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C-series polysialogangliosides are expressed on stellate neurons of adult human cerebellum

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Until now 'c-series' polysialogangliosides were known to exist in human brain only during development and in some pathological conditions like Alzheimer's disease. Using thin-layer chromatography (TLC) and immunostaining with Q211 antibody (TLC-overlay technique) we have analysed 'c-series' gangliosides in four human cerebella (age 20, 47, 52 and 54 years). Four distinct ganglioside bands, most probably corresponding to GT1c, GQ1c, GP1c and GH1c were found to exist in the analysed brains, which is convincing demonstration of the existence of 'c-series' gangliosides in normal adult human brain. Immunohistochemical analysis was performed to locate polysialogangliosides in the analysed tissue. Q211 antibody was found to bind specifically to a single subpopulation of neurons in the molecular layer of adult cerebellum. According to their position and morphology these cells correspond to stellate neurons.

***Keywords*: gangliosides, human cerebellum, Q211 antibody, c-series gangliosides**

***Abbreviations*: TLC, thin-layer chromatography; GM3, II^{3}NeuAc-LacCer; GM2, II^{3}NeuAc-GgOse^{3}Cer; GM1, II^{3}NeuAc-GgOse$_4$Cer; GD3, II^3(NeuAc)$_2$-LacCer; GD1a, IV^{3}NeuAc, II^{3}NeuAc-GgOse$_4$Cer; GD1b, II^3(NeuAc)$_2$-GgOse$_4$Cer; GQ1b, IV^3(NeuAc)$_2$II^3(NeuAc)$_2$-GgOse$_4$Cer; GP1c, IV^3(NeuAc)$_2$II^3(NeuAc)$_3$-GgOse$_4$Cer; GT1c, II^3(NeuAc)$_3$-GgOse$_4$Cer; GQ1c, IV^{3}NeuAc, II^3(NeuAc)$_3$-GgOse$_4$Cer; GP1c, IV^3(NeuAc)$_2$II^3(NeuAc)$_3$-GgOse$_4$Cer; GH1c, IV^3(NeuAc)$_3$II^3(NeuAc)$_3$-GgOse$_4$Cer**

Introduction

Gangliosides are a heterogeneous group of glycosphingolipids present in the outer leaflet of cell membranes. They are especially abundant and diverse in the brain, but they exist in virtually all eukaryotic cells [1]. Though mostly membrane constituents, they were also found to be associated with intercellular structures [2]. As with other glycoconjugates, there is probably no general function of gangliosides, but each specific structure performs distinct functions in development, function, regeneration and aging of the nervous tissue [3, 4].

An interesting subgroup of gangliosides are the 'c-series' polysialogangliosides [5]. They were first isolated from chicken brain [6], but were subsequently also found in fishes, reptiles and some mammals [7]. In human brain they are known to exist only during fetal development, and what is very interesting, to reappear in brains afflicted with Alzheimer's disease [8–11]. The addition of 'c-series' gangliosides enhances the migration of chick neurons *in vitro* [7], and apparently at least some polysialogangliosides play an important role in axonal growth [12].

Compared with the neocortex, which contains various neuronal structures, the cerebellum is a relatively simple system. It contains only three layers with five well defined types of neurons. Recent studies on mouse cerebellum have shown that various ganglioside structures can be specifically associated with distinct layers, or even specific cell types [13, 14]. In this study we have used Q211 monoclonal antibody to examine biochemically and immunohistochemically the existence of 'c-series' gangliosides in the adult human cerebellum.

Materials and methods

Materials

Four adult human cerebella were analysed. Samples were obtained and processed according to the protocol approved by the Ethical Committee of the University of Zagreb School of Medicine. Brains originated from persons without neurological records whose death was due to accidental causes (age 20-B1, 47-B2, 52-B3 and 54-B4). Fresh tissue (12 h post mortem) was used for biochemical and immunochemical analysis. Prior to immunohistochemical

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analysis, the tissue was fixed in 4% paraformaldehyde. Q211 monoclonal antibody which specifically recognizes three sialic acids on inner galactose [6] was a kind gift of Dr Rösner (Stuttgart, Germany).

Gangliosides from two whole cerebella (B3 and B4) were extracted according to the Svennerholm procedure [15] as modified by Trbojevic-Cepe *et al.* [16]. Briefly: After homogenization, tissue was extracted with chloroform/methanol (1:2) and reextracted with chloroform/methanol/water (4:8:3). Following partition and repartition with chloroform/methanol/water in a final ratio 1:2:1.4, the upper phases were evaporated, dissolved in bi-distilled water and extensively dialysed.

