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Diffusion Barriers of the Lateral Ventricular System

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Diffusion Barriers of the Lateral Ventricular System

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B.tech. Anna University, India, 2009

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Diffusion Barriers of the Lateral Ventricular System

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ABSTRACT

Adult neurogenesis in mammalian brain has become a well-accepted phenomenon. The neural stem cells in the sub ventricular zone (SVZ) along the lateral wall of the lateral ventricle, the largest germinal center of adult neurogenesis, are maintained by the neural stem cell niche. One prominent feature of the niche is its close proximity to the cerebral spinal fluid (CSF). The role of micromolecules (such as ions, water and gases) present in the CSF and diffusing through the lateral ventricular wall and how the stem cell micro-environment is maintained is poorly understood. The cytoarchitecture of the lateral ventricular wall with a pinwheel organization of ependymal cells around astrocyte processes gives uniqueness to the lateral wall of the lateral ventricles versus other ventricle walls. The possibility of different cell junctional protein interactions that could take place here may provide us with some cues that may indiicate a preferentially diffusion taking place through the lateral walls in comparison with the medial wall of the lateral ventricles or other ventricle walls. To test this hypothesis we injected the tracer BDA (3 kDa) and NaFl (0.376 kDa) into lateral ventricles and studied the diffusion pattern. The diffusion curves generated from the injection of the two different sized tracers did not show any significant difference in signal intensities obtained from the medial and lateral regions of the lateral ventricles or between the lateral and medial walls, indicating the possibility of similar tight junctional protein complexes present along both the walls. With the help of EM, we observed the presence of tight junctions between all the cell types of the lateral and medial wall. The organization of peripheral membrane tight junction protein (ZO-1) also showed similar expression patterns between the cells of both walls of lateral ventricles. The result from the localization of tight junction proteins was consistent with diffusion analysis. Thereby, I found that despite the presence of astrocyte processes at the ventricle walls; the barrier role of the SVZ is still maintained.
1. **INTRODUCTION:**

1.1. **Adult neurogenesis:**

From late 19th century it has been believed that the mammalian central nervous system lacked the ability to give rise to new neurons soon after birth and remained the same throughout adulthood (Gross et al., 2000). But these views were challenged with the usage of H-thymidine, which preferentially labels cells undergoing DNA synthesis, labeling the proliferating cells and their progeny. Altman and Das (1965; 1966) reported the presence of new neurons using H-thymidine labeling, in the olfactory bulb, hippocampus and cerebral cortex with rat and cat models. Along with this finding the neurogenic regions of the adult brain in non-mammalian vertebrates such as song birds were also identified (Goldman and Nottlebohm et al., 1983; Alvarez-Buylla and Nottlebohm et al., 1988). With the research, the production of new neurons through adulthood became an accepted concept and primary regions where neurogenesis occurred were identified.

In the adult mammalian brain, the neurogenic niche is located primarily in two regions: one, in the sub-granular zone (SGZ) and second, in the sub-ventricular region (SVZ). The newly generated neuron from SGZ migrates to the granular layer and produce axons in the CA3 region of the hippocampus. The dentate gyrus is primarily located at this region. This zone has proven to be critical in learning and memory (Aimone et al., 2009; Clelland et al., 2009; Deng et al., 2009; Garthe et al., 2009; Kreigstein et al., 2009; Shors et al., 2009). The sub-ventricular region (SVZ) consists of a large germinal center and plays an important role in adult neurogenesis. This proliferative niche is located in the lateral walls of the lateral ventricles. The new neurons produced here fasiculate into the chains and enter the rostral migratory stream where they
migrate to reach the olfactory bulb. In the olfactory bulb, neuroblasts terminally differentiate into granular cells or the periglomerular interneurons that play an important role in fine olfaction discrimination (Gage et al., 2000; Alvarez-Buylla et al., 2001; Peterson et al., 2002; Doetsch et al., 2003; Imura et al., 2003; Alvarez-Buylla & Lim et al., 2004; Kempermann et al., 2004; Wurmser et al., 2004).

1.2. **Cytoarchitecture of adult SVZ:**

Electron microscopy imaging of the SVZ has revealed the presence of different cell types. They include the ependymal cells, astrocytes, transistory amplifying cells and the mature migrating neuroblasts (Doetsch et al., 1997; Garcia-Verdugo et al., 1998). The ependymal cells form a monolayer of barrier cells that act as a filtration system between the cerebral spinal fluid (CSF) and the brain parenchyma. These ependymal cells are first formed during the gestation day 12 and are produced all through embryonic development of the mammalian brain.

