ischemia/reperfusion injury [24], hypoxia [49], cerebrovascular and cardiovascular diseases [15], cognitive deficits and dementia [14, 25, 28] have also been described. The cellular mechanisms underlying these multiple effects of *Ginkgo biloba* can be attributed to the different components of the extract, which may act independently or synergistically.

EGB 761 has been shown to improve the fluidity and decrease deformability of neuronal membranes that may be altered in aging and several pathological situations [25, 43]. Its protective effects include upregulation of mitochondrial ND1 gene expression, which is crucial in meeting the high-energy demands of neurons [49]. Mitochondrial dysfunction implicated in aging and several neurodegenerative disorders, such as Parkinson's, Alzheimer's and Huntington's diseases [28, 49]. EGB 761 was also found to influence glucose uptake and glycogen synthesis by various cells [42, 44]. EGB's biological effects were determined to be somewhat similar to these of tyramine [35]. They include an increase in the density of muscarinic cholinergic, β-adrenergic, and thyrotropin-releasing hormone receptors [35]. The extract has been found to be able to increase the rate of acetylcholine turnover and to stimulate binding activity of ligands to the muscarinic receptors in the hippocampus [26] and also to influence brain dopaminergic transmission [43]. Chronic administration of EGB 761 inhibits stress-induced corticosterone hypersecretion through a reduction of the number of adrenal peripheral benzodiazepine receptor (PBR) sites, and suppression of its gene [1, 2, 33].

The restraint model of stress in rats, used in this study, involves no painful stimulation and combines both emotional and physical components of physiological stress [46].

There were many investigations aimed at exploration of the mechanisms of beneficial actions of EGB 761 in various dysfunctions but little is known about its effects on memory impaired by stress.

The aim of this study was to investigate efficacy of EGB 761 in prevention and treatment of the post-stress memory dysfunctions in the animal model of stress. The groups of rats chronically injected with 'equivalent' dose of corticosterone mimicking its natural stress levels [22] were ran in parallel to evaluate the contribution of this hormone to the post-stress memory disorders.

Materials and Methods

Animals

A total of 120 male Wistar rats (C. Kozłowska, Brwinów, Poland), weighing at the beginning of the experiment 150–160 g, and at the end 300–350 g, were used. They were maintained under a 12 h light/dark cycle at constant temperature 22 ± 2°C and 70% humidity and housed five per a cage in wire mesh cages, except during testing when they were housed in Plexiglas boxes with wood shavings as bedding. The experiment was carried out in four stages with thirty rats at each stage. The procedures were conducted between 8:00 and 13:00 h. The animals were fed standard lab food for rodents (LSM, Agropol, Poland) and tap water was freely accessible.

There were six experimental groups of animals: CONTROL, STRESS, CORT (corticosterone), EGB 761 (*Ginkgo biloba*), EGB 761 + STRESS, EGB 761 + CORT.

The procedures were carried out in accordance with the guidelines set by European Community Council (Directive 86/609/EEC), and were approved by the Local Ethics Commission for the Animal Experimentation.

Restraint stress procedure

Restraint was performed by daily placing animals in 20 × 7 cm plastic tubes for 2 h for 21 days [32]. There were several 3 mm holes at the far end of the tubes for breathing. They allowed plenty of air but animals were unable to move.

Drugs

Extract of *Ginkgo biloba* (EGB 761) standardized to 24% ginkgoflavonoglucosides and 6% terpenoid lactones was provided by PPHV Biofarm, Poznań. It was a light brown powder suspended in 0.5% methylcellulose and given orally by a gavage at the dose of 100 mg/kg daily for 21 days, in a volume of 1 ml/kg, 30 min before stress procedure or corticosterone injection. All animals, which did not receive EGB 761 (CONTROL, STRESS, CORT), were given orally appropriate volumes of the vehicle (0.5% solution of methylcellulose).

Corticosterone (Sigma, 5 mg/kg) was dissolved in absolute ethanol and subsequently diluted in water to the final concentration of 10% ethanol and injected subcutaneously in a volume 1.5 ml/kg [17, 45]. All animals which did not receive corticosterone (CONTROL, EGB 761, STRESS and EGB 761 + STRESS) were given subcutaneously (*sc*) appropriate volumes of its vehicle (10% ethanol).

Behavioral tests

Morris water maze

This task was adapted from the paradigm originally described by Morris [50, 52]. The water maze was a black circular pool (180 cm in diameter, 60 cm high), filled with water (26 ± 1°C) made opaque with a grain instant coffee, to the depth of 30 cm, placed in a room rich in consistently located spatial cues (door, shelves, illumination lights, posters). The pool was divided into four quadrants designated Northeast (NE), Northwest (NW), Southeast (SE) and Southwest (SW). An escape platform (9 cm in diameter) was placed in the middle of one quadrant (NW), 1.5 cm below the water surface, equidistant from the sidewall and middle of the pool. The platform provided the only escape from the water and was located in the same quadrant on every trial. Four different starting points for rats were equally spaced around the perimeter of the pool (N, E, S and W). On each of the four training days, all four start points were used once each in a pseudo-random sequence. A trial began by placing the animal in the water facing the wall of the pool at one of the starting points. If the animal failed to escape on the platform within 60 s it was gently placed there by the experimenter and allowed to stay for 15 s. The intertrial interval was 5 min. After each trial, the rats were towel and fan dried and returned to their home cages. All animals were trained one session of four trials daily for four consecutive days. Twenty four hours after the last training session, the rats underwent probe trial without the platform. The probe trial was performed by placing an animal in the water at the point S. The latency to reach the original position of the platform, the number of crossings over its location during 1 min, and time spent by rat in the target quadrant and in the opposite quadrant were measured manually.

