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Characterization of the Ependymal Barrier due to Human Aging and Injury in Murine Models

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ABSTRACT

Ventriclulomegaly, or the expansion of the ventricles in the brain, is a phenomenon associated with age and injury to the brain. The ependymal layer that encases the ventricles displays certain degrees of plasticity and regenerative ability due to its associated stem cell niche, the subventricular zone (SVZ). Previous research in the Conover Lab has shown that in the mouse, which maintains an actively proliferating SVZ into adulthood, there is an intact ependymal monolayer throughout normal aging, with maintained lateral ventricle size with some degree of stretching. In contrast, the human SVZ declines in proliferative capacity after infancy, and age-related changes to the ventricular lining can be seen. By using longitudinal MRI-analysis of ventricle sizes, I confirmed that ventriculomegaly, or the expansion in ventricle volume, is consistent in human brains, particularly after the age of 60. In order to understand the factors that influence ventricular expansion, we analyzed paired postmortem human MRI sequences with immunohistological analysis of ventricular tissue. It was found that extensive formation of gliosis is associated with ventriculomegaly, possibly compensating for the loss of ependymal cell coverage along the ventricle wall.

After seeing how aging affects the ventricle barrier, we wanted to see how the ependymal barrier integrity may be implicated in injury. By using a mouse model of repeated mild traumatic injury, we looked at the effects of injury on the ventricular lining. Results confirm that in mice, there is an increase in ependymal cell size correlated with increased ventricular size, along with an increased expression of astrocytes along the apical surface of the ventricles. These studies show the importance of the ventricular system in the brain, and further research could elucidate how it is implicated in neurodegeneration.

CHAPTER I: INTRODUCTION

The ventricular system

The ventricular system of the brain is a network of interconnecting cavities that include the two lateral ventricles, the third ventricle, the cerebral aqueduct, and the fourth ventricle. These structures encase the cerebrospinal fluid (CSF) that flows through the brain, creating a dynamic fluid system in the brain (Abbott, 2004). Beyond maintaining homeostasis of the cerebral interstitial fluid, the CSF also serves a crucial role in maintaining important nutrients in the brain, and eliminating potentially toxic catabolites from the brain (Veening and Barendregt 2010). Recent studies have also shown an interactive role that the surrounding tissue plays in maintaining the CSF. Studies on the water channels in the brain, Aquaporin-4 (AQP4) channels, show that these channels are crucial in facilitating CSF metabolite clearance in the brain. Animals without AQP4 channels on astrocytes and ependymal cells surrounding the CSF show slowed CSF flow, and reduced solute clearance from the brain (Iliff et al, 2012). The crucial role that AQP4 plays in transependymal bulk flow is important in illustrating how the ventricle and the CSF interact with each other.

The functional units in the ventricular system are single-layered cuboidal epithelial cells called the ependymal cells, which serve as a barrier between the CSF and the underlying tissue (Del Bigio, 2010; Del Bigio, 1995). These ependymal cells are ciliated, which create a current of CSF along the wall of the lateral ventricle. When there are disruptions to the ependymal surface, there are disruptions to CSF flow and ventricle enlargement, or ventriculomegaly, could occurs (Spassky et al, 2005). In addition to these

barrier functions, the ependymal lining of the lateral walls of the lateral ventricles are even more significance due to the role they play in the neurogenic niche in the mammalian brain, called the subventricular zone (SVZ).

The Subventricular Zone

The subventricular zone (SVZ) exists as a neural stem cell niche that is found adjacent to the CSF-filled lateral ventricles in the lateral wall. In mice, the SVZ has been shown to be active in producing neuroblasts that migrate in chains through the rostral migratory stream (RMS) to the olfactory bulb, where they differentiate into interneurons that ultimately integrate into the granular layer or the periglomerular layer (Kornack and Rakic, 2001). In addition, this proliferative capacity is maintained to a certain extent, as our lab has demonstrated that through aging, there is a consistent population of proliferating neural stem cells, but an overall decline in neurogenic output to the olfactory bulb (Shook et al, 2012). In the human, however, the regenerative capacity of the SVZ remains controversial. The adult human SVZ contains a hypocellular gap layer separating the ependymal lining from a dense periventricular ribbon of astrocytes (Figure 1). In 2004, a group from the University of San Francisco found that some of these astrocytes could be neural stem cells. In addition, they found rare migrating immature neurons in the RMS of adult humans (Sanai et al, 2004). In 2011, the group expanded on their work and found that only the infant human SVZ is proliferative and active in producing immature neurons before 18 months of age, but this neurogenic activity subsides in older children and by adulthood, is essentially nonexistent (Sanai et al, 2011). This drastically limits the neurogenic capacity of the SVZ in the adult human.

Ventricle changes in aging

With distinct differences in the neurogenic capability of the SVZ between humans and mice, there are also distinct differences in their regenerative capacity in response to damage along the ependymal cell layer. The regenerative capacity of the SVZ in mice has been shown to mediate moderate repair mechanisms for the ependymal layer by maintaining a relatively intact ependymal layer through aging (Shook et al, 2013). When there is extensive damage to the ependymal layer in mice, a stem cell-mediated gliosis, or "scarring", can be found (Luo et al, 2008). Our lab has shown that normal aged mice show no gliosis or ventricle expansion, and maintain a healthy, functional SVZ stem cell niche with only some stretching of the ependymal cells (Shook et al, 2013). In the adult human brain, however, the limited regenerative capacity of the SVZ hinders the potential for ependymal repair in aging (Conover et al. 2011, Sanai et al. 2011). In stark contrast to the mouse, the human ependymal layer *does* undergo changes in aging, as the volume of the lateral ventricles increases in aging and changes due to neurodegenerative diseases and injury (Höglinger et al. 2004, Scahill et al. 2003). However, the factors that influence ventricular expansion, and the effect of ventricular expansion on the integrity of the ependymal wall are not well understood.

In trying to understand the mechanisms of ventricular expansion, hydrocephalus hyh mutant mouse models have been used to identify changes to the ependymal wall integrity due to induced expansion (Bronson et al. 1990). It has been shown that ventriculomegaly is associated with distinct changes in junctional proteins (N-cadherin and connexin 43) and permeability proteins like AQP4. First, glial scarring can be found

scattered along the lateral ventricles. Interestingly, there does not seem to be any traces of N-cadherin in areas where there is glial scarring, indicating that the junctional proteins that bind the cells along the ventricle lining are lost in ependymal denudation. AQP4 is found to be upregulated throughout areas with glial scarring, potentially mediating the formation of periventricular edema (Roales-Bujan et al. 2012). It is unclear, however, how these junctional and transport proteins are implicated in humans during normal aging.

In addition, there is a lack of a clear understanding of how age-related changes to the ependymal layer are manifested in MRIs. Pathological changes have been shown to correlate with changes on the MRI in neurological conditions like depression (Thomas et al. 2002, Davis et al. 2002), and in Alzheimer's disease (Grimmer et al. 2012), but how MRIs may change in aging is still not clear. In addition, pathological changes have been shown to correlate with areas of white matter hyperintensities in T2-weighted MRI in the form of gliosis, loss of myelinated axons, and denudation of the ventricular lining (Scheltens et al. 1995; Murray et al, 2012). In patients with Alzheimer's diseases and hydrocephalus, a high occurrence of periventricular hyperintensities could be found in T2-weighted MRI scans, which could indicated areas of edema surrounding the ventricles (Fazekas et al. 1987, Zimmerman et al.1986). However, it is unclear how normal aging may contribute to these abnormalities in white matter changes. Additionally, as new technology like fluid-attenuated inversion recovery (FLAIR) MRI technology, a type of T2-weighted MRI that blocks out the fluid signals from the CSF in order to show cortical hypertensive regions more clearly, there exists a much more accurate means of measuring periventricular hyperintensities.

Ventricular changes due to Injury

Ventricle expansion is also, notably, a hallmark of brain injury in humans. It is especially well documented in single cases of traumatic brain injury (Levine et al, 2008; Youngt et al, 2002), as well as in a neurodegenerative disease that is thought to be a result of repeated mild injuries to the head, chronic traumatic encephalopathy (CTE) (Chaunhan, 2014). CTE has been most commonly found in professional athletes who participate in high-impact sports like American football, ice hockey, and boxing. More recently it has been linked with military blast injuries as well (Mez et al, 2013). This progressive neurodegenerative disease currently can only be characterized by post-mortem analysis, notably identified by frontal and temporal atrophy, axonal degeneration, and hyperphosphorylated tau in the brain. In particular, ventriculomegaly is also a hallmark of CTE (Tartaglia et al, 2014). However, these diagnosis can only be made after autopsy, making it more difficult for researchers to understand how this disease progresses. Here, we are especially interested in seeing how repeated mild injuries on the brain may affect ventricle integrity, and how this process may be different from the one seen in aging. With a better understanding of how ventricle expansion may occur in the brain due to repeated hits, it could serve as an efficient means of diagnosis in the form of MRIs.

Thus, in order to evaluate how injury and aging affects the ependymal barrier, we first performed a comparative analysis of ventricle histology and radiological data in order to better understand the relationship between ventriculomegaly in aging and corresponding changes to the ependyma. Using cross-sectional and longitudinal MRIs taken from patients with no dementia across different ages, we can determine the extent of ventricle expansion that happens with age. In addition, we (Shook et al, 2013) sought to

perform post-mortem tissue analysis of the ventricular lining, and compared it to post-mortem MRI in order to investigate whether glial scarring may be correspondent to ventricle expansion. In order to assess how repeated mild traumatic brain injury affects the ventricular lining, we used a mouse model of rmTBI taken from a collaboration with a group at Wayne State School of Medicine in order to replicate the effects of repeated injury on humans. From this mouse model, we wanted to evaluate whether there is an associated ventricle expansion due to hits to the brain, and whether there may be a glial response to injury in the form of glial scarring. This multidisciplinary approach to look at physiological changes in the brain due to aging and injury is novel in nature, and would ultimately lead to direct applications in early diagnosis of neurodegenerative diseases using MRI technology.

Figures:

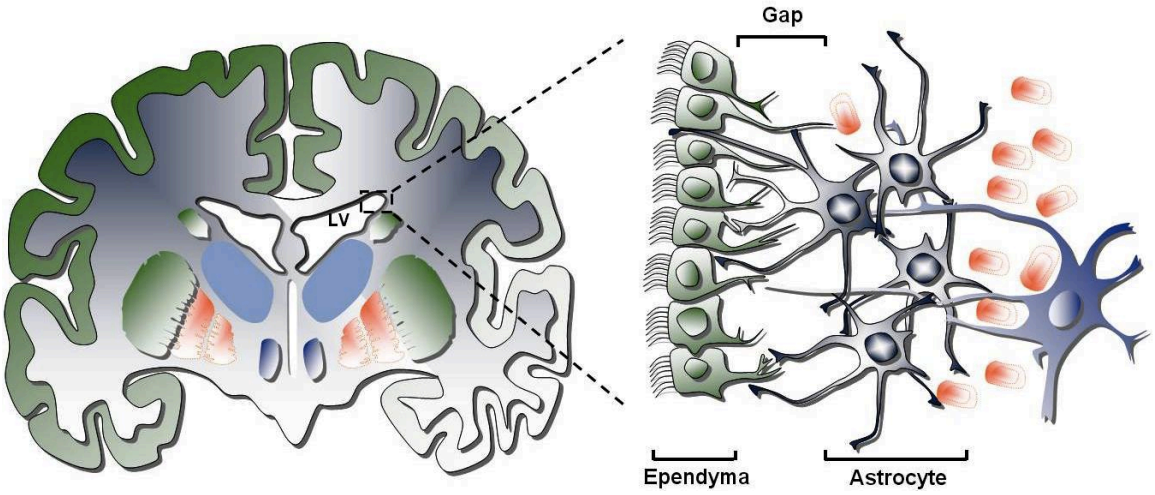

Figure 1. Organization of the human SVZ. Adapted from Arias-Carrion et al, 2008. At the surface, multicilliated ependymal cells line the lateral ventricles. Underneath, there is an hypocellular gap region filled with astrocytic processes that extend from the dense region of astorcytes underneath, called the astrocytic ribbon.

Chapter II. Aging in Humans

Methods:

Ventricle Analysis of Human MRI

In order to evaluate the extent of ventricle expansion in humans through aging, we evaluated human datasets with longitudinal and cross-sectional MRI images. High-resolution T1-weighted MRI image sets were obtained from the Open Access Series of Imaging Studies (OASIS) database (www.oasis-brains.org). Only male and female subjects who remain non-demented (clinical dementia rating < 0.5) during the duration of their participation in the database were analyzed from a longitudinal dataset as well as a cross-sectional dataset. For the longitudinal dataset, only the first scans were taken from each individual for analysis (Marcus et al, 2009). Additional brain scans were obtained from Yale University Radiology department. Patients were clinically diagnosed as non-demented, and acquisitions were made as part of routine checkups and/or mild neurological problems.

In total, 70 individuals were analyzed and broken down into six age groups: 19-39 (n=23), 40-60 (n=6), 61-60 (n=10), 70-79 (n=13), 80-89 (n=13), and >90 (n=5).

