# Correlation between the cerebralization, astroglial architecture and blood-brain barrier composition in Chondrichthyes

Ph.D. thesis

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**Introduction**

Representing a separate radiation of vertebrates, Chondrichthyes underwent a unique brain evolution. They display a wide range of cerebralization, differences in the glial architecture and in the composition of the blood-brain barrier. Some groups of Chondrichthyes, and also some of their brain parts were neglected in previous neuroanatomical studies and only few immunohistological techniques were applied in order to describe the glial pattern of cartilaginous fishes.

Class of cartilaginous fishes (Chondrichthyes) comprises two major divisions (subclasses), the Elasmobranchii (sharks, skates and rays) and the Holocephali (chimaeras or ratfishes or ghostsharks). The living elasmobranchs comprise four major superorders: Squalomorphii, Galeomorphii, Sqatinomorphii, and Batoidea, according to Compagno (1977), while the Holocephali are represented today by only one extant order with three families.

According to a recent study within each of gnathostomata radiations - chondrichthians, actinopterygians, and sarcopterygians- two types of organization may be found in the brains of the various species: type I (’laminar’) and type II (’elaborated’). In type I brains, which is characteristic in squalomorph sharks and in holocephalan, the neuronal cell bodies not or only partially migrated away from the periventricular matrix. The brain ventricles are large, while the brain wall is relatively thin. In type II brains, which is characteristic in galeomorph sharks and in batoids, the neurons migrate extensively and therefore these brains are generally relatively larger in size and more complex as compared with brains exhibiting laminar organization. More individual nuclear groups are formed, the ventricles are of reduced size, whereas the brain wall has thickened.

Comparison of cerebralization in chondrichthyan fishes with that of other vertebrate groups has been made by constructing ‘minimum convex polygons’ to enclose data points in a double logarithmic scale of a brain weight/body weight plot by Jerison (1973). The same method was applied to additional data, by Northcutt (1977, 1978, 1981) and Smeets et al. (1983). According to such analysis, chondrichthians exhibit a wide range of cerebralization. The brain weight/body weight ratios in batoids and galeomorph sharks are two to six times larger than in squalomorph sharks. Within the superorder Batoidea (skates and rays), Rajiformes (skates) have relatively low brain weight/body weight ratios, whereas Myliobatiformes show the highest brain weight/body weight ratios known for elasmobranchs.

Horstmann (1954) reported that there was a principal difference between the elements of the astroglial system in sharks and rays. In skates and rays the preponderant glial elements are true astrocytes, (non-ependymal stellate-shaped cells), such as in birds and mammals. However, in sharks the preponderant glial elements are ’tanycytes’, i.e. thin and elongated, fiber-like and usually radial cells of ependymal origin, as in reptiles and in the majority of anamniotes.

In the present study, I investigated three different astroglial markers, glial fibrillary acidic protein (GFAP), S-100 protein, and glutamine synthetase. GFAP is the most widely used marker of astroglia, and the anti-mammalian GFAP antibodies have been applied successfully in other vertebrates, including cartilaginous fishes. These studies were confined to the demonstration of cross-reactivity of chondrichthyan GFAP with mammalian anti-GFAP antibodies *in vitro*, and in some tissue samples. Astrocytes have not always proven immunoreactive to

GFAP in birds and mammals, therefore using additional astroglial markers, glutamine synthetase and S-100 protein was necessary. Preliminary experiments demonstrated that these methods can be applied successfully in cartilaginous fishes, and similar results for S-100 protein were reported by Chiba (2000).

Chondrichthians exhibit differences in the blood-brain barrier, therefore their gliovascular connections are especially interesting. While in sharks and rays, the blood-brain barrier is formed by perivascular glia, in chimaeras endothelial cells are responsible for this function, as in other vertebrates.

The dystroglycan complex (DGC) is the most important laminin receptor beside integrins. It is required for the stabilization of gliovascular structure, and for the maturation and functional integrity of the blood-brain barrier. Linking cells to basement membranes, the complex was originally described in muscle but it also occurs in the brain and other organs. The components of the DGC are well known in the electric organ of *Torpedo* but it is not known whether these proteins are present also in the vessels of cartilaginous fishes. The complex is responsible for the distribution and anchoring of the water-pore channel protein, aquaporin-4. Aquaporins (AQP4 and AQP9) play a crucial role in volume homeostasis.

