UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA

DOCTORAL SCHOOL

PHD THESIS SUMMARY

ASSESSMENT OF THE ROLE OF AQUAPORIN 4 IN THE BALANCE OF WATER AND SOLUBLE FRACTION OF AMYLOID Aß40 IN THE CENTRAL NERVOUS SYSTEM

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Introduction

In this study, we analyzed the role of aquaporin 4 in two of the most common neurological pathologies: Alzheimer's Disease and Stroke, both diseases being the apanage of the elderly.

Alzheimer's disease is the most important and the most common degenerative disease of the CNS and represents between 50 and 75% of all dementia cases. It has been observed that the distribution of dementia worldwide varies according to socio-economic and cultural factors, but the prevalence of AD is more important in the developed countries, compared to the developing ones (Ferri et al., 2005). Due to personality disorders, dysnomia, temporal-spatial disorientation and amnesia, in the end the patient becomes completely immobile, isolated and mute. Finally, the death of these patients occurs due to heart disease, malnutrition or secondary infections.

The main morphological changes that occur in Alzheimer's disease are the accumulation of amyloid Aβ and neurofibrillary tangles. The amyloid can be found as plaques at the level of cerebral cortex but also as cerebral amyloid angiopathy, by depositing it in the walls of the blood vessels. Amyloid deposits can be diffused when they continue to diffuse with the surrounding brain or compact when they are well delimited by surrounding nerve tissue (Wisniewski et al., 1989).

Stroke gains the lead in frequency and importance among all neurological disorders of the adult. Stroke causes about 7.8 million deaths worldwide annually and accounts for about 13% of all causes of

death. According to the latest comprehensive review of the World Health Organization (WHO) since 2004, stroke is found in the first five causes of death regardless of social category. The most important risk factors for stroke are: atrial fibrillation, type 2 diabetes, high blood pressure, hyperlipidemia and smoking.

In this thesis I have shown in animal models that inhibition of AQP4 causes a delay in the perivascular drainage of Aβ, causes increase of its accumulation around the blood vessels, especially those with smaller diameter, thus favoring the deposit of amyloid, characteristic of Alzheimer's disease. We also evaluated the expression of the two aquaporins at the level of the CNS by immunofluorescence and found an increased expression of AQP4 at the level of the pia mater ; the high expression of AQP1 I encountered at the white matter level. Both aquaporins were expressed in the perivascular end-feets of astrocytes.

KEYWORDS: Alzheimer's disease, Stroke, Amyloid Aβ 40, Aquaporin 4.

GENERAL PART

1. ANATOMY AND HISTOLOGY OF THE CNS

The nervous system can be structurally divided into the central nervous system and peripheral nervous system. The central nervous system is composed of the brain and spinal cord and the peripheral nervous system is made up of the cranial nerves, spinal nerves and associated ganglia.

Both the brain and spinal cord are protected by bone structures, respectively the brain box and vertebral tube. In addition to bone protection, the CNS is also protected by three conjunctival membranes, called meninges. The brain is the part of the central nervous system located in the cranial box and is composed of: the brainstem (spinal cord, bridge and mesencephalon), the cerebellum, the diencephalon (the thalamus, the metathalamus, the subthalamus, the epithalamus and the hypothalamus) and the telencephalon (Felten et al., 2016).

From histological point of view, the central nervous system consists of parenchyma and stroma. The parenchyma of the central nervous system contains all the nerve cells and the stroma contains fine connective elements, glial cells and blood vessels. The parenchyma is limited to the surface by a layer of astrocytic external glia limitans and in depth by an ependymal epithelium. Between these limits the parenchyma is structured into two different tissue areas as functional and morphological: gray and white. The gray matter is made up of dendrites, neuronal bodies, neuroglial cells, the initial non-myelinated portion of the axons and a rich system of blood vessels. The white matter is composed of myelinated axons arranged in cords or bundles.