Total ventricular volume analysis

In order to ascertain the total volume enclosed by the lateral ventricles, semi-automatic segmentation of the lateral ventricles was performed using ITK-SNAP (Yushkevich et al., 2006), followed by quantification of total volume and surface area. In ITK-SNAP, pre-processing of the images was performed prior to automatic isolation of the lateral ventricle, including setting an lower threshold for image intensity at 30% of total intensity range. For automatic segmentation, lateral ventricle expanding balloon force was

set to 3.0 and detailed curvature force was set to 0.20, with 125 iterations at step size 1 was often sufficient to isolate the lateral ventricle. If expansion into the third ventricle occurred, less iteration were used and/or post-segmentation separation of the third ventricle from the region of interest was manually performed. Mean averages in age categories were calculated.

Using the longitudinal dataset, segmentations of the lateral ventricles at discrete time points for one patient could be overlaid on top of each other using MANGO (Kochunov et al, 2002) in order to show how much expansion is happening along the lateral wall, and where that expansion is.

Subsegmentation of the lateral ventricle

In order to evaluate where expansion may be more prone to occur along the ventricular wall, subsegmentation was performed, breaking down the lateral ventricle into 5 discrete regions in order to look at regional expansion. These regions are the lateral wall, the dorsal wall, the ventral wall, the trigone area, and the temporal horn (Figure 2A). In total, 22 individuals with longitudinal datasets were evaluated to determine which regions were more susceptible to expansion. Segmented lateral ventricles were from different time points were overlaid on top of each other as described above, and using the slicing tool in ITK-SNAP, we could determine how much expansion occurred in each region (Figure 2B). Volume results for expansion in each region were then obtained for analysis.

Wholemount analysis of post-mortem tissue

Human tissue obtained from the Harvard Brain Tissue Resource Center and the University of Connecticut Department of Anatomic Pathology and Laboratory Medicine were fixed in 10% formalin upon delivery to our laboratory at the University of Connecticut. After rinsing with 0.1M PBS to rid of excess formalin in the tissue, wholemounts of the lateral wall of the lateral ventricles were extracted from the brains. After wholemounts were obtained, they were processed for immunofluorescence. A combination of the following markers was used to understand gliosis and ependymal cell makeup of the lateral ventricles. Beta-catenin (b-catenin), a cell-adhesion protein marker, was used to outline ependymal cells. Glial fibrillary acidic protein (GFAP), an intermediate filament protein that is expressed throughout the central nervous system, was used to illustrate astrocytes (Luo et al, 2008). Aquaporin-4 (AQP4) was used to illustrate both ependymal cells as well as potential areas of scarring. The following antibodies were used: mouse anti-b-catenin (1:250; BD Biosciences, San Jose, CA, USA); goat anti-GFAP (1:250; Santa Cruz Biotechnology); rabbit anti-AQP4 (1:400; Sigma-Aldrich). Dissected whole mounts were coverslipped with Aqua-Poly/Mount (Polysciences) and imaged on a Leica TCS SP2 confocal laser-scan microscope.

Two subjects (Subject 1 = 82 years, Subject 2 = 86 years) underwent both post-mortem histological analysis as well as post-mortem MRI. Wholemounts of all regions of the lateral ventricle were obtained (Subject 1: 36 15 mm x 8 mm sections and Subject 2: 18 15 mm x 8 mm sections). In additional, several coronal sections were taken from the superior-most region of the anterior lateral ventricle to undergo haematoxylin and eosin staining, as described (Luo et al, 2008).

Postmortem MRI

Postmortem MRI was performed on brains after acquisition from Dr. Qian Wu from the UConn Health Center Department of Pathology. After autopsy, the brains were fixed and suspended in 10% formalin and high resolution 3D T1-weighted MP RAGE MRIs were taken. Using similar procedures outlined above, the lateral ventricle was segmented using ITK-SNAP, and volumetric readings along with the surface areas were recorded using 3D Slicer (www.slicer.org) (Federov et al, 2012).

FLAIR MRI segmentation and analysis

Longitudinal Fluid attenuated inversion recovery (FLAIR) MRI datasets were obtained from Yale University as well as from OASIS. In order to evaluate the extent of perivascular white matter (PWM) in the MRI scans, manual segmentation of PWM was done on 3D Slicer (Federov et al, 2012). Using a threshold evaluation of MRI intensities, white matter was defined as the upper 15% of the upper threshold, while the lateral ventricles was defined as the lower 30% of the upper threshold. Using different colors to differentiate the ventricles from the PMW, as well as at different time points, 3D modeling of the FLAIR MRI scans could be visualized, as shown in Figure 3. Using this method, ventricle as well as PMW volume data could be obtained for individuals from each longitudinal data point.

Results:

Expansion in Humans through Aging

Using a combination of data from the "Open Access Series of Imaging Studies"(OASIS) database (Marcus et al, 2007) and from Yale University, we gathered a cohort of cross-sectional MRI dataset as well as longitudinal datasets in order to evaluate the extent of ventricle expansion in aging. Using the cross-sectional database from OASIS, we were able to gather data on 70 individuals ranging from 20-100 years old. By using semi-automated segmentation followed by 3D reconstruction of the lateral ventricles, the lateral ventricle volumes were collected (Figure 4). A dramatic age-related increase in total volume could be seen, especially after the age of 60. This follows relatively stable volumes throughout adulthood from 20-60. In order to evaluate the extent of volume expansion, we analyzed data from longitudinal data obtained from the OASIS database. In total, 39 individuals were evaluated (female = 24 male = 15), all of whom had at least two MRI scans at least one year apart. Total volume data was also obtained for each time point, and overlaying the ventricle reconstructions from the first time point with the next, we could effectively study where along the lateral ventricle expansion and stenosis is taking place (Figure 5A). We found that generally, ventricles expand exponentially and sequentially past the age of 60 in both males and females (Figure 5B). We also analyzed any potential differences in ventricle size of males and females, and found that there was no significance difference in ventricle expansion or size (Figure 6).

By using subsegmentation of the lateral ventricles, we hoped to identify specific regions within the lateral ventricle that may be more prone to expansion. After calculating the percent increase in volume versus baseline, and comparing the average percent increase in subjects from 60-80 years old again subjects that are 80+, we were able to see regional differences in how much expansion happened. Interestingly, the lateral wall and

the temporal horns showed a significant increase in volume between the two age groups (Figure 7), indicating that perhaps those regions are more prone to expansion later in life.

Human exhibit age-related perivascular gliosis

In order to take a closer look at how the ependymal layer is changing through aging, we collected human tissue from the Harvard Brain Tissue Resource Center as well as the University of Connecticut Health Center Department of Anatomical Pathology. First, we looked at histological slides of the ventricles taken from the UConn Health Center. Coronal sections of the lateral ventricles showed areas of intact ependymal cell as well as areas without full ependymal cell coverage (Figure 8A, B). Immunohistochemical staining for GFAP could be seen in areas without intact ependymal cell, indicating gliosis at the surface of ventricle wall (Figure 8D, E). In addition, GFAP staining showed an increase in astrocyte processes in the area right underneath the ependymal layer that is typically hypocellular in nature (Figure 8E). Using whole mount preparations of the human lateral ventricles, we looked at the surface of the ventricles to examine the ependymal layer integrity through aging. Figure 8 F-H shows a wholemount representation of an aged human ventricle wall, which consists of areas of intact ependymal (shown by the β-catenin staining in green) as well as areas of gliosis (shown by the GFAP staining in red).

Postmortem MRI with associated histological analysis shows that gliosis corresponds with ventricle expansion in humans

After showing that glial scarring and ventricle expansion is implicated with age, we sought to better understand the relationship between the two. We used a paired analysis of

post-mortem MRI and associated histological analysis of the ventricle wall in order to illustrate this relationship. Two subjects with similar ages were selected due to dramatic differences in ventricle volumes. High-resolution 3D T1-weighted MP RAGE MRI images were obtained for both after autopsy at the University of Connecticut Health Center. Semi-automatic segmentation of the high-resolution MRI was performed using ITK-SNAP, similar to the method used previously. Again, volume and surface area measurements were obtained for the lateral ventricles using 3D Slicer. Figure 9A shows a 3D model of the ventricle for Subject 1 (Age 82), who had an age-appropriate lateral ventricle size of 59241.4 mm^3. In contrast, Figure 10A shows a 3D model of the ventricle for Subject (age 86), who had a dramatically smaller ventricle size of 11279.5 mm^3, which as shown in Figure 3B is more typical for a person in his/her 20s – 40s.

Coronal sections from the anterior portion of the ventricles from both subjects underwent H&E staining. *En face* whole mount sections were also obtained from the rest of the lateral ventricle wall for immunofluorescence analysis in order to study the ventricular wall surface. In Subject 1, coronal sections show areas of diminished ependymal coverage (Figure 9B) while in Subject 2, we see complete ependymal coverage (Figure 10B). Wholemount sections for Subject 1 showed consistent ependymal coverage along the inferior portion of the ventricle, as shown by long expanse of β-catenin stained ependymal cells (Figure 9C,D). Along the dorsal side of the ventricle, areas of compromised ependymal can be seen especially at the anterior region (shown in yellow in Figure 9D) where there is absent or diffuse β-catenin staining but no indications of GFAP positive gliosis. Large areas of GFAP+ gliosis along with small “islands” of GFAP+ gliosis can be found through out the ventricle (shown in red in Figure 9D). Representative sections are shown in Figure 9C in

order to show areas of intact cuboidal ependymal cells (stained by β-catenin) that exhibit classic cobblestone appearance with little to no change in cell size. Areas of gliosis (stained by GFAP) can be shown in red, and found in areas without complete ependymal cell coverage.

In contrast, wholemount preparations of the lateral ventricle from Subject 2 revealed intact ependymal cell coverage along the entire lateral wall of the lateral ventricle (Figure 10B,C in green); we did not detect any gliosis along the lateral wall.

Using FLAIR MRI as a clinical diagnosis of ventricle integrity and edema.

With the establishment that glial scarring is implicated in age-related changes in the integrity of the lateral ventricles, we sought to find a clinical implication of this histological phenomenon. Postmortem fluid attenuated inversion recovery (FLAIR) MRI matched with histological tissue analysis has shown that periventricular hyperintense regions on the FLAIR MRI corresponds with increased astrogliosis, loss of myelination, and increased microglia activation in the tissue surrounding the ventricles. (Murray et al, 2013). Thus, by using FLAIR MRIs as a clinical assessment tool to measure the extent of gliosis along the ventricular wall, we could potentially better understand the effects of aging on ventricle integrity. Non-demented longitudinal MRI datasets were obtained from Yale University. Figure 11 shows a representation of how we could quantify not only the expansion in ventricles, but also the expansion in volume of the periventricular hyperintensities over time. This methodology could be used with a larger cohort of longitudinal FLAIR MRIs to determine the progression of hyperintensity changes. Using subsegmentation techniques

as mentioned, we could also effectively evaluate whether hyperintensities may correspond to areas that are more prone to expansion.

Aquaporin4 water channels are upregulated in areas of gliosis along the ventricle wall

Seeing that periventricular edema is associated with age by looking at FLAIR MRIs (Leys et al, 1990), we wanted to better characterize the influence of fluid flow in ventriculomegaly. In our lab's model of glial scarring through injections of neuraminidase (as described in Shook et al, 2013), we studied expression levels of Aquaporin channel markers. More specifically, Aquaporin 4 (AQP4), is expressed at low levels in the brain, and found at the perivascular endfeet as well as the outline of the basolateral surface of the ependymal cells (Haj-Yasein et al, 2011; Illiff et al, 2012). AQP4 expression is also found to increase during gliosis (Sofroniew, 2009). When examining areas of scarring in both humans (Figure 12B) and mice (Figure 12A), we found that AQP4 was upregulated throughout the areas of scarring, and found at normal levels as areas of consistent ependymal cell coverage.

Discussion:

The ventricular system is crucial in our understanding of the brain through the aging process. The neuroregenerative portion of the brain, the subventricular zone (SVZ) is located along the lateral wall of the lateral ventricle (Conover and Shook, 2011). While its neuroregenerative capabilities diminish substantially in humans after early development, its potential role in response to aging and injury is implicated in mice, where it has been

shown to repair some ependymal cells as well as mediate the formation of glial "scarring" after extensive damage to the ependymal layer (Luo et al, 2008).

Our finding that ventricle expansion is related to aging is supported by extensive clinical evidence (Scahill et al, 2012; Carmichael et al, 2007; Kii et al, 1998). In addition, our work on studying longitudinal MRIs show that ventricle volumes expand sequentially in humans. We also show that in many aged humans, histological sections of the ventricle surface reveal spans of denudated ependymal cells, where some denudated areas show $GFAP^+$ cells in place of the lost ependymal cells. Thus, in order to understand the pathological basis of this expansion, we paired post-mortem MRI analysis with histological whole mount and coronal section analysis. We report the first analysis of ventriculomegaly-related gliosis along the ventricle wall. We cannot show that gliosis is necessarily related to age, as the two subjects studied had similar ages. However, with distinctively different ventricle sizes, this demonstrates that gliosis could be greatly implicated in other neurological conditions such as traumatic brain injury that have ventriculomegaly as an associated clinical observation (Mez et al, 2013).

