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Neuroinflammation, oxidative stress, and blood-brain barrier (BBB) disruption in acute Utah electrode array implants and the effect of deferoxamine as an iron chelator on acute foreign body response

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Abstract

The use of intracortical microelectrode arrays has gained significant attention in being able to help restore function in paralysis patients and study the brain in various neurological disorders.

Electrode implantation in the cortex causes vasculature or blood-brain barrier (BBB) disruption and thus elicits a foreign body response (FBR) that results in chronic inflammation and may lead to poor electrode performance. In this study, a comprehensive insight into the acute molecular mechanisms occurring at the Utah electrode array-tissue interface is provided to understand the oxidative stress, neuroinflammation, and neurovascular unit (astrocytes, pericytes, and endothelial cells) disruption that occurs following microelectrode implantation. Quantitative real time polymerase chain reaction (qRT-PCR) was used to quantify the gene expression at acute time-points of 48-hr, 72-hr, and 7-days for factors mediating oxidative stress, inflammation, and BBB disruption in rats implanted with a non-functional 4×4 Utah array in the somatosensory cortex.

During vascular disruption, free iron released into the brain parenchyma can exacerbate the FBR, leading to oxidative stress and thus further contributing to BBB degradation. To reduce the free iron released into the brain tissue, the effects of an iron chelator, deferoxamine mesylate (DFX), was also evaluated.

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Data Availability

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

Keywords

Utah arrays; blood-brain barrier (BBB) disruption; neuroinflammation; oxidative stress; iron chelator; deferoxamine mesylate (DFX)

1. Introduction

Chronically implanted microelectrode arrays hold tremendous promise to further our understanding of the nervous system through basic research [1–3] and to restore lost sensory inputs and motor outputs through brain-machine interfaced (BMI) devices [4–11]. Long-term stability and inconsistent functional performance are the critical barriers for neural electrode arrays that have, upon implantation, presented limitations in their effective clinical use in therapeutic or prosthetic applications [12–16]. Acute insertion trauma [17], tissue inflammation [14, 18–27], blood-brain barrier (BBB) disruption [23, 28–31], and encapsulation [20, 21] can cause the performance of chronic implants to degrade significantly over time [12, 15, 32, 33]. Following an electrode implant, BBB disruption leads to further hemorrhaging, edema, ischemia, microglial activation, and pro-inflammatory cytokine secretion [30] and has been indicated as a key determinant affecting the chronic electrode-tissue interface stability [23, 29]. The BBB provides a physical and metabolic barrier that regulates cerebral homeostasis [34, 35] and is composed of endothelial cells that are connected by an extensive network of complex tight junction and adherens junction proteins [36–40], which influence junction organization of the barrier [41]. Tight junctions are extremely critical to BBB permeability [38, 42, 43]; they consist primarily of the occludin, claudin, and zonula occludens family of proteins [35, 39], of which occludin and claudin-5 restrict paracellular transport across the BBB [42, 43]. Adherens junctions, which consist of the cadherin and catenin family of proteins, are required for the correct organization of tight junctions [44], signaling crosstalk [45], and overall vascular homeostasis [41]. The expression of these proteins is tightly regulated and closely associated with changes in BBB integrity and permeability [35].

The neurovascular unit (NVU), which forms and maintains the BBB and enables the regulation of blood flow in the cerebral vasculature, consists of the endothelial cells, pericytes, astrocytes, microglia, and neurons [46–48]. Vasculature damage causes disruption of the various components of the NVU resulting in microglial activation, oxidative stress, inflammation, and retrospectively, mitochondrial dysfunction [46, 47, 49–52]. Vascular disruption also causes a sudden increase of iron levels in the brain tissue due to erythrocyte/hemoglobin entry [53] where increased iron levels can exacerbate oxidative tissue damage via Fenton chemistry [53–56]. Additionally, hemolysis occurs after bleeding and hemorrhaging, such as that due to electrode implant injury, resulting in hemoglobin degraded products, such as free iron, to be released through heme-oxygenase-1 activity [55, 57]. Through a sequence of reactions called the Fenton reactions, iron plays a central role in causing excitotoxicity and neuronal injury [54, 55]. These reactions yield reactive oxygen species (ROS) which are toxic and lead to oxidative stress, cell death, lipid peroxidation, further worsening BBB disruption [53, 55, 58–61]. In the case of an electrode implant, a chronic, microbleeding implantation site results in sustained local iron overload, which has