Object recognition task

The basic design of the object recognition equipment was similar to that described by Ennaceur and Delacour [21]. The apparatus was an open box made of wood 63 (length) × 45.5 (width) × 44 cm (height). The objects to be discriminated were made of glass or ceramics and existed in duplicate. Their weight was such that rats could not displace them. The apparatus was placed in a soundproof dimly-lit (40 lux at the level of the apparatus) room.

On the day preceding experimental day, all animals were allowed to explore the box for 1 min. They were then given two test sessions separated by a 48 h interval. Throughout the experiment, no cleaning of the box was allowed, in order to saturate it with olfactory stimuli.

Each session comprised two trials. In the first trial (T1), one object-stimulus, the sample (A), was placed at the rear wall of the box in a location equidistant from the back corners. During the second trial (T2), a new object (B) was added, and each object was placed in one of the back corners. The object (A) presented during T2 was a duplicate of the sample presented in T1 in order to avoid olfactory trails. From rat to rat, the role (sample or new object) and the position of the two objects during T2 were counterbalanced and randomly permuted. A different pair of objects was used for each session. If O1 and O2, were the two objects of each pair, for half of the rats, O1 was the sample (A) and O2 the new object (B). For the other half, the roles of O1 and O2 were opposite. These precautions were taken to reduce object and place preference effects. The objects apparently had no natural significance for rats and had never been associated with reinforcement.

At the beginning of each trial, the rats were placed near the center of the front wall of the box with their heads in opposite direction from the object. The respective duration of T1 and T2 was 5 and 3 min. Intertrial interval was 1 h.

The measure was the time spent by rats in exploring objects during T1 and T2. Exploration of an object was defined as follows: directing the nose at a distance < 2 cm to the object and/or touching it with the nose. Turning around or sitting on the object was not considered as an exploration.

Let (A) be the time spent in exploring the sample during T1, (A') and (B) the times spent, respectively, in exploring the sample and the new object during T2 the following variables were analyzed: A and A' + B as the measures of the exploration of both objects during T1 and T2 respectively; and B-A', as memory index reflecting the discrimination between the new and the familiar objects. Moreover as B-A' may be biased by the differences in overall levels of exploration, the variable B-A'/B + A' was computed.

Open field

Locomotor exploratory activity was measured in an open field, which was a square white floor measuring 100 × 100 cm divided by eight lines into 25 equal squares and surrounded by a 47 cm high walls [10]. Four plastic bars, 20 cm high, were designed as objects of possible animal's interest and fixed perpendicularly, parallel to each other, in four different line crossings in the central area of the floor. A single rat was placed in the center of the floor for 1 min for adaptation. Subsequently, crossings, rearings and bar approaches were counted manually for 5 min.

Elevated 'plus' maze

Anxiety was evaluated in an elevated 'plus' maze (constructed of gray colored wooden planks) consisting of two open arms measuring 50 cm (length) × 10 cm (width) and two closed arms [50 cm (length) × 10 cm (width) × 40 cm (height)], covered with a removable lid, such that the open or closed arms were opposite to each other [40]. The maze was elevated to a height of 50 cm from the floor. A rat was placed for 5 min in a pretest arena (60 × 60 × 35 cm constructed of the same material) prior to exposure to the maze. This step allowed for the facilitation of the exploratory behavior. Immediately after the pretest exposure, the rats were placed in the center of the elevated 'plus' maze facing one of the open arms. During 5 min test period the following measures were taken: the number of entries into the open arms and the time spent in the open arms. An entry was defined as entering into one arm with all four feet. An increase in open arms entries and increase in time spent in open arms were interpreted as an index of potential anxiolytic activity.

Statistical analysis

The statistical significance of the results was computed by one-way analysis of variance (ANOVA I) followed by Newman-Keuls test. F – ratios, degrees of freedom and p values are reported only for signifi-

cant differences. The latencies to reach platform in the Morris water maze during acquisition were analyzed by two-way (groups × days) analysis of variance (ANOVA II). The times spent by rats in target *vs.* opposite quadrants were compared with the Student's *t*-test.

Results

Effects of EGB 761 on spatial learning in Morris water maze

Figure 1 shows the mean daily latencies to reach the hidden platform by stressed and corticosterone-treated rats, given or not EGB 761, compared with these receiving only the vehicles.

