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An Analysis of the Association Between Ependymal Integrity and Ventriculomegaly

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An Analysis of the Association Between Ependymal Integrity and Ventriculomegaly

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Thanks to my family for their long-distance encouragement.

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Abstract

The ventricles of the brain are lined by a monolayer of ciliated ependymal cells that act as an interface between the circulating cerebrospinal fluid and the underlying tissue. Along the lateral walls of the lateral ventricles, the ependymal cells have additional significance as they are considered one of the cell types that comprise the subventricular zone (SVZ) neurogenic niche. Since lateral ventricle expansion is a well documented occurrence in not only a variety of neurological disorders but also with normal aging, it is important to consider the effect of this expansion on the ependyma. By combining MRI-based 3-D reconstructions of the lateral ventricles with thorough immunohistochemical evaluation of the lateral walls of aged human brains, we confirmed that ventriculomegaly is associated with a severely compromised ependyma and gliosis at the ventricle surface. To determine whether any directionality exists between gliosis and ventricle enlargement, we induced ependymal gliosis in mice, who do not display either gliosis or ventricular expansion with age under normal circumstances. We found that not only are the resulting scars phenotypically similar to those observed in humans, but that the lateral ventricles of mice with widespread scarring caused by neuraminidase have increased volume compared to saline-injected controls. This work establishes a link between periventricular gliosis and enlarged ventricle volumes and supports the hypothesis that damage to the ependyma and the resulting scarring can affect ventricle volume.
INTRODUCTION/BACKGROUND

Brain Ventricles

The ventricular system of the brain is a network of communicating cavities that include the two lateral ventricles, the third ventricle, the cerebral aqueduct, and the fourth ventricle. The choroid plexus, a structure comprised of epithelial cells located inside the ventricles, produces the cerebrospinal fluid (CSF) that fills these hollow spaces.

The ventricles are not merely static fluid-filled cavities; rather, the CSF-filled ventricles are a dynamic pressure system that has biophysical and biochemical effects on the surrounding parenchyma (Johanson et al. 2011; Sakka et al. 2011). In addition to the well-known action of hydromechanical protection of the brain, the CSF plays a crucial role in maintaining homeostasis of cerebral interstitial fluid, as well as elimination of potentially toxic catabolites, regulation of electrolytes, and circulation of active molecules and neuropeptides (Mori et al. 1990; Veening and Barendregt 2010). The entire ventricular system is lined by a single layer of cuboidal epithelium called the ependyma (Del Bigio 1995; Lennington et al. 2003; Conover and Shook 2010). This monolayer of multi-ciliated cells acts as an interface between the CSF and underlying tissue (FitzGerald et al. 2002; Del Bigio 2010).

In addition to these functions, the ependyma of the lateral walls of the lateral ventricles have even more significance due to the role they play in a specialized niche that allows for adult neurogenesis, the subventricular zone.
Adult Neurogenesis

The idea that new neurons can be generated in the adult brain has only been broadly accepted by the scientific community fairly recently. For decades, it was believed that the adult brain was an unchanging organ, unable to regenerate or create any new neuronal connections, until work done by Joseph Altman in the 1960’s suggested that neurogenesis occurred in the subgranular zone (SGZ) in the dentate gyrus of the hippocampus as well as in the subventricular zone (SVZ) at the forebrain (Altman 1962; Altman and Gopal 1965; Altman and Das 1967). However, this discovery of adult neurogenesis was greeted by such a large amount of skepticism and outright rejection that the field essentially stalled. It wasn’t until years later as other scientists began documenting neurogenesis in songbirds, reptiles, and mammals that interest was re-ignited (Goldman and Nottebohm 1983; Perez-Caellas et al. 1996; Garcia-Verdugo et al. 2002). While there is continuing research on whether areas of the brain such as the neocortex, striatum, and amygdala demonstrate neurogenic activity (Gould, 2007), there is definitive evidence that neurogenesis in the adult mammalian brain persists in two regions: the SGZ of the hippocampus, and in the SVZ along the lateral walls of the lateral ventricles (Gonzalez-Perez 2012).

The Subventricular Zone Niche

The SVZ contains the largest capacity for neurogenesis in the adult mammalian brain. Four cell types contribute to the architecture of the SVZ—neuroblasts (type A cells), ependymal cells (type E cells), transit-amplifying progenitor cells (type C cells), and astrocytes (type B cells) (Imura et al. 2003; Doetsch et al. 1997, 2003; Conover et al. 2008, 2010). An additional cell type, the stem cells of the SVZ, have been identified as a subpopulation of astrocytes that have an apical process with a primary cilium that contacts the ventricle/CSF and a basal process that
The subventricular zone has five primary cell types: ependymal cells, neural stem cells, Type C cells, neuroblasts, and astrocytes. A monolayer of ciliated ependymal cells (yellow cells) separate the underlying cells, including astrocytes (green cells) and type C cells (blue cells) from the CSF. Incorporated into the monolayer are the apical processes of the neural stem cells (purple cells), which also basally contact the blood vessels (orange).
extends to the blood vessels underlying the niche (Fig i) (Doetsch et al. 1999; Lennington et al., 2003; Conover and Shook 2011). In an en face view of the lateral ventricle wall, pinwheel structures comprised of ependymal cells arranged around clusters of the GFAP+ apical processes of neural stem cells can be seen (Mirzadeh et al. 2008).

