In the first stage of the project, intact chondroitin/dermatan sulfate (CS/DS) glycosaminoglycan chains, as part of proteoglycans, were obtained. These structures, belonging to the extracellular matrix of the central nervous system, were extracted and purified from wild type mouse brain.

Initially, proteoglycans from mouse brain were extracted and purified by DEAE anion exchange chromatography based on negatively charged glycosaminoglycans. Extraction and purification procedure consisted of incubation of brain tissue in 4M guanidinium chloride, resuspension of the supernatant in a solution containing 150 mM NaCl, followed by a stage of separation by anion exchange chromatography. Because of their negative charge, glycans are attached to stationary phase represented by DEAE-Tris-Acryl M. Elution from the column of interest glycans was performed in the concentration gradient, eliminating nonspecific bound compounds, finally collecting the proteoglycans using 1M NaCl elution buffer. The next step in this process was removal of NaCl by dialysis followed by sample concentration. The saccharides from intact long chains of chondroitin sulfate and dermatan sulfate were further separated from proteic core by β elimination.

These free chains have undergone processes consisting purification by rechromatography on anion exchange concentration gradient using the same stationary phase, followed by precipitation in ethanol and nitrous acid digestion. These purification methods have been removed other impurities eluted together with compounds of interest in the first phase of chromatography. The workflow of the experimental process is depicted in figure 1.
Figure 1. Schematic presentation of extraction, purification, preparation, analysis and structural identification through advanced mass spectrometry and hyphenated techniques of brain CS/DS. (Zamfir AD, Flangea C, Serb A, Sisu E, Zagrean L, Rizzi A, Seidler DG. Brain chondroitin/dermatan sulfate, from cerebral tissue to fine structure: extraction, preparation, and fully automated chip-electrospray mass spectrometric analysis. Methods Mol Biol., 2012, 836, 145-159)

At the end of this stage we obtained pure hybrid chondroitin sulfate/dermatan sulfate chains that can be subjected to further analysis procedures by advanced mass spectrometry techniques. The entire protocol was published in Proteoglycans. Methods and Protocols; Methods in Molecular Biology, vol 836, Springer Protocols, Humana Press Publishing House, 2012.
In order to be analyzed by high performance mass spectrometry techniques, pure hybrid glycans obtained as long chains must be depolymerized with enzymes specific for different types of structures. In the case of hybrid chains of chondroitin sulphate/dermatan sulfate, the form found in biological tissues, were chosen different types of lyase enzyme having a high specificity. Thus, the link between N-acetyl galactosamine (GalNAc) and glucuronic acid (GlcA) was cleaved using chondroitin AC lyase while the connection between GalNAc and iduronic acid (IdoA) was cleaved by B lyase. Following the action of both enzymes to eliminate a water molecule, results a double bond between carbon 4 and carbon 5 of IdoA and GlcA from the nonreducing end. The structures obtained have always 18 Daltons less, corresponding to a water molecule. Throughout the mass spectrometry experiments, the 18 Daltons represent a tag for the nonreducing end, this difference generated by the removal of a water molecule produce a specific marker of hexuronic acid at the nonreducing end.

Oligosaccharide mixture obtained after enzymatic digestion was subjected to separation by size exclusion, getting separated disaccharides, tetrasaccharides, hexasaccharides, octasaccharides and decasaccharides.

