Distribution of sialoglycoconjugates – gangliosides and PSA-NCAM in the brain of two venomous snakes: *Vipera ammodytes* and *Vipera berus bosniensis*

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Abstract

The Bosnian adder (*Vipera berus bosniensis*) and the horned viper (*Vipera ammodytes*) are two venomous snake species with different ecological preferences. The Bosnian adder occurs in a range of habitats and is endemic to the Balkan Peninsula, while the horned viper thrives in dry, rocky areas with little vegetation. The horned viper is best known for its highly venomous venom, making it the most dangerous of the European vipers. The aim of this study was to compare the expression and distribution of complex gangliosides and to identify migratory zones in the brain of Bosnian adder and horned viper. Immunohistochemistry was performed using specific antibodies for the major brain gangliosides (GM1, GD1a, GD1b, GT1b) and PSA NCAM and analysed in different brain regions. Both snake species showed expression of all four complex gangliosides with similar distribution patterns. GD1b was the most prominent ganglioside expressed in all brain structures, while GM1 showed varying distribution between the species. The strongest expression of PSA NCAM was observed in the periventricular zones of the telencephalon, suggesting that these areas are associated with neurogenesis, whereas other regions with lower expression may serve as migratory zones. In addition, it is important to note that the specific distribution of gangliosides and PSA NCAM may be influenced by factors such as brain region, developmental stage, and species-specific characteristics.

Key words: *gangliosides; PSA-NCAM; brain; venomous snake*

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Introduction

Sialic acid is an essential component of glycoconjugates in the glycocalyx, and it plays an important role in cellular processes such as cell adhesion, immune response, and cell signalling. Its presence and modifications contribute to the functional diversity and complexity of glycans in different biological contexts (Schauer, 2009; Schnaar et al., 2014). Polysialic acid neural cell adhesion molecule (PSA NCAM) and gangliosides are molecules that play important roles in neuronal development and cell adhesion in the nervous system. Although different molecules, both gangliosides and PSA NCAM can be considered components of sialoglycoconjugates as they contain sialic acid residues in their structures. Gangliosides are a type of glycolipid found mainly in the outer leaflet of cell membranes, including neuronal membranes, and are involved in cell signalling, cell adhesion, and other cellular processes (Lopez and Schnaar, 2009) while PSA NCAM refers to a polysialic acid modification of the neural cell adhesion molecule (NCAM), a glycoprotein that plays a critical role in neurogenesis and neuron migration (Gascon et al., 2007).

The distribution of gangliosides and PSA NCAM varies among animal species. The types and abundance of gangliosides may differ depending on developmental stage, tissue type, and species-specific characteristics (Ohashi, 1979; Viljetic et al., 2011; Cutillo et al., 2020). The specific ganglioside profile may vary among brain regions and cell types. Further, different animal species may show variations in the types and proportions of gangliosides in their nervous systems. The distribution of sialoglycoconjugates has been thoroughly studied in the mammalian brain, whereas there are almost no studies on their expression and distribution in the snake brain. Gangliosides such as GM1, GD1a, GD1b, and GT1b are widely distributed in the mammalian brain. With their wide species diversity and evolutionary relationship to mammals, reptiles provide excellent models for studying the structural and functional evolution of neural circuits in vertebrates. Comparative studies of reptilian brains have aimed to identify similarities or homologies at multiple levels, including brain regions, circuits, and cell types.

Fish and amphibians exhibit high levels of adult neurogenesis and have a remarkable ability to effectively regenerate neural structures, including the brain, spinal cord, retina, and olfactory system. In comparison, the regenerative abilities of reptiles, birds, and mammals are limited. Although these groups have some regenerative capabilities, they are significantly limited compared to the regenerative potential observed in fish and amphibians.

The aim of this study was to identify and compare the distribution of complex gangliosides and the presence of migratory zones expressing PSA-NCAM in the brain of two venomous viper species: Bosnian adder (*Vipera berus bosniensis*) and horned viper (*Vipera ammodytes*).

Material and methods

Animals

In this study, the brains of two adult venomous vipers were used: Bosnian adder (*Vipera berus bosniensis*) and horned viper (*Vipera ammodytes* Fig. 1). These two species have opposite environmental preferences: the Bosnian adder inhabits a range of habitats and is

Figure 1. Bosnian adder (*Vipera berus bosniensis*) – left and horned viper (*Vipera ammodytes*) – right. (Source: iNaturalist)

endemic to the Balkan Peninsula, while the horned viper thrives on dry, rocky slopes with sparse vegetation. In addition, the horned viper is notorious for its potent venom, which has earned it the reputation as the most dangerous of the European vipers.