These ventricle-contacting stem cells generate the intermediate progenitors (type C cells), which differentiate into proliferative neuroblasts. The new neuroblasts generated in the SVZ migrate forward in chains along the lateral ventricle and then through the forebrain following a restricted path termed the rostral migratory stream (RMS) (Doetsch et al. 1999). The RMS ends at the olfactory bulbs, where neuroblasts differentiate into two types of local interneurons and express mature neuronal markers (Luskin, 1993; Doetsch and Alvarez-Buylla 1996; Gheusi et al. 2000).

While large amounts of data are available on the subventricular zone of adult rodents, the degree of activity in the subventricular zone of humans is not as well defined. The adult human SVZ has a unique organization that consists of the ependyma, a hypocellular gap region of undetermined purpose that is hypothesized to be the residual pathway of migrating neuroblasts, and a highly organized ribbon of astrocytes that may be the location of neural stem cells in humans (Fig ii) (Sanai et al. 2004). However, whether migratory neuroblasts are present or not is still subject to debate. While some groups describe no evidence of neuroblasts in the human SVZ or RMS, other researchers report contradicting data that demonstrates the presence of small numbers of migrating neuroblasts in the SVZ and RMS (Sanai et al. 2004; Curtis et al. 2007; Wang et al. 2011). Investigations into this area are made more difficult by the realities of human-based studies: the logistics involved in obtaining human tissue are often complex, only endpoint analysis is feasible for most human studies, and individual variation is highly likely.
Fig ii. The organization of the human subventricular zone
(Adapted from Sanai et al. 2004)

On the right, DAPI staining shows the gap layer between the ependyma and the ribbon, a structure thought to be the corridor for migrating neuroblasts. On the left, GFAP staining shows the astrocytic ribbon that is characteristic of the human subventricular zone.
among humans. Because of these obstacles, investigation of adult neurogenesis in humans continues to be an uphill battle.

**Ependymal Damage**

There is evidence that an intact ependyma is important in the proper maintenance of the surrounding neuronal tissue as well as the SVZ. In aged humans, areas of ependymal denudation are associated with the severity of periventricular white matter lesions as visualized by MRI (Fernando et al. 2006) while in hydrocephalic human fetuses, the destruction of ependyma results in a disorganization of the neurogenic subventricular zone and abnormal migration of neuroblasts (Dominguez-Pinos et al. 2005). Studies in mice have indicated that intact ependyma and access to CSF may be necessary for SVZ neurogenesis. One of the age-related changes that have been shown to occur in the cytoarchitecture of the murine SVZ is stenosis of the ventral walls of the lateral ventricles. The walls gradually adhere together and eventually fuse, resulting in a loss of ependymal cells in the areas of fusion (Luo et al. 2006; Shook et al. 2012). During this normal process of ventricular stenosis, it has been demonstrated that the process of ventricular adhesion not only results in a loss of ependymal cells, but in degradation and ultimate loss of the neurogenic niche underneath (Shook et al., 2012).

There are many potential causes of damage to the ependyma in humans during both fetal and adult life. Ependymal cells are particularly susceptible to infection by viruses such as herpes simplex, cytomegalovirus, and the mumps (Takano et al. 1999; Kawasaki et al. 2002; Braun et al. 2006), while hydrocephalus and non-syndromic ventricular expansion can greatly damage the ependyma (Sarnat 1995; Del Bigio 1995). Many neurological disorders such as schizophrenia, Alzheimer’s disease, and traumatic brain injury are associated with an increase in volume of the lateral ventricles (Juuhl-Langseth et al. 2012; Palha et al. 2012; Poca et al. 2005; Daneshvar et al. 2006).
2011). However, in addition to pathological ventriculomegaly, there is a well-documented ventricular expansion associated with normal, non-impaired aging (Pfefferbaum et al. 1994; Sowell et al. 2007; Raz et al. 2010).

Any noteworthy increase in ventricular volume would be expected to compromise the form and function of the ependymal lining, though the exact response and progression of damage to the ependyma is not well documented. In the present study, we combine information on the ventricle volume obtained from MRI-based 3-D reconstructions of the lateral ventricles with a thorough histological evaluation of the lateral walls of aged human brains to examine to what extent ependymal integrity is compromised by ventricular expansion.

**Modeling Ependymal Damage in Mice**

Because extensive ependymal loss and resulting gliosis does not occur normally in mice (Luo et al. 2008), in order to study the consequences of expansion and periventricular gliosis, scarring must be induced in a mouse model. This can be done using the enzyme neuraminidase.

