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The Effects of Aging and Injury on the Murine Ependymal Barrier and Subventricular Zone

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ABSTRACT

Previous research has shown that numerous age-related changes occur in the subventricular zone (SVZ) and diminish the neurogenic output of the region. Here, various age-related changes in the structure of the lateral ventricle, the ependymal barrier, and the SVZ were examined. It was found that the walls of the lateral ventricles move together, adhere, and eventually fuse. This results in a degradation of the SVZ. Other changes associated with aging were found, including ependymal cell stretching, heightened, ependymal repair, changes in CSF flow velocity and dynamics, and altered ependymal cell polarity. These changes may be related to the process of intraventricular fusion. Similar age-related changes did not occur in other parts of the ventricular system. The effects of injuries, including repetitive, mild traumatic brain injury (rmTBI) and Neuraminidase injections were also investigated. Preliminary studies on rmTBI did not produce an effect on the volume of the lateral ventricle, but did increase the prevalence of intraventricular fusion. However, further analysis is needed. Neuraminidase injection resulted in a significant increase in the volume of the lateral ventricles. Taken together, these results demonstrate the lateral ventricle's unique susceptibility to changes associated with the aging process.

INTRODUCTION

History of Stem Cell Research

Research into stem cells has become one of the most prolific scientific endeavors in recent memory. The unique properties of these cells bring about countless potential applications that advance scientific knowledge and medical care.

Research into the science behind stem cells began as early as 1908, when the possibility arose that the different cell types within blood were derived from the same precursor. At this time, the Russian-American histologist Alexander Maximov is credited with coining the term "stem cell" in his description of blood cell lineages. Amongst the numerous stem cell pioneers in the 1960s was the Canadian duo Ernest McCulloch and James Till. Till and McCulloch injected irradiated mice with bone marrow and observed that nodules formed in the spleens of the mice that were proportional to the number of cells that were injected. The researchers speculated that each nodule was the product of a single marrow cell, which was later identified as a hematopoietic stem cell.

Currently, three types of stem cells have been identified. The first and most well known type is the embryonic stem cell, which is derived from the blastocyst during embryonic development. Embryonic stem cells are pluripotent, meaning they can form nearly all cell types, including all three germ layers. Second, there are adult stem cells, which are found in select regions in adults. They are multipotent, meaning they can form all of the cell types within the organ system in which they are found. Lastly, researchers have developed induced pluripotent stem cells, which are reprogrammed somatic cells that gain pluripotency through viral transfection of certain genes. These cells were first created in 2006 by Shinya Yamanaka, who received the Nobel Prize for his work in 2012. Embryonic, adult, and induced pluripotent stem

cells all share the two defining properties of stem cells. First, these cells are capable of self-renewal, which allows the cells to undergo multiple divisions while maintaining itself in an undifferentiated state. Second, all stem cells have a degree of multipotency, meaning they are able to differentiate into several distinct cell types. It is these unique properties that distinguish stem cells from all other cell types and make them such popular and promising research targets.

The following work focuses on adult stem cells. Adult stem cells are found in niches, or specialized regions, throughout the body. Mammalian adult stem cell niches have been found in the skin, intestines, stomach, muscles, bone marrow, and brain. All of these niches share similar characteristics that allow them to harbor adult stem cells.

First, the stem cells are all found along some type of central hub, or somatic niche cell, that allows the stem cells to receive activating or suppressing signals (Figure 1). The hub can be a basement membrane (such as in the skin and muscle niches), a fluid filled space (such as the ventricular surface in the subventricular zone brain niche), or an open space (such as the lumen in the intestinal niche).

Second, niches support adult stem cells by allowing them to undergo either symmetric or asymmetric division. In symmetric division, the adult stem cell divides to form two adult stem cells. It requires continuous attachment to the hub for both the mother cell and its two daughter cells. Asymmetric division, on the other hand, results in the formation of one adult stem cell and one differentiated cell. Only the adult stem cell remains attached to the hub.

Finally, niches provide molecular support to adult stem cells by providing a microenvironment that limits proliferation and differentiation by promoting quiescence. In a quiescent state, a stem cell will not be actively in the cell cycle, but is poised for division if the

appropriate signals are received. The quiescent state is beneficial for the niche itself, as it prevents stem cells from undergoing frequent cellular divisions and accruing genetic mutations.

Neurogenesis

Within the central nervous system, only two adult stem cell niches have been conclusively shown to exist: the subventricular zone (SVZ) and the subgranular zone (SGZ). These niches serve to produce new neurons and glia within their respective regions of the brain. The history of research into these two areas of neurogenesis is a valuable story, as it demonstrates how difficult it can be to overturn a strongly held scientific dogma.

The presence of neurogenesis within the adult mammalian brain was long-thought to be impossible. Santiago Ramon y Cajal, the renowned Spanish histologist and so-called "father of neuroscience", thought that the nervous system was incapable of regeneration and was instead in a fixed state after development. In the book Estudios sobre la degeneración y regeneración del sistema nervioso [Studies on the degeneration and regeneration of the nervous system], Ramon y Cajal stated, ""In adult centers the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated" (1914). This axiom of "no new neurons after birth" was accepted for much of the 20^{th} century.

The American neurobiologist Joseph Altman was the first researcher to demonstrate the presence of neurogenesis in the adult mammalian brain. Altman gave the first depiction of neurogenesis in both the dentate gyrus, in 1962, and the subventricular zone, in 1969. In the latter paper, Altman injected 30 day-old rats with thymidine-H^3, a thymidine analog that allows proliferating cells to be visualized, and sacrificed the rats at varied intervals. He made observations at four important time points based on these injections. First, in rats killed less than 24 hours after the thymidine-H^3 injection, the labeled cells were found along the lateral ventricle.

By three days after injection, the majority of cells were found in the middle portion of what Altman called the rostral migratory stream. By the sixth day, the cells were found in the subependymal layer of the olfactory bulbs. Lastly, by day twenty, the cells had integrated into the internal granular layer of the olfactory bulbs (Altman, 1969). These observations make up the basis for the current understanding of subventricular zone-mediated neurogenesis.

The scientific community, however, largely ignored Altman's findings on neurogenesis. The response to his findings was a combination of justifiable critique and skepticism, ignorance to valid results, and direct hostility (Kempermann, 2005). Pasko Rakic, a neuroscientist at Yale University, was the most vocal opponent of early neurogenesis findings. Rakic questioned the validity of research that used thymidine analogs, as evidence suggests that the analogs may label dying cells in addition to healthy, proliferating cells. Rakic's arguments, however reasonable, were seen by many to be overly aggressive. These researchers have gone so far as to suggest that Rakic held back the field of neurogenesis by multiple years (Costandi, 2012). In the few years after Altman's publications, the only researcher to agree with his findings and publish similar results was Shirley Bayer, Altman's wife. Bayer showed that the neuronal population in the dentate gyrus continues to grow throughout adulthood using volumetric and thymidine-H^3 data (Bayer, 1982).

It was not until the 1990's that olfactory bulb neurogenesis was re-discovered by three independent researchers. First, Frank Corotto and colleagues used 5-bromo-2'-deoxyuridine (BrdU), a thymidine analogue that is incorporated into proliferating cells during s-phase, to investigate cellular division in the SVZ. They showed that proliferating cells from the SVZ migrated to the olfactory bulb and expressed mature neuronal markers (Corotto et al. 1993). At the same time, Marla Luskin used a β-galactosidase retrovirus to show how the progeny of SVZ

precursors traveled to the olfactory bulb and differentiated into two types of interneurons (Luskin, 1993). Carlos Luis and Arturo Alvarez-Buylla of Rockefeller University reinforced the link between olfactory bulb neurogenesis and stem cell biology in the adult brain. They showed that SVZ-precursors labeled with thymidine-H^3 are able to differentiate into neurons and glia *in vitro*, one of the two main properties of stem cells described earlier (Lois and Alvarez Buylla, 1993).

It is important to note that the previously described research was exclusively conducted in rodents. The presence of neurogenesis in adult humans remains a heavily debated area of research. In 1998, Fred Gage and Peter Eriksson found evidence for hippocampal neurogenesis in five adult cancer patients that had received injections of BrdU. The researchers found cells that were labeled with both BrdU and immature neuronal markers in the patients' hippocampi. These cells, however, were not shown to be functional (Eriksson et al. 1998). These findings, however, should be treated with caution because, as previously stated, dying cells may incorporate BrdU.

The presence of neurogenesis in the human subventricular zone remains an open question as well. In 2004, researchers at the University of California San Francisco showed that the human SVZ contains a unique organization of astrocytes, termed the "ribbon". These astrocytes may be similar to neural stem cells, yet they saw no evidence of new neurons migrating from the region in adults (Sanai et al, 2004). This study was contradicted by a report from Peter Eriksson and colleagues, who demonstrated that neuroblasts migrate to the olfactory blub via an extension of the lateral ventricle in adult humans (Curtis et al. 2007). Additionally, a research group from Fudan University in China found that a small number of neuroblasts exist within the SVZ and RMS in adult humans. They did not, however, find any neuroblasts in the olfactory bulbs (Wang

et al. 2011). Overall, there are mixed results regarding the presence of subventricular zone-mediated neurogenesis in humans. It may be the case that any neurogenesis that may be present is not robust enough to be easily detected. It is also possible that adult neurogenesis is simply a nonfunctional remnant of an ancestral system that is not useful to humans. Regardless, the search for a conclusive answer to the question of human neurogenesis will be difficult to find because of the complications involved in human tissue research. The lack of available tissue, limited experimental possibilities, and cost to investigate cellular processes in the human brain will continue to hold the field back.

The Murine Subventricular Zone

In mice, the subventricular zone is found along the lateral walls of the lateral ventricles. The region is made up of four cell types: astrocytes, transitory amplifying progenitors (Type C cells), neuroblasts, and ependymal cells (Figure 2). A subpopulation of SVZ astrocytes has been identified as the neural stem cells of the region. These cells extend a basal process to blood vessels underlying the SVZ and an apical process that contacts the ventricle (Conover and Shook, 2011).

The SVZ is separated from the ventricle by a monolayer of ependymal cells. These cells are simple cuboidal epithelium-like cells and bind together using adherens junctions. Additionally, ependymal cells are multiciliated and help produce microcurrents in the cerebrospinal fluid as it flows through the lateral ventricles. They also have microvilli that absorb CSF. The apical, astrocytic processes of the neural stem cells are interspersed between ependymal cells. A single NSC process extends one primary cilium into the ventricle.

Neural stem cells in the subventricular zone divide to form at least one Type C cell. These Type C cells give rise to multiple neuroblasts, which exit the SVZ via the rostral

migratory stream (RMS). Here, neuroblasts organize into chains that lead to the olfactory bulb, where they differentiate into interneurons.

In both the injured and aging subventricular zone, neural stem cells have also been shown to participate in an alternative form of repair. By tracking BrdU-labeled SVZ neural stem cells over time, Luo et al. (2008) demonstrated that neural stem cells can integrate into the ependymal wall. These cells then acquire similar antigenic and morphological properties to ependymal cells, such as s100β immunoreactivity and the formation of adherens junctions with neighboring ependymal cells. This integration occurs in 2-year old (elderly) mice and can be induced in 2-month old (young adult) mice by injecting the toxin neuraminidase into the lateral ventricle (Luo et al. 2008). In the next sections, additional age-associated changes in neural stem cells and the subventricular zone will be discussed.

Age-Related Changes in SVZ-Mediated Neurogenesis

The degree to which neurogenesis in the subventricular zone declines during the aging process has been a subject of much recent research. Numerous studies have shown that the number of proliferating cells in the SVZ is reduced by 50-75% (see Shook et al. 2012). Additionally, the number of BrdU-positive neuroblasts that reach the olfactory bulb decline by around 75% in aged mice. This decline is correlated with a reduced ability to discriminate fine odors (Enwere et al. 2004). In order to assess whether these changes in neurogenesis were the results of alterations to the neural stem cell pool, we (Shook et al. (2012)) performed a spatiotemporal analysis of SVZ neural stem cell organization and output. Whole mount dissections were performed, during which the medial wall of the lateral ventricle was removed and the surface of the lateral wall was exposed. This preparation allowed immunohistochemistry to be performed on the lateral wall surface.

First, we showed that the neural stem cell pool declines in a uniform fashion across the entire lateral ventricle in aging. Mizradeh et al. (2008) demonstrated that, on the surface of the lateral ventricle, neural stem cells exhibit a unique 'pinwheel' architecture, with the neural stem cell's apical process surrounded by several ependymal cells (see Figure 1). Using immunohistochemistry to label the pinwheel structures in young adult (3-months old), adult (6-months old), older adult (1-year old) and elderly (2-years old) mice, we showed a significant decline in the number of pinwheel units with age (Figure 3).

To further assess changes in the neural stem cell population, the number of neural stem cell processes within each pinwheel was determined. This was accomplished by counting the number of gamma-tubulin + primary cilium in the core of a given pinwheel. It was found that the number of neural stem cells per pinwheel declines significantly with age (Figure 4). Taken with the previous data on pinwheel decline, the number of neural stem cells per square millimeter declines by roughly 73% in 1-year old mice and 86% in 2-year old mice, when compared to 3-month old mice (Figure 4D).

