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TIME-COURSE OF GLIAL CHANGES IN THE HYPERHOMOCYSTEINEMIA MODEL OF VASCULAR COGNITIVE IMPAIRMENT AND DEMENTIA (VCID)

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Abstract—Vascular cognitive impairment and dementia (VCID) is the second leading cause of dementia behind Alzheimer's disease (AD) and is a frequent co-morbidity with AD. Despite its prevalence, little is known about the molecular mechanisms underlying the cognitive dysfunction resulting from cerebrovascular disease. Astrocytic end-feet almost completely surround intraparenchymal blood vessels in the brain and express a variety of channels and markers indicative of their specialized functions in the maintenance of ionic and osmotic homeostasis and gliovascular signaling. These functions are mediated by end-foot enrichment of the aquaporin 4 water channel (AQP4), the inward rectifying potassium channel Kir4.1 and the calcium-dependent potassium channel MaxiK. Using our hyperhomocysteinemia (HHcy) model of VCID we examined the time-course of astrocytic end-foot changes along with cognitive and neuroinflammatory outcomes. We found that there were significant astrocytic end-foot disruptions in the HHcy model. AQP4 becomes dislocalized from the end-feet, there is a loss of Kir4.1 and MaxiK protein expression, as well as a loss of the Dp71 protein known to anchor the Kir4.1, MaxiK and AQP4 channels to the end-foot membrane. Neuroinflammation occurs prior to the astrocytic changes, while cognitive impairment continues to decline with the exacerbation of the astrocytic changes. We have previously reported similar astrocytic changes in models of cerebral amyloid angiopathy (CAA) and therefore, we believe astrocytic end-foot disruption could represent a common cellular mechanism of VCID and may be a target for therapeutic development. © **2016 Published by Elsevier Ltd on behalf of IBRO.**

Key words: neuroinflammation, astrocyte, dementia, cerebrovascular, microhemorrhage.

INTRODUCTION

Vascular contribution to cognitive impairment and dementia (VCID) is widely considered to be the second most common cause of dementia after Alzheimer's disease (AD), accounting for 20–30% of cases (Levine and Langa 2011). In addition, VCID occurs as a co-morbidity with other common dementias including AD, where it is estimated to occur in as many as 40% of all cases (Bowler et al. 1998; Kammoun et al. 2000; Langa et al. 2004; Snyder et al. 2015). Over twenty years ago, hyperhomocysteinemia (HHcy) was identified as an independent risk factor for stroke and vascular disease (Refsum et al. 1998). HHcy is also associated with pathologically confirmed VCID and AD (Clarke et al. 1998) and is now accepted as a risk factor for AD (Beydoun et al. 2014). We have established a hyperhomocysteinemia (HHcy) model of VCID in wildtype, C57BL6, mice. Dietary induction of HHcy is achieved by the elimination of B6, B12 and folate from the diet and enrichment with methionine (Troen et al. 2008). The mice develop cognitive impairment, cerebral microhemorrhages and neuroinflammation in response to the diet (Sudduth et al. 2013).

Astrocytes compose 50% of the cells of the brain and play several key roles in maintaining the health of the neurons. In particular, they buffer potassium to regulate the excitability of the neurons (Newman et al. 1984; Simard and Nedergaard 2004; Wallraff et al. 2006). This potassium buffering is also thought to contribute, at least in part, to the process of neurovascular coupling; i.e., the process of matching local cerebral blood flow to the local neuronal activity (Dunn and Nelson 2010; Witthoft et al. 2013), however, the potassium buffering role in neurovascular coupling remains disputed (Metea et al. 2007). To perform the critical function of potassium buffering and osmotic homeostasis, astrocytes ensheath the cerebrovasculature with specialized processes called end-feet. The astrocytic end-feet express a variety of channels and markers indicative of their specialized functions in the maintenance of ionic and osmotic homeostasis and gliovascular signaling (Simard and Nedergaard 2004). The channels enriched at the astrocytic end-feet are the aquaporin 4 water channel (AQP4) (Amiry-Moghaddam et al. 2003), the inward rectifying potassium channel Kir4.1 (Butt and Kalsi 2006) and the calcium-dependent potassium channel MaxiK (also known as the BK channel) (Price et al. 2002). AQP4 and Kir4.1 are almost exclusively expressed by astrocytic end-feet (Simard and Nedergaard 2004). The MaxiK channel is primarily in the astrocytic end-feet, with some expression by astro-

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Abbreviations: AD, Alzheimer's disease; AQP4, aquaporin 4 water channel; CAA, cerebral amyloid angiopathy; HHcy, hyperhomocysteinemia; MMP9, Matrix metalloproteinase 9; VCID, vascular cognitive impairment and dementia.

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cyte processes that are not associated with the vasculature (Farr and David 2011).