Neuraminidases are a large family of enzymes found in viruses and bacteria. Interestingly, neuraminidase, along with another surface protein called hemagglutinin, is used in the classification of influenza type A viruses (WHO Memorandum, 1980). Depending on the particular serotype of hemagglutinin (H) and neuraminidase (N) present, the viruses are categorized and given names such as the notorious H1N1 (swine flu) or H5N1 (avian flu).

In the CNS, sialoglycoproteins are involved in cell adhesion and function in maintaining the integrity of the ependymal monolayer (Kuchler et al. 1994; Figarella-Branger et al. 1995). Neuraminidase hydrolyzes terminal sialic acid residues to cleave glycosidic linkages (von Itzstein, 2007). When introduced to the mammalian ventricular system, the cleaving of the
sialoglycoproteins of ependymal cells results in denudation of individual cells and a degradation of the ependymal wall (Grondona et al. 1996). Low doses of neuraminidase cause small levels of ependymal denudations that can be repaired by stem-cell-mediated mechanisms, while higher doses of the enzyme result in such severe amounts of ependymal loss that the repair mechanism is apparently overwhelmed, and instead of ependymal repair, astrocytic gliosis is instead observed at the ventricle surface (Luo, 2008).

For our work, we use this neuraminidase-induced model of ependymal gliosis in mice to investigate if this periventricular gliosis can lead to ventriculomegaly.

**Current Work**

The study presented here addresses the relationship between lateral ventricle volume and periventricular gliosis along the ventricle surface. We first examined the ventricle lining in human tissue in the first reported use of en face whole mount preparation and imaging of human ependyma. We then used two human brains as case studies, selected for their dramatically different ventricle volumes, and combine 3-D reconstructions from post-mortem MRI’s with extensive immunohistochemistry to assess the connection between enlarged ventricles and ependymal gliosis in aged humans. In order to continue our investigation it was necessary to develop a mouse model of periventricular gliosis, which was evaluated for its application to our studies. As evidence indicated that the model was suitable, we utilized the mouse model to test whether extensive ependymal damage and the resulting gliosis would result in an increased ventricular volume.
METHODS AND MATERIALS

**Human Tissue Immunohistochemistry**

Postmortem human brain tissue (hemispheres and slices) was obtained from the Harvard Brain Tissue Resource Center (McLean Hospital) and from the UCHC Department of Anatomic Pathology and Laboratory Medicine. Tissue was fixed in 10% formalin (minimum 2 weeks, maximum 6 weeks), rinsed thoroughly in 0.1M PBS and wholemounts of the lateral ventricle lateral wall were processed for antigen retrieval and immunohistochemistry. Wholemounts of all regions of the lateral ventricle (Subject 1: 18 15mm x 8mm sections and Subject 2: 36 15mm x 8mm sections) were prepared as described above. Coronal sections were taken from the superior-most region of the anterior lateral ventricle to investigate the integrity of the ependymal lining. For immunoperoxidase labeling, 8µm paraffin sections were processed using a Bond Max autostainer (Leica Microsystems). Hematoxylin and eosin staining was performed as previously described (Luo et al. 2008).

**Human Lateral Ventricle Volume Measurements**

For evaluation of lateral ventricle volumes across age, high-resolution MRI sets were obtained from the Open Access Series of Imaging Studies (OASIS) database (www.oasis-brains.org) (Marcus et al. 2007, Marcus et al. 2010). Only male and female subjects remaining non-demented (clinical dementia rating < 0.5) throughout the length of the study were analyzed (Marcus et al. 2007). MRIs of younger individuals (non-demented subjects age 20 to 34-years) were obtained from Yale Medical School. In total, 70 individuals were analyzed in six different age groups: 19-39 (n=23), 40-69 (n=6), 60-69 (n=10), 70-79 (n=13), 80-89 (n=13), and >90 (n=5). The lateral ventricles were defined on high-resolution MRI T1-weighted images using a
semi-automated 3-D segmentation tool, ITK-SNAP (www.itksnap.org) (Snippert et al. 2010). Pre-processing of the images was performed prior to automatic isolation of the lateral ventricle, including setting an upper threshold for image intensity at 30%. For automatic segmentation, lateral ventricle expanding balloon force was set to 3.0, detailed curvature force set to 0.20, and 125 iterations at step size 1 was sufficient to isolate the lateral ventricle. Volumes were rendered using Slicer3D [www.slicer.org]. Longitudinal MRI sets (OASIS) were used from healthy subjects that underwent MRI scanning in three or more sessions spanning multiple years (Marcus et al. 2010).

**Human Post-Mortem MRI**

Post-mortem MRI was performed using a 1.5 Tesla MR unit (Siemens Avanto). A contiguous thin section (1.3mm), 3-D T1-weighted MP RAGE sequence was performed on formalin-fixed brains of two individual subjects. Subject 1 (male, 86 years) died of cardiac arrest and Subject 2 (82 years) was diagnosed with Alzheimer’s disease. Lateral ventricle volumes were processed and rendered, as described above.

