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GINKGO BILOBA EXTRACT EGB 761 OR TROLOX C PREVENT THE ASCORBIC ACID/FE^{2+} INDUCED DECREASE IN SYNAPTOSOMAL MEMBRANE FLUIDITY

CHARLES RAMASSAMY, FRANCK GIRBE, YVES CHRISTEN* and JEAN COSTENTIN

Unité de Neuropsychopharmacologie Expérimentale, U.R.A. 1170 du C.N.R.S., Faculté de Médecine & Pharmacie de Rouen, 76803 Saint-Etienne du Rouvray, FRANCE

**Institut IPSEN, 30 rue Cambronne, 75015 Paris cedex 15*

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The ability of synaptosomes, prepared from striata, to take up ^{3}H-dopamine declined rapidly during incubation at 37°C, in an oxygenated Krebs-Ringer medium with 0.1 mM ascorbic acid. Ascorbic acid was responsible for this decrease. Its effectiveness after a 60 min incubation was concentration dependent from 1 μM and virtually complete for 0.1 mM. Furthermore, a decrease of synaptosomal membrane fluidity was revealed by measurements of fluorescence polarization using 1,6-diphenyl-1,3,5-hexatriene. This decrease was potentiated by Fe^{2+} ions (1 μM). In contrast, it was prevented by the Fe^{2+} ion chelator, desferrioxamine (0.1 mM), by the Ginkgo biloba extract EGb 761 [2-16 μg/ml], as well as by the flavonoid quercetin (0.1 μM). This preventive effect was shared by trolox C (from 0.1 mM). It is concluded that peroxidation of neuronal membrane lipids induced by ascorbic acid/Fe^{2+} is associated with a decrease in membrane fluidity which, in turn, reduces the ability of the dopamine transporter to take up dopamine.

KEY WORDS: Membrane fluidity, Ginkgo biloba extract (EGb 761), diphenyl-hexatriene (DPH), dopamine uptake, synaptosomes, ascorbic acid/Fe^{2+}

INTRODUCTION

We have recently reported[1] that, during a long-term incubation of synaptosomes a dramatic decrease in ^{3}H amine uptake (^{3}H-dopamine for synaptosomes prepared from striatum, ^{3}H-5-HT for synaptosomes prepared from cortex) occurred in the presence of ascorbic acid. This effect has been related to the generation of free radicals operated by the couple ascorbic acid/Fe^{2+} ions. It was observed that the suppression of either ascorbic acid or the complexation of Fe^{2+} ions by desferrioxamine prevented the decrease in ^{3}H-amine uptake. In addition, free radical scavengers (trolox C or the Ginkgo biloba extract EGb 761, acting by its flavonoid fraction) also prevented the decrease in ^{3}H-amine uptake. We tested EGb 761 since it has been claimed to display free radical scavenger properties[2,3]. Our interest in EGb 761 was strengthened by its therapeutic use for treatment of cerebral disorders resulting from ischemia, anoxia, cerebral degenerative diseases[4,5] in which free radicals may participate. One of the consequences of free radical generation is lipid peroxidation of neuronal membranes. Lipid peroxidation has been reported to alter membrane structure as determined by electron spin

resonance[6]. Under physiological conditions a balance between oxidant and anti-oxidant processes results in a low level of lipid peroxidation allowing an optimal functioning of ionic transports, enzymatic activities or receptor recognition by their ligands. Modifications in membrane fluidity induced by lipoperoxidation could affect these parameters[7,8]. In synaptic transmission some parameters such as release of amines may change in response to modifications in membrane fluidity.

Since the recently cloned DA carrier[9,10] seems to function as a channel, possibly alternately opened inside towards the cytosol and outside towards the synaptic cleft, one is prompted to imagine that a decrease in membrane fluidity could alter its functioning and thereby dopamine (DA) uptake. This hypothesis led us, in the present study, to compare the modifications in ^{3}H DA synaptosomal uptake and in membrane fluidity under peroxydative conditions (as provided by the couple Fe^{2+}/ascorbic acid). A simultaneous decrease on both parameters was observed, and we then tried to prevent them using trolox C[11] and the Ginkgo biloba extract EGb 761.

MATERIALS AND METHODS

Animals

Male Swiss albino mice, 25–30 g (CD1; Charles River, Saint Aubin lès Elbeuf, France) were used. The animals were housed in a well-ventilated room, at an ambient temperature of 22°C, with a 7 am–7 pm light-dark cycle. Food and water were available ad libitum.