To address whether these stem cells are lost uniformly across the surface of the lateral ventricle, a spatial analysis was performed. The surface of the lateral ventricle was binned into five regions: anterior dorsal, anterior ventral, posterior dorsal, posterior ventral and middle ventricle (Figure 5E). It was found that there was no significant difference between the five regions in either the decline in number of neural stem cells or the decline in number of pinwheels (Figure 5).

Next, it was investigated whether the decline in olfactory bulb interneuron production differed between the types of interneurons. Six types of olfactory bulb interneurons are produced from the neuroblasts migrating from the SVZ. In the olfactory bulb granular layer,

interneurons are either deep, superficial, or CalR+ interneurons, while in the glomerular layer, interneurons are either CalR+, CalB+, or TH+ interneurons. Using immunohistochemistry and EdU injections, a uniform decline in the formation of each subtype was found (Figure 6). The percentage of each EdU+ subtype produced did not change with age. This was concordant with the spatially uniform decline in neural stem cells discussed earlier.

Finally, the rate at which aging neural stem cells undergo mitosis was investigated. In order to accomplish this, each neural stem cell's apical process was tracked down to its nucleus using serial reconstructions (Figure 7A). The cellular division marker Phosph-histone H3 (PH3) was used to identify dividing neural stem cells. The number of dividing neural stem cells was found to remain constant throughout aging (Figure 7C). After combining this with the data on the declining numbers of total neural stem cells shown earlier, the percentage of mitotic neural stem cells was shown to be significantly higher in 1- and 2-year old mice, when compared to 3-month old mice (Figure 7D). This suggests that the age-related decline in SVZ neurogenesis occurs downstream of neural stem cell activation, such as in Type C cell division or neuroblast migration. The SVZ appears to be compensating for these changes by increasing the percentage of mitotic neural stem cells.

Taken together, previous research on neurogenesis, aging, and the subventricular zone guided the formation of numerous questions, which helped lead the research that will be the focus of this paper. These general questions included: What other age-related changes are occurring in the subventricular zone? How could these changes affect neural stem cells and neurogenesis? Do similar changes occur in other ventricular systems? How does the aging brain compare to a brain that underwent traumatic brain injuries? The research that follows attempts to provide answers, or at least clues towards possible answers, to these questions. The results

will be broken up into individual sections, each with its own methods and results subsections. A general discussion regarding overarching themes will then follow.

EXPERIMENTS

Stenosis and Fusion of the Lateral Ventricle

Methods

Animals: Male CD-1 mice were purchased from Charles River and aged in our vivarium. All animal procedures were performed under protocols approved by the Institutional Animal Care and Use Committee of the University of Connecticut and conforming to National Institutes of Health guidelines.

Immunohistochemistry: Mice underwent transcardial perfusion with 0.9% saline, followed by 4% paraformaldehyde (Electron Microscopy Science) in PBS. Brains were fixed overnight in 4% paraformaldehyde at 4°C and then washed in PBS three times (20 min each wash) before cutting into 40–50 μm coronal sections with a vibratome (VT-1000S; Leica). Tissue was then permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 10 min, blocked in 10% horse serum (Invitrogen) in PBS/1% Triton X-100 for 1 h, and incubated with combinations of the following primary antibodies: goat anti-doublecortin (DCX) (1:250; Santa Cruz Biotechnology); rabbit anti-s100β (1:500; Dako); rabbit anti-Caspase 3 (1:250, Abcam); mouse anti-TGFβ (1:500, Abcam); and mouse anti-glial fibrillary acidic protein (1:500; Abcam). 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. In order to verify the results, control tissue was stained with only secondary antibodies. Sections were coverslipped with Aqua-Poly/Mount (Polysciences) and imaged on a Carl Zeiss Axio Imager M2 microscope with Apotome (Carl Zeiss) using Hamamatsu ORCA-R2 digital camera C10600.

For analyses using the whole mount technique, mice were perfused with 0.9% saline alone. The brains were dissected according to the procedure from Mizradeh et al. (2010). Subsequent immunohistochemistry was performed via the same protocol described above for coronal sections. Imaging of whole mount slides was performed on a Leica TCS SP2 confocal laserscan microscope.

3-D Reconstructions: After imaging coronal sections, the lateral ventricles were traced with contours using the StereoInvestigator software. Different contours were drawn around open ventricles, areas of intraventricular adhesion, and areas of ependymal cell loss. Contours were then uploaded into the Neurolucida Explorer software, in which 3-D models were constructed.

Results

Numerous age-related changes have been shown to occur in the cytoarchitecture of the SVZ. Previous work in our laboratory suggested that, in older mice, stenosis of the ventral walls of the lateral ventricles occurs. The walls move together and eventually fuse, resulting in a loss of ependymal cells (Luo et al. 2006). Here, the age-related progression of stenosis and fusion were investigated.

First, it was found that the degree of ventricle stenosis increases with age. Areas of open ventricle space were defined as areas where s100β+ were in contact with open space, not other ependymal cells. Adhesion was defined as the presence of either a bilayer or monolayer of s100β+ ependymal cells that linked areas of open ventricle. Finally, fusion was defined as any area between two regions of open ventricles that was entirely void of s100β+ ependymal cells. Older mice were associated with more ventricle adhesion and fusion in the anterior horn of the lateral ventricle. Areas that had adhesion present in younger mice were entirely fused in older mice. This indicated that the process occurs in a continual fashion, from stenosis to adhesion to

fusion (Figure 8a). In younger mice, there are more areas of open ventricle space (outlined in white), while in older mice, there are more areas of adhesion (blue) and fusion (pink).

These colored outlines, which are called contours, were arranged in the Neurolucida Explorer computer program to create three-dimensional reconstructions of lateral ventricles in young and old mice (Figure 8b-d). It can be seen in these representative images that the area of adhesion/fusion greatly increases with age.

This change in area was quantified using whole mount dissections, as described above. The surface area of intact ependymal cells, which have not undergone adhesion or fusion, was calculated in young (3-month old), middle-aged (6-month old), older adult (1-year old), and elderly (2-year old) mice. In order to make this calculation, the surface area of adhesion/fusion was subtracted from the total surface area (Figure 9a). This percentage of ventricle surface area with intact ependymal was then normalized to the percentage found in 3-month old mice. It was found that the area of intact ependyma decreased between each successive age group (Figure 9b). This difference became significant between 3-month old and 2-year old mice ($p<0.05$, one-way ANOVA with Bonferroni's multiple comparison post hoc test; $n=5$).

Additionally, previous results from our laboratory indicated that the subventricular zone degrades in areas of fusion (Luo et al. 2006). Here, these observations were confirmed using immunohistochemistry. In area of fusion, again defined as a lack of s100β+ ependymal cells, there were no DCX+ neuroblasts along the lateral side of the lateral ventricles (Figure 10d). This confirms the idea that, in areas of complete fusion, the SVZ degrades and neurogenesis ceases. This means that it is likely that neural stem cells need contact with cerebrospinal fluid in order to survive as stem cells and form new neurons. This observation raises numerous questions. The fate of the neural stem cells that no longer proliferate is unknown. The stage of neurogenesis

during which the process first breaks down is also unknown. Taken with the previous findings on the decrease in open ventricle surface area, it can be concluded that the functional area of the subventricular zone decreases over time. This likely contributes to the subventricular zone's age-related decline in neurogenesis (Shook et al. 2012).

The mechanism underlying the progression from stenosis to adhesion to fusion was briefly investigated. The primary goal was to decipher what happens to ependymal cells at the point of adhesion/fusion. First, it was investigated if the ependymal cells were undergoing apoptosis as the walls fused. This possibility was disproved using Caspase 3 immunohistochemistry, as Caspase 3 did not appear to be up-regulated at the site of fusion (Figure 11). It is possible, however, that apoptosis is occurring at the sight of fusion under the mediation of a different Caspase protein.

Next, it was investigated if the ependymal cells transitioned to a different cell type at the site of ventricle wall fusion. This type of cellular transition occurs at numerous points in development, including the formation of the palate of the upper lip. In this process, the two sides of the palate come together to form an epithelial seam, similar to the adhesion step observed in the lateral ventricles. This seam then disintegrates to create a single, confluent area of tissue. There are two main theories regarding the mechanism underlying this disintegration process, as reviewed by Nawshad (2008). The first possibility is apoptosis, in which the epithelial cells along the seam undergo Caspase-mediated programmed cell death. The second possibility is termed epithelial-mesenchymal transition (EMT). In this process, epithelial cells with an apical-basal polarity undergo a phenotypic transition, lose their polarity, and become mesenchymal cells. Both of these possibilities have received widespread support and it is now believed that the epithelial seam requires both processes to disintegrate.

Relating this process to lateral ventricle fusion, it was investigated whether EMT could play a role in the loss of ependymal cells observed in areas of fusion. Previous studies have shown that transforming growth factor beta (TGF-β) is required for EMT to take place (Nawshad 2008). Here, TGF-β immunohistochemistry was used to examine the area of fusion. It was found that in areas of fusion, there was a slight up-regulation of TGF-β (Figure 12). Taken with the previous results on the lack of positive Caspase-3 staining, the mechanism underlying lateral ventricle wall fusion remains uncertain. It is likely that fusion is occurring by a similar, if not identical, mechanism to palatal fusion. Future studies may help shed more light on this process.

Ependymal Cell Density and Repair Along the Lateral Wall

Methods

Animals: Male CD-1 mice were purchased from Charles River and aged in our vivarium. All animal procedures were performed under protocols approved by the Institutional Animal Care and Use Committee of the University of Connecticut and conforming to National Institutes of Health guidelines.

Immunohistochemistry: Mice were perfused with 0.9% saline alone. The brains were dissected according to the procedure from Mizradeh et al. (2010). Tissue was then permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 10 min, blocked in 10% horse serum (Invitrogen) in PBS/1% Triton X-100 for 1 h, and incubated with combinations of the following primary antibodies: rabbit anti-beta catenin (1:500, Abcam) and rabbit anti-gamma tubulin (1:500, Abcam). 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. In order to verify the results, control tissue was stained with only secondary

antibodies. Sections were coverslipped with Aqua-Poly/Mount (Polysciences) and imaged on a Leica TCS SP2 confocal laser-scan microscope.

EdU: Mice were injected i.p. with 3 daily injections of EdU (Invitrogen) at 150mg/kg body weight to label dividing cells. Following a 6-week chase, label-retaining cells in the SVZ were examined.

Results

Previous results from our laboratory indicated that the dorsal section of the lateral ventricle might undergo an age-related expansion in volume. If this were the case, it would be expected that the ependymal cells in that area would undergo changes caused by the increased CSF volume. It was therefore investigated whether the density of ependymal cells changes as a result of the aging process.

To accomplish this, whole mount tissue from 3-month old, 6-month old, 1-year old, and 2-year old mice was stained with beta-catenin and gamma tubulin to label ependymal cells and their ciliary filaments (Figure 13a). Ependymal cell density was calculated at four distinct regions relative to the area of adhesion/fusion: anterior ventral, anterior dorsal, posterior dorsal, and middle (Figure 13b-c). The number of ependymal cells per square millimeter was calculated for each region and age group. In the anterior ventral, anterior dorsal, and middle regions, there was no change in the density of ependymal cells across the four age groups (Figure 13d,e,g). In the posterior dorsal region, however, an age-related decrease in cell density was found (Figure 13f). This decrease was statistically significant between the 3-month old and 2-year old mice ($p<0.05$; n=3,1□way ANOVA with Bonferroni's multiple comparison post test).

Next, it was investigated if the rate ependymal repair differed with respect to these different regions. For this experiment, ependymal repair was defined as a label-retaining SVZ

cell that was integrated into the ependymal wall (Figure 14a-b). This type of cell is believed to be a neural stem cell that took on ependymal cell characteristics as it integrated into the wall (Luo et al. 2008). The label used in this experiment was EdU, a thymidine analog similar to BrdU. The number of s100β+, EdU-retaining cells in the ependymal wall was counted at five different regions relative to Bregma (Figure 14d). It was found that there were more EdU+ ependymal cells in the region -0.01 to -0.6mm to Bregma than any of the other regions analyzed (Figure 14c). This region corresponds to the posterior dorsal region in which ependymal cell density was found to decrease in aging. This difference was found to be statistically significant ($p<0.05$; n=3,1□way ANOVA with Bonferroni's multiple comparison post test).

Age-related Changes in CSF Flow and Ependymal Polarity

Methods

Animals: Male CD-1 mice were purchased from Charles River and aged in our vivarium. All animal procedures were performed under protocols approved by the Institutional Animal Care and Use Committee of the University of Connecticut and conforming to National Institutes of Health guidelines.

CSF Flow: Mice were perfused with 0.9% saline. Brains were removed, placed in a sucrose solution, and dissected according to the whole mount procedure discussed earlier. The lateral wall was transferred to a new petri dish with fresh sucrose solution and pinned down to limit movement. A pedal-operated syringe was placed horizontally over the lateral ventricle wall that allowed India Ink to be ejected onto a given spot on the ventricle surface. A video camera stationed above the petri dish captured the subsequent movement of the ink across the ventricle surface. The flow velocity was analyzed afterward using the distance traveled by the ink and the

time needed to travel that distance. The lateral ventricle whole mounts were washed with PBS and then analyzed using immunohistochemistry.

