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Normal cochlear lateral wall permeability to fluorescent macromolecules

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NORMAL COCHLEAR LATERAL WALL PERMEABILITY TO FLUORESCENT MACROMOLECULES

By

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A Capstone Project submitted in partial fulfillment of the requirements for the degree of:

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Approved By:
Kevin K. Ohlemiller, Ph.D., Capstone Project Advisor

Abstract:* *Damage to the stria vascularis resulting in leakage of macromolecules from the capillaries has been indicated in research as undermining the endocochlear potential. Results indicate normal paracellular diffusion and caveolae-mediated transcytosis, suggesting normal permeability that does not necessitate pathology or undermine the endocochlear potential.

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Abstract

The existence of an endocochlear potential in the inner ear is suggested to require nearly complete control over the passage of both large and small molecules from the capillaries of the stria vascularis. Passage of molecular tracers such as horseradish peroxidase, Immunoglobulin G and albumin from the strial capillaries into the strial lumen under certain conditions has been interpreted by some investigators as evidence for abnormal leakage around capillary endothelial cells, or some other type of abnormal transport process. Any degree of pericellular leakage is suggested to injure the stria and undermine the generation of an endocochlear potential.

In the present study, the authors assessed the permeability of fluorescently tagged molecular tracers applied to young, healthy CBA/J and C57BL/J6 mice using fluorescence confocal microscopy. Imatinib, a tyrosine kinase inhibitor, was then applied immediately prior to the injection of the macromolecules and changes in permeability were judged. The results suggest robust paracellular passage of small molecules and transcellular transport of protein tracers under unstressed, undamaged conditions. These findings support a surprisingly high degree of normal permeability of strial capillaries that does not constitute pathology nor coincide with a drop in the endocochlear potential.

Introduction

Generation of the Endocochlear Potential

The mammalian cochlea is comprised of a fluid-filled tubular structure, the membranous labyrinth, that subdivides the cochlea into three fluid filled compartments: scala vestibuli, scala media, and scala tympani. Scala vestibuli and scala tympani are filled with perilymph, while the scala media is filled with endolymph (Hibino, Nin, Tsuzuki, & Kurachi, 2010). The ionic composition of perilymph is similar to cerebrospinal fluid and plasma. Perilymph is high in

sodium and chloride, with some bicarbonate and some potassium. The perilymph between the scala vestibuli and scala tympani differ in that scala vestibuli has a slightly higher potassium level and lower sodium level. The endolymph within the scala media is a unique extracellular fluid that resembles intracellular fluids. It has high levels of potassium and chloride, with some bicarbonate, and little sodium (Salt, n.d.).

The endolymph is maintained at a positive DC voltage of approximately 80-100 mV relative to perilymph, depending on species. This potential is known as the endocochlear potential (EP). The EP is generated by the stria vascularis, and is critical to hair cell transduction and normal auditory function (Hellier, Wagstaff, O'Leary, & Shepherd, 2002). The cochlear hair cell bodies are bathed in perilymph, while their apical membranes and stereocilia are exposed to endolymph. When a hair cell responds to sound, the EP increases the driving force of the potassium (K+) and calcium (Ca2+) into the cell, increasing the sensitivity of transduction. After K+ passes through the hair cells, it may return to the spiral ligament via one of two routes. It may diffuse through the perilymph into the inferior spiral ligament, or be transported through the gap-junctional network of Deiter's cells and supporting cells in the lateral organ of Corti. Potassium then flows from the spiral ligament into the stria vascularis, where it is actively moved into the endolymph by ion pumps within the fibrocytes and the stria itself. Within the stria (Hibino, 2010), the generation site of the EP is considered to be the intermediate cell membrane, and specifically, the KCNJ10 potassium channel. Large outward K+ currents from strial intermediate cells constitute the diffusion current that lies at the heart of the EP (Wangemann, 2002).

Fundamentally, strial operation and EP generation requires strict control of compartmental ion concentrations, especially K+. There are three compartments that are assumed to be 'ion tight', at least in the sense that most ion movement is controlled. The first is

scala media, with its very large luminal boundary in part consisting of strial marginal cells, Reisners membrane, and the reticular lamina. The second is the intra-strial space, where K+ levels are kept extremely low by uptake of K+ into marginal cells. The third is often assumed to be capillary walls, so that ion flow in and out of capillaries must be controlled or the EP may be 'shorted out' by unregulated current flow across capillary walls (Trune, 2010).

Role of the Endocochlear Potential

The EP is critical to normal cochlear function. In 2011, Jacob, Pienkowski and Fridberger demonstrated the role of the EP for maintaining the geometry and mechanical and electrical properties of the organ of Corti. When they manipulated the EP, the reticular lamina was displaced such that the inner portion up to the second row of outer hair cells was pulled toward the basilar membrane, and the outer portion, including the third row of outer hair cells and Hensen's cells, were pulled in the opposite direction. This decreased vibrations in response to acoustic stimulation on the inner portion of the reticular lamina, and caused a 10-fold increase in vibrations for the outer portion of the reticular lamina. These observations underscore that the cochlear amplifier itself depends on the EP.

Knockout and transgenic mice lacking an EP are profoundly deaf, indicating its role for hearing (Hibino, 2010). The endolymph is maintained an approximately +80-100 mV in mammals, while the inner and outer hair cells rest at approximately -45 and -60 mV, respectively. This electrochemical gradient increases the driving potential for outer haircell receptor currents by a factor of ~2 (Patuzzi, 2011). The spontaneous firing rate of afferent fibers attached to inner hair cells is highly sensitive to changes in EP. An increase in EP can depolarize the inner hair cells, resulting in a rise in the spontaneous firing rate of the afferent fibers. Conversely, a decrease in EP can hyperpolarize inner hair cells and reduce afferent firing rates.

A hyperpolarization of 20 mV is sufficient to significantly impact the release of neurotransmitters from hair cells (Patuzzi 2011).

Cochlear Vasculature

The cochlear lateral wall is made up of the spiral ligament and the stria vascularis. The stria vascularis is a three layered epithelium. The luminal layer is made up of marginal cells, whose apical membranes are bathed in endolymph. The abluminal layer consists of basal cells. Sandwiched between these are the intermediate cells – the generation site for the EP. These are pigment producing cells that migrate into position during development. The basal cells, intermediate cells and the fibrocytes of the spiral ligament are connected via gap junctions to form an electrical syncytium, vital to potassium recycling (Nin et al., 2008).

Circulation via the capillaries enables the cochlea to receive essential cell nutrients and remove waste products. The stria vascularis has one of the highest metabolic rates per gram of any body tissue. Since the organ of Corti is avascular, its metabolic needs must be met by strial and ligament capillaries. Therefore, strial circulation must support not only the needs of the organ of Corti, but the stria itself. Therefore, strial circulation must support not only the stria itself, but also the needs of the organ of Corti (Patuzzi, 2011).

Metabolites may move across the strial capillary boundaries via active or passive processes. In passive transport, the diffusion of a molecule is driven by a chemical gradient, where a molecule diffuses from a region with a high concentration to a region with a lower concentration. Passive movement cannot occur against a concentration gradient. Passive transport may be carrier mediated or non-mediated, meaning that dedicated transport proteins are involved. An active transport process refers to transport of molecules against a concentration gradient, and it frequently requires ATP or other energy sources (Voet, Voet & Pratt, 2008).

Capillary Leakage and Molecular Transport

As was stated, ion movement across strial capillary walls is assumed to be under tight control, without which the EP cannot exist. Capillary permeability generally refers to movement of ions, macromolecules, and water across capillary boundaries, whether actively or passively. 'Leakage' is often used to refer to permeability, and may or may not be used to describe a pathological process. An abnormal increase in permeability, may occur as a result of damage or death of endothelial cells, or a change in the oncotic pressure. These may be due to drug reactions, sepsis, allergic reactions, infections, tumors, and other events (Fishel, Chandrakanth, & Barbul, 2003). Macromolecular tracers have commonly been used to assess permeability. Various macromolecules covalently bound to electron dense or fluorescent moieties are injected into the vasculature and permeability is judged, based on the distribution of the tracer. In instances where the macromolecule is judged not to be permeable, the animal may be subjected to an insult (e.g., noise trauma or ototoxins). If permeability of the macromolecule is observed, it is called leakage and damage to the structure is assumed.