2. EPIDEMIOLOGY AND PHYSIOPATOLOGY OF ALZHEIMER'S DISEASE

Dementia is a clinical syndrome caused by neurodegeneration and is characterized by progressive deterioration of cognitive ability and capacity for independent living. Clinical manifestations include memory impairment and at least one of the following: aphasia, agnosia, apraxia and disorders of complex motor functions. It is a priority in the field of health and social assistance for many high-income countries.

AD represents about 50% -75% of all dementia cases. Cerebrovascular dementia accumulates an additional 10% -20%, followed by dementia with Lewy bodies (LBD, 10% -15%), frontotemporal degeneration (FDT, 5% to 15%), mixed dementia (10% -15%) and others causes (from 2% to 5%) (Kawas, 2003).

The distribution of dementia in the world seems to vary according to cultural and socio-economic differences. Interestingly, the overall prevalence of dementia in general and AD, in particular, appears to be higher in developed countries than in developing ones (Ferri et al., 2005).

The risk factors for AD fall into two categories: modifiable and non-modifiable risk factors. The modifiable risk factors include diabetes, hypertension (HTA), dyslipidemia, obesity, smoking, low physical activity, cerebral hypoperfusion, stroke, depression, head trauma, contusions. Among the non-modifiable risk factors are: age, sex, family history, race, Down syndrome, cerebral amyloidosis (Hebert et al., 2013, Seshadri et al., 1997, Alzheimer's, 2016).

Macroscopic changes in Alzheimer's disease are represented by the expansion of the grooves and the atrophy of the gyrus, especially in the medial temporal regions, but may also affect the medial parietal and temporal

regions (the hippocampus). A significant extension of the ventricular system may also be present, being affected especially the lateral ventricles. The morphological changes within this disease are characterized by accumulation of amyloid peptides in the cerebral parenchyma as plaques but also in the walls of the blood vessels as cerebral amyloid angiopathy but also by the presence of neurofibrillary glands composed of the aggregation of the protein in the extensions and neuronal bodies.

3. EPIDEMIOLOGY AND PHYSIOPATOLOGY OF ISCHEMIC SROKE

Stroke represents the neurological disease with the highest frequency and importance of all the neurological diseases of the adults; it is the fifth leading cause of death and a major cause of disability worldwide (Writing Group et al., 2016). In Europe, the incidence of brain injury varies from country to country, with about 100-200 strokes per 100,000 inhabitants per year, which is a huge burden on the economy. Romania is one of the top ten countries in the world as the incidence of stroke. Stroke mortality is six to seven times higher in our country than in the United States and three to four times higher than in the rest of the European Union (EU) countries. These negative statistics do not relate to the economic level of our country but to the health system in Romania where primary and secondary prevention are missing (Pavaloiu and Mogoanta, 2017). Statistics show that the maximum incidence of stroke occurs in 75% of cases after age 65, the associated age, with a much more difficult recovery after stroke (Brown et al., 2003, Badan et al., 2003, Markus et al. a ., 2005) .

Risk factors for stroke are divided into two categories: modifiable risk factors and non-modifiable risk factors. The category of non-modifiable risk

factors includes: age, sex, race and genetics; and from the category of modifiable risk factors are: HTA, smoking, alcohol consumption, obesity, diet, physical activity, dyslipidemia.

The pathophysiology of stoke consists of two processes: the first is the loss of glucose and oxygen supply due to vascular occlusion and the second is represented by the changes in cellular metabolism that ultimately lead to the disintegration of cell structures and their membranes.

PERSONAL CONTRIBUTION

4. THE ROLE OF PERIVASCULAR SPACES IN LYMPH CELL MIGRATION IN THE CNS

Introduction: Virchow-Robin spaces are the perivascular areas that surround the cerebral blood vessels in their course from the subarachnoid space to the cerebral parenchyma. Physiologically these areas can only be observed microscopically but when they are dilated they can also be seen on MRI images (Hirabuki et al., 1994). Tuberculosis is a chronic granulomatous infection caused by Mycobacterium tuberculosis. Infection of the central nervous system with Mycobacterium tuberculosis occurs in the form of a subacute or chronic meningitis. Tuberculomas may also be present, and may sometimes be interpreted as space replacement lesions (Rock et al., 2008) Tuberculous meningitis may be the only manifestation of TB or may occur at the same time as pulmonary or disseminated disease (Sharma et al., 2012) . Another consequence is the occurrence of vasculitis in the vertebro-basilar territory, the middle cerebral artery and the Willis arterial circle (Misra et al., 2011).

