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Exploring Mechanisms of Persistent Cognitive Deficits in Mice with Transient Hyperhomocysteinemia

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THESIS

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By

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Abstract

Hyperhomocysteinemia (Hhcy) has been found in the elderly and is considered a risk factor for several neurological diseases, especially Alzheimer's and Vascular Dementia. Previous studies have found an increase in Blood-Brain Barrier (BBB) leakage, glial activation, neuronal damage, and cognitive deficit with elevated levels of plasma homocysteine. Lowering homocysteine could be an easy and inexpensive way to reduce the risk of dementia. But it is still not known for sure if reducing plasma homocysteine levels can recover cognitive function. In our transient Hhcy model, 28 mice were given Hhcy inducing or nutritionally matched control diet for 8 weeks and then returned to normal chow for 4 weeks. The Hhcy mice had a normal level of homocysteine at the end of the 4 weeks but showed persistent deficits in spatial learning and memory. We hypothesized that the transient Hhcy mice had BBB leakage, glial activation, and loss of synapses, which were responsible for observed cognitive deficits. We found no significant difference in albumin leakage in the hippocampus between the control group and Hhcy group. The hippocampal levels of the microglial marker P2Y12 and the synaptic protein PSD95 were also not significantly different from the control group. The only statistically significant effect observed was an increase in hippocampal marker of astrocytes, alongside increases in some parameters of vascular staining (branch length and branch points). The increased branching profile of the cerebral blood vessels could be a compensatory mechanism to offset the injury in Hhcy or a consequence of the BBB repair activity.

Acknowledgements

This work would not have been possible without the help and support of my lab members. Thank you, Dr. Van Eldik, for allowing me to be a part of this amazing lab family. I cannot express how much I appreciate you for giving me an opportunity to learn from, and work with such dedicated people. David, thank you for your valuable and consistent support and guidance. Your work ethic and commitment has inspired me. You have been an amazing teacher, and I truly appreciate that you encouraged me to ask and answer my own questions from the very beginning. Danielle, thank you for training a rookie student into a not-so-clueless member of the lab. I am always amazed by your sense of ownership, professionalism, and attention to details. I have always tried to work as neatly and efficiently as you, and regard you as my research godmother. Ed, thank you for reminding about all the important things beyond the lab and school. Ms. Amy, Ms. Verda and Dr. Josh, thank you for attending to and answering my questions, worries and mishaps in the lab no matter how busy you were. Your conversations, chuckles, your enthusiasm for science, and the food you brought, made my long days in the lab much more enjoyable.

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1. INTRODUCTION

1.1 Hyperhomocysteinemia (Hhcy)

Alzheimer's disease (AD) and Vascular Dementia (VD) make up 80% of the dementia cases in western countries (Rizzi et al., 2014). One of the risk factors for both AD and VD is high levels of an amino acid called homocysteine (Zhuo et al., 2011; Nilsson et al., 2010). When levels of homocysteine rise above 15 μM, the condition is known as Hyperhomocysteinemia (Hhcy) (Zhang et al., 2016). Homocysteine (Hh) is an amino acid found naturally in the body. Hh level can rise due to mutations, diet-related factors or renal failure. Mutations that cause loss of enzymes such as Cystathionine Beta Synthase (CBS) or Methylenetetrahydrofolate reductase (MTHFR) result in severe accumulation of Hh in people with these mutations, Hh level rises above 100 μM, and the condition is known as severe Hhcy. Hh level can also rise due to a lack of B vitamins. Lack of B vitamins leads to an increase in the level of Hh to 10-100μM which is classified as mild-moderate Hhcy (Hainsworth et al., 2016).

Mild-moderate Hhcy is the more common form of Hhcy found in elderly individuals (Hainsworth et al., 2016). Hh levels rise with age (Shelhub et al., 1993) and have been reported to cause a cognitive deficit in the elderly (Ravaglia et al., 2003). Hhcy accelerates the worsening of cognitive function in people with dementia (Oulhaj et al., 2010). Hhcy has also been associated with an increased rate of hippocampal atrophy (Clarke et al., 1998) and stroke (JAMA 2002; Khan et al., 2008). Most of the actions of moderate Hhcy are vascular (Hainsworth et al., 2016). Elevated levels of Hh cause BBB leakage (Tyagi et al., 2005; Kamat et al., 2016), microglial and astrocyte activation, increased inflammation (Zou et al., 2010; Kamat et al., 2013) and neuronal degeneration (Kalani et al., 2014; Kuszczyk et al., 2009; Park et al., 2010).

While increased levels of Hh can negatively impact several components of the CNS, the levels can be brought down easily with vitamin B supplements. Vitamin B supplements and normal Hh level restoration has been shown to slow down hippocampal atrophy and, in some cases, restore cognitive deficits (Smith et al., 2018). Hence, Hhcy is considered a modifiable risk factor for AD and VD.

1.2 Vascular changes in Hhcy

The blood-brain barrier (BBB) is a key feature of the cerebral vasculature. BBB tightly regulates the CNS homeostasis and protects the brain from systemic inflammation, toxins, and disease. BBB is composed mostly of endothelial cells (EC) and is sheathed by pericytes and astroglial endfeet. Tight junctions in between the endothelial cells and the basement membrane help further regulate the influx of molecules into the brain (Abbott et al., 2006; Hawkins and David 2005) Loss of BBB has been reported in neurodegenerative diseases and is associated with loss of cognitive function (Zlokovic, 2008; Yamazaki and Kanekiyo, 2017). Leaky BBB has been found in animal models of Hhcy (Rhodehouse et al., 2013). Hhcy can cause degeneration of pericytes, and degeneration of mitochondria in endothelial cells (Kim et al., 2002). Matrix metalloproteinases (MMPs) and astrocytes are activated in Hhcy (Lominadze et al., 2012). MMPs dissolve the proteins and tight junction (TJ) proteins such as occludin and claudin 5 in the basement membrane (Agrawal et al., 2006; Rosenberg and Yang, 2007;

Yang et al., 2007), while the activated astrocytes can affect and reduce the levels of TJ proteins such as occludin (Wosik et al., 2007).