During embryonic development of the mammalian brain the ependymal cells are produced from the neural progenitor cells known as the radial glial cells (RG). They divide both symmetrically (to produce more RG cells) and asymmetrically to give rise to various cell types. These neural progenitor cells can be identified by their distinct morphology with one of their processes contacting the pial surface and the other, contacting the ventricular surface. But these cells are lost in the adult mammalian brain. However, a subtype of the postnatal astrocytes, produced from the radial glia maintains this unique morphology by having their apical process at the ventricles and basal process extending towards the underlying blood vessels. Along with this, they may divide both symmetrically (to produce more B1 cells) and asymmetrically (to produce
more C cells). This subtype of astrocytes identified as the B1 cells, are termed as the putative neural stem cells (NSCs) in the adult brain. NSCs give rise to different cell types, one of which being the transitory amplifying cells, that are interspersed between the chains of migratory neuroblasts in the SVZ and to some extent in the RMS (Doetsch et. al (2003)).

1.3 The role of ependymal cells in maintaining the niche:

The unique SVZ microenvironment consists of scaffolding protein matrix along with a high degree of intercellular and intracellular signaling that controls neurogenesis in this region (Luo et al., 2006). This distinct environment somehow maintains the neural stem cells; as studies have shown that transplanting NSCs into the brain outside the niche results largely in differentiate glial cells (Mill et al., 2009, Kokovay et al., 2008; Zhao et al., 2008). Hence, we realize that it is very important to understand how this niche is structured and what factors makes it unique when compared to other regions.

One of the prominent cell types of the niche are the ependymal cells. They play a critical role in forming a structural barrier at the ventricular surface, and also act as a sensor of CSF components and osmotic pressure. The CSF, contains the growth factors that affect adult neurogenesis, such as transforming growth factor-α (TGF-α; Seroogy et al., 1993), basic fibroblast growth factor (bFGF) (Hayamizu et al., 2001) and amphiregulin (Falk & Frisen et al., 2002), produced by the choroid plexus. The absorption of ions and transport of factors is actively regulated by the ependymal cells from the CSF into the brain parenchyma (Riquelme et al., 2008).
Ependymal cells also form tight and adherens junctions with their neighboring cells. They form a barrier wall selectively allowing molecules from the CSF to diffuse into the niche. The contribution of these molecules diffusing into niche in maintaining its proliferative capacity is unknown.

1.4 **What makes the lateral wall unique?**

Lateral wall of the lateral ventricle, is made up of a monolayer of the multiciliated ependymal cells (Bruni et al., 1985). In this wall GFAP+ apical processes of astrocytes have been identified. The ependymal cells often spiral around the astrocyte processes and make up a characteristic pinwheel structure, as shown in figure 1 (Mirzadeh et al., 2008). This pinwheel organization is unique to the lateral wall and is not present in the medial wall or in the third ventricle (as shown in the figure below). Hence in the lateral wall, the cell contacts present between E-E (two ependymal cells), E-B1 (ependymal – astrocyte process) and B1-B1 cells (two astrocyte processes) form a barrier of different cellular organization, contrasting the E-E cellular barrier found along the medial wall of the lateral ventricles. Due to the possibility of varied junctional contacts that could be made between the cells at the lateral wall, it is important to understand their contribution to barrier function.
Figure 1: Schematic of ependymal cells arranged in pinwheel organization present in the anterior region of the lateral wall. The ependymal cells are identified by \( \beta \)-catenin (green), which delineates the cell membrane and \( \gamma \)-tubulin (red) showing the presence of basal bodies. The ependymal cells spiral around the astrocyte processes (yellow). The astrocyte processes are identified by the \( \gamma \)-tubulin (red) that shows the presence of primary cilium.

1.5 Possible interactions between the different cell types of the lateral wall

Cells in a barrier system interact and adhere to one another to maintain their proper function. The junctions between these cells are made up of transmembrane protein complexes present as continuous strands that forms intercellular junctional contacts with neighboring cells. The protein complexes play an important role in maintaining the polarity of the cell, along with modulating various other signaling mechanisms. These junctions can be of different types such as:

1. Tight junctions
2. Adherens junctions
3. Gap junctions
Of the different junctions, tight junctions play an important role in maintaining a selectively permeable barrier wall.

### 1.5.1 Tight Junctions:

Tight junctions are intercellular protein complexes that help in adhering the neighboring cells together.

They provide various advantages to the tissue such as:

1. **Formation of a semi-permeable barrier:** The membrane-bound protein complexes of one cell form a tight seal with the extracellular domain of the protein molecules, from the adjacent cell. This interaction facilitates the formation of a semipermeable barrier for the movement of molecules that has both ion-specific and size-specific restrictions.

