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The *Ginkgo biloba* extract, EGb761, increases synaptosomal uptake of 5-hydroxytryptamine: in-vitro and ex-vivo studies

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Abstract—The *Ginkgo biloba* extract (EGb 761) added to a synaptosomal fraction prepared from mice cerebral cortex modified [^{3}H]5-hydroxytryptamine ([^{3}H]5-HT) uptake in a biphasic manner. Between 4 and 16 μg mL^{-1} EGb 761 increased significantly the [^{3}H]-5-HT uptake (maximum +23%). A similar increase was also obtained when synaptosomes were prepared from the cortex of mice treated orally with EGb 761, either acutely (100 mg kg^{-1}, 14 h and 2 h before death) or semi-chronically (2 × 100 mg^{-1} kg daily for 4 consecutive days). The in-vitro increase in [^{3}H]5-HT uptake induced by EGb 761 was not observed in the presence of 10^{-6} M clomipramine, a 5-HT-uptake inhibitor. EGb 761 did not increase [^{3}H]dopamine uptake by synaptosomes prepared from striatum of mice. We investigated different fractions of EGb 761 in order to determine the compounds inducing the increase in [^{3}H]5-HT uptake. The BN 52063 extract (corresponding to the EGb 761 devoid of flavonoid substances) did not increase [^{3}H]5-HT uptake. The Cp 202 extract (corresponding to the EGb 761 devoid of terpenic substances and containing mostly flavonoid substances) increased [^{3}H]5-HT uptake. Among the flavonoids, quercetin has been tested and had no effect on the [^{3}H]5-HT uptake. Since at the usual therapeutic doses of EGb 761, the effective concentrations of the components responsible for this increase are likely to be reached in the brain, one may suggest that this effect could contribute to the therapeutic effect of EGb 761.

Ginkgo biloba extract EGb 761 is used therapeutically to increase peripheral and cerebral blood flow, for treatment of certain ischaemic cerebral diseases and neurological disorders (see review by DeFeudis 1991). We have previously reported that EGb 761 dose-dependently inhibited synaptosomal uptake of [^{3}H]dopamine, [^{3}H]choline, and [^{3}H]5-hydroxytryptamine ([^{3}H]5-HT) (Ramassamy et al 1990). These inhibitory effects were obtained in the presence of high concentrations of EGb 761 (in the 1 mg mL^{-1} range), therefore their specificity is questionable. In addition, such high concentrations are unlikely to be reached in the brain following the usual therapeutic doses of EGb 761 ($\approx$120 mg per day of dry extract). Thus we were prompted to investigate the effects of lower concentrations of the extract. We observed that EGb 761 increased synaptosomal uptake of [^{3}H]5-HT, in the 10 μg mL^{-1} concentration range. The aims of the present study were to determine the dose-response curve of this effect, to investigate whether this effect was also observed with synaptosomes prepared from cortex of mice treated orally, either acutely or semi-chronically, with EGb 761 to check whether a similar increase could be observed in the [^{3}H]dopamine uptake by synaptosomes prepared from striatum, and to specify the fraction of the EGb 761 which induces the increase in [^{3}H]5-HT uptake.

Materials and methods

Animals. Male Swiss albino CD1 mice, 20–25 g (Charles River, Saint Aubin lès Elbeuf, France), were housed (20 mice per Makrolon box, 31 × 28 × 20 cm) in a well ventilated room, at an ambient temperature of 22°C, with a 12 h light-dark cycle. Food and water were freely available.

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Uptake of [^{3}H]dopamine and [^{3}H]5-HT by synaptosomes. A crude synaptosomal fraction (S1) was obtained by homogenization (Potter-Elvehjem, clearance 80–130 μm, with teflon pestle) of the striata or cortex in 10 volumes of ice-cold 0·32 M sucrose containing pargyline (10^{-4} M) followed by centrifugation (1000 *g*, 10 min, 2°C). The sedimented material (cell debris and nuclei) was discarded.

Portions of the supernatants (50 μL) were preincubated (37°C) with 100 μL EGb 761 at different concentrations or 100 μL buffer for controls and with 800 μL of Krebs–Ringer phosphate buffer (NaCl 103 mM, $CaCl_2$ 1 mM, $MgCl_2$ 1 mM, KH_2PO_4 1 mM, $NaHCO_3$ 27 mM, glucose 5·4 mM, ascorbic acid 10^{-4} M), gassed (95% O_2 – 5% CO_2) for 30 min before use. Then 50 μL of [^{3}H]dopamine (20 nM, final concentration) or 50 μL of [^{3}H]5-HT (20 nM, final concentration) was added.

