Distribution of Major Brain Gangliosides in Olfactory Tract of Frogs

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ABSTRACT

Gangliosides are major cell-surface determinants in the central nervous system (CNS) of vertebrates, found both in neuronal and glial cell membranes. Together with cholesterol and glycosylphosphatidylinositol (GPI) – anchored proteins, gangliosides are involved in organization of plasma membrane microdomains. Based on biochemical studies, frog brain was previously described as having low quantities of gangliosides and their distribution pattern in specific brain regions was unknown. Using highly specific monoclonal antibodies generated against four major brain gangliosides (GM1, GD1a, GD1b and GT1b), we examined the distribution of these molecules in CNS of four different species of frogs (Rana esculenta, Rana temporaria, Bufo bufo and Bufo viridis). We also studied the distribution of myelin- associated glycoprotein (MAG), an inhibitor of axonal regeneration, which is a ligand for gangliosides GD1a and GT1b. Our results show that ganglioside GD1a is expressed in neurons of olfactory bulb in all studied animals. In the brain of Rana sp., GD1a is expressed in the entire olfactory pathway, from olfactory bulbs to amygdala, while in Bufo sp. GD1a is restricted to the main olfactory bulb. Furthermore, we found that most of myelinated pathways in frogs express MAG, but do not express GD1a, which could be one of the reasons for better axon regeneration of neural pathways after CNS injury in amphibians in comparison to mammals.

Key words: gangliosides, myelin-associated glycoprotein (MAG), membrane microdomains, amphibians

Introduction

Gangliosides are a versatile class of glycosphingolipids particularly abundant in the vertebrate central nervous system (CNS). There are more than a hundred different ganglioside structures based on variations in their glycan and ceramide moieties. Gangliosides are actively involved in formation of plasma membrane microdomains[1]. Ganglioside glycans typically extend from the membrane surface and can participate in molecular interactions responsible for cell-cell recognition, modulation of receptor responses and fine tuning of signaling[2].

Early biochemical studies noted significant differences in ganglioside expression between animal species[3]. The most pronounced difference exists between the lower and higher vertebrates. Teleostea, Anurans and Urodela are characterized by low total content of lipid-bound sialic acid, which is predominantly presented on polysialogangliosides. Different genera show greater quantitative and qualitative variations than species in the same genus[4] and some of the major gangliosides are synthesized along unique pathways[5]. Inhibition of enzymes in-

volved in ganglioside biosynthesis obstructs morphogenetic processes in *Xenopus laevis* embryos[6].

Birds and mammals have higher total content of lipid-bound sialic acid which is predominately found on four major gangliosides: GM1, GD1a, GD1b and GT1b and synthesized along the same branched pathway[7]. Knocking out some of the key enzymes in ganglioside biosynthesis in mice does not affect morphogenesis and is not lethal in early development[8], although it interferes with myelin maintenance[9] and affects certain signaling pathways[10–12].

In higher vertebrates, gangliosides GD1a and GT1b function as receptors for myelin-associated glycoprotein (MAG)[13], one of the major inhibitors of axon regeneration in adult mammalian CNS. MAG is expressed in CNS of most vertebrates, from amphibians to mammals[14]. MAG-like proteins, different in size and glycosylation, exist in Teleosts, but their inhibitory function on axonal outgrowth is overcome by growth promoting molecules[15]. These differences may contribute to the observation that Teleosts are more successful in their recovery from spinal cord injury and other CNS lesions than are mammals. On the other hand, amphibians – the first terrestrial vertebrates, recover from optic nerve lesions[16], but not from spinal cord injury[17].

The aim of our study was to determine the anatomical localization of MAG and its glycolipid ligands, gangliosides GD1a and GT1b, in amphibian brain. Because of the observed interspecies variability of ganglioside content in amphibians[4] we studied four different species of frogs: *Rana esculenta, Rana temporaria, Bufo bufo* and *Bufo viridis*. Our selection was based on preferable ecological conditions, i.e. *Bufo sp*. as terrestrial and *Rana sp*. as aquatic.

Materials and Methods

For the immunohistochemical study of major brain gangliosides in frog brain we used three brains from each of the following species: *Rana esculenta, Rana temporaria, Bufo bufo* and *Bufo viridis*. Experimental procedure was approved by Ethical Committee of School of Medicine, »J. J. Strossmayer« University, Osijek (no. 219-0061194-2158) and Croatian Ministry of Culture, Directorate for Nature Protection. All animals were caught in lower area of river Drava (near the city of Osijek).

