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Strial capillary permeability studied with fluorescent tracers in inbred mice

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STRIAL CAPILLARY PERMEABILITY STUDIED WITH FLUORESCENT TRACERS IN INBRED MICE

By

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Abstract: ***The stria vascularis generates the endocochlear potential (EP), which relies on the maintenance of ionic boundaries. Strial and spiral ligament capillary permeability to FITC-conjugated dextrans and other tracers was assessed in mice of different strains, with and without prior systemic application of mannitol. Mannitol appeared to increase strial capillary permeability to 4 kDa FITC-dextran and effects of mannitol were clearest for post-injection times of less than 2 hours. Present results agree with previous work in suggesting that cochlear capillaries are very 'leaky' under normal conditions.***

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Abstract

Cochlear stria vascularis is responsible for mediating the passage of ions, such as Na^+, K^+, and Cl-, to and from endolymph. The controlled flow of these ions, particularly K^+, generates the endocochlear potential (EP), which is necessary for normal cochlear transduction. Strial function requires fine control of ion concentrations, in part through the maintenance of highly selective ion boundaries around scala media and the stria itself. It has often been argued that highly selective boundaries must also exist around the capillaries that course through the stria. Molecular tracers have frequently been used to investigate the permeability of strial capillaries under normal conditions and following inflammation-related injury. Results from such studies have been used to support the claim either that strial capillaries are highly selective, or that strial capillaries are naturally very leaky. The present study assessed the cochlear lateral wall capillary permeability to fluorescently tagged molecular tracers (4 kDa and 150 kDa fluorescein isothiocyanate-dextran) (FITC-dextran), and .02 um diameter FluoSphere carboxylate beads applied to young, healthy CBA/J and C57BL/6J mice, as determined using confocal microscopy. In some experiments, the hyperosmotic agent mannitol was applied 30 minutes, 1 hour, 2 hours, or 5 hours prior to the injection of the tracers, and changes in permeability were judged. The results support the idea that strial capillaries are normally leaky to a wide variety of ions and macromolecules, and that variation in strial capillary permeability has no implications for hearing.

Introduction

Cochlear Fluids and the Endocochlear Potential

The mammalian cochlea is a fluid-filled structure with three tubular compartments: scala vestibuli, scala media, and scala tympani. Scala vestibuli and scala tympani are continuous at the helicotrema and are filled with perilymph, a typical extracellular fluid which is low in K^+, and with similarities to cerebrospinal fluid and plasma (Wangemann, & Schacht, 1996). Scala media is filled with endolymph, which is uniquely similar in the body to an intracellular fluid, high in potassium (K^+), low in sodium (Na^+), and low calcium ($Ca2^+$) content.

The fluid content of scala media is largely determined by the stria vascularis, which also generates the endocochlear potential (EP), which provides part of the electromotive force driving sound-generated currents through cochlear hair cells. Accordingly, variations in endolymphatic ion concentration influence the EP and transduction currents (Dallos, 1996). The endolymph contains 150 mM K^+, and relatively little Na^+ or $Ca2^+$. Cochlear hair cells are surrounded around their basolateral membranes by perilymph, with their stereocilia protruding into endolymph. When sound is presented, fluid motion within the cochlea is transmitted into electrochemical transduction through the influx of K^+ into the hair cells. After entering the hair cells, K^+ crosses membranes through K^+ channels and reaches the lateral cochlear wall. K^+ is then 'recycled', reaching the cochlear lateral wall either through perilymphatic or through a gap-junctional network that joins cells in the lateral organ of Corti (Wangemann, 2002). K^+ is then transported through the cellular network joining fibrocytes of the spiral ligament ultimately to the intrastrial space (Hibino, Nin, Tsuzuki, & Kurachi, 2010). The essential step in EP generation is thought to be a high level of K^+ flux across a high resistance posed by KCNJ10 K^+ channels in the membranes of strial intermediate cells (Hibino et al. 2010). This current requires

a very low level of K^+ within the intrastrial space, which condition depends on rapid uptake of K^+ by strial marginal cells.

Cochlear lateral wall vasculature

The stria vascularis is so-named because it is highly vascularized. Strial and ligament capillaries likely supply the metabolic requirements of both the stria as well as the somewhat remote organ of Corti. The organ of Corti is avascular; therefore the strial and ligament vessels are thought to supply glucose and metabolites to the organ (Patuzzi, 2011). Endothelial cells of strial and ligament capillaries are joined by tight junctions that are presumed to form an ion-tight barrier that is critical to strial function (Shi, 2010). Thus critical metabolites within strial capillaries such as glucose must escape from the capillaries and be actively taken up by marginal cells or basal cells before they can be released into endolymph or perilymph, respectively.

The Blood-strial barrier

The 'blood-labyrinth barrier' is a broad term for capillary permeability limits on the passage of molecules from blood plasma to either endolymph or perilymph. The more restrictive term 'blood-strial barrier' refers to passage of molecules from strial capillaries to the intra-strial space. Certain specific pathologies may represent dysfunction of this barrier. Examples include strial edema that may result from autoimmune inner ear disease and noise exposure (Ruckenstein, Keithley, Bennet, Powel, Baird, & Harris, 1999; Duvall & Robinson 1987; McMenomey, Russell, Morton, & Trune, 1992), and possibly Meniere's disease, which often includes endolymphatic hydrops (swelling of scala media and distention of Reissners membrane) (Duvall et al. 1987; Tagaya et al. 2011).

Capillary composition

Capillary makeup generally includes endothelial cells, which form capillary walls, and closely associated basement membrane, which may support mechanical stability and slow the passage of some macromolecules such as proteins (Gratton, Meehan, Smyth, & Cosgrove, 2002). Tight junctions joining endothelial cells prevent the passage of polar molecules larger than ~180 Da from simply passing between cells (Neng, Zhang, Kachelmeier & Shi, 2012). Thus, transport across capillaries is generally described as trans-cellular (across endothelial cells) or paracellular (between cells). Other capillary-associated cells, such as pericytes and macrophage-type melanocytes may modulate both modes of transport. Perivascular resident macrophages (PVMs) are an additional specialized cell type found in the brain and potentially stria. PVMs play an important role in immunological defense and repair by scavenging invading microorganisms and dead cells. During tissue inflammation, PVMs produce inflammatory cytokines and participate in vascular repair following tissue insult (Cue, Yin, & Benowitz, 2009). Shi (2010) reported that PVMs reside in the blood-labyrinth barrier surrounding strial capillaries. Previously found in the intra-strial fluid-blood barrier, PVM/Ms were also found in the blood-labyrinth barrier within the three ampullae of the semicircular canals, utricle, and saccule of the vestibular system (Zhang, Zhang, Neng, & Shi, 2013). Similarly, PVM/Ms intertwined with endothelial cells and pericytes in the vestibular system. Injections of bacterial lipopolysaccharide created an inflammatory response, causing the PVM/Ms to arrange in an irregular pattern along capillary walls, thereby increasing vascular permeability and leakage. PVM/Ms are suggested to be necessary for blood barrier integrity and initiating local inflammatory responses (Zhang et al. 2013).

Capillary Leakage and Molecular Transport

In environments such a brain or stria, resident cells require proper ionic media to perform their function. Uncontrolled passage of any molecule into the pericapillary space will also draw water and may cause edema. 'Capillary permeability' expresses the ease of movement of ions, macromolecules, and water across capillary boundaries. 'Leakage' is a general term for improper escape of any molecule from a capillary, and is generally used in the sense of pathological events. Blunt trauma, infection, and certain drugs may result in an abnormal increase in permeability. To study these events, fluorescently-tagged macromolecular tracers have often been applied. A wide variety of these, varying in size, charge, and hydrophilic or lipophilic character can be tracked from vasculature to tissue, providing an indication of both 'porosity' of capillaries, and whether the transport occurs across endothelial cells or between them. FITC-conjugated dextrans have been used to distinguish active transport from passive paracellular leakage in many studies.

Metabolites can move across strial capillary boundaries by active or passive transport processes, depending on type. Molecules naturally move from an area of high concentration to an area of lower concentration without need of an energy source. Lipophilic molecules freely diffuse across endothelial cells, according to their gradient, while hydrophilic molecules and proteins require transport. Active transport is required to move any molecule against a concentration gradient, and requires an energy source. Caveolae-mediated transcytosis is a major route of active transport by capillaries whereby membrane-bound vesicles termed caveolae shuttle molecules independently, in clusters, or by forming a trans-cellular channel. Transcytosis selectively transports materials for purposes of immune defense, nutrient absorption, and plasma membrane biogenesis (Bernd, 2010).

The problem—or not—of strial capillary leak

Noise exposure, inflammation, ototoxins, and aging have all been associated with decreased EP, although the prevalence of this as a contributor to hearing loss is not clear (Ohlemiller 2009, 2015). For example, in a study by Hellier, Wagstaff, O'Leary, and Shepard (2002), animals were administered kanamycin and a loop diuretic (sodium ethacrynate or furosemide). Changes in the appearance of stria vascularis were accompanied by a temporarily reduced EP. In the acute phase, loop diuretics cause strial edema that may be related to altered strial capillary permeability and dysregulation of K^+ in the intrastrial space. Several studies by Shi and colleagues (e.g., Shi, 2009; Pan & Zhang, 2006) involving the application of Evans blue or fluorescently tagged proteins such as albumin have claimed that noise exposure increases paracellular leakage in a manner that promotes hearing loss. Mouse models of chronic genetically-linked cochlear inflammation (Trune, 2010) show a reduced EP that has been associated with elevated levels of IgG in the strial pericapillary space. It has been claimed that this is evidence of leakage that is causally related to the EP reduction. The problem with such studies is that they ignore the prospect of increased active transport of these tracers that may be adaptive, not pathological. At present, there exists no solid evidence for strial paracellular capillary leakage that is causally related to hearing loss. There are two primary issues here that we will consider separately. First is the issue of *how* a given tracer escapes strial capillaries. Second is the issue of whether it *should matter*.