Materials and methods: In this study we used nerve tissue from a patient diagnosed with tuberculous meningitis. After macroscopic examination, tissue was fixed and processed for paraffin embedding at the Department of Pathology, Emergency County Hospital of Craiova, and prepared for immunohistochemistry (IHC) at the Research Center for Microscopic Morphology and Immunology, University of Medicine and Pharmacy of Craiova. After fixation in 10% neutral buffered formalin, all tissue fragments were sectioned with a microtome, and sections stained with Hematoxylin – Eosin (HE) for basic histopathological diagnosis. Brain sections were further processed for IHC using the anti-collagen IV [mouse, Dako (Glostrup, Denmark), code M0785, 1:50], anti-leukocyte common antigen [LCA, cluster of differentiation 45 (CD45)] (mouse, Dako, code M0701, 1: 100), or the anti-smooth muscle actin (mouse, Dako, code M0851, 1: 100) primary antibodies. After antigen retrieval by microwaving in citrate buffer, pH 6, and peroxidase blocking, the primary antibodies were incubated on the sections overnight, at 4 ° C. The next day, after thorough washing, an anti-mouse goat secondary antibody linked to Horseradish peroxidase (HRP) was added on the slides for 30 minutes (Nichirei Bioscience, Tokyo, Japan), the enzyme was visualized with 3,3'-Diaminobenzidine tetrahydrochloride hydrate (DAB) (Dako), and the slides were coversliped with an xylene based medium (Sigma-Aldrich, St. Louis, MO, USA) .

Results: On microscopy, the lung reconfirmed the active disease, showing a generalized purulent and mononuclear mixed alveolitis, with large confluent caseating necrotic areas surrounded by chronic granulomatous reaction, with epithelioid cells and Langhans giant cells. On the microscopic examination of the brain in the region of the lateral sulcus, the leptomeninges and the upper cortical layers exhibited

a polymorphonuclear and mononucleated exudate, with fibrin co-existence, hemorrhages and caseous necrotic areas. No Langhans giant cells could be found here, but toward the middle cortical layers, the affected region was surrounded by numerous reactive astrocytes. Septic emboli could be identified in larger subcortical vessels. The inflammatory exudate was not, however, limited to the meninges and surrounding cortex, but extended around vessels throughout the regional cortex, without involving the white matter beneath . We Identified mononuclear inflammatory cells in the brain parenchyma , dilated perivascular spaces and cells that appeared to dissector ed the vascular walls . In an attempt to clarify this observation, we further performed IHC for collagen IV, and in multiple instances this clearly delineated vacuolated and saccular spaces in the thickness of the vessel walls surrounded by collagen IV and containing round cell-looking nuclei. Dilated perivascular spaces also contained lymph cells, and small vessels without a clear-cut dilated perivascular space also showed this phenomenon. Immunostaining for smooth muscle actin also confirmed that these nuclei appear to dissect the muscle wall, that these cells were not smooth muscle cells, and that these spaces were distinct from the perivascular spaces.

Discussions: Tuberculous meningitis is the rarest extrapulmonary localization of TB (with an incidence of 5–15%), and the most severe. Especially in developing countries, tuberculous meningitis has high mortality and morbidity rates, the second largest incidence in the world being found in China (Bartzatt, 2011). Some studies have been postulated that immune complexes can become entrapped in the vascular basement membrane, but not complete immune cells (Carare et al., 2013), but our studies have shown that the influx of immune cells into the brain in a case of meningitis with encephalitis also traps lymph cells into the thickness of the

vascular basement membrane, besides the classical cuffing around the blood vessels (Rosu et al., 2018).