2. **Demarcate the polarity of the cells:** The formation of tight junction complexes precedes asymmetric distribution of both the apical and basolateral composition of the proteins and phospholipids. It also plays a very important role in restricting the movement of the different lipids to their distinct domains, thereby forming a distinction between the apical and basolateral membrane and giving the cell polarity (Mandel et al., 1993).

3. **Role in cell proliferation and differentiation:** Tight junction proteins regulate self-assembly by controlling transcription and localization of tight junction proteins. In addition, the proteins at tight junctions are involved in proliferation and differentiation as shown in the figure 2 (Annu. Rev. Cell Dev. Biol. 2006.22:207-235).
**Figure 2:** (Annu. Rev. Cell Dev. Biol. 2006.22:207-235)

aPKC, atypical protein kinase C; CDK4, cell division kinase 4; MAGI1, membrane-associated guanylate kinase with inverted domain structure; PKA, protein kinase A; RalA, Ras-like GTPase; Tiam1, T-lymphoma invasion and metastasis; VASP, vasodilator-stimulated phosphoprotein; ZO, zonula occludens; ZONAB, ZO-1-associated nucleic acid–binding protein.

These tight junctions are classified based on their different protein families:
1. Transmembrane proteins
2. Peripheral membrane proteins
3. Integral membrane proteins

1.5.1.1 Transmembrane proteins:

Transmembrane proteins form a tight seal by reaching across the junction and connect the membranes of the adjacent cells. Three families of the protein complexes make up the transmembrane proteins, they are:

a) Occludins
b) Claudins
c) Junctional adhesion molecules.

a) Occludins: Occludins form one of the constituents of the intermembrane strands (Furuse et al., 1993). Occludin consists of four transmembrane domains, two extracellular loops, and two intracellular domains (Feldman et al., 2005).

Occludin interacts with peripheral membrane proteins such as zonula occludens ZO-1, ZO-2 and ZO-3, which in turn interact with the actin cytoskeleton for the localization of the occludin at the tight junction.

These occluding protein complexes have a lot of functional significance with paracellular permeability. This was observed by expressing the C-terminal truncated form of occludin in MDCK11 cell lines which showed increased paracellular permeability (Balda et al., 1996). By expressing occludins in occludin-null fibroblasts, increased cell adhesion was also observed (Itallie & Anderson et al., 1997). From these studies, we understand the role of occludins in maintaining the paracellular permeability and cell adhesion.
Apart from this, occludins also take part in various signaling mechanisms of cell proliferation as shown in the Figure 2. Overexpressions of occludins have shown to suppress the Raf-1-induced tumor growth (Li & Mrsny et al., 2000; Wang et al., 2005). Occludins are also involved in RhoA activation through GEF-H1/Lfc, a guanine nucleotide exchange factor associated with the tight junction protein complexes.

b) Claudins: Claudins also belong to the transmembrane protein family with four transmembrane domains, two extracellular and two intracellular domains. Despite the topological similarities seen between the occludins and claudins, there exist no sequence similarities between them.

Claudins family consists of 24 members. Mostly two of these members are expressed together in a tissue specific manner. They play an important role in inducing cell-cell adhesion, which is observed by expressing claudin-1 and claudin-2 in L-fibroblasts lacking tight junction. These claudins when overexpressed in fibroblasts showed increased cell adhesion and made cell contact at the regions where claudins were expressed (Furuse et al., 1998). Also when occludins and claudins were coexpressed in L fibroblasts, occludins were recruited by the claudins in the tight junction strands, showing the importance of the claudins.

Claudins form the tight seal, by heterotypically interacting with the adjacent cells. They also interact homotypically with the claudin proteins of the same cell (Furuse et al., 1999). They have conserved PDZ domain in the C terminus through which they interact with peripheral membrane containing proteins, ZO-1, ZO-2 and ZO-3 (Roh et al., 2002; Tsukita et al., 2001).

Claudins have tissue-specific expression pattern i.e. different members of claudins are expressed in different tissues. To parallel this, different members of the claudin family also have different
functional significance. This is observed through the mutations in claudin-16 in a human disease syndrome, where the normal paracellular permeability of calcium and magnesium were affected (Hou et al., 2005; Simon et al., 1999). Ectopic claudin 4 expression in the epithelia causes a decreased paracellular conductance by reducing sodium permeability (Itallie et al., 2001).

Claudins 1, 3, 5 are expressed in the ependymal cells of the third ventricle (Mullier et al., 2010). It will be interesting to know if they are also expressed by the ependymal cells along the lateral ventricles and what their contribution to barrier function is at the lateral ventricular surface.

c) Junctional Adhesion Molecules (JAM): JAMs belong to the immunoglobulin superfamily and have a single transmembrane domain. They interact with the scaffolding proteins such as ZO-1 and stabilize the tight junctions at the periphery. These form cell-cell contact and help in maintaining the barrier wall.