Thin-layer chromatography (TLC) and immunostaining was performed using a 'sandwich method' on silica gel 60-coated glass plates (Merck, Darmstadt, Germany) with double separation in fresh solution chloroform/methanol/12 mmol l^{-1} $MgCl_2$/13.3 mol l^{-1} NH_3 (60:30:9:0.5) [17]. The samples were applied symmetrically on two halves of the plate. After separation, the plate was broken into two identical halves. Gangliosides on the one half were visualized by resorcinol spraying, and the other half was immunostained with the Q211 antibody [6]. The immunostaining procedure (overlay technique) was carried out according to Müthing and Mühlradt [18]. Briefly: The silica gel was fixed with 0.2% polyisobutylmethacrylate in n-hexane (Plexigum P28; Rohm, Germany). After blocking with 1% bovine serum albumin, the plate was incubated for 4 h with Q211 antibody (hybridoma supernatant) diluted 1:2 with PBS. After 1 h incubation with anti-mouse IgM conjugated to alkaline phosphatase (Sigma, St. Louis, USA), ≮ and then five washes in PBS, phosphate ions which inhibit alkaline phosphatase were removed by rinsing the plate twice in 0.1 M glycine, 1 mM $ZnCl_2$, 1 mM $MgCl_2$, pH 10.4. Bound antibody was visualized with 0.05% (w/v) 5-bromo-4-chloro-3-indolylphosphate dissolved in the same buffer.

The immunohistochemical analysis were performed on parasagittally-oriented paraformaldehyde-fixed tissue blocks taken from the posterior wall of the primary fissure of the right cerebellar hemisphere. Blocks were cryoprotected in graded sucrose (4%, 8% and 16% sucrose in PBS) and frozen in propane chilled with liquid nitrogen. Sections were cut at 10 µm with a Cryostat 1720 cryomicrotome (Leitz, Germany) and mounted on gelatin covered glass slides.

Air-dried cryosections were incubated three times in freshly prepared 0.1% $NaBH_4$ in PBS, and rinsed with PBS. Nonspecific binding was blocked with 1% BSA in PBS, and the sections were washed with 0.05% Tween 21 in PBS. After overnight incubation (4 °C) in 100 µl Q211 antibody diluted 1:5 with 1% BSA in PBS, the sections were rinsed with PBS and stained for 2 h with a 1:50 dilution of secondary DTAF conjugated anti-mouse secondary antibody (Dianova, Germany). Sections were then washed with PBS and cell nuclei were stained with the DNA-specific stain 4′,6-diamidine-2-phenylindole dihydrochloride (DAPI; Boehringer Mannheim, Germany). 100 µl of 1:1000 diluted stock solution (0.01% DAPI in PBS) was used per section (10^{-5}% final concentration). Sections were washed again and embedded in Mowiol (Hoechst, Germany).

Bound DTAF-labeled antibodies, as well as nuclei stained with DAPI, were examined under a fluorescence microscope (Axioskop, Zeiss, Germany) equipped with a camera (MC 63 A, Zeiss). Specific sets of filters were used for each stain to achieve selective excitation adequate to the maxima of absorption/emission of DTAF (495 nm/528 nm) and of DAPI (368 nm/488 nm). The sections were photographed on a professional black and white negative film (Kodak TMAX 400 ASA, Eastman Kodak Co., USA).

Results and discussion

Gangliosides were extracted from two complete fresh human cerebella and separated by thin layer chromatography. Samples were stained with resorcinol (non-specific staining for gangliosides) and immunostained with Q211 antibody which specifically recognizes 'c-series' gangliosides (Figure 1). Six major and some minor ganglioside fractions present in human cerebellum are clearly visible. The displayed pattern is highly reproducible and is in widespread use as a ganglioside standard in our laboratory. The identity of specific ganglioside bands was determined by comparison with the commercial standards (data not shown). Immunostaining of the same samples demonstrated the existence of 'c-series' gangliosides in adult human cerebellum. Four distinct bands are visible, which most probably correspond to GT1c, GQ1c, GP1c and GH1c gangliosides [8].

After demonstrating that 'c-series' gangliosides exist in the normal adult human cerebellum, we were interested

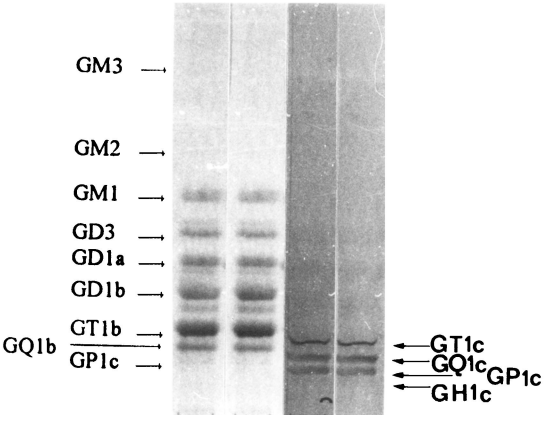

Figure 1. Thin layer chromatography and immunostaining of cerebellar gangliosides. Gangliosides from two adult human cerebella (B3 and B4) were separated by TLC and stained with resorcinol (first two lanes) and Q211 antibody (second two lanes). 5 µg of total gangliosides were applied for resorcinol staining and 10 µg for immunostaining.

