Distribution of β-dystroglycan and aquaporin-4 in the ependyma and subventricular zone

PhD Thesis

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1. Introduction

Current study concentrates on the thin layer of the vertebrate central nervous system that is described in the literature as ventricular and subventricular zone. The information on this region was enormously expanded during the last decade that is mainly due to its role played in neurogenesis.

In recent years studies by Mercier et al. (2002, 2003) described a fine network formed by laminin. This network extends from the basal lamina of cerebral vessels to the ependyma through the subventricular zone (SVZ) and supposed to have role in functional plasticity and neurogenesis by serving as a pathway for citokines and growth factors

The dystrophin-glycoprotein complex (DGC) is important in the formation of the connection between basal lamina and cells and in the regulation of intracytoplasmatic processes. B-dystroglycan has a central position in the DGC and anchors among others the aquaporin-rich cell membrain domains to the laminin of the extracellular matrix (ECM) therefore seems to determine the distribution of aquaporin-4 (Ap4). Our aims were to investigate the aforementioned connection along the ventricular system and in some specialized region such as the circumventricular organs.

The ECM that interlaces the neuroglia and forms connection with the vascular system may have prominent role in pathological conditions (for instance brain oedema, stroke) and in their prevention, as well. For this reason we focused on the connection of the basal lamina-network and neuroglia situated in the ventricular and subventricular zone.

2. Aims of the study

The main objectives of the current theses were as it follows:

1) The role of β-dystroglycan in relation of the fracton-system described by Mercier.

2) What are the reginoal differences along the ventricular system (for instance the territory of the circumventricular organs)?

3) What is the correlation between the expression of β-dystroglycan and aquaporin-4 immunopositivity?

The data are primarily obtained from mature rats but we tried to extend our investigations to developing animals and other vertebrate species.

3. Methods

Our expriments were carried out on 20 mature rats (Wistar, 250-300g), 3 mature chickens (Hunnia broiler), 3 gerbils *(Meriones unguiculatus)*, 3 mature rabbits (*Oryctolagus cuniculus domestica)* and on 1 whip stingray (*Dasyatis akajei*). In case of developmental research we used rat embryos (14, 16, 18 and 20 day old ones), early postnatal animals (neonate, 2, 4, 7, 9 day old ones) and young rats (15, 19, 20, 21, 30 day old ones).

The majority of the slices were processed for immunohistochemical investigations, both immunofluorescent and avidin-biotin-complex methods were applied. The great majority of the fluorescent slices were examined by confocal laser scanning microscope (Radiance-2100, BioRad, USA). Photos were taken in perpendicular planes in order to evaluate the extent of colocalization of antibodies.

Electonmicroscopical investigations were performed according to two different methods, such as a) pre-embedding immunohistochemical reaction against β-dystroglycan and b) electronmicroscopical observations without immunohistochemical reactions.

4.Results

4.1. The structure of β-dystroglycan-immunopositive (β-Dg-ip) globules:

The immunohistochemical reaction detected round β-Dg-ip dots along the ventricular surface. Their diameter was between 1 and

2 μm, and they were aligned strictly in the same layer, 6 to 8 μm from the ventricular surface, following each other with distances 3 to 5 μm. In contrast to the cross-sections of vessels, they were solid, without lumina. In order to determine their 3-dimensional structure and distribution, sections were made in coronal, sagittal and horizontal plane. Their globular structure was concluded from the same round appearance of each plane of section therefore they have been termed 'globules'. Similar globules were visible when immunohistochemical reaction was carried out by either fluorescent or ABC method. No 'globules' were found in the absence of primary or secondary antibody.

4.2. The distribution of Ap4 and other components of DGC in the globules:

The territory of β-Dg-ip globules were Ap4-ip, as well. The colocalization was proved by photos taken on dual labelled slices in perpendicular planes.

Utrophin and α-dystrobrevin were also situated in the globules similar to β-dystroglycan. A1-syntrophin was not found in globules but it was localized in the cytoplasm of ependimocytes adjacent to their cell-membrane.

Double labelling studies clearly proved the colocalization of α-dystrobrevin with β-dystroglycan and utrophin. Double labelling study emphasized the different localization of β-dystroglycan and α1-syntrophin.

4.3. Electronmicroscopic investigations

When the ultrastructural localization of b-dystroglycan

was investigated, the diaminobenzidin precipitation was found in the basal part of the ependymal cells, at infoldings, cisterns, or labyrinthine membranous systems In some cases the structure was preserved enough to demonstrate its original characteristics. The size of this labyrinthine system was similar to that of 'globules' in the fluorescent microscopic photos. The complexity of these structures

was various.In some cases their communication with the narrow intercellular clefts and extraependymal space was visible.