Treatments × days ANOVA II of all latencies with the repeated measures for factor 1 revealed significant differences between the groups [$F(5,53) = 3.71$, $p < 0.006$], a day effect over the four-day training period [$F(5,156) = 217.08$, $p < 0.0001$] and no significant groups × days interaction. *Post-hoc* analyses indicated that the stressed rats treated with EGB 761 reached the hidden platform statistically significantly ($p < 0.01$) earlier than the stressed animals. The results show that rats in all groups learned the water maze task with only slightly different effectiveness, and that certain deleterious effects of stress and corticosterone was abolished by EGB 761.

ANOVA I of the latencies to reach the previous location of the platform during the recall probe trial yielded $F(5,51) = 2.79$ ($p < 0.05$) showing significant differences between the groups (Fig. 2). *Post-hoc* comparisons revealed that the rats treated with EGB 761 reached the position significantly ($p < 0.05$) earlier than control rats. Also, the animals which were stressed ($p < 0.05$) or treated with corticosterone ($p < 0.05$) but pretreated with EGB 761, reached original platform position significantly earlier than the respective vehicle-pretreated groups.

Similarly, ANOVA I of the numbers of crossings over the platform location showed highly significant differences between the groups; $F(5,51) = 5.57$ ($p < 0.001$) (Fig. 3). Rats treated with EGB 761 ($p < 0.05$) visited the location significantly more often than control animals, similarly as stressed ($p < 0.001$) or corticosterone-injected ($p < 0.01$) but EGB 761-pretreated animals.

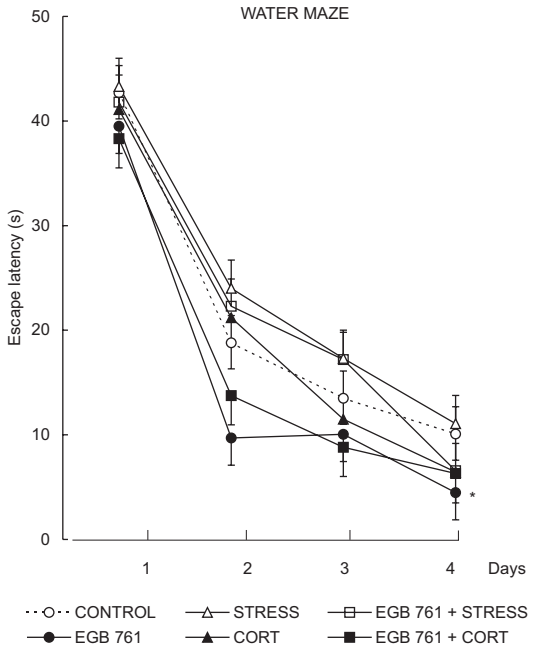

Fig. 1. Effects of chronic stress (2 h/day for 21 days), chronic corticosterone (5 mg/kg *sc* for 21 days) and EGB 761 (100 mg/kg/orally for 21 days) on the mean escape latencies during acquisition of the spatial navigation task. The control group received 0.5% solution of methylcellulose (1 ml/kg/day orally and 30 min later of ≤ 10% ethanol diluted in water 1.5 ml/kg/day *sc* for 21 days). Values are the means of 10 subjects and SEM * $p < 0.01$ EGB 761 *vs.* STRESS group

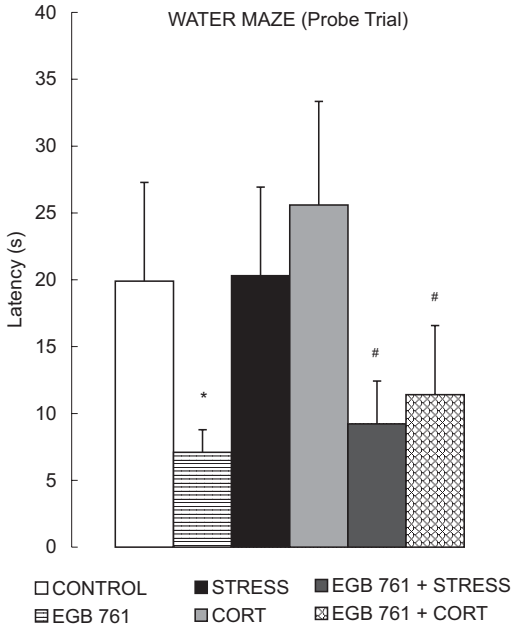

Fig. 2. Effects of chronic stress (2 h/day for 21 days), chronic corticosterone (5 mg/kg *sc* for 21 days) and EGB 761 (100 mg/kg/orally for 21 days) on the latencies in reaching the original position of the platform in the probe trial on Day 5. The control group received 0.5% solution of methylcellulose (1 ml/kg/day orally and 30 min later of ≤ 10% ethanol diluted in water 1.5 ml/kg/day *sc* for 21 days). Columns represent the means ± SEM of the values obtained from n = 10 rats. * $p < 0.05$ *vs.* CONTROL; # $p < 0.05$ *vs.* the respective STRESS and CORT groups