**Mouse Intraventricular Neuraminidase Injections**

To introduce neuraminidase into the lateral ventricle, 3-4 month old male CD1 mice were anesthetized using isoflurane in 2% oxygen and then positioned in a stereotaxic apparatus (Stoelting). Body temperature was maintained using heating pads. A syringe loaded with 1µL (50mU) of diluted neuraminadase from *Clostridium perfringens* (Roche Diagnostics) was slowly lowered into the right lateral ventricle using the following coordinates relative to bregma: bregma: 0.0mm, lateral: 0.75mm, ventral: 2.25mm. The neuramindase was injected at a rate of 0.5µL/min. Saline injections into the ventricles of littermates were done as a control. All animal
procedures were performed under protocols approved by the Institutional Animal Care and Use Committee of the University of Connecticut and conform to National Institutes of Health guidelines.

**Mouse Tissue Immunohistochemistry**

At 2 months after neuraminidase injection, mice were deeply anesthetized with isoflourane and then perfused transcardially with 0.9% saline followed by 3% paraformaldehyde (PFA) in PBS. Brains were removed and fixed in 3% PFA overnight at 4°C. The brains were sectioned into 50 µm section using a Leica vibratome and then processed for immunohistochemistry.

Free floating brain sections were blocked and permeabilized for 1 hour at room temperature in a solution of 10% horse serum in PBS/0.1% Triton X-100. Sections were incubated overnight (12 hours) with the following primary antibodies diluted in 10% horse serum in PBS/0.1% Triton X-100: goat anti-GFAP (1:250, Dako), rabbit anti-AQP4 (1:400, Sigma), mouse anti-beta catenein, and mouse anti-s100B (1:200, Dako). After completing three fifteen-minute washes in PBS, sections were incubated with the corresponding Alexa Fluor dye-conjugated secondary antibodies (Invitrogen) for 1 h at room temperature and cell nuclei were labeled with DAPI. Sections were mounted on slides and coverslipped with Aqua-Poly/Mount (Polysciences) and dried overnight. Slides were imaged on a Carl Zeiss Axio Imager M2 microscope with Apotome (Carl Zeiss) using Hamamatsu ORCA-R2 digital camera C10600 and processed using ImageJ and Adobe Photoshop CS2.

**BrdU**

Free-floating 50 µm brain sections (A/P coordinates 0.5-1.4mm, relative to bregma) were conducted by the following procedure: 2 N HCl was used to dissociate the DNA followed by
neutralization (sodium borate) then blocking and permeabilization (10% horse serum in PBS/0.1% Triton X-100). The floating brain sections were incubated overnight (12 hrs) at 4°C using Rat BrdU primary antibody diluted in 10% horse serum in PBS/0.1% Triton X-100. Sections were washed three times for five minutes in PBS and incubated for 2 hours at room temperature with donkey secondary antibodies (Alexa Fluor, 1:1000). Sections were again washed three times for five minutes in PBS, mounted and dried overnight.

**Mouse lateral ventricle reconstructions and volume measurements**

After brain sections where mounted on slides sequentially anterior to posterior, they were imaged using StereoInvestigator (MicroBrightField) and then contours were traced around the lateral ventricles, beginning at the first section with visible ependyma and ending when the lateral ventricles converged on the third ventricle. Different contours were drawn around the anterior lateral ventricle, open ventricles around the area of fusion, and the posterior lateral ventricle. The contours were uploaded into Neuroludica Explorer software and compiled into three-dimensional reconstructions. The 3-D models created allowed us to obtain ventricle volume measurements.
RESULTS

Humans Display Age-Related Lateral Ventricle Expansion and Periventricular Gliosis

To examine ventricle volumes across the lifespan, cross-sectional MRI data from young, middle-aged and non-demented older adults were obtained from the ‘OASIS’ dataset (Marcus et al. 2007). Automatic segmentation followed by 3-D reconstruction of the lateral ventricles revealed age-related increases in total volume and surface area (Fig. 1A, B). Utilizing longitudinal data from the OASIS collection, which included 150 subjects aged 60-95 years (Marcus et al. 2010), we observed volume increases over time in both males and female individuals (Fig. 1C, D), as has been previously reported (Pfefferbaum et al. 1994; Sowell et al. 2007; Raz et al. 2010).

In light of this ventricular expansion documented to accompany aging, we next utilized human tissue samples to evaluate the integrity of the ependymal lining through aging. We processed and examined aged lateral ventricle tissue samples collected by the Harvard Brain Tissue Resource Center and the University of Connecticut Health Center, Department of Anatomical Pathology. Both coronal sections and 12mm x 12 mm wholemount preparations of the lateral ventricle lateral walls were evaluated. Tissue samples were thoroughly examined and then categorized as having: (1) intact ependymal cell coverage, (2) an intermediate phenotype with disorganized ependymal cells showing reduced and diffuse β-catenin staining (marker of adherens junctions) and the presence of limited surface astrocytic processes or (3) dense areas of gliosis (Table 1). In coronal histology sections, staining for GFAP indicated that regions without an intact ependymal lining showed astrocytic gliosis at the ventricle surface (Fig. 1G). Analysis of the apical surface of the lateral ventricles revealed both zones of contiguous ependymal cell coverage and regions of extensive gliosis at the ventricle surface (Fig. 1H-K). These studies indicate surface gliosis at regions of lost ependymal cell coverage and this phenotype was prominent with increased age and presence of enlarged ventricles.
A