When specimens were investigated without immunohistochemical reaction, but applying uranyl acetate and lead citrate stainings on the ultrathin sections, the labyrinth seemed to be more narrow, but full of moderately electrondense material, which continued on the basal surface of the ependymocyte.

The β-Dg-ip globules were situated in the corners between ependymocytes where at least 3 ependimocytes were adjacent. Five to six of such β-Dg-ip globules could be measured along the circumference of each ependimocyte.

4.4. The distribution of globules along the ventricular system

The β-Dg-ip 'globules' were ubiquitous in the ventricular system, including the cerebral aqueduct and the central canal, except the ventral part of the third ventricle, where they were completely missing. The boundaries of this area: anteriorly between the retrochiasmatic area and the arcuate nucleus as it is represented at P2100 μm (P stands for posterior to the bregma) in coronal section; posteriorly the caudal end of the inframamillary recess (P4500 μm in coronal section); ventrally it comprises the whole median eminence, and extends into the mamillary recess.

4.5. Position of globules to the ependyma and astrocytes

Dual labelling for GFAP and β-Dg visualized faintly the ependyma and revealed that the β-Dg-ip 'globules' were positioned on the basal (contra-luminal) part of ependymocytes. Their occurrence did not depend on the height of ependymocytes, i.e whether they were flat, cuboideal, or –in the spinal cord- columnar. Astrocyte processes parallel to the ventricular surface formed a continuous GFAP-ip 'cordon' on the contra-ependymal side of the β-Dg-ip globules. Some of these fine processes connected them. Within this 'cordon' vessels were not found. This arrangement was found in the 4th ventricle and in the central canal, too.

The above-mentioned 'globule-free' area of the third ventricle corresponded to that, where tanycytes lined the ventricle. Here, the GFAP–ip astrocytic processes parallel to the surface were missing, too.

4.6. The circumventricular organs: median eminence

Dual labelling of β-Dg and Ap-4 visualized no globules in the region of the median eminence – in line with our previous results. B-Dg-ip globules were present only where ependymocytes lined the ventricular surface. The tanycytes of the median eminence were Ap4-immunonegative whereas the tanycytes situated dorsolaterally were Ap4-immunopositive.

4.7. The circumventricular organs: the subcommissural organ

Despite the intensive Ap4-immunopositivity of the ependyma of the third ventricle the region of the subcommissural organ proved to be Ap4-in. Besides the GFAP was also missing from this organ in concordance with our earlier results. B-dystroglycan and Ap4 dual labelling shown no globules in the subcommissural organ.

4.8. The distribution of globules in different vertebrate species

Observations made in chicken brain did not differ from that of made in rat regarding with distribution of β-dystroglycan and Ap4. The ependymal layer was labelled more intensively in case of the lateral ventricle, third ventricle and the fourth ventricle than the adjacent neuropil by Ap4, however, the ependyma of the central canal was Ap4-immunonegative.

The β-Dg-ip globules were found in the other examined mammals (mouse, gerbil (*Meriones unguiculatus)*, rabbit (*Oryctolagus cuniculus domestica)*] and they were also distributed along the ventricular system covered by ependymocytes of the whip stingray (*Dasyatis akajei*). The globules were missing in case of the

gerbil's third ventricle covered by tanycytes similar to our observations made in rat.

4.9. The expression of β-dystroglycan and aquaporin-4 in certain circumventricular organs of chicken

The whole thickness of the median eminence was Ap4-immunonegative, whereas an intensive Ap4-signal was detected in the hypothalamus. The Ap4-immunostaining delineated the long processes of tanycytes situated there. The Ap4-negative immunostaining of the subcommissural organ was in sharp contrast with the otherwise Ap4-ip lining of the third ventricle. The ependyma of the paraventricular organ was Ap4-in and the β-Dg-ip globules were missing similar to the subcommissural organ.

4.10. Studies on perinatal rats: the subcommissural organ and the median eminence

Ap4 immunopositivity was not detected until the embryonic (E) 16th day. During the late fetal period (E18-20) the subcommissural organ preved to be Ap4-ip whereas the Ap4-immunopositivity of the neighourin ependyma was weak and did not surpass that of the adjacent brain substance. At birth, on the contrary, the subcommissural organ was Ap4-in and the neighbouring ependyma shown intensive Ap4-immunopositivity. Similar tendencies were observed in case of the median eminence: the prenatally Ap4-ip ependymal layer was postnatally Ap4-in. From the 7th postnatal day the Ap4-immunolabelling was similar to the adult one.

4.11. Studies on older postnatal rats – the appearance of the globules

The β-Dg-ip globules were not observed in rats until the 19th postnatal day. From the 20th postnatal day they appeared along the ventricular system and by the thirtieth postnatal day their distribution was similar to the adult one.