Immunohistochemistry: The wholemounts were permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 10 min, blocked in 10% horse serum (Invitrogen) in PBS/1% Triton X-100 for 1 h, and incubated with combinations of the following primary antibodies: rabbit anti-beta catenin (1:500, Abcam) and rabbit anti-gamma tubulin (1:500, Abcam). 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. In order to verify the results, control tissue was stained with only secondary antibodies. Sections were coverslipped with Aqua-Poly/Mount (Polysciences) and imaged on a Leica TCS SP2 confocal laser-scan microscope.

Results

Previously, it was unknown if the flow of cerebrospinal fluid through the lateral ventricles changes as a result of the aging process. To study CSF flow dynamics, we created a model that allows for the injection of India ink onto the surface of a lateral ventricle whole mount. After perfusion, the tissue was kept in a sucrose solution to provide energy for still-functioning motile cilia. Each whole mount was then placed under a hypodermic needle filled with India Ink. The ink was ejected onto a spot on the surface of the whole mount and a video camera above captured the movement of the ink created by the beating cilia. The video recordings were then used to analyze flow dynamics.

First, the average velocity of the flow of cerebrospinal fluid across the lateral ventricles was calculated in 3-month old and 2-year old mice. It was found that the velocity declines in an age-related fashion (Figure 15). The difference between the two age groups was found to be

statistically significant ($p<0.05$; n=4,1□way ANOVA with Bonferroni's multiple comparison post test).

Next, the directionality of CSF flow across the lateral ventricles was investigated. Upon examining the videos of the India ink experiment, it was observed that there was no age-related change in the direction of CSF flow at most points along the ventricle surface. Above the area of adhesion/fusion, however, a backwards microcurrent was observed in each 2-year old sample that was not present in any of the 3-month old samples.

As shown in Figure 16, when the ink was injected posterior and dorsal to the area of stenosis in 3-month old mice, the ink flowed in both the ventral direction and around the area of stenosis to the anterior end of the ventricle (Figure 16a). In 2-year old mice, however, the ink flowed in only the ventral direction (Figure 16b). This indicated that there was an aberrant area of flow above the area of fusion. To investigate this further, the ink was injected at two spots above the area of stenosis. At the more anterior point, the ink flowed in both the ventral and posterior directions (Figure 16c). When injected at the more posterior point, the ink flowed in the posterior direction only (Figure 16d). This demonstrated that in 2-year old mice, there is an aberrant current above the point of fusion. At that point, the ink flows in the opposite direction as would be expected from observations in younger mice.

To investigate this point of aberrant flow further, the tissue used in the India Ink experiment was immunostained with β-catenin and γ-tubulin, which stain the outline of ependymal cells and the microtubules of motile cilia, respectively. Previous studies have shown that the organization of cilia on the surface of ependymal cells is indicative of the direction in which the cilia beat (Mizradeh et al. 2010). It has also been shown that this polarity is set up by a combination of hydrodynamic forces and planar cell polarity proteins (Guirao et al. 2010).

Therefore, it was examined whether the polarity of ependymal cell above the area of fusion exhibited the age-related difference expected from the India Ink experiments.

In 3-month old mice, it was found that the ependymal cells at the point above the area of fusion all had the same ciliary orientation. This was indicated by the position of γ-tubulin-stained punctae within each ependymal cell (not shown). In 2-year old mice, however, there was a group of ependymal cells above the area of fusion oriented opposite to the rest of the cells. The majority of the cells in this location were oriented in the anterior direction (Figure 17a). A small subgroup of cells, however, was oriented in the posterior direction (Figure 17b, c). This is concordant with the flow dynamics observed in these ventricles: a slight posterior current above the area of adhesion/fusion.

Analysis of the Remainder of the Ventricular System

Methods

Animals: Male CD-1 mice were purchased from Charles River and aged in our vivarium. All animal procedures were performed under protocols approved by the Institutional Animal Care and Use Committee of the University of Connecticut and conforming to National Institutes of Health guidelines.

Immunohistochemistry: Mice underwent transcardial perfusion with 0.9% saline, followed by 4% paraformaldehyde (Electron Microscopy Science) in PBS. Brains were fixed overnight in 4% paraformaldehyde at 4°C and then washed in PBS three times (20 min each wash) before cutting into 50 μm coronal sections with a vibratome (VT-1000S; Leica). Tissue was then permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 10 min, blocked in 10% horse serum (Invitrogen) in PBS/1% Triton X-100 for 1 h, and incubated with the following

primary antibodies: mouse anti-GFAP (1:250, Abcam) and rabbit anti-s100β (1:500, Abcam). 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. In order to verify the results, control tissue was stained with only secondary antibodies. Sections were coverslipped with Aqua-Poly/Mount (Polysciences) and imaged on a Carl Zeiss Axio Imager M2 microscope with Apotome (Carl Zeiss) using Hamamatsu ORCA-R2 digital camera C10600.

EdU: Mice were injected i.p. with 3 daily injections of EdU (Invitrogen) at 150mg/kg body weight to label dividing cells. Mice were sacrificed 1 day after the final injection.

3-D Reconstructions: After imaging coronal sections, the ventricles were traced with contours using the StereoInvestigator software. Different contours were drawn around the ventral third ventricle, dorsal third ventricle, cerebral aqueduct, and fourth ventricle. Contours were then uploaded into the Neurolucida Explorer software, in which 3-D models were constructed.

Results

After observing numerous age-related changes in the lateral ventricles, a brief analysis was conducted on age-related changes in the remainder of the ventricular system: the third ventricle, cerebral aqueduct, and fourth ventricle. Tissue was acquired from 3-month old and 2-year old mice and comparisons were made between these two groups.

First, general observations were made regarding differences between the two age groups. As shown by representative images from the dorsal third ventricle and the cerebral aqueduct, there were minimal differences found (Figure 18a-d). Neither group showed any type of adhesion or fusion between the ventricle walls. Both groups had an intact ependymal wall along

within each ventricular segment and the shapes of the ventricles were maintained. Additionally, EdU staining did not show any proliferation observed along the walls of the ventricles.

In order to calculate if there was an age-related difference in the overall volume of these pieces of the ventricular system, three-dimensional reconstructions were made from the imaged sections. It was found that the total volume of the system did not change between 3-month old and 2-year old mice. Additionally, the volumes of the individual segments did not change with age (Figure 18e).

The Effects of Repetitive, Mild Traumatic Brain Injury

Methods

Injury Model: Mice were provided by the Kune laboratory at Wayne State University. The animals underwent the repetitive, mild traumatic brain injury model described in Kane et al. (2012). Head injuries were produced once a day for five consecutive days. 20 days after the last hit, the mice were sacrificed.

Immunohistochemistry: Each brain was cut into 50 μm coronal sections with a vibratome (VT-1000S; Leica). Tissue was then permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 10 min, blocked in 10% horse serum (Invitrogen) in PBS/1% Triton X-100 for 1 h, and incubated with the following primary antibodies: mouse anti-GFAP (1:250, Abcam) and rabbit anti-AQP4 (1:500, Abcam). 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. Sections were coverslipped with Aqua-Poly/Mount (Polysciences) and imaged on a Carl Zeiss Axio Imager M2 microscope with Apotome (Carl Zeiss) using Hamamatsu ORCA-R2 digital camera C10600.

3-D Reconstructions: After imaging coronal sections, the ventricles were traced with contours using the StereoInvestigator software. Different contours were drawn around the anterior lateral ventricle, open ventricles around the area of fusion, and the posterior lateral ventricle. Contours were then uploaded into the Neurolucida Explorer software, in which 3-D models were constructed.

Results

The effects of repetitive, mild TBI (rmTBI) on the lateral ventricles were examined next. Specifically, the volume of the lateral ventricle was calculated in TBI and control brains to examine if rmTBI results in an expansion of the ventricles. This was accomplished using 3-D reconstructions of the lateral ventricles (Figure 19a,b). It was hypothesized that rmTBI would result in a slight increase in volume when compared to controls.

It was found that there was no difference between the volume of the lateral ventricles in rmTBI mice and the volume in control mice (Figure 19c). Within each segment of the lateral ventricle, there was also no difference in volume. There was, however, a difference in the degree of intraventricular fusion between the two groups. The rmTBI group had a larger area of fusion than the control group (Figure 19d). As shown by the representative reconstructions, the control brains had a much greater area of open, non-fused ventricle (Figure 19a,b). This difference that was nearly significant ($p=0.054$, one-way ANOVA with Bonferroni's multiple comparison post hoc test; $n=5$).

The Effects of Neuraminidase on Ventricular Volume

Methods

Animals: Male CD-1 mice (aged 1.5 to 2 years) were purchased from Charles River and aged in our vivarium. All animal procedures were performed under protocols approved by the Institutional Animal Care and Use Committee of the University of Connecticut and conforming to National Institutes of Health guidelines.

Neuraminidase Injections: Neuraminidase from Clostridium perfringens (Roche Diagnostics, Indianapolis, IN) was dissolved in saline at a concentration of 0.5 mg/ml. Each animal was anesthetized with isofluorane and placed in a stereotaxic apparatus. Diluted neuraminidase (1 ul) was delivered unilaterally to the anterior lateral ventricle using a stereotaxically-positioned capillary needle and a microinjector.

Immunohistochemistry: Mice underwent transcardial perfusion with 0.9% saline, followed by 4% paraformaldehyde (Electron Microscopy Science) in PBS. Brains were fixed overnight in 4% paraformaldehyde at 4°C and then washed in PBS three times (20 min each wash). Each brain was cut into 50 μm coronal sections with a vibratome (VT-1000S; Leica). Tissue was then permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 10 min, blocked in 10% horse serum (Invitrogen) in PBS/1% Triton X-100 for 1 h, and incubated with the following primary antibodies: mouse anti-GFAP (1:250, Abcam) and rabbit anti-AQP4 (1:500, Abcam). 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. Sections were coverslipped with Aqua-Poly/Mount (Polysciences) and imaged on a Carl Zeiss Axio Imager M2 microscope with Apotome (Carl Zeiss) using Hamamatsu ORCA-R2 digital camera C10600.

3-D Reconstructions: After imaging coronal sections, the ventricles were traced with contours using the StereoInvestigator software. Different contours were drawn around the

anterior lateral ventricle, open ventricles around the area of fusion, and the posterior lateral ventricle. Contours were then uploaded into the Neurolucida Explorer software, in which 3-D models were constructed.

Results

In order to further elucidate the effects of injuries on the lateral ventricles, the toxin Neuraminidase was injected into the lateral ventricles of aged mice. Neuraminidase cleaves sialoglycoproteins from ependymal cells, causing a degradation of the ependymal wall (Grondona et al. 1996). It was found that injection of Neuraminidase resulted in an increase in the volume of the ipsilateral lateral ventricles when compared to control animals (Figure 20). This difference was statistically significant ($p<0.05$, n=4,1□way ANOVA with Bonferroni's multiple comparison post test). As expected, the volume of the contralateral lateral ventricles of the injected mice was higher than control animals, yet lower than the ipsilateral ventricles. When the lateral ventricle was segmented into different regions, it was found that an increase in volume in the posterior region was most responsible for the increase in total volume. The difference in posterior volume between the ipsilateral injected ventricles and control ventricles was found to be statistically significant ($p<0.05$, n=4,1□way ANOVA with Bonferroni's multiple comparison post test).

DISCUSSION

Here, numerous age-related changes to the subventricular zone, ependymal barrier, and lateral ventricle structure were shown to occur in mice. There are a few possible explanations for the phenomena observed. First, each these changes could be independent of one another. The stretching of ependymal cells posterior and dorsal to the area of fusion, for instance, could be unrelated to the changes in CSF flow dynamics. This explanation, however, does not take into account the systematic nature of the relationship between the subventricular zone, the ependymal barrier that guards it, and the entire lateral ventricle surrounding it. All of these components are part of a larger, intertwined system that governs the flow of cerebrospinal fluid, the integrity of the ependymal wall, and neurogenesis. It is therefore reasonable to speculate that these changes may be related to one another.

The most likely explanation for the observed changes relates back to the first finding in this study. It was initially shown that the area of adhesion/fusion increases with age (Section I). This increase was significant, as the area of fusion nearly tripled between 3-month old and 2-year old mice. As this area of fusion is expanding with age, it is reasonable to assume that related changes will occur in the regions closest to this expansion.

It is possible, for example, that as the wall fused with itself, the flow of cerebrospinal fluid around that area was disturbed. In this case, CSF flows from the middle of the lateral ventricle to the area above fusion, then around the anterior horn. A disruption would most likely occur where it first comes in contact with the newly fused walls, which would be above the area of fusion. This disruption in flow may have caused the cilia of the ependymal cells to re-orient themselves, as it has been shown that the flow of cerebrospinal fluid help orient ciliary beating (Guirao et al. 2010). These re-oriented cilia would be the same ones observed in the previously

shown experiments above the area of fusion (Section III). They would then produce the altered CSF currents shown to occur above the area of stenosis (Section III).

One possible consequence of this altered CSF flow is an increased fluid pressure exerted on the cells in the surrounding area. The turbulent conditions created by forward flowing CSF and the newly formed back current may exert increased pressure on the ependymal cells in that area. This idea is corroborated by the observation that cells posterior and dorsal to the area of fusion are prone to significant age-related stretching (Section II). The subventricular zone may respond to this change in cellular density by increasing the rate of ependymal repair in that area. This increased rate was seen posterior and dorsal to the area of fusion, as predicted (Section II). This same region, posterior to the area of fusion, underwent the greatest volume increase upon treatment of Neuraminidase (Section VI).