In addition, we tried to relate our research on the gliosis along the ventricle wall to a clinical correlate in the form of MRI scans. While traditional T1-weighted MRIs can easily show enlarged ventricles, they are ineffective at indicating the extent of gliosis and ependymal compromise along the ventricle walls. T2-weighted MRIs, on the other hand, serve a more pathological purpose for radiologists, as hyperintense regions on a scan indicate implicated changes in fluid flow. Fluid-attenuated inversion recovery (FLAIR) MRI is a type of T2-weighted MRI, except the cerebral spinal fluid signals are suppressed, thus only showing fluid changes in the subcortical and cortical regions in the brain. Thus, FLAIR

MRI has been used as a better diagnostic alternate to T2-weighted MRI when detecting various types of neurological diseases (De Coenes et al, 1992). These lesions serve as important indicators of disease, and while clear pathological correlations with hyperintensities found within FLAIR MRI scans have been inconsistent, it has been established that perivascular hyperintensities may be indicative of edema, loss of ependymal coverage, demyelination and gliosis (Gibson et al, 2010; Matsusue et al, 2006; Fazekas et al, 1993; Van Swieten et al, 1991). Extensive research has implicated the presence of increased hyperintensities on T2-weighted MRI as correlative to aging, cognitive decline, and age-related mental disorders such as dementia or stroke (Fazekas et al, 1987; Grimmer et al, 2012; Scheltens et al, 1995; Van Swieten et al, 1991; Kang et al, 2013). Thus, better understanding how FLAIR MRIs are implicated in aging is crucial in serving as a clinical link to the pathology of gliosis and edema along the ventricular wall. Here, we demonstrate that segmentation and consequent volumetric analysis of FLAIR-MRI is possible, and could potentially be used on a larger cohort of patients with longitudinal scans in order to better characterize changes to periventricular hyperintense regions in the brain. Ultimately, this would provide us with a better understanding of when and how glial scarring may be formed in aging. Similar to how we showed that ventricle expansion seems to progress drastically after the age of 60, there could be a similar progression as to how hyperintensies form along the ventricles. This could be instrumental in bettering clinicians' interpretation of FLAIR MRI results, and help to better diagnose neurodegeneration in the brain.

Figures:

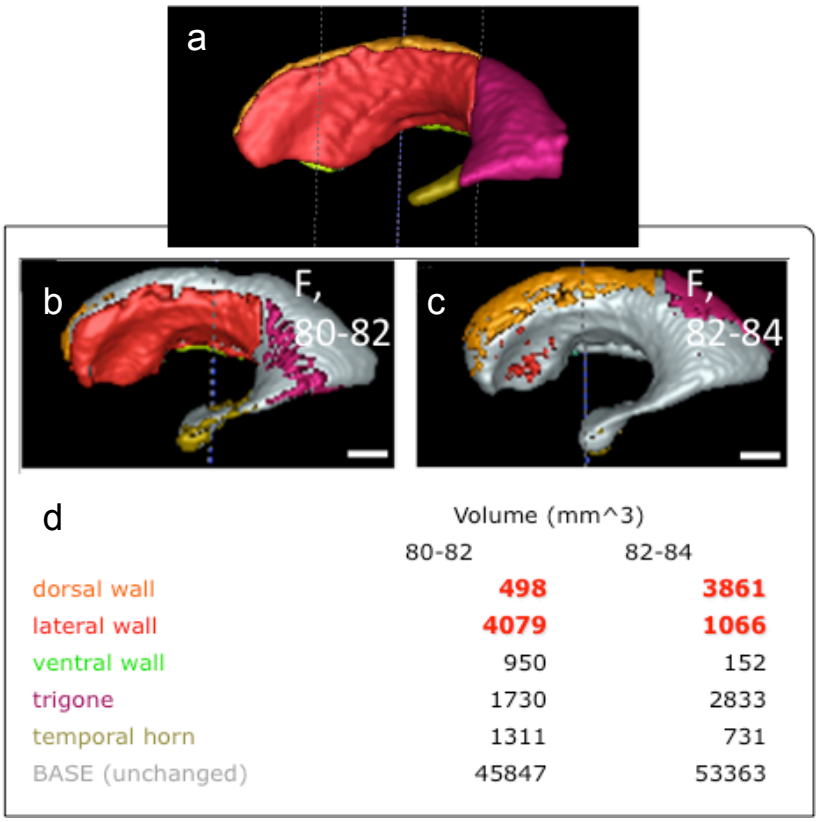

Figure 2. Subsegmentation of the lateral ventricle expansion. (a) A schematic of the lateral wall broken into five discrete regions: dorsal wall (orange), lateral wall (red), ventral wall (green), trigone (purple), and temporal horn (gold). (b) A representation of subsegmentation on a patient from age 80-82, where there is greater expansion in the lateral wall. (c) At age 82-84, the same patient as in (b) showed greater expansion along the dorsal wall. (d) Corresponding volume data, showing that the lateral wall expanded initially, after which the dorsal wall expanded.

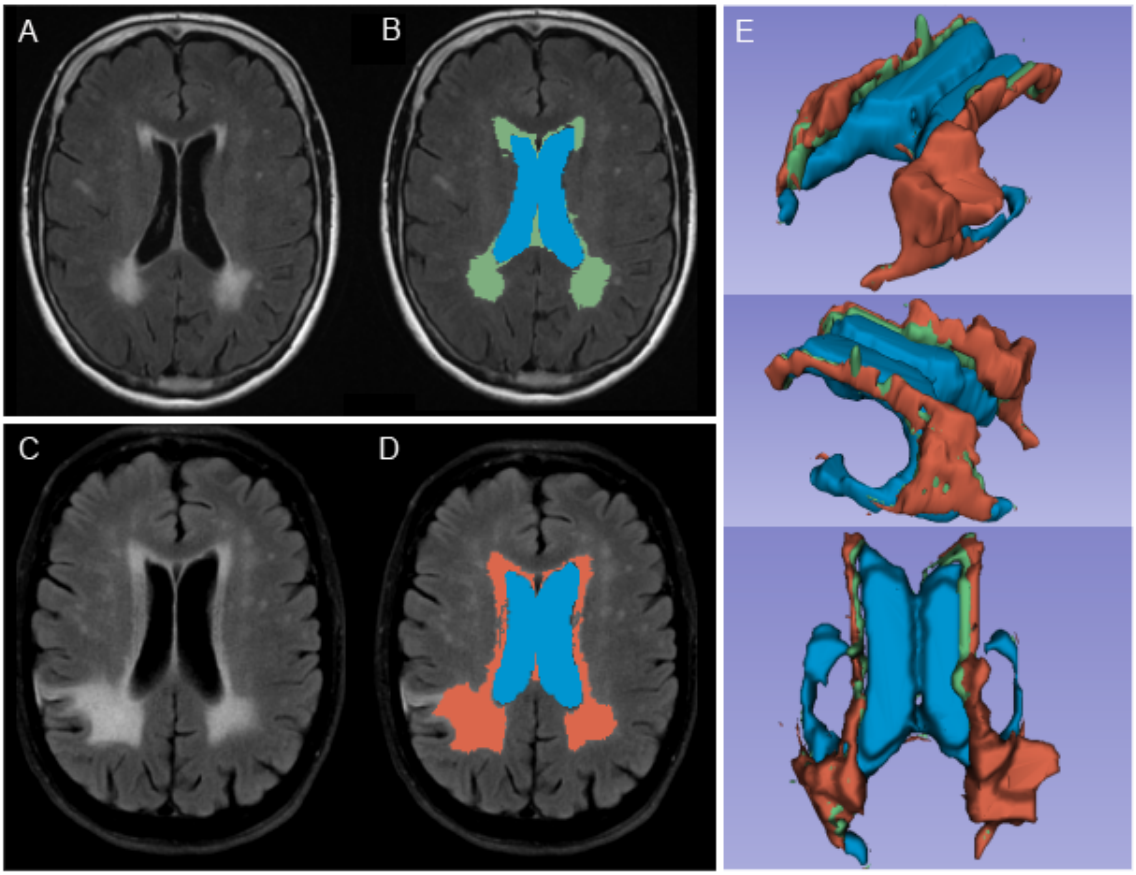

Figure 3. Segmentation and 3D modeling of periventricular white matter (PWM) from FLAIR MRI scans from 2 time points. (A) FLAIR MRI from subject at 77. (B) Using 3D Slicer, PWM is shown in green, and the lateral ventricle is shown in blue. (C) FLAIR MRI from subject at 80. (D) PWM is shown in orange, and the lateral ventricle is shown in blue. (E) 3D modeling of changes in PWM volume in aging, especially in dorsal region

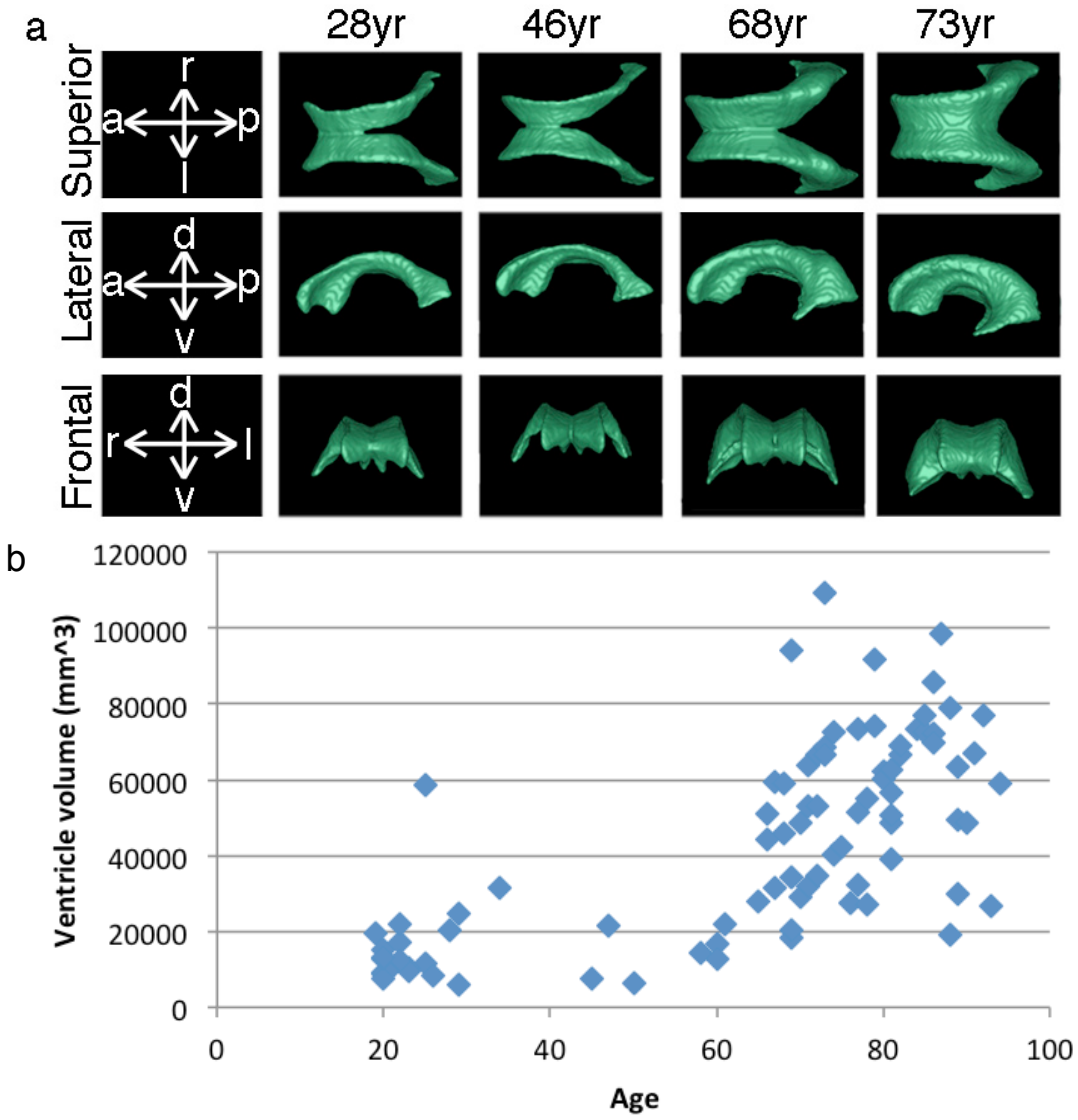

Figure 4. Cross-sectional data showing ventricle expansion in humans throughout aging. (a) Age-related, representative 3D reconstructions of lateral ventricles as visualized on 3D Slicer (b) Ventricle volumes at different ages from 20-94 (n=70).