5. INHIBITION OF AQUAPORIN 4 DECREASES INTRACEREBRAL PERIVASCULAR DRAINAGE OF AMYLOID AB40

Introduction: Aβ is produced during neuronal activity, by APP, a membrane protein that acts as a signaling receptor (Bero et al., 2011, Neve and McPhie, 2007) . In physiological conditions, APP is cleaved by α-secretase, which impedes Aβ formation, and the resulting carboxy-terminal fragment is then cleaved by γ-secretase. The resulting products do not aggregate (Chow et al., 2010).

Materials and methods: This study was performed on 16 male C57BL / 6J (N = 8 for ex vivo studies and N = 8 for in vivo studies) mice . Before to intracranial injection of fluorescently labeled amyloid Aß 40 some of the animals included in the study received intraperitoneally the dose of the TGN-020 aquaporin-4 inhibitor, and brain labeled with Vessels Were Sulfurodamine 101 to be visualized. I followed the perivascular drainage of Aβ 40 for 20 minutes using a 7MP Zeiss two-photon laser-scanning microscope, then the mice were anesthetized, decapitated and half of the brain was prepared for fluorescence microscopy and the other half was prepared for electron microscopy.

Results: In vivo imaging confirmed that Aβ40 rapidly diffused into the surrounding neuropil, with a small number of vessels showing an accumulation of Aβ40 peptide around them, mostly around the injection site and almost immediately after the injection began, and with some being visible for up to 30 minutes afterwards. After systemically injecting TGN-020 AQP4

inhibitor, prior to Aβ40 injection, in vivo imaging clearly showed more vascular accumulation of Aβ40 peptide compared to the untreated group.

Next, we were interested to evaluate whether the drainage of Aβ occurs uniformly for vessels of all sizes. As such we decided to measure the diameter of the vessels showing Aβ deposits. In animals treated with the AQP4 inhibitor, Aβ accumulation was greater around smaller vessels (8.366 +/- 0.821 μm) compared to controls (13.17 +/- 1.532 μm) (p = 0.0164). If we looked for the vessels that had no Aβ deposits around them, there was no difference between their average diameters for treated and respectively, untreated animals (5.192 ± 0.289 μm / 5.558 ± 0.246 μm), (p = 0.33) There were no differences microscopically visible morphology of blood vessels in treated and untreated animals, as seen on the paraffin-embedded hemispheres, without acute bleeding and no inflammatory infiltrate hidden in the vessel walls.

Discussions: In the present study, we have shown for the first time that AQP4 inhibition directly decreases amyloid Aβ drainage through the glymphatic pathways, and thus its downregulation or age-related decrease at the level of the perivascular astrocytic end-feets would greatly contribute to the accumulation of Aβ in the brains of LOAD patients. Indeed, a number of astrocyte changes have been described in AD mouse models and human pathology, such as detachment of astrocyte end-feet from the BBB, reduction of end-foot metabolism including loss of astrocytic glucose transporter 1, AQP4, as well as an overall impaired perivascular drainage of solutes (Wilcock et al., 2 009, Merlini et al., 2011, Hawkes et al., 2011).

The impact of age-associated AQP4 deficits on Aβ clearance is even more pronounced in long-standing deficits compared to the present experiment, as it has been shown that during sleep the glymphatic clearance is

increased with up to 60% allowing, under normal conditions, an optimal solute clearing from the brain (Xie et al., 2013) .

6. DISTRIBUTION OF AQUAPORINS 1 AND 4 IN THE CNS

Introduction : The aquaporins (AQP) are channels that are selectively permeable to water, first identified in the early 1990s . These proteins have different functions and localizations in the human body . The main aquaporins present in the brain are AQP1, AQP4 and AQP9, but recent studies have identified AQP3, AQP5, and AQP8 .

Materials and methods: We have utilized here brain tissue from patients who died of non-CNS related causes . The patients had been admitted and followed-up in the department of Neurology, Clinical Hospital of Neuropsychiatry, University of Medicine and Pharmacy of Craiova, Romania. After macroscopic examination, tissue blocks were sampled from all major isocortical areas, routinely processed for paraffin embedding, then 4μm-tick sections were cut and flattened on poly-L-lysine coated glass slides. Hematoxylin and eosin staining with firmed no obvious histopathological changes in the cortex and white matter, ie slight patchy gliosis, stasis and isolated perivascular and pericellular edema . Frontal, temporal, parietal and occipital tissue blocks were selected from each patient and further processed for immunohistochemistry. The antibodies we used were anti-AQP1, anti-AQP4 and anti-GFAP. We also performed double immunofluorescence reactions for both aquaporins and for GFAP and aquaporins.

