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IMPLICATIONS OF WATER CHANNEL PROTEINS (AQUAPORINS AND RELATIVES) IN EPILEPSIES

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ABSTRACT. The abundant evidence linking epilepsy with hydroelectrolytical changes in epilepsy reported in the medical literature for over 9 decades is reviewed. Our work on this topic is presented, including the detection by NMR of a decreased water permeability of the red blood cells (RBCs) from children with epilepsy compared with control children. This was interpreted as a generalized membrane defect in epilepsy. The subsequent program of research performed by Gh. Benga's group in Cluj-Napoca led to the discovery of the first water channel protein (WCP) in the RBC membrane, protein later called aquaporin1 (AQP1). A lot of WCPs (AQPs and relatives) have been identified later by various authors, in the body of humans and many other species. The physiological roles of WCPs from the central nervous system (CNS) and their implications in epilepsy and other neurological diseases are reviewed, including the information published in the last months.

Keywords: *water channel proteins, aquaporins, epilepsy, paediatric neurology, NMR*

INTRODUCTION

Epilepsies are among the most frequent Central Nervous System (CNS) disorders, caracterized by seizures, affecting approximately 1-2% of the world's population [1]. The seizure is the most frequent symptom (after headache) in paediatric neurology [2]. The neurophysiological basis of a seizure is the spontaneous repetitive discharge of a hyperexcitable aggregate of neurons [3, 4].

Abundant evidence linking epilepsy with water metabolism was reported in the medical literature for over 9 decades and was reviewed by many authors [5-7]. Some observations and studies are discussed below. Frisch and Walter [8], Keith et al. [9] and Helmholtz [10] reported a relationship

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between seizure frequency and water intake in epileptic patients. Moreover, the antiseizure effects of various diuretics were reported by the middle of the 20th century, the topic being reviewed later [11, 12].

McQuarrie [13, 14] in epileptic children and Teglbjaerg [15, 16] in epileptic adults performed the first investigations under carefully controlled conditions. Both groups concluded that fluid restriction reduced seizure frequency, while hyperhydration led to an exacerbation of seizures. McQuarrie et al. [14] studied the balance of water and minerals (sodium, potasium, calcium and phosphorus) in children with epilepsy and found that "a positive water balance was followed by convulsive seizures" and when seizures occured potassium was increased in urine "with a striking increase in the potassium to sodium ratio", while during periods with no seizures sodium was the predominant mineral in urine. The authors concluded: "This apparent 'leakage' of potassium from the cells... may indicate an innate weakness in the retaining membranes, presumably of the cells of the central nervous system. It is possible that abnormal amounts of water and even sodium ions entry at the same time". "The data recorded are tentatively interpreted as favoring the view that an inherent defect in the mechanism for regulating permeability of the brain cell membranes is characteristic of the epileptic state". Schneider [17] observed that in children and adults suffering from *petit mal* epilepsy the exacerbation of *petit mal* events is associated with reduction in urinary volume, and that clinical remission coincides with an increase in water excretion (spontaneous, drug-induced or hormone induced). Reynolds [18], using radioisotopic methods, reported some changes in the whole body distribution of water and sodium in epilepsy, with a magnitude proportional to the frequency of seizures. He concluded that disturbances in body water and sodium are linked in some way to the aetiology of epilepsy. Moreover, comparing two groups of patients, one with idiopathic epilepsy and the other with focal epilepsy, he found "a striking similarity between the two groups of patients for all the biochemical variables" measured, considering that his study supports the concept of an essential unity between the idiopathic and focal epilepsy.

Tower [19] incubated slices of human epileptogenic cortex, which were unable to take up K^+ and extrude excess Na^+ in a similar way as the control cortical samples. He found the same changes in the ability to handle Na^+ and K^+ in cortical slices from cats in which epilepsy had been induced by megimide or methionine-sulfoximine. Tower [19] considered that the characteristic instability of the excitable membrane in epileptogenic neurons is a consequence of an impairment of operation of the Na^+ and K^+ transport system, which is "the final common expression of epileptogenicity".

On the other hand, there were many observations of water intoxication (WI) in non-epileptic individuals. Ferrier [20] described the symptoms of WI: the first include headache, blurred vision, polyuria, vomiting, tremor, followed by

muscle cramps, ataxia, delirium, stupor, coma, and then convulsions can follow. WI caused by polydipsia (compulsive water drinking) was described in schizophrenics, major motor seizures being present in about 80% of cases [21]. There are also experiments in animals. Rowntree [22] reported that WI in mammals produce “extremely severe toxic manifestations” including seizures. Other studies confirmed that ADH and water loading produce seizures in otherwise normal mammals: rats [23-25], rabbits [26].

Wasterlain and Torack [27] described (apparently for the first time) astrocytic swelling and enlarged extracellular space in compact white matter in the absence of any vascular damage or blood brain barrier (BBB) breakdown in brain edema associated with WI. Other reviews regarding the brain volume regulations and brain excitability in response to changes in osmolality have been published [28-31].