1.5.1.2 Peripheral membrane protein:

Peripheral membrane proteins bind the transmembrane protein to the actin cytoskeleton through their intracellular domain. The peripheral membrane proteins allow the transmembrane proteins to organize in the membrane and initiate signaling processes. They also stabilize their junctional interaction with their neighboring cell through the cytoskeletal attachment provided by the peripheral membrane proteins.

Zonula Occludin (ZO): Zonula Occludins belong to the family of peripheral membrane proteins. ZO proteins thus act as scaffolding proteins. They interact and bind to their binding partners, such as the transmembrane proteins and also the cytoskeletal proteins. The ZO proteins bind to the c-terminus of the occludins as well as the claudins. The C-terminus regions of the
claudins interact with the ZO proteins and bind to them through the first PDZ domains on the ZOs (Itoh et al., 1999).

There are three isoforms of Zonula occludins, ZO-1, ZO-2 and ZO-3. The ZO-1 isoform interacts with the other isoforms of ZO-2 and ZO-3 via its second PDZ domain. ZO-1 is found to be localized in the epithelial and endothelial cells. Apart from these cell types, ZO-1 is also present in non-epithelial cells such as schwann cells, astrocytes and fibroblasts.

ZO-1 plays an important role in the assembly and functioning of the tight junctions. ZO-1 knockout mice show delayed formation of the tight junctions (Sheth et al., 1997, Umeda et al., 2004). ZO-1 is also required in the formation of a functioning barrier wall. T84 epithelial cells showed reduced barrier function when there was a reduction of ZO-1 expression (Youakim & Ahdieh et al., 1999). ZO 1 interaction with claudins and occludins is shown in the figure 1.
1.6 Tracers to study the permeability:

I. **Sodium Fluorescein (NaFl):** Fluorescein sodium salt has been utilized in studies as a drug model, as a penetrating dye and also as a fluorescein tracer. These molecules have the following chemical structure, with a molecular weight of 376.27 Daltons. Sodium fluorescein has an effective diffusion radius of 5 Å. It is used as a control and is expected to show similar levels of permeability between the two walls of the ventricles. The structure of NaFl is seen as below.

![Structure of Sodium Fluorescein](image)

II. **Biotinylated Dextran Amine (BDA):** Dextrans are polysaccharides that diffuse across a membrane as a linear molecule, and have a molecular weight of 3kDa. This tracer molecule provides various advantages to be used as a tool in understanding various *in vivo* processes. Some of its advantages are:

- BDA reliably yields good results from pressure injection.
- It is easy to make small and well-defined injections of BDA.
- The morphological detail of the labeling is often exquisite.
- Availability of wide range of molecular size.

- The simplicity of the visualization procedure.

Of all the above advantages, the availability of this tracer in different size ranges helps us to choose a tracer, larger than the control tracer that freely diffuses between the junction proteins. Thus, we choose 3kDa BDA that has the effective diffusion radius (EDR) of 12 Å. It is a Stokes radius calculated from the average of hydrodynamic radius of a molecule, obtained from viscosity and translation diffusion coefficients of the molecule in solution. This radius is closest to the true molecular radius if the molecule takes up the shape of a sphere in the solution. Since our tracer diffuses as a linear molecule comparing the permeability as a function of the Stoke’s radius would not be accurate. Still, we choose this tracer because it is one of the prominent tracers used in many in vivo diffusion studies. In vivo diffusion studies have shown prominent occlusion of the tracer, 3kDa BDA during the formation of tight junction in the developing choroid plexus of Monodelphis domestica (Liddelow et al., 2009).
Table 1: The fluorescent tracers used for diffusion measurements:

<table>
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<tr>
<th>Tracer</th>
<th>Mw (Da)</th>
<th>Shape</th>
<th>Volume injected (μL)</th>
<th>Circulation time in CSF</th>
<th>Rate (μL/min)</th>
<th>Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Fluorescein (NaFl)</td>
<td>376</td>
<td>Spherical</td>
<td>2</td>
<td>Perfused 5 minutes after injection</td>
<td>0.25</td>
<td>Fluorescein directly coupled to NaFl</td>
</tr>
<tr>
<td>Biotinylated dextran amine (BDA)</td>
<td>3000</td>
<td>Linear</td>
<td>2</td>
<td>Perfused 5 minutes after injection</td>
<td>0.25</td>
<td>Fluorescent labeled secondary antibody bind to BDA</td>
</tr>
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1.7 Experimental Focus:

The neural stem cells in the sub-ventricular zone, SVZ are required in maintaining the adult neurogenesis. Hence, it becomes important to understand how this stem cell niche, is maintained. One of the possibilities through which these an environment could be maintained through signaling molecules from the cerebro-spinal fluid (CSF) that circulates right next to the SVZ. We know that some factors that maintain this niche are actively transported across the barrier wall from the CSF to the SVZ niche (Riquelme et al., 2008). Although there is high degree of active transport taking place through the lateral wall into the SVZ, it is important to note that the lateral wall itself is very unique. The astrocyte processes of the B1 cells contact the lateral ventricular wall unlike any other ventricular barrier walls. The presence of different cell types in the lateral wall of the lateral ventricles, suggests various junctional interactions (as discussed before) to be present between them. This led us to believe that there could be a different permeability in the lateral ventricles that may contribute to maintaining the SVZ niche.

