Gangliosides GM1 and GD1b are not polarized in mature hippocampal neurons

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Received 1 July 1999; received in revised form 19 August 1999

2. Materials and methods

Abstract Analysis of the binding of cholera toxin to ganglioside GM1 in both living and fixed neurons, and comparison with the distribution of defined axonal and dendritic proteins, demonstrates that ganglioside GM1 is distributed in a non-polarized manner over the axonal and dendritic plasma membranes of mature, cultured hippocampal neurons. Likewise, ganglioside GD1b is also distributed in a non-polarized manner. These results suggest that a recent report [Ledesma, M.D. et al. EMBO J. 18 (1999) 1761–1771] proposing that ganglioside GM1 is highly enriched on the axonal versus dendritic membrane of hippocampal neurons may need to be re-evaluated.

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Key words: Ganglioside; Cholera toxin; Neuron; Axon;

Dendrite; Polarity

1. Introduction

The sialic acid containing glycosphingolipids (GSLs), the gangliosides, are enriched in neuronal tissues, where they have been proposed to play a number of important roles [1]. Biochemical and immunohistochemical analyses have demonstrated that gangliosides are distributed throughout the embryonic and adult brain, although specific gangliosides are restricted to specific neuronal cell populations at different stages of development [2,3]; this presumably reflects one or other aspect of their function in particular neurons. In contrast, there is less evidence that gangliosides are restricted to specific neuronal membrane domains, i.e. axons or dendrites. Three gangliosides (GD3, GD1\alpha and GM1b) have been reported to be restricted to Purkinje cell dendrites (reviewed in [3]), and until recently, there was no evidence that any ganglioside is restricted to the axonal membrane of any neuronal cell. However, a recent study [4] proposed that ganglioside GM1 is highly enriched in the axonal compared to the dendritic membrane of mature, cultured hippocampal neurons. This suggestion was based on analysis of the binding of a fluorescent derivative of cholera toxin (CT), which binds with high affinity to ganglioside GM1, to young and mature neurons. Since previous studies have not observed a preferential localization of ganglioside GM1 to axons [5], we have reexamined CT binding to both living and fixed [6] neurons. In contrast to the recent report [4], we find no evidence that ganglioside GM1 is distributed in a polarized fashion in mature, cultured hippocampal neurons.

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Hippocampal neurons were cultured on glass coverslips suspended over a glial monolayer, as described [7–10].

The distribution of GM1 was determined using a fluorescent-derivative of CT, Bodipy-CT, prepared by conjugation of CT (Calbiochem, La Jolla, CA, USA) with Bodipy succinimidyl ester (Molecular Probes, Eugene, OR, USA), according to the manufacturer's instructions. Neurons (plated at a density of 6000 cells/13 mm coverslip) in Hanks' balanced salt solution (HBSS) were incubated with 10 nM Bodipy-CT (dissolved in 10 mM HEPES pH 7.4, containing 0.1% (w/v) bovine serum albumin (BSA)), at 13-16°C for 30 min [5,11] and immediately examined using either a Plan Neofluar 40×/1.3 n.a. or a Neofluar 100×/1.3 n.a. oil objective of a Zeiss Axiovert 35 microscope equipped with a filter for Bodipy fluorescence and photographed using a Contax 167MT camera and Kodak Tmax p3200 film. Alternatively, neurons in HBSS were incubated with Bodipy-CT (25 nM, in 0.1% (w/v) BSA, 10 mM HEPES pH 7.4) for 1-2 min on ice, prior to fixation and permeabilization with methanol (-20°C, 5 min) [4]. Neurons were subsequently incubated with 3% (w/v) BSA in HBSS and then incubated for 1 h at 25°C with an anti-MAP-2 antibody (1:200) (a dendritic protein [12]), or with an anti-GAP 43 antibody (1:1000) (an axonal protein [13]). An indocarbocyanine (Cy3) conjugated goat anti-mouse antibody (Jackson Immunoresearch Laboratories) was used for detection.

CT was iodinated as described [14] to a specific activity of 57 Ci/mmol. Neurons (25 000 cells/13 mm coverslip) were incubated with ¹²⁵I-CT (10 nM, 16°C, 30 min), and after washing, coverslips were placed in vials and the amount of bound ¹²⁵I-CT determined using a percentage.