Animals were deeply anesthetized and decapitated. Dissected brains were immersed in cold 4% paraformaldehyde (w/v) in 10 mM sodium phosphate buffer (PBS). After 24 hours of fixation, tissue was cryoprotected in 10% sucrose (w/v) in PBS for additional 24 hours. Brains were frozen in cold 2-methylbutane and kept on –80°C.

For each studied species, we obtained cryosections in each of the three planes: horizontal, coronal and sagittal. Serial free-floating sections were pretreated in 0.6% hydrogen peroxide (v/v) in PBS to inhibit endogenous peroxidases. Blocking was performed in 1% bovine serum albumin (w/v) with 5% goat serum (v/v) (Invitrogen, Carl-

sbad, CA, USA) and 1% Triton X-100 (v/v) (Sigma-Aldrich, St. Louis, MO, USA) in PBS for 2 h. Triton X-100 was omitted in all steps in the case of anti-ganglioside immunohistochemistry. In the case of MAG and SMI 312 immunohistochemistry, Triton X-100 was used just in the blocking step and was omitted from diluted antibodies. Incubation with primary antibody was 16 h. We used highly specific monoclonal antibodies to gangliosides GM1, GD1a, GD1b and GT1b in concentrations of 0.1–0.7 μg/mL (Seikagaku, Tokyo, Japan). For the detection of myelinated fibers anti-MAG monoclonal antibody 513 was used (Chemicon, Temecula, CA) in concentration of 5 μg/mL. For the detection of fiber tracts we used an antibody against pan-axonal neurofilament marker, SMI 312 (Sternberger Monoclonals Incorporated, Baltimore, MD, USA) in dilution of 1: 10 000. Secondary antibody used was biotin-SP-AffiniPure Goat Anti-Mouse IgG (H+L) (Jackson Immunoresearch Labs., West Grove, PA, USA) in concentration of 1 μg/mL followed by Vector Elite peroxidase kit (Vector Laboratories, Burlingame, CA, USA) and developed with SIGMA*FAST*™ DAB with Metal Enhancer (Sigma-Aldrich, St. Louis, MO, USA). Sections were then mounted on slides; air dried and scanned with Nikon Super CoolScan 9000 ED scanner before coverslipping. After coverslipping with Vectamount (Vector Laboratories, Burlingame, CA), microscopic images were acquired using an Olympus D70 camera mounted on Zeiss Axioskop 2 MOT microscope. Multiple images were first assembled in CorelDraw 12 software and afterwards uniformly adjusted for contrast, intensity and brightness.

BDA tracing of the olfactory tract was performed on two additional animals of *Rana esculenta* species. Under anesthesia, each animal was unilaterally injected with 5 μL of 10 % (w/v) 10,000 MW biotinylated dextran amine (BDA) (Invitrogen, Carlsbad, CA, USA) diluted in PBS. After two weeks animals were sacrificed, brains were dissected, fixed and frozen as previously described. Biotinylated tracer was detected on serial cryosections of the entire brain with Vector Elite peroxidase kit and developed with SIGMA*FAST*™ DAB with Metal Enhancer.

Free floating sections from species found to be positive for GD1a (*R. esculenta, R. temporaria, B.bufo and B. viridis*) were subjected to sequential solvent extractions to remove lipids as follows: 30% ethanol, 70% ethanol, 96% ethanol, 100% ethanol, xylene, chloroform : methanol (1:2, v/v), chloroform : methanol (1:1, v/v), chloroform : methanol (2/1, v/v). After extraction, rehydration was performed by incubating in the same solutions in reverse order, and sections were subjected to immunohistochemistry with anti-GD1a as described above.

Results and Discussion

Expression of gangliosides GM1, GD1a, GD1b and GT1b was studied qualitatively using immunohistochemistry on brains of *Rana esculenta, Rana temporaria, Bufo bufo* and *Bufo viridis* cut in all three planes (coronal, sagittal and horizontal). Of the four gangliosides

studied, we found immunoreactivity only to ganglioside GD1a in brains of *Rana sp.* and *Bufo sp.* In these frogs ganglioside GD1a was strongly expressed either in the main olfactory bulb or throughout telencephalic structures transmitting different modalities of olfactory clues[18].

Rana esculenta had strong expression of ganglioside GD1a in the main and accessory olfactory bulb. The major projections from mitral cell layer of the main olfactory bulb i.e. medial and lateral olfactory tract strongly expressed GD1a and could be followed through medial and lateral cortices to medial septal nuclei and amygdala, respectively. Furthermore, the lateral olfactory tract and its synaptic connections that occupy uppermost third of the lateral cortex also strongly expressed ganglioside GD1a. Particularly distinct staining of GD1a was found in medial amygdaloid nuclei, the major projection site of accessory olfactory bulb[18] (Figure 1).