2.2 Surgical Procedure

A custom made, 1-mm long, 400 μ m spaced, 4 $\times$ 4, non-functional Utah microelectrode array (UMEA) with parylene-coated silicon shanks was used in this study. Isoflurane was used to anesthetize the animals, and stereotactic surgery was performed under deep anesthesia. A midline incision was made on the rat's skull, and the periosteum was scraped to expose the skull surface. Upon identifying the bregma, four stainless steel screws were drilled on the skull to support the head cap. A craniotomy (~4mm x 4mm) was drilled over the sensorimotor cortex (1mm lateral and 2mm posterior to the bregma). Care was taken during the drilling process by stopping every 10–15 seconds and flushing the skull surface with sterile saline so as not to cause heating of the brain tissue during the drilling procedure. A 25-gauge sterile needle angled at ~45° was used to puncture the dura and a microscissor was used to cut and reflect the dura onto the sides to expose the cortical surface. The UMEA was carefully placed on the cortical surface at the craniotomy location and then inserted into the cortex using a pneumatic inserter (Blackrock Microsystems, Inc, UT). The pneumatic inserter was stereotactically positioned on top of the array, which drops a weight inside the inserter wand to displace a chuck at the bottom of the wand by 1mm to insert the electrode array. The craniotomy was then covered with a 25 μ m thick silastic sheet, and dental acrylic was used to cover the skull surface, which served as the head cap.

2.3 DFX and safety of DFX administration

Animals in the treatment group received DFX (100 mg/kg in sterile water, intraperitoneal) as commonly reported in animals and humans [66, 67, 75]. The animals received drug injections one hour following surgery and then every 12-hours for the duration of the implant. The safety of DFX administration was assessed by performing liver and kidney function tests on animals (n=5/group) for only the 7-day time-point (Table 2). DFX was administered as described previously for 7 days. Blood was drawn from the tail vein at day 7 to evaluate kidney and liver function. Since DFX is metabolized primarily by the liver [76], drug toxicity, if present, would lead to an increase in the liver enzyme levels, such as those of aspartate aminotransferase (AST) and alanine transferase (ALT) corresponding with a decrease in the albumin levels, indicating hepatic pathology. The blood chemistry levels indicated that DFX administered for 1 week did not cause any kidney or liver toxicity as compared to control animals (untreated). Safety of DFX administration has also been previously reported in the literature [77–81].

2.4 Quantitative Real-Time PCR

Quantifying mRNA using real time polymerase chain reaction (RT-PCR) remains a powerful technique for expression analysis, which allows for fast, accurate, and highly sensitive quantification of gene expression. The method also allows screening of a large number of genes simultaneously as compared to other protein analysis methods such as Northern or Western Blots. A similar protocol as reported before [82] was used for RNA extraction, reverse transcriptase, and cDNA amplification. Brain tissue samples harboring the electrode array were weighed and homogenized with 1mL of PureZol (BioRad, CA) and the lysate sample was allowed to rest at room temperature for dissociation of nucleoprotein complexes to occur. Nano Drop ND-1000 (Thermofisher Scientific) was used to determine the RNA