Bodipy-CT binding to neurons was quantified as follows: Neurons (plated at a density of 1000-2000 cells/cm²) were incubated with Bodipy-CT (10 min, 37°C) in recording medium (phenol red-free, riboflavin-free minimal essential medium, supplemented with glucose (30 μM), pyruvate (1 mM), kynurenic acid (500 μM), trolox (20 μM), and N-acetylcysteine (60 μM)), rinsed briefly and sealed into a microscope chamber with fresh recording medium. Living cells were imaged at room temperature on a Leica DM-RXA microscope equipped with a 40×/1.0 n.a. Plan Apo oil objective. Cells were selected for analysis using phase contrast microscopy to prevent photobleaching. Fluorescence images were acquired with a Princeton Instruments chilled CCD camera with a Sony interline chip (2 s exposure) and stored for offline analysis. Images were mathematically corrected for uneven illumination and background fluorescence using Metamorph software (Universal Imaging). Isolated segments of axons or dendrites were selected, traced and the average pixel value was measured for each segment. Isolated dendrites were identified by the absence of axons continuing past their tips.

The distribution of ganglioside GD1b was examined using monoclonal antibody B17 (provided by Dr. Israel Pecht, Department of Immunology, Weizmann Institute of Science), which binds specifically to GD1b [15], as described [6,8]. Living cells were incubated with B17 (1:100 dilution, 20 min, 37°C), fixed in formaldehyde (4% (w/v), 37°C for 20 min) and detected using a Cy3 conjugated goat anti-mouse antibody.

3. Results

The localization of ganglioside GM1 was initially examined

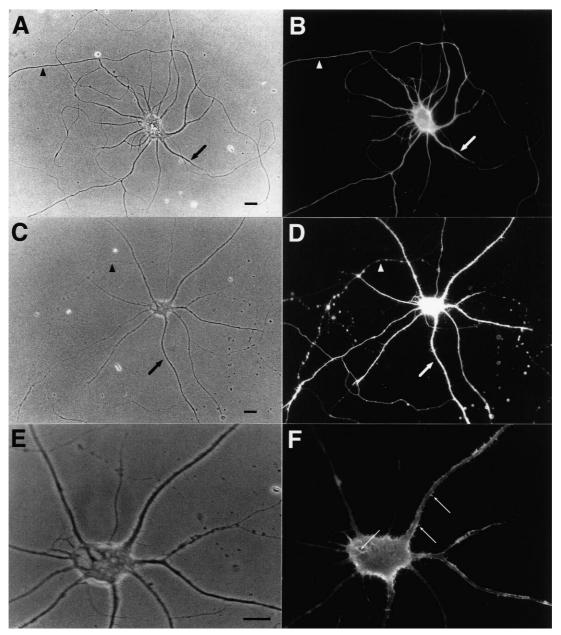


Fig. 1. The distribution of Bodipy-CT on the surface of living hippocampal neurons. After incubation with Bodipy-CT, 10 day old neurons were immediately examined using either a $40 \times (A-D)$ or a $100 \times (E$ and F) objective. The left-hand panels are phase contrast micrographs, and the right-hand panels are immunofluorescence micrographs. In panels A-D, axons are indicated by arrowheads, and dendrites by arrows. Panels E and F show the same cell as in panels C and D, but at higher magnification, in which both dendritic filopodia and somatic filopodia (indicated by arrows) can be resolved; Bodipy-CT labeling is observed on these structures. Bar = $10 \mu m$.

Table 1 Effect of fixation methods on ¹²⁵I-CT binding to neurons

Effect of fixation methods on Tell binding to ficurous	
Fixation method	¹²⁵ I-CT bound per coverslip (cpm)
Unfixed	16883 ± 2374
Methanol (-20°C for 5 min)	10079 ± 2250
Formaldehyde (37°C for 20 min)	10046 ± 783
Formaldehyde and methanol	10557 ± 740
Formaldehyde and Triton X-100 (0.25%, 37°C, 5 min)	3177 ± 560

Living neurons were incubated for 30 min at 13–16°C with 10 nM ¹²⁵I-CT, prior to fixation by the indicated methods and analysis of ¹²⁵I-CT binding.