Ethical approval

The research protocol and animal husbandry were conducted in accordance with Directive 2010/63/EU (European Union 2010) on the protection of animals used for scientific purposes. The animals were handled in accordance with the protocol approved by the Ethics Committee of the Faculty of Medicine Osijek and the Ministry of Culture, Directorate for Nature Protection of the Republic of Croatia. The study was conducted as a part of the project "Lipid rafts and glycoconjugates in development and regeneration of CNS" (br. 219-0061194-2158); Principal investigator Prof. Marija Heffer, PhD.

Sample collection

Animals were deeply anaesthetised and decapitated. The dissected brains were immersed in pre-cold 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), fixed for the following 48 hours, cryoprotected up to 20% sucrose, frozen in cold 2-methylbutane, and stored at -80°C for further use.

Immunohistochemical analysis

Brains were sectioned in the coronal plane on a cryostat (Leica, CM3050S, Germany). Immunohistochemistry was performed on 35 μm thick serial free-floating sections, with all steps performed at a temperature of 4°C. Endogenous peroxidases were blocked with 1% hydrogen peroxide/PBS, followed by 2-h incubation in blocking solution (1% bovine serum albumin and 5% goat serum in 0.1 M PBS). Sections were incubated overnight with the following primary antibodies in blocking solution: anti-GM1 (1:1000); anti-GD1a (1:2000); anti-GD1b (1:2000); anti-GT1b (1:10000), anti- PSA-NCAM (Chemicon) diluted 1:1000, anti-NeuN (Chemicon) diluted 1:2000, anti-SMI312 (Stenberg Monoclonals) diluted 1:10 000. The specificity of IgG antiganglioside antibodies has been published previously (Schnaar et al., 2002). The antibody NeuN for detection of neurons and SMI 312 as a marker for myelinated fibres were used for easier orientation on brain sections. The secondary antibodies

(biotinylated goat anti-mouse immunoglobulin G (IgG) or biotinylated goat anti-mouse immunoglobulin M (IgM) antibodies) (Jackson Immunoresearch) were used at a dilution of 1:500 in blocking solution. Washed sections were incubated in tertiary complex, developed with chromogen, mounted on slides, and air dried. The slides were scanned using NikonScan and ScanScope. Images were adjusted for contrast, intensity, and brightness.

Results

Immunohistochemistry with specific antibodies against GM1, GD1a, GD1b, and GT1b showed that all four complex gangliosides are expressed with similar distribution in the brains of both snakes. The most dominant ganglioside in both species is GD1b, which is present in all brain structures from the rostral telencephalon, tectum mesencephali, and hypothalamus to the caudal telencephalon, caudal tectum mesencephali, cerebellum, and rhombencephalon. GD1a is detected in the telencephalon and tectum of both species, whereas GT1b has a similar distribution to GD1b but is less expressed. GM1 showed a different distribution between the two species, with weak expression in Bosnian adder though evenly distributed in all brain structures, while in horned viper GM1 is expressed in the midbrain and cerebellum (Figs. 2, 3).