We also studied the expression of myelin-associated glycoprotein (MAG) in *Rana sp.*, which was expressed in the dorsal part of the main olfactory bulb, fibers running just below GD1a-stained superficial layer of lateral cortex and fibers running from the main olfactory bulb to medial cortex. On the other hand, medial and lateral olfactory tract, accessory olfactory bulb and stria medullaris thalami were completely devoid of MAG (Figures 2 and 3). Interestingly, stria medullaris thalami, where medial and lateral olfactory tracts combine, was stained with anti-GD1a. To further support our data, we used BDA to trace projections from the main olfactory bulb i.e. lateral and medial olfactory tract, and found that both are well stained with GD1a and neither one is stained with MAG (data not shown).

The same pattern of distribution of ganglioside GD1a was also found in *Rana temporaria* central nervous system (data not shown). In both *Rana* species, GD1a was completely extractable with organic solvents from all brain structures, confirming the lipid nature of the antibody epitope (data not shown).

In *Bufo bufo*, immunoreactivity to ganglioside GD1a was limited to mitral and granule cell layer of the main olfactory bulb[19] (Figure 4). MAG was strongly expressed in medial cortex and periventricular layer of the lateral cortex, but it was absent from medial and lateral olfactory tract. We obtained the same result in the CNS of *Bufo viridis*.

Earlier biochemical studies of *Rana temporaria*[21] showed that major gangliosides of frog brain, separated by thin layer chromatography (TLC), are comprised of a slow migrating component, which is probably GT1b, and one faster component that migrates between GD1a and GD1b. The same result was obtained on the whole brain extract of *Rana catesbeiana*[22]. The content of gangliosides in *Rana pipiens* brain myelin preparation[23] was very low, with TLC bands migrating close to GM1 and between GD1a and GT1b. All of these biochemical studies are similar in their conclusions: the content of gangliosides in amphibian brain is very low, and the gangliosides that are detectable do not migrate on TLC the same as major mammalian or chicken brain gangliosides. The reason for the different migration could be in the length of the ceramide anchor and it has been found that the most common fatty acid component of ceramide anchor in *Rana catesbeiana* is hydroxy- and nonhydroxy- C24:1 fatty acid[24], while direct analysis of major brain ganglio-
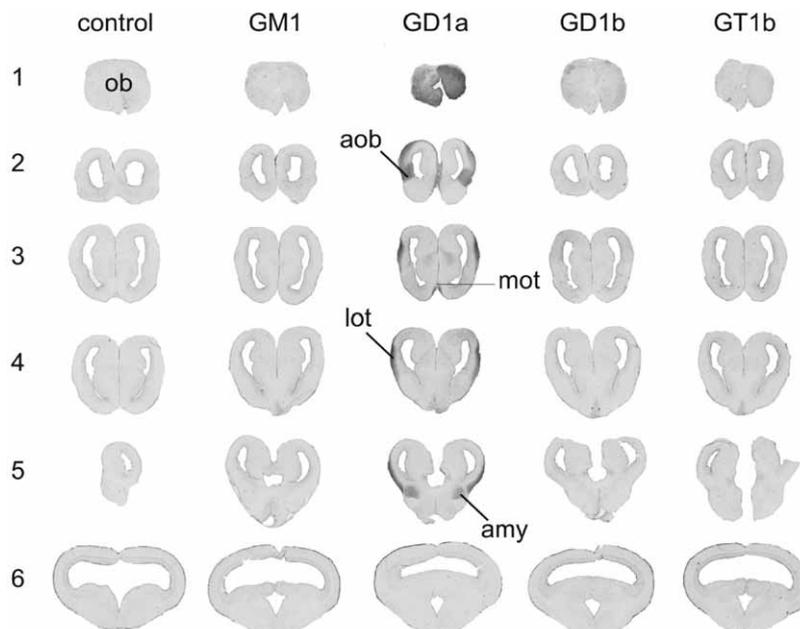