Tab. 1. Effects of chronic stress (2 h/day for 21 days), chronic corticosterone (5 mg/kg *sc* for 21 days) and EGB 761 (100 mg/kg orally for 21 days) on the object recognition in Session 1. The control group received 0.5% solution of methylcellulose (1 ml/kg/day orally and 30 min later ≤ 10% ethanol diluted in water 1.5 ml/kg/day *sc* for 21 days). Variables describe object recognition (see text). Values are the means from 9–10 subjects and SEM. (in parentheses). * $p < 0.05$, ** $p < 0.01$, *vs.* CONTROL. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ *vs.* respectively, STRESS or CORT

Variables	Treatment					
	CONTROL	STRESS	CORT	EGB 761	EGB 761 + STRESS	EGB 761 + CORT
A	39.2 (6.91)	19.38 (3.93)*	22.4 (3.86)*	40.8 (6.98)	60.4 (5.68)$^{\#\#\#}$	45.3 (5.59)
A'	16.89 (3.05)	16.4 (3.22)	13.9 (2.83)	21.22 (1.89)	15.2 (2.71)	16.5 (2.78)
B	22.33 (2.86)	12.6 (2.51)	14.3 (2.69)	33.11 (2.89)*	33.89 (3.99)$^{\#\#\#}$	28.8 (2.6)$^{\#\#}$
B-A'	5.44 (1.93)	–5.25 (1.85)**	0.4 (1.54)*	13.89 (2.24)*	17.56 (0.84)** $^{\#\#\#\#}$	12.3 (2.14)* $^{\#\#\#}$
B + A'	39.33 (5.56)	34.5 (5.14)	28.2 (5.34)	54.3 (4.18)*	51.33 (5.43)$^{\#}$	43.3 (4.28)$^{\#}$
B-A'/B + A'	0.16 (0.062)	–0.12 (0.005)**	0.05 (0.0074)	0.21 (0.0049)	0.33 (0.068)$^{\#\#\#\#}$	0.32 (0.0059)$^{\#\#}$

These results showed that EGB 761-treated rats recalled the original position of the platform significantly better than all the remaining animals.

EGB 761 ($p < 0.0001$), EGB 761 + STRESS ($p < 0.01$) and EGB 761 + CORT ($p < 0.001$) groups of rats swam significantly longer time in the target quadrant of the maze during recall test than in the opposite one (Fig. 4). The remaining 3 groups swam comparable times in both quadrants.

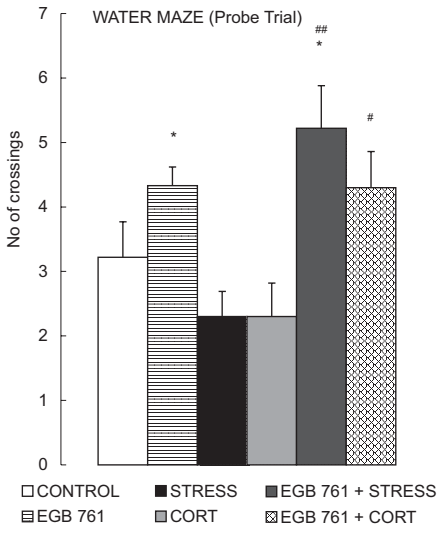

Fig. 3. Effects of chronic stress (2 h/day for 21 days), chronic corticosterone (5 mg/kg *sc* for 21 days) and EGB 761 (100 mg/kg/orally for 21 days) on the number of crossings over the original position of the platform in the probe trial on Day 5. The control group received 0.5% solution of methylcellulose (1 ml/kg/day orally and 30 min later of ≤ 10% ethanol diluted in water 1.5 ml/kg/day *sc* for 21 days). Columns represent the means ± SEM of the values obtained from n = 10 rats. * $p < 0.05$ *vs.* CONTROL; # $p < 0.01$, ## $p < 0.001$ *vs.* the respective STRESS and CORT groups

Effects of EGB 761 on recognition memory

ANOVA I of the times spent by rats in exploring the sample object A in T1 yielded $F(5,49) = 6.83$ thus showing significant ($p < 0.0001$) differences between the groups (Tab. 1). *Post-hoc* comparisons made with Newman-Keuls test revealed significant decrease in the exploratory activity in stressed ($p < 0.05$) and corticosterone-treated ($p < 0.05$) rats. EGB 761 while having no effect on its own reversed statistically significantly the effect of stress ($p < 0.001$) and also (insignificantly) that of corticosterone.