Results: We first evaluated the relative disposition of AQP1 and AQP4 in the cortex and white matter of control individuals, with unclear CNS-associated pathology (Rosu et al., 2019). Most of the AQP4 was expressed, as

already described (Mogoanta et al., 2014) in the pia mater, petechial in the cortical astrocytes, and denser in the white matter astrocytes. When we evaluated AQP1 in the cortex, there were only a few astrocyte-like cells stained, and apparently with little / no colocalization with AQP4 . We intended to see the most common cellular expression for AQP1 and evaluated it concomitantly with GFAP.

We next intended to see the most frequent AQP1 cellular expression, and thus we evaluated its expression concomitantly with GFAP. In all cases, there was complete closeness of the two signals, as is the case of AQP4. Since both are membrane-bound proteins, aquaporin signal is surrounding the GFAP cytoskeleton, and this was the expression pattern throughout the analyzed images.

Discussions: This ancient family of proteins, aquaporins, are present in all life forms, showing their importance in maintaining normal physiology of all organisms. They are present in a larger number in multicellular organisms compared to unicellular organisms, where there are only a few (Heymann and Engel, 2000, Zardoya, 2005).

The major roles played by aquaporins in the nervous system are neuroexcitation, astrocyte migration, and facilitating water movement into and out of the central nervous system (Arcienega et al., 2010).

AQP 1 is present at the levels of the choroid plexuses and plays a part in CSF secretion and AQP 4 is present in ependymal cells and subependymal astrocytes, especially on the perivascular end-feets and plays a role in CSF absorption (Nielsen et al., 1997, Mogoanta et al., 2014, Rash et al., 1998, Popescu et al., 2017).

7. EXPRESSION PATTERNS OF AQUAPORINS 1 AND 4 IN STROKE

Introduction: In the world, stroke represents the leading cause of death in developed countries and the prevalence of this condition is increasing slightly (Kim et al., 2010). Ischemic stroke is the most common type, represents 80% of all stroke patients and occurs from a sharp decrease in blood flow to a particular region of the brain; neurological dysfunctions are the main consequence of this disease (Richard Green et al., 2003). In this article, we focused on describing the location of AQP1 and AQP4 in stroke and on observing their degree of colocalization in different areas such as the perilesional scar, perilesional white matter, control cortices and white matter areas.

Materials and methods: We have used here brain tissue from eight patients with confirmed ischemic pathology and from five patients who died of non-central nervous system related causes. These patients had been admitted and diagnosed in the Department of Neurology, Clinical Hospital of Neuropsychiatry, University of Medicine and Pharmacy of Craiova, Romania, and their biological material had been included in the brain bank project that is undergoing in the Department of Histology from University of Medicine and Pharmacy of Craiova . The average ages were 67.13 ± 5.03 years for stroke patients, and $6\,6.4 \pm 7.02$ years for control cases. We have made classic histological stains such as hematoxylin-eosin but also simple, double and triple immunofluorescence techniques. The antibodies used by us in this study were: anti-AQP1, anti-AQP4, anti-Collagen IV and anti-GFAP.

Results: We first evaluated the immunoexpression of AQP1 versus AQP4 on serial sections from perilesional tissue, in both the cortex and white matter. In both the control tissue and all the regions we analyzed in the brains

of stroke patients, the expression of AQ P4 was higher than that of AQP1. Next, we compared comparatively the expression of the two AQPs, in fluorescently stained sections from perilesional cortices. Thus, while AQP4 was intensely expressed by the pia mater and by focal astrocytes in the cortex itself, AQP1 was almost non-existent at the level of the pia, and with only sparse astrocytes being positively co-labeled by this marker. An interesting finding was that while most AQP1 signal co-localized with GFAP, not all AQP4 signal came from GFAP-labeled astrocytes.