Route of passage

There exists a large literature on capillary permeability in a wide range of tissues (e.g., Pan & Zhang, 2006; Shi, 2009; Hashimoto, Seki, Miyasaka, & Watanabe, 2006). Capillaries in various tissues may perform quite different functions that tolerate—or require—different degrees of leakiness. At one extreme are capillaries in the kidney loop of Henle. These are highly fenestrated and possess no tight junctions. Their purpose is to pose essentially no barrier to the passage of macromolecules. At the other extreme are brain capillaries, which carry out little active transport and possess highly selective tight junctions. According to Sakagami and colleagues (Sakagami, Fukazawa, Kitamura, Doi, & Matsunaga, 1991; Sakagami, Sano, Tamaki, & Matsunaga, 1984), strial capillaries are intermediate between these, performing active transport of large molecules such as horseradish peroxidase (HRP) at a high rate. They made no arguments about the leakiness of strial capillary tight junctions, other than to show that HRP does not normally pass between endothelial cells.

What one may argue from the movements of tracers in the stria depends on the charge, hydrophobic/lipophilic character, and size of the tracer. Canis, Arporchaynon, Messmer, Suckfuell, Olzowy, & Strieth, (2010) and Chen et al. (2009) applied FITC conjugated to 500 kDa and 2 MDa dextrans, respectively, to study blood flow in the brain and found that under normal conditions, the tracers remained within the vasculature and no leakage occurred. Xu, Watanabe, & Komatsuzaki (1994) used HRP to probe capillary permeability in cochlear lateral wall of healthy mice and found leakage outside of the strial capillaries. Hashimoto et al. (2006) contradicted these findings, stating that HRP leakage only occurred as a result of strial damage. Shi (2009) assessed changes in capillary permeability following noise exposure using immunoglobulin G (IgG). The study suggested that IgG leakage into the intrastrial fluid resulted

from acoustic trauma. Such findings have typically been placed into a pathological context, asserting a causal link between such leakage and hearing loss. This assertion has largely gone unchallenged.

Does it matter?

It is worth considering what risks are posed by strial capillary leakage. First, strial capillaries serve the metabolic needs of not only stria, but probably also the rather remote organ of Corti (e.g., Ohlemiller 2015). Thus, chronic leakiness may better serve the efficient transfer of metabolites. As to exactly what strial capillaries may be trying to keep out, this is less clear. Most arguments focus on K^+, which must be kept at low levels in the intrastrial space for EP generation. However, according to one estimate (Wangemann, 1996), intrastrial K^+ is much higher than that in plasma. Moreover, the electrical potential in the intrastrial space is essentially the EP. Thus neither the concentration gradient nor the electrical gradient favors net K^+ flux from strial capillaries into intrastrial space. If not K^+, a small ion whose regulation is critical for EP generation, it is not clear exactly what other larger molecule is likely to require strict control. As we will consider, even hyperosmotic shock that leads to pronounced strial swelling does not seem to affect the EP.

Promoting paracellular leak with systemic mannitol

If strial capillary leak promotes EP reduction and hearing loss, then systemic compounds that promote paracellular leakage should have this effect. Mannitol (MW 182.2 Da) is one example. Mannitol is primarily used as a therapeutic intervention to reduce cerebral edema (e.g., Goodman & Gilman, 1975). The primary mechanism is that it elevates the osmotic strength of

plasma, thus drawing water out of surrounding tissues. The effectiveness of this treatment appears dependent on an intact blood-brain barrier that inhibits the penetration of mannitol into cerebral intercellular spaces. From its size, mannitol would be expected to leak out of capillaries between endothelial cells. Moreover, mannitol is widely taken to promote paracellular leakage by altering the geometry of endothelial cells. Mannitol should therefore promote paracellular leakage of compounds that would not normally easily pass through tight junctions.

Mannitol has been used both clinically and experimentally in the cochlea (Duvall, Hukee & Santi 1981; Mangat & Hartl 2015). Because of its diuretic effects, it has been shown to reduce the effects of endolymphatic hydrops in a manner similar to glycerol. When applied in animals, it causes strial edema similar to that caused by furosemide and ethacrynic acid. Edema of the stria can be caused by many different manipulations (e.g., severe acoustic trauma, ototoxics) and is usually temporary. In 1981, Duvall, Hukee, and Santi investigated osmotic changes and membrane permeability as possible causes of strial edema. The purpose of the study was to determine the effects of intravenously administered hypertonic mannitol on strial ultrastructure. Mannitol caused swelling of the intrastrial space that may result from differences in osmolarity between the blood plasma and the intrastrial fluid, or between intrastrial fluid and endolymph. Assuming mannitol is able to escape into the intrastrial space by leaking through tight junctions, the intrastrial fluid would be rendered hyperosmotic. Water might then be drawn from either capillaries or the endolymph, causing the stria to draw in water across marginal cells until the osmolarity equalizes (Duvall et al., 1981).

Study Aims

The present study follows a series of studies in the Ohlemiller laboratory exploring strial

capillary leak in mice (Dwyer 2010; Henson 2013). Those showed non-passage of large carboxylate beads (≥ 0.02 um), but extensive transfer from capillaries of fluorescein and proteins such as IgG, HRP, and albumin under normal conditions as well as noise exposure. Fluorescein is typically taken to exit capillaries by a paracellular route. It is this route that seems most likely to represent 'uncontrolled' and maladaptive capillary leakage, and most likely to correspond to strial dysfunction. The purpose of the present study was to assess the permeability of strial capillaries in the living mouse to fluorescently tagged dextrans of various sizes under normal and hyperosmotic conditions. Fluorescent tracers were applied to healthy, normal mice from two different strains, and permeability was assessed in terms of the tracer dispersion pattern in flat-mounted segments of stria and spiral ligament. Mannitol, a hyperosmotic agent, was then applied 30 minutes, 1 hour, 2 hours, or 5 hours prior to the same tracers. We reasoned that the mannitol should increase strial capillary permeability and lead to paracellular leakage of the tracers. We also predicted that EP measures (conducted in parallel by K. Ohlemiller) would show a normal EP under the same conditions.

Methods

Animals

Male and female C57Bl/6J (B6) and CBA/J inbred mice were used (See Table 2). Mice were between 8 and 24 weeks of age at the time of the experiments. Three to 5 mice from each strain were used for each test condition (See Table 2). All procedures were approved by the Washington University Institutional Animal Care and Use Committee.

Surgical Procedure

Mice were deeply anesthetized with a 50 mg/kg intraperitoneal injection of sodium pentobarbital. When the pedal reflex could no longer be elicited, the heart was surgically exposed, and 0.1 ml of tracer/vehicle was injected into the left ventricle. The object of this procedure was to capture normal circulatory events in the living animal. Tracer was allowed to circulate for 5 minutes before the cochleae were removed and stored in fixative. For some experiments, 5 g/kg mannitol in 0.9% NaCl was administered via intraperitoneal injection at 30 minute, 1 hour, 2 hour, or 5 hour time intervals prior to tracer injection. A subset of animals was used to test the role of active transport processes in the dispersion patterns of tracer. To accomplish this, animals were fixed transcardially using 4% paraformaldehyde in 0.1 M phosphate buffer prior to the introduction of tracer, in order to shut down all active transport and leave only paracellular pathways. For these 'pre-fixation' experiments, a modified syringe needle catheter was inserted into the left ventricle. The catheter was then used to administer 10 ml of 4% paraformaldehyde in phosphate, followed by 0.5 ml of the selected tracer/vehicle. Animals that did not maintain a steady heartbeat for 5 minutes after the tracer injection or showed signs of hypoxia or instability were not included in the study.

Following perfusion, the cochleae were then quickly extracted from the skull in fixative, 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 removed from the cochleae and mounted on a microscope slide using Permount containing 4',6'diamidino-2-phenylindole (DAPI) as the mounting medium. Samples from both cochleas were pooled for each animal. Slides were sealed using clear nail polish and stored away from light at 4 degrees Celsius.

Tracers

Tracers used included: 4 kDa and 150 kDa fluorescein isothiocyanate-dextran (FITC dextran), and 0.02 um FluoSphere carboxylate beads. These were selected because they have been used in previous studies investigating paracellular capillary leakage. Each tracer has a known diameter and hydrophilic/hydrophobic character, allowing for strial pore size to be interpreted and route of capillary exit to be inferred: 4 kDa FITC (1.4 nm diameter, hydrophilic); 150 kDa FITC (8 nm diameter, hydrophilic); .02 um carboxylate bead (20 nm diameter, partially soluble and tends to form an emulsion).

Confocal Immunofluorescence Microscopy

Tissue samples were assessed using a Zeiss LSM 700 multiphoton confocal microscope. For each mouse, images were collected from one basal segment and one apical segment, and assessed using a 20x objective. For each fluorescent tracer, laser gain and intensity were held constant across preparations to maintain consistent appearance and background levels. See Table 1 for tracer information.