Superior

28yr  46yr  68yr  73yr

Lateral

Frontal

B

Cross Sectional

Volume (mm$^3 \times 10^4$)

Age (years)

C

Longitudinal

Male  Female

Volume (mm$^3 \times 10^4$)

Age (years)

D

Left Lateral  Superior  Right Lateral  Inferior

87-88  88-92

Base Volume  Expansion

E

F

G

GFAP

H

β-catenin

GFAP

I

J

K

14
Figure 1. Increase in ventricle volume as well as periventricular gliosis is associated with age. (a) Representative MRI-based 3-D reconstructions of different age groups. (b) A cross sectional sampling of MRI scans from the OASIS dataset demonstrates the increased ventricle volume associated with age. The ventricular volumes of the two brains presented as Subject 1 and 2 (see Fig. 2 and 3) are denoted with red markers. (c) Longitudinal data shows expansion occurring over time in both male and female individuals. (d) MRI-based reconstructions show expansion occurring in specific regions over time. (e) H&E staining of coronal sections clearly show areas devoid of an intact ependymal layer (bracket). (f) Enlarged region denoted in (e) shows regions lacking ependymal cells and (g) immunohistochemical analysis reveals that areas without an ependymal monolayer have GFAP+ processes at the ventricle surface. (h) Wholemount preparation of the ventricle surface shows that while some areas have intact ependyma (i), those areas are bordered by regions of ependyma with interspersed GFAP+ processes (j) and other areas where ependymal cells are completely absent, leaving only astrocytic gliosis (k). Scale bars, 500μm (e) and 100μm (f).
Table 1. Summary of tissue analysis: ependymal coverage versus scanning

<table>
<thead>
<tr>
<th>Age</th>
<th>Gender</th>
<th>Cause of death</th>
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<td>Male</td>
<td>pancreatic cancer no metastasis</td>
<td>SA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IA</td>
<td>IM</td>
</tr>
<tr>
<td>61</td>
<td>Female</td>
<td>pneumonia (Alzheimer's)</td>
<td>SA</td>
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<tr>
<td></td>
<td></td>
<td>IA</td>
<td>IM</td>
</tr>
<tr>
<td>77</td>
<td>Female</td>
<td>cardiac arrest</td>
<td>SA</td>
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<tr>
<td></td>
<td></td>
<td>IA</td>
<td>IM</td>
</tr>
<tr>
<td>83</td>
<td>Male</td>
<td>myocardial infarction</td>
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<td></td>
<td>IA</td>
<td>IM</td>
</tr>
<tr>
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<tr>
<td></td>
<td></td>
<td>IA</td>
<td>IM</td>
</tr>
<tr>
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<td>Female</td>
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<td></td>
<td></td>
<td>IA</td>
<td>IM</td>
</tr>
<tr>
<td>91</td>
<td>Female</td>
<td>cardiac arrest</td>
<td>SA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IA</td>
<td>IM</td>
</tr>
</tbody>
</table>

Subject 1

| 82  | Female | pneumonia (Alzheimer's) | SA | SM | SP |
|     |        | IA | IM | IP |

Subject 2

| 86  | Male   | myocardial infarction | SA | SM | SP |
|     |        | IA | IM | IP |

Lateral Ventricle Region Key

- SA: Superior anterior
- A: Central anterior
- IA: Inferior anterior
- SM: Superior middle
- M: Central middle
- IM: Inferior middle
- SP: Superior posterior
- P: Central posterior
- IP: Inferior posterior

Color Key

- Green: Intact ependyma
- Yellow: Stretched ependyma
- Red: Dense astrocytic scarring
- White: Not available

Post mortem intervals range from 11-28 hours
**Enlarged ventricles are associated with periventricular gliosis in aged humans**

To examine directly the relationship between lateral ventricle expansion and glial scarring at the ventricle surface in human tissue, we paired postmortem MRI scans with histological analysis of periventricular tissue. Two age-matched subjects were selected based on dramatic differences in lateral ventricle volumes. A contiguous thin section (1.3mm), 3-D T1-weighted MP RAGE sequence was performed on the formalin-fixed brains of the two subjects. Automatic segmentation of the lateral ventricles defined on high-resolution MRI was performed using ITK-SNAP (Snippert et al. 2010) and volumes rendered using Slicer3D.

