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Prevention by a *Ginkgo biloba* Extract (GBE 761) of the Dopaminergic Neurotoxicity of MPTP

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Abstract—In mice implanted subcutaneously with osmotic minipumps releasing the neurotoxic agent *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) for 7 days (105 μg h^{-1}/mouse) ($\approx$ 100 mg kg^{-1} day^{-1}) a significant reduction ($\approx$25%) in the striatal dopaminergic nerve endings was observed. This neurotoxic effect was prevented by the semi-chronic ingestion of a *Ginkgo biloba* extract for 17 days (GBE 761, $\approx$ 100 mg kg^{-1} day^{-1}). The high concentrations ($\approx$ 1 g L^{-1}) at which GBE 761 in-vitro either prevented the uptake of [^{3}H]dopamine by synaptosomes prepared from striatum, or prevented the specific binding of the pure dopamine uptake inhibitor [^{3}H]GBR 12783 to membranes prepared from striatum suggests that the prevention of the MPTP neurotoxicity does not depend on an inhibition of the MPTP uptake by dopamine neurons. This is also suggested by the lack of prevention of the in-vitro striatal binding of [^{3}H]GBR 12783 administered i.v. at a tracer dose, in mice pretreated for 8 days with GBE 761 (100 mg kg^{-1} p.o.) and receiving a supplementary gastric administration of GBE 761 (100 mg kg^{-1}) 1 h before testing. Similar treatment with GBE 761 did not modify the toxicity for dopamine neurons of 6-hydroxydopamine (20 μg) directly injected into the striatum of rats.

Parkinson's disease is characterized by the degeneration of the dopaminergic nigrostriatal pathway, as indicated by the severe loss of substantia nigra neurons (Escourolle et al 1971) and by the decrease in striatal dopamine (DA) concentration (Ehringer & Hornyckiewicz 1960). Several lines of evidence have suggested that this may be the result of long term exposure to subthreshold doses of a neurotoxic factor which is either exogenous, or is endogenously synthesized (Calne & Langston 1983; Langston 1987), specifically directed against nigral DA neurons. The *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism in young drug users has strongly contributed to the conception of this hypothesis (Burns et al 1983; Langston et al 1983; Heikkila et al 1984a). MPTP is transferred through the blood brain barrier into the central nervous system; it is oxidized mainly by the type B monoamine oxidase (Heikkila et al 1984a,b; Langston et al 1984; Sundstrom & Jonsson 1986) and after its conversion into *N*-methyl-4-phenylpyridinium (MPP^+), it is taken up by neurons causing their degeneration (Javitch et al 1985; Keller & Da Prada 1985). The possibility that formation of free radicals might be involved in the development of this degeneration (Cohen 1987; Grimes et al 1987) is supported by the enhancement of MPTP-induced depletion of striatal DA by an inhibitor of superoxide dismutase (Corsini et al 1985).

In order to select drugs which could exert protective effects against agents toxic for nigrostriatal DA neurons, we have used MPTP slowly and semi-chronically released from an osmotic minipump subcutaneously implanted in mice.

We report here the protection exerted by a *Ginkgo biloba* extract (GBE 761). Its effects have been investigated because it is widely used in cerebral senescence, whereas several reports indicate its action on DA neuronal metabolism (Rapin & Le Poncin-Laffite 1979) as well as its free radical scavenging properties (Pincemail & Deby 1986). A protective activity of GBE 761 against MPTP neurotoxicity having been observed, we have begun to investigate the mechanism underlying this protection by determining whether it was the consequence of an inhibition of the neuronal toxin uptake.

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Materials and Methods

Animals

Male Sprague Dawley rats, 220–250 g, and male Swiss albino mice, 25–30 g, CD1 (Charles River, Saint Aubin lès Elbeuf, France), were housed (5 mice per makrolon box 25 × 20 × 13 cm; 5 rats per makrolon box 31 × 28 × 20 cm) in a well ventilated room, at an ambient temperature of 22°C, with 0700–1900 h light-dark cycle. Food was freely available and water was available according to the experimental conditions.

Minipump implantation in mice

2001 osmotic minipumps (Alza Corporation, Palo Alto, CA, USA) were filled with 200 μL of either distilled water (controls) or MPTP solution (42 mg, 200 μL). For implantation as well as for removal of minipumps, mice were anaesthetized with pentobarbitone (40 mg kg^{-1} i.p.). A small incision (about 4 mm) was made in the skin of the median part of the back, through which the minipump was subcutaneously introduced. The incision was closed with an iron clip. The hourly rate of infusion was 0·5 μL, corresponding to 105 μg of MPTP ($\approx$ 100 mg kg^{-1} day^{-1}). The minipump was removed 7 days after implantation.