Analysis of tetrasaccharide fraction depolymerized with AC lyase I noticed a number of atypical sulfation patterns. To confirm this structure, the trisulfated tetrasaccharide was submitted to a fragmentation procedure represented by collision-induced dissociation (CID) during infusion by chip-based nanoelectrospray mass spectrometry. This methodology outlined a new structure, unidentified until now, namely \([4,5\Delta \text{GlcA-GalNAc (4S) -Ido (2S, 3S) -GalNAc}]\) (figures 2, 3 and 4). Fragmentation in multiple stages allowed us to discover such new molecules and introduce them in the scientific literature. However, to confirm this structure were necessary supplementary experiments.
Figure 2. Fully automated chip (-) nanoESI HCT MS² of the [M-H]⁻ at m/z 997.30 corresponding to hybrid trisulfated-[4,5β-GlcAGalNAc(4S)-IdoA(2S,3S)-GalNAc]. MS² by CID. Inset: proposed structures and the fragmentation pathways. The ions diagnostic for trisulfated-[4,5α-GlcA-GalNAc(4S)-IdoA(2S,3S)-GalNAc] are marked by *; the ions diagnostic for trisulfated-[4,5β-GlcA-GalNAc-IdoA(2S,3S)-GalNAc(4S)] are marked by #; the ions common for both structures are unmarked. (Flangea C, Petrescu AJ, Seidler DG, Munteanu CV, Zamfir AD. Identification of an unusually sulfated tetrasaccharide chondroitin/dermatan motif in mouse brain by combining chip-nanoelectrospray multistage MS(2) -MS(4) and high resolution MS. Electrophoresis, 2013 34, 1581-1592).
Figure 3. Fully automated chip (-) nanoESI HCT MS$^3$ of the [M-H]$^-$ at m/z 794.28 assigned to C$_3$** in MS$^2$. MS$^3$ by CID. Inset: proposed structure and the fragmentation pathway. (Flangea C, Petrescu AJ, Seidler DG, Munteanu CV, Zamfir AD. Identification of an unusually sulfated tetrasaccharide chondroitin/dermatan motif in mouse brain by combining chip-nanoelectrospray multistage MS(2) -MS(4) and high resolution MS. Electrophoresis, 2013 34, 1581-1592).
Figure 4. Fully automated chip (-) nanoESI HCT MS$^4$ of the [M-H]$^-$ at m/z 353.04 assigned to Y$_1$ in MS$^3$. MS$^4$ by CID. Inset: zoomed aream/z (50–350). (Flangea C, Petrescu AJ, Seidler DG, Munteanu CV, Zamfir AD. Identification of an unusually sulfated tetrasaccharide chondroitin/dermatan motif in mouse brain by combining chip-nanoelectrospray multistage MS(2) -MS(4) and high resolution MS. Electrophoresis, 2013 34, 1581-1592).

These experiments were performed in 2013 in the next stage of the project. Being a newly discovered molecule was necessary to demonstrate the postulated structure using another mass spectrometry technique based on nanoelectrospray infusion having another separation principle of molecules. In this regard was employ quadrupole time-of-flight mass spectrometer (QTOF). **Fragmentation could demonstrate in this case the existence of [4,5Δ GlcA-GalNAc (4S) -Ido (2S, 3S) -GalNAc]** (figure 5) and this research and discovery was

Figure 5. (-) nanoESIQTofMS/MS of the [M-2H⁺]²⁻ at m/z 498.04 corresponding to hybrid trisulfated-[4,5_-GlcA-GalNAc(4S)-IdoA(2S,3S)- GalNAc]. MS/MS by CID using Ar as the collision gas. Collision energy within (8–30) eV. Inset: proposed structures and the fragmentation pathways. (Flangea C, Petrescu AJ, Seidler DG, Munteanu CV, Zamfir AD.
Identification of an unusually sulfated tetrasaccharide chondroitin/dermatan motif in mouse brain by combining chip-nanoelectrospray multistage MS(2)-MS(4) and high resolution MS. Electrophoresis, 2013 34, 1581-1592).

Hyphenated techniques for high performance mass spectrometry methods can be applied to separate both online and off-line structures depending on the degree of sulfation. One such technique is capillary electrophoresis. Coupling on-line capillary electrophoresis with mass spectrometry, unlike offline version offers many advantages in terms of time of analysis, determination accuracy and significantly smaller sample volume. Description of the method and the protocol was published in 2013 in Mass Spectrometry of Glycoproteins: Methods and Protocols; Methods in Molecular Biology, Vol 951, Springer Protocols, Humana Press Publishing.

The research was continued with the study of glycan expression in brain belongs to the group of gangliosides, performing a comparative analysis of the sensory and motor cortex. Both regions were subjected to the same protocol and analyzed under identical conditions. Unlike the sensory cortex, motor cortex could be observed in the presence of tetrasialylated structures. Here were also identified several species fucosyl, and acetylated ganglioside with trihydroxylated sphingoid bases. These structural differences may be related to the different functions performed by the two regions, but to clarify this issue further studies are needed.

For fragmentation was chosen simply charged species at m/z 1718.89 identified only in the motor cortex, which corresponds, according to mass calculated, to Fuc-GM1 (d18: 1/20: 0). This structure was chosen for detailed characterization because no other fucosylated monosialilated tetraose was observed in the sensory cortex and, in addition, this molecule not investigated until now. Conditions for tandem mass spectrometry (MS/MS) were chosen to achieve a fragmentation to allow the characterization of ganglioside species with sialic acid and fucose attached to prove their position. The analysis of MS/MS illustrates the binding of sialic acid and fucose to the inner galactose as well as confirm the type of ceramide (d18: 1/20: 0) (figure 6).