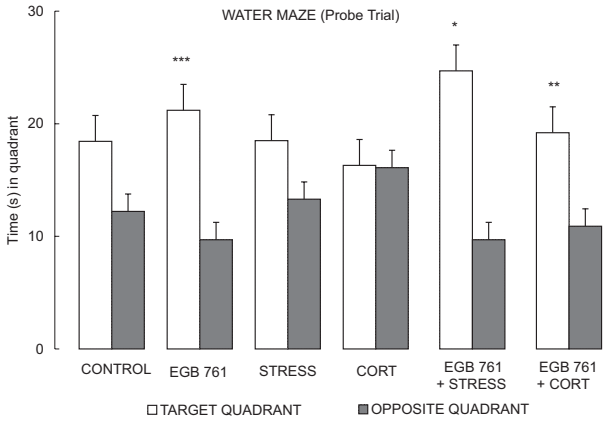

Fig. 4. Effects of chronic stress (2 h/day for 21 days), chronic corticosterone (5 mg/kg *sc* for 21 days) and EGB 761 (100 mg/kg/orally for 21 days) on the times spent by rats in the target quadrant as compared to that spent in the opposite quadrant during the probe trial on Day 5. The control group received 0.5% solution of methylcellulose (1 ml/kg/day orally and 30 min later of ≤ 10% ethanol diluted in water 1.5 ml/kg/day *sc* for 21 days). Columns represent the means ± SEM of the values obtained from n = 10 rats. * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$

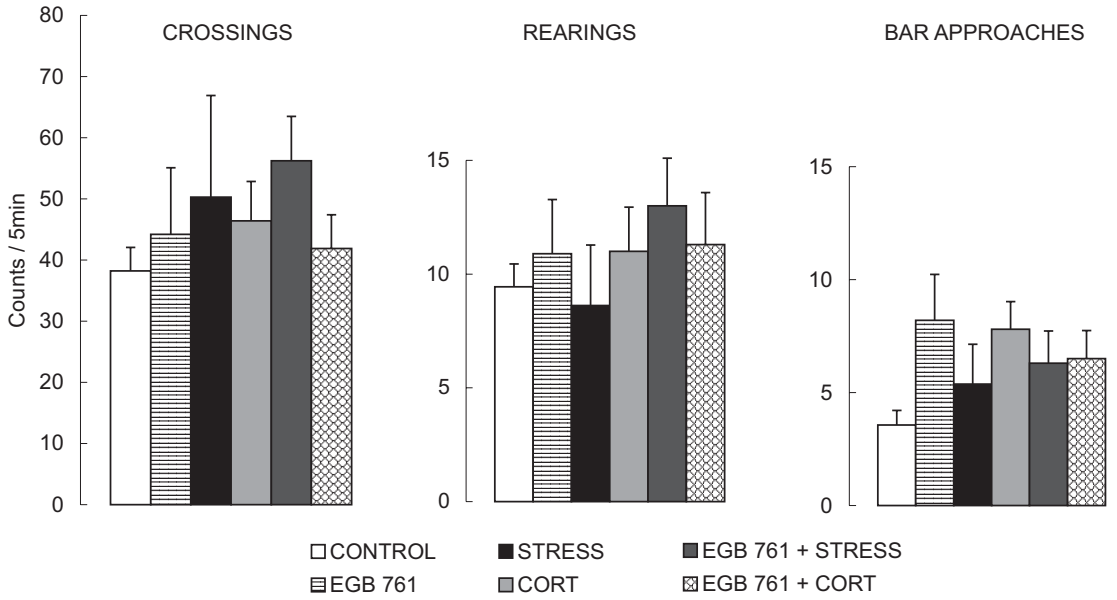

Fig. 5. Effects of chronic stress (2 h/day for 21 days), chronic corticosterone (5 mg/kg *sc* for 21 days) and EGB 761 (100 mg/kg/orally for 21 days) on the number of crossings, rearings and bar approaches in the open field. The control group received 0.5% solution of methylcellulose (1 ml/kg/day orally and 30 min later of ≤ 10% ethanol diluted in water 1.5 ml/kg/day *sc* for 21 days). Columns represent the means ± SEM of the values obtained from n = 10 rats

ANOVA I of the times of exploration of the duplicate of object A (A') in T2 revealed no statistically significant differences between the groups.

ANOVA I of the times of exploration of the new object B in T2 yielded $F(5,49) = 10.08$, revealing significant ($p < 0.0001$) differences between the groups. Further comparisons between the groups showed that the rats treated with EGB 761 were statistically significantly ($p < 0.05$) more active than controls. Stressed ($p < 0.001$) and corticosterone-treated ($p < 0.01$) but EGB 761-pretreated animals maintained their exploratory activity at the levels of the animals given only EGB 761which, on the other hand, were statistically significantly higher than those in all the remaining groups.

ANOVA I of the discrimination times between the new and familiar objects (B-A') yielded $F(5,49) = 14.89$ thus showing significant ($p < 0.0001$) differences between the groups. *Post-hoc* comparisons made with Newman-Keuls test revealed significant improvements of the object recognition memory in EGB 761-treated group as compared with control ($p < 0.05$). Similarly, EGB 761 + STRESS ($p < 0.0001$) and EGB 761 + CORT ($p < 0.001$) groups displayed better discrimination/recognition performance than the respective STRESS and CORT groups. Stressed ($p < 0.01$) or corticosterone-treated rats ($p < 0.05$) exhibited impairment of recognition memory in comparison with the CONTROL group. In addition, stressed animals stayed with the familiar object longer than with the new one displaying neophobia, which was effectively abolished by EGB 761.