Fig. 1. Distribution of gangliosides GM1, GD1a, GD1b and GT1b in coronal sections of Rana esculenta brain. Negative control was performed by omitting primary antibody (control). ob – olfactory bulb, aob – accessory olfactory bulb, mot – medial olfactory tract, lot – lateral olfactory tract, amy – amygdala. Scale bar = 4000 μm.
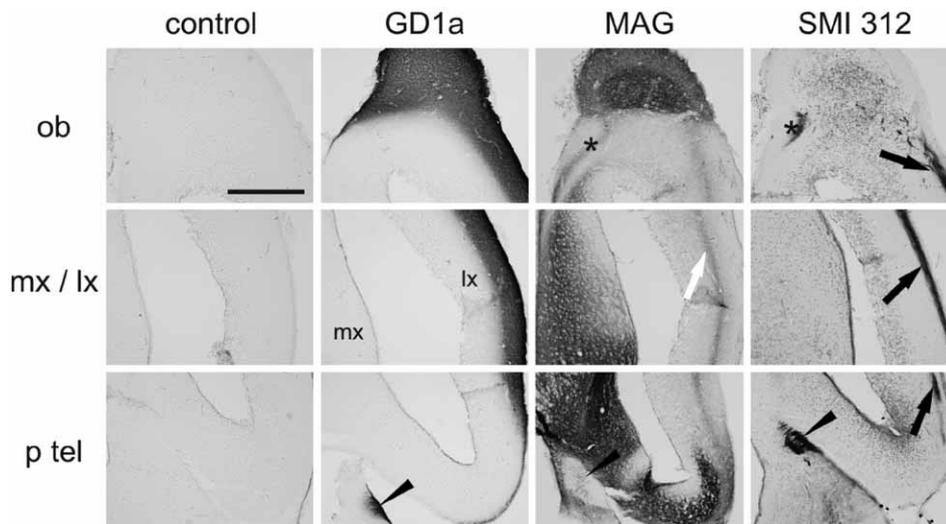
Fig. 2. Distribution of ganglioside GD1a and its ligand, myelin-associated glycoprotein (MAG), in horizontal sections of Rana esculenta olfactory bulb (ob) and telencephalon (dorsal part). Negative control was performed by omitting primary antibody (control). Stria medullaris thalami (black arrowheads) and lateral olfactory tract (black arrows) are defined with SMI312 antibody. Asterix denotes medial olfactory tract. White arrow denotes thalamic fibers. ob – olfactory bulb, mx – medial cortex, lx – lateral cortex, p tel -posterior telencephalon. Scale bar = 500 µm.

sides in mouse hippocampal tissue by mass spectrometry imaging technique shows predominance of C20:0 and C18:0 fatty acid[25].

Our results show that, among the major brain gangliosides of vertebrates, GD1a is the predominant ganglioside in CNS of *Rana sp*. and *Bufo sp*., whereas we did
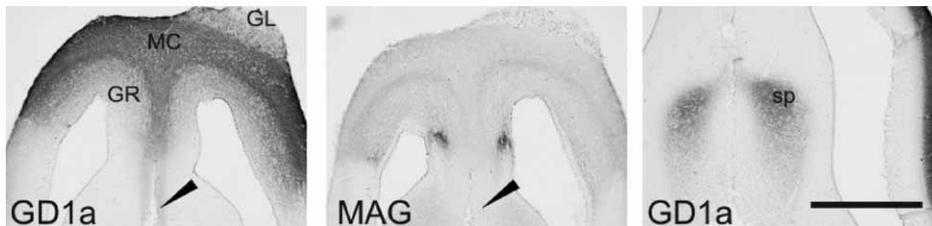

Fig. 3. Distribution of ganglioside GD1a and its ligand, myelin-associated glycoprotein (MAG), in horizontal sections of Rana esculenta olfactory bulb and telencephalon (ventral part). Black arrowheads denote medial olfactory tract. GL – glomerular cell layer of olfactory bulb, MC – mitral cell layer of olfactory bulb, GR – granule cell layer of olfactory bulb, sp – septal nuclei. Scale bar = 500 µm.
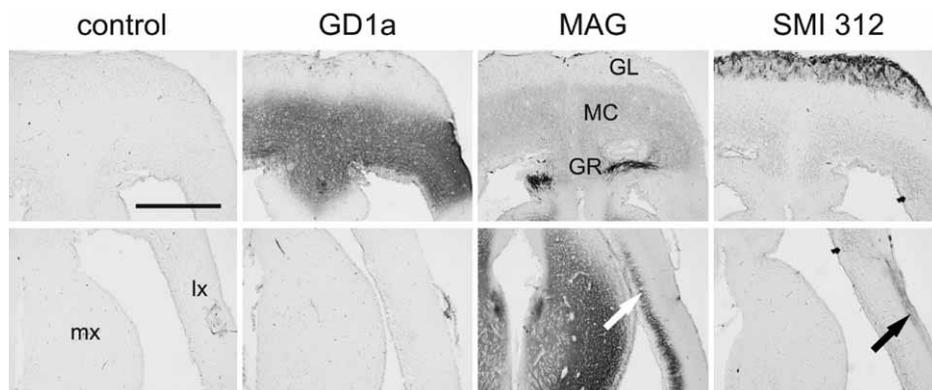