Clearly, the lateral ventricles, in specific the region posterior to the area of fusion, are prone to age-related changes. These changes, however, do not appear to occur elsewhere in the ventricular system. Upon analysis of the 3rd ventricle, cerebral aqueduct, and 4th ventricle, no such changes in volume, structure, or proliferation occur outside of the lateral ventricle. Therefore, the lateral ventricle has unique characteristics that make it uniquely susceptible to age-related changes. One possible example of such a feature is related to the flow dynamics of cerebrospinal fluid. As mentioned, the choroid plexus in the lateral ventricles produces the majority of CSF. It then flows to the 3rd ventricle, cerebral aqueduct, and 4th ventricle. If there were a change in CSF production, the lateral ventricle would be the first area to feel the effects of the associated changes in fluid flow and pressure. The lateral ventricle could buffer these changes before they effect the rest of the ventricular system. The adaptability in structure of the lateral ventricle, therefore, may be a way to buffer changes in CSF production.

A similar line of reasoning may be used to explain the finding that repetitive, mild traumatic brain injury results in an increase in fusion. rmTBI could result in a decrease in CSF production, causing more fusion to occur to buffer the changes in CSF volume and equalize the pressure of CSF. Alternatively, the increased fusion could be the result of heightened inflammation in the tissue surrounding the lateral ventricles. This could cause more force to be exerted on the walls of the ventricle, causing them to compress into the ventricular cavity.

The unique changes that occur in the lateral ventricles may highlight a possible novel function of the SVZ. If the lateral ventricle is indeed subject to special changes, the SVZ may have developed along the walls of the lateral ventricle to provide a stem cell niche to help buffer said changes. As shown above, neural stem cells from the SVZ integrate themselves into the ependymal barrier at the site of greatest change. This role of the SVZ could help explain why the stem cell niche is found along the lateral walls of the lateral ventricles, rather than closer to the olfactory bulb, where most of its progeny end up.

It is important to note that many of the age-related changes demonstrated above have also been shown to occur after the induction of a stroke. Young et al. (2012) showed that ependymal cell stretching, altered ependymal cell polarity, a decrease in velocity of CSF flow, and disruptions in the directionality of CSF flow all occur one to two weeks after middle cerebral artery occlusion-induced stroke in 3-month old mice. This indicates that, as seen in previous studies, the SVZ reacts in similar ways to the aging process and injury models.

Moving forward, there are numerous experiments that could be performed to better understand the results shown here. First, the ability of neural stem cells to acquire nutrients and growth factors from the CSF could change in tandem with the numerous changes described above. This may be a relevant in determining the causes of age-related declines in neurogenesis.

Secondly, the genetic and structural characteristics of ependymal cells could be examined in the lateral ventricle and elsewhere in the ventricular system. Comparisons could be made to help discern the mechanism underlying the lateral ventricle's unique susceptibility to structural changes. Additionally, in order to examine the potential role of the SVZ as a buffer for age-related changes in the lateral ventricles, neurogenesis could be knocked out. If the lateral ventricles underwent more significant changes in the knockout condition, it would support the hypothesis that the SVZ has a role as a buffer.

Similarities and differences between the mouse SVZ and the human SVZ would also be beneficial to investigate. The human SVZ and ependymal barrier are very understudied, so there is much to be learned about the region and how it changes over time. For example, preliminary results from our laboratory indicate that ependymal cells are lost in various regions along the walls of the lateral ventricles in elderly humans. This ependymal loss appears to be associated with an increase in reactive astrogliosis along the wall and, ventriculomegaly, an increase in ventricular volume. Further investigations into this area will likely produce important results in the field of aging research.

On a final note, the research presented here deals almost exclusively with the effects of the normal aging process. The field of aging research is growing at rates not seen since the boom in cancer research in the late 20th century. Hopefully, researchers will be able to find treatments for devastating neurodegenerative diseases, such as Alzheimer's disease, Amyotrophic lateral sclerosis (ALS), and Parkinson's disease. But at what end will we be satisfied with our progress? Will research move away from age-related diseases and instead focus on extending the human lifespan? I believe that the answer to these questions lies in the idea of successful aging. This field focuses on the positive aspects of the aging process and the

overall well-being of older adults. Aging research should be considered a success when the great majority of people are able to grow old in a healthful, positive manner, free of any major diseases. Obviously, everyone's life must eventually come to a conclusion. Research should not focus on delaying this inevitable end, but should instead attempt to improve the physical and mental well-being of individuals as they grow older.

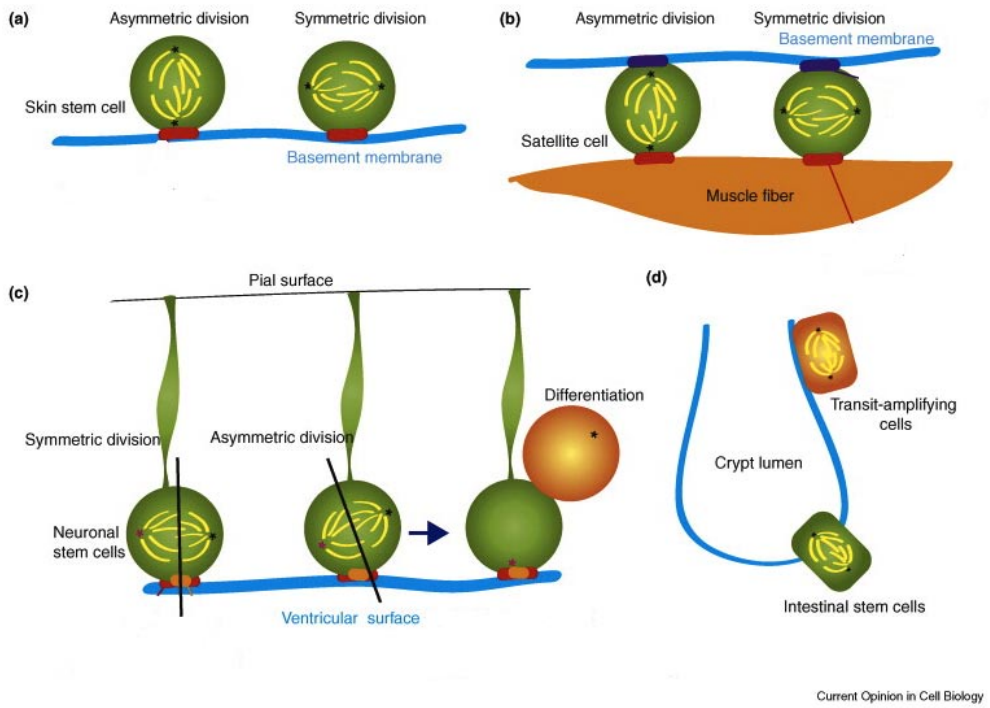

Figure 1: Stem cell niches and division (Adapted from Yamashita, 2010)

Various stem cell niches are shown, including the skin (A), muscle (B), subventricular zone (C), and intestinal crypt (D). The directionality of symmetric and asymmetric division is also shown.

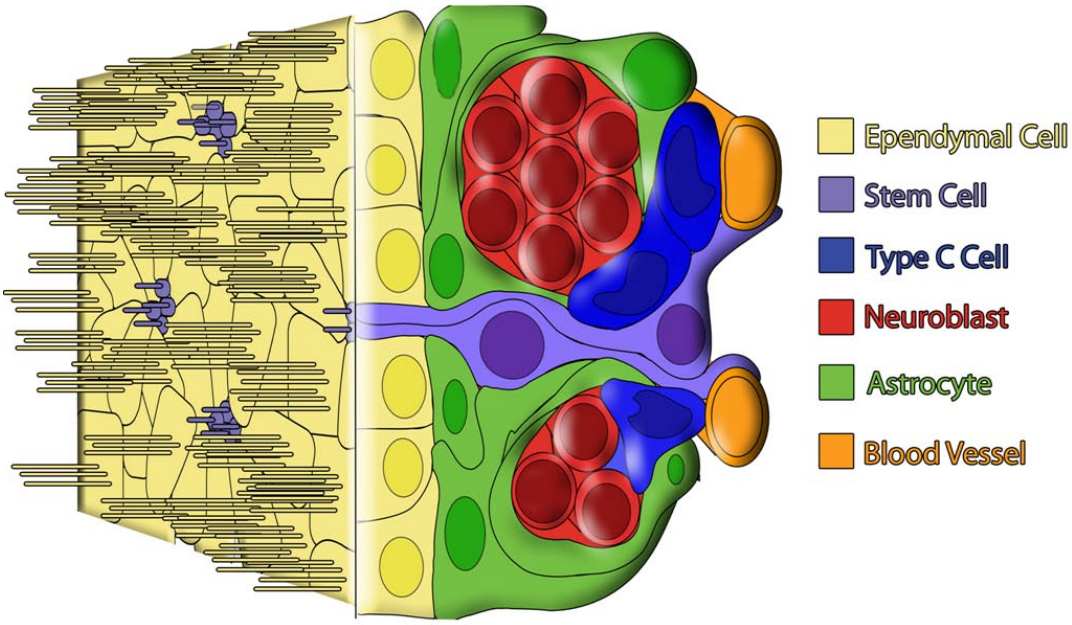

Figure 2: The cytoarchitecture of the murine subventricular zone

The subventricular zone has five primary cells types: ependymal cells, neural stem cells, Type C cells, neuroblasts, and astrocytes. The neural stem cells extend a process through the ependymal barrier into the lateral ventricle.

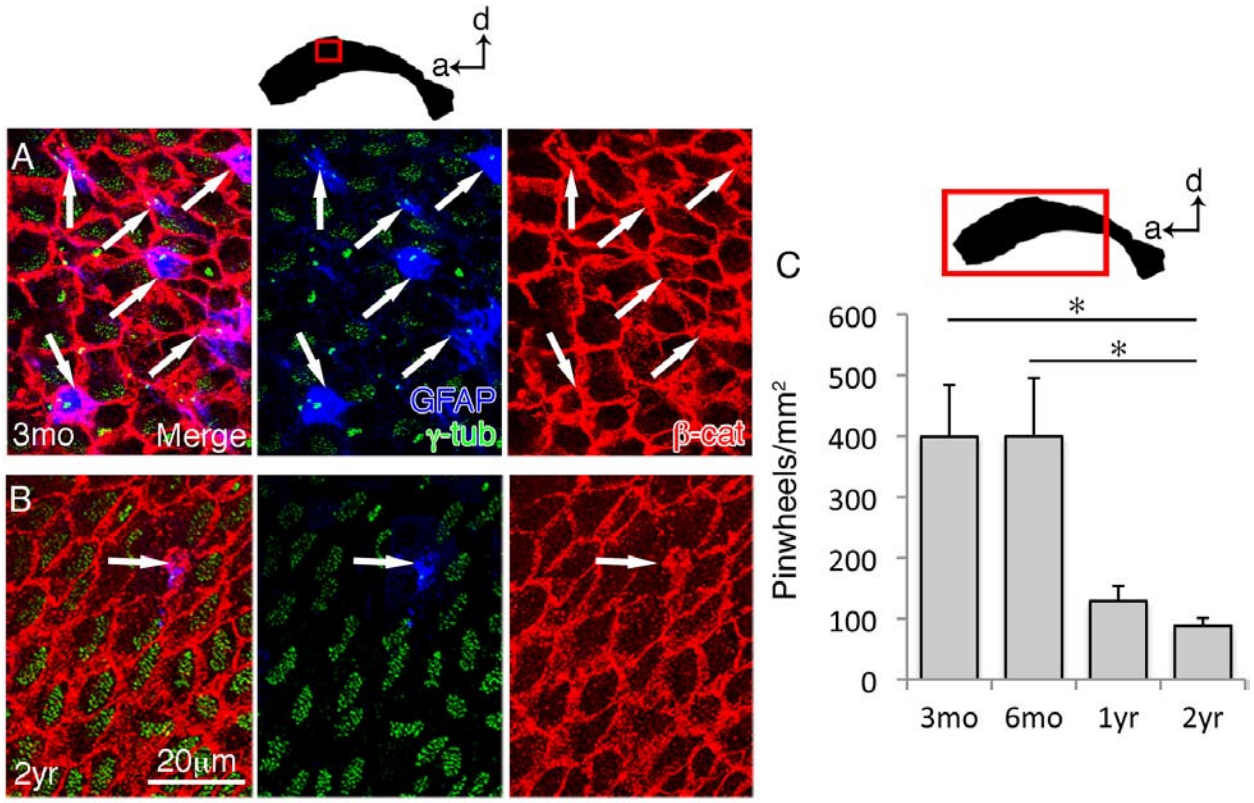

Figure 3: The number of pinwheels on the ventricle surface declines with age.

Pinwheels with NSC cores can be seen in 3-month old (A) and 2-year old (B) mice. The number of pinwheels per square millimeter declines significantly with age (C).