Thus, through high performance mass spectrometry techniques could be identified not only structural difference between the two brain regions but made possible the identification of a first Fuc-GM1 type isomer (d18: 1/20: 0) with fucose and sialic acid attached to the same galactose. These data reveal that acetylation, fucosylation and sialylation contribute greatly to the performance of specific brain functions, in general, and sensory and motor cortex, in particular. The results were published in 2013 in Australian Journal of Chemistry (Mass spectrometry of gangliosides from human sensory and motor cortex. Flange C, Fabris D, Vukelić Ž, Zamfir AD. Aus. J. Chem., 2013; 66: 781-790.).
A number of such structures with different monosaccharides attachment and configuration of various carbohydrate residues could provide answers in elucidating the mechanisms of senescence, degeneration and tumorigenesis. Further investigation of structures present in various physiological and pathological states by high performance mass spectrometry techniques are required to enable identification of the fine changes in molecules, undetected by other methods.

Identification and discovery of new structures in the previous stages such as chondroitin/dermatan sulfate tetrasaccharides was continued following composition and structural characteristics of longer chains. The research was focused on octasaccharides because hybrids fraction of chondroitin sulphate/dermatan sulfate have double number of monomers than species investigated successfully before.

Octasaccharides were extracted and purified from wild type mice brains of 14 and 4 weeks old. Besides structural characterization, to follow and discover some differences in terms of glycosaminoglycans expression at different ages for future studies of the structures effects on brain development and maturation.

In the case of molecules identified in fraction from 14 weeks mice is observed a total number of 26 structures of which 9 are undersulfated, 5 are regular sulfated and 12 are oversulfated. Of these, 9 saturated species shows a relatively equal distribution in terms of the number of sulfate groups. Chains identified in 4 weeks mice shows 14 species of which 4 undersulfated structures, one regular sulfated and 6 oversulfated structure. It can be seen only one undersulfated saturated octasaccharides. Comparative examination of the two spectra reveals the increasing number of saturated species with age but also a variation in the number of sulfate groups. Also, there has been a diversification in terms of SO$_3$ group distribution along chains, oversulfated species belong to the 4 weeks old mice which have only 7 or 8 sulfate groups while molecules from 14 weeks old mice display 5, 6, 7 and 8 SO$_3$ groups. From our data we can observe a variety of structural enrichment in aging, modification along the chain as well as in real non-reducing end of the intact CS/DS chain.
MS analysis of glycosaminoglycans from 14 weeks old mice revealed 14 structures of which 6 are undersulfated, 3 are regularly sulfated and 3 are oversulfated. We could not observe saturated chains at this age. This aspect can be explained by the absence of iduronic acid at the real non-reducing end at this age. It can also be observed in addition a undersulfated hexasaccharide. In 4 weeks mice brains were identified 11 structures from which 5 are undersulfated, 2 are regularly sulfated and 4 oversulfated. Noticing in the two comparative profiles at different ages, can be seen an increase number of undersulfated species and decreases the number of oversulfated saccharides with brain maturation and development. Also, in the same brain, during the maturation process, the real nonreducing end will not contain saturated iduronic acid, although there are arguments that at younger ages can have iduronic acid in composition, supported by hexasaccharide [IdoAGalNAc (GlcAGalNAc) 2] (0S) at m/z 1154.44.

Among the structures, the most interest is undersulfated and oversulfated species because their changing during development is one of the consequences of complex transformations in central nervous system. To demonstrate this aspect, octasaccharides pentasulfated molecule from 14 weeks mice digested with AC lyase was chosen for fragmentation experiment.

The MS fragmentation technique used was collision induced dissociation (CID) applied to species detected at m/z 478.06 as [M - 4H]⁺. MS² spectrum (Figure 7), MS³ (Figure 8), and MS⁴ (Figure 9) shows the structure of [4,5Δ-GlcA (S) GalNAc (S) -IdoAGalNAc (S) -IdoAGalNAc (S) -IdoAGalNAc (S)].
Figure 7. CID MS² fragmentation of [M-4H⁺]⁺ detected at m/z 478.06 as [4,5Δ-GlcAGalNAc(IdoAGalNAc)₃](SS). Inset: proposed structure and fragmentation pathway.