Quantification

No statistical analysis was used in evaluation, due to the qualitative nature of the data collected. Images were processed using Volocity, and evaluated for presence/absence, degree, and location of fluorescence in the intrastrial space. Central tendencies were deduced from trends that appeared across at least 3 animals per strain/condition, and at least 3 pieces inspected per animal.

Results

Tracer dispersion patterns

In otherwise untreated animals, the 4 kDa FITC-dextran tracer was only weakly present in the intrastrial space, but clearly outlined strial capillaries (Figs. 1 and 2). Permeability of this tracer under normal conditions appeared to be independent of mouse strain and gender, and did not appear to vary based on location in the cochlea (apical/basal). Extra-capillary tracer signals were too weak to suggest whether any tracer was taken up by basal, intermediate, or marginal cells. The 150 kDa FITC-dextran tracer appeared only within strial capillaries, as well as some ligament vessels (Fig. 6).

Effect of mannitol on tracer dispersion patterns

Mice that received mannitol, followed by 4 kDa FITC-dextran showed bright fluorescence throughout the stria (Figs. 1 and 2). Mannitol increased capillary permeability to the tracer independent of mouse strain and gender, and did not appear to vary based on location in the cochlea (apical/basal). Radial views (Fig. 5) suggest that the tracer fills the intrastrial space and is taken up by basal, intermediate, and marginal cells. Comparison of strial thickness in mannitol/vehicle animals indicated that the intrastrial space is swollen, consistent with previous work (Duvall et al., 1981). Mannitol did not alter the dispersion pattern of either 150 kDa FITC-dextran or the .02 um fluorophore (Figs. 6 and 7). Comparison of dispersion patterns for different tracers places a rough estimate of the size of paracellular openings produced by mannitol somewhere between 1.4 nm and 8 nm in diameter.

Differences in 4 kDa tracer dispersion were noted between CBA/J and B6 mice were hinted by results following mannitol (Fig. 8). The B6 mice appeared to have slightly increased

strial uptake of the tracer versus CBA/J mice. Reasons for this suggested strain difference should be investigated further.

Possible strain effects

Differences in 4 kDa tracer dispersion between mannitol and control conditions were also noted in the spiral ligament vessels in CBA/J and B6 mice (Figs. 9 and 10). Mice treated with mannitol appeared to have slightly more uptake of the tracer in the spiral ligament vessels as compared to control mice.

Dependence on time of mannitol injection

Mannitol was administered at 30 minutes, 1 hour, 2 hours, or 5 hours prior to tracer injection in B6 mice. Tracer uptake was greatest at 30 minutes and 1 hour intervals, while only slight tracer uptake was seen at 2 hour and 5 hour intervals. Radial views demonstrate that strial edema appeared to be greatest when mannitol was administered 1 hour prior to the tracer (Figure 11).

Spatial patterns of tracer distribution

For both mannitol and control conditions, 4 kDa FITC-dextran fluorescence seemed greater in ringed patterns that are consistent with some retention by the basement membrane that lines strial capillaries (Fig. 12). Figure 12 shows the tracer dispersion in the intrastrial space and basement membrane 1 hour after mannitol.

Effect of prior fixation on tracer dispersion patterns

Fixation prior to the injection of 4 kDa FITC-dextran in B6 mice did not impact the dispersion pattern. The tracer was still readily found in the intrastrial space (Figure 4).

EP measurements

EP measurements were taken on CBA/J and B6 mice within 1-1.5 hrs hour after mannitol was administered. Results were consistent with normal control animals (Fig. 3). This supports previous work (Duvall et al., 1981) suggesting that acute strial edema and increased strial capillary permeability following mannitol injection (present work) did not affect strial function, and thus are unlikely to negatively impact hearing. While there did not appear to be any significant reduction in EP due to mannitol in B6 mice, CBA/J mice demonstrated a slight yet significant reduction in EP due to mannitol. This could suggest possible strain differences.

Discussion

These experiments were designed to test the extent of cochlear lateral wall vascular permeability to macromolecular tracers following administration of mannitol. The use of two different inbred mouse strains also allowed us to test for potential strain differences. Our observations suggest that strial capillaries are 'leakier' than spiral ligament capillaries under normal and hyperosmotic conditions, consistent with previous work (Sakagami, Matsunaga, & Hashimoto, 1982). The suggested appearance of strial edema in response to mannitol likewise was not associated with a reduction in the EP. Therefore, this increase in strial capillary paracellular permeability may not affect hearing sensitivity.

While it is difficult to prove that any molecule or tracer escapes capillaries by a particular mechanism, the overwhelming weight of the literature favors the interpretation that mannitol leaks out of capillaries between capillary endothelial cells. In the process, mannitol reportedly alters the dimensions of endothelial cells, thus promoting leakage of large macromolecules such as 4 kDa FITC-dextran. Based on our results, the paracellular 'holes' created by mannitol may be somewhere between 1.4 nm and 8 nm in diameter.

In some respects our results are not especially surprising. As stated, glycerol, a hyperosmotic agent similar to mannitol, has been used to determine the presence of cochlear hydrops and corroborate a diagnosis in patients with Meniere's disease (Lutkenhoner & Basel, 2013). In the case of Meniere's, the addition of a hyperosmotic agent often improves hearing. This seems unlikely to be the case if the dominant effect of hyperosmotic agents was to promote strial dysfunction.

The present results build on a long line of experiments indicating that strial capillaries are normally permeable to a wide range of macromolecules, including several proteins (albumin, HRP, IgG) (Xu et al., 1994; Shi, 2009) and small polar molecules (mannitol, fluorescein) (Duvall et al., 1981; Henson, 2013). Proteins appear to be actively transported across endothelial cells, while the smaller tracers, based on the weight of previous work, leak between endothelial cells. For any tracer, dispersion is much more delimited for spiral ligament capillaries, so that these two capillary beds clearly behave quite differently. Why two capillary beds (e.g., stria versus brain) possessing tight junctions should function so differently is not clear. Anatomic differences include the presence of glial cells only in brain, and potentially different density of pericytes and other regulatory cells. Functional requirements that differ between these include the presence of a high, positive intrastrial potential, a normal intrastrial K+ concentration that

exceeds that in brain (Wangemann, 1996), and the need for metabolites such as glucose to readily escape capillaries as an adaptation to serving the needs of remote cells (hair cells).

Conclusion

Strial capillaries in control B6 and CBA/J mice showed few signs of paracellular leakage 4 kDa and 150 kDa FITC-dextran. When mannitol was administered, 4 kDa FITC-dextran dramatically filled strial cells and the intrastrial space, although capillaries of spiral ligament still indicated little leakage of tracer. These trends applied regardless of mouse gender or apical/basal location, although it appeared that transport in CBA/J mice was less robust than in B6. Under no circumstances were 150 kDa FITC-dextran or 0.02 um beads clearly observed outside of capillaries, placing an upper bound on the size of openings produced by mannitol. Our results indicate that strial capillary leakage occurs in response to a hyperosmotic agent, and are consistent with a large literature favoring very active strial capillary leak both across and between capillary endothelial cells. The EP is uncompromised by mannitol-induced strial capillary leak. On this basis, we question pathophysiologic scenarios according to which noise, inflammation, or other events promote uncontrolled strial capillary leak and consequent EP reduction and hearing loss.

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Table and Figure Appendix:

Macromolecule	Abbreviation	Molecular weight	Emission wavelength	Source
fluorescein isothiocyante - dextran	FITC	4 kDa	520 nm maximum	Sigma Aldrich
fluorescein isothiocyante - dextran	FITC	150 kDa	520 nm maximum	Sigma Aldrich
FluoSpheres®, Sulfate Microspheres, yellow-green, 2% solids, 0.02 um	0.02 um carboxylate beads		505/515	Invitrogen

Table 1: The 3 tracers utilized, including the name, abbreviation, molecular weight, emission wavelength, and source from which the tracer was obtained.

Macromolecule	Condition	Strain	Number of Animals
150 kDa fluorescein isothiocyanate-dextran (FITC dextran)	Control	B6	5
		CBA/J	3
		-	-
	Mannitol Prior to Macromolecule	B6	3
		CBA/J	0
		-	-
4 kDa fluorescein isothiocyanate-dextran (FITC dextran)	Control	B6	4
		CBA/J	3
	Mannitol Prior to Macromolecule	B6	5
		CBA/J	3
	Fixative Prior to Macromolecule	B6	3
		CBA/J	0
0.02 um diameter FluoSphere carboxylate beads	Control	B6	3
		CBA/J	3
		-	-
	Mannitol Prior to Macromolecule	B6	3
		CBA/J	0
		-	-