RESULTS AND DISCUSSION

Our first studies of hydroelectrolytic changes in child epilepsies

Considering all the above mentioned researches, as well as the local conditions where I was working, I choosed to study hydroelectrolytic changes in child epilepsies as topic for my Ph.D. Thesis [32]. In those years I was Junior Lecturer at Discipline of Child Psychiatry (DCP), Institute of Medicine and Pharmacy (I.M.F.) Cluj-Napoca (to become later “Iuliu Haţieganu” University of Medicine and Pharmacy – U.M.F. – Cluj-Napoca). DCP was functioning together with Discipline of Child Neurology (DCN) in the same Clinic of Child Neuropsychiatry (C.C.N.) Cluj-Napoca, the building belonging to the Cluj Children Hospital. I also worked in the laboratories of Discipline of Medical Biochemistry (DMB) of I.M.F., collaborating with my husband (Gheorghe Benga, Senior Lecturer) and with other distinguished faculty members of DMB. We published some papers regarding the hydroelectrolytic changes in child epilepsies. In one of these [33] we measured the concentrations of Na and K in the red blood cells (RBCs) of 35 children with epilepsy and 25 control children. We found a statistically significant lower concentration of Na and a higher concentration of K in children with epilepsy compared with the control children. We wrote: “These changes observed in case of RBCs of children with epilepsy may indicate the perturbation of mechanisms which maintain the polarization of cell membrane. Tower [19] by measurements on human brain showed that in case of epileptogenìc cortex there is a deficiency of mechanisms which maintain the normal distribution of Na and K across the cell membrane. Our data showed changes of the distribution of Na and K in

RBCs, i.e. at distance from the organ implicated in the generation of convulsive discharges (brain). This makes us to suggest a more general perturbation of the mechanisms which ensure the intra and extracellular concentrations of Na and K in other cells of the body. Our results are in agreement with data of Reynolds [18] who found by radioisotopic methods general changes of distribution of Na and water in the body of patients with epilepsy" [33].

The collaboration with Vasile V. Morariu begins

One day in 1976 my husband told me that he has incidentally met Vasile V. Morariu, his former classmate in the high school (Liceul "Emil Racoviţă" Cluj), who returned from Australia in 1973, where he worked three years as a Ph.D. student (1969-1972) at Canberra National University. Vasile graduated the Faculty of Physics at "Babeş-Bolyai" University (U.B.B.) Cluj-Napoca. After graduation (1966) Vasile Morariu was employed as researcher at the Institute for Stable Isotopes, which soon changed the name to Institute for Isotopic and Molecular Technology (ITIM) Cluj-Napoca (to become in 1999 The National Institute for Research and Development of Isotopic and Molecular Technologies – INCDTIM – Cluj-Napoca). In 1976 Vasile Morariu was researcher at ITIM, leading a group oriented to biophysics, using NMR as the main technique of investigation. My husband has just returned from England, after working 12 months as Post-Doctoral Fellow in the laboratory of Professor Dennis Chapman (a famous scientist in the field of biological membranes), learning NMR, spin labelling ESR and other biophysical techniques to study protein-lipid interactions in biomembranes. Vasile Morariu mentioned to Gh. Benga that he knew a paper of two Australian researchers who used NMR to investigate the water diffusion through the red blood cell (RBC) membrane [34]. Gh. Benga said that we are very interested in such measurements, taking into account the topic of my Ph.D. Thesis [32] and the research we have already performed regarding the hydroelectrolytic changes in children with epilepsies [33].

As described by Professor Petre T. Frangopol [35] in Australia Vasile Morariu has done outstanding NMR work on water adsorbed on silica, elucidating the origin of the "abnormal" water, discovered by some Russian scientists, who considered that it is a special polymerized water. Famous laboratories from the U.S.A. and U.K. have reproduced the experiments of the Russians and many international conferences took place on the subject. It was speculated that under certain conditions the water on Terra could polymerise and the life on our planet could be in danger! Vasile Morariu suggested to his Ph. D. supervisor (Professor R. Mills) to choose the study of "abnormal" water as his Ph.D. work project. His suggestion was accepted and Vasile Morariu

discovered that in fact the polymerization of the “abnormal” water is due to the impurification with silicic acid [36]. Other scientists produced the polymerization with other impurities, so the scientific confrontation (with possible major political and military implications) between the two superpowers (U.S.A. and U.S.S.R.) vanished!