After incubation, the uptake was stopped by dilution with ice-cold Krebs–Ringer buffer (4 mL) followed by vacuum filtration through Whatman GF/B filters. Each tube was rinsed and the filters washed twice with 4 mL ice-cold Krebs–Ringer buffer. Filters were dried for 1 h in a ventilated incubator (60°C) and were placed in minivials containing 4 mL Aqualyte (J. T. Baker Chemical, Deventer, Holland). The radioactivity was determined by liquid scintillation spectrometry. Nonspecific uptake was determined at 0°C. The specific uptake was expressed as fmol (mg protein)$^{-1}$. The protein concentration was determined by the method of Lowry et al (1951) with bovine serum albumin as the standard.

Ex-vivo effect of EGb 761 on [^{3}H]5-HT uptake by synaptosomes. Animals were deprived of food 2 h before the administration of EGb 761. For acute treatment, mice received 100 mg kg^{-1} EGb 761 via a gastric cannula 14 and 2 h before they were killed; controls received tap water. For semi-chronic treatment, mice received 2 × 100 mg kg^{-1} per day EGb 761 for 4 consecutive days. Mice were killed 16 h after the last administration. Immediately after death, a synaptosomal fraction was prepared from the cortex of each mouse. The [^{3}H]5-HT uptake was measured after a 5-min preincubation without [^{3}H]5-HT and a 5-min incubation with [^{3}H]5-HT.

Chemicals. The *Ginkgo biloba* extract EGb 761 was prepared by the Henri Beaufour Institute. EGb 761 is a well-defined and complex product prepared from leaves of *Ginkgo biloba*. The leaves are dried and subjected to a 15-step extraction procedure, commencing with acetone-water mixture under partial vacuum. The final extract is standardized to contain 24% of flavonoid glycosides (ginkgo flavones glycosides) and 6% terpene lactones which are characteristic of *Ginkgo* and have a unique structure (ginkgolides, bilobalides) (Drieu 1986). The dried extract was diluted in Krebs–Ringer phosphate buffer and resulted in a clear solution when tested in-vitro. The Cp 202 extract (in Henri Beaufour Institute's nomenclature) corresponds to EGb 761 devoid of terpenoid substances. This dried extract was diluted in Krebs–Ringer phosphate buffer and resulted in a clear solution. The BN 52063 extract (in Henri Beaufour Institute's nomenclature) is the EGb 761 devoid of flavonoid substances. This dried extract was diluted in Krebs–Ringer phosphate buffer and

ethanol (0·05%) and resulted in a clear solution. Quercetin and pargyline were purchased from Sigma. [^{3}H]Dopamine (15 Ci mmol^{-1}) and [^{3}H]5-HT (9·3 Ci mmol^{-1}) were purchased from Amersham (Les Ulis, France).

Statistics. Differences between groups were determined by Dunnett's *t*-test (Dunnett 1955) for the in-vitro studies or by the Student's *t*-test for the ex-vivo studies.

Results

In-vitro effects of various Ginkgo biloba *extracts (EGb 761, Cp 202, Bn 52063) on [^{3}H]5-HT synaptosomal uptake.* Increasing concentrations of EGb 761 modified in a biphasic manner the [^{3}H]5-HT uptake by synaptosomes prepared from cerebral cortex of mice. Between 4 and 16 μg mL^{-1}, EGb 761 significantly increased (+23% of the control value) [^{3}H]5-HT uptake. From 32 μg mL^{-1}, EGb 761 dose dependently decreased the amine uptake. This decrease became significant from 500 μg mL^{-1}. The inhibition was approximately 80% for the highest tested concentration 2 mg mL^{-1} (Fig. 1). EGb 761 (4–16 μg mL^{-1})-induced [^{3}H]5-HT uptake by synaptosomes was almost completely blocked by clomipramine (10^{-6} M) (Fig. 1).

The effects of other *Ginkgo biloba* fractions and of quercetin are shown in Fig. 2. The Cp 202 fraction, at concentrations between 1·25 and 5 μg mL^{-1}, significantly increased [^{3}H]5-HT uptake (+23%). The BN 52063 fraction, at concentrations between 0·25 and 10 μg mL^{-1} did not significantly modify [^{3}H]5-HT uptake, as compared with controls. Quercetin (0·1–5 μg mL^{-1}) had no effect on [^{3}H]5-HT uptake.

