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Authors
von Euler, G
Fuxe, K
Bondy, SC

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Ganglioside GM₁ prevents and reverses toluene-induced increases in membrane fluidity and calcium levels in rat brain synaptosomes

G. von Euler¹, K. Fuxe¹ and S.C. Bondy²

¹Department of Histology and Neurobiology, Karolinska Institutet, Stockholm (Sweden) and ²Department of Community and Environmental Medicine, University of California, Irvine, CA 92717 (U.S.A.)

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INTRODUCTION

The effects of exposure to ganglioside GM₁ and to toluene in vitro upon synaptosomal integrity have been examined using fluorescence polarization of two probes: 1-[4(trimethylamino)phenyl]-3,5,5-hexatriene (TMA-DPH) and 1,6-diphenyl-3,5,5-hexatriene (DPH) to measure membrane anisotropy, and the fluorescent indicator fura-2 to assay levels of cytosolic calcium ([Ca²⁺]). The anisotropy of both TMA-DPH and DPH was decreased by toluene, implying increased membrane fluidity. The decrease in TMA-DPH but not in DPH anisotropy was prevented by pretreatment with GM₁ in concentrations as low as 10 µM. This is not an additive interaction since 10 µM of GM₁ alone did not significantly modulate TMA-DPH anisotropy. When the GM₁ treatment succeeded the addition of toluene the decrease in anisotropy of both probes was reversed. Toluene treatment increased [Ca²⁺], in a dose- and time-dependent manner. This increase could partially be both prevented and reversed by treatment with 50 µM of GM₁. These effects may reflect an additive interaction, since this concentration of GM₁ alone reduced [Ca²⁺]. The present results show that toluene increases membrane fluidity and intracellular calcium levels. These effects may be counteracted by the endogenous compound GM₁.

MATERIALS AND METHODS

Adult male rats (CR1CD), 3–4 months old, weighing 290–340 g were used.

Synaptosomal preparation

Brains were rapidly removed and the forebrain was dissected out on ice, weighed and homogenized in 10 vols. of 0.32 M sucrose at 0 °C. The homogenate was centrifuged (1500 g, 10 min) to give a post nuclear supernatant which was layered over 1.2 M sucrose and centrifuged for 25 min (250,000 g). The interphase band was removed and layered over 0.8 M sucrose and centrifuged again for 25 min at 250,000 g8. The purified synaptosomes were resuspended in HEPES buffer (pH 7.4) at 0.15 g-equiv/ml corresponding to a final concentration of 120–140 µg protein/ml. The HEPES buffer was composed of (in mM): NaCl 125, KCl 5, NaH₂PO₄ 1.2, MgCl₂ 1.2, NaHCO₃ 5, Glucose 6, CaCl₂ 1, HEPES 25; and adjusted with NaOH to pH 7.4.

Membrane order

Synaptosomal membrane order was evaluated by fluorescence polarization studies using two probes. 1,6-Diphenyl-1,3,5-hexatriene (DPH) is a polar lipophilic molecule capable of penetrating into and through inner lipid-rich membrane layers38. 1-[4(trimethylamino)phenyl]-3,5,5-hexatriene (TMA-DPH) is a related compound with a polar-constituent that causes the molecule to be aligned at the outer surface of limiting membranes with the polar head at the hydrophilic surface, while the non-polar body penetrates the lipid interior26.24.

Synaptosomes were prepared as described above and were incubated at 37 °C in the presence of TMA-DPH (30 min) or DPH...
and allowed to equilibrate for 30 min at 37 °C prior to measuring fluorescence intensity and polarization in a water-jacketed cuvette holder maintained at 37 °C (Aminco SPF-500 spectrofluorometer, American Instrument Co., Urbana, Ill., U.S.A.). An excitation wavelength of 360 nm (bandwidth 10 nm) was used with a determination of emission at 430 nm (bandwidth 10 nm). Corrections for light scattering (membrane suspension minus probe) and for fluorescence in the ambient medium (after pelleting membranes) were made.

Fluorescence anisotropy \( r \) was determined by the formula \( r = I_{VV} - I_{HH} [I_{VV} + 2I_{VH}] \): \( I_{VV} \) is the fluorescence intensity with excitation and emitted light polarized vertically and \( I_{HH} \) is the intensity obtained with a vertical orientation of the exciting polarizer with the emitted fluorescence passing through a horizontal polarizer. Total fluorescence intensity \( F = I_{VV} + 2I_{VH} \). Relative microviscosity is proportional to \( r/n - 1 \), where \( r \) is the maximal limiting anisotropy of the probe; 0.362 for DPH and 0.39 for TMA-DPH. A correction factor \( G \) for instrument asymmetry was also made using \( G = I_{VV}/I_{HH} \) where \( I_{VV} \) is fluorescence intensity with horizontal excitation light and emitted light read vertically, and \( I_{HH} \) is the corresponding value with the entire light path horizontally aligned. This compensates for the sensitivity of the detection system toward vertically and horizontally polarized light. All values of \( I_{VV} \) were multiplied by \( G \) in the calculation of \( r \). All corrections made amounted to less than 6% of the original unmodified readings.