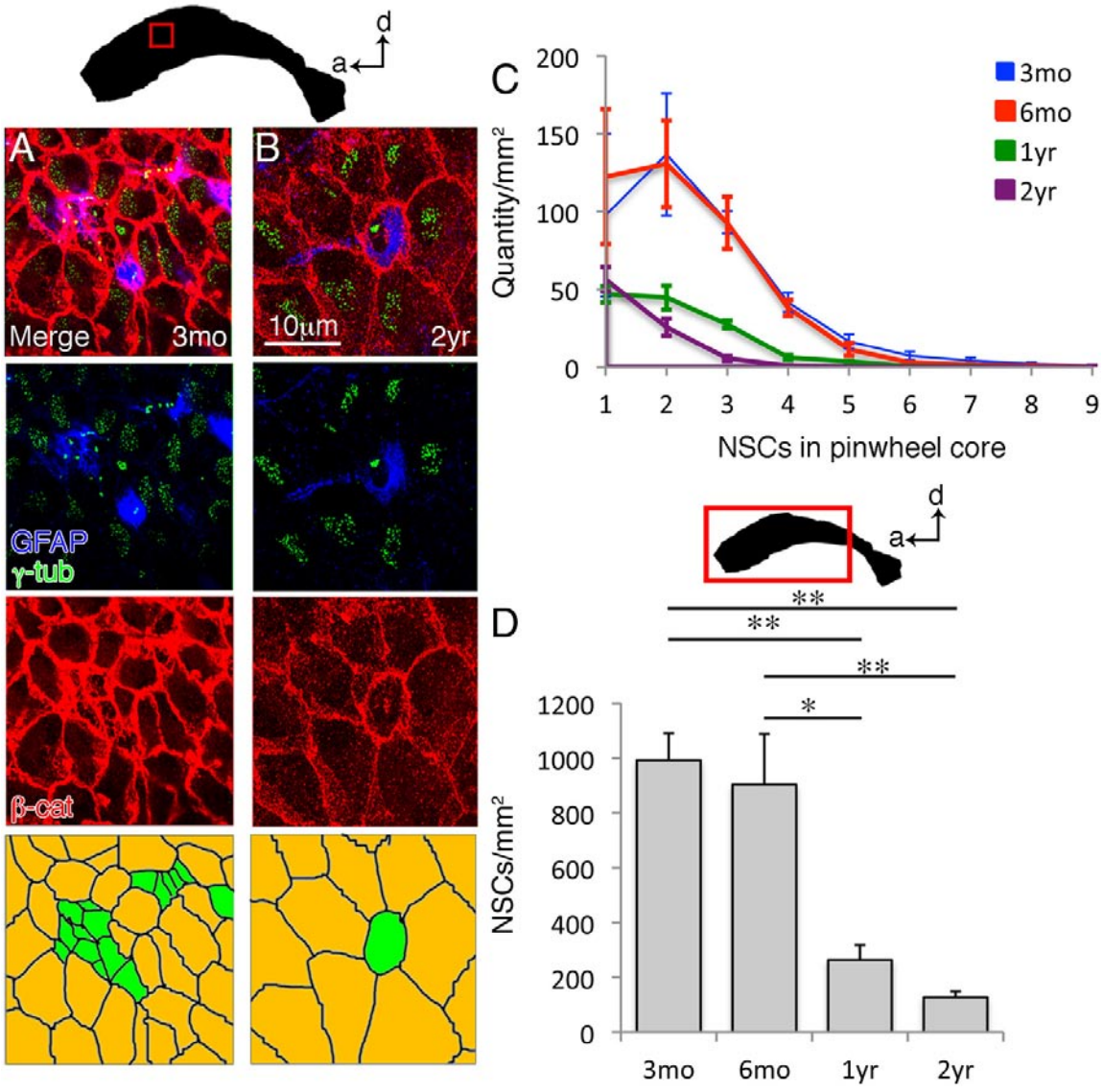

Figure 4: The number of stem cells per pinwheel and total number of stem cells decline with age.

The number of NSCs per pinwheel can be seen in 3-month old (A) and 2-year old (B) mice. The number of pinwheels per core declines with age (C). Taken together with previous data, this shows that the total number of neural stem cells per square millimeter declines significantly with age (D).

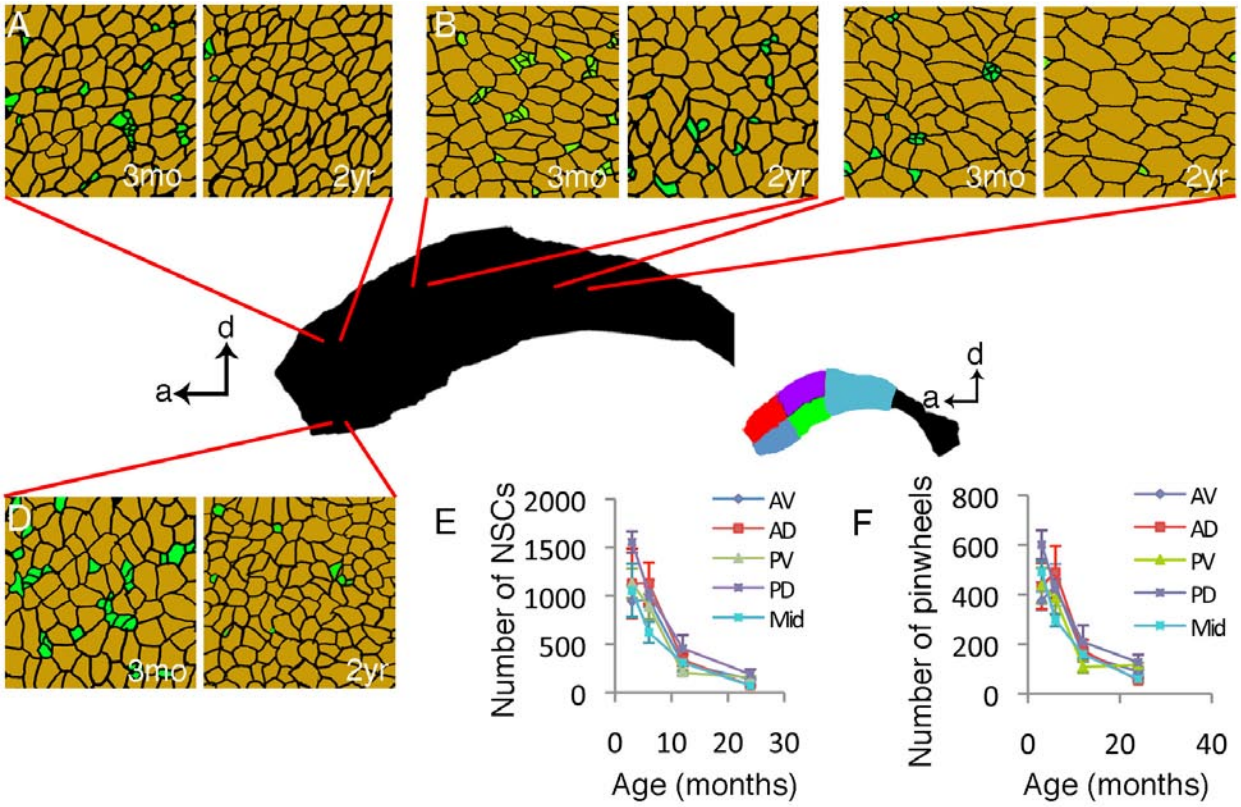

Figure 5: Age-related decline in neural stem cell number is not region-specific.

The number of NSCs and pinwheels were analyzed in four different regions across the surface of the lateral ventricle (A-D). The number of neural stem cells (C) and number of pinwheels (D) did not differ between the regions.

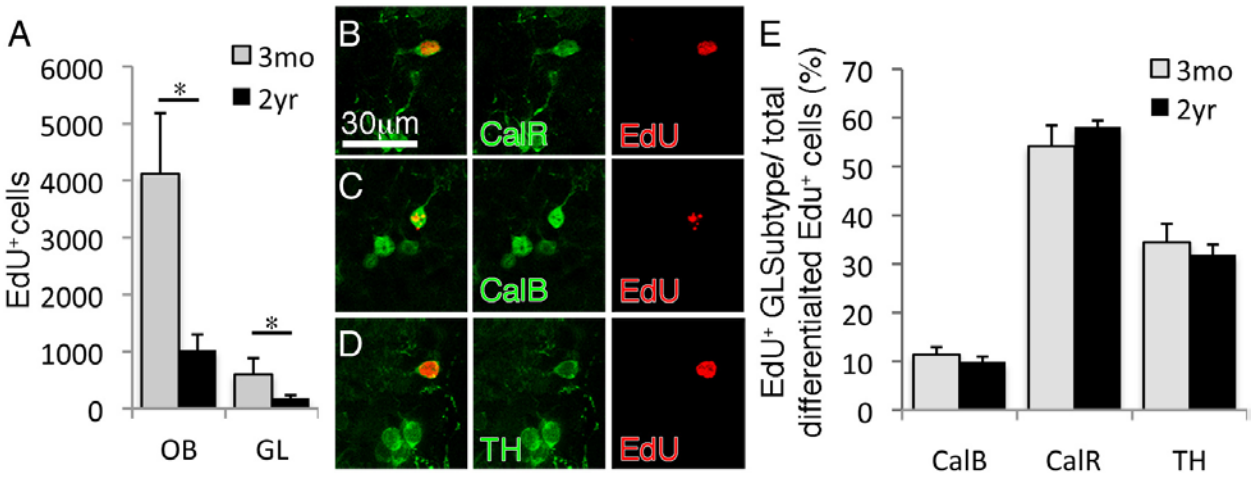

Figure 6: Olfactory bulb interneurons experience a uniform, age-related decline.

The number of EdU+ interneurons in the olfactory bulb decline significantly with age (A). The ration of glomerular layer subtypes produced by the SVZ does not change with age (B-E).

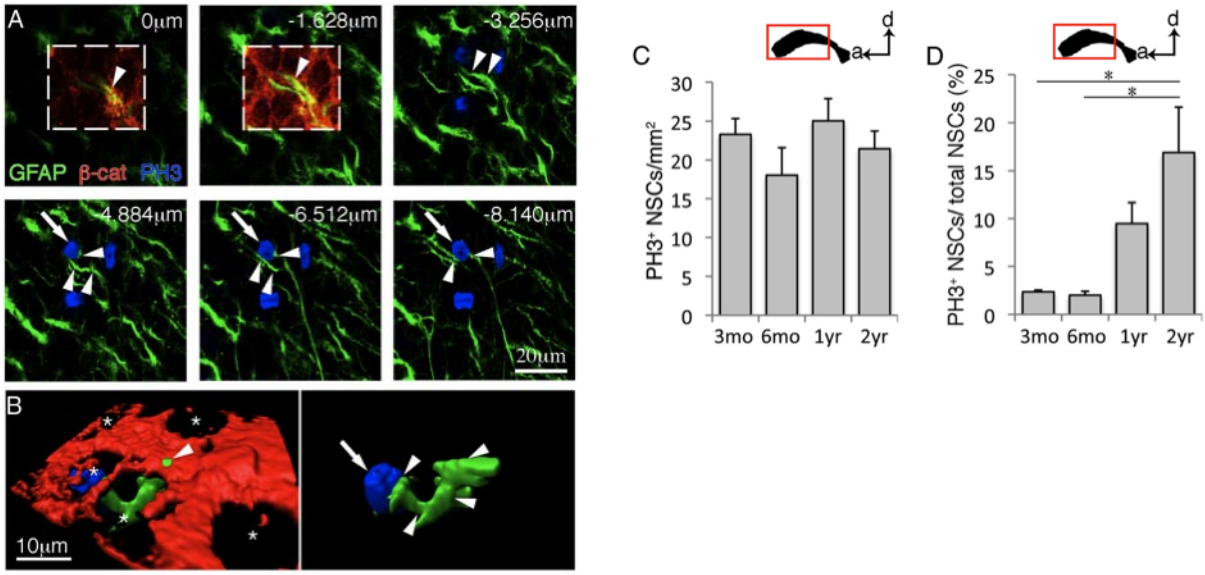

Figure 7: The percentage of neural stem cells undergoing mitosis increases with age.

Dividing neural stem cells were identified by their GFAP+ process and PH3+ nucleus (A-B). The total number of PH3+ NSCs was found to remain unchanged throughout aging (C), meaning the percentage of mitotically active NSCs increased significantly with age (D).