To test this hypothesis, we used 3-month old mice of the CD1 strain and injected two different tracer- BDA and NaFl, into the right lateral ventricle and immediately sacrificed the mice to study the distance of permeability of the tracer in the lateral and medial walls of the lateral ventricle using immunolabelling and other imaging techniques. The results obtained from this experiment were further validated using immunolabelling of the junctional protein ZO-1. Further, tight junction structures were also examined using electron microscopy.

2. MATERIALS AND METHODS:
2.1 **Animals and Tissue preparation:**

Nine male CD1 mice, purchased from Charles River, were used in this study. All the experiments were carried out in accordance with the protocol directed by the Institutional Animal Care and Use Committees (IACUC).

2.2 **Stereotaxic Injection:**

NaFl was used as the control tracer and 3% 3kDa BDA was used as an experimental tracer for this study. The intracerebroventricular injection (i.c.v) of the tracer molecules involved two sets of 3-month old CD1 mice (i.c.v; n=3) for each tracer.

These adult mice were placed in a stereotactic frame after being anesthetized with isoflurane in 2% oxygen. Throughout the surgery the mice were constantly under anesthesia using isoflurane in 2% oxygen. A burr hole was drilled 0.85 mm lateral to the bregma and 2.5 mm deep relative to dura, according to the mouse brain atlas (Paxinos and Franklin, 2001).

A hamilton syringe filled with 2µL of NaFl or 2µL of 3% 3kDa BDA was slowly inserted into the right lateral ventricle of the brain. Using an infusion pump, the tracers were injected into the mice at the rate of 0.25µL/min. The needle from the Hamilton syringe was removed 1 minute after the entire amount of tracer was injected. The mice were perfused immediately following the injection.

2.3 **Gravity-Facilitated Perfusion:**

Immediately after the intracerebroventricular (i.c.v) injection the animals were maintained in the anesthetized state with an intraperitoneal (i.p.) injection of avertin (2.5%; 0.025mL per gram mouse mass) followed by transcardial perfusion with 25mL of 0.9% saline, which flushed the
vascular system. Following this, the fixation process was carried out using 25mL of 4% Paraformaldehyde, PFA.

The brains were quickly removed and post-fixed with 4% PFA at 4°C overnight to prepare them for sectioning using the Leica vibratome. PFA-fixed brains were washed 3 times for 20 minutes in PBS and sectioned (A/P coordinates 0.5-1.54mm, relative to bregma) to obtain 50µm coronal sections. All the brain sections were collected in the 24 well plates.

### 2.4 Immunohistochemistry:

Free-floating brain sections were permeabilized with 0.1% Triton X-100 in PBS for 10 min. Following this the tissue were blocked with 10% horse serum in PBS/1% Triton X-100 for 1 hour at room temperature. Sections were incubated overnight with the following primary antibodies: rabbit anti-β catenin, (1:100); rabbit anti-γ tubulin, (1:500); mouse anti-GFAP, (1:500); rabbit anti-s100β (1:1000) and mouse anti-ZO-1 (1:10).

The next day, sections were washed three times in PBS and incubated for 1 hour at room temperature with fluorescent-labeled secondary antibodies: Alex Fluor 488 donkey anti-mouse (1:500), Alexa Fluor 568 donkey anti-rabbit (1:500) and Alex Fluor 647 donkey anti-mouse (1:500). Secondary antibody staining alone was used as a control for BDA injections. The sections were mounted on gelatin-coated slides and cover slips were placed over the sections. The slides were allowed to dry overnight. All slides were imaged using a Leica TCS SP2 confocal laserscan microscope or Zeiss Axio imager M2 microscope, using HAMAMATSU ORCA-R2 digital camera C10600.

### 2.5 Whole mount dissections:
The whole mount dissection protocol was adapted from Mirzadeh et al. (2008 and 2010). The sections were immunostained with rabbit anti-β catenin (1:100), rabbit anti-γ tubulin (1:500), mouse anti-ZO1 (1:10). The coverslip was placed over the whole mount with aquapolymount and imaged on a Leica TCS SP2 confocal laserscan microscope.