purity and concentration for each tissue sample. After RNA extraction, Superscript First-Strand Synthesis System (BioRad, CA) was used for reverse transcriptase. The cDNA was then stored at -80°C until used for qRT-PCR. For running qRT-PCR, a solution consisting of 10ml of ssadvanced universal mix (BioRad, CA), 3 ml of cDNA, 1 ml of respective gene primer (BioRad, CA), and 6ml of sterile water was used for each well on the PCR plate. Each gene was run in duplicate wells, and GAPDH was used as the housekeeping gene for all samples. All experimental tissue samples were normalized with respect to control tissue samples from unoperated animals, which served as the baseline values for gene transcription. The control and experimental tissue sample C_q values were first normalized with respect to the sample GAPDH C_q values, producing C_q ($C_q = C_q(\text{target gene}) - C_q(\text{reference gene i.e. GAPDH})$). The C_q value was calculated by normalizing each experimental C_q to its control sample C_q value ($C_q = C_q(\text{target gene}) - C_q(\text{control sample})$). The relative fold changes in mRNA values with respect to unoperated animals were calculated using the $2^{(-C_q)}$ method [83]. A two-fold or higher change in gene expression was considered significant [84]. The relative fold change values are sensitive to the C_q values obtained from the housekeeping gene, i.e. GAPDH. An average C_q value for GAPDH was calculated for all plates for each experimental group (DFX-treated, un-treated controls, and unoperated controls) at each time-point and no statistical difference was found across the groups indicating that GAPDH values obtained across PCR plates were stable and its expression can be used as an internal control. Table 3 provides a summary of the gene transcripts used to study oxidative stress, neuroinflammation, and the BBB.

2.5 Statistics

Two-way analysis of variance (ANOVA) was used to determine whether there was a difference in the fold change values of the mRNA expression for each gene between the control and DFX groups or across time. The Shapiro-Wilk test for normality was used before applying ANOVA. If there was a significant difference ($p < 0.05$) in either row or column, one-way ANOVA followed by Holm-Sidak post hoc comparison test was used to find which groups were significantly different. Significant interaction between groups for a small number of genes was observed. For those genes, an ANOVA with simple effects was re-ran to evaluate if the main effect(s) was statistically significant. If they were found to be significant, a post hoc test was used to find which groups (within time or drug vs untreated) were different.

3. Results

3.1 Oxidative stress

3.1.1. Generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS): A panel of genes was monitored for their roles in the production of reactive oxygen and nitrogen species (Figure 1). Significant upregulation was present for several substrates involved in the NADPH oxidase (NOX) complex, such as CYBB, DUOX1, NCF1, NOX4, and NOXA1, leading to its production of ROS. CYBB and NCF1 expression was similar for both control and DFX groups, except at the 72-hr time-point, where control groups' expressions were significantly higher than the DFX group. NOX4 transcription levels were significantly higher at the 48-hr time-point for the DFX group;

however, the levels quickly declined and remained similar (~5-fold) for the 72-hr and 7-day time-points. NOXA1, as an activator of NOX activity, increased in expression to ~4-fold from 48-hr to 7-days for both groups. DUOX1 and AOX1 followed similar trends in expression profiles. DUOX1 was upregulated throughout the study, and its expression level increased at each time-point. AOX1, although not as abundant as DUOX1, significantly increased for both groups, (>2-fold). IL17A (>50-fold) was included as its function can coincide with NOX mediated cell disruption (Figure 3A). Nitric oxide synthesis (NOS) enzymes were monitored as routes for reactive nitrogen species production within neuronal tissue (nNOS or NOS1) and macrophages (iNOS or NOS2). NOS1 showed slight upregulation across time and was significantly reduced at 48-hr for the DFX group. NOS2 transcript synthesis was much more rampant for both groups, especially 72-hr after implantation where fold-changes reached ~800-fold. However, NOS2 levels reduced drastically by 7-days in both groups.

3.1.2. Antioxidants: Several genes responsible for their antioxidant properties that are involved in the reduction of heme, hydrogen peroxide, and superoxide anions were also monitored (Figure 2). HMOX1 transcript levels were consistently high throughout the study in both groups, suggesting significant heme degradation. DFX administration induced significant upregulation in nine of the antioxidants studied at 48-hr post-implant, including FTH1, GPX1, GPX4, MPV17, PRDX1, PRDX2, PRDX3, SOD1 and SOD2. All of the mentioned genes except for FTH1 and MPV17 followed a significant decrease in expression from 48-hr to 72-hr post implant within the DFX group. In fact, the fold-change was significantly downregulated (indicated by the more prominent dotted line) for GPX4, PRDX1, PRDX2, PRDX3, SOD1, and SOD2. The following antioxidant genes expressed similarly across the control group, where they remained at or suppressed below baseline values: GPX4, PRDX1, PRDX2, PRDX3, SOD1, and SOD2. FTH1 modestly upregulated only at 72-hr for the control group but remained upregulated across time with DFX. GPX1 upregulated to ~3-fold by 72-hr and 7-days for the control group. MPV17's regulation stayed below 2-fold; however, at 48-hr, there was significance between the groups and a significant upregulation by 7-days for the control group. For both control and DFX groups, SCARA3 modestly upregulated by 2-fold at 72-hr and 7-days. At 48-hr, DFX groups were at baseline values for SCARA3, which was significantly lower than the control group. SOD3 showed no changes in regulation values with or without DFX treatment.