Synaptosomal Preparations

A crude synaptosomal fraction (S1) was obtained by homogenization (Potter-Elvehjem, clearance 80–130 μm) of the cortex in 10 volumes of ice-cold, 0.32 M sucrose[12] containing pargyline (0.1 mM), followed by centrifugation (1000 g, 10 min, 2°C).

Incubation Conditions

The 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) was gassed (95% O_2, 5% CO_2) for 30 min prior to use. Aliquots of the supernatant (50 μl) were pre-incubated (37°C) with 900 μl of buffer. ^{3}H-Dopamine (^{3}H-DA) at a final concentration of 20 nM (50 μl) was then added.

3H-Dopamine Synaptosomal Uptake

After an incubation period in the presence of the amine (5 min), the uptake was stopped by dilution with ice-cold Krebs-Ringer buffer (4 ml) followed by vacuum filtration through filters (Whatman GF/B) previously soaked with Krebs-Ringer phosphate buffer. Each tube was rinsed and filters were washed twice with 4 ml

ice-cold Krebs-Ringer buffer; they were then dried for 1 h in a ventilated incubator (60°C). Filters were put 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 under similar conditions. The specific uptake was expressed as fmoles/mg of protein. The protein concentration was determined by the method of Lowry *et al.*[13].

Fluorescence Polarization

Synaptosomes were incubated with or without ascorbic acid (0.1 mM) for 60 min at 37°C. Fluorescence emitted under polarized light by synaptosomes prepared from mouse striatum was determined using the 1,6-diphenyl-1,3,5-hexatriene (DPH) probe dissolved in tetrahydrofuran (THF) (DPH reportedly measure fluidity at membrane depths corresponding to those of fatty acid side chains and between lipid bilayers)[14]. Then synaptosomes were incubated with 1 μM DPH in Krebs-Ringer, pH 7.4, at 37°C for 60 min. Fluorescence was determined in a Shimadzu RF 5000 fluorescence spectrophotometer, equipped with rotating polarizing filters. Excitation and emission wavelengths of 360 nm and 430 nm, respectively, were used[15-16]. The cuvette, under stirring, was maintained at 37°C with a circulating water bath. Polarization (P) was calculated directly from the following equation:

$$P = (Ivv - Ivh^{\cdot}(G))/(Ivv + Ivh^{\cdot}(G))$$

where *Ivv* is the fluorescence intensity with excitation and emitted light polarized vertically and *Ivh* is the fluorescence intensity with a vertical orientation of the exciting polarizer and the emitted fluorescence passing through a horizontal polarizer. A correction factor for instrument asymmetry was made using $G = Ihv/Ihh$ where *Ihv* is fluorescence intensity with horizontal excitation light and emitted light polarized vertically and Ihh corresponds to the value with the excitation and emitted light polarized horizontally. All values of Ivh were multiplied by G in the calculation of P, in order to compensate for the sensitivity of the detection system towards vertically and horizontally polarized light.

Statistical Analysis

Differences between groups were determined either by analysis of variance (ANOVA 2-ways), by Dunnett's-t-test or by Student's-t-test.

Drugs

The Ginkgo biloba extract (EGb 761) was prepared by IPSEN Institute from leaves of Ginkgo biloba. The final extract of EGb 761 was standardized to contain 24% of flavonoid glycosides (ginkgo flavone glycosides) and 6% terpene lactones (ginkgolides, bilobalides)[17]. EGb 761 was dissolved in Krebs-Ringer phosphate buffer.

Quercetin, tetrahydrofuran (THF) and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Sigma (France); trolox C was purchased from Aldrich; desferrioxamine was a generous gift from Ciba-Geigy and, ^{3}H-DA (12 Ci/mmol) was obtained from Amersham (Les Ulis, France).

RESULTS

Influence of Experimental Conditions on the Emitted Fluorescence

Addition of ascorbic acid (0.1 mM) to a solution of DPH (1 μM) in THF, at 37°C, did not modify the spontaneous emitted fluorescence. Thus the fluorescence polarization value (P) for DPH alone was 0.060 ± 0.006 while in the presence of ascorbic acid it was 0.058 ± 0.001. Similar observations were made at 0°C with $P = 0.053 \pm 0.009$ for DPH alone and P = 0.058 ± 0.008 for DPH in the presence of ascorbic acid.