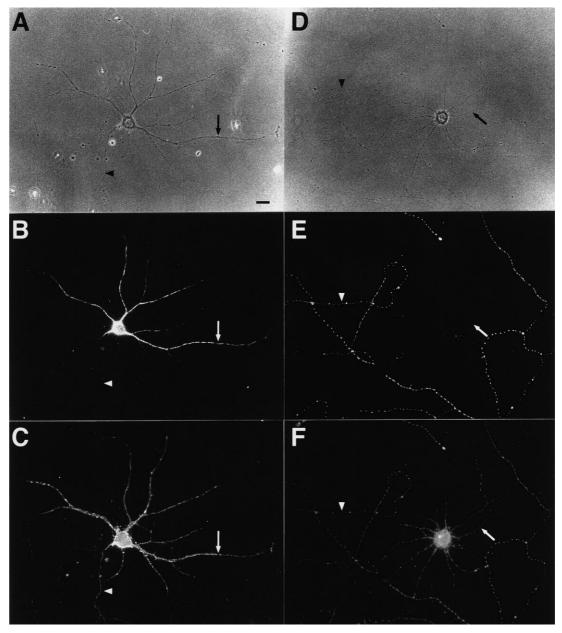


Fig. 2. Comparison of the distribution of GM1 with dendritic and axonal marker proteins. Neurons were labeled with Bodipy-CT (C and F) on ice, followed by incubation with either anti-MAP-2 (B) or anti-GAP 43 (E) antibodies. Panels A and D are phase contrast micrographs and panels B, C, E and F are immunofluorescence micrographs. Axons are indicated by arrowheads, and dendrites by arrows. MAP-2 is localized to dendrites, and GAP 43 to axons, whereas Bodipy-CT binds to all processes (see text for more details). Bar = $10 \mu m$.

in living hippocampal neurons (i.e. neurons that had not been fixed) under conditions in which no endocytosis occurs [5]. In 10 day old neurons, Bodipy-CT labeled both the short, thick processes (identified as dendrites, see below) and also the long, thin processes (identified as axons, see below) (Fig. 1A–D) and in no cases were neurons observed in which only a subset of processes was labeled; similar results were obtained with 11-14 day old neurons. Occasionally, a GM1-negative cell was observed. In some cells, dendritic filopodia appeared to be labeled when neurons were examined at low magnification (using a $40\times$ objective, Fig. 1C,D), which was clearly apparent at higher magnification ($100\times$ objective, Fig. 1E,F). This excludes the possibility that the dendritic labeling is actually due to axons which run in close apposition to dendrites, as is

sometimes observed when examining the distribution of axonal proteins [9]. Moreover, small filopodia on the soma were also labeled by Bodipy-CT (Fig. 1E,F).

To unambiguously confirm the identity of the processes as either axons or dendrites, the distribution of the axonal marker protein, GAP 43 [13], and the dendritic marker protein, MAP-2 [12], was examined subsequent to labeling with Bodipy-CT under similar conditions to those used by Ledesma et al. [4]. MAP-2 was detected in a subset of processes, corresponding to dendrites (Fig. 2A,B), whereas Bodipy-CT labeled all processes, including those labeled by MAP-2 (compare Fig. 2B,C). Likewise, GAP 43 was detected in a subset of processes, corresponding to axons (Fig. 2D,E), whereas Bodipy-CT labeled all processes (compare Fig. 2E,F). The 'dotty/

Table 2 Quantification of Bodipy-CT binding to axons and dendrites

	Bodipy-CT binding (arbitrary fluorescence units/pixel)		Ratio dendrites/axons
	Axons	Dendrites	
Experiment 1	176.3 ± 5.6 (11)	338.0 ± 15.5 (7)	1.9
Experiment 2	$355.5 \pm 12.0 (8)$	$483.8 \pm 11.6 (7)$	1.4

Values represent the mean \pm S.E.M. of average pixel values for measured segments from 14 cells (selected from two separate cultures). Only isolated segments of dendrites or axons were measured. The number of processes measured is indicated in parentheses. Total length of neurites analyzed was 840 μ m for dendrites and 1120 μ m for axons.