The first results obtained in collaboration with Vasile V. Morariu

Vasile Morariu and Gh. Benga took the decision to start immediately the NMR measurements of water permeability of RBCs in children with epilepsy compared with control children. When my husband informed me about his discussion with Vasile, I told him that Cornelia Morariu, Vasile’s wife, is intern in paediatrics, performing a 6 months rotation in the C.C.N. working with me. We met all four in the following days and Vasile explained us the principle of the NMR method of Conlon and Outhred [34] for measuring water permeability of the RBC membrane. The method involves addition of a paramagnetic solution ($MnCl_2$) to the plasma and measurement of the spin-spin relaxation time (T_2) of the RBC water proton. The spin-spin relaxation time of water inside the isolated RBCs is about 140 ms and is much longer than the time required for water to exchange across the membrane (the water exchange time, T_{ae}), which is about 10 ms. If the relaxation time in plasma is made much shorter than the exchange time (by adding the paramagnetic ion Mn^{2+}), the observed relaxation time of the RBC (T_{2b}) is dominated by the exchange process through the membrane. The spin-spin relaxation time is evaluated from a logarithmic plot of the nuclear spin-echo as a function of the time interval 2 ĩ where ĩ is the time interval between the radiofrequency pulses. When the system is characterized by a single relaxation time, the plot is a straight line and the relaxation time is the reciprocal of the slope. For a system characterized by two relaxation times (as for the blood doped with Mn^{2+}) the plot consists of two lines and the relaxation times are calculated from the slopes of these lines. The value of T_{ae} is inversely related to the water permeability (P_d) of RBCs.

We started the work immediately. I selected the patients, helped by Cornelia Morariu, while Vasile Morariu and Gh. Benga performed the laboratory work. All blood samples (obtained by venous puncture, in heparinised tubes) were numbered and transported by Gh. Benga to ITIM, where Vasile Morariu and his co-workers performed the NMR measurements, without knowing which blood samples are from children with epilepsy and which are from control children. Patients were 24 children with epilepsy aged 1-12 years and controls were 24 children aged 2-16 years. The patients included 11 children with

idiopathic *grand mal* (GM), one with idiopathic GM plus *petit mal* (PM) and 12 children with focal epilepsies (7 with focal GM, 3 with minor attacks, 2 with temporal lobe epilepsy). In all children with epilepsy the exchange time of water through the RBC membrane (T_{ae}) was longer than in control subjects. The measurements were made at different temperatures and concentrations of Mn, and two methods were used to measure spin-spin relaxation time: the 90^0 ĩ 180^0 method and the Carr-Purcell-Meiboom-Gill (CPMG) method (which eliminates errors due to the inhomogeneity of the magnetic field and the molecular diffusion in field gradients) [37]. There were no significant differences in T_{ae} values between idiopathic and focal epilepsies. High values of T_{ae} were found in patients who had seizures every day and in whom the attacks were poorly controlled by anticonvulsant therapy. It was also found that the value of T_{ae} during the seizure was not higher than in the interictal period. This indicated that the low water permeability of RBCs in epilepsy is a permanent alteration (not a transient one). The abnormal water permeability was found in both untreated and treated patients, i. e. was not related to the anticonvulsant therapy. An alteration (decrease) of the permeability to water of RBCs in children with epilepsy was the most likely explanation for our findings. We realized immediately the possible important significance of our findings, as we had already studied extensively the publications regarding the NMR (Vasile Morariu) and epilepsy (myself and my husband). In October 1976 a manuscript was sent to *Nature*, in December 1976 was accepted to be published without changes and in February 1977 it appeared [38]. The idea of a generalised membrane defect in epilepsy was discussed as follows: “Abnormalities in functions of erythrocyte membranes are considered to indicate a generalised membrane defect [39, 40]. Within certain limits a close qualitative and quantitative analogy is possible between the stability of the membrane of the erythrocyte and that of the neuron [41, 42]. Fritz and Swift [43] have shown (by the NMR method we used) that for the frog nerve the exchange rate of the water protons between the intra and extracellular environments is lower for the depolarised nerve than for the polarised nerve. That is, the water permeability of the membrane is smaller in the depolarised state than in the resting state...We suggest that decreased permeability in erythrocytes of epileptics may reflect a membrane defect in all tissues and may be an expression of the individual predisposition in epilepsy; it might be of particular importance in the nervous system. Further studies on erythrocyte membranes in epilepsy may give clues to the understanding of the membrane defect in molecular terms” [38]. The authors were Gheorghe Benga and Vasile V. Morariu, while the contributions of myself and Cornelia Morariu were mentioned as: “We thank Drs. Ileana Benga and Cornelia I. Morariu for helpful discussions and for blood samples”.

The results obtained in the following decades regarding the water permeability of membranes and the significance for epilepsies

In the following decades our group continued the research on this line, in agreement with the background and interests of each of us. Vasile Morariu and Gh. Benga evaluated the NMR method of Conlon and Outhred [34] and found that some improvements and standardizations are necessary, including: the calculation of the RBC membrane diffusional permeability to water (P_d) from the water exchange time; the study in detail of the effects of temperature and pH on P_d; the calculation of the activation energy ($E_{a,d}$) of the membrane diffusional permeability to water [44-47].

I continued to publish articles regarding the hydroelectrolytic changes in child epilepsy based on our work [48, 49] and to follow the implications of water channel proteins in epilepsies and other neurological disorders.