**Aims**

The aims of the present study were:

1) To get a better view on the astroglial architecture of elasmobranchs, the present study supplements the former impregnation based studies and GFAP immunohistochemistry by the immunohistochemical detections of glutamine synthetase and S-100 protein.
2) To extend examination to other representatives and important groups of Chondrichthyes, such as Myliobatiformes (because of their large brain), and Holocephali (because of the different blood-brain barrier), on which no glial architectural study has been done as yet.
3) To study the rhombencephalon in greater detail, which has been hardly covered in previous glial studies.
4) To reveal some characteristic features of the gliovascular connections in cartilaginous fishes: investigating the presence and distribution of the proteins of dystroglycan complex, such as dystroglycan (DG), dystrobrevin, dystrophin, syntrophin and utrophin, in addition to AQP4 and AQP9.
5) To discern correlation between cerebralization types, glial architecture and the immunohistochemical markers of blood-brain barrier.
6) To highlight evolutionary changes of astroglia in chondrichthyes.

In appendix:

7) To discuss the significance of the enlarged brain parts in ecological perspectives.

**Materials and Methods**

**Sample collection**

Every important chondrichthyan group was represented in our study:

*Callorhinchus milii*: Holocephali; *Squalus acanthias*: Squaliformes; *Pristiophorus cirratus*: Pristiophoriformes; *Cephaloscyllium laticeps*, *Scyliorhinus canicula*: Carchariniformes; *Dipturus whitleyi*, *Raja miraletus*, *Raja clavata*: Rajiformes; *Torpedo marmorata*: Torpediniformes; *Dasyatis pastinaca*, *Myliobatis australis*,

*Mobula japanica*: Myliobatiformes. The species were identified using the identification keys of Last and Stevens (1994). The gender, body length, body weight and in situ brain morphology have been documented. The specimens obtained from fishermen were caught as by-catch, and the post-mortem time prior to fixation was 2 to 3 hours.

**Histological processing**

**Tissue fixation**

None of the animals have been perfused. Brains were removed on site and immersion-fixed in 4% paraformaldehide and after 24 hours the fixative was changed and the brains were stored for two more weeks. The large-sized *Mobula japanica* brains have been immersion fixed in AFA fixative (90ml 80% ethanol, 5ml formalin, 5ml glacial acetic acid, Northcutt, 1977, 1978), which penetrates well, and after 24 hours the brains were placed into 70% ethanol for transportation.

**Tissue embedding**

For embedding the following methods were applied:

- **Agarose**: Before sectioning the brains were embedded in agarose (5g agar was dissolved in 100ml cold water, stirred and warmed up until it was completely dissolved) After removing the meninges, the brains were placed into blocks of hot agarose, and then cooled in a refrigerator. Because of the large size of *Mobula japanica* brains, it was impossible to use the agarose embedding technique.

- **Paraplast**: From each species represented by more than one specimen, immunohistochemical reactions were also performed on materials embedded in paraplast (Sigma, Paraplast Plus P3683). A detailed description is given below for Mobula brains but the method was also applicable with slight modifications for the smaller sized brains.

The large-sized *Mobula japanica* brains were dehydrated by storing in 80 % ethanol for 7 days, 90 % ethanol for 7 days, 96 % ethanol for 2 x 7 days, 100 % ethanol for 3 x 1 days, 1 % celloidin methylbenzoate for 1 and 2 days, and xylene for 2 x 1 hours. Before embedding the brain was stored in paraplast: xylene mixtures (1: 2, 1:1 and 2:1), each for half an hour, followed by paraplast for 12 hours, than for 1 day twice. The brains were embedded in paraplast (Sigma, Paraplast Plus P3683).

**Sectioning**

From the brains embedded in agarose serial coronal sections (60-100 μm thick) were cut with a Vibratome and the sections were floated in phosphate buffer (0.1 M, pH 7.4).

From the brains embedded in paraplast serial coronal sections (10 μm thick) were sectioned by a Reichert microtome and mounted on slides coated with albumin or gelatine.

Where it is mentioned in the text, semithin sections were also prepared, following dehydration in an ascending dilution series of alcohol and propylene oxide, and embedding in epoxy resin (Durcupan), cut by Reichert ultramicrotome, and stained with toluidine blue.

