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Prevention of chemically induced changes in synaptosomal membrane order by ganglioside GM_1 and α-tocopherol

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Synaptosomal membrane order has been studied by analysis of light depolarization by fluorescent dyes intercalated within membranes following exposure to various environmental toxicants. Two probes were explored: 1,6-diphenyl-1,3,5-hexatriene (DPH), signaling predominantly from the lipid-rich membrane core, and 1-[4-(trimethylamino)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH), reporting from the more hydrophilic membrane surface. Chlordecone, a neurotoxic insecticide, decreased the anisotropy of either dye and this change could be prevented by prior treatment of synaptosomes with ganglioside GM_1 but not α-tocopherol. Exposure to an iron-ascorbic acid oxidizing mixture enhanced synaptosomal membrane order and this effect was blocked by preincubation with α-tocopherol but not ganglioside GM_1. While these interactions may have partially reflected additive anisotropy changes, the protective agents were also effective at concentrations where they did not in themselves modulate membrane order. Methyl mercuric chloride at concentrations up to 100 μM had no discernable effect upon membrane order. It is suggested that these changes in membrane order may underlie some of the previously reported variations in the content of ionic calcium and in the leakiness of synaptosomes.

Introduction

Several neurotoxic agents are believed to act in part by increasing levels of free ionic calcium within cytoplasm [1–5]. Such an elevation may impair cell function by a variety of means [6–8]. Isolated synaptosomes constitute a useful system in which to study these events. In the case of organochlorine insecticides and organometal compounds, there is a good correlation between the in vivo neurotoxicity of a chemical within one of these classes and its ability to elevate cytosolic calcium, $[Ca^{2+}]_i$ [9–11]. Chemically-induced increases in $[Ca^{2+}]_i$ are frequently accompanied by increased loss to the extracellular fluid of the intracellular fluorescent dye (fura-2) used to assay calcium levels. Pretreatment of synaptosomes with agents such as ganglioside GM_1 or α-tocopherol can wholly or partially protect against the induced changes in $[Ca^{2+}]_i$. These compounds also reduce the induced loss of fura-2 from synaptosomes [12]. Both of these potentially protective agents have some efficacy in mitigating neurotoxic damage effected in the intact animal [13–15]. In the case of α-tocopherol, the protective effect is believed to be due to its antioxidative properties, while the mechanism of ganglioside GM_1-induced amelioration of chemically-induced damage is not as clearly understood.

The coincidence of elevated $[Ca^{2+}]_i$ and increased membrane permeability suggests that elevated synaptosomal 'leakiness' may allow entry of calcium across the synaptosomal limiting membrane, activating the major phospholipid degradative enzymes (phospholipases) and leading to compromised integrity. This study was designed to ascertain the extent to which changes in $[Ca^{2+}]_i$ and membrane permeability could be accounted for in terms of altered membrane order. While synaptosomal preparations represent a rather heterogeneous assembly, they have been of great utility in the study of presynaptic events. However, the changes in membrane order reported here may reflect the properties of cerebral plasma membranes in general since they cannot be precisely attributed to a single component. We have

Abbreviations: TMA-DPH, 1-[4-(trimethylamino)phenyl]-6-phenyl-1,3,5-hexatriene; DPH, 1,6-diphenyl-1,3,5-hexatriene; THF, tetrahydrofuran; *r*, anisotropy; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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employed two fluorescent probes, one of which is lipophilic and primarily reports from the lipid core of the membrane (1,6-diphenyl-1,3,5-hexatriene, DPH) [16] while the other (1-[4-(trimethylamino)phenyl]-6-phenyl-1,3,5-hexatriene, TMA-DPH) possesses a cationic group which confines the dye to the superficial hydrophilic membrane domain [17,18]. The neurotoxic agents methyl mercuric chloride and chlordecone have been utilized. In addition, a free-radical inducing iron and ascorbic acid mixture deleterious to synaptosomal integrity has been investigated. This latter state also induces synaptosomal 'leakiness' but does not elevate $[Ca^{2+}]_i$ (unpublished observations).