The reconstructions showed that Subject 1 (82 years) had an age-appropriate lateral ventricle volume (59241.4mm$^3$) (**Fig. 2A**) (Subject 1 volume indicated in red on graph in **Fig. 1B**). The corresponding lateral ventricle tissue was then processed using antibodies against GFAP to visualize areas of gliosis at the ventricle surface and β-catenin, which marks the adherens junctions between ependymal cells and will consequently reveal areas of intact ependyma. Both coronal sections and 15mm x 8mm *en face* wholemount samples were analyzed, effectively covering the entire lateral ventricle lateral wall. Hematoxylin and eosin (H&E) staining of coronal slices revealed some attenuated ependymal cells at the ventricle surface as well as areas totally devoid of ependymal coverage (**Fig. 2B**). Immunohistochemical analysis of wholemount preparations of the ventricle wall revealed that while some regions had intact ependymal cell coverage, other regions presented attenuated, limited ependymal coverage and the anterior, posterior and superior lateral ventricle surfaces were comprised of large, GFAP$^+$ gliotic expanses (**Fig. 2C, D**).
To provide contrast with Subject 1, MRI-based 3-D reconstructions of Subject 2 (86-years) showed a ventricle volume (11279.5mm$^3$), a value more typical of a 20-40 year old (Fig. 3A) (Subject 2 volume indicated in red on graph in Fig. 1B). Whole mount preparations of the lateral ventricle from Subject 2 revealed intact ependymal cell coverage of the entire lateral wall of the lateral ventricle, and the corresponding H&E staining of the tissue surrounding the ventricle also displayed an intact, structured ependyma. (Fig. 3B, C). These studies revealed a striking distinction in periventricular cytoarchitecture that appears to be associated with ventricle enlargement.
Figure 2. Large ventricular volume is associated with widespread gliosis at the ventricle surface. (a) Utilizing MRI-based 3-D reconstructions, the resulting ventricular volume for Subject 1 was found to be in the normal range for an 82-year old. (b) The H&E staining of periventricular tissue revealed attenuated ependymal cells (arrows) as well as areas devoid of ependymal coverage. (c) Representative images from the extensive immunohistochemical analysis of the ventricle surface revealed that while some areas with normal ependyma were present, large expanses without intact ependyma that instead showed gliotic scarring at the ventricle surface. (d) Schematic of entire lateral wall of the lateral ventricle, with red indicating areas of astrocytic gliosis, yellow indicating attenuated ependymal covering, and green representative of intact ependyma. Scale bars 100\(\mu\m\) (b), 40\(\mu\m\) (c).
Figure 3. **Lower ventricle volume associated with an intact ependyma.** (a) MRI-based 3-D reconstructions revealed that the ventricle volume of Subject 2, an 86-year old, was in the range of 20-40 year olds. (b) H&E staining revealed a robust ependymal monolayer while (c) wholemount preparations and immunohistochemistry also showed uninterrupted ependyma with no surface gliosis observed. Scale bars 100μm (b), 40μm (c).
Neuraminidase causes scarring at ventricle surface in mice that is phenotypically similar to the gliosis in humans

Investigating the connections between periventricular gliosis and ventricular expansion poses a challenge, as neither of these conditions is observed in mice under normal conditions (Luo et al. 2006, Shook et al. 2012). However, damage to the ependymal cell monolayer can be induced by intraventricular injection of high concentrations (100-500 ng/µl) of the enzyme neuraminidase, which cleaves sialoglycoproteins involved in cell adhesion and causes ependymal denudation (Luo et al. 2008). After a unilateral injection of neuraminidase, immunohistochemistry reveals extensive gliosis along the ventricle surface (Fig. 4A). Instead of a healthy ependymal layer and a normal neurogenic subventricular zone, there were instead large areas lacking intact ependymal coverage and obliteration of the SVZ niche. In these places where excessive ependymal denudation had occurred, GFAP+ areas of gliosis were observed (Fig 4A). Varying degrees of ependymal damage can be generated by changing the concentration of neuraminidase injected. We found that 500 ng/µl resulted in ventricle surface scarring similar to that found in aged humans (Fig. 1). Immunohistological analysis of the neuraminidase-induced damage along lateral ventricle wall showed an increase in astrocyte number and density at the ventricle surface in the form of scars (Fig. 4A), appearing qualitatively comparable to the scars observed in aged human ependyma (Fig 1). This mouse model provides us with the means to investigate the connection between periventricular gliosis and ventriculomegaly further study the implications of ventricle surface gliosis.

To investigate whether proliferating astrocytes contribute to the gliosis at the ventricle surface, dividing cells were labeled with injections of the thymidine analog BrdU on days 3, 4, and 5 after neuraminidase treatment (Fig. 4B). The tissue was processed and coronal sections of the lateral ventricle lateral wall were analyzed. BrdU+ astrocytes were observed along the ventricle surface at sites of ependymal cell denudation (Fig. 4C), indicating reactive gliosis at sites of extensive injury.
Figure 4. Neuraminidase-induced scarring at the ventricle surface in mice.