Another important feature of cerebral vasculature is the cerebral blood flow (CBF). CBF and perfusion are maintained by molecules secreted by endothelial cells and astrocytes that affect the tone of the vessels. The endothelial cells help dilate the arteries by the action of endothelial nitric-oxide synthase (eNOS) (Katusic and Austin, 2014). Astrocytes also regulate activity-dependent blood flow to the neurons by acting on smooth muscle cells of the vessels and pericytes (Attwell et al., 2010; Gordon et al., 2011). Hhcy results in damaged endothelial cells and pericytes, vessel hypertrophy, and loss in vessel distensibility (Kim et al., 2002; Baumbach et al., 2002), and all of these conditions can lead to decrease in CBF. Impaired vasodilation in the cerebral vasculature has been reported in Hhcy mice (Dayal et al., 2004) caused by decreased levels of eNOS (Faraci et al., 2003, Zhang et al., 1998). BBB leakage in Hhcy can also affect the CBF. A decrease in CBF can lead to cognitive impairment and brain volume atrophy (Everson-Rose and Ryan, 2015). Lowered CBF is also an early marker of Alzheimer's disease (Toree, 2002).

1.3 Glial activation and synaptic loss

Microglia are scavengers in the CNS that remove dead and dying cells. They are also the first line of defense in the CNS and play an important role in synaptic plasticity (Napoli and Neumann, 2009; Wu et al., 2015). Under normal conditions, microglia are in a resting state with their processes spread out constantly sampling their environment (Davalos et al., 2005; Nimmerjahn et al., 2005). Microglia are activated by injury, dead and dying cells or cytokines (Napoli and Neumann, 2009; Nimmerjahn et al., 2005; Griffin et al., 1998; Giulian et al., 1998). Microglia are activated by anything foreign to the CNS environment, for example blood proteins like fibrinogen (Adams et al., 2007). When activated, microglia retract their processes and assume an amoeboid shape. Activated microglia release nitric oxide, arachidonic acid, and cytokines into their surrounding environment (Nishioku et al., 2010; Yang et al., 2015; Vegeto et al., 2001; Liu and Hong, 2003). In the presence of BBB disruption, activated microglia can downregulate the TJ proteins and exacerbate BBB leakage (Almutairi et al., 2016). Microglia activated by blood protein fibrinogen has also been also found to phagocytose synapses (Merlini et al., 2019) Activated microglia have been found in Hhcy model animals (Zou et al., 2010) as well as in-vitro. In Hhcy animals, the disrupted BBB, as well as reduced NO, could activate microglia and induce proinflammatory behavior (Katusic and Austin, 2014; Faraci et al., 2003).

Astrocytes make up ~50% of the cells in the CNS. They play an important role in maintaining the ionic homeostasis, directing blood flow to the active neurons, maintaining the BBB, and are required for the formation and maintenance of the synapses (Simard and Nedergaard, 2004; Alvarez et al., 2013; Zonta et al., 2003; Christopherson et al., 2005; Ullian et al., 2001; Ullian et al., 2004). Reactive astrocytes can help restore synapses (Emirandetti et al., 2006; Tyzack et al., 2014) or target synapses for elimination (Stevens et al., 2007). Astrocytic end-feet wrap the blood vessels and contain channels such as aquaporin4 (AQP4) and the inward rectifying potassium channel (Kir4.1) that are responsible for potassium and osmotic homeostasis (Butt and Kalasi, 2006; Amiry-Moghaddam et al., 2003). Increased GFAP levels due to Hhcy have been found in vitro (Longoni et al., 2018). Hhcy animals also have reduced levels of AQP4, Kir4.1 and

dystrophin-1 (Dp71) that anchor the Kir4.1and AQP4 channels to the end-foot membrane of the astrocytes (Sudduth et al., 2017). Hence, activated microglia and astrocytes could be in part responsible for a loss of synapses and cognitive deficit in Hhcy animals.

1.4 Rationale for current study

The effect of Hh on vascular integrity, brain volume and cognition has been known for some time now. Hhcy can also be easily reversed by supplementation with B vitamins. And yet there is no consensus whether normalizing the Hh levels will lead to the recovery of cognitive deficit. In our transient model of Hhcy, the mice were placed on Hhcy diet for 8 weeks and then a normal diet for 4 weeks. In the end, despite having normal levels of plasma Hh (<5 μmol/L), the mice still had deficits in spatial learning and memory as assessed in the radial arm water maze and novel place recognition tasks (**Figure 1**). Based on the study of the current literature, we hypothesized that BBB leakage due to transient Hhcy is associated with persistent glial inflammatory changes and synaptic loss. To test our hypothesis, we performed IHC to investigate changes in GFAP and P2Y12 expression in the hippocampus (as markers of activated astrocytes and microglia), as well as the level of albumin in the hippocampal tissue (as a marker of vascular dysfunction). We also stained for Dp71 (dystrophin) to examine other vascular changes in Hhcy brain. Finally, we performed Western bot with the hippocampal tissue homogenate to check the level of synaptic protein PSD95.

