Targeting Reactive Astrocytes in Vascular Dementia: Investigation of Neuronal-Astrocyte-Vascular Interactions

Neuroscience Insights
Volume 19: 1–4

DOI: 10.1177/26331055241255332

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ABSTRACT: Historically known as neuronal support cells, astrocytes are now widely studied for their close structural and functional interactions with multiple neural cell types and cerebral vessels where they maintain an ideal environment for optimized brain function. Under pathological conditions, astrocytes become reactive and lose key protective functions. In this commentary, we discuss our recent work in The Journal of Neuroscience (Sompol et al., 2023) that showed Ca2+ dysregulation in reactive astrocytes, as well as hyperactivation of the Ca2+-dependent protein phosphatase calcineurin (CN) and the Nuclear Factor of Activated T Cells (NFATs), in a diet-induced hyperhomocystienemia (HHcy) mouse model of Vascular Contributions to Cognitive Impairment and Dementia (VCID). Intravital multiphoton imaging coupled with whisker stimulation was used to explore astrocyte Ca2+ signaling and neurovascular function under active phase, fully awake conditions. Interestingly, evoked Ca2+ transients in individual astrocytes were greater, even though intercorrelated Ca2+ signaling across networks of astrocytes was impaired in HHcy mice. Blockade of astrocytic CN/NFAT reduced signs of astrocyte reactivity, normalized cerebrovascular function, and improved hippocampal synaptic strength and hippocampal dependent cognition in HHcy mice, revealing a previously unrecognized deficit regarding neuron-astrocyte-vascular interactions. These findings strongly support the use of astrocyte targeting strategies to mitigate pathophysiological changes associated with VCID and other Alzheimer's-related dementias.

KEYWORDS: Vascular dementia, astrocyte, neurovascular function, synapse, Alzheimer's disease

RECEIVED: February 1, 2024. **ACCEPTED:** May 1, 2024.

TYPE: Commentary

FUNDING: The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: Work supported by: Grants from the National Institutes of Health; Grants AG027297 and AG078116 to Dr. Christopher Norris; AG074146, NIH-UL1TR001998, UK-NRPA, and UK-ACR to PS. The Hazel Embry Research Trust; and the Sylvia Mansbach Endowment for Alzheimer's Disease Research.

DECLARATION OF CONFLICTING INTERESTS: The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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COMMENT ON: Pradoldej Sompol, Jenna L. Gollihue, Blaine E. Weiss, Ruei-Lung Lin, Sami L. Case, Susan D. Kraner, Erica M. Weekman, John C. Gant, Colin B. Rogers, Dana M. Niedowicz, Tiffany L. Sudduth, David K. Powell, Ai-Ling Lin, Peter T. Nelson, Olivier Thibault, Donna M. Wilcock and Christopher M. Norris. Targeting Astrocyte Signaling Alleviates Cerebrovascular and Synaptic Function Deficits in a Diet-Based Mouse Model of Small Cerebral Vessel Disease. Journal of Neuroscience 8 March 2023, 43 (10) 1797-1813; DOI: https://doi.org/10.1523/JNEUROSCI.1333-22.2023

Astrocyte Reactivity in Alzheimer's Disease (AD) and Vascular Contributions to Cognitive Impairment and Dementia (VCID)

Astrocytes become reactive under pathological conditions where their molecular, cellular, metabolic, morphologic, and functional phenotypes undergo substantial remodeling.[1] These changes can disrupt interactions with other brain cells as well as cerebrovasculature. Morphologic signs of astrocyte reactivity were reported more than 100 years ago in the first documented case of AD. Since then, reactive astrocytes have been noted as a major pathologic marker in most other forms of acute and chronic neurodegeneration, as well. However, the underlying molecular mechanisms and function of reactive astrocytes remain understudied particularly in more insidious AD-related dementias, including VCID. In our recent work published in *The Journal of Neuroscience* (Sompol et al 2023), we investigated the functional impact of reactive astrocytes in mice under dietary conditions (methionine enrichment with low levels of folate, vitamin B6 and vitamin B12) to induce hyperhomocysteinemia (HHcy), a well-established risk factor for stroke and other forms of cerebrovascular pathology. Importantly, mice exposed to HHcy exhibit progressive hallmarks of VCID including small cerebrovessel pathology, vascular inflammation, cerebral hypoperfusion, and cognitive impairment. While astrocytes in HHcy diet mice don't appear overly reactive (minimal changes in GFAP expression) they do show alterations in endfeet processes that interact extensively with the cerebrovasculature.