Ex-vivo effects of EGb 761 on the synaptosomal uptake of [^{3}H]5-HT. EGb 761 was administered intragastrically (100 mg kg^{-1} of dried extract freshly dissolved in distilled water) 14 and 2 h before the animals were killed and synaptosomes were prepared from cerebral cortex. [^{3}H]5-HT uptake, during the 5 min incubation period, was significantly higher than in controls (+25%) (Table 1).

In another experiment, a dried EGb 761 extract freshly dissolved in distilled water was administered intragastrically (100 mg kg^{-1}) twice a day, with a 2 h interval, for 4 consecutive days. Animals were killed 1 h after the final dose and synaptosomes were prepared from cerebral cortex. [^{3}H]5-HT uptake during the 5 min incubation period was significantly higher than in controls (+30%) (Table 1).

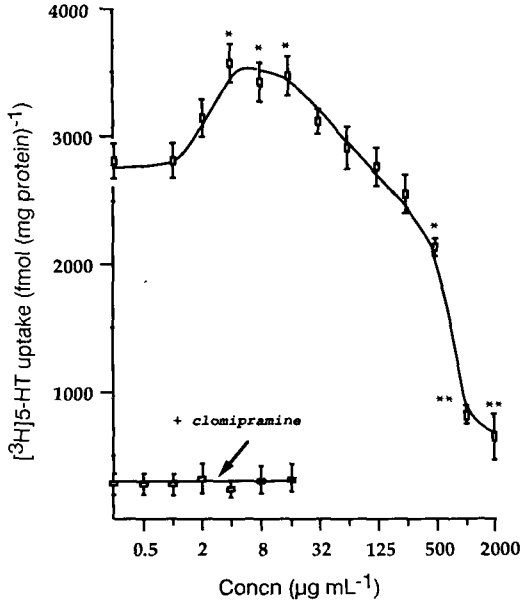

FIG. 1. Effects of EGb 761 on [^{3}H]5-HT synaptosomal uptake. Synaptosomes prepared from cerebral cortex of mice were incubated with increasing concentrations of EGb 761 5 min before the addition of [^{3}H]5-HT (20 nM final concentration). The incubation was then continued for 5 min. Means ± s.e.m. of 3 experiments in triplicate. *$P < 0·05$ compared with controls. **$P < 0·01$ compared with control.

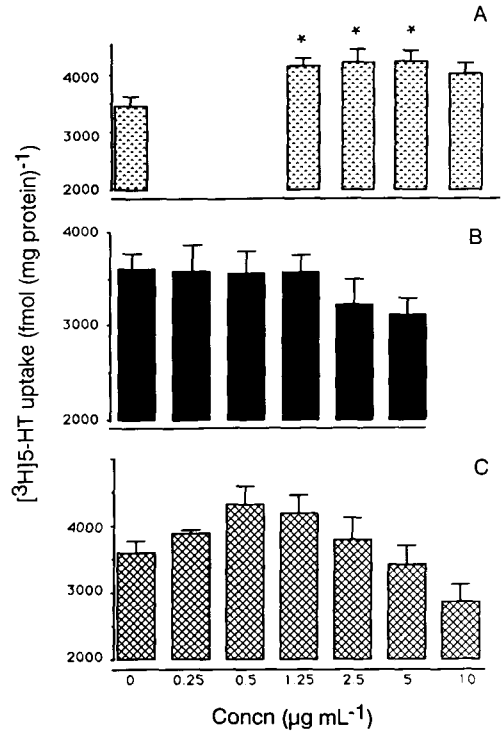

FIG. 2. Effects of A.Cp 202, B. BN 52063 or C. quercetin on [^{3}H]5-HT synaptosomal uptake. Synaptosomes, prepared from cerebral cortex of mice, were incubated with increasing concentrations of Cp 202, quercetin or BN 52063, for 5 min before the addition of [^{3}H]5-HT (20 nM final concentration). The incubation was then continued for 5 min. Means ± s.e.m. of 3 experiments in triplicate. *$P < 0·05$ compared with control.

Table 1. Effect of an acute or a semi-chronic oral administration of EGb 761 on [^{3}H]5-HT synaptosomal uptake.