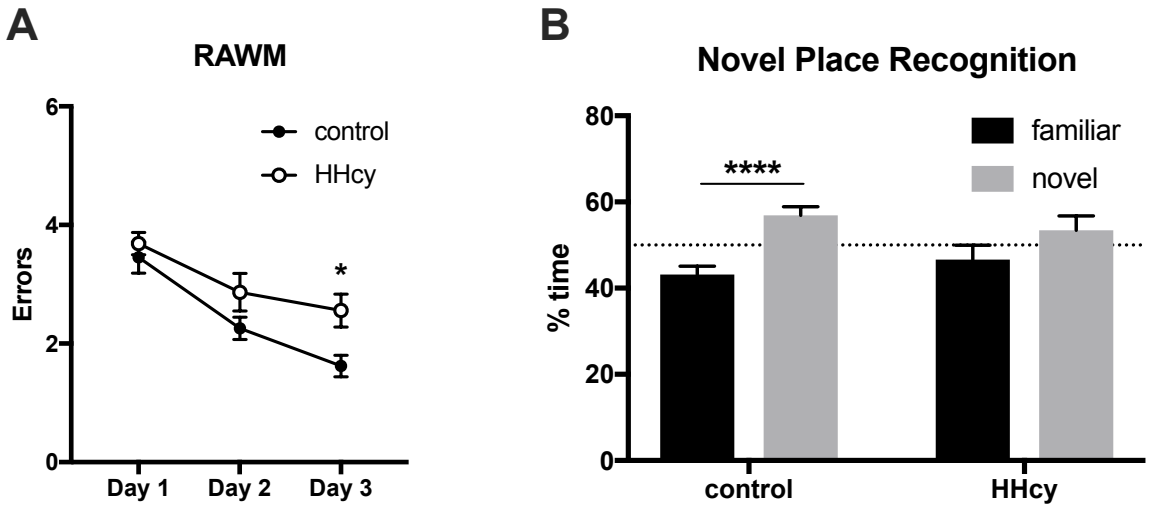

Figure 1. Persistent hippocampal-associated memory deficits after transient hyperhomocysteinemia (Hhcy). (A) Mice were assessed for spatial learning deficits in the radial arm water maze. Although both control and Hhcy groups showed improvement in the task, the Hhcy group committed significantly more errors by day 3. *$p < .05$ versus WT control, two-way repeated measures ANOVA with Sidak's multiple comparison tests, n = 14 per group. (B) To assess short-term memory, mice also were tested in the novel place recognition task with a 5-minute inter-trial interval. As expected, the WT control mice spent significantly more time with the object in the novel location. The Hhcy group showed no preference. ****$p < .001$ versus familiar, multiple t-tests with Bonferroni-Sidak correction, n = 14 per group.

2. METHODS

2.1 Animals

Tissues from 28 male and female C57BL/6J mice were used for the analyses. For induction of Hhcy, mice received a specially formulated diet that lacks vitamins B6, B12, and folate, and is supplemented with excess methionine (Harlan Teklad TD97345). The mice were put on this diet for 8 weeks and then returned to standard rodent diet for 4 weeks and sacrificed (Hhcy). Half of the mice received 8 weeks of a nutritionally-matched diet with normal levels of B vitamins and methionine (Harlan Teklad TD01636) before returning to standard rodent chow (control).

2.2 Fluorescence Immunohistochemistry (IHC)

Free-floating IHC was performed and tissues were stained for microglial activation (P2y12: Rabbit anti-P2Y12; AnaSpec #AS-55043A, 1:3000, Secondary antibody: Alexa 633 Goat antirabbit Invitrogen #A21070, 1:1000), astrocytes (GFAP; Rat anti-GFAP; Dako #Z0334, lot #00059585, 1:3000, Secondary antibody: Goat anti-Rat 633; Invitrogen #A11036; lot 997761, 1:1000), blood vessels (Dystrophin Abcam #15277, 1:1000; Secondary: Alexa 568 1:500).

Free floating IHC general procedure

The tissues went through three 10-minute washes in 1x PBS. They were then blocked for 60 minutes at room temperature in the block buffer (10% Goat serum, 0.2% TX-100 in 1xPBS (PBST)), and then incubated with the primary antibody overnight at 4°C. The next day, the tissues went through three 10-minutes washes in 1x PBST on shaker. They were then incubated with secondary antibodies at room temperature for 1-2 hours. Next, they went through three 10 minutes washes and two 5 minutes washes in 1x PBST. The tissues were then mounted on slides and either further stained or cover-slipped. The slides were stored at -20°C.

Sudan Black staining

The mounted tissues were stained with 0.01% Sudan black solution in 70% ethanol. First, the tissues were incubated with 70% ethanol for 5 minutes and then incubated in the Sudan black solution for 5 minutes. Next, the slides were quickly rinsed twice with 70% ethanol solution and washed for 5 minutes in PBS. These tissues were allowed to dry for at least an hour before they were cover-slipped.

3,3'-Diaminobenzidine (DAB) Immunohistochemistry

The tissues went through three 10-minute washes in 1x PBS at room temperature (RT). To remove the endogenous peroxidase activity, the tissues were then incubated in 0.3% peroxide made in methanol for 30 minutes. Next, they went through three 10-minute washes in PBST and then were incubated in block solution (10% donkey serum, 0.2% TX-100 in PBS) for an hour at RT. They subsequently underwent overnight incubation in primary antibody (Goat anti-albumin, Bethyl Labs #A90-234A, 1:10,000) at 4°C. The next day the tissue was washed 5x in PBS for 5 minutes each on a shaker and then incubated in secondary antibody (Biotin Donkey anti-goat, JAX #705-035-147, 1:500) for 1 hour at RT. After another 5x 5-minute washes in PBS, the samples were incubated for 1 hour at RT in ABC solution (Vector #PK-6100) and subsequently washed 3x for 10 minutes each in PBS. During the last wash, DAB solution was freshly made: 0.05% DAB

in 1X PBS with 0.03% hydrogen peroxide. This solution was added to the tissue at RT and the development was watched closely. The tissue was incubated in DAB for 81 seconds and then quickly dipped in double distilled (dd) water to quench the reaction. The tissue was then washed again with 1x PBS for three times, each wash 10 minutes long. The tissue was then mounted, allowed to dry overnight, counterstained with methyl green, and then cover-slipped.

Methyl Green Counterstaining
The mounted slides were washed in dd water for two minutes and then placed in 1x methyl green solution (0.125g zinc chloride salt in 100 ml of 0.1M sodium acetate buffer) for 1 minute at RT. The slides were then dipped in dd water and then dipped in 0.5% acetic acid in acetone 15 times. The slides were then dehydrated, and refractive index matched by dipping them consecutively in 70% ethanol for 3 minutes, then 95% ethanol for 3 minutes, 100% ethanol for 5 minutes and then finally in citrus clearing solvent (citrisolv) (Richard Allan scientific, REF: 8301) for 5 minutes. The slides were held in citrisolv until they were ready to mount. They were then cover-slipped with hardset DPX mounting medium and allowed to dry for 48 hours before cleaning and scanning.

Table 1. List of the primary and secondary antibodies used for imaging and analysis.