Pathologic leakage of macromolecules has often been suggested to be correlated with a reduced EP. Chen et al. (2009) concluded that fluorescein isothiocyanate dextran leakage is associated with severe vascular disruption and severe tissue injury. Rybak, Weberg, Whitworth and Scott (1992) demonstrated a transient reduction in EP that correlated with furosemide-induced edema, and recovery correlating with reversal of edema. The authors suggested that the edema was the cause of the reduction in the EP. Hellier et al. (2002) demonstrated permanently damaged organ of corti structures and stria vascularis, along with a transiently depressed EP following the administration of kanamycin and a loop-diuretic. They offered multiple explanations for the transient depression of the EP, including K+ leakage.

Active Transport and Imatinib

Caveolae-mediated transcytosis is a commonly utilized method of active transport by capillaries (Lin, 2009). In this form of transcytosis, pit formations termed caveolae in the endothelial cell membranes transport cargo across the cell body. Caveolae can exist as a fully invaginated caveolae, a cluster of multiple caveolae, or can join to form a trans-cellular channel (Bernd, 2010). Tyrosine kinases are a family of proteins that are critical to signaling, triggering the active process (van Nieuw Amerogen, van Delft, Vermeer, Collard, & van Hinsbergh 2000). Imatinib is a tyrosine kinase inhibitor, and therefore, interrupts and prevents the process of transcytosis. Other tyrosine kinase inhibitors have been demonstrated with successfully preventing active transport of various molecules (van Nieuw Amerogen et al., 2000; Triuppathi, Song, Bergenfeldt, Sass & Malik, 1997)

Macromolecules and Capillary Leakage in the Literature

Strial capillary permeability has been evaluated both under normal conditions and under conditions of pathology utilizing a variety of tracers, including but not limited to: horseradish peroxidase (HRP), immunoglobulin G (IgG), albumin, and fluorescein isothiocyanate dextran (FITC-Dextran). In 1996, Xu, Watanabe and Komatsuzaki assessed the permeability of capillaries in healthy mice to HRP. The authors cited leakage in 18% of their samples at 10 minutes, and increasing leakage quantity over the 30 minute, 1 hour and 2 hour samples in healthy mice. It was further speculated that HRP may be reabsorbed back into the blood circulation. While the mice were healthy, the authors termed the HRP presence outside the capillaries “leakage.”

Hashimoto, Seki, Miyasaka and Watanabe (2006) sought evidence of increased capillary permeability due to drill vibration in middle ear surgeries. Occasionally, sensorineural hearing

loss results from middle ear surgeries. One proposed etiology is damage to the stria vascularis from the vibrations of the drills. The study assessed drill duration and associated leakage of HRP from the capillaries of the stria. Hashimoto et al. (2006) reported that HRP leaked from the capillaries specifically as a result of vibration induced strial damage, based on results showing increased leakage with increasing durations of drilling. The authors then applied steroids prior to administering drilling in the middle ear, and their results suggested that steroids reduced the pathological leakage of HRP from the capillaries. Contrary to Xu, Watanabe and Komatsuzaki (1994), Hashimoto et al.'s results suggested that HRP is only permeable under conditions of pathology, and is associated with hearing loss.

Pan and Zhang (2006) assessed the role of cyclic AMP in strial capillary permeability using Evans blue and albumin. When cyclic AMP levels were increased using cholera toxin, the authors observed a significant decrease in the transfer of both tracers across endothelial cells. When cyclic amp was decreased using katlex, the authors observed a significant increase in transfer of the tracers across endothelial cells. Ahlstrom, Thalamm, Thalamm & Ise (1975) suggest that cyclic AMP is inversely related to Na+/K+-ATPase activity, so that increasing cyclic AMP-inhibits Na+/K+ATPase activity. The findings from Pan and Zhang imply that an active process is involved in the transport of the molecules; however, they did not discuss the possible mechanism(s) of capillary transport.

Shi (2009) assessed changes in capillary permeability by tracing the movement of IgG in mouse stria in conjunction with noise exposure. The animals that were not noise exposed demonstrated no leakage of IgG from the strial capillaries. The animals that were noise exposed showed leakage of IgG from the capillaries into the intrastrial space. This study suggested that

healthy capillaries are impermeable to IgG, and that the appearance of IgG outside of the strial capillaries represents pathology.

FITC Dextran has been used to distinguish active transport from passive paracellular leakage in different systems, including the inner ear. Canis et al. (2010) applied FITC to the vasculature of normal, healthy mice to assess cochlear blood flow. This study was based on the assumption that FITC stays within the capillaries, and does not leak out actively or via a paracellular route in normal animals. Chen et al. (2009) studied blood flow in the brain, and demonstrated that FITC remained within circulation under normal conditions, but leaked into surrounding tissue and spaces significantly following a stroke.

Li, Wang and Steyger (2011) investigated the pathway by which gentamicin leaves the cochlear vasculature and ultimately reaches the hair cells. This pathway is of particular interest due to the ototoxicity of gentamicin. The cochlear dispersion of fluorescently-tagged Texas red-conjugated gentamicin (GTTR) was monitored by confocal microscopy in normal mice and following noise exposure, since noise and ototoxicity are suggested to have a synergistic effect. Systemically applied GTTR was observed in the intrastrial space and was taken up by marginal cells in animals that were not noise exposed. The marginal cells immediately adjacent to strial capillaries showed a greater uptake of the GTTR. Noise exposure increased the uptake of GTTR by marginal cells, and in marginal cells further away from the strial capillaries. Significantly, the authors noted that vasodilation was not a prerequisite for enhanced trafficking of the GTTR. Based on their results, the authors argued that active mechanisms are involved both in the exit of GTTR from strial capillaries and the uptake of GTTR by marginal cells.

Armulik et al. (2010) specifically addressed the involvement of transcytosis in the transport of a series of fluorescently tagged macromolecules across the capillaries in the brain of

mice possessing different numbers of pericytes. The authors utilized cadaverine alexa fluor-555, dextran, immunoglobulin G, and horseradish peroxidase. In mice with an abnormally reduced number of pericytes, all of the tracer molecules were found the escape the capillary lumen. When the drug Imatinib was applied prior to the macromolecules, it prevented or reduced the permeability of albumin, horseradish peroxidase, immunoglobulin G, and dextran. Significantly, this study implies a role for active transport. The application of Imatinib, a typrosine kinase inhibitor, and the subsequent impact on permeability, suggests transcytosis as the active process involved in transport of the molecules.

Study Aims

The purpose of the present study was to assess the permeability of strial capillaries in the living mouse to fluorescently tagged molecular tracers in such a way that paracellular leakage and active transport could be distinguished, and transcytosis could be investigated as the possible active transport mechanism. Fluorescently-tagged molecules were first applied to healthy, normal mice from two different strains, and permeability was assessed. Imatinib, a tyrosine kinase inhibitor, was then applied prior to the same tracers. We reasoned that the imatinib should inhibit transcytosis, thus supporting a role for this active transport mechanism in purported stial capillary ‘leakage’. Changes in permeability were judged by the dispersion of tracers, viewed by confocal microscopy.

Methods

Deeply anesthetized mice were intracardially injected with the selected tracer, with or without imatinib, in saline. The perfused volume was allowed to circulate for 5 minutes. The animal was then perfused with fixative, and the stria vascularis and spiral ligament were

extracted from the cochleae and mounted on a slide for confocal imaging. In some experiments, fixative was perfused prior to the tracer, which we expected to halt any active transport process.

Tracers utilized for the present study (see Table 1) included: gentamicin-texas red conjugate (GTTR), sodium fluorescein (NaFl), fluorescently tagged albumin (albumin), fluorescently tagged Immunoglobulin G (IgG), CY3 tagged horseradish peroxidase (HRP). All of the molecules are water soluble. These were selected because they have been utilized frequently in the literature for studies investigating the stria vascularis, strial permeability, and strial pathology (Armulik et al., 2010; Chen et al., 2009; Hashimoto et al., 2006; Pan & Zhang, 2006; Shi, 2009; Xu, Watanbe & Komatsuzaki, 1994). They also represent a wide range of molecular weights, listed in Table 1.