Alterations in Astrocyte Ca^{2+} Signaling in the HHcy Diet Model

To begin our assessment of astrocyte function under VCID-related pathologic conditions, we used intravital 2 photon imaging to observe spontaneous and evoked astrocytic Ca2+ transients, as Ca2+ dysregulation is associated with astrocyte reactivity in several neurodegenerative conditions including Huntington's disease (HD),[2] amyotrophic lateral sclerosis (ALS),[3] stroke,[4] and AD.[5-7] We targeted the genetically encoded Ca2+ indicator GCaMP6 to barrel cortex astrocytes using AAV vectors equipped with a GFAP promotor. Imaging was performed on fully awake mice to avoid confounding effects of anesthesia on both cellular activity and cerebral blood flow. Customized MATLAB-based data processing and algorithms were used for region of interest (ROI) segmentation, trace extraction, activity and networking analyses. The effects of HHcy on astrocytic Ca2+ transients were complex and

depended on resting versus active state signaling as well as the level of analysis: that is, single cell versus network wide activity. Spontaneous activity was only modestly affected by diet. Transients were similar in amplitude and frequency, though individual transients occurred and dissipated a little more quickly in HHcy mice. Whisker stimulation elicited robust activity across astrocyte networks in both diet groups. However, HHcy was associated with fewer active ROIs compared to controls, as well as a loss of correlated activity among ROIs. Nonetheless, evoked Ca2+ transients in individual ROIs (ie, individual astrocytes) were larger and significantly faster (shorter rise and fall durations) in HHcy mice. We also looked at Ca2+ signaling specifically in astrocyte endfeet, which ensheathe much of the cerebrovasculature. Similar to previous reports on fully awake mice, endfeet Ca2+ transients occurred subsequent to dilatory responses in immediately adjacent cerebrovessels and the latency between vascular responses and endfeet Ca2+ peaks was reduced in the HHcy group. The results suggest that coordinated communication across astrocyte networks is impaired by HHcy, though Ca2+ signaling at the individual cell level may be augmented.

The Calcineurin (CN)/NFAT Pathway is Hyperactive in HHcy Mice and Leads to Increased GFAP Promoter Activity