We also considered the influence on the emitted fluorescence of the order in which DPH or ascorbic acid were added to the synaptosomal preparation. In a first set of experiments, the fluorescence was measured after 1, 2 or 3 hour incubation periods of synaptosomes with DPH. Regardless of the incubation periods, the emitted fluorescence P was roughly similar (0.277 ± 0.004; 0.288 ± 0.003; 0.284 ± 0.004; M ± SEM of 5 determinations made in triplicate).

When DPH and ascorbic acid were simultaneously added to the synaptosomal preparation, the fluorescence $P = 0.296 \pm 0.005$ did not differ significantly from that obtained without ascorbic acid (0.287 ± 0.003). When ascorbic acid was added 1 h before the probe and maintained during the 1 hour of its incorporation, P became 0.322 ± 0.004, that differed significantly ($p < 0.001$) from each preceding values (M ± SEM of 5 determinations made in triplicate). When DPH was added 1 hour before ascorbic acid the increase in fluorescence induced by the latter was completely prevented ($P = 0.288 \pm 0.004$ without ascorbic acid and $P = 0.288 \pm 0.003$ with ascorbic acid, M ± SEM of 5 determinations made in triplicate).

These data suggested that DPH (in THF solution) may oppose the synaptosomal peroxidative process induced by ascorbic acid. Peroxidative conditions were thus established over a 1 hour period preceding the addition of DPH to the incubation medium.

Effect of DPH and THF on 3H-DA Uptake

The separate influence of the DPH solvent, THF, as well as that of DPH dissolved in THF, on the ability of synaptosomes to take up ^{3}H-DA with a 1 h preincubation period under normal or in peroxidative (ascorbic acid 0.1 mM) conditions was considered. We observed that under non peroxidative conditions, THF by itself exerted a slight but significant decrease in synaptosomal ^{3}H-DA uptake. In contrast, under peroxidative conditions, both THF alone and DPH dissolved in THF reduced the ascorbic acid-induced decrease in ^{3}H-DA uptake (Figure 1).

Influence of the Incubation Period in the Presence or in the Absence of Ascorbic Acid on Synaptosomal Membrane Fluidity

When synaptosomes were incubated in a Krebs Ringer medium with ascorbic acid (0.1 mM) for different periods, a time dependent decrease in their membrane fluidity was evidenced with the fluorescent probe DPH ($P = 0.298 \pm 0.004$ at the 5^{th}, $P = 0.310 \pm 0.003$ at the 20^{th}, $P = 0.317 \pm 0.003$ at the 40^{th} and $P = 0.318 \pm 0.005$ at the 60^{th} min of incubation; $r = 0.44$, $P < 0.001$). However, in the absence of ascorbic acid a smaller decrease in membrane fluidity was observed, which reached a plateau after the 20^{th} min of incubation ($P = 0.282 \pm 0.002$ at the 5^{th} and $P = 0.293 \pm 0.003$ at the 20^{th} min).

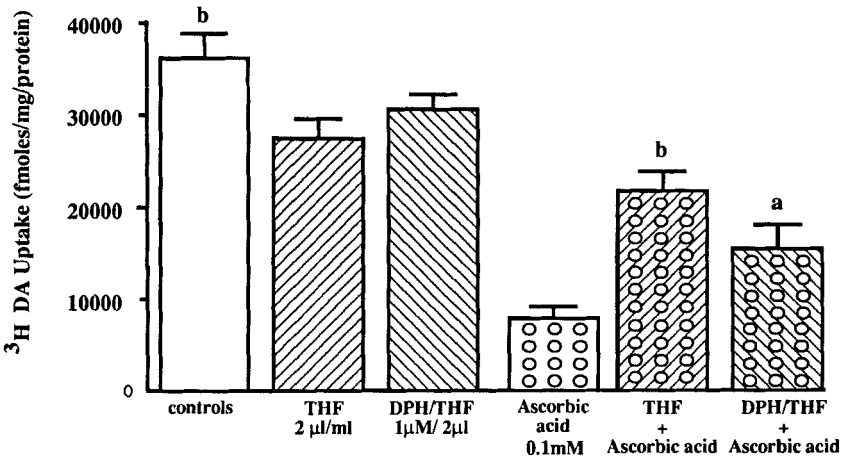

FIGURE 1 Effects of THF or DPH dissolved in THF associated or not with ascorbic acid on synaptosomal ^{3}H DA uptake. Synaptosomes were incubated during 60 min in the Krebs-Ringer medium with or without ascorbic acid (0.1 mM) in the presence of THF (24.6 mM final concentration) or DPH dissolved in THF (1 μM final concentration of DPH). ^{3}H DA (20 nM final concentration) was added for a further 3 min period of incubation, followed by the measure of its synaptosomal uptake. Means ± S.E.M of 3 experiments performed in triplicate. Differences between groups were determined by *Student's t-test*. *a*: $P < 0.05$ as compared to controls with ascorbic acid. *b*: $P < 0.001$ as compared to controls with ascorbic acid.