Animals

Male and female CBA/J and C57BL/6J (B6) inbred mice were used. Mice were between 8 and 24 weeks of age at the time of our experiments. At least 3 mice from each strain were used to test all five fluorescent tracers with and without imatinib. All procedures were approved by the Washington University Institutional Animal Care and Use Committee.

Surgical Procedure

Mice were deeply anesthetized with a typically 0.05 ml injection of sodium pentobarbital. The heart was then surgically exposed, and a modified syringe needle catheter was inserted into the left ventricle. The catheter was then used to administer the selected tracer or the same tracer+imatinib in 0.1 ml saline, either followed or preceded by 10 ml of fixative (4% paraformaldehyde in PBS) per the experimental condition. If the animal did not maintain a steady heartbeat for 5 minutes after the tracer injection, or showed signs of hypoxia, it was not included in the study. The cochleae were then extracted from the skull, the stapes was removed,

a small hole was created in the apex, and the cochleae were immersed in fixative for 24 hours. The stria and spiral ligament were then separated from the cochleae and mounted on a microscope slide, utilizing permount containing 4',6'diamidino-2-phenylindole (DAPI) as the mounting medium. Slides were then sealed using clear nail polish.

Confocal Immunofluorescence Microscopy

Tissue samples were assessed utilizing a Zeiss~~s~~ LSM 700 multiphoton confocal microscope. For each mouse, images were collected from one basal segment and one apical segment, and assessed using a 20x or 63x objective. The pinhole diameter was consistently optimized for the objective being utilized. For each fluorescent tracer, laser gain and intensity were held constant across preparations to maintain consistent appearance and background levels.

Quantification

Image assessment was qualitative in nature; no statistical analysis was used to evaluate the data collected. Images were processed using the computer program Volocity, and evaluated for presence/absence of fluorescence in the intrastrial space, and when possible, presence of the tracer within the particular strial cells.

Results

Tracer dispersion patterns

NaFl, Albumin, and IgG, were all readily found within the intrastrial space after perfusion, demonstrating a high degree of permeability to these tracers under normal conditions. This is shown in Figure 1(A, C, E), where in the fluorescent green of the macromolecular tracer is apparent throughout the tissue sample. Permeability of these tracers under normal conditions appeared to be independent of mouse strain and gender, and did not vary with cochlear apical/basal location. By virtue of the strial layer where they appeared, NaFl and albumin

appeared to be taken up by the intermediate and marginal cells (Figure 1 B, D, F). GTTR was generally not as widely dispersed (Figure 2A) and most often appeared retained within the capillary walls, or in the spaces near capillaries. Figure 2B shows the a side view of the stria with the luminal side facing upward. The walls of the capillaries are illuminated with the GTTR, as well as the intrastrial space, indicating permeability of the molecule. HRP readily escaped the strial capillaries for all tissue samples for B6 mice, but was not found in any segments examined from CBA/J mice, depicted in Figure 3. For the B6 mice, permeability of HRP appeared to be independent of gender, and did not vary with basal/apical location. Due to resolution limitations, it could not be determined whether strial basal cells took up the tracer in most experiments.

For all of the mice that received NaFl, the tissue samples exhibited fluorescence within the spiral ligament. In approximately half of mice that received IgG and Albumin, fluorescence was imaged within the spiral ligament. HRP was infrequently visualized in the spiral ligament. GTTR was never imaged in the spiral ligament. Due to resolution limitations, the pathway by which the fluorescence reached the spiral ligament could not be assessed with the present experimental design. However, the smallest tracers (NaFl and GTTR) are small enough that they might be expected to pass through the gap junctions that connect intermediate cells, basal cells, and ligament fibrocytes.

Effect of prior fixation on tracer dispersion patterns

Fixative was perfused prior to the tracer in a small series of experiments. Our assumption in doing this was that the fixative would shut down any active transport process, but would not affect any paracellular leak process. When fixative was applied prior to the injection of NaFl in B6 mice, it did not impact the dispersion pattern. The macromolecule was still readily found in the intrastrial space, and appeared to be taken up by the intermediate and marginal cells. When

fixative was applied prior to the injection of NaFl in two CBA/J mice, permeability was eliminated in both segments examined from one CBA/J mouse. However, permeability appeared unaffected for the other CBA/J (Figure 4). Applying fixative prior to albumin and IgG reliably reduced or prevented detectable tracer dispersion (Figure 5). The tracer could be found within the strial capillaries, but not within the intrastrial space.

Effect of Imatinib on tracer dispersion patterns

Imatinib had no effect on the permeability of NaFl for B6 mice (Figure 6); For all B6 mice, NaFl was found in the intrastrial space, and appeared to be taken up by the intermediate and the marginal cells following the application of Imatinib. However, Imatinib effectively eliminated permeability to NaFl in CBA/J mice (Figure 6).

Imatinib effectively prevented permeability to IgG in CBA/J mice (Fig. 7). For B6 mice, results were inconclusive. For three of six segments imaged, it appeared qualitatively that the Imatinib reduced the permeability relative to the control mice. Some images in B6 mice following the application of Imatinib suggested that IgG was retained within endothelial cells of the capillaries (Figure 7C). Interestingly, Imatinib completely eliminated detectable levels of HRP from B6 strial samples (Figure 8).

Discussion

These experiments were designed to test the extent of cochlear lateral wall vascular permeability to commonly applied macromolecular tracers, and the process by which they may exit capillaries. The use of two different inbred mouse strains also allowed us to test for potential strain differences (genetic modification) of these processes. Our observations suggest that strial capillaries are ‘leakier’ than ligament capillaries, moreover that they are surprisingly ‘leaky’ by both passive paracellular diffusion and active trans-endothelial transport. Thus

supposedly pathologic appearance of macromolecular tracers in the intrastrial space need not signal pathology, nor any process coincident with reduction of the EP. The greater surprise to us was the suggestion that these presumed fundamental processes may be subject to genetic modification. This has implications for variation in how the stria of animals and humans may respond to environmental challenges.

Transcytosis and Molecular Transport in the Stria Vascularis

Our use of Imatinib was intended to test for the role of transcytosis in strial macromolecular transport. Transcytosis is a means by which the endothelial cells of the capillaries actively transport materials across cell boundaries, enabling the existence of unique environments on either side of the cell. This process is commonly used for nutrient absorption, plasma membrane biogenesis, and immune defense. (Bernd, 2010). Two types of transcytosis have been described – pinocytotic or phagocytotic. There are two morphologically different pinocytotic pathways: caveolae-mediated and clathrin-mediated. Blood capillaries are a well-known site for caveolae-mediated transcytosis. This process transports proteins from the blood across the endotheslium to the tissue interstitium, and removes modified proteins from the blood (Lin, 2009). In caveolae mediated transcytosis, pits in the membranes of endothelial cells named caveolae are used to transport cargo from basal to apical surfaces of cells, and vice versa. Caveolae can exist as fully-invaginated caveolae, can cluster to form various arrangements of multiple caveolae, or can join to form a trans-cellular channel. (Bernd, 2010).

Transcytosis requires one or more tyrosine kinases, which in turn can be inhibited by imatinib. Another tyrosine-kinase inhibitor, genistein, has also been shown to reduce brain capillary permeability to various molecules such as HRP, IgG and albumin (van Nieuw Amerogen et al., 2000; Triuppathi et al., 1997). Tyrosine kinase proteins and inhibitors are not

suggested to be involved in paracellular transport or modification of paracellular pathways (van Nieuw Amerogen et al., 2000). Therefore, an effect on strial capillary permeability seen with Imatinib implicates caveolae-mediated transcytosis, and not paracellular diffusion.

Our results indicate that the capillaries were permeable to HRP and IgG under normal conditions. Following the application of Imatinib, the permeability was modified for all three tracers in a strain-dependent fashion. This finding is consistent for a role of active transport with all three tracers. For B6 mice with HRP and for CBA/J mice with IgG, the process likely transcytosis. For CBA/J mice and NaFl, the Imatinib effectively prevented permeability, but had no effect on the B6 mice. This is consistent for an active transport process, likely transcytosis, for NaFl with CBA/J mice. For B6 mice with IgG, it is possible that another active transport mechanism not prevented by Imatinib is being utilized; however, further research is needed validate the strain divergence, and to distinguish what appears to be different cellular processes.