speckled' appearance of the labeled processes appears to be a result of either the fixation/permeabilization protocol (methanol, -20°C for 5 min) since analysis of both MAP-2 and GAP 43 labeling under different fixation conditions [9,16] did not give this labeling pattern, or of the low temperature used for Bodipy-CT binding since a similar pattern was not observed upon Bodipy-CT labeling to living neurons (Fig. 1A–D). A reduction in the fluorescence intensity of Bodipy-CT was consistently observed in neurons fixed with methanol compared to those examined alive. This was quantified by analyzing the amount of ¹²⁵I-CT [5] bound to hippocampal neurons after various fixation protocols; a significant reduction in binding was observed after all fixation methods (Table 1). Thus, as previously demonstrated [6], commonly used fixatives and per-

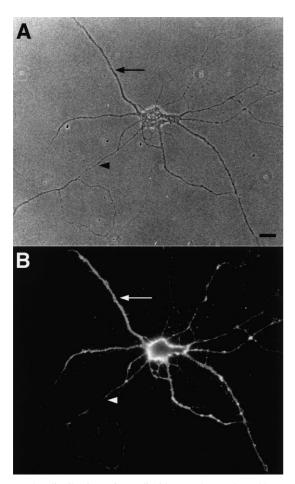


Fig. 3. The distribution of ganglioside GD1b. 10 day old neurons were incubated with an anti-GD1b antibody and examined by phase contrast (A) or fluorescence microscopy (B). Axons are indicated by arrowheads, and dendrites by arrows. Bar = $10 \mu m$.

meabilizing agents can significantly reduce the amount of cellsurface gangliosides, presumably by extracting them from the membrane.

The density of Bodipy-CT binding to axons and dendrites was quantified from images of living neurons. In two experiments, no evidence was obtained for preferential distribution of Bodipy-CT binding to axons. In contrast, a slight enrichment was observed over the dendritic surface (Table 2).

Finally, the distribution of a 'b-series' ganglioside, GD1b, was examined in 10 day old neurons using an anti-GD1b antibody [15]. Similar to younger neurons [8], GD1b was detected on both axons and dendrites (Fig. 3A,B).

4. Discussion

The suggestion that GSLs (including gangliosides) are enriched on the axonal membrane was first raised based on comparative studies between epithelia and neurons [17]. In epithelia, GSLs are enriched on the apical compared to the basolateral membrane, a similar polarized distribution was suggested for neurons, with the axonal membrane considered analogous to the apical membrane of epithelia and the dendritic membrane analogous to the basolateral membrane [18]. However, it is now clear that there are many exceptions to this suggestion [19], in particular with respect to apical proteins, which when expressed or detected in neurons, are often found equally distributed between dendrites and axons [20]. This also appears to be the case for gangliosides GM1 and GD1b, which although enriched in the apical domain of epithelia (at least in the case of GM1 [21]), are uniformly distributed over the axonal and dendritic surface of cultured hippocampal neurons (this study).

The differences in the distribution of GM1 observed in the current study and in that of Ledesma et al. [4] could be due to a number of reasons. (a) The conditions for CT binding used by Ledesma et al. [4] involve very short times of incubation (1–2 min on ice) with a relatively high concentration of CT, whereas in the current study, longer times of incubation were used with lower concentrations of CT. It is likely that Ledesma et al. [4] were examining CT binding under non-equilibrium conditions. (b) The apparent distribution of gangliosides can be altered depending on fixation conditions [6]. Indeed, we observed a significant reduction in the amount of membrane-bound ¹²⁵I-CT with the fixation conditions used by Ledesma et al. [4], compared to unfixed cells. (c) In the current study, it is possible to unambiguously distinguish axons from dendrites, both by phase contrast microscopy and also by the use of specific axonal and dendritic marker proteins. Although Ledesma et al. [4] suggest that 89% of GM1 is present in axons, little detail is given of how this analysis was performed. In contrast, we observe a small enrichment of CT

binding on dendrites. Based on the current and on earlier studies in somewhat less mature neurons [5], we conclude that GM1 is not polarized to axons but rather distributed over the axonal and dendritic surfaces, as appears to be the case for many other gangliosides [3] and GSLs, including ganglioside GD1b.

Acknowledgements: This work was supported by Grant 95-254 from the US-Israel Binational Science Foundation. We thank Rivi Zisling (Rehovot) and Jon Muyskens (Portland) for help in preparing and maintaining the hippocampal cultures.

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