5. Discussion

5.1 The connection between globules and the DGC

To our knowledge this is the first description of such globules' containing dystroglycan and other DGC components in the basal part of the ependymocytes. The colocalizations of a-dystrobrevin with both b-dystroglycan and utrophin suggest the co-localization of β-dystroglycan with utrophin, as well.

Globules were not found in the third ventricle where tanycytes formed the ependyma. The boundary of the occurence of globules matched to the boundary between the 'light cell zone with tanycytes' and 'light cell zone without tanycytes' (Mitro and Palkovits 1981).

The separate localizations of a1-syntrophin and β-dystroglycan within the same cells seems to be surprising, but DGC without syntrophin has been found in the brain (Moukhles and Carbonetto, 2001), as well as syntrophin without dystroglycan in muscles (Cote et al., 2002). The presence of other syntrophins (β1,β2,γ1,γ2, Gorecki et al., 1997; Piluso et al., 2000; Tinsley et al., 1994) however, has not yet been ruled out.

5.2. The distribution of aquaporin-4 and its connection with globules

Concerning the general distribution of Ap4-immunoreactivity, our results are in accordance with the former descriptions (Jung et al. 1994; Frigeri et al. 1995; Nielsen et al. 1997, Venero et al. 2001). Ap4-immunoreactivity was found throughout in the CNS, but a more intense labelling in the perivascular and subpial endfeet (glia limitans).

The ependymocytes of the lateral ventricle, third ventricle, cerebral aqueduct and fourth ventricle consisted of Ap4 (Venero et al. 1999, Mack et al. 1987) whereas that of situated in the central canal did not. Besides the Ap4-immunostaining was confined to the

basolateral cell membrane of ependymocytes in line with studies of Frigeri et al. (1995) and Nielsen et al. (1997). Novel finding in our study that the aforementioned results were observed in chickens, as well.

The β-Dg-ip globules and the Ap4 were colocalized in general, however, in the central canal β-Dg-ip globules were observed without Ap4. According to our conclusion the Ap4 is not an obligatory concomitant feature of the β-Dg-immunpositivity since we found Ap4 where β-Dg was not present (for instance dorsolaterally to the median eminence).

5.3. The connection between globules and the membrane labyrinths

The intra-ependymal localization of the globules was proved by pre-embedding electron microscopic immunohistochemical reaction against β-dystroglycan. At this level the globules proved to be membrane labyrinths inthe basal part of the ependymal cells. In a relatively thick section these may appear as globules. Former electron microscopic studies described bulbous labyrinthine extrusions (basement membrane labyrinths) on the basal surface of ependymocytes (Booz et al., 1974; Leonhardt, 1970). Stained by the period-acid-bisulfite aldehydthionine-method these looked like globular or irregular spots under light microscope and may correspond to the β-Dg-ip globules. In our nonimmunostained but uranyl- and lead-stained materials the labyrinths were similar to that shown by Reichenbach and Robinson (1995).

The appearance of globules between P19 and P30 that was detected by β-Dg antibody correlates well with the light microscopic observations of Booz et al. (1974) on the development of basal membrane labyrinths. However, in this earlier study the anlagen of the labyrinth system was observed by electron microscope from P12 as lamellar structures.

5.4 The connection between globules and the fracton system

The functional relevance of laminin receptors on the basal surface of ependymocytes is suggested by former data. Reichenbach

and Robinson (1995) following Leonhardt (1970) and his co-workers (Booz et al., 1974) considered them as remnants of an embryonic vascular network (i.e. their basal laminae) in the SVZ. According to Mercier et al. (2002, 2003) a delicate laminin-network originates from the basal lamina of capillaries near the ventricular surface. It spans the subependymal layer and sends off branches to the ependyma in a fashion quite similar to the fractal system. For this reason it was termed 'fracton'. We suggest that β-dystroglycan in the globules (i.e. at the basal labyrinth) may tether the laminin 'fractons' to the ependyma.

A question may arise, why globules are not found in the tanycytes lined part of the third ventricle. Radial glia, which lines the ventricular system during development, maintains direct connections with the basal lamina of pial surface. Later radial glia is replaced by ependyma. The ependymocytes, that have no long basal processes with direct connection to the pial surface, retain the connections to the pia mater in the form of laminin 'fractons' to the vessels, and *via* the vessels, to the pia mater. Laminin-receptor β-dystroglycan is, therefore, accumulated at the basal part of ependymocytes, in the basal labyrinth, which appears like globules in immunostained material, under light microscope. Tanycytes preserve their long processes contacting directly the pial lamina basalis, therefore no laminin-binding globules are necessary in their cell bodies.