Genetic Background and Active Transport

The strains we used, CBA/J and B6, are divergent age related hearing loss, noise related hearing loss and the effects of noise on the EP. When hearing thresholds are the primary consideration, B6 mice are considered to be highly noise vulnerable, whereas CBA/J mice are relatively noise resistant. However, when the EP is considered, CBA/J mice may be more vulnerable. Given a single, intense noise exposure, Ohlemiller and Gagnon found that the EP of B6 mice was unchanged while the EP in CBA/J mice was transiently depressed (2006).

The results of the present study suggest that fundamental cellular processes, how things get out of the capillaries, may have genetic influences. Albumin and GTTR did not appear to demonstrate strain differences for control mice. At this time, data is being collected regarding the effect of Imatinib on these macromolecules. HRP was not permeable in the control CBA/J mice,

but it was permeable for the B6 mice. This permeability was effectively prevented by the application of Imatinib for the B6 mice. This result suggests that the method of transport for this macromolecule in normal control mice depends on the genetic strain. For B6 mice, the strial capillaries actively transport HRP utilizing transcytosis. For CBA/J mice, the macromolecule is neither actively nor passively transported. To verify that the capillaries are not permeable to HRP for CBA/J mice, further studies over longer time periods are recommended.

CBA/J and B6 mice demonstrated uniform permeability to NaFl in control animals, and this permeability was prevented for CBA/J mice, but not for B6 mice with the application of Imatinib. This result suggests that the method by which NaFl is transported for CBA/J and B6 mice is fundamentally different. The prevented permeability with Imatinib for CBA/J mice indicates transcytosis. It is possible that for B6 mice, either a different active process not affected by Imatinib, or a passive paracellular permeability, is responsible. Similarly, CBA/J and B6 mice demonstrated permeability to IgG in young, healthy controls. With the application of Imatinib, permeability was effectively prevented for CBA/J mice, while results were mixed and inconclusive for the B6 mice. Further research is needed to elucidate these strain divergences.

GTTR and Permeability

The pathway of GTTR entry into the cochlea was investigated by Wang & Steyger in 2009. These authors imaged GTTR over time and its appearance in different areas in the cochlea. GTTR was identified in the stria vascularis at 30 minutes, 1 hour and 3 hours post-injection. 30 minutes was the earliest post-injection time assessed. Their findings indicated that GTTR was trafficked into the cochlea and subsequently the hair cells via the strial capillaries, through the marginal cells. GTTR is of particular interest due to its cytotoxicity, and more specifically, ototoxicity.

In the present study, GTTR was assessed specifically within the stria vascularis. A shorter time window of 5 minutes was utilized. Our results correlate well with the findings from and Wang & Steyger (2009), suggesting that GTTR is actively transported across the strial capillaries. GTTR was taken up by the endothelial cells of the capillaries and was present in the intrastrial space in healthy control animals. Results demonstrated uptake of GTTR by the marginal cells in only approximately half of the images. This finding may be due to the limited time window used, consistent with the time-dependent distribution of GTTR throughout the cochlea noted in Wang & Steyger (2009). Further studies focused on the stria vascularis over longer time periods would be beneficial for verifying the uptake of GTTR by marginal cells as the route by which it enters the endolymph and haircells of the cochlea.

Endothelial Cell Uptake of Macromolecules

In their 2008 study, Dai and Steyger demonstrated uptake of GTTR by the endothelial cells of the capillaries. The finding of macromolecule uptake by endothelial cells of the capillaries is consistent with an active transport mechanism versus passive paracellular permeability. Similar imaging patterns were identified in the GTTR samples for our B6 and CBA/J mice. Further, this imaging pattern was identified in some, but not all of the B6 mice with Imatinib injected prior to IgG. This result further indicates the active transport of GTTR across the strial capillaries. For IgG, it is possible that an active transport mechanism not specificially inhibited by Imatinib is being utilized in B6 mice; however, further studies are needed to understand the strain divergence in the results of the IgG and Imatinib.

Endocochlear Potential and K+ Permeability

K+ is a significantly smaller molecule than our smallest molecule used, NaFl. Our results demonstrated that NaFl moves across the capillary boundaries in control mice, and may be either

passive or active depending on the genetic strain. Therefore, K+ is likely recycled, at least in part, through a passive mechanism. Other findings in the research support this possibility. Indirect evidence is provided by studies that have demonstrated permanent damage to the stria vascularis, whether by noise trauma or by ototoxic insult, and transient depression of the EP. The EP recovers, but the damage to the structures persists, suggesting that the EP is not dependent on normal active function of the cells (Hellier et al., 2002; Xiong et al., 2011).

Patuzzi (2011) reported that the EP collapses when the electrogenic pumping of the stria vascularis is interrupted, either by hypoxia, loop diuretics or interruption of blood flow. Immediately after, the EP drops as low as -40 mV. During this time, a passive diffusion gradient takes over and restores the EP 0 mV. This finding demonstrates that at least to some extent, passive processes play a role in the maintenance of the EP and transport of K+ ions across the cell layers of the stria vascularis.

Conclusions

In healthy control animals, NaFl, Albumin, IgG, and GTTR demonstrated permeability in the strial capillaries. It was imaged in the intrastrial space regardless mouse gender or genetic strain, and was present in both basal and apical regions. The tracer appeared to be taken up by intermediate and marginal cells. HRP appeared to be permeable in B6 mice, but not CBA/J mice. Imatinib impacted the permeability of NaFl in a strain dependent fashion; permeability was prevented in CBA/J but not B6 mice. Imatinib prevented permeability for CBA/J mice for IgG, but results were mixed and inconclusive for B6 mice. Imatinib effectively prevented the permeability of HRP for B6 mice, the strain that demonstrated permeability in controls. The findings with the Imatinib are consistent for normal paracellular transport in the absence of

pathology in a strain-dependent fashion for NaFl, and active transcellular transport in a strain-dependent fashion, likely transcytosis, for the macromolecules IgG and HRP.

Our results indicate that most strial capillary leakage, including instances associated with inferred strial pathology, in fact represents a controlled and likely adaptive process, namely transcytosis. Significantly, our results are further consistent for an active role of genetics in the fundamental cellular process of capillary permeability. These findings are significant for studies that have been performed utilizing these tracers, and drawing conclusions about pathology based on leakage of the aforementioned molecules. Studies of strial capillary leakage have often involved explicitly pathologic conditions. Often, the EP has been assumed to be reduced, but it most cases, it has not been directly measured. Endothelial cell transport by transcytosis does not necessarily imply any process that would be expected to reduce the EP. Thus the EP should not be assumed to be compromised in all preparation wherein plasma macromolecules are detected in the intrastrial space.

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Table and Figure Appendix:

Name	Abbreviation	Molecular Weight	Source
sodium fluorescein	NaFl	376.3 Da	Sigma Aldrich
Fluorescently tagged immunoglobulin G	IgG	150 kDa	Sigma Aldrich
fluorescently tagged albumin	Albumin	66 kDa	Sigma Aldrich
CY3 tagged horseradish peroxidase	HRP	40 kDa	Sigma Aldrich
gentamicin-texas red conjugate	GTTR	477 Da	Oregon Health and Sciences University
imatinib mesylate	Imatinib	589.72 Da	Novartis

Table 1: The six tracers utilized, including the name, abbreviation, molecular weight in daltons (Da) or kilodaltons (kDa), and the source from which the tracer was obtained.