	[^{3}H]5-HT uptake (fmol (mg protein)$^{-1}$) by synaptosomes prepared from cortex of mice	
Treatments	Saline	EGb 761
Acute	1704 ± 72	2113 ± 195[a]
Semi-chronic	2801 ± 58	3662 ± 99[b]

In the acute experiment, saline or EGb 761 (100 mg kg^{-1}) was administered intragastrically to mice 14 and 2 h before death. In the semi-chronic experiments, saline or EGb 761 (100 mg kg^{-1}) was administered intragastrically twice a day with a 2 h interval, for 4 consecutive days; animals were killed 16 h after the last administration and synaptosomes were prepared from cerebral cortex. [^{3}H]5-HT uptake was measured after a 5 min incubation period in the presence of [^{3}H]5-HT (20 nM, final concentration). [a]$P < 0·05$, [b]$P < 0·01$ compared with respective saline controls (10 mice per group). The synaptosomal fraction prepared from each mouse was tested in triplicate.

In-vitro effects of EGb 761 on the synaptosomal uptake of [^{3}H]dopamine. [^{3}H]Dopamine uptake by synaptosomes prepared from striatum of mice was not modified by EGb 761 at concentrations between 2 and 16 μg mL^{-1}; [^{3}H] uptake was 21547 ± 122 and 21116 ± 215 fmol (mg protein)$^{-1}$ in the absence and in the presence of 16 μg mL^{-1} EGb 761, respectively (means ± s.e.m. of 3 experiments in triplicate).

Discussion

[^{3}H]5-HT uptake by mouse cortex synaptosomes is increased at low concentrations of EGb 761 and decreased at high concentrations. The latter effect is non-specific as it also occurred for choline and dopamine (Ramassamy et al 1990). The increased [^{3}H]5-HT uptake at lower concentrations of EGb 761 (10–20 μg mL^{-1}) could have a therapeutic relevance. The effect is also seen after oral administration indicating that substances responsible can reach the brain at an effective concentration. While the effective dose of EGb 761 (100 mg kg^{-1}) is much greater than doses given to man, it should be pointed out that effective doses in mice are usually greater than in man and that the drug was much diluted during the preparation and incubation of synaptosomes. Thus the EGb 761-induced increase in the [^{3}H]5-HT synaptosomal uptake might occur at therapeutic doses. A similar increase in [^{3}H]5-HT uptake has been described with the antidepressant drug tianeptine, both ex-vivo on [^{3}H]5-HT synaptosomal uptake (Fattaccini et al 1990) and in-vivo by microdialysis (Whitton et al 1991), although the drug did not increase the [^{3}H]5-HT uptake in-vitro (Mennini et al 1987). The activity of some antidepressants seems linked to their ability to inhibit the neuronal 5-HT uptake; however, the antidepressant activity of tianeptine appears well-documented. Tianeptine and EGb 761 are effective in the learned helplessness (Porsolt et al 1990) and mice-despair (Clostre et al 1988) tests; this could suggest a link between the increase in 5-HT uptake and the effectiveness of these drugs in these tests, which would be predictive of an antidepressant activity.

The 5-HT uptake complex is clearly involved in the synaptosomal storage of radioactivity, since the 5-HT-uptake inhibitor, clomipramine, suppressed both the synaptosomal concentration of [^{3}H]5-HT and its increase by EGb 761. We have verified that the increase in [^{3}H]5-HT synaptosomal uptake was neither an artefactual consequence of an increase in the free concentration of [^{3}H]5-HT in the incubation medium (due to a prevention by EGb 761 of its non-specific binding to the surface of incubation vials) nor an increase in the non-specific binding of [^{3}H]5-HT to the filters (data not shown).

The increase in synaptosomal [^{3}H]5-HT uptake, induced by EGb 761, was shared by the Cp 202 fraction, which mostly contains flavonoid substances. This fraction was effective at lower doses (> 1·25 μg mL^{-1}) than EGb 761 (> 4 μg mL^{-1}), suggesting the involvement of flavonoid substances. However, quercetin, one of the most prominent flavonoids in the Cp 202 fraction, was ineffective, and other flavonoids or substances present in the Cp 202 extract, such as proanthocyanidines, must be involved. The BN 52063 fraction, devoid of flavonoid derivatives and containing mostly terpenes, was ineffective.

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