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Combined effects of aquaporin-4 and hypoxia produce age-related hydrocephalus

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ABSTRACT

Aquaporin-4, present in ependymal cells, in glia limiting and abundantly in pericapillary astrocyte foot processes, and aquaporin-1, expressed in choroid plexus epithelial cells, play an important role in cerebrospinal fluid production and may be involved in the pathophysiology of age-dependent hydrocephalus. The finding that brain aquaporins expression is regulated by low oxygen tension led us to investigate how hypoxia and elevated levels of cerebral aquaporins may result in an increase in cerebrospinal fluid production that could be associated with a hydrocephalic condition. Here we have explored, in young and aged mice exposed to hypoxia, whether aquaporin-4 and aquaporin-1 participate in the development of age-related hydrocephalus. Choroid plexus, striatum, cortex and ependymal tissue were analyzed separately both for mRNA and protein levels of aquaporins. Furthermore, parameters such as total ventricular volume, intraventricular pressure, cerebrospinal fluid outflow rate, ventricular compliance and cognitive function were studied in wild type, aquaporin-1 and aquaporin-4 knock-out animals subjected to hypoxia or normoxia. Our data demonstrate that hypoxia is involved in the development of age-related hydrocephalus by a process that depends on aquaporin-4 channels as a main route for cerebrospinal fluid movement. Significant increases in aquaporin-4 expression that occur over the course of animal aging, together with a reduced cerebrospinal fluid outflow rate and ventricular compliance, contribute to produce more severe hydrocephalus related to hypoxic events in aged mice, with a notable impairment in cognitive function. These results indicate that physiological events and/or pathological conditions presenting with cerebral hypoxia/ischemia contribute to the development of chronic adult hydrocephalus.

1. Introduction

Cerebrospinal fluid (CSF) is the main component of the extracellular fluid in the central nervous system (CNS) [1–6]. Apart from its important protective function cushioning the brain and spinal cord against mechanical injury, this fluid represents an important pathway for clearance of waste from neural tissue, and to some extent the delivery of nutrients, hormones and gases [7]. CSF directly communicates with brain interstitial fluid (IF), and in doing so, plays an important role in maintaining the homeostasis of the external fluid bathing glia and neurons. Regulation of brain fluid content (ions and all other solutes) and volume is critical for the normal functioning of the CNS, which is highly sensitive to changes in the osmolarity and hydrostatic pressures of CSF and IF. In a normal stationary state, there must be an equilibrium between the rates of CSF production and absorption in order to avoid conditions that may trigger hydrocephalus and even parenchymal edema.

It has been suggested that brain aquaporins (AQPs), particularly AQP4 and AQP1, play an important role in CSF homeostasis, and a simplistic view of their functions associates AQP1 with CSF secretion and AQP4 with its absorption [8–11]. Their distribution, with AQP1 expressed in epithelial cells of the choroid plexus [12–15] and AQP4 present in ependymal cells bordering the intraventricular compartments and glia limitans and particularly abundant in pericapillary astrocyte foot processes [8,10,16], provides arguments to propose that both proteins take part in the homeostasis of CSF.

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Nevertheless, AQP water permeability is associated with general fluid movement among the different brain compartments, namely, blood, CSF and IF, but can also be expected to participate in the pathophysiology of brain fluid disorders. Alterations in the expression of these proteins are associated with the development of brain edema and hydrocephalus. In AQP1-null mice, the lack of AQP1 was associated with a potential beneficial effect on edema produced by a cerebral lesion [14], and kaolin-induced hydrocephalus [17]. Likewise, other studies have suggested an important role for AQP4 in animal models of edema and adult hydrocephalus [8,18–23]. Specifically, for instance, AQP4 participates in the clearance of extracellular brain fluid toward the paravascular space in interstitial edema [24,25]. Further, higher AQP4 expression was observed at the blood-brain barrier (BBB) and blood-CSF interfaces in pharmacologically-induced models of adult hydrocephalus [24,26]. Moreover, AQP4$^{-/-}$ mice, developed greater ventriculomegaly and higher intracranial pressure (ICP) after kaolin injection, suggesting an adaptive role of AQP4 to resolve the hydrocephalic situation [27].

Idiopathic normal pressure hydrocephalus (iNPH), also commonly called chronic adult hydrocephalus, is a neurological disorder associated with aging characterized by ventriculomegaly and cognitive deficits [28]. Although alterations in CSF production, movement or drainage have been linked with this disorder, the pathophysiological mechanisms underlying this disease are unknown. Apart from the body of evidence that suggests an involvement of cerebral AQPs in CSF homeostasis and adult chronic hydrocephalus, hypoxia has also been associated with the pathophysiology of iNPH [29,30]. In this context, the findings that cerebral AQP expression is altered by hypoxic or ischemic conditions [31–34] and by aging [35], allow us to study how possible modifications of cerebral AQP expression produced by these conditions could alter CSF homeostasis causing or aggravating adult chronic hydrocephalus.