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

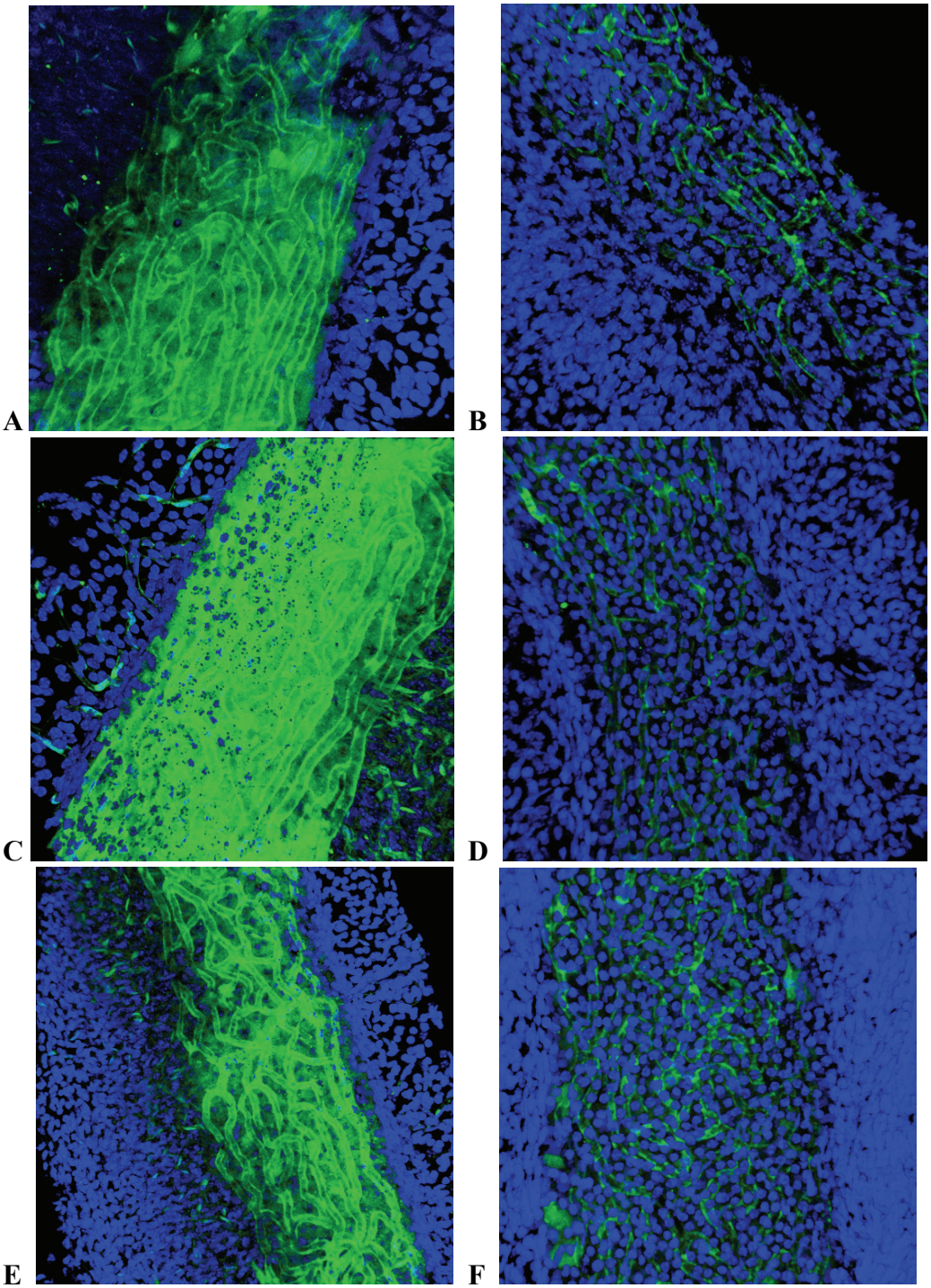


Figure 1: Strial capillary permeability to 4 kDa FITC-dextran in B6 mice. Panels (A), (C), and (E) show tracer dispersion 1 hr after mannitol in the stria vascularis. Panels (B), (D), and (F) show tracer in control mice. Mannitol appears to greatly promote capillary permeability to tracer.

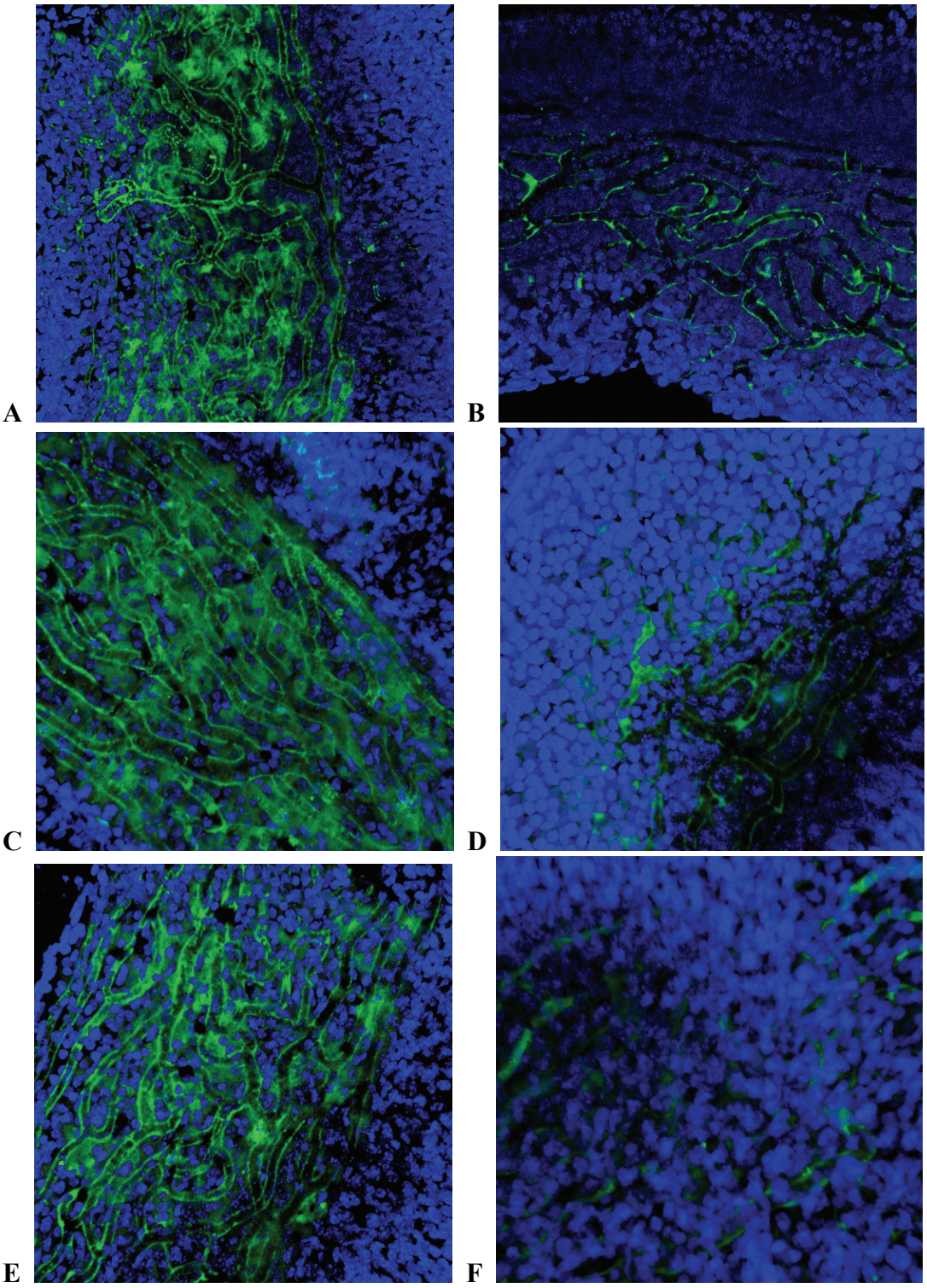


Figure 2: Strial capillary permeability to 4 kD FITC-dextran in CBA/J mice. Panels (A), (C), and (E) show tracer dispersion 1 hr after mannitol in the stria vascularis. Panels (B), (D), and (F) show tracer in control mice. Mannitol appears to greatly promote capillary permeability to tracer.

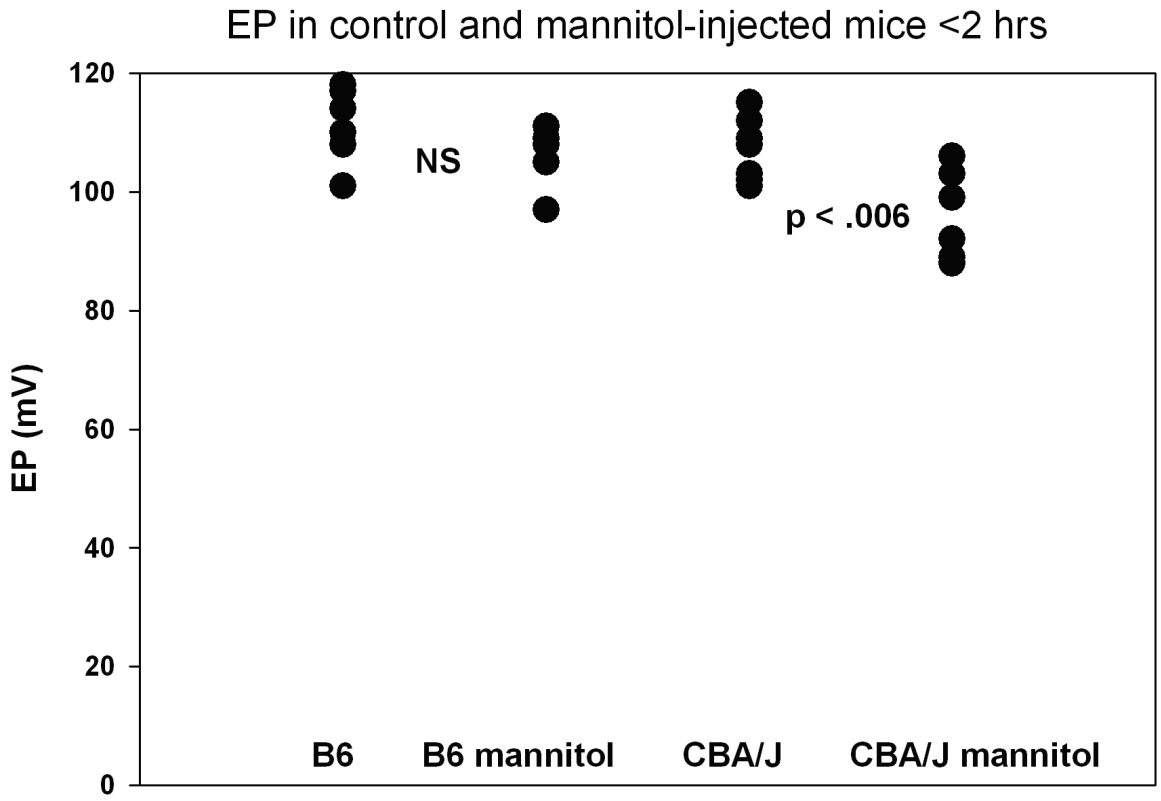


Figure 3: EP recordings obtained at peak mannitol effect (1 hour). There did not appear to be any significant reduction in EP due to mannitol in B6 mice. CBA/J mice demonstrated a slight yet significant reduction in EP due to mannitol.