Materials and Methods

Preparation of synaptosomes

Adult male CR 1 CD rats (Charles River Breeding Laboratories, Inc., Wilmington, MA) 4 to 5 months old, weighing 290 g to 350 g were used. Rats were decapitated, the brains excised quickly on ice and the whole brain except the cerebellum and pons-medulla dissected out. Synaptosomes were made by the modification of Dodd et al. [19] of the method of Gray and Whittaker [20]. Briefly, after homogenization in 10 volumes of cold 0.32 M sucrose, the homogenate was centrifuged (1900 $\times g$, 10 min, 0–4°C) and the supernatant laid over 1.2 M (10 ml). After high speed centrifugation (250 000 $\times g$, 25 min), the layer at the interface was collected, diluted 2.5-fold with 0.32 M sucrose and laid over 8 ml 0.8 M sucrose. After centrifugation again at high speed, the synaptosomal pellet was suspended in Hepes buffer (pH 7.4) to give a tissue concentration of 0.15 g-equivalent/ml (about 1.6 mg/ml of protein). The composition of Hepes buffer was (millimolar): NaCl, 120; KCl, 2.5; NaH_2PO_4, 1.2; $MgCl_2$, 0.1; $NaHCO_3$, 5.0; glucose, 6.0; $CaCl_2$, 1.0; and Hepes, 10.

Labeling with fluorescent probes

Three 1-ml aliquots of the synaptosomal suspension were each diluted with 5 ml of Hepes buffer (pH 7.4) and centrifuged (12 500 $\times g$, 8 min). The pellets were resuspended in 4 ml Hepes buffer (pH 7.4) and then combined. One 2-ml aliquot was incubated with 5 μM DPH for 30 min at 37°C. Another 2-ml aliquot was incubated with 5 μM TMA-DPH for 15 min at 37°C. The DPH and TMA-DPH probes were dissolved in tetrahydrofuran (THF) and THF-water, 1 : 1 ratio, respectively. Following incubation, the labeled synaptosome suspensions were kept on ice.

Fluorescence polarization

For fluorescence measurements, 0.5-ml aliquots of each fluorescent labeled synaptosome suspension were centrifuged in a microcentrifuge (16 000 $\times g$, 2 min). The synaptosomal pellet was resuspended in 2 ml of warm (37°C) Hepes buffer and allowed to equilibrate at 37°C for 10 min before fluorescent measurements were taken.

After control levels of fluorescence were determined, the agents were added and incubation was continued. Fluorescence was determined 15 min later. Synaptosomes were preincubated for 5 min with or without protective agents before addition of chlordecone or Fe/ascorbate. Fluorescence was determined 10 min later. Chlordecone and α-tocopherol were added in dimethyl sulfoxide; control samples received an equivalent concentration of solvent (maximal final concentration was 0.5% v/v). Corrections for light scattering (membrane suspension minus probe) were made. Fluorescence in the ambient medium (after pelleting membranes) was negligible.

Fluorescence measurements were performed on a Farrand MK1 spectro-fluorometer. Fixed excitation and emission polarization filters were used to measure fluorescence intensity both parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the polarization phase of the exciting light. I_{VV} corresponds to both vertically polarized excitation and emission while I_{VH} corresponds to vertically polarized excitation and horizontally polarized emission. Excitation and emission wavelengths of 360 nm and 430 nm, respectively, were used with the band width of both monochromators at 10 nm. Cuvette temperature was maintained at 37°C with a circulating water bath.

In measuring anisotropy, a correction factor (G) for instrument asymmetry was considered. The G factor is the ratio of the sensitivities of the detection system for vertically and horizontally polarized light: $G = S_V/S_H$ [21]. S_V and S_H are the sensitivities of the emission channel for the vertical and horizontal components, respectively. The G factor was measured using horizontally polarized excitation. Its magnitude was sufficiently low and invariable that it was not taken into account. Fluorescence anisotropy (r) was determined [16] by the formula:

$$r = (I_{\parallel}) - (I_{\perp})/(I_{\parallel}) + 2(I_{\perp})$$

Total anisotropy was sometimes divided into static and dynamic elements by use of the equation, formulated by Van der Meer et al. [22]: $r_\infty = r_o(r_s)^2/[r_o r_s + (r_o - r_s)^2/m]$ where r_s is measured steady state anisotropy, r_o is the maximal anisotropy and r_∞ is the static component. The dynamic component r_f is thus equal to $r_s - r_\infty$. A membrane order function S, analagous to that obtained from electron spin resonance studies, was also derived [23]: $S^2 = r_\infty/r_o$. The maximal anisotropy (r_o) of DPH was taken as 0.362 [16], and that of TMA-DPH as 0.39 [17].