2.6 **Electron microscopy:**

The electron microscopy images were processed using the protocol adapted from Luo et al. (2003). The images were analyzed using Adobe Photoshop CS2.

2.7 **Quantification of signal intensity:**

All the images obtained from injected tracer NaFl and BDA were analyzed using Image J software.

2.8 **Statistical significance:**

Statistical analysis was performed using a two-tailed unpaired Student’s t test. The sample size used for this analyses was n=3. The level of significance was set at p < 0.05.

3. **RESULTS:**
**NaFl permeates evenly through lateral and medial wall of the lateral ventricles**

Sodium fluorescein (NaFl) is a fluorophore that contains sodium salt. The effective diffusion radius of NaFl is 5 Å. The fluorophore in NaFl has an absorption spectrum at 494 nm and emission spectrum at 521 nm. Using epifluorescence microscopy to capture the emission spectrum, the rapid diffusion of NaFl from the lateral ventricles into the brain parenchyma can be visualized. NaFl is a good control tracer, diffusing evenly through the two walls of the lateral ventricles when an intracerebroventricular injection was administered into the right brain of the adult male mouse. All the experiments from henceforth were done in collaboration with Brett Shook and Jessica Lennington. Results from the diffusion curve obtained from mice (n=3) shows no significant difference in the diffusion of the control tracer between the lateral and medial wall of the lateral ventricle.
Figure 1: NaFl diffuses evenly through lateral and medial wall. NaFl was injected into the right lateral ventricle as shown in the schematic (top right corner). Panel A, D and G shows the diffusion of NaFl signal forming a gradient from the wall of the ventricles. At the wall of the ventricles, NaFl was not localized in any cell type. Panel H is the schematic of the lateral ventricles in a coronal section with CC–Corpus Callosum, AC–Anterior Commissure, LW–Lateral Wall, MW–Medial Wall, D–Dorsal region, V–Ventral region. Scale bars=100µm.
Dorsal region:

Ventral region:

Figure 2: Diffusion curve from NaFl (n=3). NaFl signal intensities obtained at various distances away from the wall of the lateral ventricles showed no significant difference between the lateral and medial region. Similar pattern was observed both in the dorsal and ventral region of the lateral ventricles. In Panel A, the boxed region shows the background intensity of NaFl that was subtracted from the NaFl intensity at different distances to obtain the true value.
**3KDa BDA diffuses evenly through lateral and ventral wall**

Biotinylated dextran amine (BDA) is a large molecule made up of linear polysaccharide chains. BDA is occluded by the tight junctions found between the cells of the choroid plexus (Liddelow et al., 2009). Hence, BDA was used as an experimental tracer to observe if there exists a differential permeability between the two walls, which may be a result of differential junctional protein expression. BDA was detected through streptavidin coupled with FITC (fluorescein isothiocyanate). Streptavidin binds specifically to biotin molecules of BDA. FITC has an excitation spectrum at 495nm and emission spectrum at 521 nm. Epifluorescence microscopy captures the signal from FITC and was used to detect streptavidin-bound BDA. The results from this experiment show that BDA is passively and actively transported at the wall of the ventricles. The number of cells that actively take up BDA in the lateral wall is comparable to that of the medial wall. Results from the diffusion curve (n=3) show no significant difference in the signal intensity of BDA between the lateral and medial walls. Hence we conclude that there is no significant permeability difference for BDA between the two walls.
Figure 3: **BDA is localized within the cell and at the periphery of the cell.** Panel A, D, I and L shows the BDA (green) signal intensity. White arrows indicate BDA signal, at the periphery of the cells showing that it could possibly diffuse in between cells. Red arrows indicate BDA signal, localized within cells showing the uptake of the experimental tracer. Yellow arrows indicate the GFAP+ processes (blue) associated with the ependymal cells (red). Scale bars=50µm.
Dorsal region:

![Image of signal intensity graph]

2b Internal control:
Strep only

Ventral region:

![Image of signal intensity graph]

**Diffusion curve for BDA (n=3).** BDA signal obtained from the FITC–labeled streptavidin which was bound to BDA was used to generate the diffusion curve. The intensity of the BDA signal at different distances away from the wall was plotted on the curve. Panel A, shows the signal intensity from FITC-labeled streptavidin only. The intensity of the background at x=72, 276 and 540 μm was obtained. This intensity was subtracted from the raw signal intensity (BDA + streptavidin), which was higher than the background was used to obtain the true signal. True signal intensity was used to plot the diffusion curve shown above. No significant difference was seen from the results obtained from both the walls. Scale bar=50 μm.
Characteristics and coding of the cell types in the ventricular wall