3.2 Inflammatory response

All the pro-inflammatory markers (CCL3, CXCL1, CXCL2, IL1A, IL1B, IL6, IL17A and TNF) tested in this study were upregulated at the various evaluated time-points (Figure 3A) and had been reported, except for CCL3 and IL17A, for the 6-hr and 24-hr time-points previously [82]. For both the control and DFX-treated groups, these inflammatory markers depicted a sharp initial (48-hr) increase in the gene regulation, likely due to implant-induced trauma. For the control group, these markers generally remained upregulated (CXCL2 (>40-fold), IL1A (>2-fold), and IL1B (>20-fold)) at subsequent time-points tested in this study with no significant differences between time-points. For the DFX-treated group, IL1A levels remained below 2-fold at 48-hr, 72-hr, and 7 days. The IL1B levels remained similar across groups and at all time-points. IL6 levels were at first upregulated (~10-fold) similarly but did

reduce to baseline values at the 7-day period for both groups. TNF values remained similar (~5-fold) at 48-hr and 72-hr for both groups. There was a significant decrease in TNF from 72-hr to 7-day in the control group, where the fold change was <3-fold for both groups. The most upregulated were CCL3, CXCL1, CXCL2, and IL17A. Interestingly, CCL3 and CXCL1 expressed significantly higher at 48-hr for the DFX-group, but their levels decreased significantly at subsequent time-points. By 7-day, these levels were lower for DFX-group.

Toll-like receptors (TLRs) shuttle inflammatory factors across cell membranes, increasing the subsequent native inflammatory response [85, 86]. Both TLR2 and TLR4 were transcribed (~5-fold and ~3-fold, respectively) at different time-points and their level of expression was unaffected by DFX administration (Figure 3B). DUSP1 can act in relation to TLR activity and was found to be suppressed at 72-hr and 7-days (Figure 3B). Caspases (CASP) are generally involved in programmed cell death [87]. CASP1 remained upregulated (>2-fold) for the control group across time however, was significantly higher for the DFX groups at 48-hr (Figure 3C). The DFX group showed similar trends in CASP1, CASP3, and CASP7, where CASP3 and CASP7 quickly returned to similar values as the control group (<2-fold) at subsequent time-points (Figure 3C).

3.3 Neurovascular unit dysfunction:

3.3.1 Paracellular BBB disruption and dysfunction: The genes that regulate the tight junctions (CLDN5, OCLN, ZO1, and ZO2) and the adherens junctions (CDH1 and CDH5), responsible for creating the barrier properties of the BBB [35], were monitored across different time-points and groups (Figure 4A). Baseline values (no fold change) are represented by a thin dotted line, while a second, more prominent dotted line on the graph indicates a value of 0.5-fold, which corresponds to a downregulation of 2-fold indicating significant BBB-disruption. For the control group, CLDN5 and OCLN were transcribed similarly from 48-hr to 7-day. CLDN5 in the DFX-group was significantly downregulated at 48-hr followed by an increase in transcription by 7-day, which indicates a less permeable barrier by 7-day. The tight junctions primarily limit the paracellular transport, among which the claudins (CLDN5) and occludin (OCLN) plays a significant role in limiting the paracellular transport across the BBB [39, 40, 88], so its downregulation following an injury indicates a permeable barrier, whereas an upregulation at subsequent time-points suggests an over-expression of this protein, restoring barrier properties of the BBB. Together with claudins, occludin (OCLN) is an important member of the tight junctions contributing to BBB stability and permeability [42]. For the DFX-treated groups, OCLN followed a similar trend as CLDN5, and its transcription was significantly higher than the control group by 7-day (Figure 4A). The zonula occludens (ZO) family of proteins are implicated to be scaffolding proteins in the tight junction where they play a role in restricting permeability as well as assembly of the tight junctions [39, 88]. ZO1's gene regulation peaked at 48-hr for control animals; however, in DFX-treated animals ZO1's transcription followed a similar trend as CLDN5 and OCLN and became significantly upregulated at 7-day relative to other time-points as well as compared to the control group (Figure 4A). ZO2's gene expression only slightly upregulated in both groups at all time-points except for at 48-hr, where the expression was significantly larger for the control group.