Gh. Benga started an extensive program of research aimed to identify the pathway by which the water molecules cross the membrane. Several important aspects had to be studied until the final goal was achieved: NMR measurements of the effects on P_d of various inhibitors, of chemical modification of membrane proteins, measurements on resealed ghosts (prepared by a special procedure: hemolysis to remove hemoglobin and then restoring the membrane integrity), labelling, by a radioactive inhibitor, of the protein involved in water permeability, identification of this protein by polyacrylamide gel electrophoresis etc [50-54]. Obviously, to perform all these procedures there were many requirements: the appropriate laboratories (including those for work with radioactive compounds), the equipment (centrifuges, electrophoresis equipment, NMR spectrometer, a lot of reagents), and people with experience in laboratory work and authorized to work with radioisotopes and funds to purchase all reagents and equipment. In many of these aspects Professor Frangopol played an important role, in his position as Chief of a Center of Radiochemical Production at “Horia Hulubei” National Institute for Research& Development in Physics and Nuclear Engineering (in Romanian Institutul de Cercetare-Dezvoltare pentru Fizică şi Inginerie Nucleară “Horia Hulubei”, abbreviated as IFIN-HH). He provided financial support from his grants when we started the collaboration with Vasile Morariu, since the NMR measurements at ITIM required the covering of all expenses (including the salaries of researchers). Professor Frangopol obtained grants from The Academy of Medical Sciences (A.M.S.) and other sources and included Vasile Morariu and his team on the grants. Later Professor Frangopol helped Vasile Morariu and Gh. Benga to get their own grants from A.M.S. Moreover, he played an essential role in the acquisition by Gh. Benga of an NMR spectrometer manufactured at IFIN-HH.

The spectrometer was installed at Cluj-Napoca by the experts from IFIN-HH in a laboratory of the newly founded Discipline of Cell Biology (DCB) which Gh. Benga was appointed to chair. The experts came to Cluj-Napoca and instructed Gh. Benga's team how to use the NMR spectrometer. With another grant from A.M.S. (obtained this time by Gh. Benga) a module of calculations of relaxation times was made by the experts of IFIN-HH, who came again to Cluj-Napoca and installed the module.

After almost a decade of hard work, the water channel protein (WCP) in the human RBC membrane was identified by Gh. Benga's group. The discovery of the first WCP was really achieved in 1985 when the first landmark paper by Benga and coworkers was sent for publication to the prestigious American journal *Biochemistry*, which accepted the publication without changes [53]. The second landmark paper was published by Benga and coworkers in 1986 in a well known European journal [54]. In 1988 the group of Peter Agre in Baltimore purified by chance the protein, calling it CHIP28 (CHannel forming integral membrane protein of 28kDa) having no idea of its function [55]. Gh. Benga presented the novelty of the discovery of his group in reviews published before 1990 [56, 57]. The group of Agre found the water transport property of CHIP28 only in 1992 [58].

The protein identified in Cluj-Napoca was the first water channel discovered. Other WCPs were discovered in 1993: in a plant [59] and in the kidney [60]. The name of aquaporins was proposed for the WCPs [61] and CHIP28 was named aquaporin 1 (AQP1).

In a few years it became obvious that a large family of WCPs exists, with three subfamilies: aquaporins (AQPs), aquaglyceroporins, and S-aquaporins. Moreover, it was discovered that actually the WCP family (with all three subfamilies) belongs to a superfamily of Membrane Intrinsic Proteins (MIPs). The superfamily includes also MIPs with no identified channel activity. MIP is an acronym first used for MIP 26 (Major Intrinsic Protein of 26 kDa) of lens fiber cells in the eye [62]. Later, the number of reviews and of articles on WCPs and their relatives published in various journals continued to increase, since the presence and roles of such proteins in all kinds of species on Terra (from prokaryotes to plants, animals and humans) have been revealed. Lots of reviews have been published in the first decade after the discovery of WCPs [63-70].

Moreover, special issues of prestigious journals [71-74], proceedings of world congresses [75], multi-authored books [76] were dedicated to the newly discovered proteins. Over 300 WCPs were discovered so far and their structures (going down to the molecular level) for a number of them have been elucidated, as described in many reviews [67, 73] and several chapters in ref. [76].

As discussed in ref. [73] a WCP may be defined as a transmembrane protein that has a specific three-dimensional structure with a pore that provides a pathway for water permeation across biological membranes. In order to understand the subsequent description I recommend to access freely the following link: https://iubmb.onlinelibrary.wiley.com/doi/10.1002/iub.156 and look at Fig. 1.

WCPs and MIPs have a relatively small size, most are less than 300 amino acids. Both the NH_2 terminus and COOH terminus are hydrophylic and located in the cytosol. The pore is formed by two regions in the amino acid sequence, called *NPA boxes* (or *motifs*), with three amino acid residues (asparagine-proline-alanine, NPA) and several surrounding amino acids. The NPA boxes have been called the “signature” of WCPs. There are six transmembrane domains (TMD), highly hydrophobic, with α-helix structure and five connective loops. The α-helices are named from the N-end succesively H1, H2, H3, H4, H5 and H6, and the loops are named A, B, C, D, and E. The TMDs and the loops form a *core* (embedded in the lipid bilayer), to which two “legs” (represented by the cytosolic N- and C-ends) are attached. NPA boxes are located in the loops B and E, which are rather hydrophobic in nature and have short (half) helices HB and HE. The six TMDs (tilted at about 30^o with respect to the membrane normal) form a right-handed bundle enclosing the *channel* (*pore*) formed by the NPA motifs and the short tetramer helices HB and HE, bended into the six-bundle and connected to the center of the bilayer. This structure is called the *aquaporin fold.* So the channel (pore) is a narrow tunnel in the center of the molecule that has at the extracellular and cytoplasmic faces funnel-shaped openings (atria or vestibules). In the membranes (natural or reconstituted proteoliposomes with purified proteins) WCPs are in the form of *tetramers*, as shown by freeze-fracture electron microscopy (EM). AQP1 tetramers are held together by extensive interactions between helices and loops of the monomers. Each monomer, however, has its own channel, functionally independent.