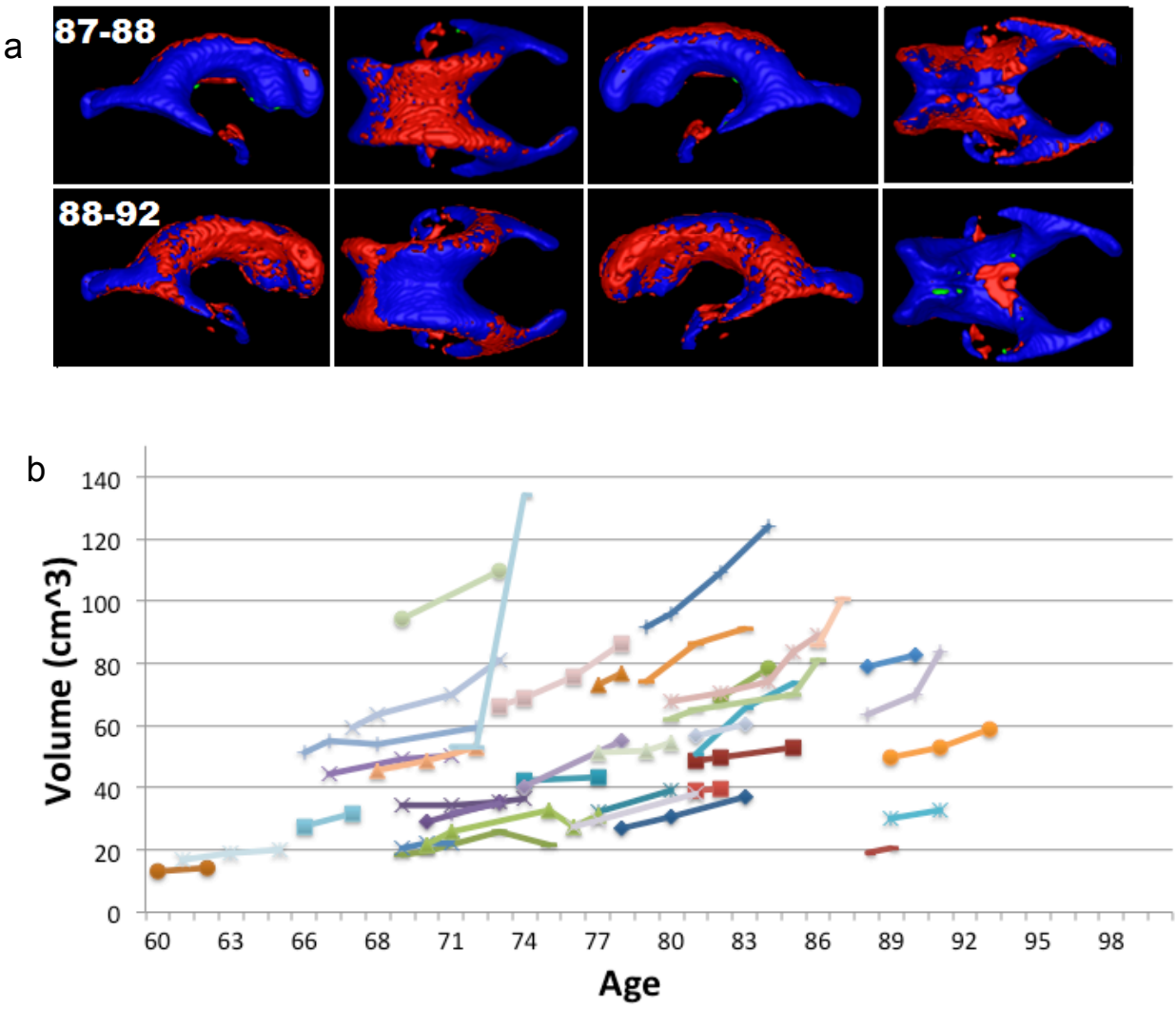

Figure 5. MRI longitudinal analysis of volumetric expansion in adults 60+.
(a) Schematic of expansion analysis on lateral ventricle volumes taken at two time points for one subject. Top row: expansion within 1 year span from ages 87-88. Bottom row: expansion within 4 year span from ages 88-92. (b) Summary of expansion data on longitudinal MRIs (n=39) taken from OASIS database (Washington University in St. Louis). We see that with aging, lateral ventricle volume expansion increases overall.

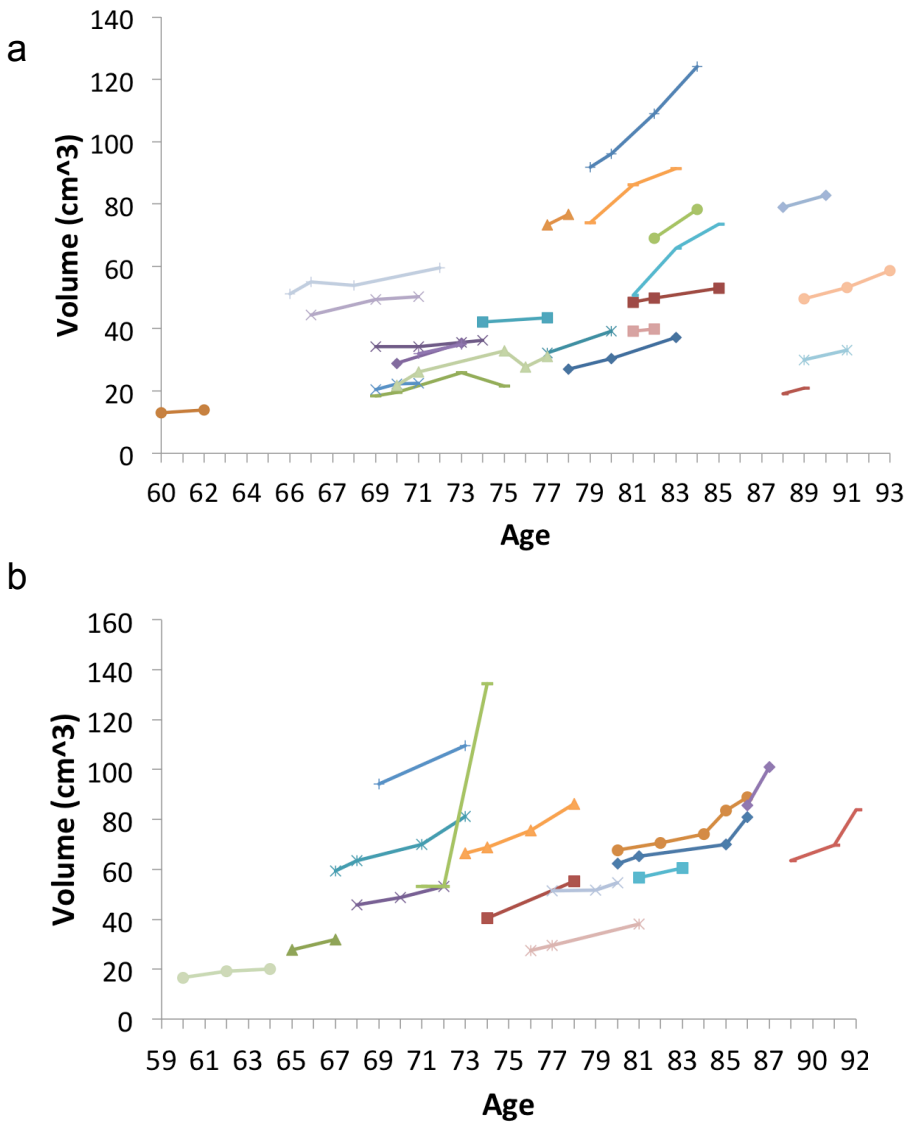

Figure 6. Comparison between male and female ventricle expansion with age. Little differences can be found between the male and female cohort. (a) Female (n=24). (b) Male (n=15).

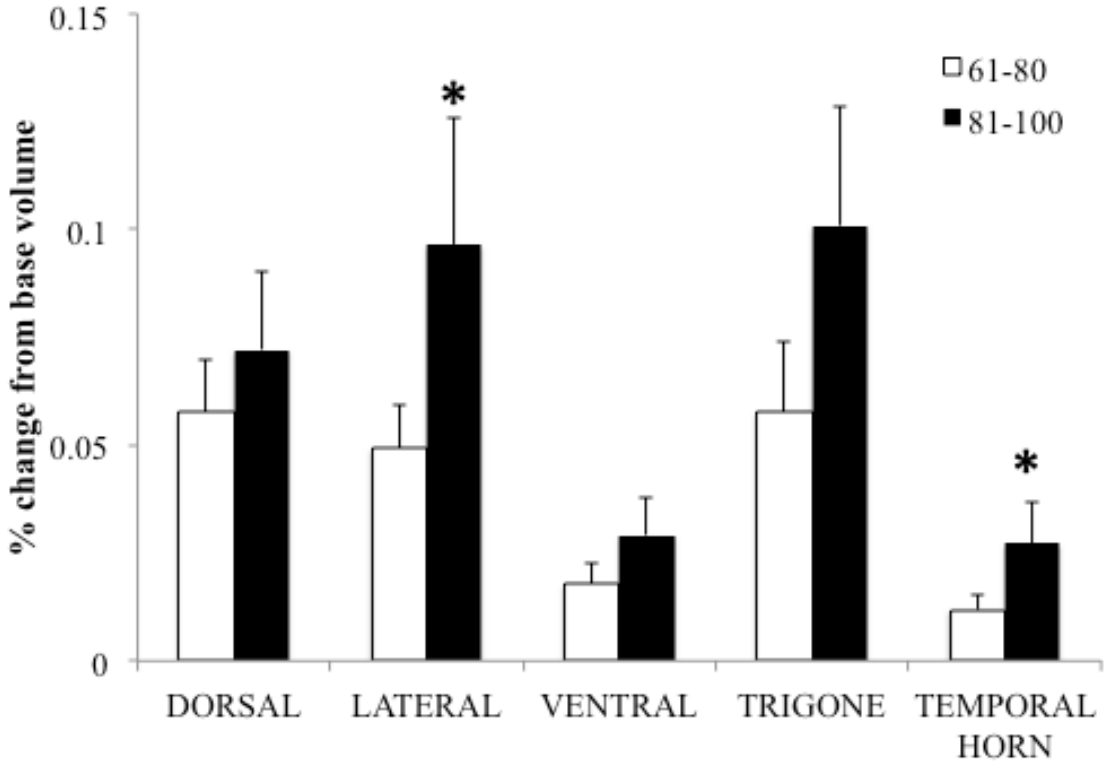

Figure 7. Subsegmentation of the lateral ventricle expansion volumes. Percent increase in volume was calaculated for each time point. Patient percent increase in volumes were averaged for each of the 5 subsegmented regions, and compared between those from ages 61-80, and ages 81-100. Student T-test was performed to test for signifiance. Here, the lateral wall and the temporal horns were significantly different in volume.

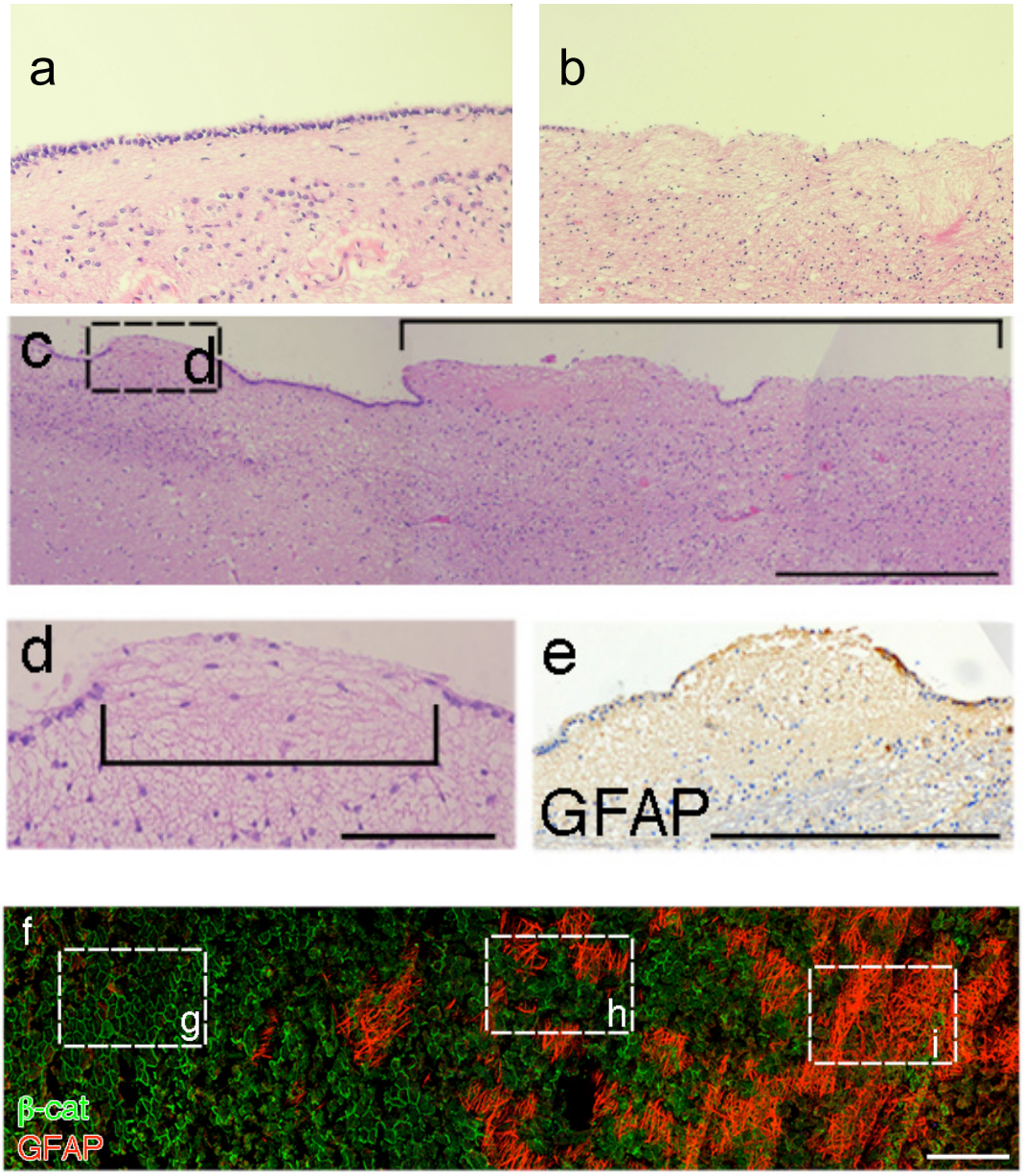

Figure 8. Human histological sections of the lateral ventricles show areas of intact ependymal coverage as well as areas lacking ependymal cell that stain positive for GFAP. Adapted from Shook et al, 2013 (a) Periventricular, coronal section from 55 year old stained with H&E shows regions where an intact ependymal is present. (b) Periventircular coronal section from 77 year old stained with H&E shows large expansions of dedudated ependymal coverage along with a diminished gap region. (c) Periventricular, coronal section from a 77yr old stained with H&E shows limited regions of intact ependyma and large areas devoid of ependymal cells (bracket). (d) Interposed between regions of intact ependyma were areas devoid of ependymal cells. (e) Immunohistochemical analysis revealed that gaps in the ependyma are filled with $GFAP^{+}$ processes (DAB staining). (f) Wholemounts of the ventricle surface (60^{+}-years) revealed areas of (g) intact ependyma