(a) Two weeks after introduction of neuraminidase, the ventricle surface shows discontinuities in the ependymal layer that are filled by GFAP+ processes. (b) BrdU was administered on days 3, 4, and 5 after neuraminidase to label any dividing cells. (c, d) Areas at the ventricle surface devoid of ependymal cells (marked by S100β) but positive for GFAP also show a large number of BrdU positive cells incorporated, indicating that reactive astrocytes are involved in the gliosis at the ventricle surface. Scale bars, 100μm (a, c), 40μm (d).
Aquaporin 4 is upregulated in areas of periventricular gliosis in both human and mouse

To examine periventricular scaring and compromised CSF-interstitial fluid homeostasis in our neuraminidase-induced gliosis model, we analyzed aquaporin-4 (AQP4) expression patterns. AQP4 is the principal water channel of the brain and is expressed on astrocytic end-feet at cerebral blood vessels, the basolateral membrane of ependymal cells, and the pia mater (Frigeri et al. 1995; Filippidis 2012). The distribution of these water channels suggests that it plays an important role in maintaining the water balance of the brain at the blood-CSF and blood-brain barriers (Amiry-Moghaddam et al. 2003). AQP4 expression is upregulated in reactive astrocytes, contributing to astrocyte migration and scar formation in response to trauma (Saadoun et al. 2005; Iacovetta et al. 2012). Deletion of AQP4 has been shown to reduce the effects of cytotoxic edema and improve outcome after stroke (Sofroniew 2009; Zador et al. 2009). Therefore, changing expression patterns will indicate alterations to CSF-interstitial fluid regulation (Papadopoulos & Verkman 2007; Haj-Yasein et al. 2011; Iliff et al. 2012). Following neuraminidase treatment, we found substantially enhanced expression of AQP4 in regions of periventricular scarring in mouse tissue (Fig. 5A). Similarly, high levels of AQP4 were detected in human tissue at sites of glial scarring, but not in uninjured tissue (Fig. 5B). These studies indicated that our neuraminidase model closely replicates the ventricle surface glial scarring found in aged humans.
Figure 5. An upregulation of AQP4 in areas of periventricular gliosis occurs in both mice and humans.

(a) Following intraventricular injection of neuraminidase, areas of gliosis (GFAP staining) show increased expression of AQP4. Ependymal cells show low levels of AQP4 staining in non-scarred regions. (b) Similarly, areas of surface gliosis in human tissue show increased expression of AQP4. Low levels of AQP4 are detected on ependymal cells in areas of intact ependyma. Scale bar, 50μm (b).
Compromised ependymal cells show upregulation of GFAP in both human and mouse

Work done by Francis Szele’s group has shown that a mouse model of experimental stroke causes ependymal cells to assume features of reactive astrocytes post-stroke, including extending small processes and exhibiting an upregulation of GFAP (Young et al. 2013). We observed a very similar phenotype in neuraminidase-injected mouse ependyma, as individual ependymal cells appeared to be strongly expressing GFAP and small processes extending outward were visible (Fig. 6A). Interestingly, ependymal cells with the same phenotype were present in aged human tissue of Subject 1 (Fig. 6B). These ependymal cells, while still clearly outlined by β-catenin junctions, presented heightened levels of GFAP and small “tails.” Though more investigation is needed to understand what exactly these GFAP+ cells represent, how long they persist, and if their distribution is significant, it is reasonable to suppose that the upregulation of GFAP is a response of ependymal cells to stress. The observation of this response to injury in both human and mouse tissue further validates our mouse model of ependymal damage.
Figure 6. Compromised ependymal cells in mouse and human display characteristics of reactive astrocytes.

(a) In areas of mouse ependyma still intact after injections of neuraminidase, certain ependymal cells show and upregulation of GFAP as well as small processes extending outwards. (b) In tissue from Subject 1 (see Fig. 2), whole mounts of the ependymal wall reveal ependymal cells exhibiting the same characteristics. Scale bar, 50μm (b).
**Extensive ventricle gliosis results in ventricle expansion in mice**

We next examined whether a direct relationship exists between gliosis at the lateral ventricle surface and ventricle enlargement and if it is possible that injury and loss of ependymal cells lining the ventricle results in ventriculomegaly, independent of neurodegeneration or other injury to the brain. To address this question, a high dose of neuraminidase (500 ng/µl) was injected unilaterally into the lateral ventricle of young adult mice. After a two month period, the brains were collected and coronally sectioned. On inspection, the lateral ventricles of the mice injected with neuraminidase appeared larger than the lateral ventricles of mice injected with saline (**Fig. 7A**). Contours were traced around the lateral ventricles then uploaded into Neurolucida Explorer software where they were then compiled into three-dimensional reconstructions (**Fig. 7B**). The 3-D models created allowed us to obtain ventricle volume measurements which revealed that the lateral ventricle volumes of mice injected with high doses of neuraminidase were significantly larger than that of both mice injected with the same volume of saline as well as uninjected littermates (**Fig. 7C**). Gliosis persisting at 2 months after introduction of neuraminidase to lateral ventricles was verified using immunohistochemistry (**Fig 7D**).
Figure 7. Extensive gliosis results in lateral ventricle enlargement in mice.