NeuN and SMI 312 were positive in the rostral tectum mesencephali, cerebel-

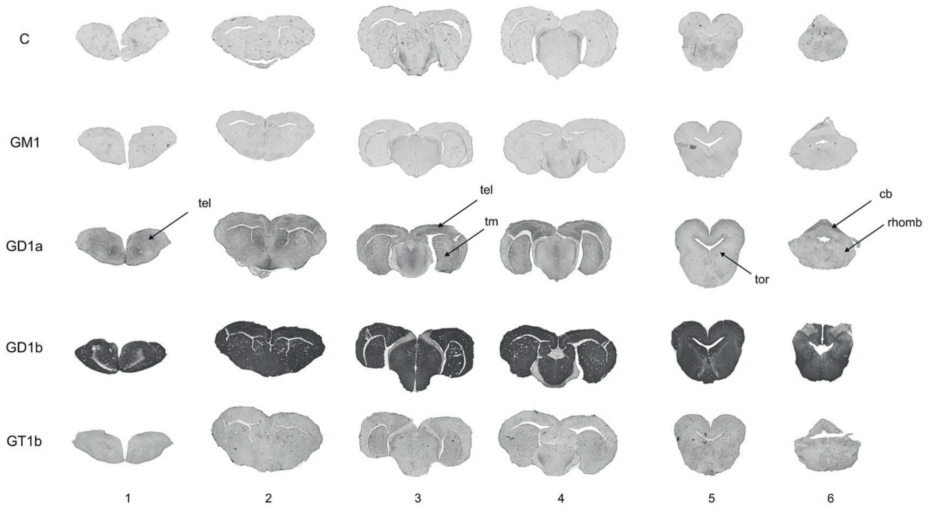

Figure 2. The distribution of GM1, GD1a, GD1b and GT1b in the Bosnian adder (*Vipera berus bosniensis*). The figure shows representative images of immunostaining for GM1, GD1a, GD1b and GT1b in coronal sections (1-6). The primary antibody was omitted from the negative control (C). Numbers denote brain regions where sections were made. 1,2) rostral telencephalon; 3) telencephalon, tectum mesencephali, and hypothalamus; 4) caudal telencephalon, tectum mesencephali; 5) rostral tectum mesencephali; 6) cerebellum and rhombencephalon.

Abbreviations: tel- telencephalon; tm- tectum mesencephali; tor- torus semicircularis; cb- cerebellum, rhomb- rhombencephalon.

lum, rhombencephalon, and spinal cord but not in the telencephalon. Compared with these two markers, the distribution of positive zones of PSA-NCAM was correlated with antibodies of NeuN and SMI 312, but was also strongly expressed in the telencephalon, where these two markers were distributed in more limited areas. The strongest expression of PSA-NCAM was found in the periventricular zones of the telencephalon, suggesting that these are areas where new neurons are born, whereas other areas with lower expression are probably migratory zones (Figs. 4 and 5).

Discussion

This study is the first report on the expression and distribution of gangliosides and PSA NCAM in the brain of two venomous snakes: Bosnian adder (*Vipera berus bosniensis*) and horned viper (*Vipera ammodytes*). As in other vertebrates, the brain of snakes can be divided into three main regions: the forebrain, midbrain, and hindbrain. The forebrain is responsible for controlling various functions, including taste, smell, and sensorimotor integration. It includes the telencephalon and the diencephalon. The midbrain is involved in the control of neuroendocrine functions and visual processing. Finally, the hindbrain controls functions such as hearing, balance, and physiological homeostasis (Divers and Stahl, 2019).

Both snake species have similar distributions of complex gangliosides, with

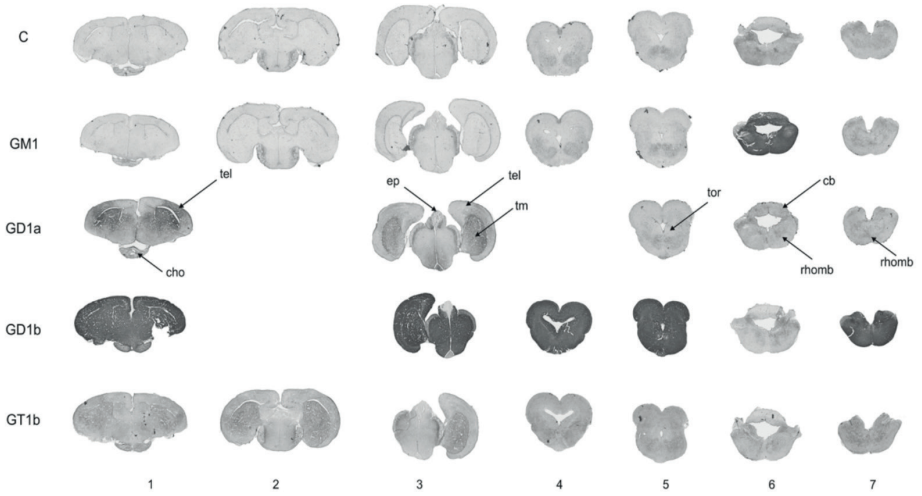

Figure 3. The distribution of GM1, GD1a, GD1b, and GT1b in the horned viper (*Vipera ammodytes*). The figure shows representative images of immunostaining for GM1, GD1a, GD1b, and GT1b in coronal sections (1-7). The numbers denote the brain regions where the sections were made. 1) rostral telencephalon; 2) telencephalon, tectum mesencephali and hypothalamus; 3) caudal telencephalon, tectum mesencephali; 4) rostral tectum mesencephali; 5) caudal tectum mesencephali and torus semicircularis; 6) cerebellum and rhombencephalon; 7) rhombencephalon.

Abbreviations: tel- telencephalon; cho- chiasma opticum; tm- tectum mesencephali; ep- epiphysis cerebri; tor- torus semicircularis; cb- cerebellum, rhomb- rhombencephalon.