and (h, i) large expanses of GFAP+ processes (glial scar). Magnifications show (g) contiguous sheets of ependymal cells, (h) ependymal cells and small GFAP+ clusters, and (i) regions of extensive gliosis. Scale bars, 500µm (c, e) and 100µm (d), 200µm (f).

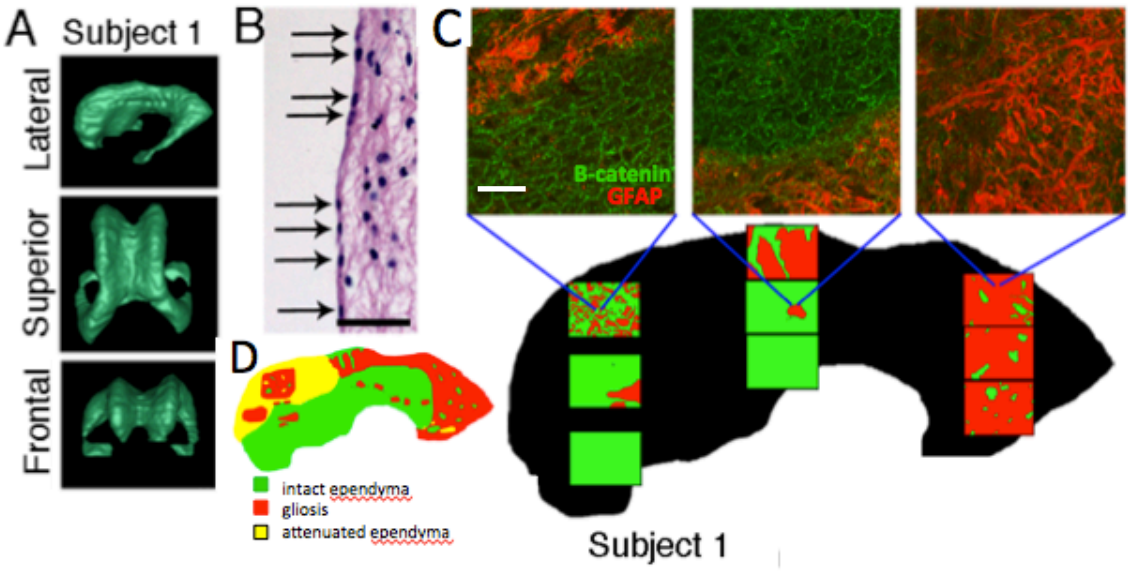

Figure 9. Subject 1 shows enlarged ventricle associated with large expanses of gliosis. Adapted from Shook et al, 2013 (A) 3D reconstruction of the postmortem MRI for Subject 1 (82 years), showing a typical, enlarged ventricle. (B) H&E staining of coronal sections show that the ependymal lining for Subject 1 contains areas of attenuated ependymal cells (C) Immunohistological examination of wholemount preparations of the lateral wall revealed that Subject 2 had large areas of gliosis, but also had areas in the ventral aspect showing intact ependyma. (D) Coded schematic of entire lateral wall of the lateral ventricle, with red indicating areas of astrocytic gliosis, yellow indicating a compromised ependymal lacking a distinct layer of cuboid ependymal cells, and green indicating intact ependymal. Scale bars, 100 µm (B); 40 µm, confocal image (C).

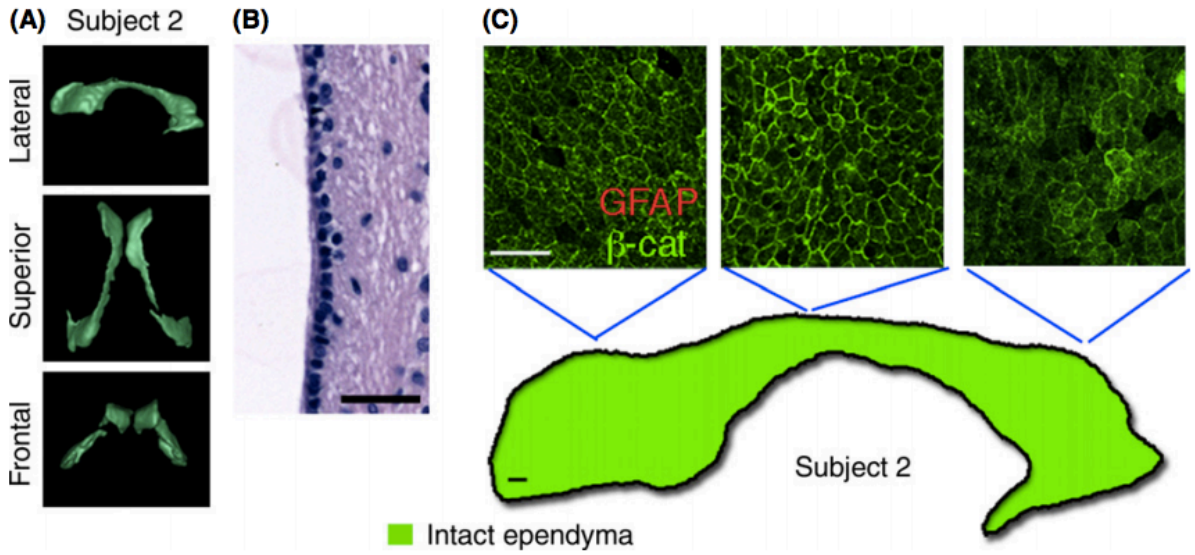

Figure 10. Subject 2 shows intact ependyma spanning the entire ventricle surface corresponding with small ventricle volume. (A) MRI-based 3D reconstructions of the lateral ventricle for Subject 2. (B) H&E staining revealed a robust ependymal monolayer, and (C) immunohistochemistry of whole-mount preparations showed uninterrupted ependymal cell coverage with no surface gliosis. Scale bars, 100 μm (B); 40 μm, confocal image (C); 1 mm, schematic (C).

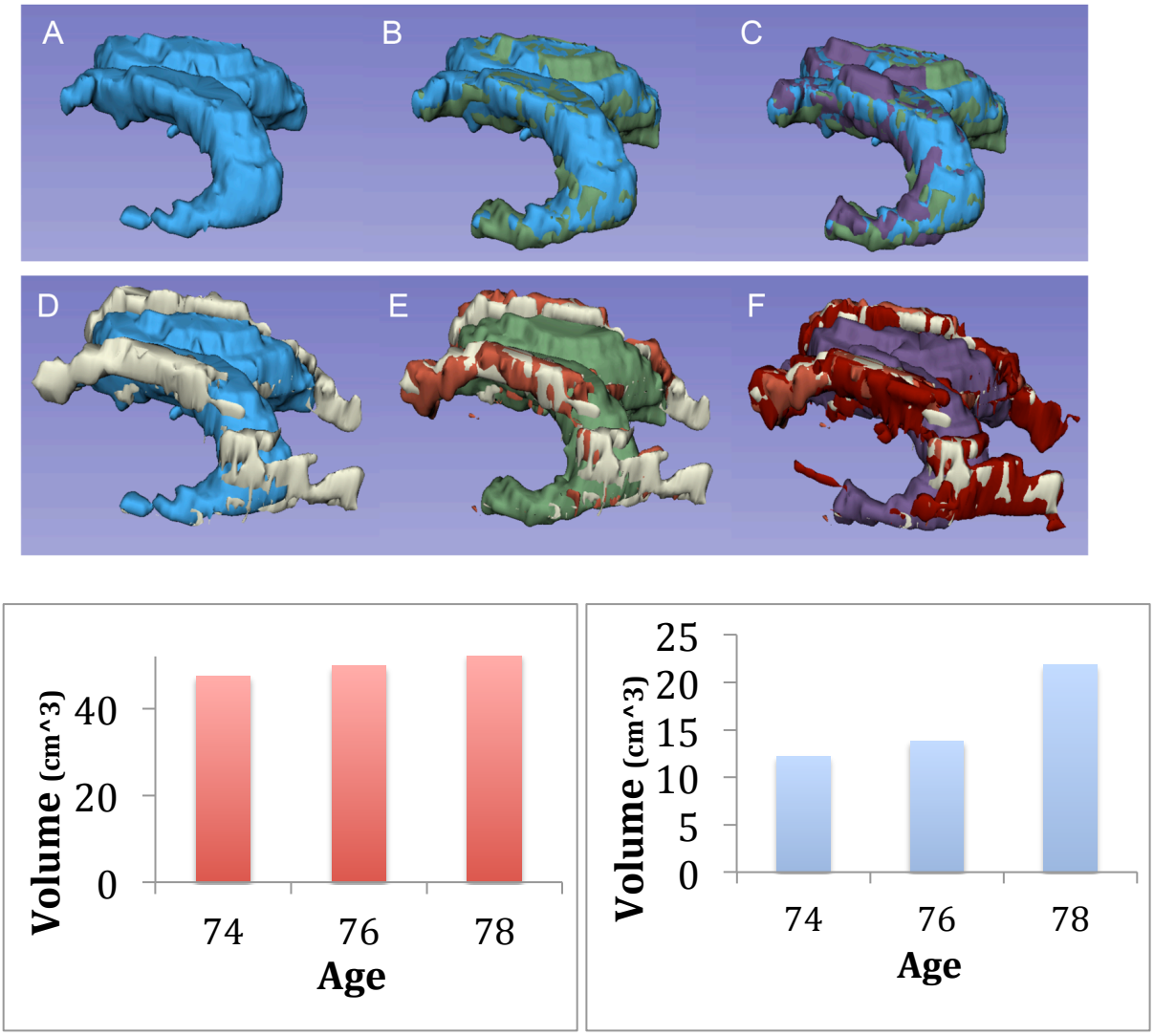

Figure 11. Volume analysis of longitudinal FLAIR MRI. (A-C) Lateral ventricle volume from subject at 74 (blue),expansion at 76 (green), and at 78 (purple). (D-F) Periventircul white matter volume from subject at 74 (white), expansion at 76 (orange), and at 78 (dark red). (G) Quantified lateral ventricle volume expansions within a 4 year range (h) Quantified periventircular white matter volume changes show correspnoding increases in periventricular white matter hyperintense areas.