Statistical analysis

Differences between groups were assessed by Fisher's least significant difference test after one-way analysis of

variance. The acceptance level of significance was $P < 0.05$ using a two-tailed distribution.

Chemicals

The probe DPH and α-tocopherol succinate were obtained from Sigma Chemical Co. (St. Louis, MO). TMA-DPH was obtained from Molecular Probes (Junction City, OR). Chlordecone was obtained from Radian Corp. (Austin, TX). Ganglioside GM_1 was kindly donated by Fidia Corp.

Results

(a) Chlordecone

Chlordecone treatment of synaptosomes led to a decrease in anisotropy at each of the sites that the two fluorescent probes were, respectively, reporting from (Figs. 1 and 2). The observed changes were greatest in the DPH signal, reporting primarily from the membrane core.

Pretreatment of synaptosomes with 100 μM ganglioside GM_1 was found to block the decrease in DPH and TMA-DPH anisotropy caused by chlordecone (Fig. 1). However, such an effect may have been merely additive since 100 μM ganglioside GM_1 itself increased membrane order. In some cases, synaptomes were centrifuged down after incubation with ganglioside GM_1, and resuspended prior to addition of chlordecone. This did not alter the results obtained, suggesting that these two compounds do not interact in solution. Other studies have shown that GM_1 ganglioside is capable of causing membrane lipid chains to assemble in a more rigid manner, as visualized by electron paramagnetic resonance techniques [24] and increased fluorescence polarization [25,26]. For this reason, a concentration of ganglioside GM_1, 10 μM, that did not alter the depolarization signal using either fluorescent probe was also used in the pretreatment of synaptosomes prior to the addition of chlordecone. This concentration of

TABLE I

Static and dynamic components of fluorescence anisotropy of DPH and TMA-DPH in synaptosomes exposed to varying conditions

Each value derived from 4–6 separate determinations ± S.E. * $P < 0.05$ that value differs from that of control (Student's two-tailed *t*-test). Incubation conditions described in Experimental section and Figs. 1 and 2.

	Control	Fe/ascorbate	Chlordecone
DPH			
r_s	0.211 ± 0.001	0.288 ± 0.004 *	0.183 ± 0.002 *
r_∞	0.180 ± 0.001	0.212 ± 0.005 *	0.146 ± 0.013 *
r_f	0.031 ± 0.001	0.026 ± 0.001 *	0.037 ± 0.001 *
S	0.70 ± 0.01	0.77 ± 0.02 *	0.64 ± 0.02 *
TMA-DPH			
r_s	0.267 ± 0.001	0.306 ± 0.005 *	0.25 ± 0.003 *
r_∞	0.246 ± 0.001	0.295 ± 0.005 *	0.231 ± 0.004
r_f	0.021 ± 0.001	0.011 ± 0.001 *	0.020 ± 0.001 *
S	0.79 ± 0.01	0.87 ± 0.02 *	0.76 ± 0.01 *

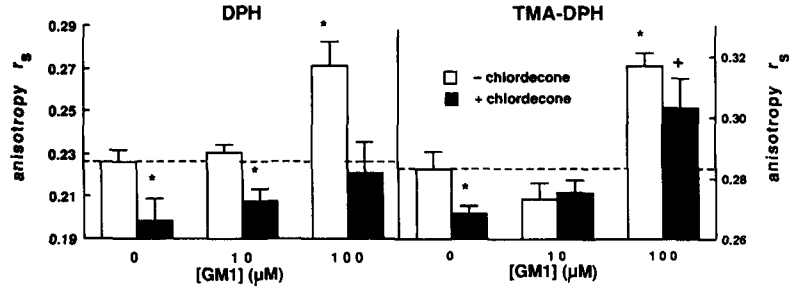

Fig. 1. Fluorescence polarization anisotropy of DPH or TMA-DPH in synaptosomes exposed to 100 μM chlordecone for 10 min. Some preparations were treated for 5 min with ganglioside GM_1 prior to the addition of chlordecone. Each column represents the mean ± S.E. of 4–7 determinations. *: differs significantly from untreated control value. +: differs significantly from corresponding value in absence of ganglioside GM_1.