**Intrasynaptosomal calcium levels**

Synaptosomes were prepared as described above and were incubated at 37 °C in the presence of fura-2 dissolved in DMSO for 10 min. They were then diluted 10 times in HEPES buffer and incubated for another 5 min. The final concentration of fura-2 was 5 \( \mu \)M. The synaptosomes were centrifuged for 8 min at 3000 g and the pellet was resuspended in HEPES buffer.

For each assay, 0.5 ml of synaptosomes was rapidly centrifuged (2 min, 13,000 g) and the resulting pellet was resuspended in 1 ml HEPES buffer at 37 °C. The buffer was as described above without NaHCO\(_3\) and NaH\(_2\)PO\(_4\) to prevent the precipitation of calcium at elevated pH (required during the determination of minimal fluorescence). The tube was rinsed with another 1 ml of HEPES buffer and the total 2 ml sample was placed in a quartz cuvette at 37 °C and left to equilibrate for 10 min. Excitation of fura-2 was at 340 and 380 nm (bandwidth 3 nm) and emission determinations were made at 510 nm (bandwidth 20 nm). Corrections for light scattering (membrane suspension minus probe) and fluorescence in the ambient medium (after pelleting membranes) were made.

**Fig. 1.** The effect of toluene on TMA-DPH and DPH anisotropy in synaptosomal membranes. The synaptosomes, preloaded with the respective fluorescent indicator, were incubated for 30 min at 37 °C with varying amounts of toluene. Data represents mean ± S.E.M. of 4 individual determinations.

**Fig. 2.** Anisotropy changes of TMA-DPH and DPH within synaptosomal membranes exposed to 2 \( \mu \)l toluene for 30 min at 37 °C. The synaptosomes were pretreated for 30 min with varying amounts of ganglioside GM\(_1\) before exposure to toluene. Data represent mean ± S.E.M. of 5–7 individual experiments. * \( P < 0.05 \) that value differs from that of untreated controls.

**TABLE I**

| Treatment       | Anisotropy (r) | DPH |
|-----------------|----------------|-----|
| TMA-DPH         |                |     |
| Control         | 0.288 ± 0.003  | 0.239 ± 0.003 |
| GM\(_1\) (10 \( \mu \)M) | 0.284 ± 0.003  | 0.232 ± 0.005 |
| GM\(_1\) (50 \( \mu \)M) | 0.297 ± 0.002* | 0.246 ± 0.002* |

* \( P < 0.05 \) that value differs from that of untreated controls.
Fura-2 into the incubation medium is added.

**RESULTS**

**Studies on membrane fluidity**

Toluene was found to decrease the anisotropy ($\chi$) of TMA-DPH and DPH in a dose-dependent way (Fig. 1). At 2 µl of toluene the change in $\chi$-value ($\Delta\chi$) of TMA-DPH and DPH were $0.017 \pm 0.003$ and $0.033 \pm 0.002$, respectively. Absence of vortexing did not allow toluene (2 µl) to act on TMA-DPH anisotropy ($\Delta\chi = 0.001 \pm 0.003$) or DPH anisotropy ($\Delta\chi = 0.001 \pm 0.003$). Ganglioside GM$_1$ (50 µM but not 10 µM) was found to increase anisotropy of both TMA-DPH and DPH (Table I).

Pretreatment with 10 µM of GM$_1$ completely abolished the reduction of TMA-DPH anisotropy induced by 2 µl toluene, without affecting the toluene-induced reduction in DPH anisotropy (Fig. 2). Following pretreatment with 50 µM GM$_1$, the inhibition by toluene on TMA-DPH anisotropy remained, whereas the toluene-induced decrease in DPH anisotropy was reduced by 50%.

The toluene-induced decrease in TMA-DPH and DPH anisotropy was reversed by subsequent treatment with GM$_1$. Ten µM of GM$_1$ counteracted the decrease in TMA-DPH and DPH anisotropy by 50%, and 50 µM of GM$_1$ reversed the decrease completely (Fig. 3). The anisotropy of treated and non-treated synaptosomes did not change over time (last measurement made after 60 min of incubation, data not shown).