Effect of Different Concentrations of Ascorbic Acid on Synaptosomal Membrane Fluidity and ^{3}H DA Uptake

The decrease in membrane fluidity observed after a 1 h incubation was proportionally related to ascorbic acid concentrations between 1 μM and 1 mM. A decrease in the ability of synaptosomes to take up ^{3}H-DA occurred simultaneously. This decrease was also proportionally related to the ascorbic acid concentrations (between 1 μM and 0.1 mM) (Figure 2).

Effect of Ferrous Ions on Synaptosomal Membrane Fluidity

The decrease in membrane fluidity elicited by ascorbic acid (5 μM) was potentiated by addition of ferrous ions (1 μM) (Figure 3, upper panel). On the other hand the decrease in membrane fluidity elicited by ascorbic acid (0.1 mM) was prevented by the ferrous ions chelator desferrioxamine (0.1 mM) (Figure 3, lower panel).

Effect of EGb 761, Quercetin and Trolox C on the Modification in Synaptosomal Membrane Fluidity

The decrease in membrane fluidity elicited by ascorbic acid (0.1 mM) was prevented by EGb 761 in a concentration dependent manner. It was also prevented in a concentration dependent manner by quercetin (from 0.3 μM) or by trolox C (from 0.1 mM) (Table 1).

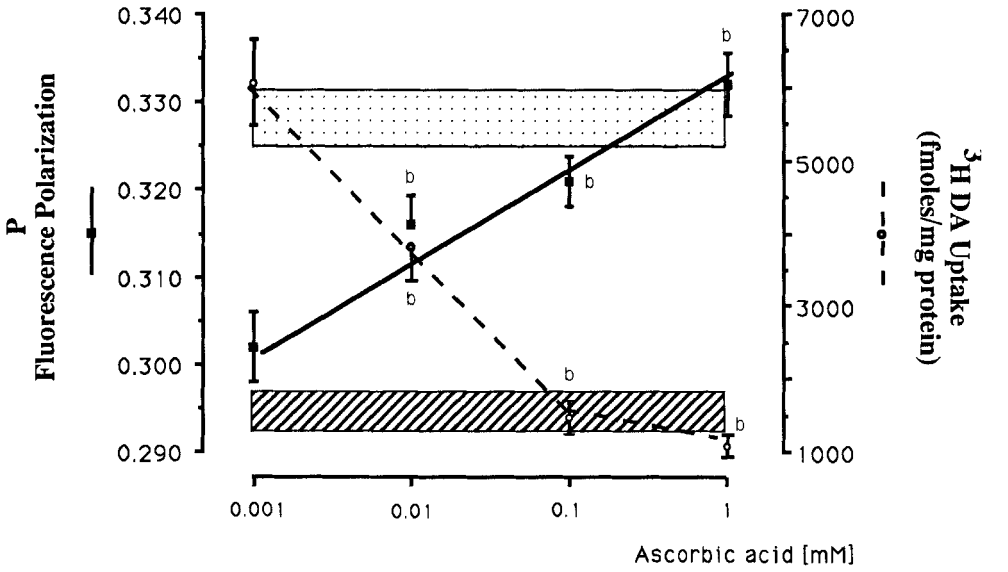

FIGURE 2 Comparison of the effects of increasing concentrations of ascorbic acid on the modifications in synaptosomal ^{3}H DA uptake and in fluorescence emitted by DPH. Synaptosomes were incubated in the presence of increasing concentrations of ascorbic acid 60 minutes before: (i) addition of ^{3}H-DA (20 nM final concentration) for a further 3 min period of incubation, followed by the measure of its synaptosomal uptake (broken line); the dotted area corresponds to the uptake ± S.E.M value of controls without ascorbic acid; (ii) addition of DPH dissolved in THF for a further 1 h period of incubation, followed by the measure of the emitted fluorescence (unbroken line); the hatched area corresponds to the emitted fluorescence ± S.E.M. value of controls without ascorbic acid. The effects obtained for the various ascorbic acid concentrations were compared using Dunnett's t-test. Means ± SEM of 3 experiments performed in triplicate. *b*: 0.01 as compared to respective controls (without ascorbic acid).