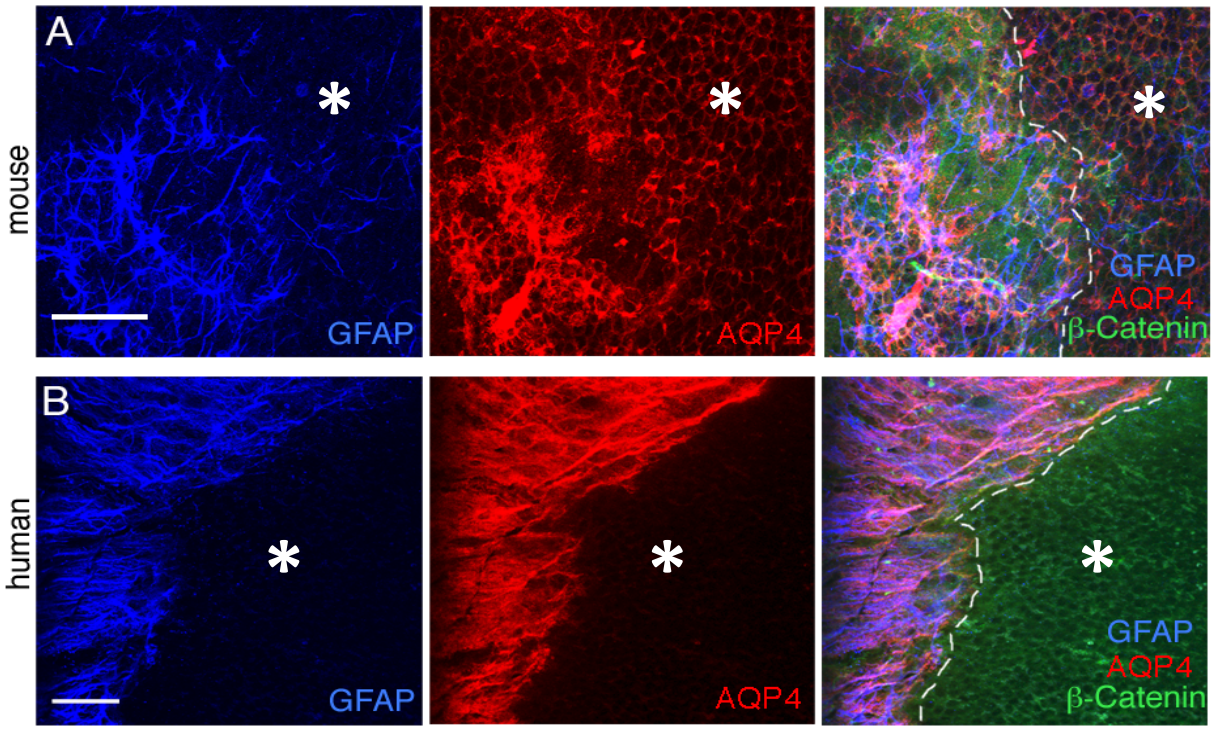

Figure 12. Aquaporin4 (AQP4) is found to be upregulated at areas of glial scarring in both humans and mice on the ventricle surface, and not in areas of ependymal coverage. Adapted from Shook et al, 2013. (A) Following intraventricular injection of neuraminidase (50 mU), areas of gliosis (GFAP+, denoted by arrows and demarcated by the dotted line) show increased expression of AQP4. Ependymal cells (*) show low levels of AQP4 staining in nonscarred regions. (B) Similarly, in human tissue, areas of surface gliosis (arrows, demarcated by the dotted line) in human tissue show increased expression of AQP4, whereas areas of ependymal cell coverage (*) are marked by b-catenin and low level expression of AQP4. AQP4 imaging was overexposed in regions of scar to show very low levels of AQP4 in intact ependymal cell layer. Scale bars, 50 μm (A); 100 μm (B).

Chapter III. Repeated Mild Traumatic Brain Injury – A Mouse Model

Methods:

A mouse model of mild traumatic brain injury from Wayne State

In partnership with Dr. Donald Kuhn from the Wayne State University School of Medicine, we have studied the effects of injury on the mouse brain, and in particular, the mouse ventricular system. The mice undergo repeated hits in Wayne state, and after perfusion and brain dissection, the fixed brains are consequently sent to the University of Connecticut for analysis. The following pertaining to the mouse injury apparatus is adapted from "A mouse model of human repetitive mild traumatic brain injury" Kane et al, 2012.

Apparatus: In order to induce injury, weights are attached to the desired mass (95g weight from a 1 m height) and put through a long PVC tube (1.5 meters in length x 20 mm in diameter). A stage is set with a piece of alumni foil, set against a Plexiglas frame that holds the mouse in place. A sponge cushion is located 10 cm below the aluminum foil to receive the falling mouse after impact.

Mouse: C57 mice are anesthetized with isoflurane for a short amount of time before being put on the aluminum foil.

Procedure: After being anesthesized, the mice were hit with the weight cranially on the scalp midline between bregma and lambda. After being hit, the mice fall freely onto a cushion (Figure 13 A,B). The mice were hit 5 times over the span of 3 days: the first two days had 1 AM and 1 PM hit, and on the last day, there was one final AM hit. After 2 hours of rest, one group of mice underwent perfusion with 0.9% saline, followed by 4% paraformaldehyde. After perfusion, the brains were extracted and fixed in 4%

paraformaldehyde overnight. Additional brains were obtained in a similar fashion 2 weeks, 1 month, and 3 months post injury (Figure 13 C). After fixation, the brains were shipped to the University of Connecticut Storrs Campus for analysis. Age-matched mice that underwent isoflurane treatment in concurrence, but without being hit, were also processed in a similar fashion.

Wholemount

Procedure: For analyses using the whole mount technique, the lateral wall of the lateral ventricle was dissected according to the procedure from Mizradeh et al. (2010) (Figure 14 A-F). Immunohistochemistry was performed using the following antibody combinations: mouse anti-b-catenin (1:250; BD Biosciences, San Jose, CA, USA); rabbit anti-AQP4 (1:400; Sigma-Aldrich); rat anti-GFAP (1:250 Invitrogen). Imaging of whole mount slides was performed on a Leica TCS SP2 confocal laser-scan microscope.

Cell Count Analysis: Ependymal cell size analysis was performed by taking cell counts of different areas within the lateral ventricle. The ventricles were divided into 6 sections – four in the anterior portion of the ventricle, one in the middle of the ventricle, and one in the posterior end of the ventricle (Figure 14 G). Using the confocal microscope, 40x images from each section were taken. From each 40x image, 9 subsections (341 u μm vs 341 μm) were divided evenly, and 3 representative sections were counted. Cells that were partially located along the left and bottom edges of the boxes were counted; those found partially along the top and right edges were not counted. Ependymal cell sizes were calculated by dividing the total area of each subsection by the average number of cells found within each subsection.

Results:

3D reconstructions of the lateral ventricles using coronal sections of the mouse brains were performed by other members of the Conover Lab. Results showed that there was a progression in the size of the lateral ventricles immediately, as well as 3 months post-injury (Figure 15). Consequently, we hypothesized that with injury, there would be an associated ependymal cell stretching and/or glial scarring. Cell counts of the 6 different regions in the lateral ventricles showed varying degrees of cell stretching along the lateral wall (Figure 16). In the anterior portion, near the areas of stenosis, there were varying degrees of ependymal cell stretching for the different time points analyzed. This could be a result of differences in extents of stenosis. In region 6, there was a more uniform cell stretch found with progressively longer time after repeated hits. In region 5, which is directly under where the brain would be hit with the weight, more uniform cell stretching could be found. When analyzing the degree of stretching as percent increase, we could see a dramatic, and sequential increase in cell size for region 5 starting from immediate post-hit to 3 months post-hit (Figure 17).

In addition to cell stretching, there was also an astrocytic response to injury. In a mouse 1-month post injury, there was the presence of glial scar found along the surface of the ventricle (Figure 18). With stretching, there was an increase in GFAP expression closer to the apical surface of the ventricle. While astrocytes in the control mice were mainly localized underneath the Aquporin-4 expressed basolateral membranes of the ependymal cells, astrocytes seem to shift up towards the apical surface of the ependymal cells, with $GFAP^+$ processes coming in line with the β-catenin stained apical membranes of the

ependymal cells (Figure 19). Ependymal cell stretching can be seen immediately post injury (Figure 19D), while the changes to the amount of astrocytes seen only later, at around 3 months post-injury (Figure 19H). This phenomenon seems to be similar to what we see in humans, when there is a loss of ependymal coverage along with an associated astrocytic shift (Figure 8A,B).

Discussion:

In this model of repeated mild traumatic brain injury, we wanted to see how injury could affect the ependymal integrity in the brain. In this model, mice undergo both blunt force impact to the head, as well as a consequential rapid rotational acceleration of the body and head, an essential characteristic known to be important for concussive injury in humans, and a factor that is missing from many existing animal models of TBI (Kane et al, 2012). In addition, despite extensive research on the role of astroglial response to injury in the hippocampus and cortex, not many look at how astrocytes are responding to injury along the ependymal lining of the ventricles. While we have observed that there are no significant changes in ependymal cell size or ventricle volume in aging in mice (Shook et al, 2013), we show here that the effects of repeated injury leads to not only ventricle volume changes but also ependymal cell size increase in this model of rmTBI.

A better understanding of how repeated injuries on the mouse ependymal lining would be crucial in elucidating how repeated injuries affect humans, both acutely, as well as chronically. Currently, the physiological basis for repeated mild traumatic brain injury is not well understood. Chronic traumatic encephalopathy (CTE), a progressive degenerative disease that can only be diagnosed post-mortem, is associated with multiple concussions.

In addition, despite it being a hallmark of the disease, how ventriculomegaly develops as a result of repeated hits is not understood (Mez et al, 2013). Because of the variability in human response to repeated injury, and the difficulties in controlling for human injury conditions, it is important to study the effects of injury on a mouse model that can accurately portray what is happening in humans. Thus, from this study, we hope to elucidate how ventricle expansion is associated with and potentially contributing to late-stage neurodegeneration in humans that have multiple concussions in life. Already, we have shown that ventricle expansion can be induced in this mouse model of rmTBI, and that there is an apparent astroglial response to injury that could be an indication of glial scarring along the ventricle wall. The timeline of when these two phenotypes appear is interesting to note. While ependymal stretching and ependymal volume increase seem be evident immediately post injury, the injury response from astrocytes doesn't seem to appear until at least 1 month post injury, with the greatest amount of GFAP+ astrocyte processes found at 3 months post injury. This apparent difference in response time could help elucidate how and when human ventriculomegaly and potential glial scarring appear due to injury. Further studies, however, would be needed in order to establish exactly how astrocytes are responding to injury, and how extensive injuries would be needed in order to induce glial scarring.

Of course, due to the differences in the regenerative capacity of the SVZ, the way that the mouse responses to injury will be inherently different from the way that the human responds to injury. However, with the presence of glial scarring, and extensive ependymal cell stretching found, it is evident that the regenerative capacity of the mouse SVZ is not enough to compensate for the damage done to the ependymal layer due to

repeated hits. Thus, when extrapolating this information and relating it to humans, it would be important to take this into consideration. Overall, further research on how injury affects the ependymal lining could be instrumental in improving our understanding of rmTBI and CTE in humans. By modeling repeated injuries in the mouse, we could extract valuable insight into how humans could potentially respond to injury.

Figures:

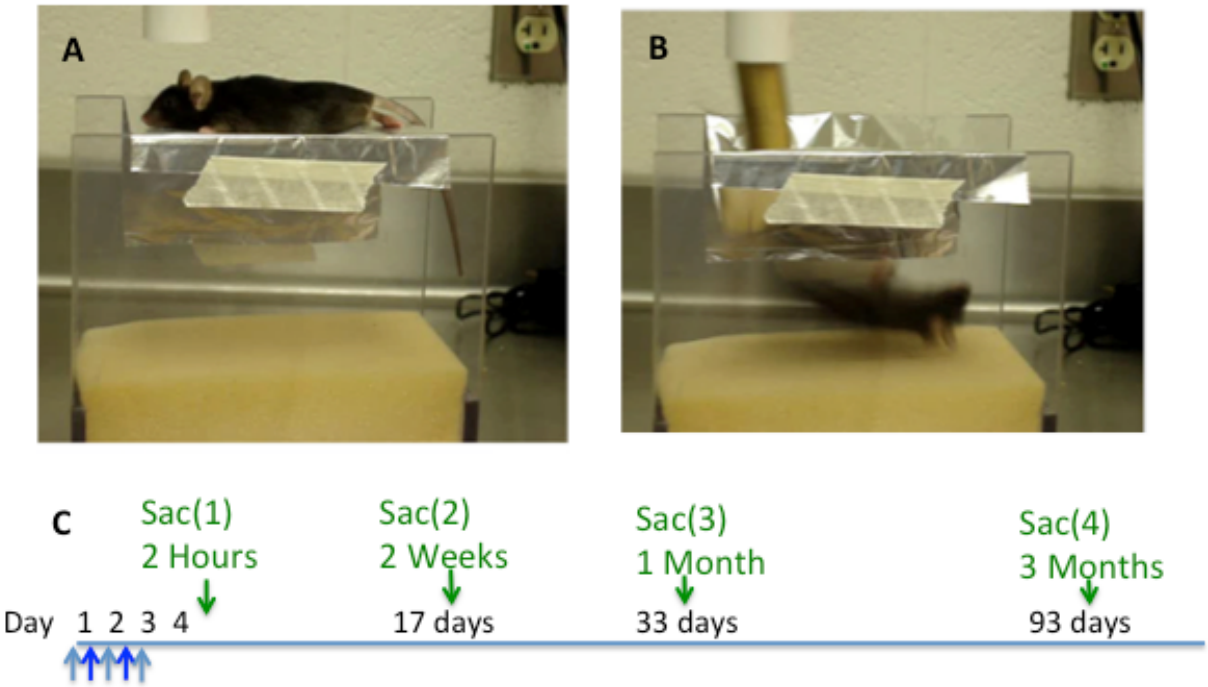

Figure 13. A mouse model of repeated mild traumatic brain injury. Adapted from Kane et al, 2012. A) A mouse is situations on a platform before hit. B) After hit, the mouse falls through and lands on a foam cushion. C) A timeline of hits (AM hits in light blue, and PM hits in dark blue) as well as when brains are dissected.