**Immunohistochemistry**

Free-floating (Vibratome) sections were pre-treated with 20% normal goat or horse serum for 1.5 hours to suppress the non-specific binding of antibodies. This

and the following steps all included a rinse in phosphate buffered-saline (PBS, Sigma, 0.01 M, pH 7.4) interposed between the changes of reagents.

The following primary antibodies were used in this study:
monoclonal anti-GFAP 1:100, mouse; polyclonal anti-GFAP 1:100, rabbit; monoclonal anti-glutamine synthetase 1:100, mouse; polyclonal anti S-100 1:100, rabbit; polyclonal anti-aquaporin 4 1:200, rabbit; polyclonal anti-aquaporin 9 1:100, rabbit; polyclonal anti-α dystrobrevin 1:100, goat; monoclonal anti-dystroglycan 1:100, mouse; monoclonal anti-dystrophin 1:2, mouse; polyclonal anti-syntrophin 1:100, rabbit; polyclonal anti-utrophin 1:10, mouse.

The liophilised immunochemicals were restituted according to the manufacturer's prescriptions, and further diluted to 1:100 in PBS containing 0.5% Triton X-100, and the sections were incubated for 40 hours at 4°C. The markers were examined in each species by using parallel series of sections. Double immunoreactions with anti-dystroglycan and anti-dystrobrevin, or anti-syntrophin were also performed.

**Visualization**

**DAB reaction on floating slides:**

The immunohistochemical reaction was developed according to the 'ABC'-method. Biotinylated anti-mouse, anti-goat or anti-rabbit immunoglobulin and avidin-biotinylated horseradish peroxidase (ABC) complex were applied subsequently, in a dilution of 1:100, for 1.5 hours, at room temperature. The immunocomplex was visualized by diaminobenzidine (DAB) reaction: i.e. by incubation in a mixture of 0.05 % 3,3'-diaminobenzidine, 0.05 M Tris-HCl buffer (pH 7.4) and 0.01 % $H_2O_2$ for 10 min, at room temperature. The sections were mounted from PBS, dried in air, and coverslipped with DePeX.

**Fluorescent reaction on floating slides:**

Fluorescent immunohistochemical reactions were also performed. This technique is insensitive to either endogenous peroxidase activity, or to endogenous biotin. The incubation with primary antibodies was the same as described above. As fluorescent secondary antibodies, Cy3 dye-conjugated anti-mouse immunoglobulin, or fluorescein-isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulin were used, respectively, in a dilution of 1:300 in PBS, for 3 hours, at room temperature. The sections were finally washed in PBS for 1 hour at room temperature and coverslipped in a 1:1 mixture of glycerol and double distilled water. The Cy3 dye emits red light (570nm) when induced by green light (550nm), whereas in the case of FITC the inducing light is blue (495nm) and the emitted light is green (519nm).

**DA reaction on paraplast slides:**

The immunhistochemical reactions on sections with paraplast were performed similarly to that on the floating sections.

Some of the sections were counterstained by cresyl violet, according to the Nissl method or with haematoxylin and eosin (HE) for orientation, and mounted in DePeX.

**Immunocontrol**

Control reactions were performed by ommitting the primary antibody. No labeled structures were found, if the primary antibody was omitted, using either

the immunofluorescent or the peroxidase method. As positive controls, the immunoreactions were performed on rat cortex or chicken cerebellum as well.

**Microscopy and charting**

The specimens were viewed under an Olympus BX51 microscope, and photomicrographs were taken by an DP50 digital camera, followed by contrast-adjustment using the Adobe 5 Photoshop program.

In case of double labeling, photomicrographs were taken under both green and blue lights, subsequently, from the same areas of alternate sections. The image pairs of different color were digitally united and slightly contrast adjusted using the Adobe5 Photoshop program.

The identification and nomenclature of brain structures are based on the descriptions of Ariëns-Kappers (1906), Northcutt (1978, 1981), Butler and Hodos (2005), and mainly Smeets et al. (1983), and Smeets (1997) on the brain of *Hydrolagus collei* (Holocephali), *Squalus acanthias*, *Scyliorhinus canicula* and *Raja clavata*. The brain of the *Callorhinchus milii* is similar to that of *Hydrolagus collei* as described by Smeets et al. (1983). To demonstrate the macroscopic structure, drawings were made with the help of a microscope slide projector apparatus, which showed the contours of the sections.