DISCUSSION

The increase in fluorescence emitted by the probe 1,6-diphenyl-1,3,5-hexatriene incorporated within the synaptosomal membrane is interpreted as being the consequence of the decrease in membrane fluidity[15]. We have verified that, in the absence of synaptosomes, ascorbic acid did not modify the fluorescence emitted by DPH. When DPH, dissolved in THF, and ascorbic acid were added simultaneously to the synaptosomal fraction, ascorbic acid was less efficient in decreasing membrane fluidity. In the same way, when synaptosomes were incubated with DPH, dissolved in THF, 60 min before the addition of ascorbic acid, membrane fluidity was not significantly modified as compared to controls without ascorbic acid. The DPH/THF-induced prevention of the decrease in membrane fluidity elicited by ascorbic acid may be explained by its incorporation within the membrane, protecting it from the lipoperoxidative effects of ascorbic acid. As far as the decrease in ^{3}H DA uptake is related to the decrease in membrane fluidity, the protection afforded by ascorbic acid appears to be the result of the solvent THF. In any case, the addition of DPH in THF stopped the alteration at the level reached before their addition. This observation indicated that ascorbic acid must be applied before the addition of DPH/THF. The fluorescence emitted by DPH was not

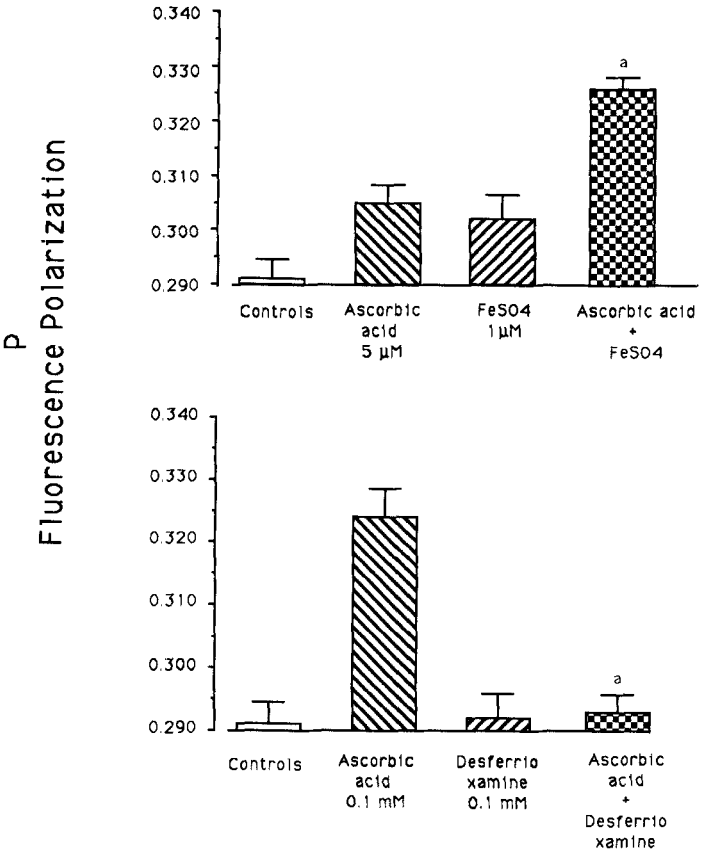

FIGURE 3 Effect of desferrioxamine and $FeSO_4$ on the decrease of synaptosomal membrane fluidity induced by ascorbic acid. Synaptosomes were incubated for 60 min with or without ascorbic acid (0.1 mM) and $FeSO_4$ (upper panel) or desferrioxamine (lower panel) before the addition of DPH (1 µM). Means ± S.E.M of 3 experiments performed in triplicate. The interactions between ascorbic acid and $FeSO_4$ or desferrioxamine were analyzed by 2-way ANOVA (*a*: $P < 0.001$).

modified between the 15th min and the 3rd hour of incubation of synaptosomes in the presence of the probe. During the 1 h preincubation period of synaptosomes, a regular time-dependent decrease in ^3H DA uptake was observed even in the absence of ascorbic acid. Its amplitude was lower than that observed, in the presence of 0.1 mM ascorbic acid[1]. In this latter case, the time course was clearly biphasic, with a slight initial decrease (until the 20th min), followed by a sharp decrease. Modifications in the emitted fluorescence did not match those observed with ^3H-DA uptake. The modification of the emitted fluorescence was only observed during the first 20 min of preincubation in synaptosomes incubated without ascorbic acid whereas in synaptosomes incubated with ascorbic acid at 0.1 mM concentration, the increase in the emitted fluorescence was observed up to the 40th min. This slight discrepancy casts some doubt on the relationship between the modifications in membrane fluidity and on the ability to take up ^3H DA. In addition, the modifications of the emitted fluorescence were relatively small whereas

TABLE 1
Effects of EGb 761, quercetin or trolox C on the decrease of synaptosomal membrane fluidity elicited by ascorbic acid.