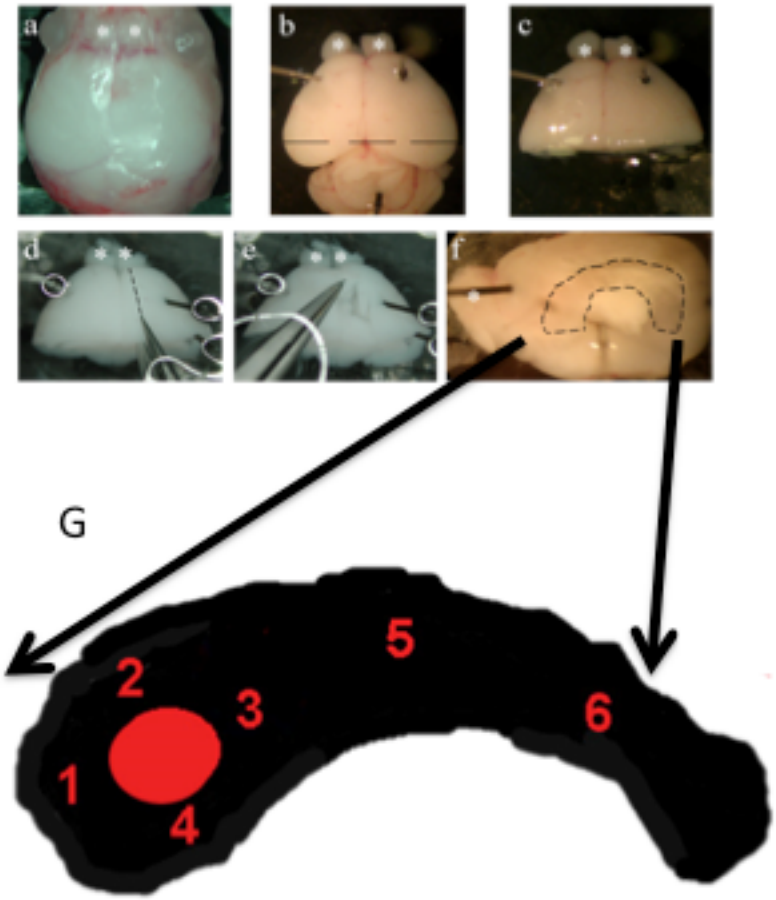

Figure 14. Wholemount dissection of the lateral ventricle and cell count analysis. (A-F) Dissection of the lateral ventricle *en-face* as according to Mizadeh et al, 2010. (G) A schematic of the lateral wall of the lateral ventricle after wholemount dissection. 40x images were taken from each of the regions shown using a confocal microscope. Regions 1-4 are found in the anterior, dorsal, posterior, and ventral regions surrounding the area of stenosis. If there was no stenosis, each region was approximated. Region 5 is found in the middle of the ventricle, and region 6 is found in the posterior portion of the ventricle. After a 40x image was taken from each region, ependymal cell counts were done in each region.

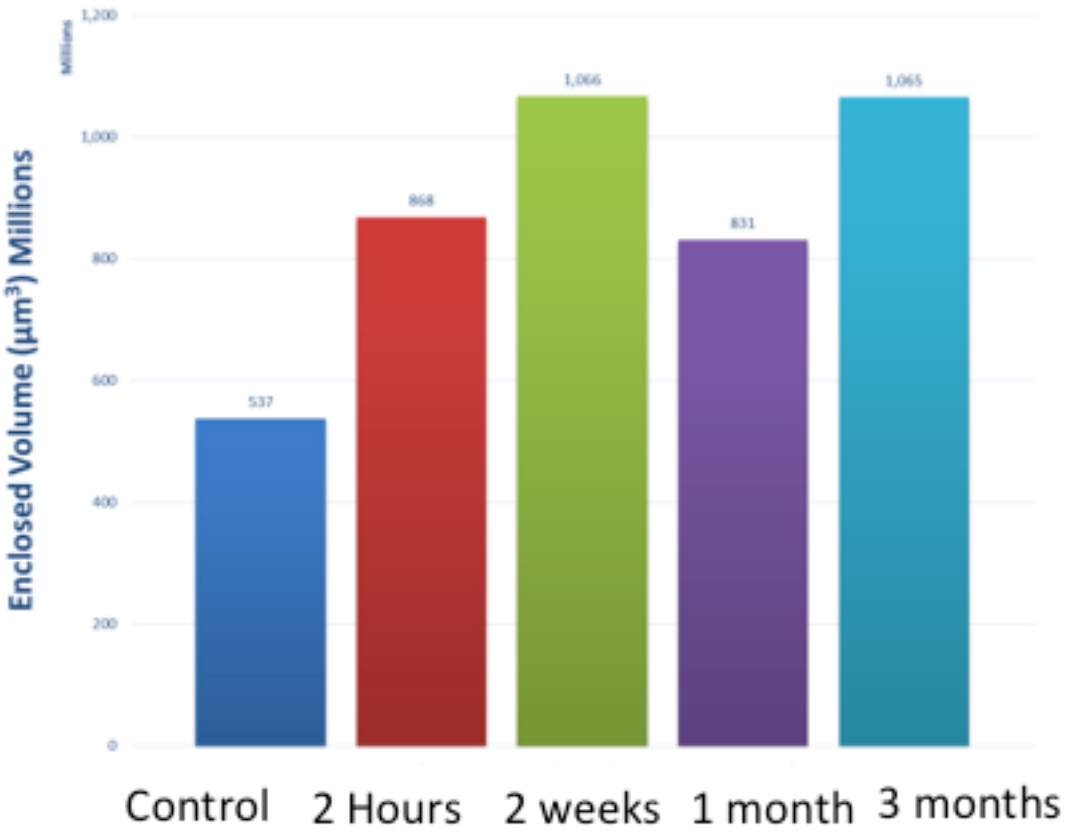

Figure 15. Ventricle volume expansion due to repeated hits. Adapted from Richard Wolferz. 3D reconstructions of the lateral ventricles were done using 50 µm coronal sections of the brain in order to obtain the lateral ventricle volume.

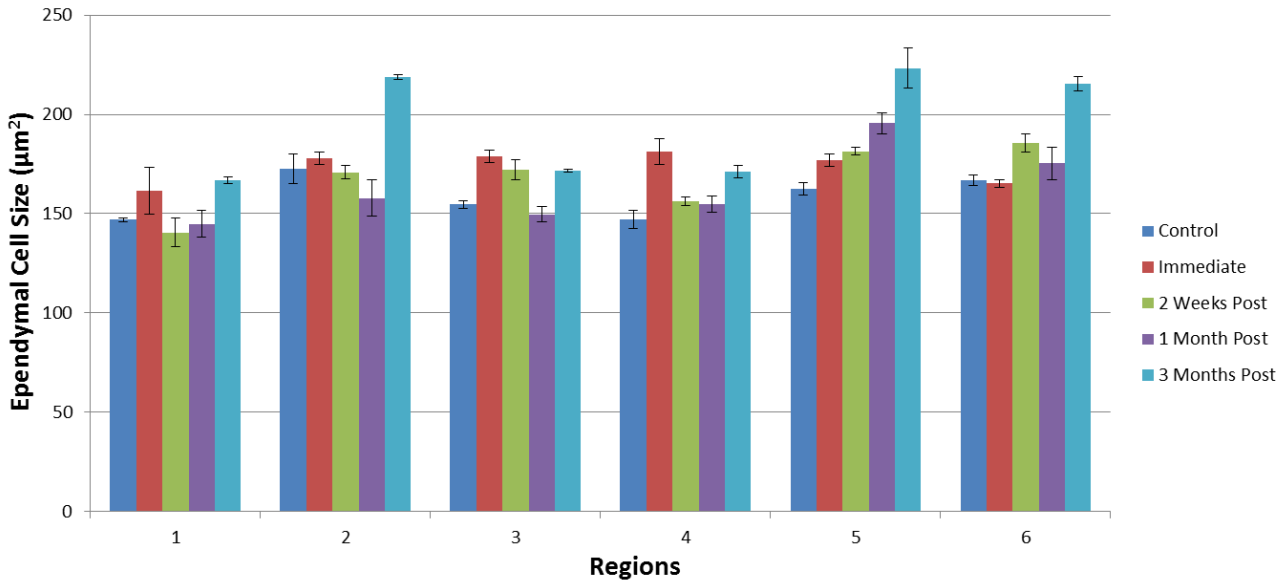

Figure 16. Whole-mount analysis of ependymal cell stretching along the lateral ventricle. (a-f) Whole mount dissection of the lateral wall of the lateral ventricle, shown by the dotted line. Asterisks mark the olfactory bulbs. (g) Schematic of cell counting analysis. 40x images were taken from each region shown, and ependymal cell sizes were evaluated by counting the number of cells in each region. (h) Average ependymal cell sizes from control (n=2) and brains from different time points post-hit (each time point, n=2).

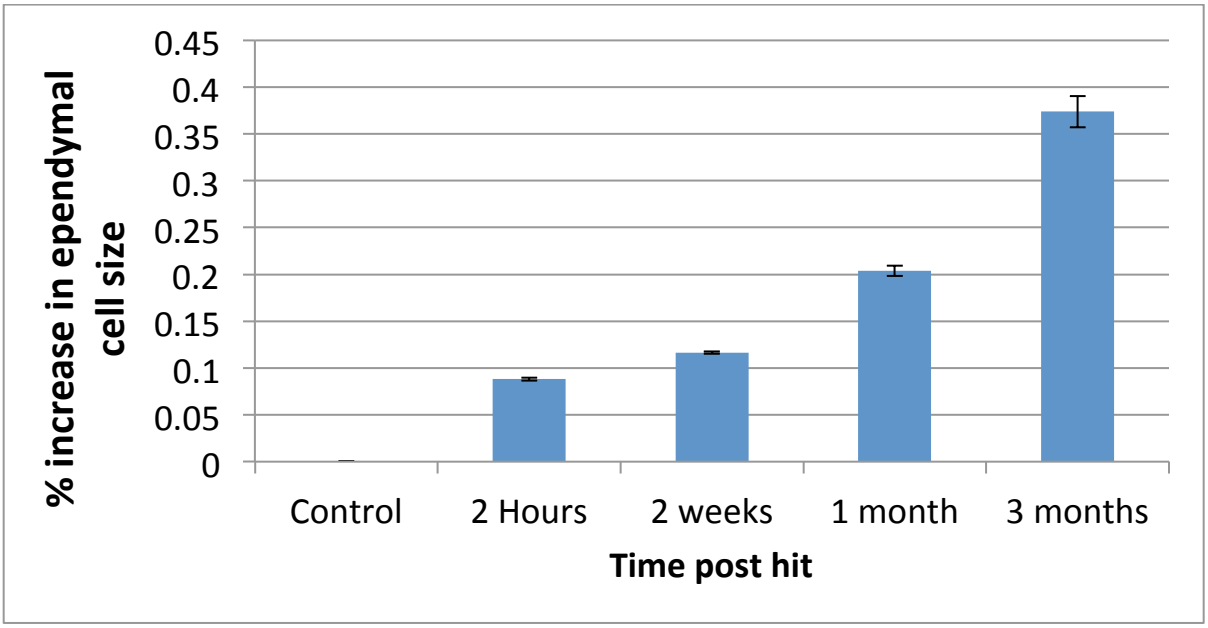

Figure 17. Percent increase in ependymal cell size in hit brains when compared against control in Region 5.