The nomenclature of WCPs uses the abbreviation AQP followed immediately by the number, (e.g. AQP1, AQP2 etc). Thirteen WCPs have been characterized in humans. Seven are aquaporins (AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8), four are aquaglyceroporins (AQP3, AQP7, AQP9 and AQP10), whereas AQP11 and AQP12 are S-aquaporins (“superaquaporins”, “subcellular aquaporins” or “unorthodox aquaporins”).

1) The AQPs were defined as water specific or water selective channel proteins, “classical”, “conventional”, “orthodox” etc. 2) The aquaglyceroporins are permeable to water, but also to other small uncharged molecules, in particular glycerol. The “signature” sequence of aquaglyceroporins is aspartic acid residue (D) in the second NPA box. 3) The S-aquaporins have little conserved amino acid

sequences around NPA boxes, being also named “aquaporins with unusual (or deviated) NPA boxes” or “unorthodox aquaporins”. The structure and function of AQP11 and AQP12 are currently poorly understood. They have less than 20% homology with other classical AQPs, indicating that they belong to a supergene family of AQPs. Details may be found in ref. [76].

In recent years, it has been found that many cellular functions of aquaporins are regulated by post translational modifications (phosphorylation, ubiquitination, glycosylation, subcellular distribution, degradation, etc.). Insight into the molecular mechanisms, responsible for regulated aquaporin trafficking and synthesis, is fundamental for the development of reliable diagnostic and prognostic biomarkers and therapeutic targets [76].

Gh. Benga had the idea to start (in Cluj-Napoca) a comparative program of studies of water permeability of RBCs in various animals. A review of the first such studies has been published [77]. He also had the idea to extend the investigations to a variety of animal species, including those living in Australia. A collaborative program of research was established with an outstanding Australian scientist, Professor Philip Kuchel (The University of Sydney). The two groups achieved exchange working visits, performing studies of the RBC water permeability of over 30 species and the program is still very active. Kuchel and Benga [78, 79] provided two new explanations for the physiological “raison d’etre” of AQPs in RBC. The first is the “oscillating sieve explanation”: the high water permeability of RBC membrane favors the energy driven membrane undulations (or oscillations) of the RBC membrane, a phenomenon also called “flickering” [80, 81]; these movements consume a minimum of energy in simply displacing water. Such membrane undulations perform a valuable role in movement of cells through capillaries. The second is the “water displacement explanation”: when ions, such as Cl^- and HCO_3^-, and solutes, such as glucose, are entering into the cells, the water molecules are displaced and exit rapidly the cell, thus obviating a change in cell volume. The molecular volume of these ions and molecules are significantly higher than that of water. In addition, Kuchel and Benga concluded [78, 79]: the parameters characterizing the water permeability of RBC appear to be a species characteristic as there are no changes correlated with the marked alteration in the habitat of the species introduced to Australia (rat, rabbit, sheep, chicken) compared with their European counterpart. The chicken and echidna RBCs have the lowest P_d values (~2×10^{-3} cm s^{-1}) and the highest values of $E_{a,d}$ (over 30 kJ mol^{-1}); this indicates that no functional AQPs are present in chicken and echidna RBCs. Human RBCs have P_d values of ~4×10^{-3} cm s^{-1} at 25 ^{0}C and ~7×10^{-3} cm s^{-1} at 37 ^{0}C with a value of $E_{a,d}$~25 kJ mol^{-1}. Large and less-active animals (cow, sheep, horse and elephant) have lower values of P_d. In contrast, small and active animals (mouse, rat, guinea pig, rabbit,

small marsupials) have P_d values significantly higher with lower $E_{a,d}$ values (from 15 to 22 kJ mol^{-1}). It appears therefore that AQPs in RBCs ensure the rate of exchange of water across the membrane required in various animals in relation to their physical activity, metabolic rate and the mean rate of circulation of their blood [77-79].

WCPs in the *central nervous system* (CNS) are of great physiological and pathological importance. The CNS is the part of the nervous system consisting primarily of the *brain* and *spinal cord*. The CNS is so named because the brain integrates the received information and coordinates and influences the activity of all parts of the body. The importance of WCPs in the brain is obvious, considering the rigid physical constraint that is imposed to the brain by the bony cranium and that ~80% of the brain is water [31, 82]. The human intracranial volume is ~1400 ml (79) and comprises the brain parenchyma (~1200 ml), intravascular (~100 ml) and cerebrospinal fluid (CSF) (~100 ml) compartments. The brain compartments include the subarachnoidian space and the cerebral ventricles. Most of the brain parenchyma water is intracellular (~1100 ml), while ~100 ml water is distributed in the extracellular space (ECS), also named the brain interstitial space (IBS) [82, 83].