Macromolecule	Condition	Strain	Number of Animals
Sodium Fluorescein (NaFl)	Control	B6	4
		CBA/J	4
	Fixative Prior to Macromolecule	B6	1
		CBA/J	2
	Imatinib Prior to Macromolecule	B6	4
		CBA/J	3
Horseradish Peroxidase (HRP)	Control	B6	3
		CBA/J	3
	Fixative Prior to Macromolecule	B6	2
		CBA/J	2
	Imatinib Prior to Macromolecule	B6	3
		CBA/J	3
Albumin	Control	B6	3
		CBA/J	3
	Fixative Prior to Macromolecule	B6	0
		CBA/J	2
	Imatinib Prior to Macromolecule	B6	0
		CBA/J	0
Immunoglobulin-G (IgG)	Control	B6	3
		CBA/J	3
	Fixative Prior to Macromolecule	B6	2
		CBA/J	0
	Imatinib Prior to Macromolecule	B6	3
		CBA/J	2
Texas red-conjugated Gentamicin (GTTR)	Control	B6	3
		CBA/J	3
	Fixative Prior to Macromolecule	B6	0
		CBA/J	0
	Imatinib Prior to Macromolecule	B6	3
		CBA/J	3

Table 2: The number of animals per strain, per condition, per macromolecule.

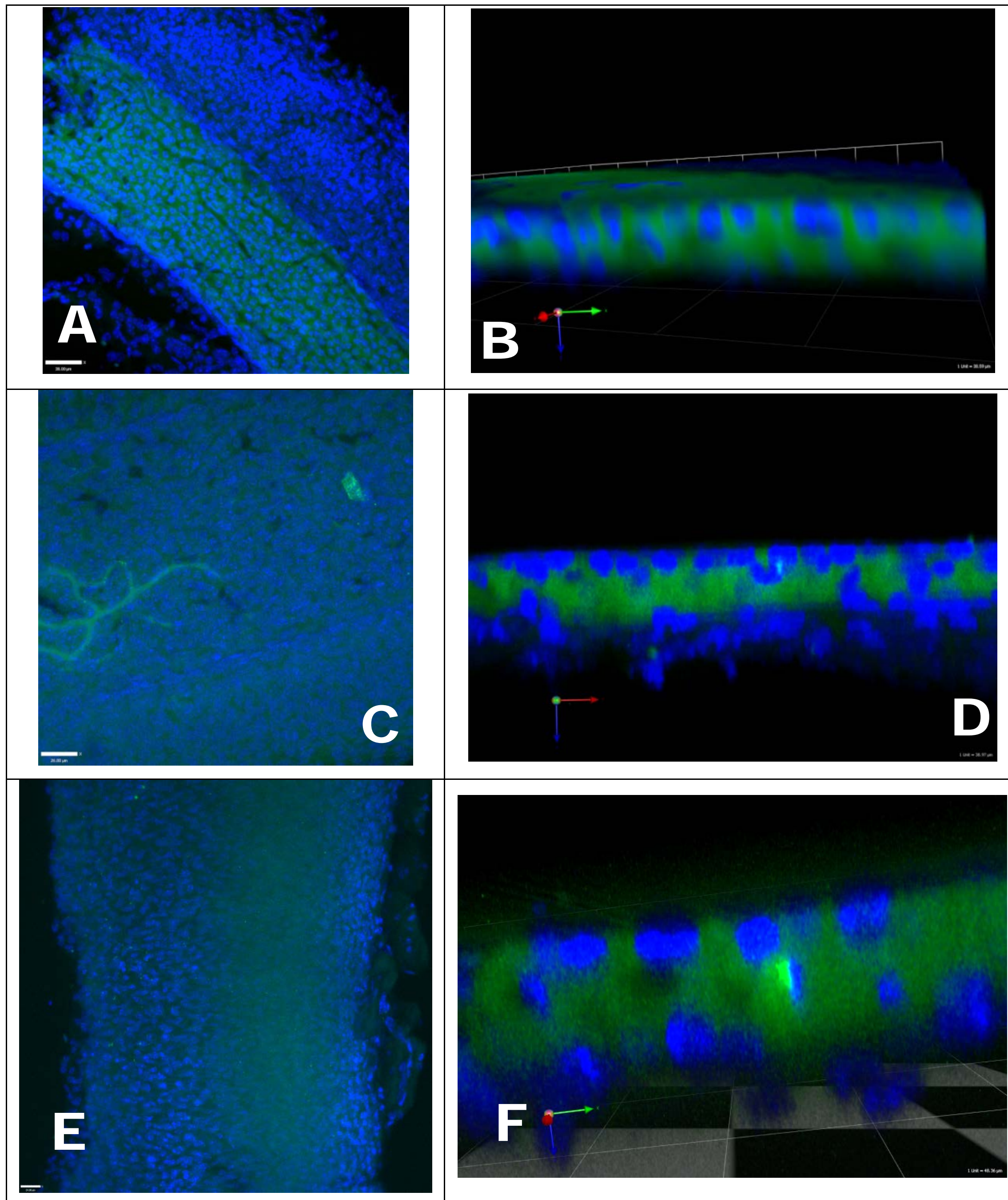

Figure 1: Panels (A), (C) and (E) show the the permeability of the NaFl, IgG and Albumin (green), respectively, in the stria vascularis. Panels (B), (D) and (F) show the same tracers with a side-on view, luminal side up. The nuclei of the marginal cells are surrounded by the fluorescent tracer, demonstrating uptake by the cell of the macromolecule. (**A**) NaFl in the stria vascularis of a B6 (**B**) End view of the NaFl in the B6 suggests NaFl taken up by intermediate and marginal cells. (**C**) IgG in the intrastrial space of a CBA/J. (**D**) End view of the stria vascularis in the CBA/J suggests IgG taken up by intermediate and marginal cells. (**E**) Albumin in the stria vascularis of a B6. (F) End view of the stria vascularis in a B6 suggests IgG taken up by intermediate and marginal cells.

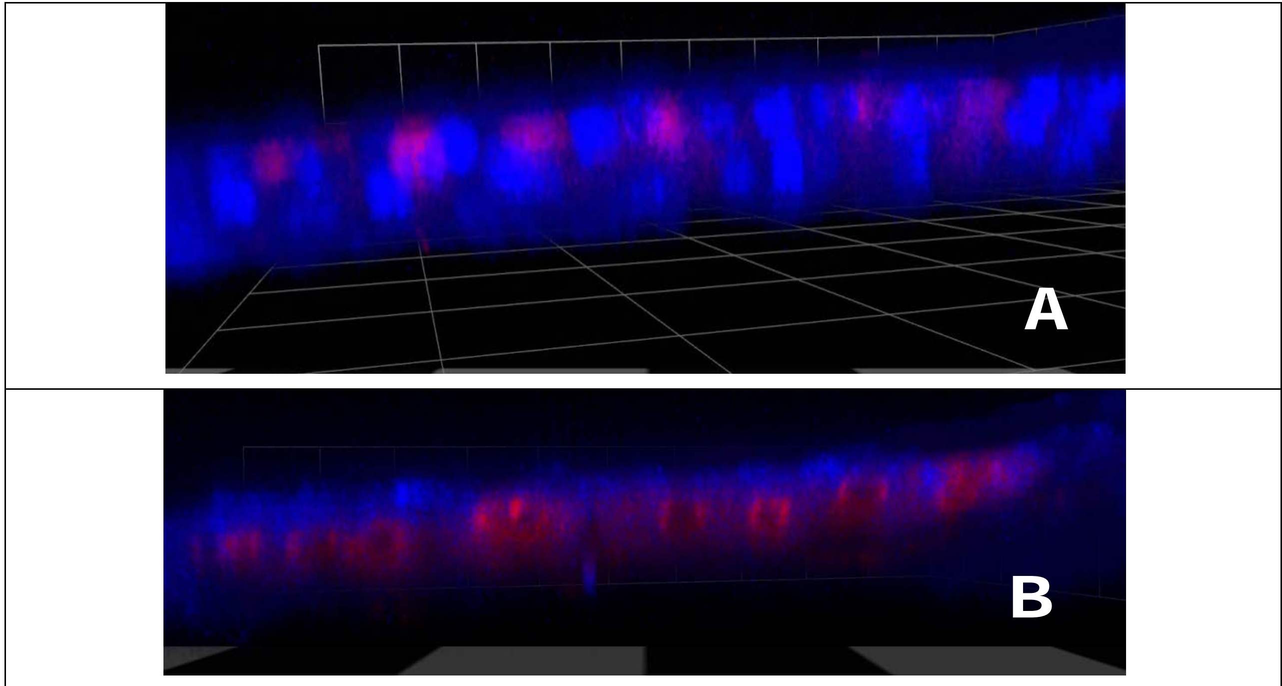

Figure 2: Figures (A) and (B) show an end-on view of GTTR in the intrastrial space, with the luminal side up. **(A)** GTTR in the stria vascularis of a CBA/J mouse. Red dots show GTTR still mostly retained in capillary walls. (**B**) GTTR in the stria vascularis of a CBA/J mouse can be seen to define capillary walls, suggesting uptake by the capillary endothelial cells, and has also diffused into the intrastrial space.