Fig. 4. Distribution of ganglioside GD1a and its ligand, myelin-associated glycoprotein (MAG), in horizontal sections of Bufo bufo olfactory bulb (ob) and telencephalon. Negative control was performed by omitting primary antibody (control). Fibers (axons) are stained with SMI 312 antibody. Black arrow points to the lateral olfactory tract. White arrow points to the thalamic fibers. GL – glomerular cell layer of olfactory bulb, MC – mitral cell layer of olfactory bulb, GR – granule cell layer of olfactory bulb, mx – medial cortex, lx – lateral cortex. Scale bar = 500 µm.

not detect any immunoreactivity to gangliosides GM1, GD1b or GT1b. The discrepancies between our study and biochemical studies could be explained by differences in the antibody binding to ganglioside epitope with a different ceramide anchor[26]. Furthermore, immunoreactivity to gangliosides also depends on the density of a particular ganglioside in the plasma membrane[27] and other components of the plasma membrane[28].

In all vertebrates olfactory bulb has the highest potential of regeneration[29,30] because of constant production of neurons and upcoming stream of migratory neurons. Regeneration of olfactory bulb and olfactory tract is a characteristic of fish and amphibian brain[31,32], but even in species where neurogenesis is limited, olfactory neurons are generated in subventricular zone and follow rostral migratory stream[33,34] toward the olfactory bulb. Our data show that MAG, an inhibitor of axonal outgrowth, is predominately expressed in different neuronal pathways than its ligand, ganglioside GD1a, in brains of *Rana sp*. This could be one of the reasons why axons of

amphibian olfactory bulb are capable of regeneration after lesion. However, our data also show that MAG and ganglioside GD1a are co-expressed in the dorsal part of the main olfactory bulb. Furthermore, other researchers have found that spinal cord, hindbrain, optic nerve and optical tectum of *Xenopus laevis* express Nogo-A[35], another myelin inhibitor of axon outgrowth. The reason why nerve regeneration still occurs in the CNS of these animals could be because of the rapid elimination of these myelin inhibitors after axonal injury[36] and their substitution with growth-promoting molecules, such as polysialylated neural cell adhesion molecule (PSA-NCAM), strongly expressed in the olfactory pathway of frogs[37].

Acknowledgements

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DISTRIBUCIJA GLAVNIH GANGLIOZIDA MOZGA U NJUŠNOM PUTU ŽABA

SAŽETAK

Gangliozidi su važne molekule staničnih membrana središnjeg živčanog sustava (SŽS) kralježnjaka, kako na membranama živčanih stanica tako i na membranama glije. Zajedno s kolesterolom i proteinima s glikozilfosfatidilinozitolnim (GPI) sidrom, gangliozidi su uključeni u organizaciju lipidnih mikrodomena (»lipidnih splavi«) u staničnoj membrani. Prethodnim je biokemijskim studijama gangliozida u mozgu žaba pokazano da njihov mozak sadrži nisku koncentraciju ovih molekula te je njihova distribucija u određenim strukturama SŽS ostala nepoznata. Koristeći visoko-specifična monoklonska protutijela na četiri glavna gangliozida (GM1, GD1a, GD1b i GT1b), proučili smo njihovu distribuciju u SŽS četiri različite vrste žaba (*Rana esculenta, Rana temporaria, Bufo bufo i Bufo viridis*). Također smo ispitali i distribuciju mijelinu- pridruženog proteina (MAG), inhibitora regeneracije aksona, za kojeg je pokazano da je ligand za gangliozide GD1a i GT1b. Naši rezultati pokazuju da se gangliozid GD1a nalazi na neuronima olfaktornog bulbusa svih proučavanih žaba. U mozgu *Rana sp.*, GD1a je nađen u cijelom njušnom putu, od olfaktornih bulbusa do amigdale, dok se kod *Bufo sp.* nalazi samo na neuronima glavnog olfaktornog bulbusa. Također smo našli da većina mijeliniziranih puteva u žaba sadrži MAG, ali ne i gangliozid GD1a, što bi mogao biti razlog za bolju regeneraciju aksona SŽS-a nakon ozljede u vodozemaca, za razliku od sisavaca.