The brain parenchyma is composed of two major cell types: neurons and glial cells. In general, glial cells are smaller than neurons. There are approximately 85 billion glial cells in the human brain, about the same number as neurons. Glial cells make up about half the total volume of the brain and spinal cord. The glia to neuron ratio varies from one part of the brain to another [84, 85].

Glial cells (Gr. *glia* = glue) are divided into two major cell classes: *microglia* and *macroglia. Microglia* (about 10% of glial cells) are small relative to macroglial cells, with changing shapes and oblong nuclei, being mobile within the brain. They subserve immunologic functions in the CNS, protect neurons, being among defense mechanisms against infectious diseases of the CNS. They are normally inactive, but, activated by any pathological change in the CNS (infection, trauma, ischaemia or neurodegeneration) can become macrophages capable of phagocytosis [84].

Macroglia include *astrocytes*, *oligodendrocytes* and *ependymocytes* (or *ependymal cells*). *Tanycytes* in the median eminence of the hypothalamus are a type of ependymal cells that descend from radial glia and line the base of the third ventricle. Tanycytes extend from the hypothalamic median eminence to the hypophyseal portal system providing a pathway whereby hormones may enter or leave CSF [84].

The total number of glial cells in the human brain is distributed into the different types with oligodendrocytes being the most frequent (45–75%), followed by astrocytes (19–40%) and microglia (about 10% or less) [85].

Astrocytes (Gr. *astron* = star) have irregular (star–shaped) cell bodies and are characterized by numerous fine leaflet-like processes, which are in intimate contact with neurons and cover non-synaptic neuronal surfaces in the CNS. In adition, astrocytes have processes called “perivascular feet” which cover 85% of the surface of capillaries in the CNS. These processes form an external glial membrane under the pia mater and an internal membrane under the ventricular ependyma. There are two main subtypes: *fibrous* astrocytes, in the white matter, which have long slender processes and many cytoplasmic filaments, and *protoplasmic astrocytes*, in the grey matter, having shorter, flattened, branched processes with few filaments. They support neurons, structurally and functionally. When damaged they hypertrophy and proliferate to form “a glial scar”. Astrocytes are the commonest source of primary malignant tumours in the CNS.

The so called “blood-brain barrier” (BBB) is primarily due to tight junctions between capillary endothelial cells. Astrocytes also contribute to the selective nutritive path between blood vessels and neurons. Astrocytes regulate the external chemical environment of neurons by removing excess potassium ions and recycling neurotransmitters released during synaptic transmission [84, 85].

Verkhratsky et al. [86] observed that since the inception of neurobiology, two centuries ago, our founding fathers of gliology had a clear vision on the active role of glia, i.e. that glia has prominent roles in pathophysiology of the brain. Awkwardly, the twentieth century brought a dominant neurono-centric approach, the starring role has been solely by neurons. This approach has been challenged by the resurgence of neurogliopathology in the past 20 years. The correct approach, however, is to emphasize that it is the interaction between neurons and glia which underlies physiology and pathology of the brain. These major cellular constituents interact, so that perturbing one will affect the other. In epilepsy, astrocytes undergo substantial pathological remodelling, which greatly affects their homeostatic capabilities and is linked to pathophysiology of this disease. In particular, the epileptic astroglial phenotype includes changes (mutations and/or expression levels) in ion channels, receptors and transporters.

The work of our group described above and the idea of a membrane defect affecting water permeability represented in fact a world priority in the field of WCPs, since it showed for the first time the medical implications of alterations of membrane water permeability. Other authors have confirmed the presence of a membrane defect involving WCPs in epilepsy. Some studies are presented below. Ottersen and coworkers [87, 88] compared the variants (SNPs, i. e. single nucleotide polymorphisms) of the genes encoding AQP4 and Kir4.1. in a group of patients with temporal lobe epilepsy and in a group

of controls. The authors found eight single SNPs in Kir4.1. gene associated with temporal lobe epilepsy and considered these findings as a further proof of the implications of astrocytes in the pathophysiology of epilepsy.

Lee et al. [89] studied the expression of AQP4 and other glial molecules (Kir4.1., glial fibrilary acid protein, glutamine synthetase) in the intra-hippocampal kainic acid (KA) model of epileptogenesis and compared the wild-type mice versus AQP4-null (animals lacking AQP4). A marked reduction of AQP4 in both astrocytic fine processes and endfeet were observed following KA status epilepticus in multiple hippocampal layers. In addition, AQP4-null mice had more spontaneous recurrent seizures than wild-type during the first week after KA status epilepticus. These results are interpreted as an indication of dysregulation of water and potassium homeostasis during early epileptogenesis.