ANOVA I of the total exploration times in T2 (B + A) reflecting overall levels of exploration yielded $F(5,49) = 3.97$, $p < 0.01$, thus showing significant differences between the groups. Further *post-hoc* comparisons revealed that the EGB 761-treated group was the most active and statistically significantly ($p < 0.05$) different from control. Stressed and corticosterone-treated groups, given EGB 761, displayed their exploratory activity at the levels comparable to control which were, however, statistically significantly higher than these of the respective stressed ($p < 0.05$) and corticosterone-treated ($p < 0.05$) groups not receiving EGB 761.

ANOVA I of the B-A'/B + A' ratios yielded $F(5,49) = 7.84$, $p < 0.0001$, showing statistically significant differences between the groups. *Post-hoc* comparisons with Newman-Keuls test revealed changes in the same directions as that of the B-A' variable virtually excluding possibility of the discrimination time bias by changes in overall levels of exploration.

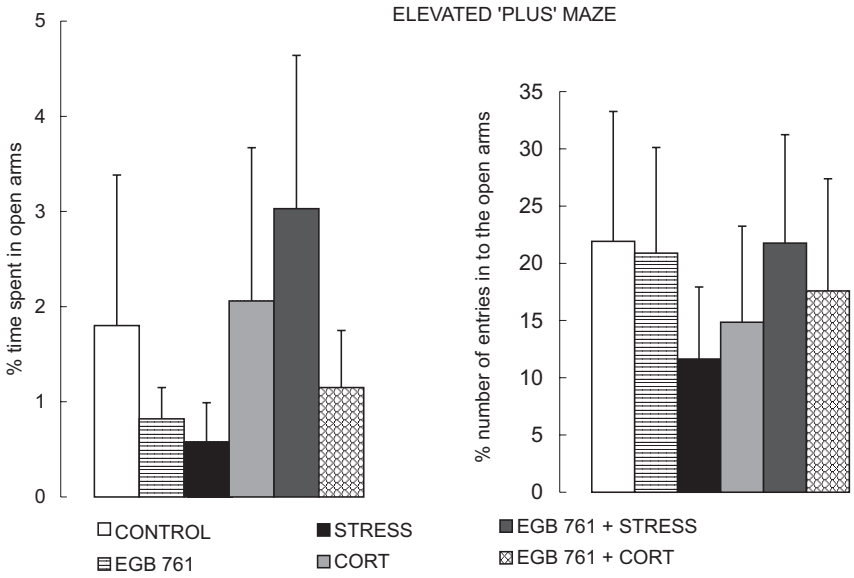

Fig. 6. Effects of chronic stress (2 h/day for 21 days), chronic corticosterone (5 mg/kg *sc* for 21 days) and EGB 761 (100 mg/kg/orally for 21 days) on the % time spent by rats in, or the % number of entries to, the open arms of the elevated 'plus' maze. The control group received 0.5% solution of methylcellulose (1 ml/kg/day orally and 30 min later of ≤ 10% ethanol diluted in water 1.5 ml/kg/day *sc* for 21 days). Columns represent the means ± SEM of the values obtained from n = 10 rats

Object recognition memory tested in Session 2 performed 48 h after Session 1 showed essentially the same but smaller differences.

ANOVA I of the times spent by rats in exploring the sample object A in T1 yielded $F(5,49) = 6.83$, showing significant ($p < 0.05$) differences between the groups (Tab. 2). *Post-hoc* comparisons made with Newman-Keuls test revealed significant increase in the exploratory activity in stressed ($p < 0.01$) EGB 761-pretreated rats in comparison with both control and stress-exposed groups.

ANOVA I of the times of exploration of the duplicate of object A (A') in T2 revealed no statistically significant differences between the groups although stressed rats pretreated with EGB 761 tended to have much better scores than all the other groups.

ANOVA I of the times of exploration of the new object B in T2 yielded $F(5,49) = 5.66$, revealing significant ($p < 0.001$) differences between the groups. Stressed ($p < 0.01$) corticosterone ($p < 0.05$)-treated and EGB 761-pretreated animals maintained their exploratory activity at statistically significantly higher

Tab. 2. Effects of chronic stress (2 h/day for 21 days), chronic corticosterone (5 mg/kg *sc* for 21 days) and EGB 761 (100 mg/kg orally for 21 days) on the object recognition in Session 2 (48 h after Session 1). The control group received 0.5% solution of methylcellulose (1 ml/kg/day orally and 30 min later of ≤ 10% ethanol diluted in water 1.5 ml/kg/day *sc* for 21 days). Variables describe object recognition (see text). Values are the means from 9–10 subjects and SEM (in parentheses). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* Control. $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ *vs.* respectively, STRESS or CORT