Target	**Primary**	**Secondary**	**Application**
Albumin	Goat anti-albumin, Bethyl Labs #A90-234A, 1:10,000	N/A	3,3′-Diaminobenzidine (DAB) Immunohistochemistry
Dystrophin (Dp71)	Dystrophin Abcam #15277, 1:1000	Alexa 568 1:500	Fluorescence Immunohistochemistry
Glial Fibrillary Acidic Protein (GFAP)	Rat anti-GFAP; Dako #Z0334, lot #00059585, 1:3000	Goat anti-Rat 633; Invitrogen #A11036; lot 997761, 1:1000	Fluorescence Immunohistochemistry
P2Y12	Rabbit anti-P2y12; AnaSpec #AS-55043A, 1:3000	Alexa 633 Goat antirabbit Invitrogen #A21070, 1:1000)	Fluorescence Immunohistochemistry
Post Synaptic Density 95 (PSD95)	Rabbit, Cell signaling #3450S, 1:10,000	Mouse, Cell signaling #3700S, 1:1000	Western Blot imaging

2.3 IHC Imaging and Analysis

The immunofluorescence slides were scanned at 20x with a Zeiss Axio Scope.A1 and the DAB slides at 20x with an Aperio ScanScope. All image files were analyzed with the HALO Image Analysis Platform (Indica Labs Ver. 2.2). Hippocampus was outlined and threshold pixel intensities were separately set for the software to quantify each stain of interest (see **Figure 2**) for an example). The total number of pixels with an intensity above the threshold was recognized by the software as positively stained. After analyzing the tissues, the area quantification algorithm of the software presented the positive staining in the form of % area of the region of interest (hippocampus). This algorithm was used for P2Y12, GFAP and albumin analysis. The branch module algorithm was used to assess vascular-related parameters for the dystrophin stain, including the total area of the branches, number of branch points and end points, total length as well as the average thickness of the branch. The person doing the analyses was blinded to the groups while outlining and analyzing the tissue. The data was then exported to MS Excel and analyzed in GraphPad Prism.

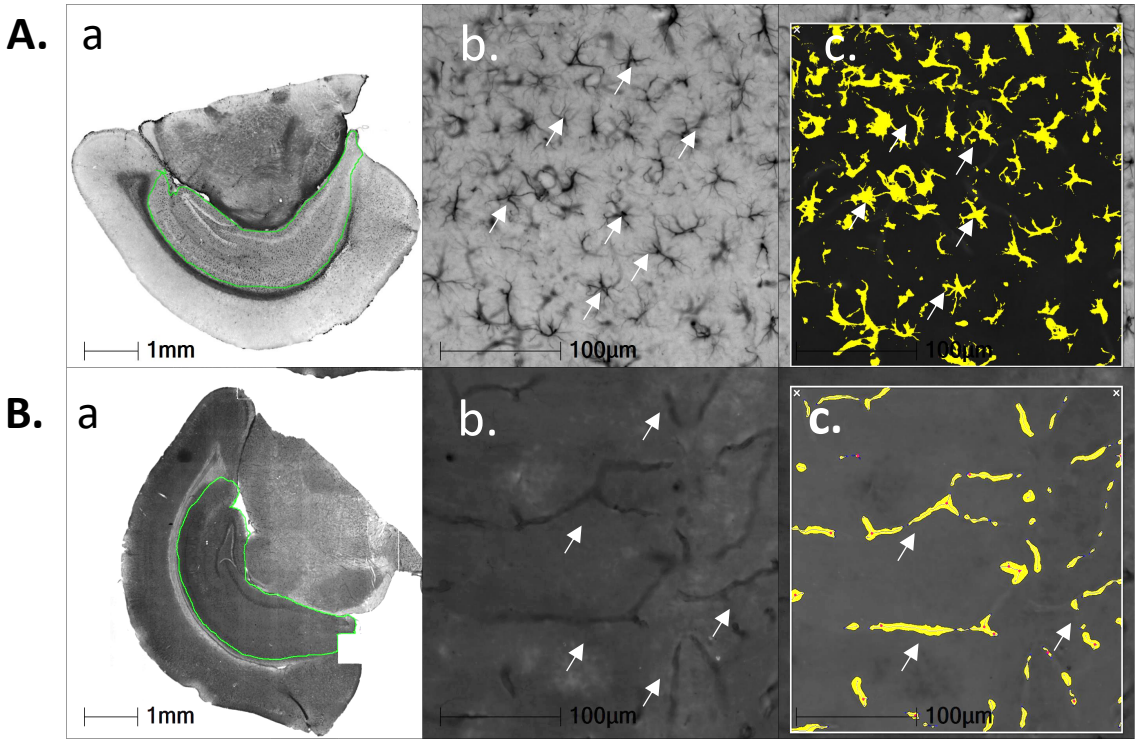

Figure 2: Analysis of the hippocampal tissue in Halo 2.2 software at 20x
A. Analysis of Glial Acidic Fibrillary Protein (GFAP) with area quantification algorithm a. Outlining the hippocampus, the green line represents the outline. b. Hippocampus at 20x with GFAP staining (arrows). c. Tuning the area quantification module to identify and quantify the area covered by the GFAP or astrocytes (yellow). **B.** Analysis of the dystrophin in the hippocampal tissue a. Outlining the hippocampus (green) b. Hippocampal tissue at 20x with the dystrophin (arrows) c. Tuning the branch analysis module to identify and quantify the dystrophin/blood vessels (yellow).

2.4. Western Blotting

Hippocampal samples were homogenized in 100 µL Pierce™ RIPA lysis buffer (Thermo Scientific, Ref: 89900) with an Omni bead ruptor (5m/s for 45 seconds), then centrifuged at 12,000xg for 20 minutes in Beckman Microfuge 18 at 4°C. Then, the supernatant was taken and aliquoted.

The protein concentration was first measured by Pierce bicinchoninic acid assay (BCA) according to manufacturer's instructions. Briefly, samples were diluted 1:6 with RIPA buffer and 20 µL of each were incubated with 200 µL of BCA reagent at 37° C for 30 minutes and compared with a bovine serum albumin (BSA) standard curve. Absorbance at 562 nm was measured with a Tecan Genios spectrophotometer and sample protein levels extrapolated from the BSA standard curve with Gen5 software. Subsequently, protein sample concentrations were normalized with the same lysis buffer and heated for 10 minutes in a water bath at 70-80°C with 1x LiCor sample buffer containing 10% beta-mercaptoethanol (BME). Fifteen µg of protein was loaded per lane in a NuPAGE Novex Bis-Tris Midi Gel, Invitrogen: 4-12% (#WG1403BX10), and run for 40 mins at 200V in MES SDS running buffer (ThermoFisher NP0002). After running, the proteins were transferred on a membrane with iBlot Transfer Device using the nitrocellulose iBLot Transfer Stack (ThermoFisher IB301001). Stacking lanes and excess gel were cut and removed before the transfer. The filter paper was pre-wet with dd water and the assembly was rolled out carefully to remove any bubbles. The transfer was performed at 20 volts for 7 minutes. After the transfer, the membrane was blocked with Odyssey blocking buffer diluted 1:2 in PBS (LiCor #927-40000) for an hour.