**Results**

The predominant element was radial ependymoglia (i.e. tanycytes) in every brain area in sharks. The radial glial processes spanned the distance between the ventricular and meningeal surfaces of the telencephalon. Their ependymal origin was clearly observed, as well as their end-feet lining the pial surface. Whereas local differences were clearly found in any species investigated, no characteristic differences in the tanycyte system were recognizable in the different species, not even between galeomorph and squalomorph sharks. Non-ependymal cell bodies were found in all of the four shark species studied, either perivascular or apovascular (i.e. non-perivascular) positions.

Immunohistochemical reaction to glutamine synthetase labeled more cells than what was observed using antibodies to GFAP or S-100 protein. Most apovascular cells had polygonal perikarya, with short, or relatively long processes. Typical astrocytes, however, similar to the mammalian and avian ones, were uncommon. Of all sharks studied, most numerous astrocyte-like cells were found in the *Pristiophorus.*

In skates (Rajiformes), the telencephalic ventricles have been reduced and radial ependymoglial processes were observed around them, in contrast to rays (Torpediniformes and Myliobatiformes) in which the lateral ventricles were obliterated. Astrocytes were found in great number in the telencephalon of each batoids. In skates, following immunohistochemical reaction against GFAP, these were observed only on the vessels and along the meningeal surface, like in our former study (Kálmán and Gould, 2001). When immunohistochemical staining of glutamine synthetase was applied, astrocytes were visualized throughout the telencephalon of skates and *Torpedo*. In *Mobula* GFAP was also expressed in both perivascular and apovascular astrocytes as well.

The perivascular glia comprised rather varied elements among sharks and batoids, in some cases even within the same species. Some perivascular glia in *Mobula* were found to contact the vessel only by their processes, a situation

commonly found in mammals. The glia limitans on the meningeal surface was also formed by cell bodies, rather than end-feet of processes, like in sharks.

In the chimaera DAB-positive astrocyte-like structures with short processes among the radial glia, were also seen in the telencephalic wall, in small and scarce groups but never attached to vessels. They were immunoreactive to glutamine synthetase and S-100 protein but not to GFAP. Some telencephalic territories could be recognized on the basis of their glial pattern, and the meningeal surface was lined, at least in part, by glial cells.

In sharks, the diencephalic and mesencephalic ventricles were large. Radial tanycytes were found around them, which formed end-feet on the meningeal surface. In the tectum, a layered structure was recognizable in some degree and astrocyte-like cells were exceptionally rare.

In skates and *Torpedo*, in the diencephalon both besides astrocytes long radial processes were found, in the Myliobatiformes only short ependymal processes, as well as in the mesencephalon. The meningeal surface of their solid tectum was lined by astrocyte-like cellular elements.

In chimaera the diencephalon had a thin wall, with a fine and dense meshwork of radial glial processes of ependymal origin and layering pattern could be distinguished which changed along the arch of the tectum.

The structure of the cerebellum was similar in each of the sharks studied: the main system consisted of radial ependymoglia, which penetrated the molecular layer like Bergmann-fibers. The meningeal surface of cerebellum was lined by their end-feet. The granular layer was enmeshed by glial processes which formed a reticular pattern with holes for the granule cells and the cerebellar glomeruli.

In skates and rays the cerebellar astroglial architecture was rather unclear on the basis of GFAP-immunohistochemistry. However, immunoreaction to S-100, and even better to glutamine synthetase, revealed a system similar to that of sharks). In *Mobula*, GFAP-immunopositive glia formed nests for the Purkinje cells and the perivascular glia was GFAP-immunopositive in the molecular layer, too. The Bergmann-glia, however, were not immunoreactive to GFAP, only to glutamine synthetase. The astroglial network of the granular eminence proved to be composed of astrocytes rather than ependymoglia. The area occupied by nerve tracts also contained numerous GFAP-immunopositive astrocytes in *Mobula*.

In Chimaera cerebellum a plexus of irregularly coursing glial processes was found in the granular eminence under higher magnification.

In the rhombencephalon the contours of cross sections were rather similar in sharks and batoids. The difference in the astroglial architecture of gray and white matters was obvious. The astroglia of the gray matter were formed by dense populations of fine processes, in the ‘reticular area’ bundles of glial processes formed loops around the groups of axons. In the white matter the axons were fasciculated by long glial processes spread from a midline glial septum like the veins of leaves. Astrocytes were not observed in this region, except in *Myliobatis* (in the ‘closed’ part of the medulla) and in *Mobula,* in which astrocytes occured in the gray matter, and also in the glial septa of the white matter.