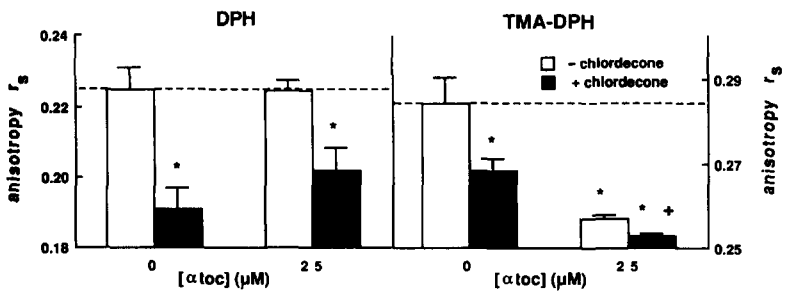

Fig. 2. Fluorescence polarization anisotropy of DPH or TMA-DPH in synaptosomes exposed to 100 μM chlordecone for 10 min. Some preparations were treated for 5 min with α-tocopherol prior to the addition of chlordecone. Each column represents the mean ± S.E. of 4–8 determinations. *: differs significantly from untreated control value. +: differs significantly from corresponding value in absence of α-tocopherol.

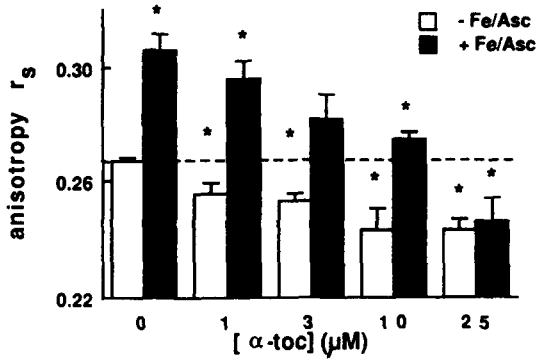

Fig. 3. Fluorescence polarization of TMA-DPH in synaptosomes exposed to 5 μM $FeSO_4$ and 100 μM ascorbic acid for 10 min. Preparations were treated with various concentrations of α-tocopherol for 5 min prior to the addition of the oxidizing mixture. Each column represents the mean of 5–8 determinations. *: differs significantly from value of untreated control.

ganglioside GM_1 prevented the reduction in TMA-DPH anisotropy caused by chlordecone. However, no similar protective effect was apparent in DPH anisotropy (Fig. 1).

Treatment of synaptosomes with 25 μM α-tocopherol did not significantly alter DPH anisotropy and depressed that of TMA-DPH. However, in neither case did this antioxidant compound change the subsequent synaptosomal response to chlordecone (Fig. 2).

(b) Methyl mercury

Incubation of synaptosomes with methyl mercuric chloride at levels ranging from 20 to 100 μM did not alter anisotropy values derived from the use of either probe (data not shown). The maximal concentrations of methyl mercury tested here were considerably higher than those previously shown to greatly elevate synaptosomal $[Ca^{2+}]_i$ and fura-2 leakage under identical incubation conditions [9,27].

Oxidizing conditions

The exposure of synaptosomes to an oxidizing mixture containing 5 μM $FeSO_{14}$ and 100 μM ascorbic acid

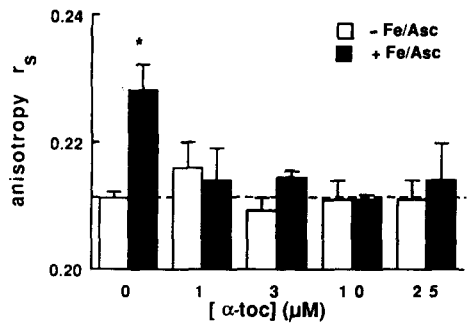

Fig. 4. Fluorescence polarization of DPH in synaptosomes exposed to 5 μM $FeSO_4$ and 100 μM ascorbic acid for 10 min. Preparations were treated with various concentrations of α-tocopherol for 5 min prior to the addition of the oxidizing mixture. Each column represents the mean of 5–8 determinations. *: differs significantly from value of untreated control.