Uptake of [^{3}H]dopamine, [^{3}H]5-HT or [^{3}H]choline by synaptosomal preparations

Crude synaptosomal fractions (S1) were obtained by homogenization (Potter-Elvehjem, clearance 80–130 μm) of the

striata or cortex in 10 volumes of ice-cold 0·32 M sucrose with pargyline 10^{-4} M, according to Snyder & Coyle (1969) followed by centrifugation (1000 *g*, 10 min, 2°C). Portions of the supernatants (50 μL) were preincubated (5 min, 37°C) with 940 μL of Krebs-Ringer phosphate buffer containing GBE 761 at different concentrations; the medium was gassed (95% O_2, 5% CO_2) for 30 min immediately before use, then [^{3}H]dopamine ([^{3}H]DA) 20 nM, final concentration (10 μL) or [^{3}H]5-HT 20 nM, final concentration (10 μL) or [^{3}H]choline 100 nM, final concentration (10μL) were added. After an incubation period (3 min for [^{3}H]DA and [^{3}H]choline or 5 min for [^{3}H]5-HT) the uptake was stopped by dilution with ice-cold Krebs-Ringer buffer (4 mL) followed by vacuum filtration (Millipore 0·45 μm filters, previously soaked with Krebs-Ringer phosphate buffer). Each tube was rinsed and filters were washed twice with 4 mL ice-cold Krebs-Ringer buffer and dried for 1 h in a ventilated incubator (60°C). Filters were placed in minivials containing 5 mL Aqualyte (J. T. Baker Chemical, Deventer, Holland). The radioactivity was determined by liquid scintillation spectrometry. Non-specific uptake was determined at 0°C in similar conditions. The specific uptake was expressed as fmol mg^{-1} of protein. The protein concentration was determined by the method of Lowry et al (1951).

Binding of [^{3}H]GBR 12783 to striatal membranes

A crude synaptosomal fraction was obtained by homogenization of striata of mice in 10 volumes (w/v) of ice-cold 0·32 M sucrose, using a Teflon-glass homogenizer. The nuclear material was removed by centrifugation (1000 *g*, 10 min, 2°C) and the supernatant was stored. The pellet was resuspended in 0·32 M sucrose and recentrifuged (1000 *g*, 10 min, 2°C). The two supernatants were combined and centrifuged at 17 500 *g* for 30 min (2°C). The resulting pellet was sonicated (microsonde diameter = 3 mm; Sonics Materials, Inc, Danbury, CT, USA) in 20 volumes of a bicarbonate buffer of the following composition (in mM): NaCl 120; NaH_2PO_4 0·2; $NaHCO_3$ 9·8. The membranes were centrifuged at 50 000 *g* for 10 min at 2°C. The supernatant was discarded and the pellet was resuspended in 20 volumes of the same medium, by sonication. The protein concentration was determined and the membrane preparation was diluted to obtain a concentration of 50 μg of membrane protein mL^{-1}. Membranes were incubated in bicarbonate buffer containing 0·01% bovine serum albumin. The binding of [^{3}H]GBR 12783 was measured after an 18 h incubation at 0°C by addition of 400 μL [^{3}H]GBR 12783 (final concentration 0·4 nM) to silicone coated tubes containing 1 mL of membrane preparation and 2·6 mL of bicarbonate buffer containing GBE 761. The non-specific binding was measured in the presence of 3 μM mazindol. The incubation was terminated by vacuum filtration through Whatman GF/B filters previously soaked for 1 h in bicarbonate buffer containing 0·5% polyethylene imine. Each tube was rinsed and the filters were washed twice with 5 mL of ice-cold bicarbonate buffer. The radioactivity remaining on the filters was measured by liquid scintillation spectrometry. The specific binding was calculated by subtracting the non specific binding from the total binding.