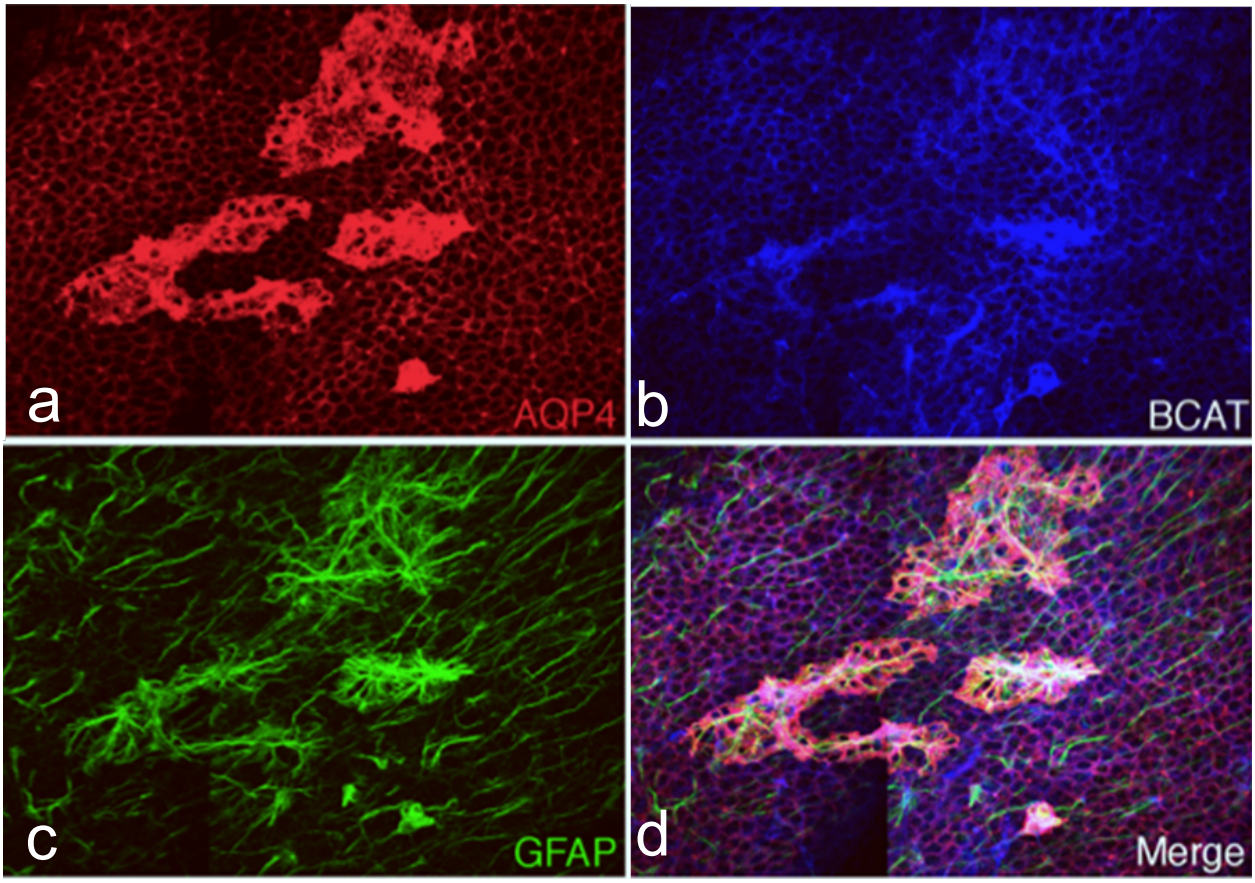

Figure 18: Presence of glial scarring along the lateral ventricle 1 month post- injury. Adapted from Meredith Halling. (a-c) Large areas of astrocytic gliosis on the lateral ventricle surface observed in wholemount. (a) AQP4 is labeled in red, and up-regulated in the presence of reactive gliosis (b) b-Catenin labels ependymal cell surface (c) GFAP is labeled in blue with up-regulation indicating astrocytosis. (d) Co-localization of GFAP and AQP4 indicate scar.

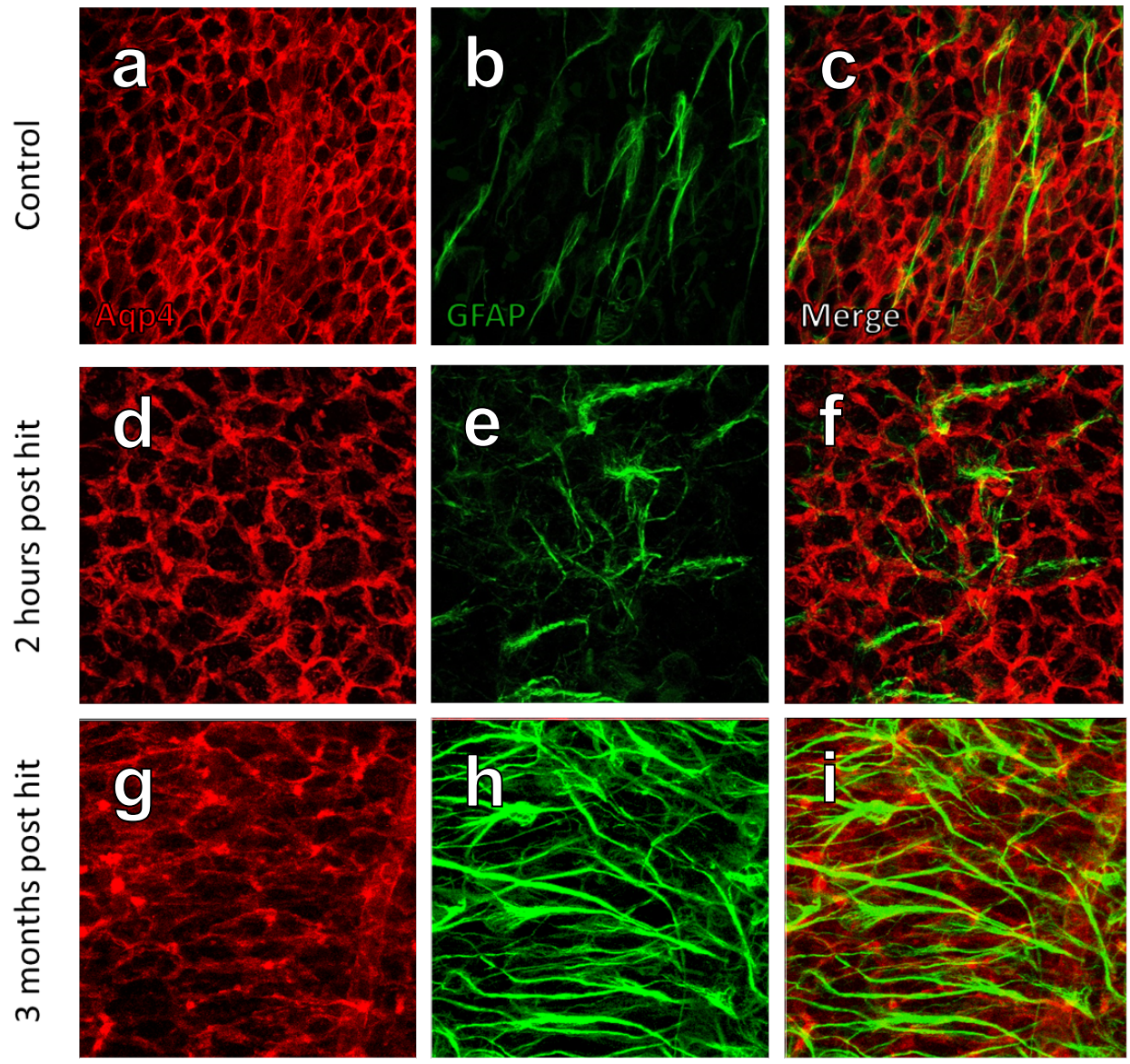

Figure 19: Ependymal cell stretching and up-regulation of astrocytes post injury using 100x confocal images taken from region 5. (a, d, g) Aquaporin 4 (AQP4) staining outline the basolateral side of ependymal cells. Cell stretching is evident post injury. (b, e, h) Glial fibrilary acidic protein (GFAP) staining outlines astrocytes found along the lateral ventricle. There is an upregulation of astrocytes at the surface of the ventricles, especially 3 months post injury (c, f, i) Co-localization of GFAP and AQP4.

Chapter IV. Conclusion

Here, we report numerous age-related and injury-induced changes to the ependymal barrier and the lateral ventricle structure in humans and mice. The first question we tried to understand is the causative relationship between the two phenomena found to be associated with aging: ventricle expansion and glial scarring. Does ventricle expansion cause glial scarring? Or does glial scarring cause ventricle expansion? This "chicken or the egg" question is one we have tried to address here. As it turns out, the answer to the question is not so clear. As a part of results reported, but not presented here, our lab has found that with neuraminidase injections in mice, which causes the degradation of ependymal wall (Grodona et al, 1996), there is the presence of glial scarring along the ventricular wall. In addition, there is an expansion in ventricle volume, but no change in total brain volume (Shook et al, 2013). This refutes what has been commonly thought to be the sole basis of ventricle expansion, namely that it is due the atrophy of the rest of the brain. However, this also does not prove that glial scarring is the basis of ventriculomegaly either. Changes in CSF production and clearance could also contribute to ventricle expansion, making glial scarring a reparative mechanism of the brain in response to the strain put upon the ependymal cells due to the expanded ventricular wall. Thus, while we cannot answer for sure whether glial scarring causes ventricle expansion or the other way around, it is clear that simply attributing ventricle expansion to be a result of brain atrophy is insufficient. In addition, in humans, by using post-mortem MRI analysis paired with histological analysis, this methodology offers us a way of directly relating glial scarring along the ventricle surface to expanded ventricle size. As ventricle expansion is a

phenomenon seen in numerous neurodegenerative diseases, such as Parkinsons, Alzheimer's, hydrocephcephalus, as well as neurological illnesses such as autism, ADHD, and schizophrenia (Palha et al, 2012), our study offers a way of looking at these diseases from the perspective of the ventricular wall, as well a potential way of understanding how glial scarring may be implicated in these diseases.

Additionally, our study on aging offers an effective baseline to understanding many of the neurodegenerative diseases mentioned. As many neurodegenerative diseases are "age-related", the definition of "normal-aging" can be hard to define. Here, we assume normal aging to be non-demented aging, with no other clinically relevant neurological disorders. By establishing how the ventricles change in normal aging, this could help clinicians in better understanding, and potentially diagnosing neurodegenerative diseases. Here, we see that aged humans develop ventriculomegaly and associated glial scarring along the ventricular surface. Unlike mice, the regenerative capabilities of the underlying SVZ do not contribute to the maintenance of the ependymal lining. Using our methodology of evaluating longitudinal MRIs, it offers a breadth of possibilities incorporating noninvasive imaging as a ways of assessing how the ventricle lining integrity may be changing. Our preliminary study on FLAIR MRIs shows the possibility of evaluating the extent of gliosis along the ventricular wall using longitudinal volumetric analysis. In addition, as we observe regional differences in the integrity of the ventricle lining using histological analysis, we have also identified certain parts of the ventricle that may be more prone to expansion using longitudinal MRIs. Better understanding of the exact correlation between areas of gliosis, ventricle expansion, and hyperintensities on the FLAIR MRI could offer significant insight into how to interpret MRI results on a clinical setting.

Indeed, the importance of using imaging techniques to diagnose neurological disorders can be especially seen in the boom in radiological research in traumatic brain injury, and in particular repeated mild traumatic brain injury, the most common type of brain injury in humans (Rutland-Brown et al, 2006). Increasingly, clinicians as well as scientists are starting to use imaging techniques such as diffusion tensor imaging (DTI) in better understanding the progression of neurodegeneration following repeated hits to the brain. DTI offers a way of evaluating axonal degeneration in the brain, which is another hallmark of CTE (Sidaros et al 2008). This could potentially change how we view CTE as a disease, as up until now, it has been a disease that can only be diagnosed post-mortem. With our study, we hope to ultimately be able to use our understanding of the changes in the ventricle system to help better diagnose a condition such as CTE. Because of the difficulties of studying a disease with such varying degrees of phenotypes in humans (Chauhan, 2014), we decided to use a mouse model of rmTBI in order to better evaluate changes to the ependymal layer due to injury. Of course, with any mouse model, it is important to take into consideration the inherent differences between humans and mice. Already, we have seen that with aging, mice seem to exhibit greater capabilities to regenerate and maintain ependymal coverage through aging. Our results show that when there are repeated hits to the brain, the effects of such impacts on the brain seem to surpass the regenerative capabilities of the mouse SVZ. As reported, the presence of glial scarring along the surface, along with ependymal cell stretching and ventricle volume increase, and increased astrocytic processes near the surface of the ventricles contrast greatly with how the murine ventricular system seem to progress under normal circumstances. Thus, while mice and humans may be inherently different when it comes to

their neurogenic niche capabilities, we could extract inferences as to how humans could potentially respond to injury. Further research, of course, is needed to understand how exactly the human ventricular system responds to repeated injuries. However, for now, it seems that an astroglial response to injury could play a role in ventricle expansion and the late-stage progression of ventriculomegaly in cases of CTE.

Moving forward, there are numerous experiments that could be done in order to better understand the results presented here. In the rmTBI model, the presence of stenosis along the ventricle surface offers an interesting challenge to the model. How does the presence of ventricle expansion affect the area of stenosis? If there is increased pressure on the ventricular system due to repeated hits, could stenosis be relieved as a result of such hits? It is interesting to note that in many of the mice studied, there were small areas of glial scarring found near areas of stenosis, perhaps indicative of the impact on the stenosed areas. In addition, how does rmTBI manifest itself in MRI scans of patients? And how does it manifest itself in FLAIR MRI scans? Further results by looking at longitudinal FLAIR MRIs in this area could effectively link what we have found in aging to rmTBI. Thus, differentiating the progression of the two processes – normal aging and injury, could offer great clinical insights.

On a final note, our research here, relating histological research and associated radiological findings, serves as a potentially powerful way of understanding neurological disorders. Too often, research labs are restricted to only understanding the clinical basis of a disease through extensive MRI analyses, or the physiological basis of a disease. Here, we have shown a potential way of relating mouse research, human tissues analysis, and radiological analysis together. As seen in our multidisciplinary way of looking at aging, we

have not only identified a limitation of the mouse model in modeling aging in humans, we have also established a clear clinical application to our research. As science become more integrative and collaborative across disciplines, we need to establish clear links between mouse research and human pathology, and extend that knowledge to the clinic. Research is ultimately aimed at the betterment of treatments and diagnosis for the human population. With that clear goal in mind, we aim to move forward with our multidisciplinary approach to looking at neurodegeneration in the brain, and in particular its affects on the ventricular system, an area of research that is generally understudied across disciplines. With this research, we hope to highlight the clinical importance of maintaining the health of our ventricular system, and it's critical role in injury and disease.

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