Lu et al. [90] examined the seizure susceptibility of AQP4-null mice following traumatic brain injury (TBI). The seizures were induced by injections of pentylenetetrazole and AQP4-null mice were compared with wild-type sham injury controls. AQP4-null mice demonstrated dramatically shortened seizure latency and increased severity grade. Morphometric analysis demonstrated a twofold reduction in astrocytosis with concomitant increase in microgliosis in injured AQP4-null mice.

Altered neuronal activity observed in AQP4 deficiency was reviewed in several publications [31, 82, 91, 92]. In animals lacking AQP4 (AQP4-null animals) the threshold for seizures is reduced while the seizure duration is prolonged. The underlying mechanism may be an impairment of K^+ homeostasis in the absence of AQP4. In addition, in hippocampal atrophy, a feature of mesio-temporal lobe epilepsy, AQP4 expression is increased in samples from atrophic hippocampi from epileptic patients. The accumulated evidence suggests that AQP4 is involved in such diverse functions as regulation of ECS volume following synaptic activation, potassium buffering, CSF circulation, interstitial fluid resorption, waste clearance, neuroinflammation, osmosensation, cell migration and Ca^{2+} signaling [82, 91-93]. The seizure phenotype data in AQP4-null mice raise the possibility that AQP4 modulation may also be effective in epilepsy therapy [94].

Consequently, in the brain, AQP4 stands as a multipurpose aquaporin, quite different from the situation in the kidney where several AQPs act in concert to regulate water transport.

Aquaporins came to the attention of neurologists mainly due to *neuromyelitis optica* (NMO) or *Devic's syndrome*, an autoimmune inflammatory demyelinating condition of the CNS, characterized by optic neuritis and myelitis.The clinical course of NMO is dominated by acute attacks. If untreated, NMO often results in blindness and tetra- or paraparesis. A significant number of

Devic's patients (70-80%) have antibodies against AQP4. The term astrocytopathy was proposed to characterize this disease. Recent reviews are available [95, 96].

The last period of collaboration with Vasile V. Morariu

After finishing the 6 months rotation in the C.C.N, Cornelia Morariu, performed a 6 months rotation in the Section of Neonatology of Cluj County Hospital. Professor Iulian Lupea (Chief of the Section) asked Vasile Morariu to measure the water permeability of RBCs from newborns. It was found that P_d was lower and $E_{a,d}$ was higher than the corresponding values of these parameters in children several years old [97]. On the other hand Gh. Benga and his co-workers found that these parameters are similar in case of RBCs from umbilical cord blood [98]. Then Vasile Morariu and Gh.Benga's group performed a detailed investigation on the age-dependence of the RBC water permeability [99]. It was found that the P_d is the lowest in the newborn, it increases in children, reaching at about 7 years a value that remains rather constant in young and mature subjects. The high permeability to water of the RBC membrane can be correlated at these ages with the ability to undertake a high level of physical activity. In elderly individuals (over 65 years) a further small, but statistically significant, increase in the P_d was observed [100]. In this case the higher RBC water permeability can be correlated with a requirement of the RBC membrane to favour the membrane undulations and the rapid entry or exit of solutes of molecular size greater that water, in conditions when the organism is less physically active, probably has lower metabolic rates and lower mean rates of blood circulation [100].

Recent knowledge about water channel proteins and their implications in epilepsies and other pathological processes in the central nervous system

Important progresses were achieved in the *genetic analyses of WCP genes,* including their localization on chromosomes; e.g. the gene of AQP4 is located in chromosome 18 (18q11.2– q12.1). The sequences of some WCP genes have been analysed (see the link: https://www.omim.org/entry/600308).

New findings were discovered regarding the *choroid plexuses* and the secretion of CSF. Anatomically, choroid plexus tissue is floating in the CSF of the lateral, third, and fourth ventricles. This tissue is well perfused by numerous villi, each having a central capillary with fenestrated endothelium.

Traditionally, the properties of the blood-brain barrier (BBB) were considered to be those of the capillary endothelium in brain. However, in contrast with capillary endothelium elsewhere in the body, the endothelium in brain capillaries is sealed with tight junctions, having a high electrical resistance and a low permeability to polar solutes. Early research unveiled, on the brain side of the BBB, ion channels and transporters capable of providing a net secretion of fluid, driven by Na^+ – K^+ ATPase. Accordingly, the BBB was proposed as a secretory endothelium, which produces the brain interstitial fluid (ISF) [101]. Recent research unveiled that the "barrier" function of the BBB is actually the result of highly regulated and complex cellular and molecular transport processes, which allow for the transport of water, solute, larger molecules and even cells [102]. According to the traditional understanding of CSF physiology, the majority of CSF is produced by the choroid plexuses, circulates through ventricles, the cisterns, and the subarachnoid space to be absorbed into the blood by the arachnoid villi. Then it was discovered that AQP1 is present in the *choroid plexuses*, being involved in the secretion of CSF. Novel insights were obtained using molecular and cellular biology tools, as well as neuroimaging. The new information, reviewed by Brinker and coworkers [103], is challenging the old concept regarding the secretion of CSF, indicating that CSF physiology may be much more complex than previously believed, astrocytes, AQPs and other membrane transporters being key elements in brain and CSF homeostasis. The new insights into the physiology of CSF circulation may have important clinical relevance for example for the understanding of hydrocephalus disorders and other brain diseases [104-106].