Probing and imaging: The membrane was probed with anti-PSD95 (Rabbit, Cell Signaling #3450S, 1:10,000) and anti-actin antibodies (Mouse, Cell Signaling #3700S, 1:1000). The membranes were incubated with primary antibodies in blocking buffer with 0.1% Tween-20 at 4°C on rocker overnight. They were subsequently washed with 1xPBST before being incubated in secondary antibodies (LiCor: IRDye 680LT Goat anti-Rabbit IgG, IRDye 800CW Goat anti-mouse IgG) at 1:10,000 for one hour at room temperature, on a shaker. Thereafter the membrane was protected from light as much as possible. It was washed 4x for 5 minutes with 1xPBST then 1xPBS and imaged using a LiCor Odyssey Infrared Imager. The PSD95 was normalized to actin for quantification with LiCor Image Studio Software.

2.5 Statistical Analysis

The data was analyzed in GraphPad Prism Version 7.0e (GraphPad Software, Inc.). Behavioral data was compared using repeated measures ANOVA with Sidak's multiple comparison tests, and multiple t-tests with Bonferroni-Sidak correction. For rest of the data, comparison between the groups were made by two-tailed student's t-tests. Data are represented in figures as Mean ± standard error of the mean (SEM). Where reported in the text, the mean is given ± the standard deviation (SD). The alpha value for significance was set to 0.05.

3. RESULTS

3.1 Hippocampal blood brain barrier leakage recovers after transient Hhcy

BBB integrity after recovery from Hhcy was examined by staining for albumin, which is excluded from the parenchyma under normal conditions. Background threshold for diffuse staining was set to the level of the no primary antibody control. As shown in **Figure 3**, there was no statistically significant difference in the percent area of the hippocampus stained by anti-albumin antibodies between the groups (Control =21.78 ± 13.623, Hhcy = 29.38±14.16, p = 0.20).

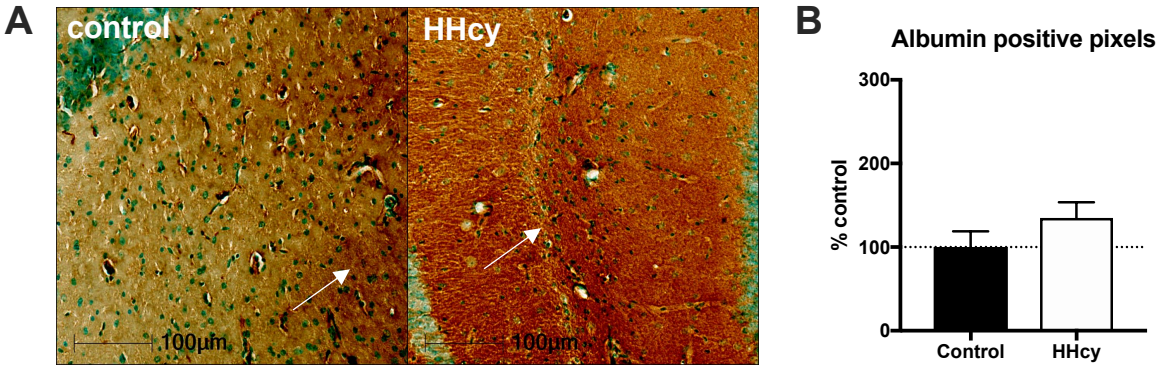

Figure 3: 3,3′-Diaminobenzidine (DAB) Immunohistochemistry and Area quantification results for albumin analysis in the hippocampus **A.** Images of albumin (arrows) stained tissues in Halo at 20x. **B.** No significant difference between mice with Hyperhomocysteinemia (Hhcy)(n=12) and control mice (n=13) for % area of the hippocampus positive for albumin (p>0.05)

3.2 Increased vascular branching and length after transient Hhcy

Vascular changes were examined by staining the tissues for dystrophin (Dp71) and analyzing the hippocampus with branch module in Halo (**Figure 4**). Dystrophin is an anchoring protein in the end-feet of astrocytes and on staining, shows the outline of the cerebral blood vessels. Hhcy mice had significantly greater number of branch points than control (Control =2621.67±1600.4, Hhcy 4719.4± 2168.5, p =0.013) and higher average branch length (Control = 61.58 mm ± 28.47, Hhcy 109.79± 51.76, p = 0.01) while there were no differences in average vessel thickness nor total vessel area between the groups (p > 0.05).

3.3 Microglial and astrocyte activation

Glial activation was studied by staining for P2Y12 (**Figure 5**) and GFAP (**Figure 6**) and analyzing the tissues through area quantification in Halo. There was a non-significant trend toward an increase in P2Y12 expression in the Hhcy versus control mice (Control =0.76±1.03, Hhcy =1.48±0.93, p = .081). There was, however, a statistically significant increase in total GFAP-positive area in the Hhcy mice versus control (Control =0.76±1.03, Hhcy =1.48±0.93, p =0.038).