	Fluorescence Polarization P
Controls	0.294 ± 0.004
Ascorbic acid	0.326 ● 0.004
Ascorbic acid	
+ EGb 761 2 μg/ml	0.313 ± 0.002
+ EGb 761 4 μg/ml	0.309 ● 0.003
+ EGb 761 8 μg/ml	0.302 ± 0.002[a]
+ EGb 761 16 μg/ml	0.285 ± 0.002[a]
Controls	0.287 ± 0.002
Ascorbic acid	0.320 ± 0.002
Ascorbic acid	
+ quercetin 0.3 μM	0.311 ± 0.002[a]
+ quercetin 0.75 μM	0.310 ± 0.003[a]
+ quercetin 1.5 μM	0.303 ± 0.002[b]
+ quercetin 3 μM	0.299 ± 0.002[b]
Controls	0.289 ± 0.002
Ascorbic acid	0.319 ± 0.004
Ascorbic acid	
+ trolox C 0.001 mM	0.319 ± 0.001
+ trolox C 0.01 mM	0.309 ± 0.003
+ trolox C 0.1 mM	0.302 ± 0.003[b]
+ trolox C 1 mM	0.279 ± 0.003[b]

Synaptosomes were incubated for 60 min without or with ascorbic acid (0.1 mM) and increasing concentrations of EGb 761 or quercetin or trolox C before the addition of DPH (1 μM). The emitted fluorescence was measured 1 h later. M ± SEM of 3 experiments performed in triplicate.

The effects obtained for each drug concentration were compared to controls with ascorbic acid by Dunnett's test. a: $p < 0.05$, b: $p < 0.01$.

modifications in ^{3}H DA uptake were great. However, small variations of membrane physical properties could result in a large modifications in its functions. Whatever may be, both phenomena are affected by the same concentrations of ascorbic acid (1 μM-1 mM) as well as the same ferrous ion concentration (1 μM). Using cholesterol to decrease membrane fluidity, Maguire and Druse[18] have also observed a decrease in ^{3}H DA uptake by rat brain synaptosomes. It may be noticed that the ascorbic acid concentrations which alter membrane fluidity or synaptosomal ^{3}H DA uptake are in the range of those found endogenously[19].

The effect of ascorbic acid on either the synaptosomal uptake of amines[1] or on the emitted fluorescence by DPH requires Fe^{2+} ions. Thus the Fe^{2+} chelating agent desferrioxamine[20] prevented this effect; on the contrary a high Fe^{2+} concentration (1 μM) potentiated the effect of a low concentration of ascorbic acid (5 μM). The decrease in synaptosomal uptake of amines as well as the increase in fluorescence emitted by DPH elicited by the couple ascorbic acid/Fe^{2+}, appear to depend on the generation of free radicals. Hydroxyl radicals[6] formed via lipid peroxidation have been reported to reduce membrane fluidity. Such an effect likely occurred when synaptosomes were exposed to an ascorbic acid/ferrous ion mixture known to display oxidizing properties[21]. Free radicals are probably responsible for these observed modifications since they are prevented by the free radical scavenge

:rolox C[11]. Its parent compound vitamin E shares this property[14]. The Ginkgo ɔiloba extract, EGb 761, also displays a protective effect. Its effectiveness may depend on its flavonoid components, since the protective effect was also displayed ɔy quercetin, which is present in EGb 761 as a glucorhamnoside ester and since flavonoids are known to display free radical scavenger properties[22-23]. In addition flavonoids could be Fe^{2+} chelating agents[22] and, by this way, might prevent generation of free radicals. Although quercetin was tested at concentrations higher :han those reached in the tested concentrations of EGb 761, its prevention of the decrease in membrane fluidity was less marked than that of EGb 761. Therefore :he prevention operated by EGb 761 cannot be simply due to this flavonoid derivative. In conclusion, the couple ascorbic acid/Fe^{2+} ions, at respective concen-:rations found endogenously, decreases membrane fluidity and reduces efficiency ɔf the amine transporter by generating free radicals. Free radical scavengers, ncluding the Ginkgo biloba extract EGb 761, exert a protective effect towards both alterations which are possibly related.

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