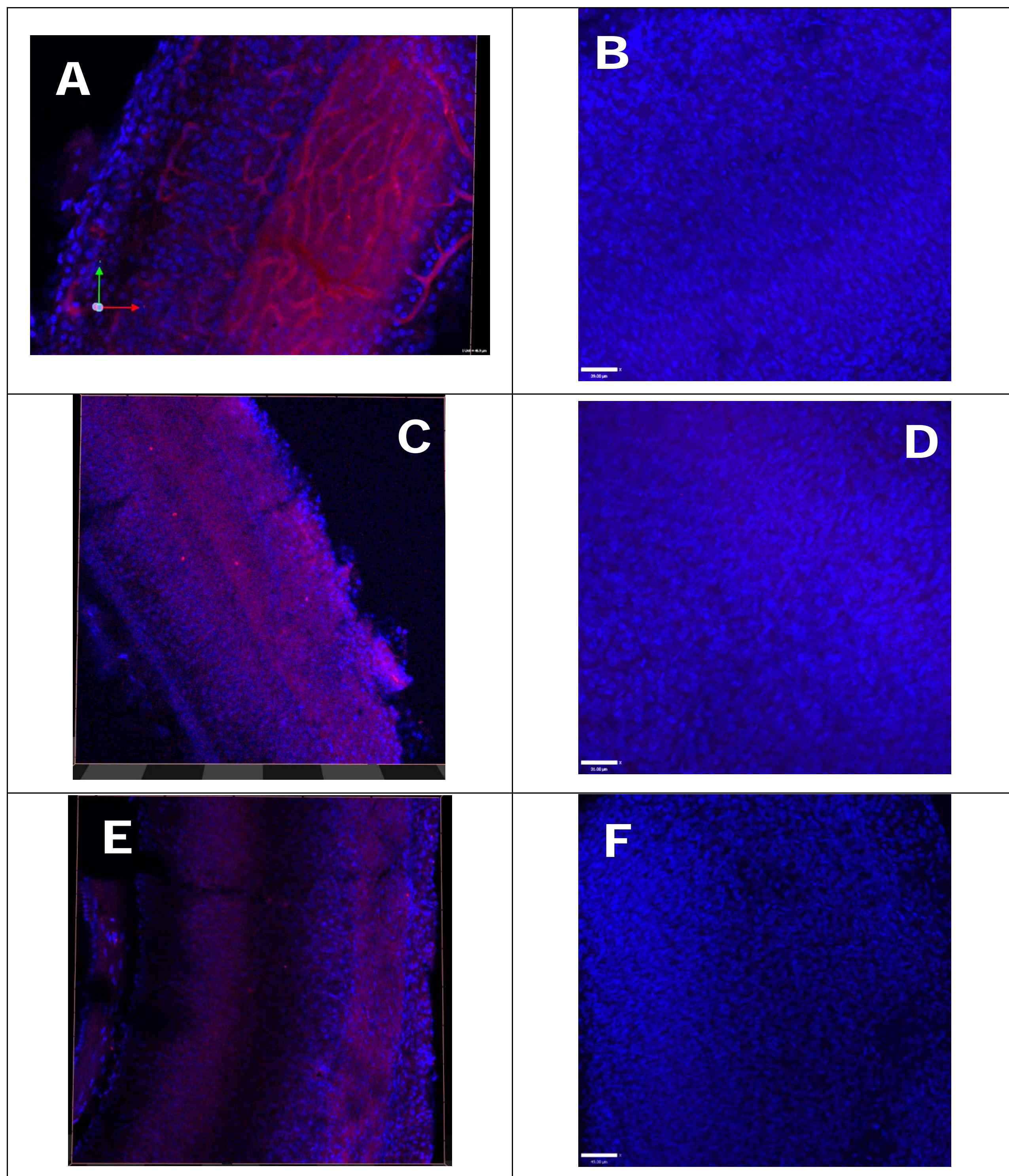

Figure 3: Mouse strain effects in are seen for HRP (red) dispersion in stria vascularis. Panels **A, C,** and **E** HRP in B6 mice show the permeability of the stria to HRP for B6 mice. Panels **B, D,** and **F** show the lack of permeability to HRP in CBA/J mice – no tracer was visualized.

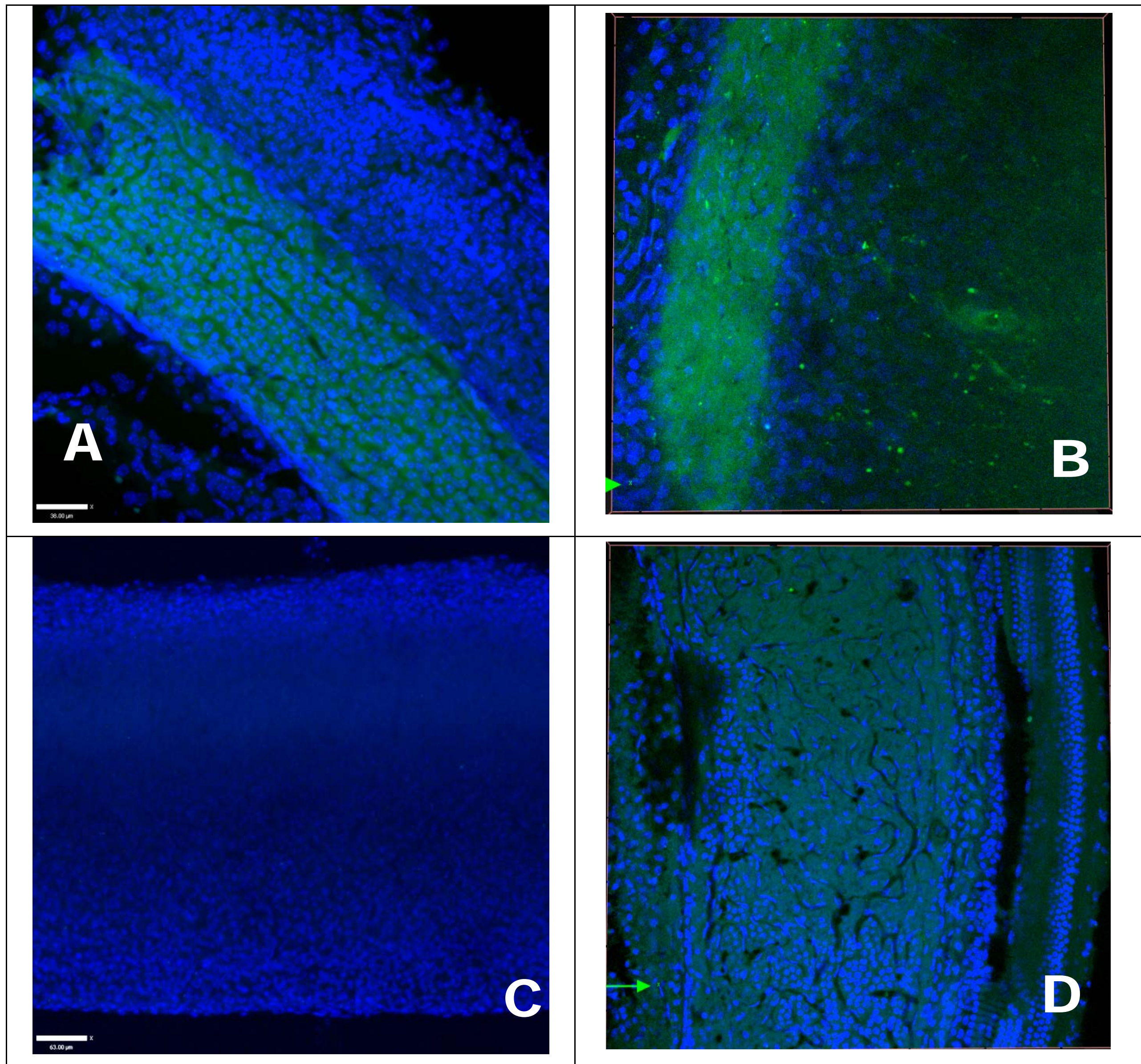

Figure 4: Imaging findings with the NaFl (green) when fixative was applied prior to the macromolecule were varied. (**A**) Typical dispersion of NaFl in a B6 strial segment (**B**) Reduced permeability to NaFl in a B6 mouse fixed prior to injection of NaFl. (**C**) Reduced permeability of NaFl in CBA/J stria fixed prior to the injection of the tracer. (**D**) Little or no effect of prior fixation for a different CBA/J mouse.