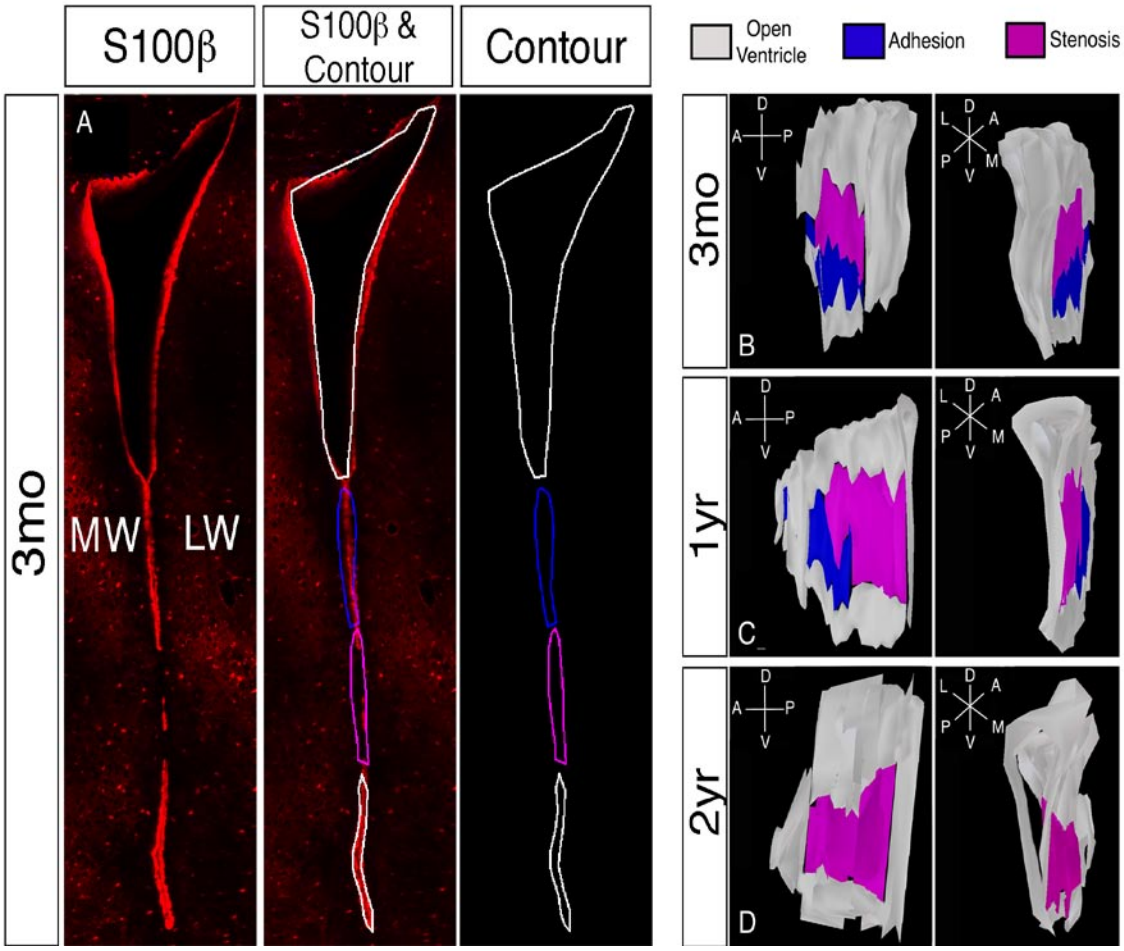

Figure 8: Stenosis progresses to adhesion and then fusion in aging mice.

In 3-month old mice, areas of open ventricle, adhesion, and fusion are seen (A). Contours drawn around these areas were made into 3-dimensional reconstructions, showing greater areas of fusion in 2-year old mice (D) than 1-year old mice (C) or 3-month old mice (B).

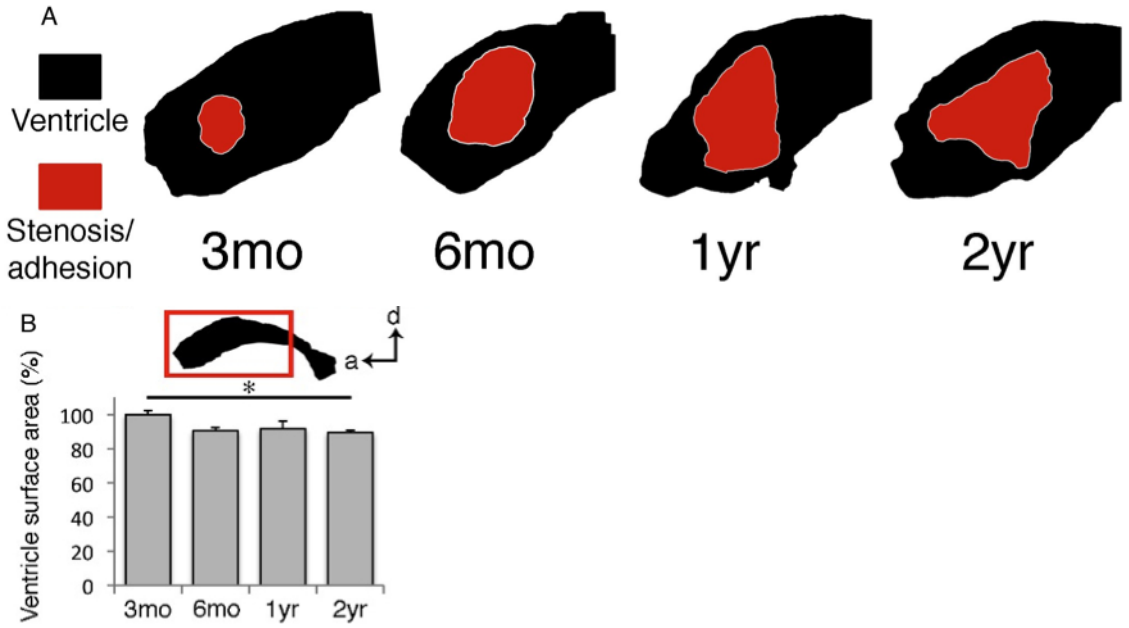

Figure 9: The anterior horn of the lateral ventricle undergoes an age-related decline in open-ventricle surface area.

Using whole mounts, the area of adhesion/fusion can be seen to increase with age (A). The remaining surface area was seen to decrease significantly between 3-month old and 2-year old mice (B).

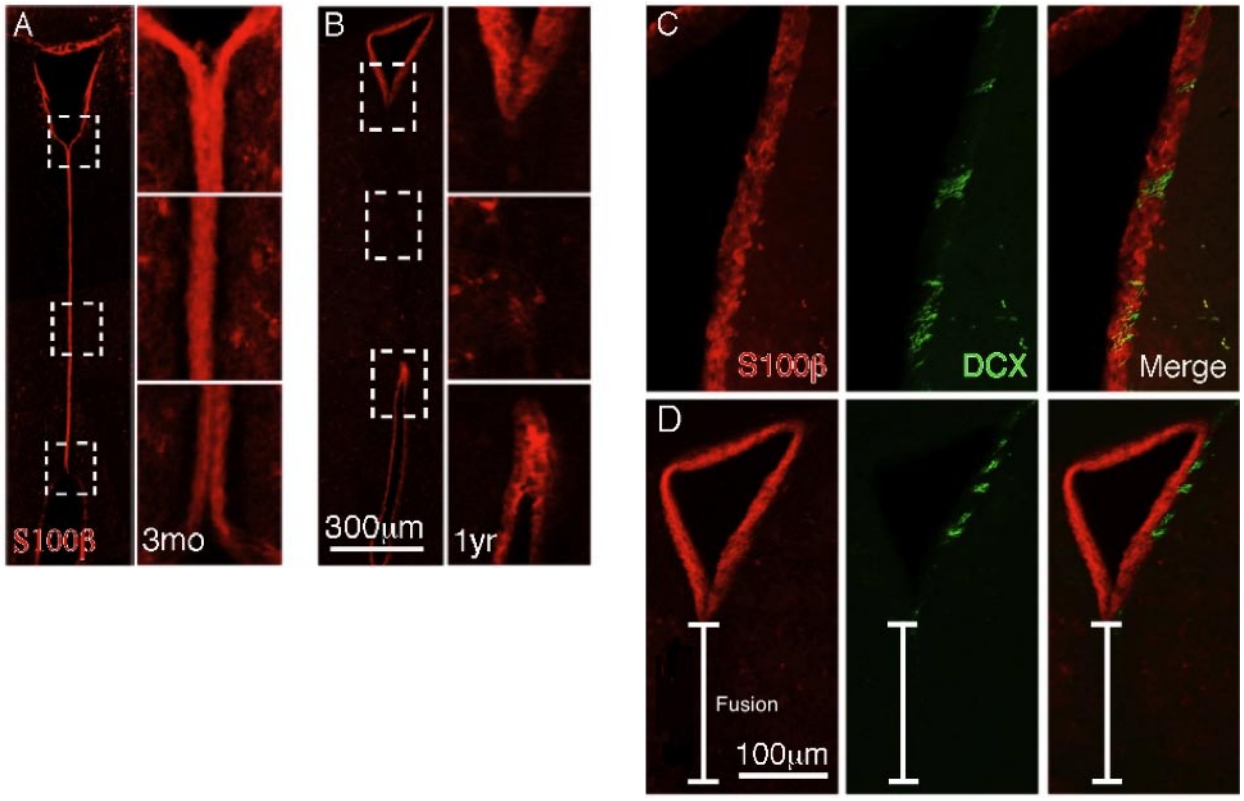

Figure 10: Fusion results in the degradation of neurogenesis.

In areas of open ventricle (A,C), DCX+ neuroblasts are present along the lateral wall of the lateral ventricles, while in areas of fusion (B,D), DCX+ neuroblasts are absent.

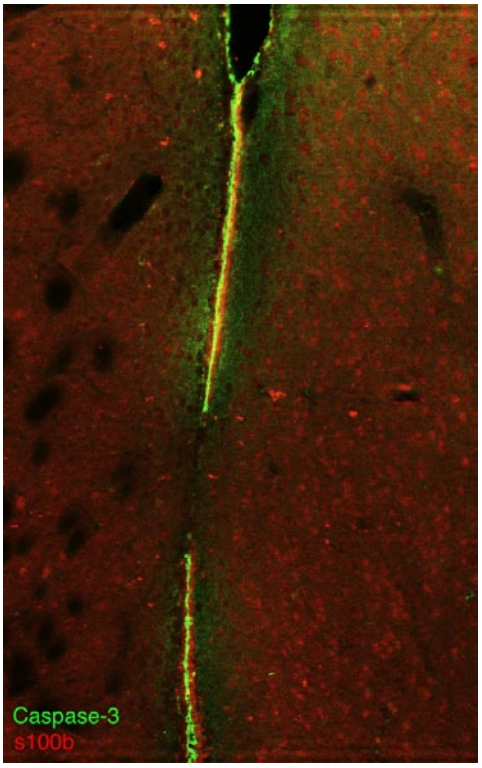

Figure 11: Caspase-3 staining showed a lack of Caspase 3-mediated apoptosis at the site of fusion.

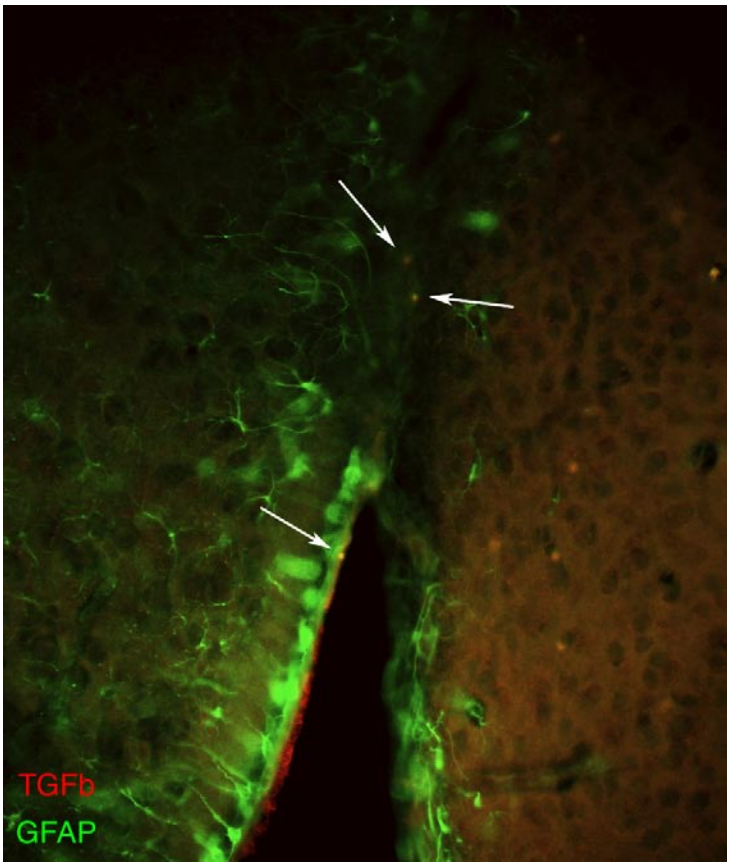

Figure 12: TGF-β staining is present in the area of adhesion/fusion.

In this 2-year old mouse, TGF-β staining was seen in the area of fusion. Arrows indicate cells with heightened TGF-β expression.