Striatal in-vivo binding of [^{3}H]GBR 12783

In-vivo cerebral binding of [^{3}H]GBR 12783 was measured according to Vaugeois et al (1990). The GBE 761 was administered in the drinking water (3 mg/5mL/day/mouse ≈ 100 mg kg^{-1}) for 3 weeks before the test. In addition, 30 min before the i.v. administration (in the tail) of the tracer dose of [^{3}H]GBR 12783 (5 μCi/mouse, 200 μL) the treated animals received through a gastric cannula 100 mg kg^{-1} of GBE 761 (5 mL kg^{-1}) and controls received tap water. Mice were killed 1 h after the [^{3}H]GBR 12783 injection and the radioactivity in striatum and cerebellum measured after sonication of these tissues in 0·9% NaCl (5 mL for cerebellum, 1 mL for striatum). A 750 μL sample of each homogenate was counted, in a minivial containing 5 mL Aqualyte, by liquid scintillation spectrometry (SL 2000, Kontron Intertechnique, Trappes, France). Protein concentrations were determined on a 20 μL sample of each homogenate. Results were expressed in d min^{-1} (mg protein)$^{-1}$.

Intrastriatal injection of 6-hydroxydopamine (6-OHDA) in rats

Rats drinking GBE 761 (≈ 100 mg kg^{-1} day^{-1}) for 7 days or tap water (controls) were anaesthetized with an i.p. injection of chloral hydrate (400 mg kg^{-1}) and placed into a stereotaxic instrument (David Kopf) to enable a unilateral striatal injection of 6-OHDA (20 μg, 5 μL, in saline plus ascorbic acid 0·02%) to be given. The coordinates used were A P: 2 mm, ML: 3 mm, DV: 6 mm according to the atlas of Pellegrino et al (1979). Following a 3 week delay, during which rats continued to drink either GBE 761 solution or tap-water with ethanol (1%), the animals were killed and their striata were rapidly removed on ice and used to prepare synaptosomes.

Drugs

The *Ginkgo biloba* extract (GBE 761) was prepared by Henri Beaufour Institute. It is a well-defined and complex product prepared from green leaves of *Ginkgo biloba*. The leaves are dried and subjected to a 15-step extraction procedure, commencing with an acetone-water mixture under partial vacuum. The final extract is standardized to contain 24% flavonoid glycosides (ginkgo flavone glycosides) and 6% terpene lactones which are characteristic of ginkgo and have a unique structure (ginkgolides, bilobalide (Drieu 1986). The dried extract was diluted in Krebs-Ringer phosphate or in bicarbonate buffer when tested in-vitro. When added to drinking tap water we used the commercial solution marketed by Ipsen (Tanakan). This hydro-alcoholic solution in tap water (final concentration 100 mg kg^{-1} day^{-1}) had, after dilution, an alcoholic content of 1°. *N*-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP) was purchased from R.B.I. (Bioblock). 6-Hydroxydopamine hydrobromide (6-OHDA) was purchased from Sigma and was dissolved in saline containing 0·1% ascorbic acid to prevent oxidation. [^{3}H]DA (20 Ci $mmol^{-1}$), [^{3}H]5-HT (9·3 Ci $mmol^{-1}$) and [^{3}H]choline (78·2 Ci $mmol^{-1}$) were obtained from Amersham (Les Ulis, France). [^{3}H]GBR 12783 (29·2 Ci $mmol^{-1}$; >98% purity) was prepared by reduction of the amide precursor with [^{3}H]diborane, by Amersham (Les Ulis, France). Mazindol was donated by Sandoz (Basel, Switzerland).

Statistics

Significant differences between groups were determined by analysis of variance (ANOVA) followed by Student's *t*-test.

The IC50 values were determined according to Litchfield & Wilcoxon (1949).

Results

Effect of GBE 761 ingestion on the lesioning effect of MPTP on striatal dopaminergic nerve endings

In mice implanted with an osmotic minipump releasing MPTP for 7 days at the rate of 105 μg h^{-1}, the decrease in [^{3}H]DA uptake by a synaptosomal fraction prepared from striatum, one week after the minipump removal was about 25%. In similar conditions mice which had access to a GBE 761 solution, from 3 days before the minipump implantation up to the time of death displayed no decrease in [^{3}H]DA synaptosomal uptake (Table 1). The daily ethanolic solution intake in control mice implanted with minipumps filled with water, and in control mice implanted with minipumps filled with MPTP, was respectively 5·5 ± 0·1 and 5·4 ± 0·1 mL. The daily GBE 761 solution intake in control and MPTP-treated was respectively 5·2 ± 0·3 and 5·2 ± 0·3 mL. Thus the daily mean intake of GBE 761 corresponded to 104 ± 0·6 mg kg^{-1} in both groups.