increased the anisotropy value of both TMA-DPH (Fig. 3) and DPH (Fig. 4). This was especially pronounced for TMA-DPH, implying that the greatest changes were at the membrane surface. α-tocopherol succinate treatment decreased TMA-DPH anisotropy in a dose-related manner (Fig. 3). When this treatment was followed by the addition of Fe/ascorbate, there was a progressive decrease in Fe/ascorbate-induced anisotropy changes (Fig. 3). This largely reflected the sum of changes induced by Fe/ascorbate or α-tocopherol acting separately. The α-tocopherol succinate is not readily oxidized prior to its enzymic deesterification and this minimizes the likelihood of a direct interaction between this compound and the Fe/ascorbate mixture in free solution. In the case of DPH, α-tocopherol alone had no influence on anisotropy (Fig. 4). However, an inhibition of oxidatively-induced changes of anisotropy was found at all concentrations of α-tocopherol studied: 1–25 μM (Fig. 4). Thus, in this circumstance, α-tocopherol appeared to exert a truly protective effect in an interactive, non-additive manner. Ganglioside GM_1 pretreatment did not affect this response to the Fe/ascorbate exposure. Although ganglioside GM_1 alone elevated anisotropy, no evidence for interaction between GM_1 and Fe/ascorbate was found with either DPH or TMA-DPH (data not shown).

Synaptosomes were incubated in a medium containing no added calcium salts. This low calcium condition has been stated to increase oxidative stress [28,29] and increase fura-2 leakage from loaded synaptosomes [27]. However, the anisotropy of DPH and TMA-DPH was not significantly altered by such treatment (data not shown).

The preincubation of synaptosomes with 10 μM deferoxamine completely blocked the effect of subsequent addition of Fe/ascorbate upon TMA-DPH anisotropy. Anisotropy changes were further evaluated in terms of dynamic and static components. This revealed that the major modulation was predominantly in r_∞, rather than r_f, implying changes in membrane order rather than fluidity (Table I). Parceling out of the anisotropy showed

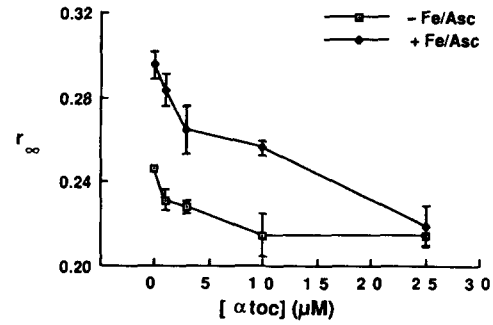

Fig. 5. Effect of oxidizing conditions upon limiting fluorescence anisotropy, r_∞, of TMA-DPH in synaptosomes. Each value is the mean of 4–8 determinations ± S.E. Incubation conditions as in Fig. 3.

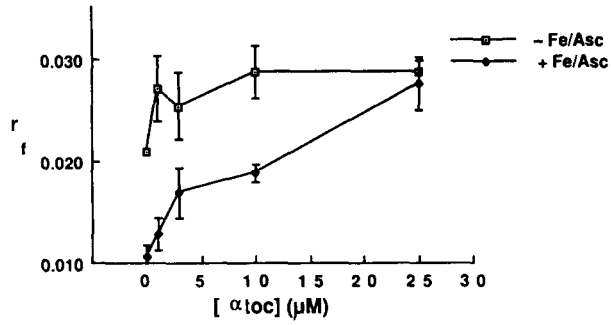

Fig. 6. Effects of oxidizing conditions upon r_f, the dynamic component of TMA-DPH anisotropy in synaptosomes. Each value is the mean of 4–8 determinations ± S.E. Incubation conditions as in Fig. 3.

that both Fe/ascorbate and chlordecone primarily affected membrane order (r_∞) while the dynamic component (r_f) was altered in an opposite direction (Table I). α-Tocopherol treatment in the presence or absence of Fe/ascorbate altered r_∞ in a manner similar to r_s, total anisotropy (Fig. 5). However, α-tocopherol effected a concentration-dependent increase in r_f when Fe/ascorbate was present, trending toward the corresponding control value (Fig. 6).

Discussion

In the case of chlordecone, there appears to be a relation between induction of synaptosomal leakiness, calcium elevation [10] and decreased anisotropy of probes (Figs. 1 and 2). Suggestion of a relation between the phenomena is enhanced by examination of the effects of potentially protective agents; ganglioside GM_1 prevents all these changes while α-tocopherol is not effective.

Methyl mercury also elevates synaptosomal leakiness and $[Ca^{2+}]_i$ [9], but has no effect on anistropy. This may be due to methyl mercury having a much more specific locus of action than chlordecone, perhaps at an ion channel [2,30].