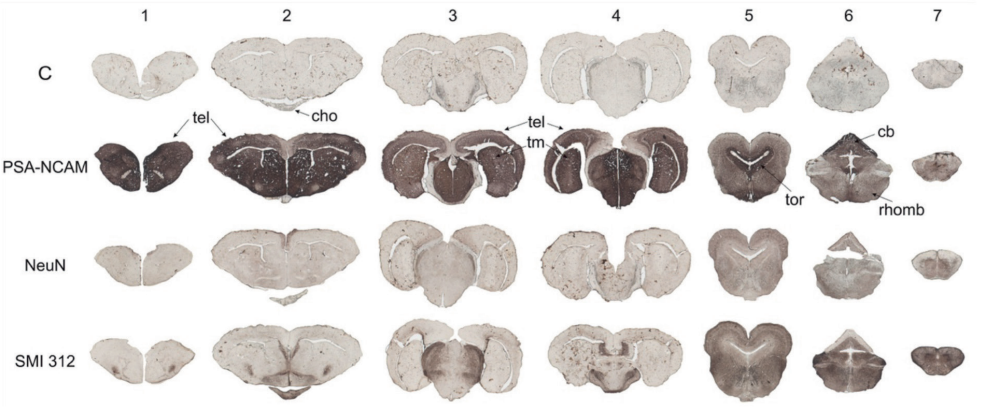

Figure 4. The distribution of migratory zones in the brain of the Bosnian adder (*Vipera berus bosniensis*). The figure shows representative images with immunostaining for PSA-NCAM, NeuN and SMI 312 in coronal sections (1-7). The numbers denote the brain regions where the sections were made. 1,2) rostral telencephalon; 3) telencephalon, tectum mesencephali and hypothalamus; 4) caudal telencephalon, tectum mesencephali; 5) rostral rectum mesencephali; 6) cerebellum and rhombencephalon: 7) spinal cord.

Abbreviations: tel - telencephalon; cho - chiasma opticum; tm - fectum mesencephali; tor - torus semicircularis; cb - cerebellum; rhomb - rhombencephalon.

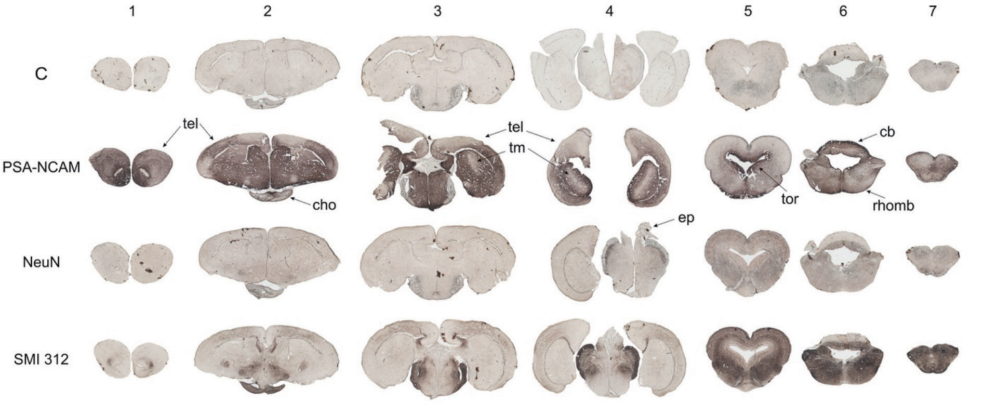

Figure 5. The distribution of migratory zones in the brain of the horned viper (*Vipera ammodytes*). The figure shows representative images with immunostaining for PSA-NCAM, NeuN, and SMI 312 in coronal sections (I-7). The numbers denote the brain regions where sections were made. 1.2) rostral telencephalon; 3) telencephalon, lectum mesencephali, and hypothalamus; 4) caudal telencephalon, tectum mesencephali; 5) rostral rectum mesencephali; 6) cerebellum and rhombencephalon; 7) spinal cord.

Abbreviations: tel - telencephalon; cho - chiasma opticum; tm - tectum mesencephali; ep - epiphysis cerebri; tor - torus semicircmaras; co - cerebellum; romb - rhombencephalon.