Effects of increasing concentrations of GBE 761 on the synaptosomal uptake of [^{3}H]DA, [^{3}H]5-HT and [^{3}H]choline

The specific uptake of [^{3}H]DA, [^{3}H]5-HT and [^{3}H]choline (Fig. 1) by synaptosomes, prepared from striatum of mice, was decreased by GBE 761 in a concentration dependent manner. The inhibition of [^{3}H]DA and [^{3}H]5-HT uptake reached a significant level from 500 μg mL^{-1} and it was virtually complete at 2000 μg mL^{-1}. For [^{3}H]choline uptake this effect became significant only at 2000 μg mL^{-1}. The IC50 values were 637 (413–984) and 803 (273–2355) μg mL^{-1} for the striatal synaptosome uptake of [^{3}H]DA and [^{3}H]5-HT, respectively. The IC50 was 745 (227–2445) μg mL^{-1} for the uptake of [^{3}H]5-HT by frontal cortex synaptosomes.

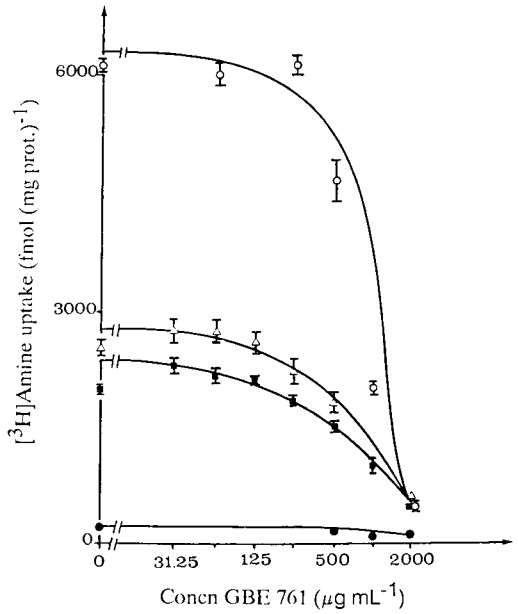

FIG. 1. Effects of increasing concentrations of the GBE 761 on the synaptosomal uptake by dopamine 5-HT and choline. Synaptosomal fractions prepared from either striatum of mice or frontal cortex of mice were incubated for 3 min with [^{3}H]DA (20 nM) or for 5 min with [^{3}H]5-HT (20 nM) in the presence of increasing concentrations of GBE 761. The specific [^{3}H]DA uptake (○——○) in controls was 6147 ± 92 fmol (mg protein)$^{-1}$; the specific [^{3}H]5-HT uptake (△——△) in controls was 2004 ± 64 fmol (mg protein)$^{-1}$ (striatum) and 2529 ± 114 fmol (mg protein)$^{-1}$ (= 100%) (cortex) (■——■); the specific [^{3}H]choline uptake (●——●) in controls was (200 ± 31) fmol (mg protein)$^{-1}$. Mean ± s.e.m. of 3 determinations made in triplicate.

Table 1. Effect of GBE 761 ingestion on the striatal dopaminergic nerve endings lesion induced by MPTP. From the third day before minipump implantation up to death 7 days after minipump withdrawal, mice of groups I and III had free access to drinking water with an ethanolic content of about 1%. Mice of groups II and IV had free access to the solution of GBE 761 of the same ethanolic content as the control solution. In groups I and II minipumps were filled with distilled water; in groups II and IV they were filled with MPTP solution. Minipumps released the drugs at 105 μg h^{-1} ($\approx$ 100 mg kg^{-1} day^{-1}). Minipumps remained in place for 7 days. Mice were killed 7 days after the withdrawal of the minipumps. The specific [^{3}H]DA uptake was determined on synaptosomes prepared from the 2 pooled striata of each mouse.

Groups and treatments	Specific [^{3}H]DA uptake fmol (mg protein)$^{-1}$
I. Alcoholic drinking water + minipump filled with water (n = 16)	1454 ± 94
II. GBE 761 solution + minipump filled with water (n = 16)	1254 ± 81
III. Alcoholic drinking water + minipump filled with MPTP (n = 16)	1029 ± 73*
IV. GBE 761 solution + minipump filled with MPTP (n = 13)	1421 ± 82

* $P < 0{\cdot}01$ as compared to group I and to group IV.