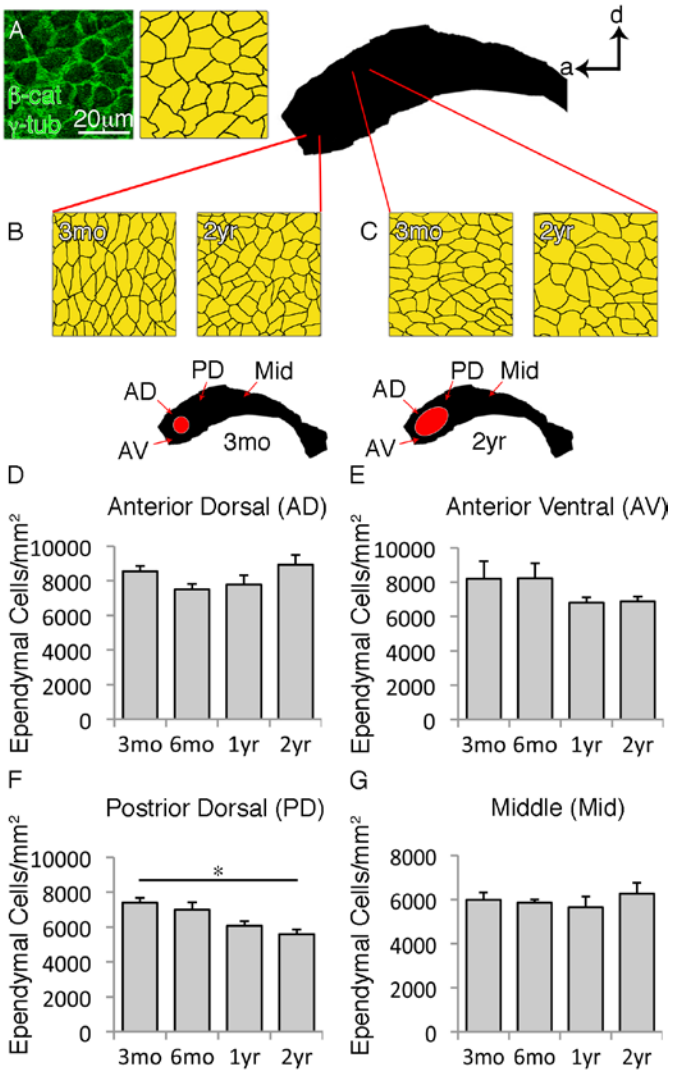

Figure 13: An age-related decline in ependymal cell density occurs posterior and dorsal to the area of adhesion/fusion.

Using whole mounts, ependymal cell density was examined in four different regions along the lateral wall relative to the area of fusion (A-C). There was no change in density across the four age groups in three of the four regions: anterior dorsal (D), anterior ventral (E), and middle ventricle (G). In the posterior dorsal region, there was a significant decline in density between 3-month old and 2-year old mice.

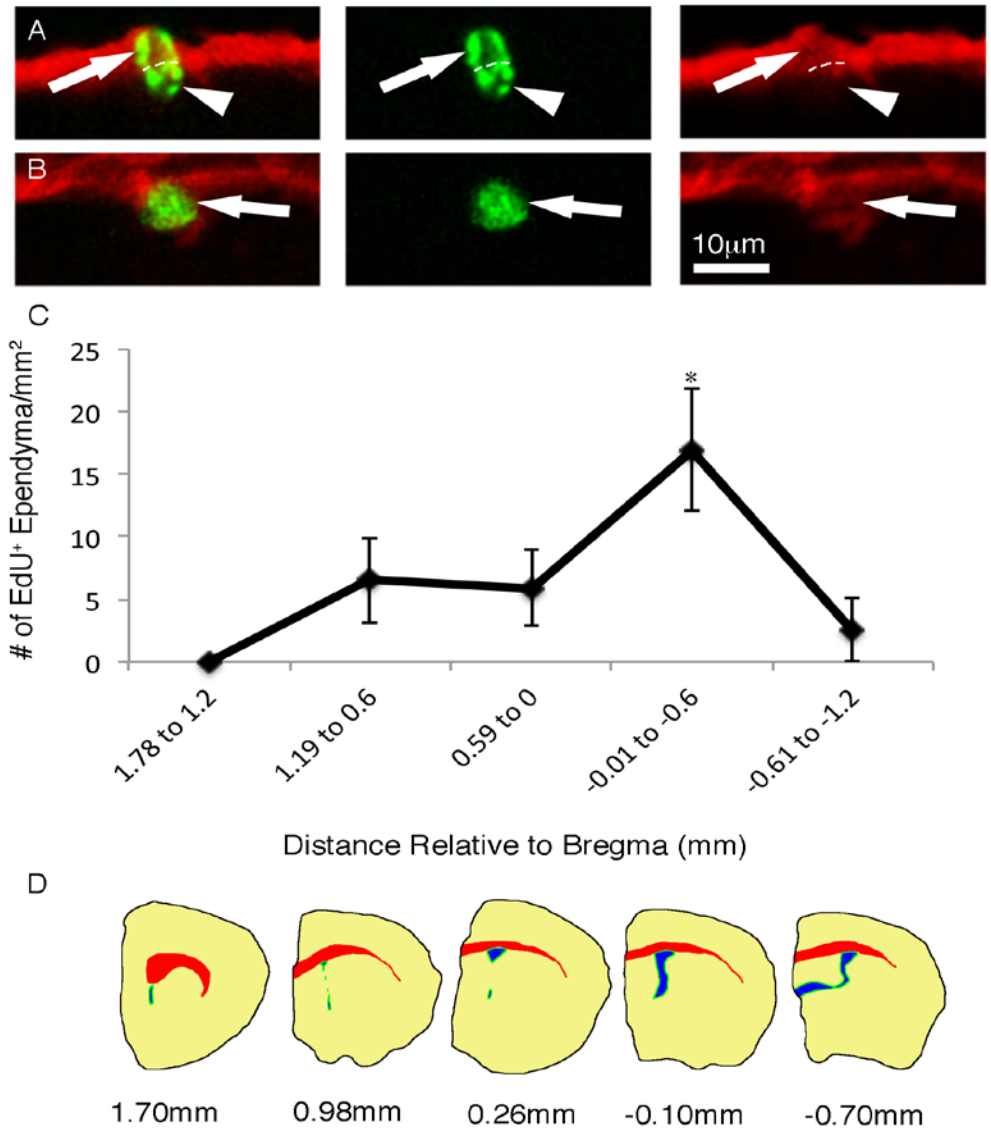

Figure 14: Ependymal repair is greatest posterior to the area of adhesion/fusion.

EdU+ nuclei can be seen to integrate into the ependymal wall and colocalize with s100β (A-B). The number of EdU+ ependymal cells was quantified at various areas relative to Bregma (D) and was found to be significantly greater posterior to the area of fusion (C).

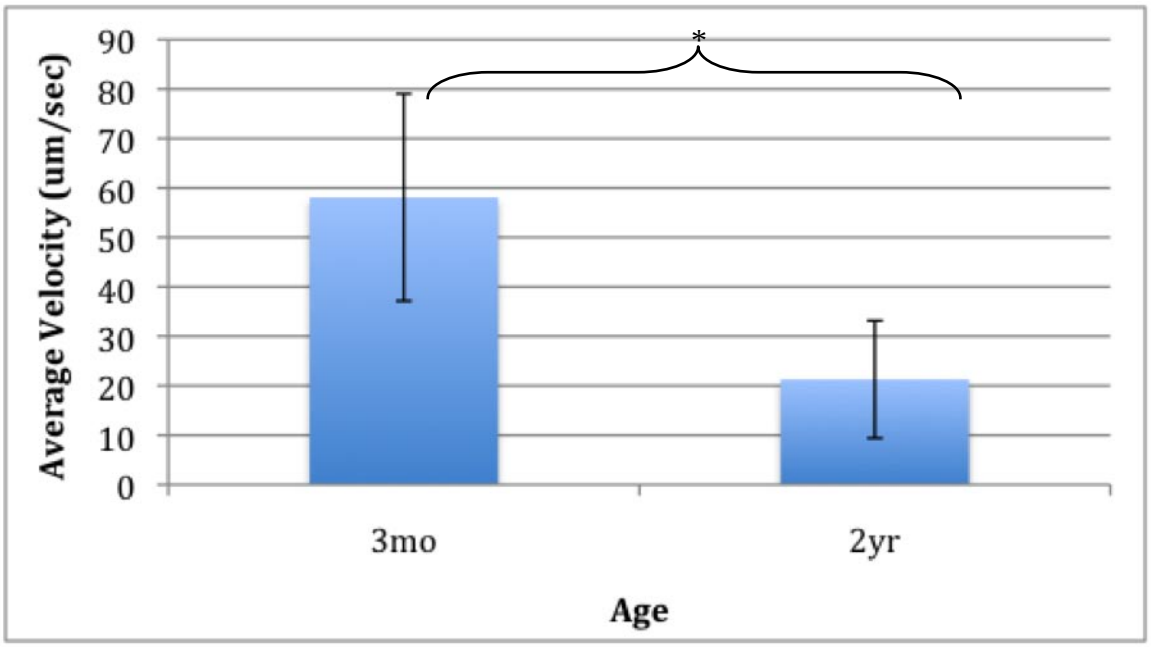

Figure 15: The velocity of CSF flow through the lateral ventricle significantly decreases between 3-month old and 2-year old mice (*=p<0.05).

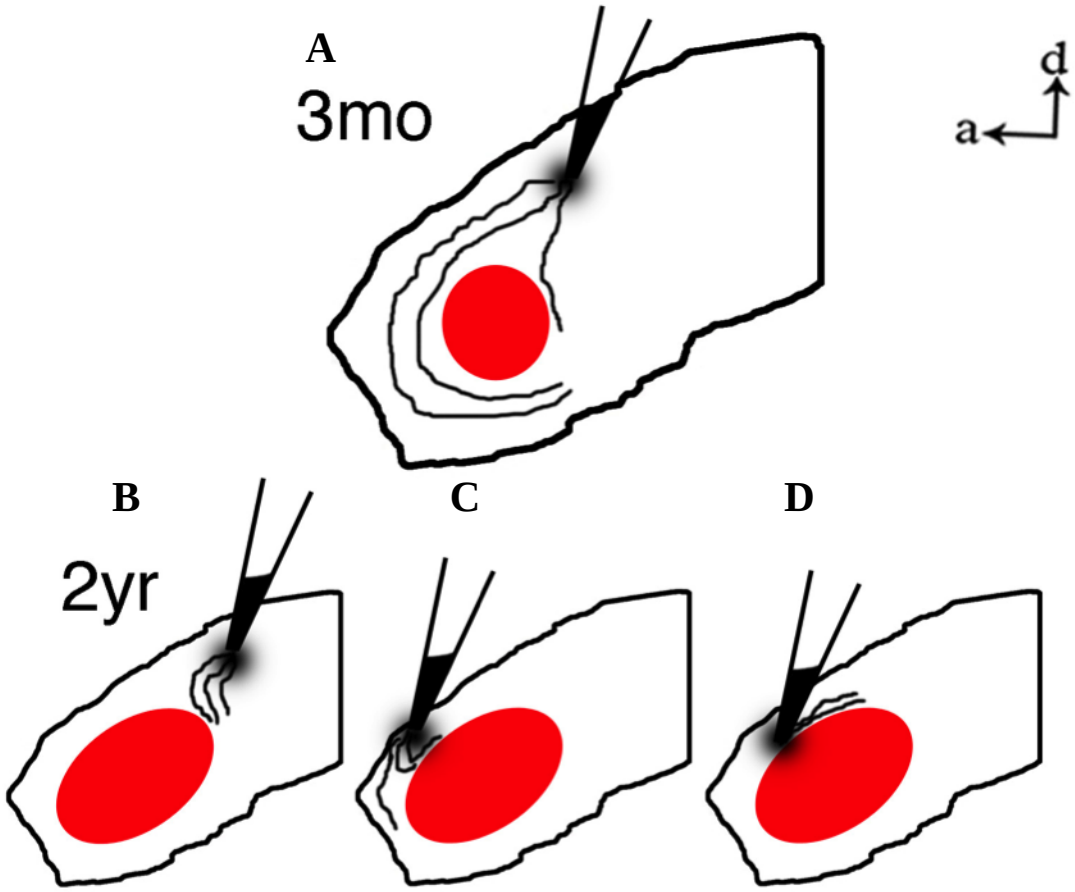

Figure 16: The directionality of CSF flow across the surface of the lateral ventricle changes above the area of fusion.

In 3-month old mice, CSF flows from the middle of the lateral ventricle, above the area of fusion, and around the anterior horn (A). In 2-year old mice, altered currents were observed above the area of stenosis (B-D).

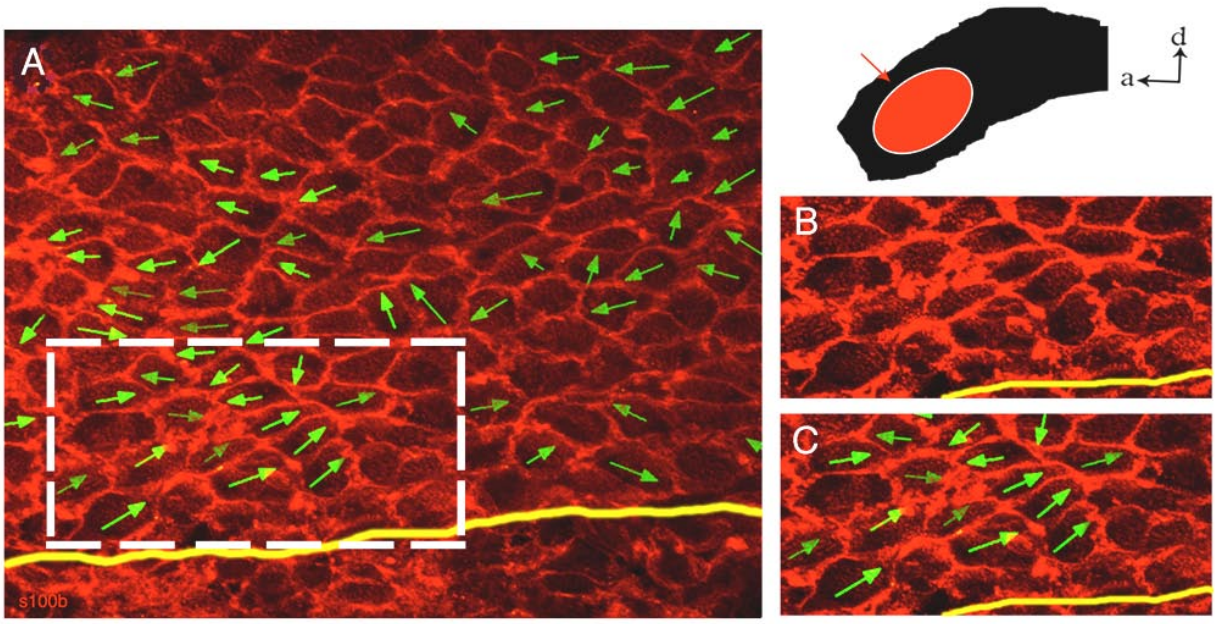

Figure 17: Above the area of fusion, a patch of ependymal cells had the opposite polarity to the cells surrounding them.