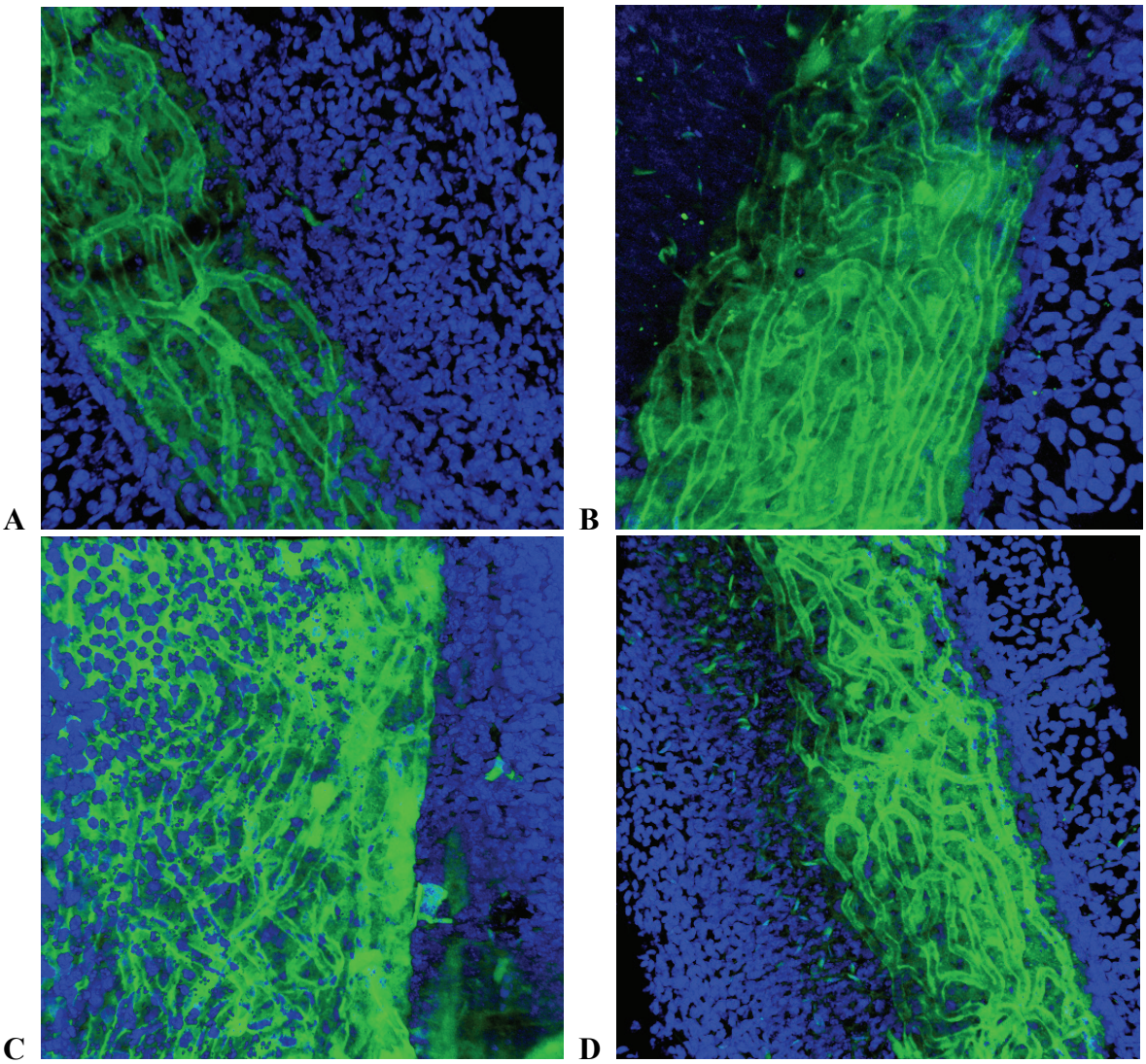


Figure 4: No effect of pre-fixation on the effects of mannitol. Panels (A) and (C) show the stria vascularis when 4% paraformaldehyde was perfuse systemically just prior to the tracer. Panels (B) and (D) show the stria vascularis when no prior-fixation was applied.

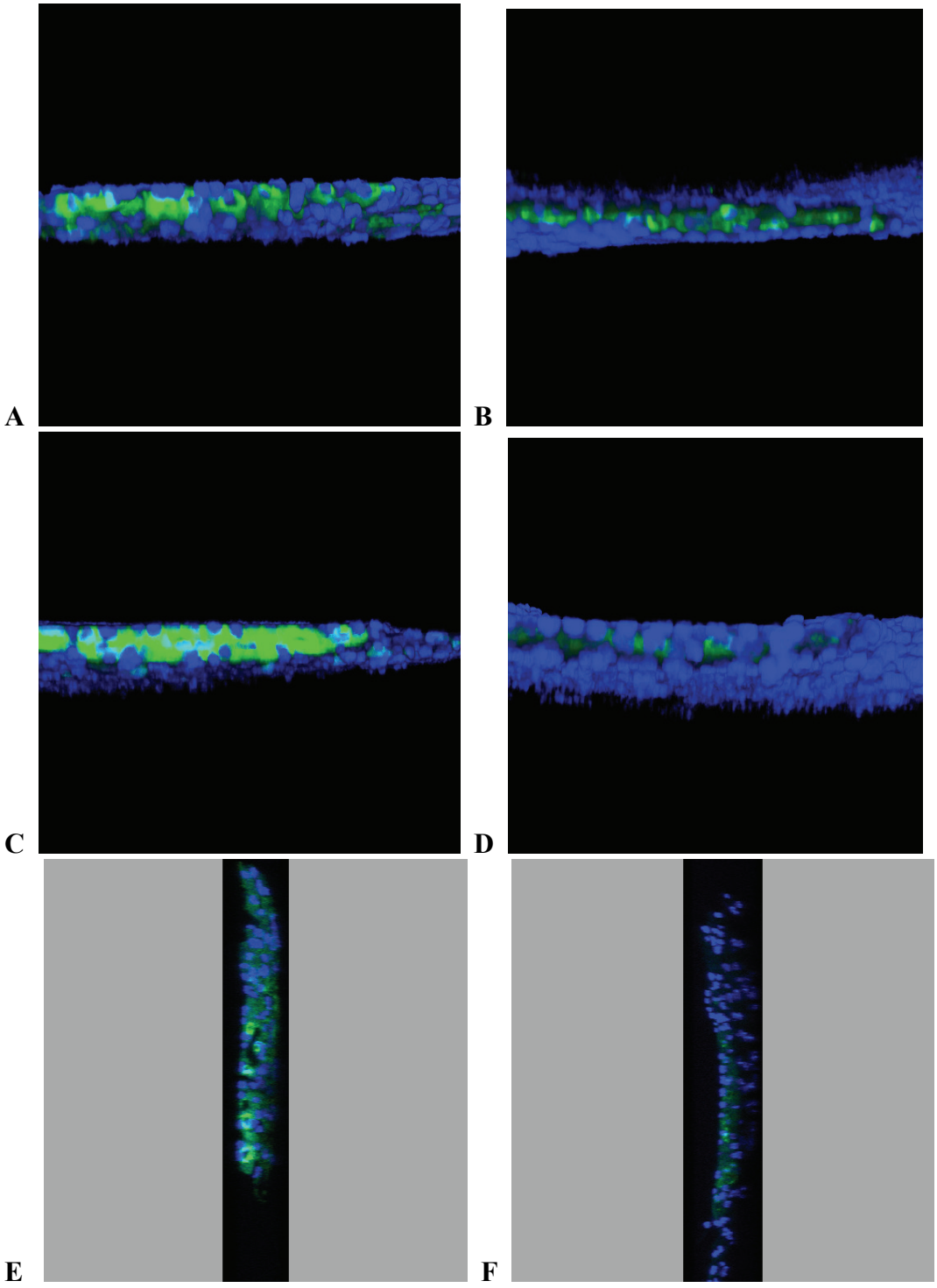


Figure 5: Radial view of 4 kDa FITC-dextran in B6 mice with and without mannitol. Panels (A), (C), and (E) show the stria vascularis when mannitol was administered prior to the macromolecule. Panels (B), (D), and (F) show the stria vascularis when no mannitol was administered. All strial cells and intrastrial space appear to contain a high level of tracer. Increased strial thickness in (A), (C), and (E) may indicate strial edema following the application of mannitol.