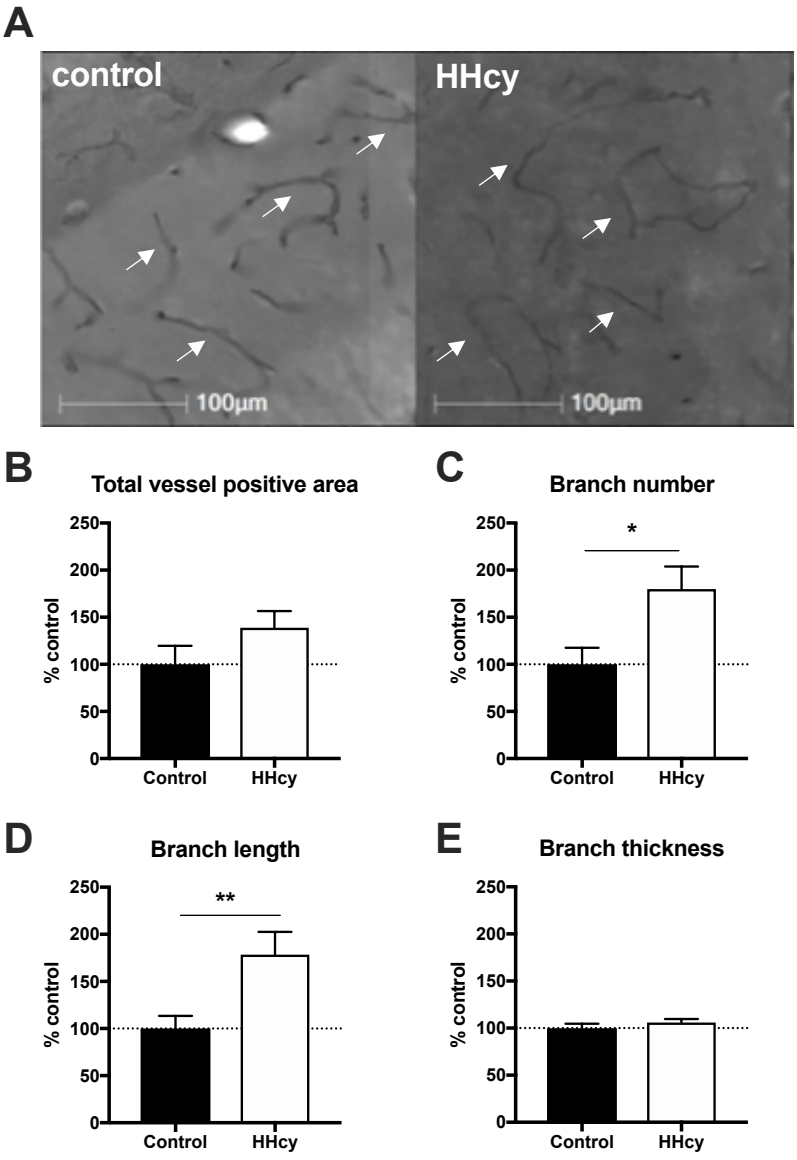

Figure 4. Fluorescence Immunohistochemistry staining and results for dystrophin analysis. **A.** Images of dystrophin (arrows) stained hippocampal tissue in Halo at 20x **B.** No significant difference in total positive vessel area between the hyperhomocysteinemia mice (Hhcy) (n=12) and the control mice (n=12) ($p>0.05$) **C.** Hhcy mice had significantly greater number of branch points than the control mice (*$p = 0.013$) **D.** Total Branch length for the Hhcy mice was significantly greater than the control mice (**$p = 0.0098$). **E.** No significant differences between the group in average branch thickness ($p > 0.05$).

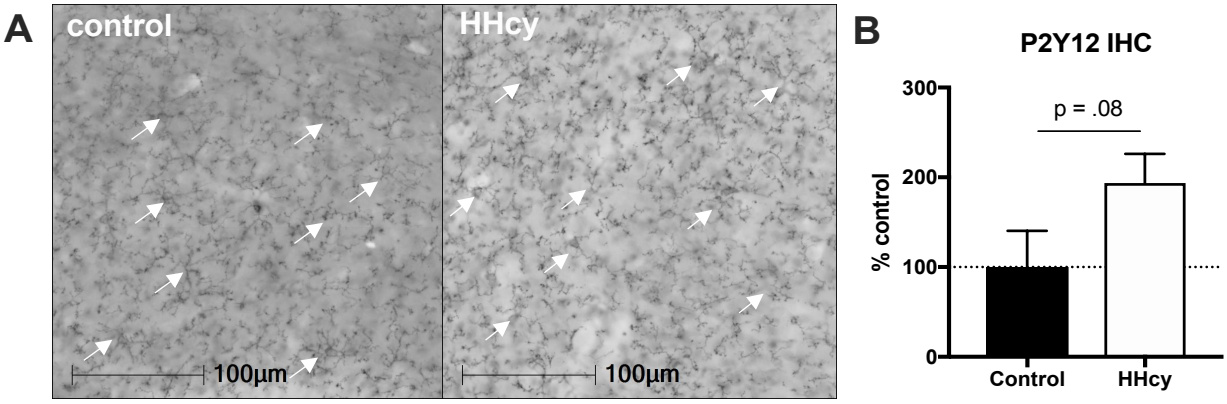

Figure 5. Fluorescence Immunohistochemistry staining and results for P2Y12 analysis **A.** Images of P2Y12 (in black, arrows) stained tissues in Halo at 20X. **B.** No significant difference in P2Y12 expression between the control (n=11) mice and mice with Hhcy (n=14) ($p>0.05$)

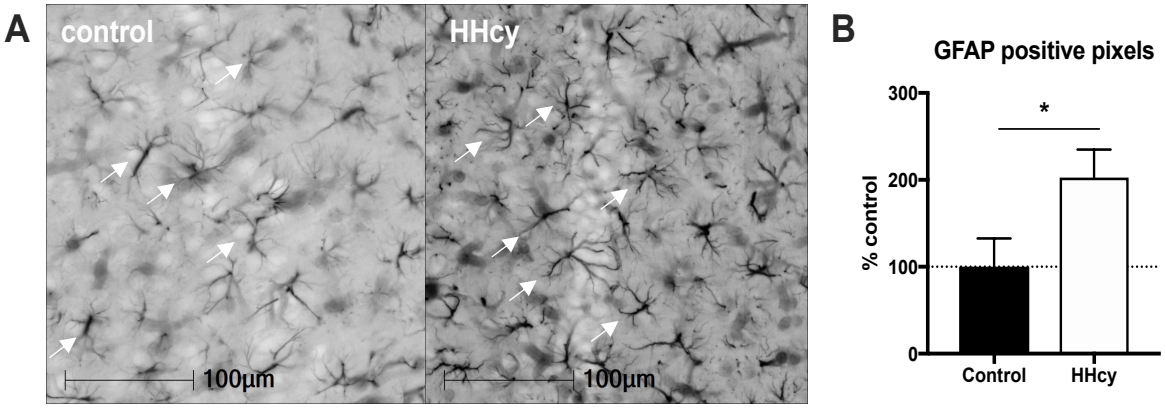

Figure 6. Fluorescence Immunohistochemistry staining and results for Glial Acidic Fibrillary Protein (GFAP) analysis **A.** Images of GFAP (arrows) stained tissues in Halo at 20x **B.** Significant increase in GFAP expression in the mice with hyperhomocysteinemia (Hhcy) (n=10) than the control mice (n= 10) ($p= 0.038$)