Detailed examination of the cytoarchitectural arrangement of the cells in the lateral and medial wall was conducted using the transmission electron microscopy images. The coronal sections of ultra thin EM micrographs are presented in figure 5. The lateral wall consists of different cell types and they were identified based on reports from Luo et al., 2006 and Doetsch et al., 1997. The astrocytes interpolated through the lateral ventricular wall were found to establish both tight and adherens junctions with the adjacent ependymal cells and other neighboring astrocytes, Figure 5A. Tight and adherens junctions were also identified at the medial wall between neighboring ependymal cells. These observations show no difference in the tight junction protein interaction between the cells of the two walls. This thereby supports our findings of similar diffusion patterns for different tracers throughout the ventricular walls.
Figure 5: Electron microscopic view of the lateral ventricular walls. Panel A, shows the lateral ventricular wall with astrocytes, interposed within the ependyma (As-Astrocyte, E-Ependyma, N-Neuroblast). Panel B, shows a single monolayer of ependymal cells with no other cell type present in the environment. Panel C shows the ependymal cells (E) with many mitochondria, lipid droplet (black arrow), basal bodies of cilia (black arrow head), microvilli, motile cilia (9+2 organization) and dense cytoplasm in coronal view. Panel D shows astrocytes with irregular nucleus and light cytoplasm. The boxed region shows the adherens junction formed by astrocytes with its neighboring cells. Panels E, F and G show E-E cells, E-As cells and As-As cells interactions respectively. All the cell types forms both adherens junctions (brackets) and tight junctions (arrow) as shown in the above figures.
Localization of tight junction protein

The results from the diffusion curve showed similar pattern of diffusion between the lateral and medial wall of the lateral ventricle. With this finding, we hypothesized that the expression pattern of the tight junction proteins is similar between the lateral and medial wall of the lateral ventricles. To investigate this hypothesis we used the marker ZO-1, a peripheral membrane protein that belonged to the tight junction protein family to label the lateral ventricular walls. The lateral wall of the lateral ventricles was organized with a pinwheel structure, the ependymal cells spirally around the astrocyte processes (Mirzadeh et al., 2008). Consistent with previous reports, pinwheel organization was identified along the lateral wall of the lateral ventricle but not medial wall. All the cell types in the lateral and medial wall expressed ZO-1 staining.
Figure 6: Localization of tight junction protein in lateral wall. The cells were visualized from whole mount preparations. A schematic in the right top corner shows the whole mount view of lateral ventricle. The Dorsal (D) and Ventral (V) regions chosen are indicated in the schematic. In Panel A-F, cells labeled for β-cat outlined individual ependymal cells (blue cells in C and F). γ-tubulin labeled multiple basal bodies found in ependymal cells allowing identification of cells as ependymal cells. Cells with a single γ-tubulin+ basal body (primary cilium) were identified as astrocyte processes. Both the cell types express ZO-1 (green) in lateral wall of the lateral ventricle. Scale bars=10µm.
Figure 7: Localization of tight junction protein in medial wall. The cells are visualized from whole mount prep. A schematic in the right top corner shows the whole mount view of lateral ventricle. The dorsal(D) and ventral(V) regions chosen are indicated in the schematic. Panel A, B, C and D shows the β-cat/γ-tub+ ependymal cells in the medial wall express ZO-1 (green).
DISCUSSION:

In this study, we used immunocytochemistry to systematically examine the distribution of the tight junction protein, ZO-1, on the walls of the lateral ventricles. Our results revealed the expression of ZO-1 protein by all the cell types present in lateral and medial wall. The localization of the tight junction protein parallels the results from the diffusion curves that exhibit similar diffusion pattern between the two walls, as shown by intracerebroventricular injections of BDA and NaFl. Our findings reveal the barrier role of the SVZ, which is unique with the presence of pinwheel organization, encompassing different cell types interacting with one another as opposed to the other ventricular walls of the brain (Mirzadeh et al., 2008). The similar diffusion pattern observed between the two walls permits us to understand the barrier role of the lateral wall, which is maintained despite the interposed NSCs within the lateral wall.

The transport of substances from the cerebrospinal fluid to the brain parenchyma has been of interest among many researchers. Detailed examination of the diffusion barriers has been established at choroid plexus and the third ventricles (Muller et al., 2010; Liddellow et al., 2009). The analyses of these diffusional barriers reveal differentially permeable domains that are unique to their function. Along with this, now we also know the size range exclusion of molecules that are not transported through the ventricular wall. This knowledge allows us to predict the possible molecules that could be transported from the CSF into the brain parenchyma. Our present study establishes the first characterization of the diffusion barrier along the lateral ventricular walls, that ensheaths the proliferative SVZ niche. Along with the diffusion pattern through the ventricle walls, our results indicate the presence of the honeycomb-like pattern of immunoreactivity for ZO-1 by the ependymal cells. This pattern of immunoreactivity is typical
of tight junction proteins (Tsukita and Furuse, 2002) and has also been identified in the cells of the choroid plexus and third ventricle (Wolburg et al., 2001; Petrov et al., 1994).