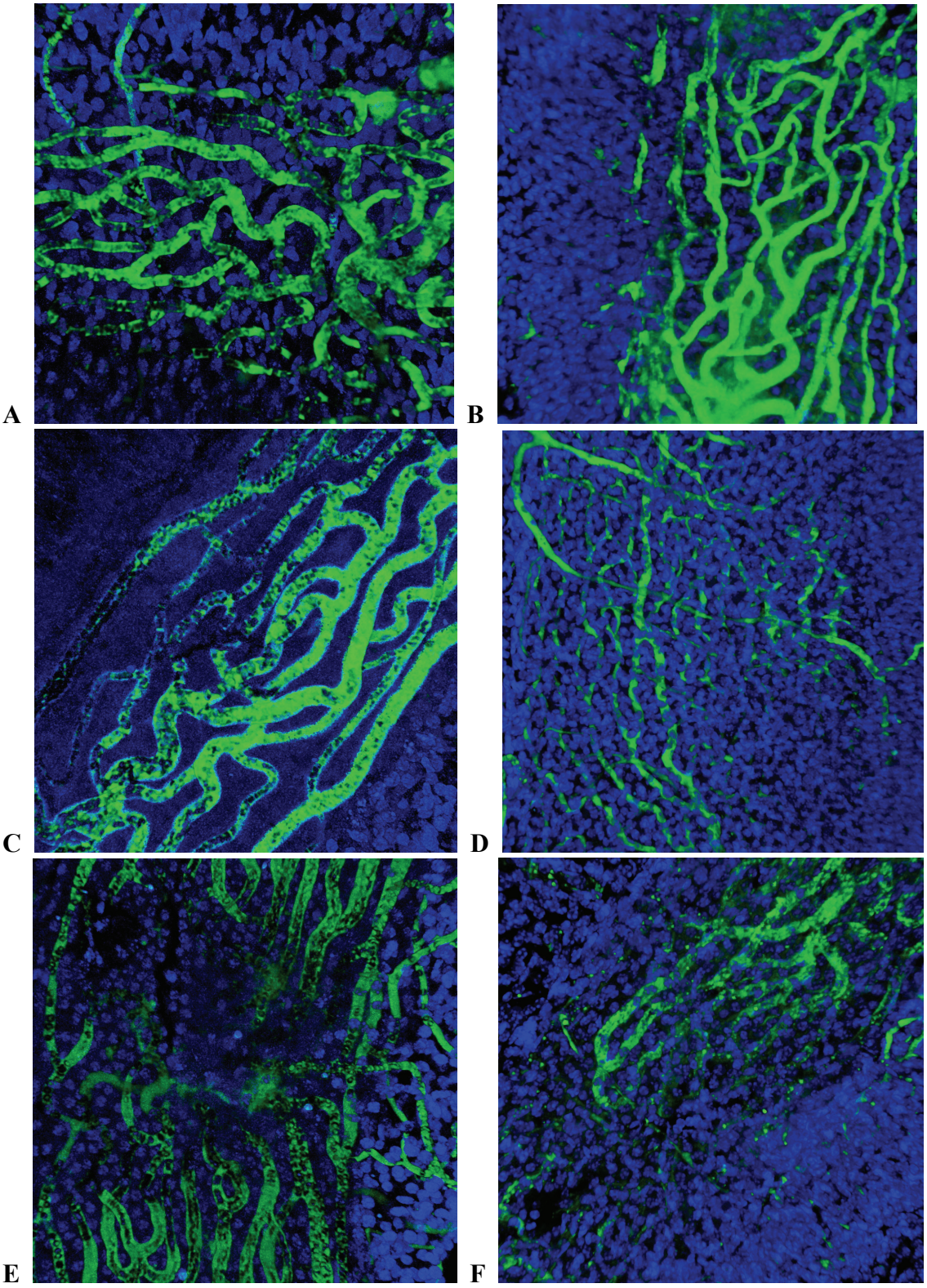


Figure 6: No strial capillary leakage of 150 kDa FITC-dextran, with (A, C, E), or without (B, D, F) mannitol. There were no apparent differences between the conditions with or without mannitol.

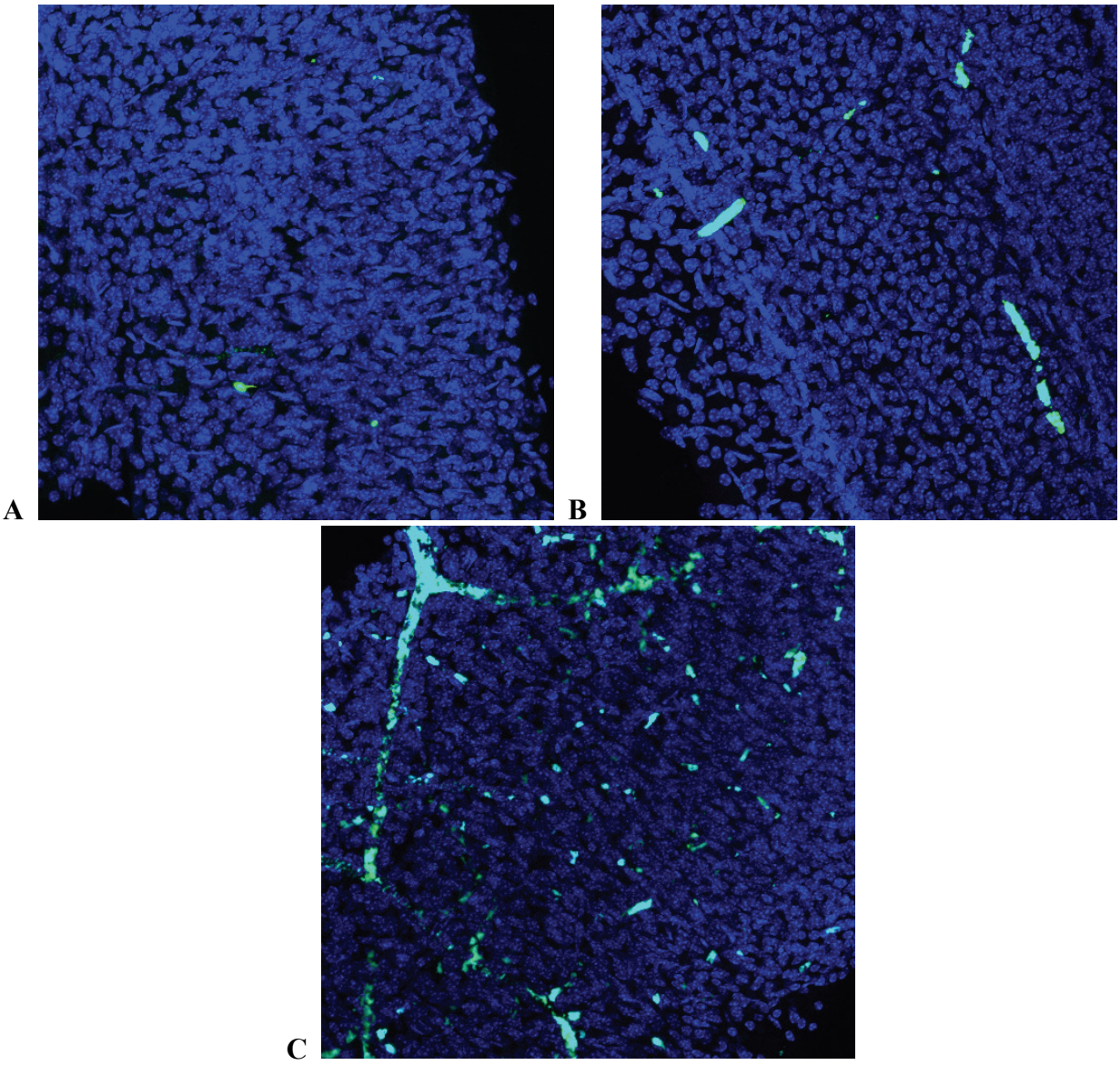


Figure 7: Lack of capillary permeability to .02 um diameter carboxylate beads in B6 stria after mannitol. Beads are only seen in capillaries. Clumping of beads contributes to spotty appearance.