After establishing alterations in astrocyte signaling in a VCID model, we next determined if astrocytes contributed to other pathophysiological features of VCID including cerebrovascular dysfunction, synapse impairments, and cognitive loss. Astrocyte signaling was modulated by targeting the calcineurin (CN)/NFAT transcriptional pathway. CN is a Ca2+ calmodulin dependent protein phosphatase that is highly sensitive to Ca2+ dysregulation arising in multiple cell types with injury and disease. Increased expression levels of CN are commonly observed in reactive astrocytes surrounding brain lesions such as amyloid deposits, microinfarcts, or sclerotic cerebrovessels. NFATs are CN-dependent transcription factors that regulate the expression of numerous cytokines, chemokines, as well as multiple Ca2+ signaling mediators. The NFAT4 isoform shows a particularly strong bias toward astrocytic expression, though it is also found in other non neuronal cells, such as pericytes. Moreover, NFAT4 levels are usually induced to a greater extent in reactive astrocytes associated with injury and disease. Consistent with these observations, NFAT4 was strongly associated with astrocytes in HHcy mice and exhibited elevated activity (ie, increased NFAT4-DNA binding). In previous work on rodent models of AD[8,9] we targeted CN/NFAT selectively in astrocytes using adeno-associated viral (AAV) vectors equipped with a GFAP promotor (Gfa2) and a transgene encoding the VIVIT peptide. VIVIT is essentially an NFAT inhibitor that mimics a CN docking site found in NFATs 1 to 4 and prevents CN-NFAT interactions in vivo, without altering CN activity per se. When administered to mice with parenchymal amyloid pathology or rats with traumatic brain injury AAV-Gfa2-VIVIT protects synaptic function, mitigates hyperexcitability, and/or stabilizes cognition. Interestingly, VIVIT may or may not affect GFAP expression itself, depending on the pathological context. For instance, in mid-age amyloid mice that show robust astrocyte reactivity, VIVIT had only modest effects on GFAP expression, but it did shift the size distribution of astrocytes in favor of smaller, less ramified cells.[9] The HHcy diet also has little effect on total GFAP levels, even though other microstructural or functional changes are apparent. As a more sensitive approach to GFAP modulation in the HHcy mouse, we instead assessed GFAP promotor activity (ie, Gfa2-dependent EGFP expression). Across a 3 month period, Gfa2 promotor activity was strongly and consistently induced in mice fed with HHcy diet as opposed to control diet. Moreover, induction of the GFAP promoter with HHcy was inhibited by VIVIT expression in astrocytes.

CN/NFAT Signaling in Reactive Astrocytes Impairs Neurovascular Coupling and Synaptic Function in HHcy Mice

Given the close association between astrocytes and the cerebrovasculature, it was especially important to determine if inhibition of reactive astrocyte signaling (with AAV-Gfa2-VIVIT) could protect cerebral vascular function in the context of progressive HHcy-dependent small vessel pathology. We first used 2 photon microscopy to assess the "leakiness" of cerebrovessels in HHcy diet mice. This was accomplished by measuring the escape of rhodamine-coupled dextran particles (40 kDa) from barrel cortex vessels across a 1 hour period. While HHcy clearly hastened the loss of interluminal rhodamine fluorescence intensity, VIVIT did not markedly affect dye leakage in the HHcy group. Unexpectedly, dye leakage in the control diet group was actually increased by VIVIT treatment suggesting a protective effect of astrocytes on blood brain barrier integrity under normal (healthy) conditions. In addition to blood brain barrier integrity, we also examined neurovascular coupling, a mechanism that the brain uses to compensate active regions with greater blood flow. Several major components of neurovascular coupling (measured in barrel cortex) were impaired in fully awake mice treated with HHcy diet. Specifically, HHcy inhibited the dilation of penetrating arterioles and slowed the velocity of red blood cell movement through downstream capillaries during air puff whisker stimulation. Each of these deficits were ameliorated by AAV-Gfa2-VIVIT. Moreover, cerebral perfusion deficits in HHcy diet mice (measured in anesthetized mice using pCASL MRI) was also prevented by VIVIT treatment.

Finally, we determined if reactive astrocyte signaling contributed to synaptic dysfunction and cognitive loss in the context of HHcy. In situ brain slices were prepared from mice treated with control or HHcy diet and electrophysiologic approaches were used to assess the basal strength and

excitability of CA3-CA1 synaptic contacts, as well as long-term potentiation (LTP). In a separate cohort of mice, behavior was assessed in an open field arena and in a Y maze task. Not surprisingly, HHcy disrupted both synaptic physiology (reduced synaptic strength, generated hyperexcitability, and impaired LTP) and behavioral performance (reduced the number of spontaneous alternations in the Y maze). Similar to our previous work in AD mouse models, treatment of HHcy mice with AAV-Gfa2-VIVIT ameliorated all of these deficits.