GD1b being the most highly expressed and evenly distributed in all brain regions of both snakes, while GM1 is dominant in the midbrain and cerebellum of the horned viper. The complex gangliosides GD1a, GD1b, and GT1b are ubiquitously expressed on neurons, whereas GM1 is present in all reptile, avian, and mammalian brains as a marker of fibres and lipid rafts (Chiricozzi et al., 2020). GD1a and GD1b are present in a specific group of non-myelinated fibres and could be used as their specific marker (Viljetić et al., 2012). Nowadays, several studies on GM1 suggest great potential in transferring the neuroprotective and neurorestorative functions of GM1 ganglioside and functional recovery of degenerated nervous system from *in vitro* to *in vivo* (Lipartiti et al., 1991; Hadjiconstantinou and Neff, 1998; Liu et al., 2015)we evaluated whether GM1 is capable of exerting antiexcitotoxic effects following its systemic administration in vivo. Newborn rats subjected to brain damage by NMDA and contemporaneously treated subcutaneously with GM1 showed significantly reduced (i. The most common animal source of GM1 remains the brain of mammals such as pig, sheep, and calf. The results of this study suggests snakes as a new potential animal model to study the role and effect of GM1.

The differences in GM1 expression and distribution could also be due to different environmental preferences. Horned vipers live in more confined and challenging conditions. Studies have shown that adult neurogenesis, the formation of new neurons in adulthood, is not limited to mammals and birds, but can also be observed in amphibians, reptiles, and bony fish. This suggests that the potential for brain plasticity and responsiveness to environmental stimuli exists in a wide variety of species. This phenomenon has been observed in a wide range of organisms, including insects such as honeybees (Groh and Rössler, 2020), other invertebrates (Groh and Rössler, 2020), mammals, fish (Salvanes et al., 2013), and reptiles (Almli and Burghardt, 2006; Nagabaskaran et al., 2021).

In general, reptiles have higher expression of complex gangliosides compared to fish and amphibians, which is related to their relatively lower regenerative potential, bringing them closer to birds and mammals in terms of regenerative capacity. Some studies suggest that b-series gangliosides are more important for the repair of damaged nerves than for the differentiation of the nervous system (Okada et al., 2002).

However, when examining the expression and distribution of PSA-NCAM migratory zones, the reptile species studied show more similarity to fish (Labak et al., 2017) and amphibians (Powers, 2016) than to mammals. PSA-NCAM, a marker associated with regenerative potential, is strongly expressed in the brains of both snake species. This observation suggests that reptiles could serve as valuable models for exploring the interplay between the maintenance of adult neurogenesis and regenerative capacity. In addition, reptiles are promising models for studying evolutionary and developmental aspects of the brain, particularly in the field of evo-devo research.

It should be noted that the specific distribution of gangliosides and PSA NCAM may be influenced by factors such as the brain region studied, developmental stage, and species-specific characteristics. Therefore, studying their distribution in different animal species could provide insights into the diversity and evolution of these molecules in the context of neuronal development and function.

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Distribucija sialoglikokonjugata – gangliozida i PSA-NCAM u mozgu dviju zmija otrovnica: *Vipera ammodytes* i *Vipera berus bosniensis*

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Bosanska riđovka (*Vipera berus bosniensis*) i poskok (*Vipera ammodytes*) dvije su otrovne vrste zmija različitih ekoloških preferencija. Bosanska se riđovka javlja u različitim staništima i endem je Balkanskog poluotoka, dok poskok obitava u suhim, stjenovitim područjima s malo vegetacije i najpoznatija je zmija po vrlo otrovnom otrovu, što ga čini najopasnijom od europskih zmija. Cilj je ovog rada bio usporediti ekspresiju i distribuciju složenih gangliozida i identificirati migracijske zone u mozgu bosanske riđovke i poskoka. Imunohistokemija je provedena pomoću specifičnih protutijela za glavne gangliozide mozga (GM1, GD1a, GD1b, GT1b) i PSA NCAM čija je ekspresija i distribucija analizirana u različitim regijama mozga. Obje vrste zmija pokazale su ekspresiju sva četiri složena gangliozida sa sličnim obrascima distribucije. GD1b je bio najistaknutiji gangliozid izražen u svim moždanim strukturama, a GM1 je pokazao različitu raspodjelu između dviju vrste. Najjača ekspresija PSA NCAM uočena je u periventrikularnim zonama telencefalona, a to sugerira da su ta područja povezana s neurogenezom, dok druge regije s nižom ekspresijom predstavljaju migracijske zone. Važno je napomenuti da na specifičnu distribuciju gangliozida i PSA NCAM mogu utjecati i drugi čimbenici kao što su: regija mozga, razvojna faza životinje i karakteristike specifične za vrstu. Stoga proučavanje njihove distribucije u različitim životinjskim vrstama pruža uvid u raznolikost i evoluciju sialoglikokonjugata u kontekstu razvoja i funkcije neurona.

Ključne riječi: *gangliozidi, PSA-NCAM, mozak, zmije otrovnice*