In 2-year old mice, an area of ependymal cells was observed with opposite polarities to the surrounding ependymal cells (A). The polarity was determined by the position of cilia on the ependymal cell surface (B,C).

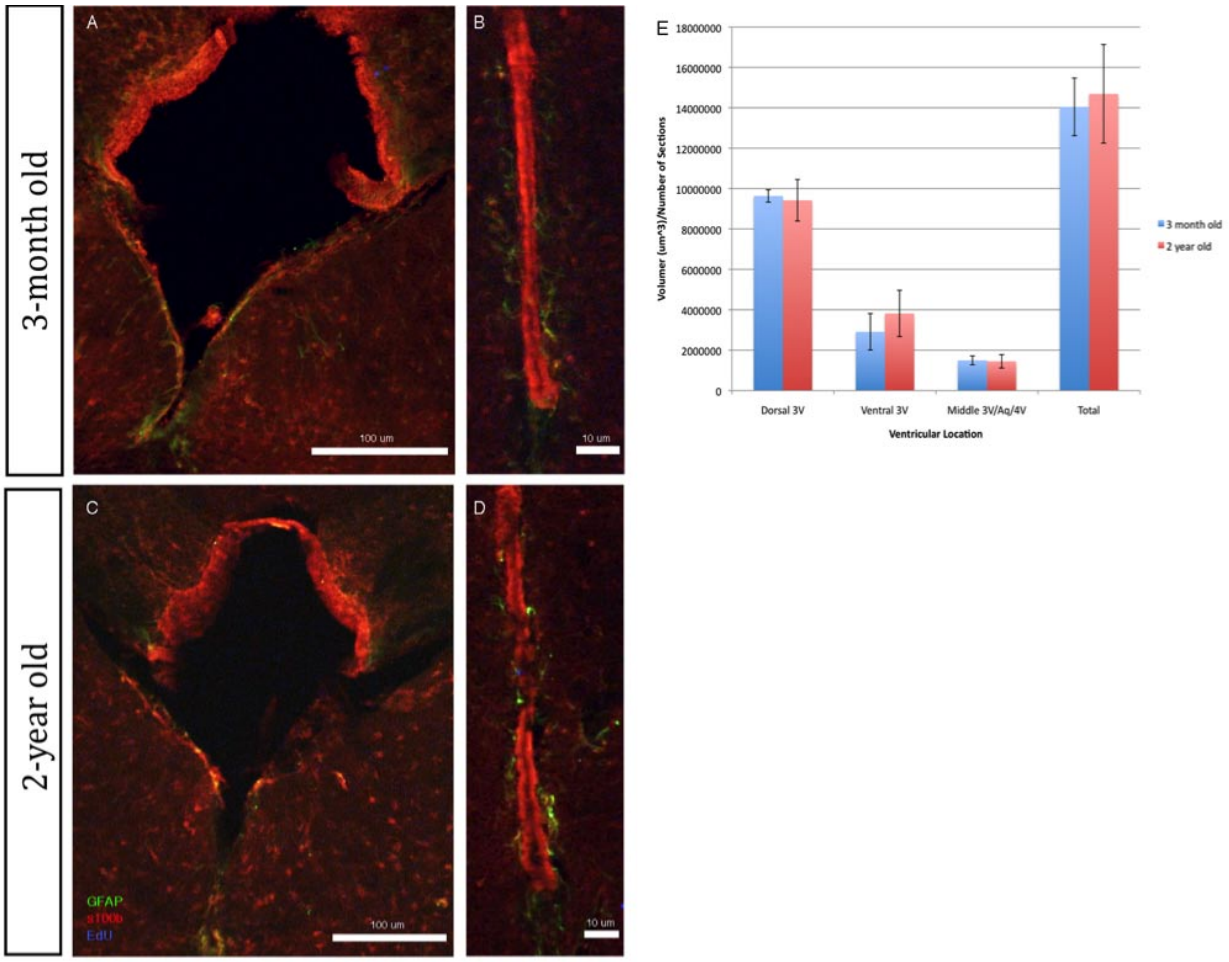

Figure 18: No differences were observed in the ependymal barrier, proliferation, shape, or volume of the third ventricle, cerebral aqueduct, or fourth ventricle.

The integrity of the ependymal barrier, structure of the ventricle, and EdU-labelled proliferation was observed in 3-month old and 2-year old mice. Representative images from the dorsal third ventricle (A,C) and the cerebral aqueduct (B,D) show the lack of changes observed. Additionally, 3-D reconstructions were used to measure the volume of each region in both young and old mice. There were no differences in volume found in any region (E).

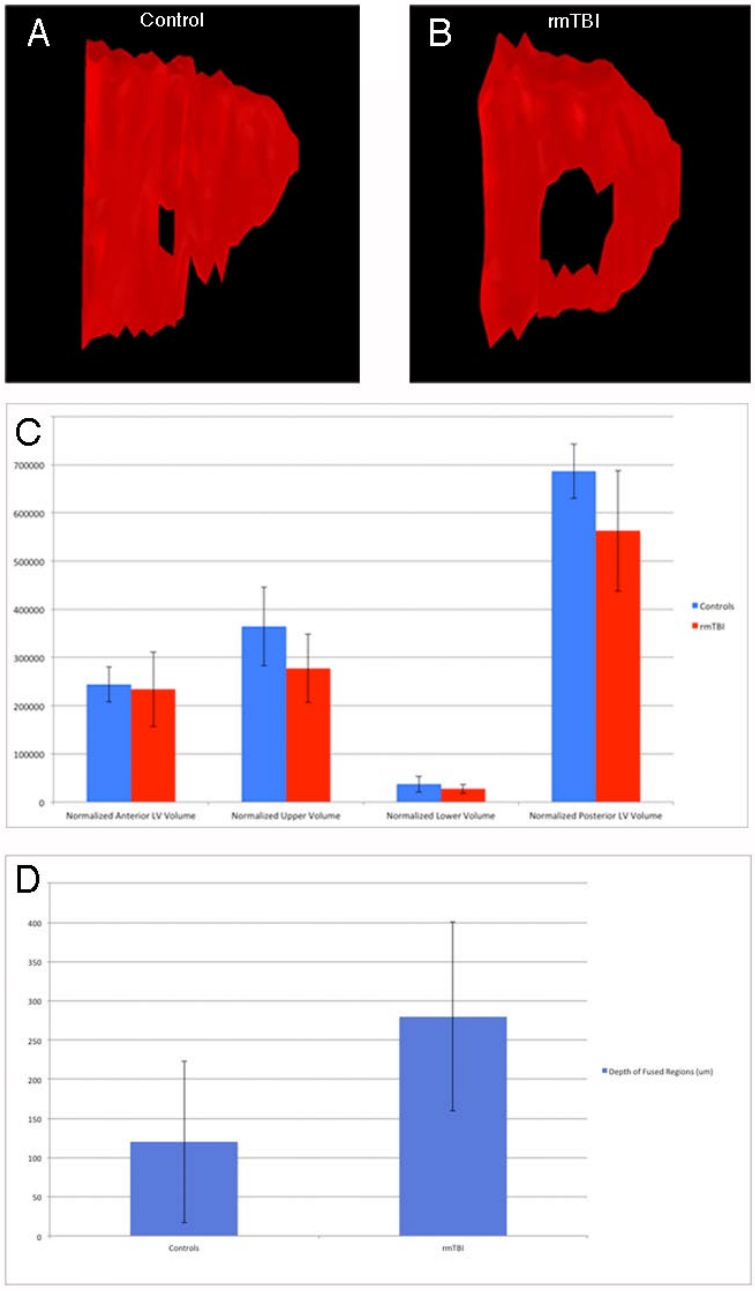

Figure 19: rmTBI results in no change in lateral ventricle volume, but does increase the depth of fusion.

3-D reconstructions were made of the lateral ventricles in control (A) and rmTBI (B) mice. No difference was found in volume in any region of the lateral ventricles (C). There was a greater degree of fusion in rmTBI mice (p=0.054) (D).

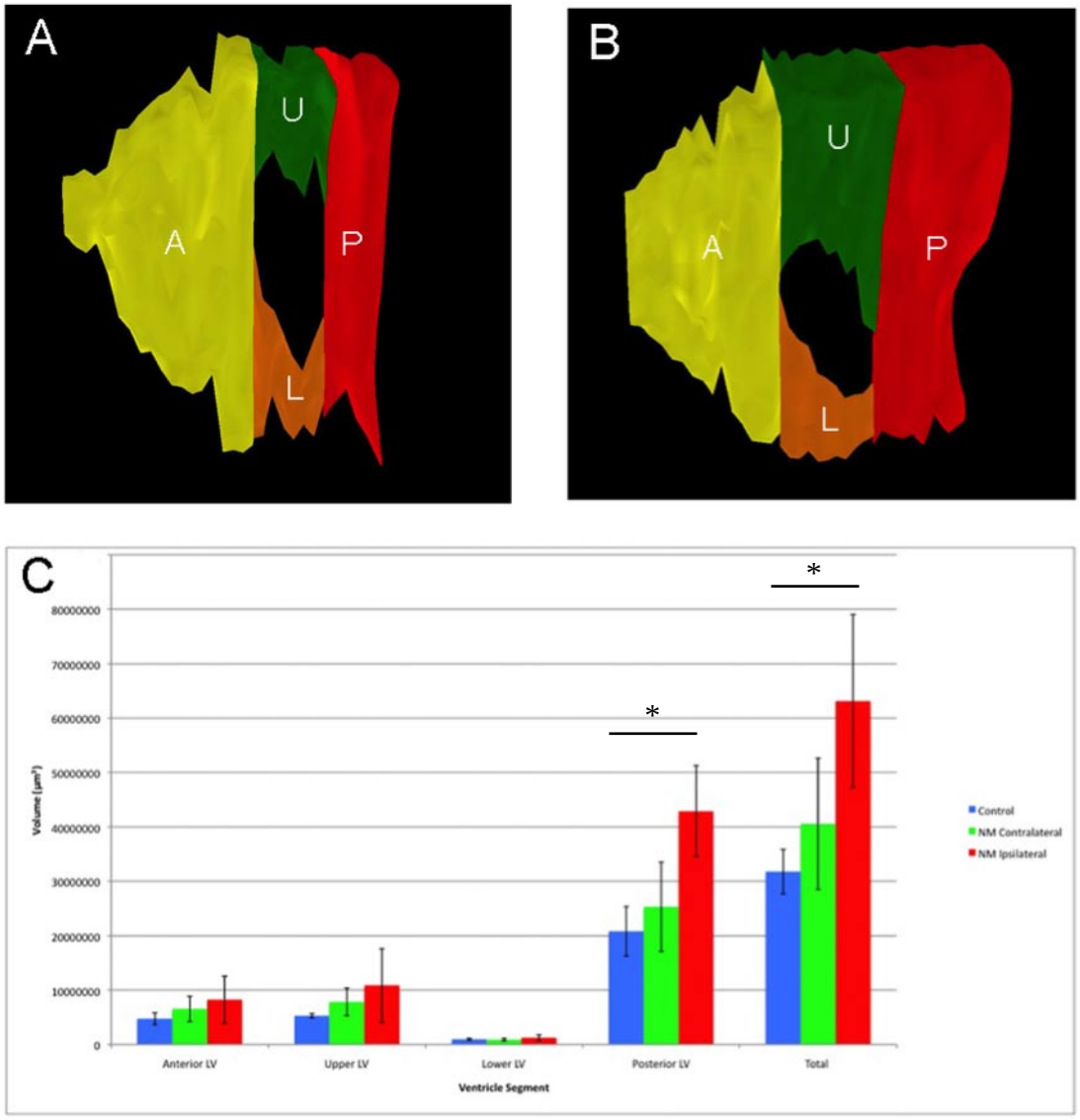

Figure 20: Neuraminidase injection results in an increase in volume of the lateral ventricle. 3-Dimensional reconstructions were made of neuraminidase injected ventricles, contralateral ventricles, and the ventricles of control littermates (A-B). A significant increase in total volume was seen between the control ventricles and the neuraminidase injected ventricles (C). When the ventricles were segmented into four different regions, the posterior lateral ventricle was found to be the only segment that had a significant increase in volume. As expected, the volume of contralateral ventricles was between the volumes of controls and injected ventricles.

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