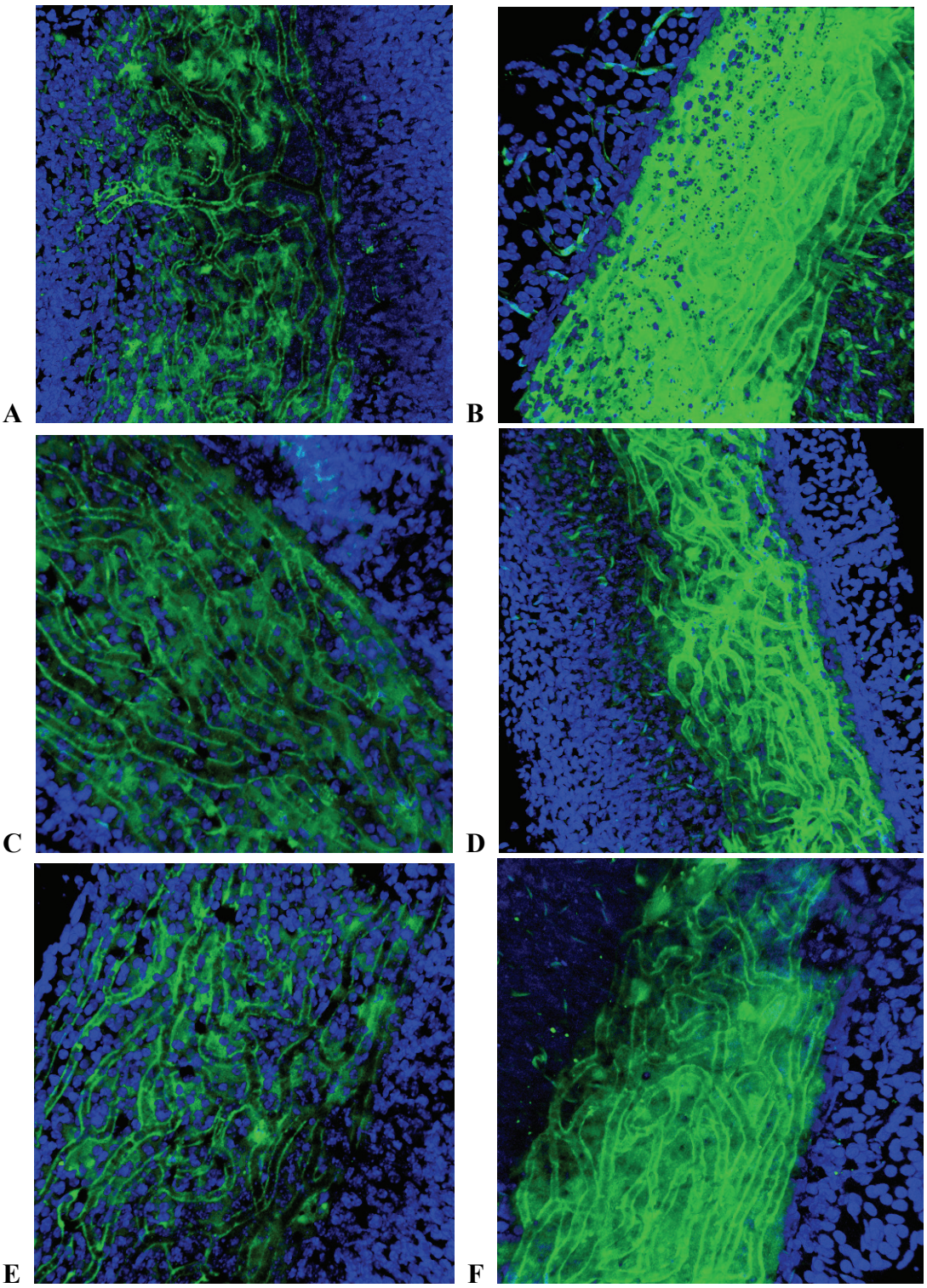


Figure 8: Possible strain differences in dispersion of 4 kDa FITC-dextran after mannitol. Panels (A), (C), and (E) show stria of CBA/J mice. Panels (B), (D), and (F) show B6 mice. B6 mice show more intense signal than CBA/J mice.

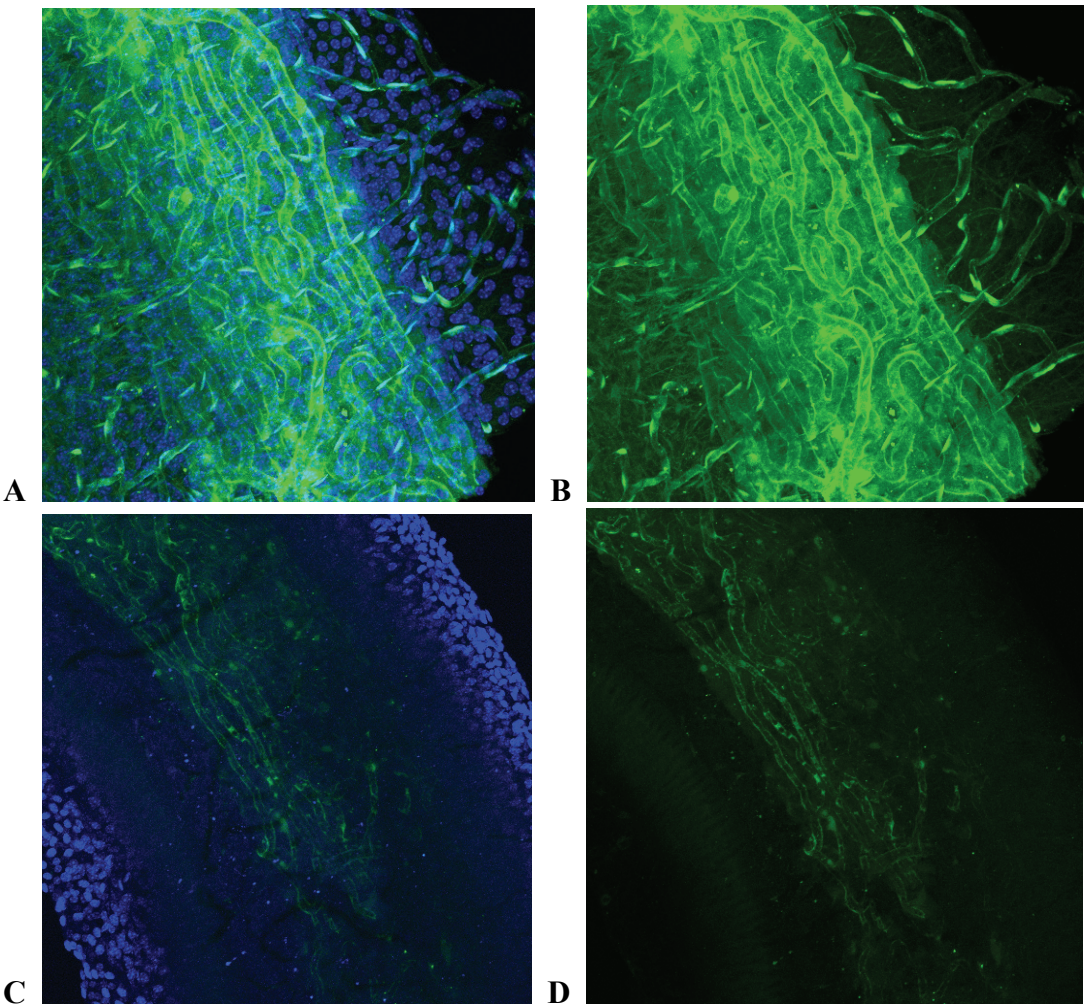


Figure 9: Stria and ligament tracer uptake differences with and without mannitol in B6 mice. Panels (A and B) show tracer dispersion between the stria vascularis and spiral ligament 1 hr after mannitol. Panels (C and D) show tracer dispersion between the same tissues in control mice. Panels (A) and (C) show the DAPI stained nuclei, whereas panels (B) and (D) only show the tracer.

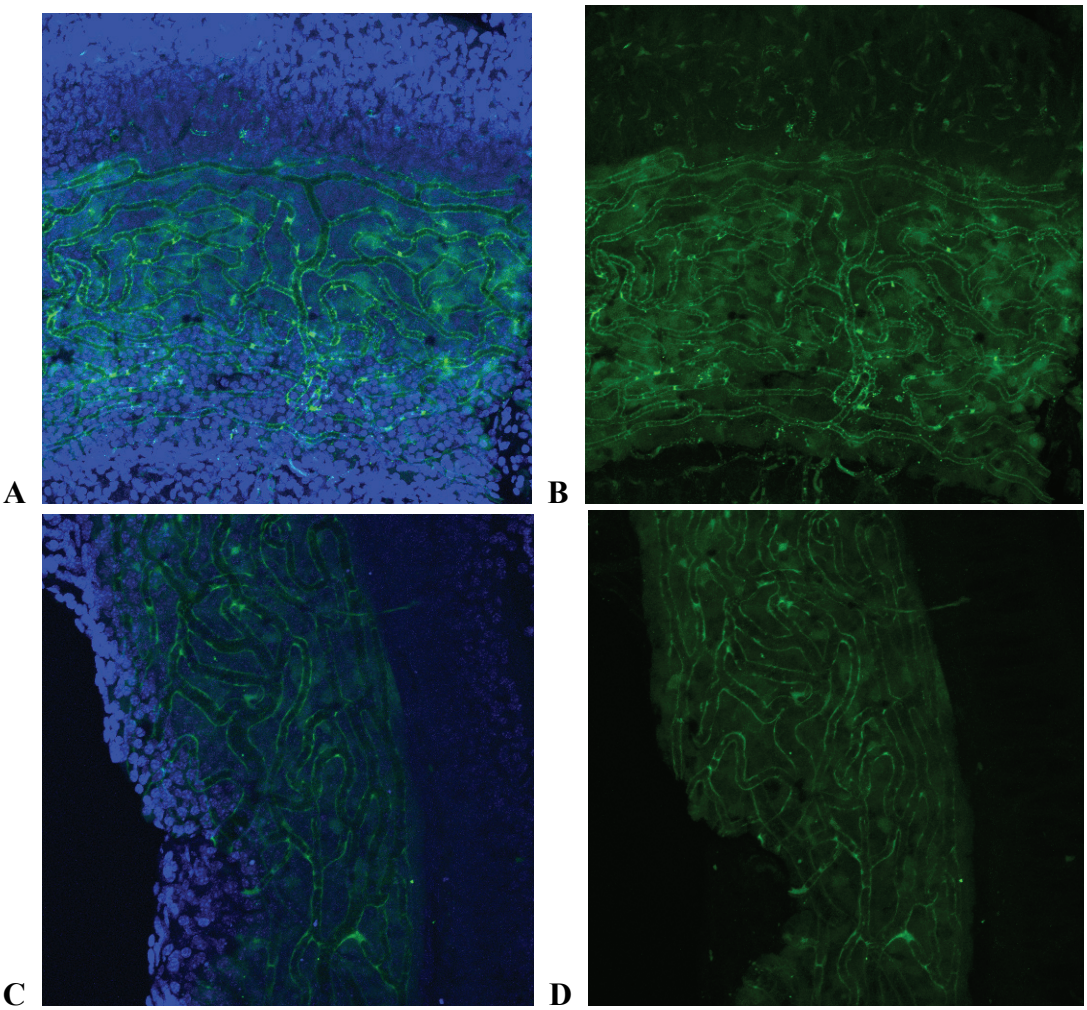


Figure 10: Stria and ligament tracer uptake differences with and without mannitol in CBA/J mice. Panels (A and B) show tracer dispersion between the stria vascularis and spiral ligament 1 hr after mannitol. Panels (C and D) show tracer dispersion between the same tissues in control mice. Panels (A) and (C) show the DAPI stained nuclei, whereas panels (B) and (D) only show the tracer.