(a) Two months after intraventricular injection of neuraminidase, lateral ventricles appear larger on inspection (V denotes ventricular space). (b) Contours traced around the ventricles complied to create 3-D reconstructions, which are then used to determine lateral ventricle volume (*denotes areas of stenosis between lateral and medial walls). (c) Ventricle volume at two months after injection is higher in mice received neuraminidase compared to mice injected with saline and uninjected littermates (n=4 for NM group, n=3 for saline and uninjected controls, *p=.025, two-tailed Student’s t-test). (d) Immunohistochemistry confirmed that gliosis at the ventricle surface, indicated by bracket, persists two months after neuraminidase injection.
DISCUSSION

Ventriculomegaly is a biomarker strongly associated with several neurological pathologies as well as with normal, unimpaired aging (Juuhl-Langseth et al. 2012; Palha et al. 2012; Poca et al. 2005; Daneshvar et al. 2011, Sowell et al. 2007; Raz et al. 2010). Though many studies have observed the ependyma while examining the effects of hydrocephalus, even speculating that a mechanism contributing to hydrocephalus could be an interruption in normal ependymal transport of fluids and solutes (Sarnat 1995), little is known about the implications of the loss of ependymal barrier in adult life. Additionally, the causal relationship between ventricular expansion and ventricle surface gliosis has not been studied.

Since any notable expansion of the ventricles is likely to be accompanied by scarring at the ventricle surface, it is an interesting exercise to attempt to piece together which abnormality arises originally. If scarring is initially caused by an event such as a head injury or a viral infection, the loss of the ependymal barrier could possibly contribute to an imbalance in water or solutes that results in an increased ventricle volume, or perhaps the difference in mechanical parameters between the organized ependyma and the stratified glial scar could produce a distention in the ventricle lining, resulting in enlargement. Conversely, expansion could potentially occur first due to an increase in CSF production, a rise in intracranial pressure, or neuronal tissue deterioration and loss, promoting ventricle expansion and necessitating astrocytic scarring of the ependyma.

It is probable that different pathologies take different routes- with some cases beginning with expansion while others initiate with scarring – to result in the same phenotype. In this study, we have produced a satisfactory mouse model to investigate the topic, and our results have
illuminated one possible path: extensive ependymal denudation and the resulting astrocytic scarring can lead to ventricular enlargement. Further investigation is needed into the mechanisms at play behind this enlargement as they are currently not understood.

Additionally, this work demonstrates an association between increased ventricle volume and loss of intact ependyma in humans. Our studies not only combine histological techniques with MRI-based 3-D reconstructions to provide informing power to MRIs and insight into the connection between ventricle volume and ependymal integrity, but use a technique not previously employed in the examination of human ependyma, the *en face* whole mount. Using the whole mount preparation to visualize the continuous ependyma of mice, we utilized neuraminidase at high dosages to create scarring at the ventricle surface in mice. The ependymal damage produced in mice was morphologically equivalent to that seen in humans, with human and mouse tissue displaying both GFAP+ ependymal cells and AQP4+/GFAP+ astrocytic gliosis.

The observation that aquaporin-4 is upregulated in areas of scarring in both mice and humans is notable for several reasons. First, it further validates our mouse model of periventricular gliosis by displaying a morphologic pattern of scarring very similar to the one observed in aged human tissue. Second, a heightened amount of aquaporin channels in areas of scarring suggests that when ependyma is replaced with astrocytic gliosis, the water balance between the CSF and the interstitial fluid could be thrown off, resulting in edema in the tissues around the ventricle or possibly a disturbance in the normal CSF/interstitial fluid concentration.

The study of ependymal integrity has value to the field of aging in the human brain. As there is much debate regarding the contribution of the SVZ in maintaining the surrounding brain environment in adult humans, it is important to understand the implications of the loss of the
organized ependymal barrier. If quiescent stem cells are present in adulthood, then it seems logical that a disruption of their niche by the introduction of CSF solutes and catabolites due to a compromised ependymal barrier could be deleterious to these cells, resulting in their eradication and the subsequent loss of any retained neurogenic capability.

In future studies, it will be important to address questions relating to the consequences of replacing the highly organized, functionally specific interface of the ependymal layer with astrocytic gliosis. Further investigation into what differences exist in expression of functional proteins such as connexin 43, GLUT1, and other transporters and junction proteins will help elucidate how the loss of the functional barrier affects the of preservation of a healthy cerebral environment. Additionally, the reactive astrocytosis and formation of a scar at the ventricle surface is likely accompanied by microglial activation, and an investigation into the role of the inflammatory response in ependymal damage and scarring could help elucidate mechanisms of expansion and implications for the neuronal tissue bordering the areas of gliosis.

While the adult ependyma could be dismissed as a residual structure with little function, it seems counter-intuitive that the highly organized ependymal monolayer would be preserved if it wasn’t contributing to the maintenance of some biological process or equilibrium of the brain. This study has demonstrated an association between ventricular enlargement and scarring at the ventricle surface in addition to establishing a relevant model of ependymal damage. Our reported observations that extensive ependymal scarring in mice results in an increase in lateral ventricle volume suggests that an intact ependyma is required to maintain a healthy ventricular environment.
References


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