The cadherin family of genes that regulate the adherens junction proteins were also monitored, specifically CDH1 (epithelial or E-cadherin) and CDH5 (vascular endothelial or VE-cadherin). The adherens junctions lie at the basal side of the endothelial cells and are involved in cell-to-cell adhesion and vascular angiogenesis among other functions [35]. CDH1 expression was significantly downregulated at 48-hr for both groups but significantly increased across time only for the DFX group (Figure 4A); there was no difference in expression between the groups at respective time-points. In contrast, CDH5 expression at 48-hr was significantly upregulated in control animals as compared with DFX-treated animals and became significantly upregulated by 7-day for both groups.

Matrix metalloproteinases (MMPs), specifically MMP2 and MMP9, were monitored at all of the above time-points (Figure 4B). Both MMP2 and MMP9 are known for their role in the degradation of extracellular matrices and junction proteins of the BBB following an injury or foreign body response [89–91]. Additionally, MMP2 is known for its delayed role in wound healing and angiogenesis [92, 93]. There was a significant increase in MMP2 levels post-implant for both the controls and DFX-treated groups. During the early time-points of 48-hr and 72-hr, MMP2 levels were similar for both groups. However, at the 7-day time-point, MMP2 levels for the DFX-treated group were significantly greater than the control group. There was an upregulation of MMP9 from 48-hr to 72-hr (~10-fold) period for controls group before decreasing to ~5-fold (not significant) at 7-day. In the DFX-treated group, there was a significant decrease in MMP9 expression. Beyond the 48-hr time-point MMP9 expression remained around 5-fold.

3.3.2 Transcellular BBB disruption and dysfunction: The neurovascular unit consists of endothelial cells, astrocytes, and pericytes, all of which are critical for monitoring both paracellular and transcellular transport across the barrier. Endothelial cells were monitored through tight and adherens junction markers as described above (Figure 4A) and through AP2A1, MFSD2A, and TFRC to monitor transcellular activity (Figure 5). AP2A1 was significantly higher than the control group at 48-hr, but immediately returned to baseline values at subsequent time-points. MFSD2A was significantly suppressed (~0.5-fold) at 48-hr for the control group and significantly upregulated between the two groups at 72-hr for the DFX treated group. TFRC was significantly downregulated in the DFX group as compared to the control group at 72-hr, and its levels were similar for both groups at 7-days.

3.3.3 Disruption of other components of the neurovascular unit: Pericytes, another important component of the neurovascular unit, communicate directly with the brain endothelial cells. RGS5, which is a specific pericyte marker in the brain was used to study the pericytes after electrode implant injury [94]. RGS5 regulation remained at baseline levels at all the time-points (Figure 6). Astrocytic activity, normally determined through GFAP, was monitored through AQP4, LCN2, and SHH gene expression, where AQP4 and SHH can communicate directly with the endothelial cells [95, 96]. Aquaporin 4 (AQP4) remained at baseline values until 72-hr, after which it increased to over 5-fold for both groups (Figure 6). Sonic hedgehog (SHH) increased in expression values solely at 48-hr for both groups, after which it returned to baseline levels at the 72-hr and 7-day time-points (Figure 6). LCN2 was secreted at significantly higher values for the DFX group at 48-hr (>300-fold). LCN2,

however, quickly reduced in value to ~40- and ~30-fold at 72-hr and 7-day, respectively, for the DFX group. For the control group, LCN2 remained over 100-fold at 48-hr and 72-hr and decreased to ~50-fold by 7-days, suggesting extremely reactive astrocytes at all acute time-points (Figure 6).