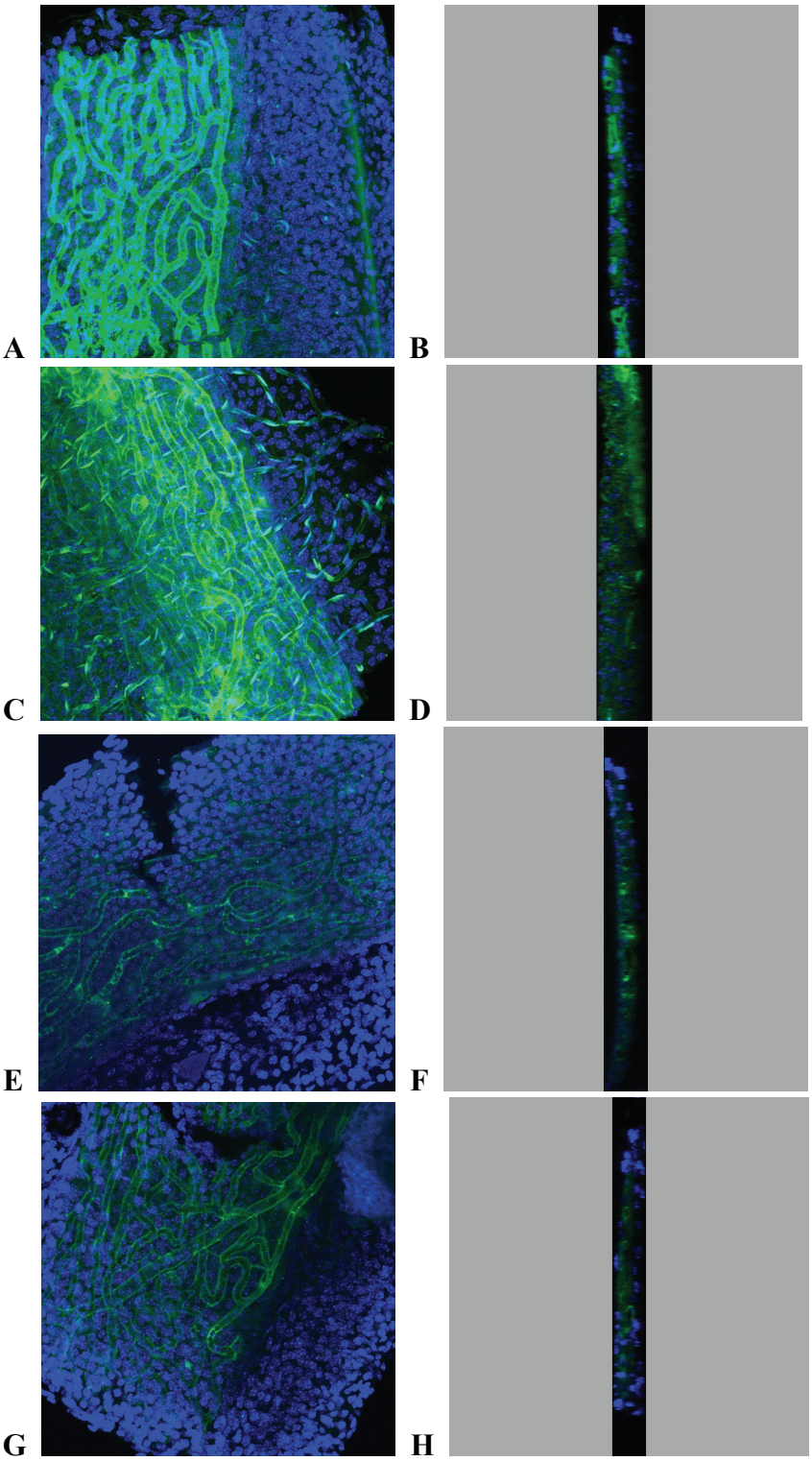
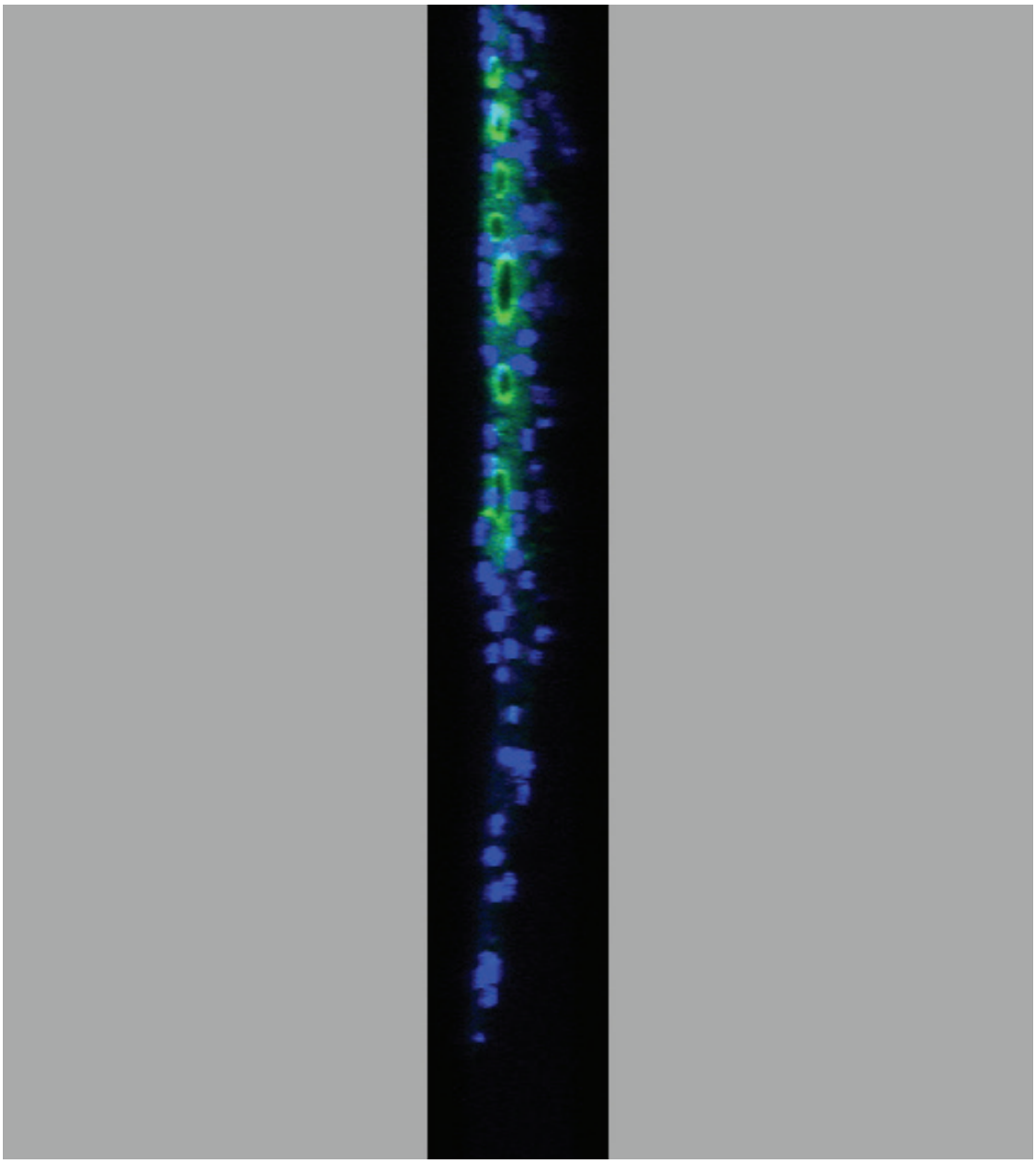


Figure 11: Whole-mount and radial view of 4 kDa FITC-dextran in B6 mice with mannitol administered at 30 minutes (A and B), 1 hour (C and D), 2 hours (E and F), and 5 hours (G and H) prior to the tracer. Strial cells have greatest tracer uptake at 30 minutes and 1 hour post-mannitol. Strial edema appears to be most severe 1 hour after mannitol administration.



A

Figure 12: Radial view of 4 kDa FITC in the stria vascularis after mannitol. Tracer may be retained by the basement membrane, but also clearly appears more widely dispersed.