In sharks, skates and *Torpedo* the spinal astroglial architecture was similar to that described in the rhombencephalon. In the *Mobula* spinal cord the white matter was also enmeshed by a system of glial septa formed by long ependymal processes, whereas the gray matter was populated by both astrocytes and fibers.

In Chimaera rhombencephalon the same three main areas could be distinguished by their glial pattern: the gray matter, the white matter and the reticular glial substance. Strong glial septum bifurcated in the midline, diverged slightly in a ventral direction, and became fan-shaped at the basal surface.

The cerebral vascular system was clearly visualized by the immunostaining against DG-β in Chondrichthyes, like in mammals, and the interspecific differences in the vascular system could also be recognized.

Dystrophin, utrophin, syntrophin, and α-dystrobrevin could be visualized in every species, but in general, the labeling was weaker than in the case of DG, and it never visualized the whole vascular system. Immunolabeling against AQP4 or AQP9 was not convincing.

**Conclusions**

1) On the whole, immunostaining to S-100, and mainly to glutamine synthetase revealed more astroglial elements, than did immunostaining to GFAP. Immunohistochemical stainings against glutamine synthetase revealed astrocytes not only in galeomorph sharks, but also in *Squalus* and *Pristiophorus* (Squalomorphii), as well as GFAP immunonegative astroglial structures in batoids.
2) From the three species studied of the Myliobatiformes order, GFAP immunopositive astrocytes were found in each brain region of *Mobula japanica* and the position of some of its perivascular glia was similar to that found in mammals.

In general, the glial architecture of *Callorchinchus milii*, a representative of Holocephali, proved to be similar to the brain of *Squalus acanthias*, a squalomorph shark of a less advanced brain type. There are, however, some features that are more advanced than in squalomorph glial structures: some telencephalic territories could be recognized on the basis of their glial pattern, and the meningeal surface is lined, at least in part, by glial cells, not just endfeet of radial glia. In the telencephalic hemispheres, astrocyte-like elements were detected, whose presence is a characteristic feature mainly in batoids and galeomorph sharks. Immunostaining of blood vessels with all glial markers was similar to that occurring in Elasmobranchii, although their blood-brain barrier is believed to be connected to different cell types. This study revealed certain advanced features in the glial pattern of *C. milii* which are more characteristic of galeomorph sharks, skates and rays, than of squalomorph sharks.

3) The difference in astroglial architecture between sharks (radial ependymoglia predominate) versus skates and rays (astrocytes predominate) was confined only to the prosencephalon and mesencephalon. Astrocytes did not prevail in conservative brain regions, such as rhombencephalon, as they did in the progressive brain regions.
4) Immunostaining against DG visualized the whole vascular system in cartilaginous fishes, even the characteristics of interspecific and regional differences. DGC and its associated proteins could be found throughout the vessels of the chondrichthyan species studied, similarly to mammals, while AQP4 and AQP9 are possibly absent, similarly to other vertebrates, excluding birds and mammals.

5) There was no specific astroglial structure to distinguish the brains of galeomorph and squalomorph sharks, there was only difference between sharks and batoids, however, it was possible to study only those sharks with thin brain walls. It seems that the astroglial structures correspond to the macroscopic structure of brain, rather than the laminar/elaborated categories. Neither the differences in their blood-brain barrier, nor the differences in their cerebralization were reflected in any differences of immunoreactivity to DG. However, in the telencephalon, the DG immunostaining could be associated, to some extent, to differences in astroglial architecture.
6) Although, the evolutionary changes of astroglia had some similarities in Elasmobranchii and Amniota, there was one meaningful difference: in Elasmobranchii astrocytes did not prevail in conservative brain regions as they did in the progressive brain regions.

In appendix:

7) The large brain size in some elasmobranch families could be connected to habitat, special termoregulation abilities and/or increased energy flow to the embryos. The enlargement of telencephalon is present in chondrichthyes that live in a 3-dimensional environment (pelagic) and this enlargement is probably related to complex social behaviours. The large, heavily foliated and asymmetric cerebellum might be associated to wide-ranging, migratory behaviour, and appears to be related to locomotor abilities and sensorymotor integration.

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