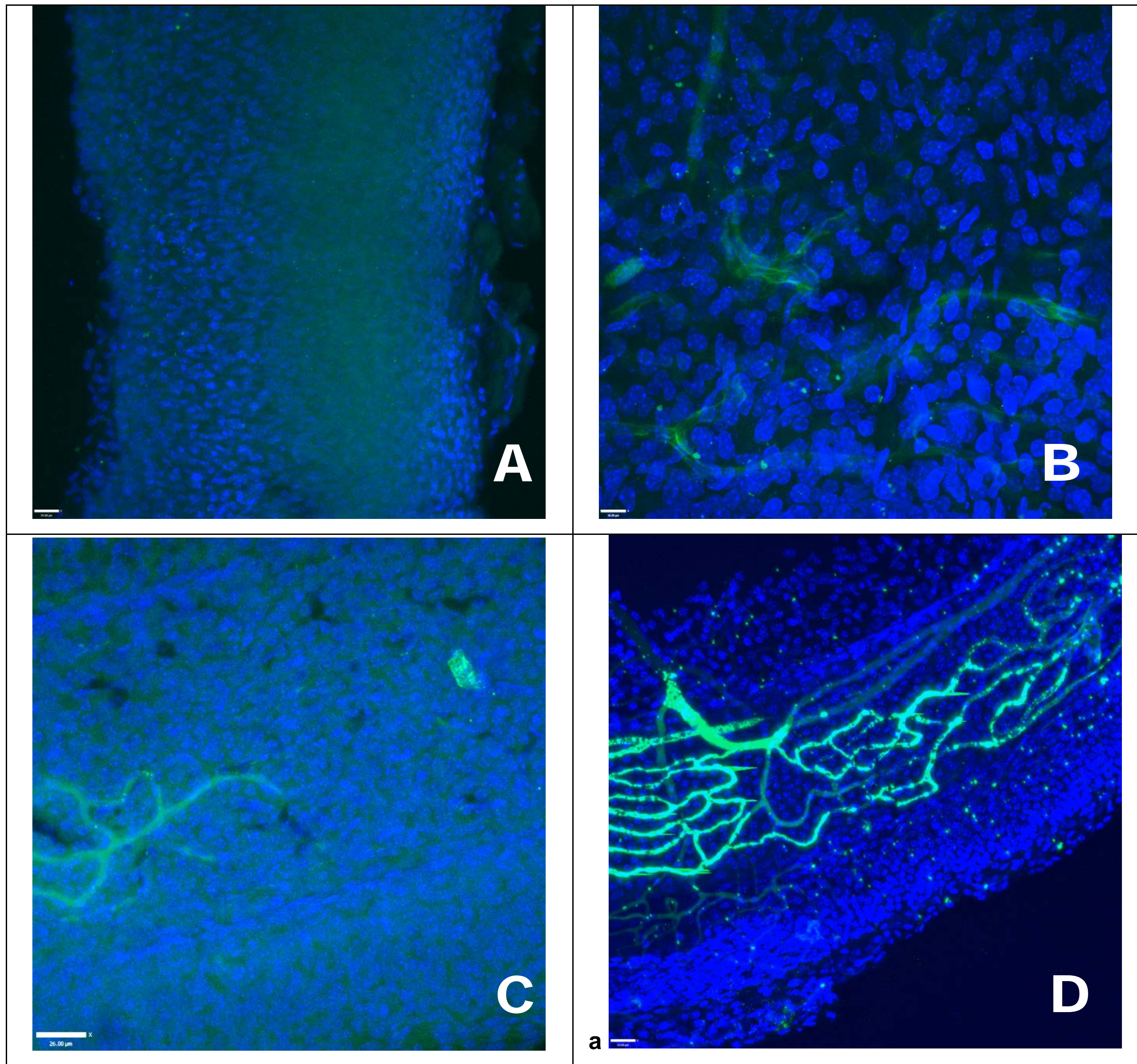

Figure 5: (**A**) Typical dispersion of albumin, shown in a B6 mouse. (**B**) Reduced permeability to albumin by fixation prior to injection of tracer in a CBA/J mouse. (**C**) Typical dispersion of IgG in a CBA/J. (**D**) Reduced permeability to IgG by prior fixation as shown in a B6.

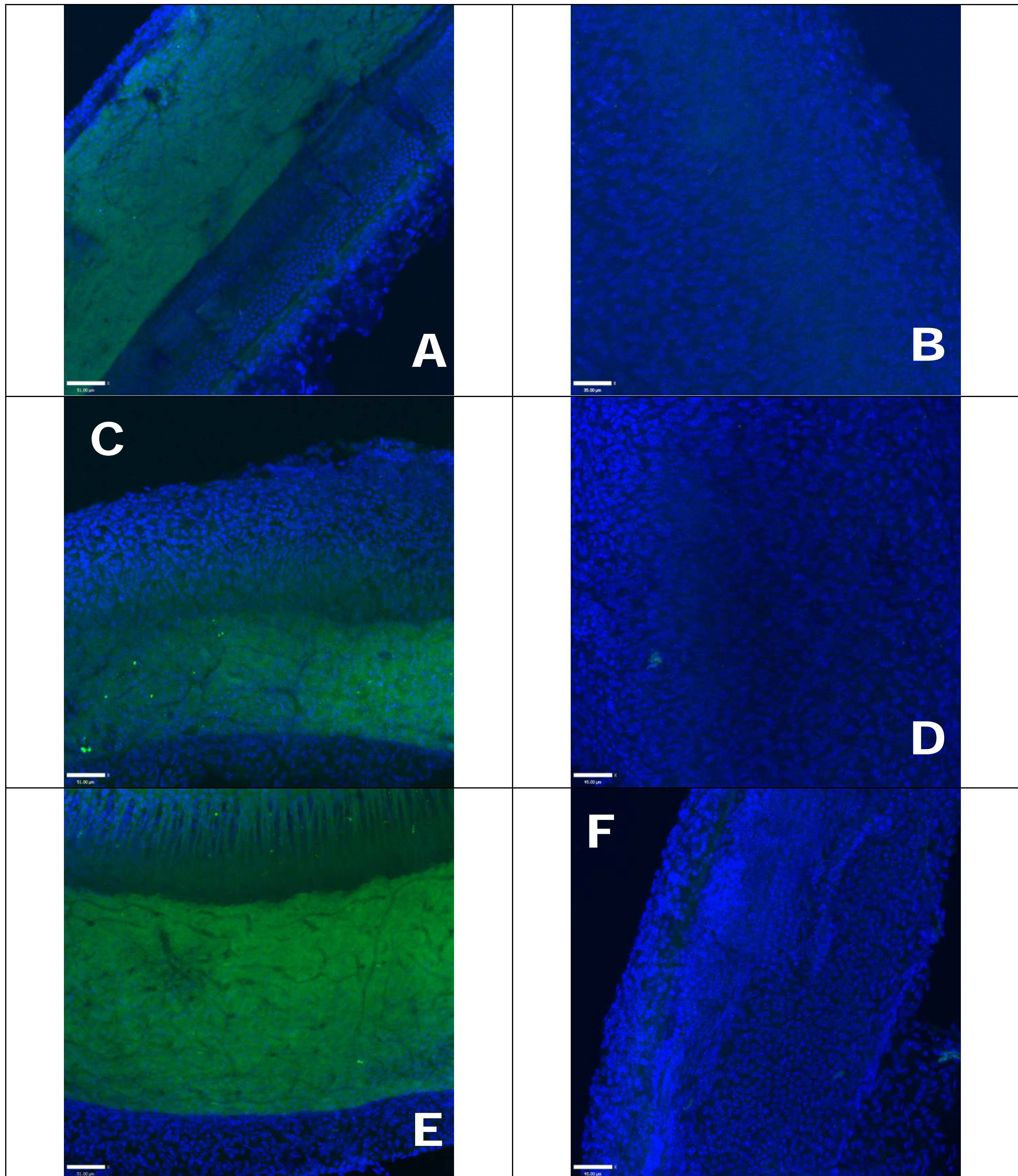

Figure 6: Mouse strain dependence was observed for the effects of Imatinib on strial capillary permeability to NaFl. Panels A, C and E show the application of Imatinib prior to the macromolecule had no effect in B6 mice. However, in CBA/J mice, Imatinib effectively prevented permeability to NaFl (**B, D, F**).