5.5 The globules in the phylogeny

According to our experiments made in different species globules were observed in those brain regions where ependymocytes were situated. Results obtained in whip stingray do not contradict this since they have astrocytic glial structure in the telencephalon. Question arises whether the similar structural feature (globules) observed in phylogenetically distant species (rat and whip stingray) is due to convergence or an ancestral common feature.

The fact that no globules were situated in regions where tanycytes were found and the tanycytes resemble the ependymoglia let us infer that the appearance of globules in whip stingray, birds and mammals is due to convergence, namely they appeared during the replacement of ependymoglia with astrocytes.

5.6 The circumventricular organs

No Ap4 or β-Dg-ip globules were found in the ependyma of the examined circumventricular organs. Concerning with the Ap4-immunonegativity of the subcommissural organ and median eminence indirect data were available in the literature (see Mack et al. 1987 about the distribution of orthogonally arrayed particles).

Our results were in concordance with the opinion that the expression of Ap4 is in inverse proprtion to the distribution of zonula occludens (Mack et al. 1987).

Results obtained in our developmental studies proved that the Ap4-immunonegativity is a secondary phenomenon in case of the subcommissural organ and the median eminence. During the late fetal period both regions shown intensive Ap4-immunopositivity that disappeared shortly after birth.

5.7. Observations made on the tanycytes

Two tanycytic populations were described in the third ventricle expressing Ap4 in a different way. The ventrally situated were Ap4-immunonegative while the dorsolateral population was Ap4-immunopositive. Observations made in rat brain were similar to that of made in chicken brain.

In the area lined by tanycytes the otherwise common subventricular GFAP-immunopositive 'cordon' of astrocytic processes (Ludwin et al. 1976, Merker 1970, Quinones-Hinojosa et al. 2006) was missing, too.

6. Summary of our results and conclusions

6.1 Summary of our results

1) We described the β-Dg-ip globules situated between the ependyma and subventricular zone and mapped their distribution along the ventricles.of rat brain

2) We examined the expression of the aquaporin-4 and DGC components in the globules (positive: α-dystrobrevin and utrophin, negative: β-dystrobrevin and α1-syntrophin). The globules were Ap4-ip except in case of the central canal.

3) The connection was proved between the globules and the earlier described membrane labyrinths (Leonhardt 1970).

4) We described the β-Dg-ip globules in different vertebrate species (mouse, gerbil, rabbit, chicken, whip stingray).

5) The ependymal area of the examined circumventricular organs (median eminence, subcommissural organ and paraventricular organ) proved to be globule- and Ap4-free area in chicken and rat, as well.

6) The Ap4-immunonegativity is a secondary phenomenon in case of the mature median eminence and subcommissural organ, during the late fetal period (E18-20) both regions were Ap4-ip.

7) The β-Dg-ip globules were appeared at the basal membrane of ependymocytes from the 20th postnatal day. These were situated in the corners between (generally three) ependymocytes.

8) We described two different tanycyte-population regarding the Ap4-expression in the third ventricle. The ventrally situated is Ap4-in while the tanycytes of the dorsolateral region were Ap4-ip. Our observations were similar made in rat and chicken brain.

6.2. Conclusions

New characteristic feature was described situated between the ependyma and the subventricular zone. This observation links two earlier discovered structures (membrane labyrinth- Leonhardt 1970, fracton system- Mercier et al. 2000). The distribution of the globules (their lack in the region of tanycytes) let us infer about the phylogenesis of the ependyma and subventricular zone. According to our hypothesis until the cell that lines the ventricle is in direct contact with the pial or perivascular ECM by its long process (like the ependymoglia, tanycyte and radial glia) there is no need of

globules. When this direct connection ceases (for instance in case of ependymocytes) the globules maintain the linkage to the perivascular ECM by anchoring the fractons

7. Publications of the author

Publications in peer reviewed papers concerning the thesis:

Adorján I, Kálmán M. 2009. Distribution of beta-dystroglycan immunopositive globules in the subventricular zone of rat brain. Glia, 57(6):657-66. IF: 4,932

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Posters concerning the theses: abstracts published in periodicals are in brackets

Adorján I, Kálmán M. 2008. Laminin-receptor dystroglycan and its associate proteins occur along the ventricular system except tanycyte-lined part of the third ventricle. FENS Conference Geneve, (FENS Abs. 207.2, 2008).

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Other publication in peer reviewed papers:

Kálmán M, Mahalek J, Adorján A, Adorján I, Pócsai K, Bagyura Z, Sadeghian S. 2011. Alterations of the perivascular dystrophin-dystroglycan complex following brain lesions. An immunohistochemical study in rats. Histol Histopathol, 26:1435-1452. IF: 2,502
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