**Studies on $[Ca^{2+}]_i$**

Toluene was found to increase the levels of intrasynaptosomal calcium in a concentration- and time-dependent way without affecting membrane leakage (Figs. 4 and 5). At 2 µl of toluene the $[Ca^{2+}]_i$ value was close to the upper limit of the assay capacity of fura-2, and at 6 µl of toluene the synaptosomes clumped together. Absence of vortexing did not allow toluene to act on synaptosomal calcium levels (data not shown). This time-dependent increase in $[Ca^{2+}]_i$ by toluene contrasted with the

**TABLE II**

| First addition      | Second addition | $\Delta[Ca^{2+}]_i$ (nM) |
|---------------------|-----------------|--------------------------|
| None                | Toluene (2 µl)  | 801 ± 201*               |
| GM$_1$ (10 µM)      | Toluene (2 µl)  | 628 ± 83                 |
| GM$_1$ (50 µM)      | Toluene (2 µl)  | 179 ± 136                |
| Toluene (2 µl)      | None            | 1463 ± 256*              |
| Toluene (2 µl)      | GM$_1$ (10 µM)  | 1546 ± 552*              |
| Toluene (2 µl)      | GM$_1$ (50 µM)  | 642 ± 213                |

* $P < 0.05$ that value differs from that of untreated controls.
GM₁-induced reduction in calcium levels (Fig. 5). Under control conditions, the [Ca²⁺]ᵢ increased slowly.

Pretreatment with GM₁ was found to prevent the toluene-induced increase in calcium levels (Table II). The effect was most pronounced at 50 µM of GM₁, at which concentration GM₁ almost completely blocked the toluene effects. Addition of GM₁ (50 µM but not 10 µM) subsequent to toluene treatment was found to reverse the toluene-induced increase of [Ca²⁺]ᵢ by 50% (Table II).

**DISCUSSION**

It was found that toluene in vitro reduces the anisotropy of both TMA-DPH and DPH in synaptosomal membranes. Our study directly shows that toluene affects and increases membrane fluidity as earlier postulated. These actions may have neurotoxic consequences in vivo, and are paralleled by effects of other organic solvents. Although statistically significant, the observed changes in anisotropy are relatively small in absolute terms. However, as is clear from enzyme studies, even minor variations in the physical form of proteins can have major functional effects. Thus changes in biological activity often have a greater magnitude than the structural alterations of the molecules that underlie such changes. However, intraperitoneal injections with toluene in vivo had no effect on membrane fluidity as measured by fluorescence polarization.

The present results confirm that GM₁ increases the rigidity of the cell membrane rather than decreases it. Already at 10 µM, a concentration at which GM₁ by itself has no effect on membrane fluidity, GM₁ is able to completely antagonize the toluene-induced reduction in TMA-DPH anisotropy. However, GM₁ is unable to prevent the actions of toluene on DPH anisotropy, perhaps due to the exclusive localization of GM₁ to the external surface of the cell membrane. However, GM₁ reversed the toluene-induced reductions of both TMA- and DPH anisotropy.

This may be the first example where treatment with GM₁ after a neurotoxic insult can block potentially deleterious changes in nerve tissue. The ability of GM₁ to reverse the DPH effects may be due to a damage to the membrane integrity by toluene, allowing GM₁ to gain access to the inner, hydrophobic part of the cell membrane.

Toluene increased intracellular Ca²⁺ levels, but interestingly, membrane leakage was not affected by toluene. Other studies have shown that toluene increases calcium uptake in synaptosomes and affects calcium regulated protein phosphorylation. Thus, the increase in calcium levels may be another mechanism underlying toluene neurotoxicity. GM₁, on the other hand, stabilized intracellular calcium levels, in contrast to the slow increase in [Ca²⁺], seen under control conditions. Thus, both the preventive and the reversal effects of GM₁ on the toluene-induced increases in calcium levels may represent an additive interaction.

The importance of vortexing for attaining the effects of toluene established in the present study emphasizes the need for standardization of vortexing protocols. Thus, it is difficult to quantitate the relation between the amount of toluene added and the resulting concentration in the membrane bilayers. The same problem is also valid for the discussion on whether the results can be transferred to in vivo situations.

In conclusion, the present study shows protective and reversal effects of ganglioside GM₁ on toluene-induced increases in membrane fluidity and intrasynaptosomal levels of free ionic calcium. It may be that treatment with GM₁ may counteract neurotoxic effects induced by toluene and other organic solvents. This suggests a potential therapeutic use of this ganglioside.

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