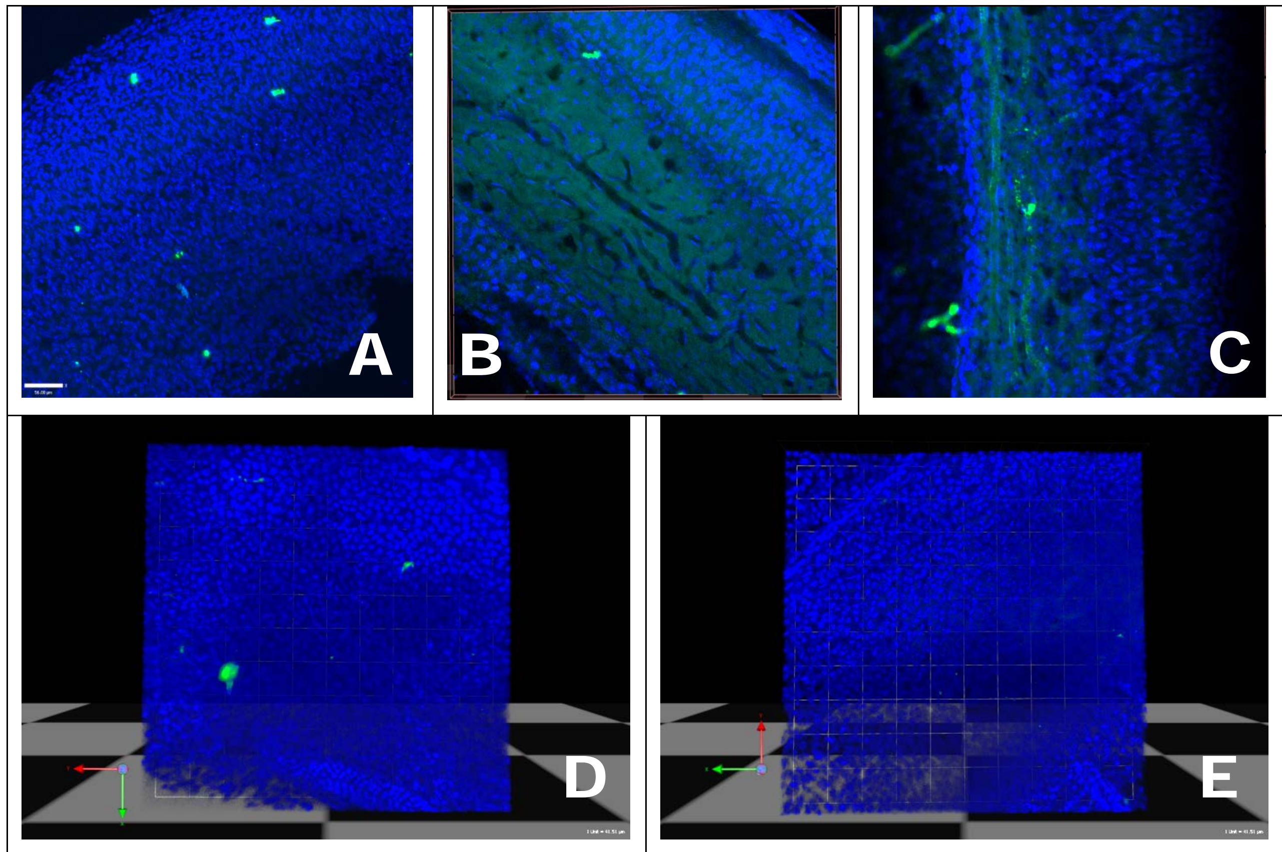

Figure 7: Strain dependence was observed for IgG and Imatinib. Imatinib applied prior to IgG in B6 mice had varied results. Panel (A) shows effectively prevented permeability, (B) shows no effect of the Imatinib, and (C) shows reduced but not prevented permeability. For CBA/J mice, Imatinib reliably prevented the permeability of IgG (**D, E**).

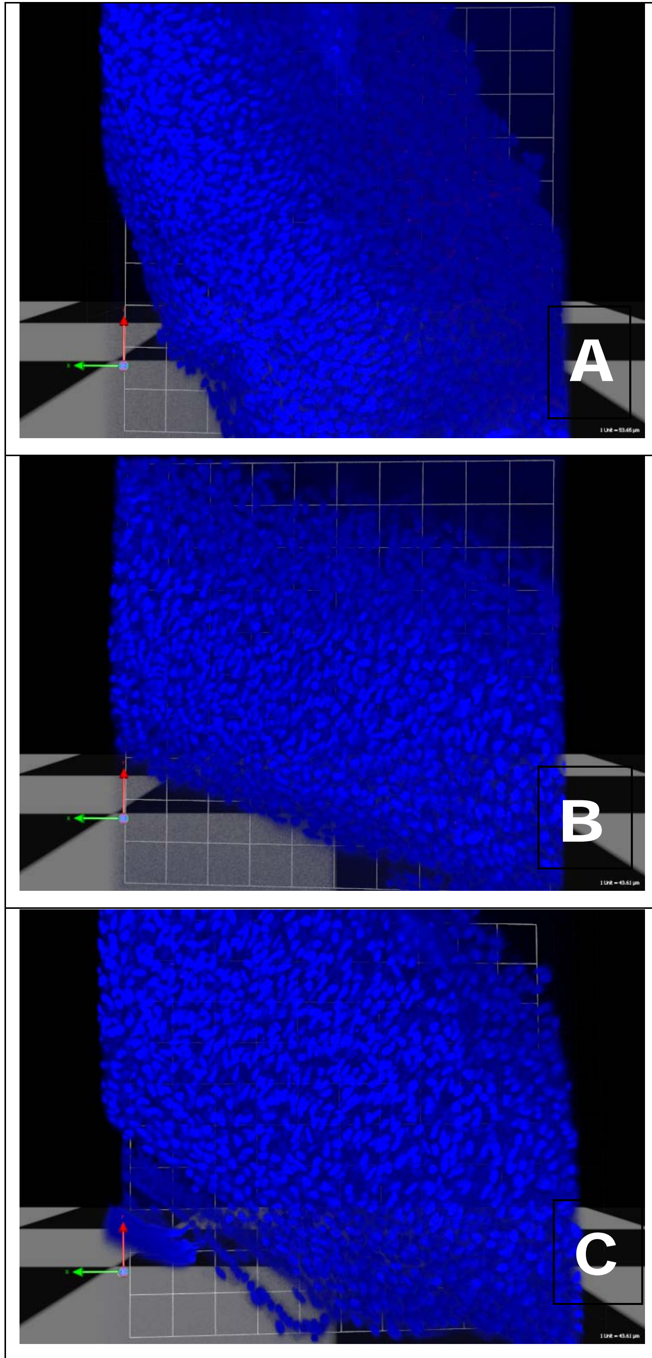

Figure 8: (**A-C**) Apparent elimination of strial capillary permeability to HRP by prior application of Imatinib in three B6 mice. No fluorescent tracer is visualized.