Variables	Treatment					
	CONTROL	STRESS	CORT	EGB 761	EGB 761 + STRESS	EGB 761 + CORT
A	24.22 (4.32)	24.75 (3.45)	28.2 (4.86)	39 (6.84)	49.78 (6.03)$^{***\#\#}$	23.16 (7.32)
A'	12.37 (2.46)	16.12 (3.04)	13.6 (1.73)	11.1 (1.31)	22.11 (4.49)	13.11 (2.14)
B	15.63 (7.71)	17.25 (2.42)	15.4 (2.83)	21.44 (2.07)	35.55 (4.68)$^{***\#\#}$	25.33 (3.97)$^{\#}$
B-A'	2.89 (1.16)	1.13 (2.86)	1.8 (1.43)	10.33 (1.87)*	13.44 (4.35)$^{*\#\#}$	11 (3.2)$^{*\#}$
B + A'	24.89 (5.42)	33.38 (4.68)	29 (4.46)	32.56 (2.91)	57.67 (8.08)$^{***\#\#}$	35.8 (5.54)
B-A'/B + A'	0.15 (0.06)	0.2 (0.11)	0.025 (0.06)	0.32 (0.05)	0.26 (0.07)	0.32 (0.05)$^{\#\#}$

levels than those in the respective groups not treated with EGB 761.

ANOVA I of the discrimination times between the new and familiar objects (B-A') yielded $F(5,49) = 3.98$ thus showing significant ($p < 0.01$) differences between the groups. *Post-hoc* comparisons with Newman-Keul's test revealed significant improvements of the object recognition memory in EGB 761-treated group as compared with control ($p < 0.05$). Similarly, EGB 761 + STRESS ($p < 0.01$) and EGB 761 + CORT ($p < 0.05$) groups displayed better discrimination than the respective STRESS and CORT groups.

ANOVA I of the total exploration times in T2 (B + A) yielded $F(5,49) = 4.43$, ($p < 0.01$), which showed significant differences between the groups. Further *post-hoc* comparisons revealed that the stressed and EGB 761-pretreated group was the most active of all the remaining groups, being statistically significantly different from control ($p < 0.001$) and stress-exposed groups ($p < 0.01$).

ANOVA I of the B-A'/B + A' ratios yielded $F(5,49) = 2.91$, ($p < 0.05$) showing statistically significant differences between the groups. *Post-hoc* comparisons with Newman-Keuls test revealed that corticosterone-treated group given EGB 761 was statistically significantly ($p < 0.01$) different from the rats receiving only corticosterone.

Interestingly, the best recognition memory and exploratory activity of all groups was seen in the EGB 761 + STRESS rats.

Effects of EGB 761 on locomotor exploratory activity of rats in open field

ANOVA of the results obtained in the open-field test yielded no statistically significant differences in the numbers of crossings, rearings, and bar approaches (Fig. 5). It means that our treatments and procedures did not appreciably affect the psychomotor or musculo-skeletal aspects of rats' performance.

Effects of EGB 761 on the behavior of rats in the elevated 'plus' maze

ANOVA of the results obtained in the elevated 'plus' maze test yielded no statistically significant differences in the times spent by rats in open arms and in the numbers of open arm entries (Fig. 6). It means that our treatments and procedures did not appreciably affect emotional aspects of rats' psychomotor performance.

Discussion

The main purpose of this study was to determine if EGB 761 can improve memory processes in control and stressed or corticosterone-treated rats.

The object recognition test employed here showed impairments of nonspatial hippocampus-dependent memory after chronic restraint stress or chronic administration of 'equivalent' dose of corticosterone, and preventive doses of EGB 761 not only normalized this post-stress (and post-corticosterone) cognitive deficits but much improved rats' performance in comparison with the control levels. This effect of EGB 761 was long-lasting as all three groups of rats receiving the drug i.e. (EGB 761, EGB 761 + STRESS and EGB 761 + CORT) had better recognition memory than the remaining three groups (CONTROL, STRESS and CORT) also in Session 2 (Tab. 2) ran 48 h after Session 1.

Although stress- or corticosterone-induced memory impairments could be found in none of the three parameters measured in Morris maze, during the probe (recall) trial such a tendency in the number of crossings over the platform location and number of visits in the target quadrant could have been observed. Interestingly, in naive, as well as in stressed or corticosterone-treated groups, EGB 761 effectively improved recall, indicating indirectly its beneficial effects also in the stress-induced impairments.

In the Morris water maze test, EGB 761 tended to improve spatial memory in control rats and in rats subjected to stress or corticosterone treatment. Notably, the animals receiving EGB 761 alone learned the water maze task significantly better than the stressed rats.

Thus, EGB 761 improved memory in control rats in both Morris water maze and object recognition tests. Most parameters of the two memory tests (the latency in reaching the original position of the platform, the number of crossings over that place and the time spent in the target quadrant compared to the opposite quadrant in the water maze as well as the object recognition) were better in the groups receiving EGB 761. In other words, EGB 761 enhanced both spatial and nonspatial hippocampus-dependent memory in all groups of animals receiving the drug.