The protection afforded by α-tocopherol pretreatment on several of the consequences of synaptosomal exposure to oxidizing conditions also suggests that the parameters studied are related. These data imply that the plasma membrane may be a significant target of diverse neurotoxic agents. Increased leakage of the anion fura-2 appears to be correlated with a pronounced change, either negative or positive, in membrane anisotropy. However, transmembrane passage of the calcium cation seems reciprocally associated with the degree of membrane order. Restoration of anisotropy values by potentially protective agents seems to be accompanied by both reinstatement of normal cell permeability and $[Ca^{2+}]_i$. Decreased membrane order caused by chlordecone (Fig. 1), ethanol and anesthetics [31] or toluene [32] is related to a greater $[Ca^{2+}]_i$; increase in membrane rigidity by peroxidative events or by ganglioside GM_1 occurs together with a somewhat diminished $[Ca^{2+}]_i$ [27].

Chlordecone caused the greatest perturbation of DPH anisotropy while Fe/ascorbate was most effective in altering TMA-DPH anisotropy. This may reflect the lipophilic nature of chlordecone and the water solubility of the oxidizing mixture. In the case of the agents potentially capable of preventing such membrane dislocations, no such relation between lipophilicity and the site of maximal effect could be made. Water soluble GM_1 predominantly influenced the DPH signal while lipophilic α-tocopherol reduced TMA-DPH anisotropy. The critical feature of these 'protective' agents may be their chemical reactivity rather than the geometry of their intercalation into membranes.

Lipid peroxidation has been reported to decrease membrane order as determined by electron spin resonance which primarily reports static structure [33]. However, using pyrene, which largely signals dynamic components of membranes [34], peroxidation increases membrane rigidity [35]. This latter change has also been reported using fluorescence polarization [36]. These different types of analyses could account for several contradictions in the literature. The limiting fluorescence anisotropy, r_∞, reflects the amplitude of probe oscillation while r_f primarily signals the rotational state of the probe. An increasingly restricted fluorophore is able to rotate faster in view of its diminishing freedom of angular motion [22]. Limitation of velocity of rotational movement (spin) may allow increased angular oscillation of the dye within the membrane (i.e., degrees of arc). This accounts for r_∞ and r_f changing in opposite directions in response to a perturbing stimulus.

Although statistically significant, the observed changes in anisotropy were relatively small in absolute terms. As is clear from enzyme studies, minor variations in the physical form of a protein can have much greater functional effects. Thus, changes in biological activity often have a greater magnitude than the structural alterations that underlie such changes. A change of membrane microviscosity of around 5% can cause a 50% change of membrane permeability to cations [37]. The transmembrane fluxes of calcium, therefore, may be significantly affected by membrane anisotropy changes of the size reported here.

Several neurotoxic compounds have been reported to modulate the microviscosity of membranes including those derived from nervous tissue [38]. In general, organic solvents including ethanol and anesthetics decrease order [39,40]. Heavy metal cations tend to increase membrane order [40,41] as does calcium [42,43] Lipid peroxidation has been reported to increase the rigidity of phospholipid bilayers [44] and erythrocyte membranes [33]. The relation of such observed changes to toxic damage in the animal is difficult to establish.

However, the amelioration of disruption of membrane integrity by protective agents described here supports the likelihood that our data are relevant to manifestations of neurotoxicity in vivo. Ganglioside GM_1 and α-tocopherol pretreatment at concentrations where they produce no detectable changes in membrane order can block the effects of agents harmful to synaptosomes. This implies some degree of specificity of the protective process, perhaps because both the toxic and protective agents act at the same membrane location. α-Tocopherol is known to protect against several types of oxidatively induced damage to the central nervous system [45,46] and the concentration range of α-tocopherol used in this study is in a similar range to the 14 μM value reported present in rodent brain [50]. Ganglioside GM_1 pretreatment has also been reported effective in blocking expression of neurotoxicant-induced damage [48,49]. Exogenous ganglioside GM_1 can be taken up by cells in culture and functionally incorporated into the plasma membrane [50] and has been shown to become bound to cell membranes following systemic injection [51]. However, the need for pretreatment with ganglioside prior to neurological insult may limit the therapeutic utility of such compounds.

General changes in membrane order may modulate the properties of specific neurotransmitter receptors [52,53] and the activity of enzymes activated by transmembrane signals [54]. Such apparently selective changes may then result in distinctive neurological dysfunction.

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