We have previously shown that cerebral amyloid angiopathy (CAA) results in AQP4 dislocalization from the astrocytic end-foot, and loss of Kir4.1 and MaxiK channels. These findings were observed in both the APPSwDI mouse model of CAA and human AD with high CAA (Wilcock et al. 2009). In the current study we examine the astrocytic end-foot markers in our HHcy mouse model of VCID along a time-course. We find that astrocytic end-feet are significantly disrupted in the HHcy model. These changes are not apparent after 6 weeks of HHcy induction, but are after 10 weeks, and are significantly worse after 14 weeks of induction. Along a similar time-course, cognitive impairment is not observed until 10 weeks after HHcy induction, and performance is worse 14 weeks after HHcy induction.

EXPERIMENTAL PROCEDURES

Animals

Seventy-two C57BL6 wildtype mice aged 3 months were placed on diet with low levels of folate, vitamins B6 and B12 and enriched with methionine (N = 12/time-point) (Harlan Teklad TD97345; Harlan Teklad, Madison, WI, USA) or a control diet that nutritionally matched the experimental diet with normal levels of methionine, folate, vitamins B6 and B12 (N = 12/time-point) (Harlan Teklad 5001C; Harlan Teklad, Madison, WI, USA). Mice received diet for 6, 10 and 14 weeks. Mice were weighed weekly to ensure no significant malnourishment was occurring due to the diet. The study was approved by the University of Kentucky Institutional Animal Care and Use Committee and conformed to the National Institutes of Health Guide for the Care and Use of Animals in Research.

Behavior testing

The two-day radial-arm water maze protocol was performed during the week prior to tissue harvest as previously published (Alamed et al. 2006). Briefly, a six-arm maze was submerged in a pool of water, and a platform was placed at the end of one arm (equipment and tracking software from Noldus Information Technology Inc., Leesburg, VA, USA). Each mouse received 15 trials per day for 2 days. The mouse began each trial in a different arm while the arm containing the platform remained the same. The numbers of errors (incorrect arm entries) were counted over a one-minute period. The errors were averaged over three trials, resulting in 10 blocks for the two-day period (blocks 1–5 are day 1 while blocks 6–10 are day 2).

Tissue processing and histology

After injection with a lethal dose of beuthanasia-D, blood was collected for plasma and the mice were perfused intracardially with 25-ml normal saline. Brains were rapidly removed and bisected in the mid-sagittal plane. The left half was immersion fixed in 4% paraformaldehyde for 24 h, while the right half was dissected into anterior cerebral cortex, posterior cerebral cortex, striatum, hippocampus, thalamus, cerebellum and rest of brain. The posterior cerebral cortex and rest of brain were combined and immediately homogenized in PBS for zymography (see detailed method below). The remaining pieces were flash frozen in liquid nitrogen and stored at −80 °C. The left hemibrain was passed through a series of 10%, 20% and 30% sucrose solutions as cryoprotection and 25 μm frozen horizontal sections were collected serially using a sliding microtome and stored floating in PBS containing sodium azide at 4 °C. Plasma samples were analyzed for Hcy levels by the clinical laboratories of the University of Kentucky.

Eight sections equally spaced 600 μm apart were selected for free floating immunohistochemistry for GFAP (Rat anti-GFAP; cat # Z0334, lot # 20023331; Dako, Carpinteria, CA, USA; 1:10,000), AQP4 (Rabbit polyclonal anti-AQP4; cat # AB2218, lot # 2697510; Millipore, Temecula, CA, USA; 1:5,000), Dp71 (Rabbit polyclonal anti-dystrophin 1, cross-reacting with Dp71; cat # Ab15277, lot # GR226781-7; Abcam, Cambridge MA, USA; 1:3,000) and collagen IV (Rabbit polyclonal anti-collagen IV; cat # Ab6586, lot # GR269410-2; Abcam, Cambridge, MA, USA; 1:500). The method for free-floating immunohistochemistry has been described previously (Wilcock et al. 2008). Sixteen sections equally spaced 300 μm apart were mounted on slides and stained for Prussian blue as described previously (Wilcock et al. 2004).

Quantitative real-time RT-PCR

RNA was extracted from the right hippocampus using the E.Z.N.A. Total RNA kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions. RNA was quantified using the Biospec nano spectrophotometer (Shimadzu, Japan). cDNA was produced using the cDNA High Capacity kit (ThermoFisher, Grand Island, NY, USA) according to the manufacturer's instructions. Real-time PCR was performed using the Fast TaqMan Gene Expression assay (ThermoFisher, Grand Island, NY, USA). In each well of a 96-well plate, 0.5 μL cDNA (100 ng, based on the RNA concentrations) was diluted with 6.5 μL RNase-free water. One microliter of the appropriate gene probe was added along with 10 μL of Fast TaqMan to each well. Target amplification was performed using the ViiA7 (Applied Biosystems, Grand Island, NY, USA). All genes were normalized to 18S rRNA and the fold change was determined using the $-\Delta\Delta$ Ct method (Livak and Schmittgen 2001).

Western blot

Approximately 60 mg of the brain powder was homogenized and protein lysates were prepared in M-per lysis buffer (Thermo Scientific, Rockford, IL, USA) containing 1% complete protease/phosphatase inhibitor (Thermo Scientific, Rockford, IL, USA). Protein concentrations were assessed using the BCA protein assay kit (Thermo Scientific, Rockford, IL, USA),