3.4 PSD95 level is unchanged from the control

Post-synaptic protein levels were examined by homogenizing hippocampi, performing Western Blot and staining for PSD95 and actin (**Figure 7**). Actin was used as housekeeping protein and the PSD95 signal was normalized to actin. No difference was observed between control and Hhcy groups (Control =4,167,839.711±732649.3201, Hhcy =3,904,196.421±752242.6852, p = 0.74).

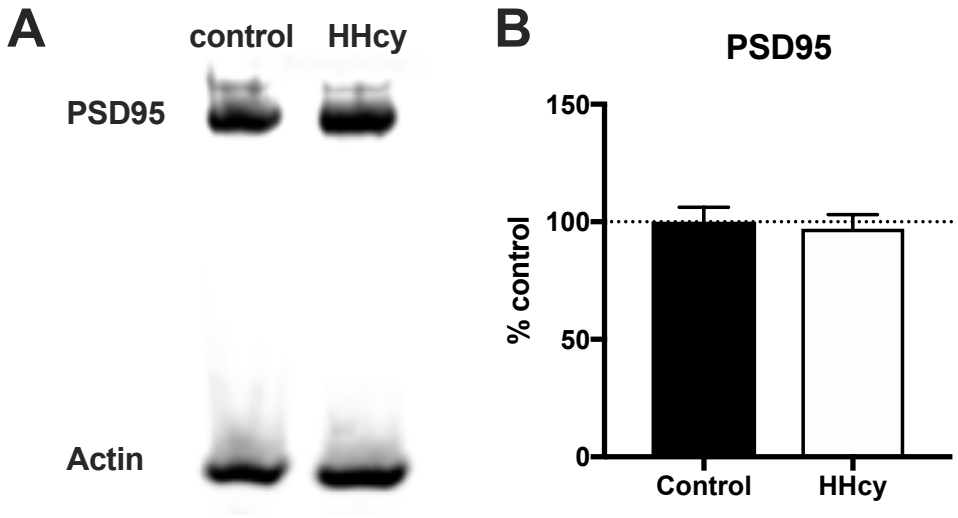

Figure 7. Western blotting result of Post-Synaptic-Density 95 (PSD 95), a post synaptic protein. A. Image of the scanned Western blot (WB) B. Results of the WB analysis. Signals were normalized to actin signals. No significant difference in PSD95 levels between the Control mice (n=7) and mice on hyperhomocysteinemia diet (Hhcy) (n=8) ($p>0.05$).

4. DISCUSSION

4.1 Summary of the results

In order to better understand why transient Hhcy can lead to persistent hippocampal-dependent cognitive deficits, we examined a number of parameters relating to BBB integrity, vascular changes, glial inflammatory activation, and synapse number. We performed DAB staining to examine the level of albumin in the hippocampal tissue and found that its level was not significantly different in Hhcy mice compared to the control mice. IHC staining also showed no significant difference in P2Y12 levels between the control mice and Hhcy mice. Interestingly, we did see a significant increase in the GFAP levels between the Hhcy mice and the control mice. The findings of the vessel branch analysis were also interesting. Even though Hhcy mice had more branch points and greater total length than the control mice suggesting increased vascular density, the % area covered by the vessels was not significantly different than the control mice. Finally, we found no difference in PSD95 levels between the groups.

4.2 Hippocampal blood brain barrier leakage recovers after transient Hhcy

BBB is dynamic and our results suggest that the BBB could be partly repaired in hippocampus after the Hh levels are restored. BBB leakage has been previously reported in mice with Hhcy (Kamath et al., 2006). Hh has been shown to increase matrix metalloproteinases (MMPs) that dissolve collagen and result in a leaky BBB. (Lominadze et al., 2012; Agrawal et al., 2006; Rosenberg and Yang, 2007; Yang et al., 2007). Levels of MMPs come back to normal after folate is supplemented in the diet (Kalani et al., 2014). MMPs could have similarly declined with the decreasing Hh levels and somewhat restored the BBB. And, while the BBB integrity is improving, the macrophages could have cleared out the albumin that had already leaked into the tissue. It is possible that this result is not representative of the overall BBB integrity. There are regional differences in the degree of angiogenesis in the brain (Patt et al., 1997) which suggests that the rate of BBB repair could be different in different regions of the brain. Albumin staining data also gives us a very narrow picture of the state of BBB. Albumin is a large protein that is cleared out rapidly from the brain. There are blood proteins smaller than albumin that could still get into the brain and take more time and effort to be cleared. Staining for blood proteins with a range of different molecular weights could give us a clearer picture of the BBB leakiness. BBB integrity could also be assessed by the levels of tight junction proteins as well as the level and condition of the pericytes.

4.3 Increased vascular branching and length after transient Hhcy

Our vascular analysis showed that in the Hhcy mice, the area and average thickness of the vessels remained similar to the control mice while the total vessel length and density increased. Vessel hypertrophy has been reported in Hhcy mice (Baumbach et al., 2002), which can help explain the changes in the vascular parameters observed. It is possible that approximately half of the hippocampal vessels in the Hhcy animals are old vessels that are hypertrophied, while the other half are thinner, newly formed vessels. This would explain why there is no change in the average thickness and area of the vessels, even though they have increased total length and branching. Increased homocysteine also has been known to decrease the expression of GLUT-1 receptors (Lee et al., 2005). Increased vascular density has been reported in rats when they are placed in reduced oxygen for 3 weeks (LaManna et al., 2014). With the decrease in GLUT-1, Hhcy

might mimic a reduced energy state similar to a hypoxic condition, which might result in increased vessel density. Increase in vascular density could also be an effect of BBB repair mechanisms or a result of the angiogenesis triggered by the BBB leak induced inflammation (Giulian et al., 1998).