A very important progress in neurosciences was the discovery of *the glymphatic system* (GS), a novel defined brain-wide perivascular transit network between cerebrospinal fluid (CSF) and interstitial solutes that facilitates the clearance of brain metabolic wastes [105]. By analogy to the lymphatic system found outside the brain, the perivascular space system was named the glymphatic system. In the GS, CSF enters the perivascular space around the arteries to the deeper brain regions, flowing into the brain parenchyma through AQP4 channels in the astrocytic end feet. The interstitial fluid within the brain parenchyma (ISF) exits through the perivascular space around the veins, thereby clearing the waste products. From the perivenous space, the ISF drains into the subarachnoid space and meningeal lymphatics of the parasagittal dura. This is the essential description of the glymphatic system [104].

The glymphatic system was proposed to contribute to lactate concentration changes in the brain, in correlation with sleep–wake cycles and sleep homeostasis. The GS dysfunction is associated with various neurological

disorders, including traumatic brain injury, BBB disruption, hydrocephalus, stroke, brain edema, epilepsy, migraine, immune cell infiltration, neuroinflammation, neuronal apoptosis, Alzheimer's disease. There is evidence for the crucial role of AQP4 in the GS, namely AQP4 facilitates glymphatic fluid transport. AQP4 is also associated with cell movement, the size of the extracellular space and connectivity between neurons.There are many reviews on GS [104-106].

I also mention a very recent review regarding structural, molecular and functional alterations of the BBB during epileptogenesis and epilepsy [107]. It may be accessed freely following the link doi:10.3390/ijms2102051. In addition to seing beautiful figures, one may understand not only the huge progresses in the field of epileptogenesis which took place in recent years, but also the advances in the therapy, the use of modern knowledge to reach finally the stage when new anticonvulsant molecules can be introduced in therapy. Such drugs are necessary all the time, since even today the clinicians are facing the difficult situations to treat patients with drug-resistant epilepsy.

CONCLUSIONS

The hydroelectrolytical disturbances are of utmost importance in epilepsy and this was proven by lots of clinical observations, laboratory investigations, studies of animal and human tissues and cells, including the use of new molecules, out of which new anticonvulsant drugs are discovered and introduced in therapy. The water channel proteins (aquaporins and relatives) play a crucial role in all these aspects.

The discovery of WCPs (aquaporins and relatives) originated from one of the studies of hydroelectrolytical changes in RBCs of children with epilepsy. The start point was the comparative NMR measurements of the RBC water permeability of children with epilepsy and control children performed by us in collaboration with Vasile V. Morariu. In the seminal paper published in 1977 in *Nature* [38], the idea of a generalized membrane defect altering (decreasing) the water permeability of membranes in epilepsy has been clearly stated. The subsequent systematic program of research planned by Gh. Benga led to the identification of the protein responsible for the water permeability of the RBC membrane. The results of the program were reported in two reference papers published in 1986 by the by Gh. Benga's group in well known international journals in the U.S.A [53] and in Europe [54]. The first water channel protein (WCP) in the human RBC membrane was actually identified by Gh. Benga's group in 1985, when the first landmark paper by Benga and coworkers was sent to the prestigious American journal *Biochemistry*,

which accepted the publication without changes [53]. The second landmark paper was published by Benga and coworkers in 1986 in a well known European journal [54].

In 1988 the protein was rediscovered by an American group led by Peter Agre in Baltimore, who purified by chance the protein, calling it CHIP28 (CHannel forming integral membrane protein of 28kDa) having no idea of its function [55]. Only in 1992 the American group discovered the function of that protein as a water channel [58]; however, they have not cited the two papers previously published by Gh. Benga's group [53, 54].In 2003, Peter Agre was awarded the Nobel Prize in Chemistry, which he shared with Roderick MacKinnon for their "discoveries concerning structure and function of channels in cell membranes".

As a final conclusion I am citing what Wolburg and co-workers [108] wrote: "The detection of water-specific membrane channels in red blood cells belong to the fundamentals discoveries in biology of the twentieth century…In 1986 and 1988, the independent groups of Gheorghe Benga and Peter Agre, respectively, discovered the water channel proteins which later were called aquaporins".

Gh. Benga proposed in 2013 the scientific term "aquaporinology" for the new domain of natural sciences which began with the discovery of the first water channel protein, later called aquaporin1 [109, 110]. Additional information may be found in ref. [111] and on the web at gheorghebenga.ro.

DEDICATIONS

This review is dedicated to two outstanding Romanian scientists: Petre T. Frangopol and Vasile V. Morariu. I would like to add my gratefulness for the many years of scientific collaboration and friendship with Professor Petre T. Frangopol and his wife Dr. Mioara Frangopol. Their support was an important stimulus for my own research outlined above.

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