In the present study, we analyzed changes in the expression of brain AQPs in young and aged animals upon hypoxic treatment. We relate such changes to parameters such as total ventricular volume measured by magnetic resonance imaging (MRI), and intraventricular pressure (IVP), CSF outflow rate and ventricular compliance measured by intraventricular recordings in live animals. Here, we demonstrate that hypoxia produces a hydrocephalic condition that could even develop into a considerably more severe hydrocephalus in aged animals, in which upregulation of AQP4 expression is critically involved. An interesting translational finding of our work is that aged mice exposed to chronic hypoxia reproduce the main symptoms of iNPH [36,37], namely, larger ventricles, a slightly elevated intracranial pressure, decreased CSF outflow and ventricular compliance, as well as cognitive deficits. Therefore, chronic hypoxic aged mice appear to be an excellent animal model for studying the pathophysiology, potential diagnostic biomarkers and new treatments for chronic hydrocephalus in adults.

2. Material and methods

2.1. Animal care and hypoxic treatments

Male C57BL/6 mice (Charles River), AQP1$^{-/-}$, AQP4$^{-/-}$ (kindly provided by Dr. A. Verkman, UCSF, CA) and wildtype (wt) littermates were housed at a controlled temperature (22 ± 1 °C) in a 12 h light/dark cycle, with ad libitum access to food and water. AQP1$^{-/-}$ and AQP4$^{-/-}$ mice were outcrossed for at least eight generations to obtain a C57BL/6 genetic background. For all experiments, mice were considered young at 2–4 months old, and aged at > 14 months old. The AQP1$^{-/-}$ and AQP4$^{-/-}$ mice were genotyped as indicated previously for AQP1 [38] and for AQP4 [38]. Mice were maintained either in normal conditions (normoxia) or exposed to hypoxia (2 or 5 days at 10% O_2) using an hermetically sealed chamber (Coy Laboratory Products, Inc., Grass Lake, MI) with continuous monitoring and control of gas concentrations, temperature and humidity as previously described [39].

The mice were sacrificed under anesthesia with a combination of 100 mg/kg ketamine (Pfizer) and 10 mg/kg xylazine (Bayer). All experiments were performed in accordance with the European Directive 2010/63/EU and the Spanish RD/53/2013 on the protection of animals used for scientific purposes. The study was approved by the Animal Research Committee of Virgen del Rocío University Hospital (26/01/2017/017; University of Seville).

2.2. RNA extraction and quantitative reverse transcription PCR analysis

Cortex and striatum total RNA were isolated using TRIzol reagent (Invitrogen) following the manufacturer's protocol. For tissues with small amounts of sample as choroid plexus and ependymal with subependymal tissue bordering the lateral and third ventricle, pools of three animals were used. These tissues were micro-dissected in ice-cold PBS under a stereoscopic binocular microscope (Olympus SZX16) from fresh brain coronal sections (thickness 1 mm, using Alto adult mouse brain matrix). Total RNA was isolated using the RNeasy Micro Kit (Qiagen). RNA was only pre-amplified, with an Ambion® WT Expression Kit (Invitrogen), in experiments where too little starting tissue was available, such as those performed with total RNA from old animals, and in the young animals used as their controls. Quantity and purity of RNA were assessed with a NanoDrop ND-1000 UV–vis spectrophotometer (Thermo Fisher), and cDNA synthesis was performed using SuperScript II RNase H$^-$ reverse transcriptase kits (Invitrogen). Relative mRNA expression levels of the genes studied were quantified using quantitative real-time polymerase chain reaction (PCR) analysis with the ABI PRISM 7500 Sequence Detection System (Applied-Biosystems), SYBR Green PCR Master mix (Applied-Biosystems) and the thermocycler conditions recommended by the manufacturer. Amplification of 18S ribosomal RNA was determined and used to normalize expression levels to compensate for variation in RNA input amounts. Primers were designed using the Primer Express software v2.0 (Applied Biosystems) and its sequences are indicated in the supplementary material (Supplementary Material-1). All samples were analyzed in triplicate.

2.3. Histological analyses

Following normoxia or hypoxia treatments, animals were intracardially perfused with 50 ml of 4% paraformaldehyde dissolved in PBS (Sigma). Brains were immediately extracted and processed for cryostat cutting as previously described [39,40]. Coronal sections (30-μm thick) were cut in a cryostat (Leica). Glial fibrillary acid protein (GFAP), AQP1 or AQP4 immunofluorescence was performed as previously described [41] using respectively monoclonal anti-GFAP (1:300; Sigma), polyclonal AQP1 (1:500; Abcam) and polyclonal AQP4 (1:100; α-Diagnostic). Anti-mouse IgG conjugated with Alexa Fluor488 or anti-rabbit IgG conjugated with Alexa Fluor568 (1:400; Invitrogen) were used as secondary antibodies. Nuclei were stained with DAPI (1:1000; Sigma). Tissue sections were mounted with Dako fluorescence mounting medium (Dako). Confocal images were acquired using a Nikon A1R$^+$ confocal microscope.

Optical density (OD) analysis was performed, for a total of five sections from each animal, covering the parietal cortex. The OD was measured from digitized images using the NIH Image software (ImageJ; NIH) as previously described [42,43].

2.4. Western blot analysis

Tissues were dissected from animals, as previously described, and frozen in liquid N_2 for protein extraction. Samples were homogenized in lysis buffer containing 50 mM HEPES, pH 7.3, 250 mM NaCl, 5 mM EDTA, 0.2% (v/v) NP-40, 5 mM dithiothreitol and 1% (v/v) of a cocktail of protease inhibitors (Sigma) and were left on ice for 5 min.