For MS³ experiments the double charged ion C₄⁻ was chosen to demonstrate atypical position of the sulfate groups link to the GlcA. The ion corresponding tetrasaccharide [4,5Δ-GlcA (S) GalNAc (S) -IdoAGalNAc (S)] is sulfated at both GalNAc molecules in addition to GlcA.

An additional argument for locating the SO₃ to GlcA is brought by the next stage of fragmentation where was chosen as the precursor ion the disaccharide from the nonreducing containing atypical sulfation and include a tag, this end represented by the presence of a double bond between the C4 and C5 due to the elimination of a water molecule during depolymerization process.
Figure 8. CID MS$^3$ of C$_4^-$ detected at m/z 498.02 in MS$^2$. Inset: proposed structure and fragmentation pathway.

Figure 9. CID MS$^4$ of C$_2^-$ detected at m/z 538.18 in MS$^3$. Inset: proposed structure and fragmentation pathway.
In these experiments, could not identify any fragmentation of ring cleavage indicating sulfate groups within the monomers. Although it can be demonstrated for the first time the existence in brain tissue of an octasaccharides sequence \([4,5\Delta \text{GlcA (S) GalNAc (S) -IdoAGalNAc (S) -IdoAGalNAc (S) -IdoAGalNAc (S) -IdoAGalNAc (S)}]\) is not yet known the role that this structure plays in physiological and/or pathological processes of a central nervous system.

Identification of structures and the discovery of new rare species in this project represent a challenge for further research that could have as a starting point in the study of the dynamics of change and maturation in normal and pathological brain development. Thus, in this project we managed to present the discovery of new structures in the brain and introduce them in the scientific literature by publishing results in journals with high impact factors. These structures are: \([4,5\Delta -\text{GlcA-GalNAc (4S) -ido (2S, 3S) -GalNAc}]\) and \(\text{Fuc-GM1 (d18: 1/20: 0)}\) isomer with sialic acid and fucose attached to the same inner galactose. In addition to these accomplishments, in this project was performed a partial brain tissue mapping in terms of glycan structure which open opportunities in the research towards the identification of molecular rearrangements in various development stages and physiological or pathological conditions of nervous system. On the other hand in this project was accomplished a bioanalytical and experimental platform that can be used in biological studies. Also, working protocols have been published in prestigious publishing houses as chapters of books, and became available to researchers in the field.

Another aspect still unknown, derived from these discoveries, is molecular signals which create the structural manifestation of these patterns, their abundance and also their possible regeneration or degeneration capacity. This project has enriched existing research data regarding the molecular morphology of the brain but also created another direction of research in the neurosciences. This opens a new opportunity in neurobiology to investigate the existence of the same chains of human brain tissue but also of their biological role.
PROJECT RESULTS (2011-2014)

1. Articles in impact factor journals

1. Flangea C, Petrescu AJ, Seidler DG, Munteanu CV, Zamfir AD. Identification of an unusually sulfated tetrasaccharide chondroitin/dermatan motif in mouse brain by combining chip-nanoelectrospray multistage MS(2) -MS(4) and high resolution MS. Electrophoresis, 2013 34, 1581-1592; impact factor: 3.161


2. Book chapters


3. Conference presentations

1. Viena, Austria, 17-19 February, 2014 la 25th Mass Spectrometry Forum

2. Siena, Italia 30.06.2013-04.07.3013 la 2nd Middle Eastern and Mediterranean Sea Region Countries Mass Spectrometry Conference

3. Berlin, Germania 10.03.2013-14.03.2013 la 46th German Society for Mass Spectrometry Conference


5. Timisoara 05.06.2013-09.06.2013 at 5th International Congress and the 31th Annual Session of the Romanian Society for Cell Biology

6. Poznan, Polonia 02.03.2012 – 08.03.2012 at conference “Joint Conference of German Mass Spectrometry Society and Polish Mass Spectrometry Society”

7. Satu Mare 13.06.2012-17.06.2012, 4th International Congress and the 30th Annual Session of the Romanian Society for Cell Biology


9. Bucuresti 18.10.2012-19.10.2012 at The 3rd Conference of the National Neuroscience Society of Romania with international CEERC-IBRO session. The paper was awarded with the 3rd Prize of the Jury

10. Arad 08.11.2012-09.11.2012, International Symposium Research and Education in Innovation Era