Future Research on Reactive Astrocytes in Mouse Model of VCID

The underlying mechanisms of reactive astrocytes under systemic HHcy conditions are not completely known. Homocysteine causes dysregulation of astrocytic Ca2+ signaling and Ca2+ sensitive CN/NFAT signaling pathway. How homocysteine regulates astrocytic Ca2+ signaling and physiology remains unclear. Several ion regulatory systems are altered by homocysteine including Na+, and K+ ATPase.[10] Imbalances of sodium and potassium influence Ca2+ homeostasis and astrocyte physiology.[11,12] On the other hand, Na+ and K+ ATPase could be activated by astrocytic Ca2+ wave.[13] Therefore, these ions are highly associated, and their equilibrium is tightly regulated. Homocysteine may also affect Ca2+ dysregulation through it's agonist actions at the NMDA receptor (NMDAR).[14] This finding implies that homocysteine is an alternative excitotoxin with concentration range at 10 to 100 uM where neuronal injury, Ca2+ influx and reactive oxygen species were found.[14,15] Alternatively, it had been reported that homocysteine stimulates neuronal NMDARs causing Ca2+ influx into the neuron and activation of ERK MAP kinase pathway linked to cell death cascades.[16] Although the effect of homocysteine on astrocyte NMDARs has yet to be elucidated, it has been reported that the GluN1 subunit of astrocyte-expressed NMDAR plays important role on receptor activation and downstream intracellular Ca2+ accumulation.[17] Astrocyte Ca2+ influx is sensitive to glutamate, NMDA and AMPA where NMDAR and AMPAR antagonists (AP5 and CNQX) can block cellular Ca2+ entry.[18,19] Elevation of intra-astrocytic Ca2+ levels triggers the release of glutamate gliotransmitter, which may, in turn, regulate neuronal NMDAR-dependent synaptic transmission. This astrocyte-neuronal interaction is consistent 1 with our present study where astrocytic Ca2+ dysregulation (observed under intravital imaging) is highly associated with neuronal hyperexcitability as indicated by a reduction of population spike threshold in the HHcy group. Though not specifically investigated in HHcy mice, glutamate dysregulation and excitotoxicity are observed in other neurodegenerative diseases and could contribute to pathophysiological conditions associated with HHcy and VCID. We previously reported profound reactive astrocyte, glutamate dysregulation and neuronal hyperexcitability in 5xFAD mice.[8] Reducing astrocyte reactivity by targeting CN/NFAT, neutralized glutamate dysregulation and improved neuronal outcomes in AD mouse models.[8] Further studies on glutamate dysregulation in hyperhomocysteinemia condition and VCID will be important follow-ups to the Sompol et al 2023 study.

Results from our group and others suggest that CN/NFAT signaling is essential for glutamate transporter (GLT-1) gene expression. Increasing astrocytic glutamate transporter is protective against glutamate spillover and neuronal excitotoxicity. Several co-signal transductions including NF-kB,[20] cAMP/PKA/CREB,[21] PI3K,[22] Yin Yang 1, and MEK/ERK[23,24] also participate in transcription processes of GLT-1. Moreover, these signaling pathways involve neuroinflammation processes that directly activate astrocytes in neurodegenerative diseases. The interaction of these signaling pathways is complex and further investigation is needed. Identifying an alternative approach or other signal transduction pathways that reduce astrocyte reactivity could potentially provide future strategies for treating AD and ADRDs.

Reactive astrocytes appear to maintain an inhospitable brain milieu in chronic AD/ADRDs and other neurodegenerative diseases. Our present study provides a possible way to neutralize dysregulation of astrocyte-neuron-vasculature interaction through modulation of the CN/NFAT4 pathway (Figure 1). However, multidirectional association of astrocytes and other brain cells such as pericytes, microglia, oligodendrocytes and other neuronal subtypes are not well classified, especially in neurodegenerative stages. These integrative cellular systems are known to involve neuroinflammation, metabolic dysfunction, dysregulation of cerebral blood perfusion and impaired neuronal viability and plasticity. The underlying mechanisms of these processes are complicated and further investigation is essential.