A major limitation of these results is that an astrocytic end-feet protein, Dp71 was used to visualize the blood vessels and quantify the vascular characteristics. Study done by Rhodehouse et al., (2013) used an endothelial marker to identify the vessels and reported no change in vascular architecture in Hhcy mice. No change in vascular density due to Hhcy was also observed in another study that used a different endothelial marker to study the vessels (Zeng et al., 2019). End-feet changes in astrocytes has also been widely reported in Hhcy (Sudduth et al., 2017). It is possible that the changes we saw could be changes in the astrocytes rather than the blood vessels. Hence, we plan to develop a reliable vascular and endothelial marker to inspect the changes in the cerebral blood vessels for future vascular studies.

Besides the changes in the hippocampus, changes in white matter area and the cortex could also be responsible for the persistent deficit in spatial learning. White matter injury is sensitive to changes in blood flow and white matter changes are correlated with loss of cognition (Wang et al., 2016; Wardlaw et al., 2013). Cortex is also involved in navigation and spatial memory (Cona and Scarpazza, 2019). Hence, future studies should re-run the analyses of the glial activation, albumin leakage and vascular changes in the cortex and the white matter of the same mice we used for this study to determine if there are regional differences in these measures.

4.4 Microglial and astrocyte activation

Elevation of GFAP levels indicates reactive astrocyte while a decrease in the level of P2y12 indicates activated microglia (Robel et al., 2009; Tozaki-Saitoh et al., 2012). We found increases in GFAP in the Hhcy mice even 4 weeks after normalization of homocysteine levels. This was in line with a previous study where increased levels of GFAP were found in Hhcy experiment in vitro (Longoni et al., 2018). Reactive astrocytes have been shown to promote angiogenesis by facilitating the adhesion of endothelial progenitor cells (EPCs) to the endothelial cells (Hayakawa et al., 2014). Hence, reactive astrocytes could be playing a constructive role at this time point and it's possible that an increase in GFAP is responsible for the decreased BBB leakage and increased vessel density and area.

While we saw increase in GFAP, we saw no difference in P2Y12 levels in the Hhcy hippocampus. Increased activation and proliferation of microglia are observed in mice with Hhcy (Zou et al., 2010). Our results suggest there might be an increase in microglial P2Y12 in response to transient Hhcy, which is interesting in that microglial P2Y12 has been shown to be important in responding to BBB leakage (Lou et al., 2016). It may be that at the timepoint measured, P2Y12 levels were already returning to baseline and this is why our results were not statistically significant. Activated microglia are known to be pro-inflammatory and cause BBB disruption (Nishioku et al., 2010; Yang et al., 2015; Gu et al., 2015; Almutairi et al., 2016). The transient nature of Hhcy and hence the transient microglial activation could help repair the BBB instead of disrupting it.

Even though we speculate that microglia and astrocytes are working to restore the BBB, it would require a separate study to pinpoint what are they actually doing. Microglia and astrocytes are both known to have pro and anti-inflammatory roles that could be either constructive or destructive (Cherry et al., 2014). To make this matter

complicated, they have multiple activation markers and both of these glial cell types interact with each other to regulate the BBB as well as synaptic proteins (Shigemoto-Mogami, 2018).

4.5 PSD95 level is unchanged from the control

Loss of synapses and neuronal degeneration have been in found in Hhcy mice (Zeng et al., 2019; Nuru et al., 2018). Loss of PSD95 and SAP97 has also been reported in Hhcy mice (Kamat et al., 2013). In our study, we found no difference in PSD95 levels between the Hhcy and the control mice. SAP97 is a postsynaptic protein that is necessary for synaptic transmission and plasticity (Howard et al., 2010). It could be that while PSD95 level has come back to normal, level of SAP97 is still low. If SAP97 is still low, even though synaptic protein levels are restored, proper connections cannot be made. This could lead to the observed cognitive deficit.

Other post-synaptic proteins such as synapsin1, PSD93 and glutamate receptor 1 (GluR1) have also been found to be decreased in Hhcy (Tanko et al., 2018; Zeng et al., 2019; Mahaman et al., 2018). Decrease in the level of several other factors responsible for memory and synaptic plasticity like dendritic spines, mushroom type spines, enzymes that phosphorylate cAMP responsive element-binding protein (CREB) and netrin1 have been found in Hhcy animals (Mahaman et al., 2018; Zeng et al., 2019; Kalani et al., 2019). Failure to recover the level of any of the above-mentioned factors could be responsible for the persistence of spatial memory and learning deficit. Hence, we will run the western blot for other synaptic proteins in the hippocampus as well as in the cortex. While the normal levels of synaptic proteins suggest that there may be no neuronal death at this time point, we might still look at apoptosis or neuronal morphology in the cortex, hippocampus and white matter to help explain the loss of spatial learning.

5. CONCLUSIONS AND FUTURE DIRECTIONS

After severe but transient Hhcy, markers of BBB integrity, glial activation, and synapse number seem to be partly restored after the Hh levels are brought back to normal. The microglia were found in their resting state and the levels of PSD95 were unchanged from the control group. Besides the elevated GFAP expression in the astrocytes, the system overall did not reflect a state of persisting inflammation. The increase in vessel branching and length, without the vessel hypertrophy that is reported in Hhcy animals (Baumbach et al., 2002), suggests that the system is in reparative mode after Hh levels are brought back to normal.

Elevated Hh is only one of the many risk factors for Alzheimer's and VD. It could be that while lowering Hh has some positive impact, it is not enough restore the cognitive function. Along with increasing Hh levels, reduction in blood flow, increase in oxidative stress, inflammation and changes in gut microbiome are also found in the elderly (Hoffman et al., 2017; Gemma et al., 2007). All of these factors are interrelated and together, they contribute to cognitive dysfunction. Hence, along with measuring cognitive status, the Hhcy clinical studies should also monitor these factors. Since the treatment of Hh is inexpensive and effective, there should also be more studies that look at the effect of normalizing Hh levels. The clinical or in-vivo studies could also add other groups that are given supplements such as tetrahydrocurcumin that is an anti-oxidant and anti-inflammatory compound (Vacek et al., 2017) besides B-vitamin supplements.

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