The tight junctions are present in the apical region of the lateral membrane. The proteins expressed at these junctions form sealing strands fusing the plasma membrane together (Tsukita et al., 2001; Tsukita and Furuse, 2002). The junction proteins that make up the paracellular barrier, forms a belt-like anchoring junction encircling the cells through their interactions with their immediate neighbors, giving rise to a honeycomb staining pattern (Coisne et al., 2005; Wolburg et al., 2001). The results from the intracerebroventricular injection of BDA and NaFl show similar diffusion patterns between the walls. The diffusion pattern, demonstrates the cells of the wall are held together at the apical membrane by similar tight junction proteins. One such protein that contributes to forming the tight junction is ZO-1 which is present in both the lateral and medial wall, Figure 4. These results are also consistent with the findings from the TEM images that show the presence of tight junctions between all the cell types of lateral and medial wall.

The intracerebroventricular injections of NaFl demonstrate that the barrier layer of the lateral and medial wall is equally permeable and uncontrolled exchange of solutes of the size range of NaFl may occur between the CSF and the brain parenchyma at both the walls. Particles of the size of NaFl with the ionic radius 4.5 Å can diffuse through the tight junction, which occludes hydrophilic molecules of the size greater than 20 Å (Anderson et al. 2001). This size correlated to the size range of water, oxygen, carbon dioxide. In contrast, BDA is effectively occluded by the tight junction protein complexes between the cells of choroid plexus in the lateral ventricles and was expected to be occluded by the tight junctions of ependymal cells in the lateral ventricles that had similar protein complexes to that of the cells of choroid plexus.
(Liddelow et al., 2009). The injection of BDA into the lateral ventricular system showed both localization of BDA within the cells and transport of BDA at the periphery of the cells. This indicates that molecules of a bigger size range than NaFl are more readily taken up by the cell (possibly through transcytosis), than through diffusion between cells, as would be expected.

Despite the similar degree of active transport that takes place along the lateral and medial wall (as shown in Figure 2) a similar amount of BDA is shown to diffuse in between the cells of the walls and get to the brain parenchyma (results from the diffusion curve of BDA; Figure 2).

The diffusion pattern along with the tight junction localization and characterization was consistent at different domains (dorsal and ventral region) along the ventricular wall. This finding at the walls of the lateral ventricular is in contrast with the third ventricular wall that shows differential permeability at distinct domains (Muller et al., 2010).

Others have reported that, the regions lacking cilia along with disrupted tight junction protein expression in the third ventricular wall show increased diffusion (Muller et al., 2010). Beating of the cilia sets in motion of the CSF flow (Sawamoto et al., 2006) and thus limits the diffusion of CSF, from the ventricles to the brain parenchyma through the ependymal sheet. This may result in the exclusion of substances near the wall which might have, otherwise, diffused through it. In the lateral ventricular region both the lateral and medial walls are uniformly ciliated and hence we do not see a differential permeability pattern at different domains as seen in the third ventricles. With aging, reports show cilia balding of ependymal cells in 22 months old mice (Luo et al., 2006). Along with the loss of the cilia, the presences of ependymal like cells are also observed at the lateral wall of the lateral ventricles (review by Conover et al., 2011). The ependymal like cells have been observed as an intermediate cell type in aged mice (22 months old) where in the loss of ependymal cells in lateral wall of lateral ventricles is partially
compensated by the SVZ repair mechanism mediated by the SVZ astrocytes that acquire the attributes of ependymal cells and finally become the ependymal cell itself (Luo et al., 2006). These factors can contribute to an increased permeability with aging and provide a potential route of entry for toxins, generally small molecules (M.W. range of 4kDa) that can diffuse in between cells, from the CSF to the brain parenchyma. Our present study on diffusion barriers will provide a proper model for comparison on such future studies.

To our knowledge, this is the first study on the diffusion barriers of the lateral and medial walls of the lateral ventricles of the adult mouse brain. The results of our immunofluorescence studies, together with dye permeability experiments and TEM image analyses, provide detailed information about the diffusion barriers between cerebrospinal fluid and brain compartment. This result did not hold true with our initial hypothesis that predicted a differential permeability between the lateral and medial wall. With these results that show similar diffusion pattern between lateral and medial ventricle walls it is interesting to note that the wall of the SVZ maintains a proper barrier.
4. **REFERENCE:**


Anderson (2001). Molecular Structure of Tight Junctions and Their Role in Epithelial Transport. Physiology, 16: 126-130