It is worth noting that vitamin B deficiency induced-systemic hyperhomocysteinemia has a big impact on brain physiology and cerebrovascular dysfunction. Our current findings emphasize that lacking of vitamin B mediates pathophysiological condition of the brain and parenchymal vessels by increasing reactive astrocytes and astrocyte calcium signaling which commonly found in AD/ADRDs. Homocystein metabolism is reduced with age and that could increase risk of common aging diseases such as cardiovascular disease, metabolic related disorders and AD. Vitamin B supplement benefits cardiovascular system, and increase cognitive function in animal and human cohorts.[25] However, it is unclear whether vitamin B supplement has beneficial effect in VCID and related neurovascular diseases. Moreover, the effect of vitamin B on astrocyte reactivity and astrocyte-neuron-vascular interaction has yet to be elucidated and further characterization is needed.

In summary, we provided evidence across multiple neuronal and cerebrovascular outcomes that inhibition of CN/NFAT activity may be a promising strategy for neutralizing harmful

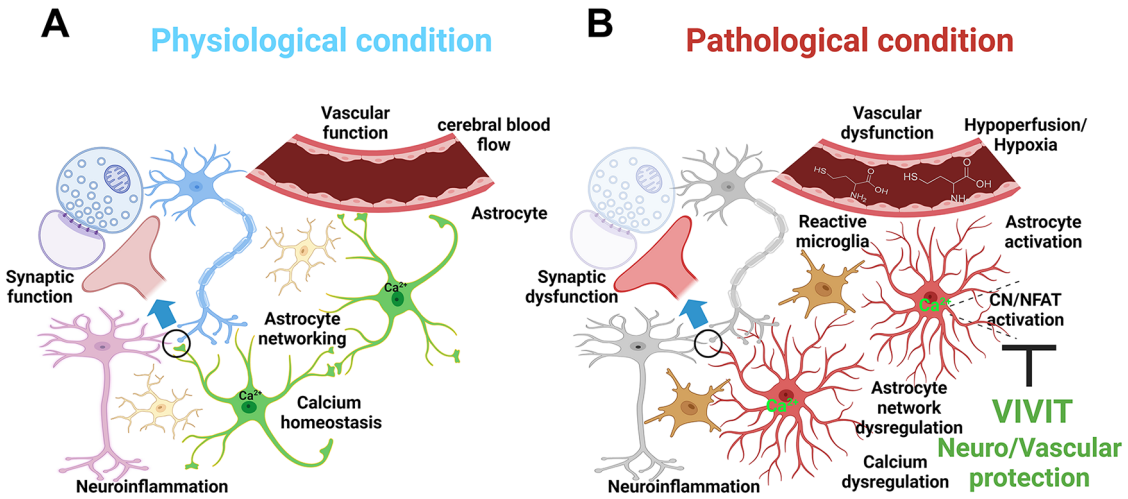

Figure 1. Schematic illustrates complexity of astrocyte interaction with brain cells and the cerebral vasculature under physiological and systemic HHcy conditions. (A) Astrocytes interact with adjacent brain cells and maintain physiological environment for optimized synaptic transmission, cerebral blood flow and brain function. These processes could be regulated, in part, by Ca2+ signaling across the astrocyte syncytium. (B) Astrocyte reactivity is evidenced by dysregulation of Ca2+ signaling and hyperactivation of the CN/NFAT pathway. Abnormal astrocyte networking and astrocyte related impairments in cerebrovascular hyperemic responses are observed. These findings are correlated with neuroinflammation, deficits of synaptic transmission and brain function. Targeting reactive astrocytes by expressing the VIVIT peptide, a blocker of CN/NFAT signaling, provides a promising neuro- and cerebrovascular protection strategy for VCID, AD, and other ADRDs. (Figure was generated and modified by Biorender.com)

reactive astrocyte and protecting against neurodegenerative processes in AD, VCID and brain injury.

Author Contribution

P.S. wrote the manuscript and prepared the figure.

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