For the proper interpretation of the present results, consideration of unspecific motor and emotional actions of the used drugs and stress is necessary. Fortunately, open field and elevated 'plus' maze tests

showed little or no effect of stress, corticosterone, and *Ginkgo biloba* extract on anxiety and locomotor exploratory activity, excluding the possibility of unspecific bias in memory tests.

Visual recognition memory is considered good animal model of that form of memory in humans. Animal tests of spontaneous recognition provide close analogy with recognition tests performed in human subjects [19]. The animal tasks are dependent on spontaneous novelty preference and assess recognition memory by measuring animal's tendency to explore novel object. Normal animals prefer to explore the novel objects more than familiar objects. From the degree of preference to exploration of the new object, it can be inferred that they retained memory of the familiar, therefore less interesting object [13, 21]. In this study, the stressed group displayed reverse phenomenon. We observed neophobia i.e. preference of familiar over the new objects. There is an evidence of important role of cortical $GABA_A$/benzodiazepine receptor complex in producing neophobia and in the processes of motor control [23]. A negative correlation between novelty-associated locomotion and specific binding of the $GABA_A$ receptor antagonist in the cingulate and prefrontal cortices, and in the ventral pallidum was described [23].

In line with our results, Beck et al. [6] demonstrated that 21days of chronic restraint adversely affected the performance of male rats in object recognition test. Similar reports were also published by other authors [2, 6, 44]. Chronic stress as modeled by 21-day restraint has been hypothesized to compromise memory processing in rats by creating a prolonged high-corticosterone level state. However, in addition to corticosterone other hormones that were not examined in most studies (e.g. catecholamines, growth hormone, prolactin) can also be profoundly involved in stress reactions [6, 40].

There are many studies investigating possible mechanisms of EGB 761 action. Important data were published by Amri et al. [1, 2] who found that treatment with EGB 761 could decrease corticosteroid levels by as much as 50%. A variety of stressful stimuli produce an overall increase in adrenocorticotropic hormone (ACTH) and corticosterone secretion [3, 8]. The numerous studies described beneficial effects of EGB 761 on cognitive functioning and adaptative processes negatively affected by GK excess [1, 44, 46]. They showed that EGB 761 might be acting through normalization of stress-elevated catecholamine and serotonin concentrations. The limbic structures affect HPA axis activity and there is considerable evidence that NE and 5-HT mediate these responses [6, 7]. Restraint stress has been reported to enhance brain NE and 5-HT [7, 48]. The catecholamine producing neurons of the brainstem which directly innervate the corticotropin releasing hormone (CRH) secreting neurons in the paraventricular nucleus of the hypothalamus, *via* the ventral noradrenergic bundle, give rise to the major stimulatory pathway of the stress-induced activation of HPA axis [7, 48]. 5-HT neurons from the raphe nuclei innervate the hypothalamic and limbic areas and are important in secretion of ACTH during stress [3, 6]. Restraint stress has been reported to enhance brain 5-HT [7] which may regulate ACTH secretion by inhibiting negative feedback of corticosteroids on CRH/ACTH axis [3]. Benzodiazepines as well as *Ginkgo biloba* actions through the negative modification of stress-induced elevation of 5-HT in the hypothalamus and plasma corticosterone are well known [48].

Prolonged exposure to stress or corticosteroids may alter the hippocampus-inhibiting tone by regulating the pharmacological properties of GABA receptors [38]. There are data suggesting that components of EGB 761 exert specific effects on adrenocortical cells by inhibiting PBR mRNA and protein expression, thus limiting the amount of mitochondrial cholesterol available for corticosteroid synthesis [1, 2]. Moreover, repeated treatment with EGB 761 and ginkgolide B (GKB) reduced the ACTH-stimulated corticosteroid production without affecting basal glucocorticoid and aldosterone formation. GKB is the only known pharmacological tool regulating PBR levels and corticosteroid synthesis [1, 2].

Apart from these considerations, precise mechanism of cognition-improving action of EGB 761 remains unknown. The above-mentioned effects of the extract on neurotransmitters are important but by no means sufficient for understanding its influence on the memory processes. The well-known vasodilatatory and oxygen consumption-increasing effects of EGB 761 may be of importance [15, 24].

Notwithstanding the considerable uncertainty about its precise mechanism of action, EGB 761 appears to be a serious candidate for a therapy of the stress-related memory deficits. Several clinical studies showing EGB 761 utility in ischemic conditions, senile dementia (including Alzheimer's disease) [28], cerebral and retinal impairment and in improving the

capacity of some geriatric patients to cope with stressful demands of daily life seem to confirm this possibility [14, 15].

In conclusion, this study provides a new perspective on protection against stress-induced cognitive disorders but a search for further effective and possibly nontoxic pharmacological tools for that purpose should continue.

Acknowledgments:
We thank Lidia Filipczak of PPHV Biofarm, Poznań for EGB 761. Edyta Sitko participated in the experiments and Agata Chwiecko and Konrad Kacejko in preparing the manuscript. This study was supported by the Medical University of Białystok (3-66 464).

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Received:
March 30, 2004; in revised form: February 17, 2005.