The most interesting phenomenon, however, was in the glial scar surrounding the necrotic core, where AQP1 expression seemed to be restricted to the immediate vicinity of the gemistocytes' membrane, with the perinuclear areas being de- voided. This feature was not observed for AQP4 in the scar regions

Discussions: We have analyzed here for the first time the immunoexpression of AQP4 versus AQP1 on serial sections through the brain from patients suffering from stroke but also through the normal brain (Rosu et al., 2019). Although the immunoexpression of both AQPs was positive in the same areas, in the astrocytes and around the blood vessels. In all areas analyzed by us, peri-liquefaction core, in the glial scar, perilesional WM and cortex the expression of AQP4 was higher than that of AQP1. It would be of interest to show in the future, the three-dimensional relationship between the blood vessels and the AQP1- respectively AQP4-positive astrocytes on scanned serial sections (Serbanescu and Plesea, 2015).

Our study, as well as other studies that have analyzed the immunohistochemical expression of AQP4 in the normal brain, have shown that it is present in glial cells with astrocyte structure and in ependymal cells. They also showed that the glial cells were stained in mesencephalon, spinal cord, thalamus and cerebellum. Just as in our study, the

neurons in the cerebellum, spinal cord and brain were not positive for these markers. The positivity of the astrocytes for AQP4 was different, so in the perivascular processes it had the highest intensity, in the pia and in the ependymal layer, data similar to those obtained by us (Nielsen et al., 1997).

8. CONCLUSIONS

In the present study was evaluated the expression pattern of AQP 4, which is the best represented water channel in the CNS, in ischemic stroke, as well as its ability to modulate the elimination of the soluble fraction of amyloid Aβ. It has been shown for the first time that inhibition of AQP4 reduces the perivascular drainage of Aβ in the brain.

We have shown in this study that when AQP4 is inhibited, there is a significant delay in Aβ drainage starting 10 to 30 minutes after injection. The accumulation of Aβ around the blood vessels is higher and is generally deposited in vessels of smaller diameter, thus explaining the deposition of Aβ in the blood vessel walls with different dysfunctions (hypertension, age-related changes).

We were also interested in evaluating the expression of aquaporins 1 and 4 in normal brain and cerebral infarction. We analyzed for the first time the expression of the two aquaporins in the cortex and the white matter in the normal brain, by means of immunofluorescence techniques. It was observed that at the level of pia mater, AQP 4 has a very high expression compared to AQP1 which is very little expressed here, and the degree of colocalization for the two markers is low. At the level of cortical astrocytes the signal for AQP4, areal expressed, we found fewer positive astrocytes for AQP1 with a weak colocalization between the two. In contrast, in the white matter, the expression of AQP1 was higher, in this area we found both positive astrocytes for both

aquaporins and astrocytes expressing only one of the aquaporins. We evaluated the expression of AQP1 with GFAP at the same time and found a perfect colocalization between the two markers.

In the perilesional cortex, on the immunohistochemistry images, we found a positive signal for the two aquaporins around the blood vessels and in the intraparenchymal astrocytes, but with a higher expression for AQP4. At the white substance level we also had a positive signal for both aquaporins, but with much more subtle differences. In the same areas we analyzed the degree of colocalization for the two aquaporins by immunofluorescence techniques. Although in most cases the expression of AQP 4 was higher than that of AQP1, we also found some areas of perilesional scarring where the expression of AQP 1 was higher.

Corroborating all the information we obtained through both microscopic images and statistical analysis, we showed that the expression of the two aquaporins increases in stroke, colocalizing with both collagen IV and GFAP, but the AQP4 values are constantly higher.

We were also concerned to evaluate the role of perivascular space in inflammatory cell drainage in the CNS. We have shown for the first time that these cells are present in the perivascular space and more, we identified them, by histological and immunohistochemical means, in pockets of the basal vascular membrane. We also found some vacuolar or sacular areas, in the thickness of the basal membrane, which were delimited by collagen type IV and contained nucleus of inflammatory cells, highlighted by immunohistochemical staining.

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