Effect of increasing concentrations of GBE 761 on the specific binding of [^{3}H]GBR 127823 to striatal membranes

In a membrane fraction prepared from striatum of mice, GBE 761 decreased the specific binding of [^{3}H]GBR 12783 in a concentration dependent manner (Fig. 2). This inhibition was significant from 62·5 μg mL^{-1} and was virtually complete at 500 μg mL^{-1}. The dose-response curve was shifted to the left relative to that of the [^{3}H]DA synaptosomal uptake inhibition elicited by GBE 761. The IC50 for each parameter was, respectively, 118 (51–276) μg mL^{-1} ([^{3}H]GBR 12783 binding) and 637 (413–984) μg mL^{-1} ([^{3}H]DA uptake).

Effect of GBE 761 ingestion on the striatal in-vivo binding of [^{3}H]GBR 12783

In mice drinking GBE 761 ($\approx$ 100 mg kg^{-1} day^{-1}) for the 3 weeks preceding the test and receiving intragastrically a supplementary dose (= 100 mg kg^{-1}), 30 min before the i.v. injection of the tracer dose of [^{3}H]GBR 12783 there was a slight, but non significant, increase in the level of radioactivity in both striatum and cerebellum, relative to the controls. The difference in the radioactivity retained in the two tissues, 1 h after the i.v. injection, was similar in controls and in mice treated with GBE 761 (Table 2).

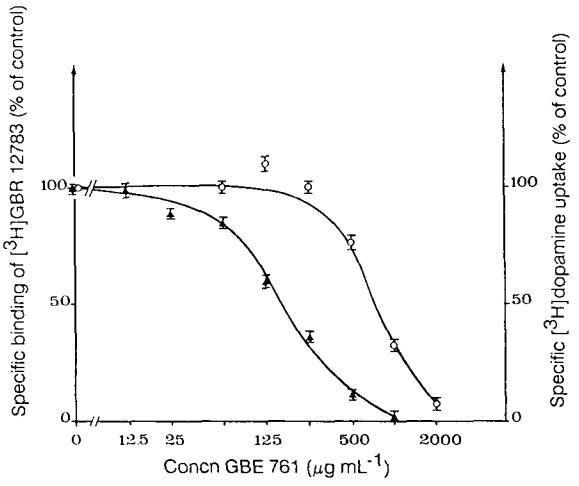

FIG. 2. Effects of increasing concentrations of GBE 761 on the specific binding of [^{3}H]GBR 12783 to striatal membrane. A membrane fraction prepared from striatum of mice was incubated with [^{3}H]GBR 12783 (0·4 nM) and increasing concentrations of GBE 761 at 0°C for 18 h (▲——▲). The specific [^{3}H]GBR 12783 binding in controls was 2134 ± 43 fmol (mg protein)$^{-1}$. For comparison with the inhibition of [^{3}H]DA uptake (O——O), the corresponding dose response curve of Fig. 1 has been included. Mean ± s.e.m. of 3 determinations made in triplicate.

Table 2. Effect of GBE 761 ingestion on the in-vivo binding of [^{3}H]GBR 12783 to striatum and cerebellum in mice. Mice were grouped 5 to a cage. GBE 761 was added to the drinking water (≈ 100 mg kg^{-1}) for 3 weeks before the test. Thirty min before the i.v. administration of the tracer dose of [^{3}H]GBR 12783 (5 μCi/mouse), the group drinking GBE 761 received through a gastric cannula 100 mg kg^{-1} (5 mL kg^{-1}) GBE 761. Controls drank tap water and received intragastrically tap water (5 mL kg^{-1}) 30 min before the i.v. injection.

	Radioactivity (d min^{-1} (mg protein)$^{-1}$)		
Treatment	Striatum	Cerebellum	Difference
Water	801 ± 211	277 ± 59	523 ± 174
GBE 761	953 ± 140	401 ± 34	552 ± 107

Mean ± s.e.m. n = 5.

Effect of GBE 761 ingestion on the 6-hydroxydopamine-induced lesion of striatal dopaminergic nerve endings

In rats drinking GBE 761 (≈ 100 mg kg^{-1} day^{-1}) for one week preceding the intrastriatal microinjection of 6-OHDA (20 μg, 5 μL) and for a further 3 weeks, there was no modification in the 6-OHDA-induced dopaminergic denervation, relative to controls. Whereas the [^{3}H]DA uptake by synaptomes prepared from the 6-OHDA injected striatum was decreased by 57·6 ± 8·5% relative to the contralateral striatum in control rats, the decrease in rats drinking GBE 761 was 53·2 ± 11·2% relative to contralateral striatum (mean ± s.e.m., n = 5 per group). The [^{3}H]DA uptake by contralateral striatum in animals injected with 6-OHDA was 6619 ± 577 fmol (mg protein)$^{-1}$ in control animals and 5639 ± 514 fmol (mg protein)$^{-1}$ in GBE 761 treated rats.