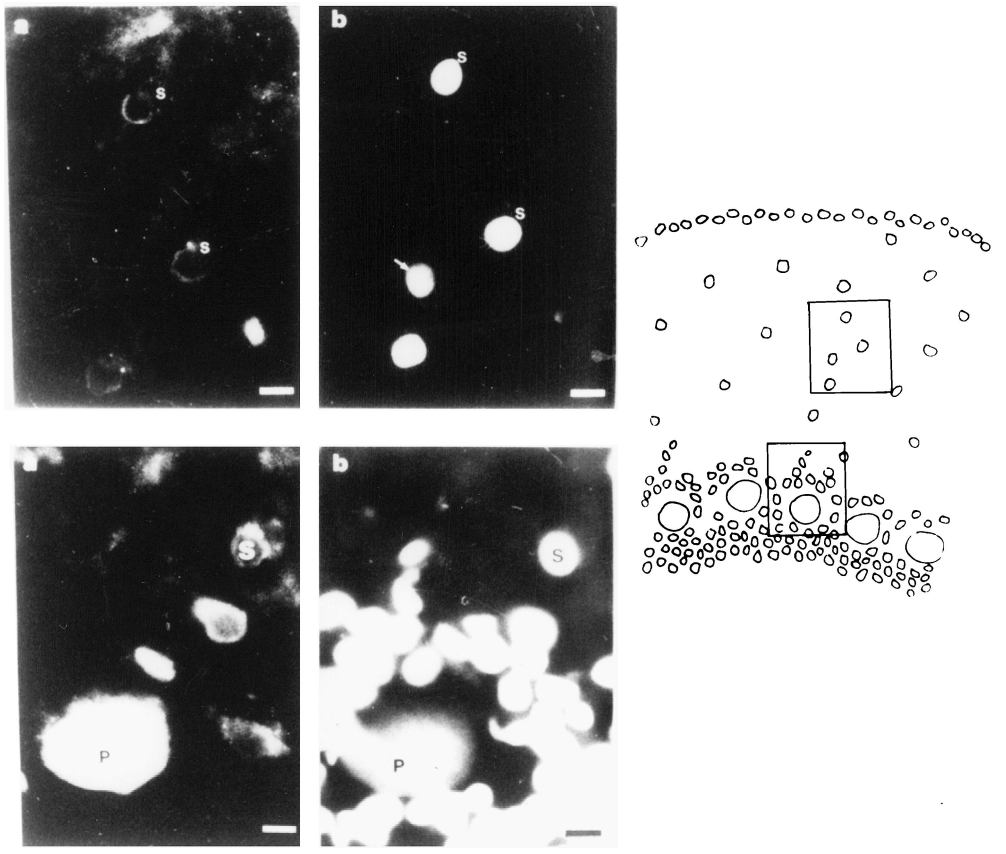

Figure 2. Immunohistochemical staining of adult human cerebellum. Adult human cerebellum slices were double stained with Q211 antibody which specifically recognizes 'c-series gangliosides' (a), and DAPI stain which stains cell nuclei (b). Two different segments of cerebellum are shown and their original positions are indicated on the drawing on the right side of the photographs. Only a single type of cells, corresponding to stellate neurons (s), was recognized by Q211 antibody. The intense staining of the Purkinje cell nuclei (P) is an autofluorescence artifact which is easily distinguishable from specific staining by its different color. (Bar = 25 μm)

to see their histochemical distribution. Since all cerebellar tissue from B3 and B4 brains had already been used, we analysed two other adult human brains from the "Zagreb neuroembryonical collection" [19]. Cerebella were prepared and immunostained as described in Materials and Methods. Results are presented in Figure 2. Surprisingly, Q211 antibody specifically recognized only a single type of cells.

There are only three types of cells above the Purkinje neurons in the molecular layer of the human cerebellum; glia cells, basket neurons and stellate neurons. Glia is known to be Q211-negative [20] and has a more fiber-like appearance throughout the whole molecular layer. Basket neurons are localized around Purkinje neurons, and were clearly negative to Q211 antibody (Figure 2). Thus the cells which were specifically recognized by the Q211 antibody could correspond only to stellate neurons.

This result confirms the existence of 'c-series' gangliosides in the adult human cerebellum, but what is even more important, their confinement to a single type of cells provides further support for the idea that gangliosides might function as specific recognition molecules in the central nervous system.

Immunohistochemical identification of the Q211 epitope in cerebellar slices, together with results from TLC analysis undoubtedly demonstrate the existence of 'c-series' gangliosides in adult human cerebellum. Moreover, they seem to be localized to a single type of cells-stellate neurons. In the context of previous reports that 'c-series' gangliosides might function in axonal outgrowth [7], this could indicate the latent plasticity of stellate neurons, a hypothesis which should be tested on cerebellum-specific neural disorders.

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