Discussion

The main finding of this study is in the suppression of the MPTP-induced denervation of striatal dopaminergic afferents by GBE 761 ingested semi-chronically.

The semi-chronic and slow release of MPTP could participate in the mechanism involved in the etiology of some cases of Parkinson's disease (see review by Langston 1987). It is now established that MPTP exerts its central neurotoxic effect in several stages: (i) after a systemic administration, it crosses the blood brain barrier in its native form; (ii) it is oxidized by monoamine oxidase B into MPP$^+$ (Heikkila et al 1984b; Langston et al 1984; Sundstrom & Jonsson 1986); (iii) this metabolite is concentrated by the DA uptake complex into dopaminergic neurons; (iv) in body cells of nigrostriatal DA neurons the neuromelanin seems to be involved in the MPP$^+$ storage (D'Amato et al 1986); (v) finally it probably destroys DA neurons by generation of free radicals (Perry et al 1985).

Thus the prevention by GBE 761 of the MPTP-induced dopamine denervation might depend on several mechanisms. Among these we have investigated whether GBE 761 acted by preventing the neuronal uptake of MPTP/MPP$^+$. For this purpose we considered the in-vitro and in-vivo effects of GBE 761 on the DA uptake complex associated with striatal dopaminergic nerve endings. The high concentrations at which GBE 761 either inhibits the [^{3}H]DA uptake by synaptosomes or competes with the specific binding of [^{3}H]GBR 12783 at the dopamine uptake complex associated with striatal dopamine nerve endings (Bonnet & Costentin 1986) do not suggest that the blockade of MPTP/MPP$^+$ uptake is responsible for the prevention of its neurotoxic effect. The in-vitro concentrations at which GBE 761 is effective (in the 1 g L^{-1} range) on these two parameters cannot be obtained in-vivo by the daily intake of GBE 761 (about 0·1 g kg^{-1}) in mice; however, the semi-chronic injection of GBE 761 could allow either one of its components, or a metabolite of one of them to accumulate in the brain displaying a DA uptake complex inhibitory effect. Against this hypothesis, we have observed that the chronic intake of GBE 761, with additional intragastric administration a short time before the in-vivo measurement of striatal [^{3}H]GBR 12783 binding, did not modify this latter process. We carried out this experiment to evaluate in-vivo the occupancy of the site which binds either reference DA uptake inhibitors or substrates of the DA uptake complex (Chagraoui et al 1987; Vaugeois et al 1990). The inhibition by GBE 761 of the [^{3}H]DA synaptosomal uptake is not a specific effect since, in the range of effective doses, the [^{3}H]5-HT uptake was also inhibited, in synaptosomes prepared from striatum (where an important participation of DA nerve endings might be expected) as well as from frontal cortex (where a participation of DA nerve endings is relatively weak) (Bonnet et al 1986). This lack of specificity is also suggested by our observation that GBE 761 inhibited the synaptosomal uptake of [^{3}H]choline, although this inhibition occurred at higher doses. At these high doses several non-specific mechanisms may be operative such as complexation of ligands by some components of GBE 761 or physicochemical modifications of the membranes. In addition there is a discrepancy between the concentrations at which GBE 761 prevented the [^{3}H]GBR 12783 binding and the [^{3}H]DA uptake; this could reinforce the suggestion of non-specificity. The GBE 761, although semi-chronically

administered, did not prevent the 6-OHDA-induced striatal lesion of dopaminergic nerve endings in the conditions selected by Cadet et al (1989) from exhibiting the attenuating effect of vitamin E. The lack of protective effect of GBE 761 in this experimental situation may depend on the acute lesioning effect of 6-OHDA which could surpass the instantaneous protective capacity of GBE 761. However, this does not exclude the possibility that GBE 761 might, like vitamin E, exert an antioxidant activity and that it might protect neurons against the toxic effects of oxyradicals produced by MPP^+. GBE 761 may also protect against MPTP-induced toxicity by interacting peripherally with the metabolism of MPTP or with its cerebral access.

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