4. Discussion

Electrode induced injury is unlike any other brain injury models where the initial insertion trauma is followed by a persistent, chronic non-healing wound. This type of injury is complex from an evolutionary standpoint, where an invasive device presents a challenge to the “immune privileged”, or restrictive nature, of the neurovascular unit (Figure 7A) within the central nervous system (CNS). In contrast to the vasculature found in the rest of the body, the brain microvascular endothelial cells that line the vasculature restricts the diffusion of substances and pathogens across the BBB through paracellular and transcellular routes (Figure 7A) [34, 35]. Implantation of intracortical electrodes initiates a foreign body response and leads to the disruption of the BBB, which exposes the brain parenchyma to pro-oxidants, pro-inflammatory factors, and blood-borne molecules. The degradation of the barrier junction proteins at acute time-points between 6-hr to 72-hr post-implant following implantation of UMEAs in the rat cortex was previously reported [82]. In this study, an extension of the analysis up to 7-days post-implant is provided as well as an inclusion of genes involved in the onset of oxidative stress, inflammation, and neurovascular unit dysfunction to provide an in-depth analysis of the acute foreign body response. Further, an iron chelator, deferoxamine mesylate (DFX), was used for its potential to attenuate the acute foreign body response by reducing the availability of free iron that is released into the brain parenchyma due to vasculature disruption and thus iron-mediated oxidative stress. The goal of the current study is (1) to show the pathophysiology of molecular events related to inflammation, oxidative stress, and BBB disruption occurring at the Utah electrode tissue interface and (2) to show the effects of using an iron chelator on acute inflammation, oxidative stress, and BBB dysfunction. In addition to studying the oxidative stress and antioxidant pathways, a detailed discussion of BBB disruption at the level of paracellular and transcellular trafficking (Figure 7A) and the interplay between the inflammation and oxidative stress to cause further BBB dysfunction is included. Figure 7B provides an overview of the various pathways evaluated in this study and how they can contribute towards the disruption of the neurovascular unit.

4.1 Oxidative Stress

4.1.1 Iron-mediated oxidative stress and DFX as an iron chelator: Iron is essential for various physiological functions that include metabolic processes, DNA synthesis, and oxygen transport that are necessary for cell survival [97, 98]. However, iron can also become toxic by forming free radicals and is thus tightly regulated within the tissue [99–101]. Vascular disruption due to electrode insertion leads to the entry of erythrocytes into the brain parenchyma, which degrades to heme and then to free iron [53, 55, 56]. An overload of free iron contributes to oxidative and inflammatory mediated cell damage through multiple routes (Figure 7B) [53, 102–104]. Both Fe^{3+} and Fe^{2+} are sources of hydroxyl radical formation, as shown below in equations (1) and (2). In equation (1), Fe^{3+}

gains an electron from superoxide and is reduced to Fe^{2+} , while superoxide is oxidized to molecular oxygen. In equation (2), also known as the Fenton reaction [56], Fe^{2+} is oxidized by hydrogen peroxide to Fe^{3+} , forming a hydroxide ion and a hydroxyl radical in the process. In order for equations (1) and (2) to occur, superoxide and hydrogen peroxide must be present in the local environment. Finally, equation (3) presents the net ionic equation, also known as the Haber-Weiss reaction [56, 105, 106].

The probable mechanism by which DFX acts as a cytoprotectant is that it chelates excessive free iron from the tissue and subsequently decreases several cellular processes linked to iron-mediated oxidative stress and inflammation [74, 76].

4.1.2 Generation of pro-oxidants: As demonstrated through the Haber-Weiss reactions, the presence of ROS could be exacerbated through the presence of free iron. Under normal physiological conditions, oxidative phosphorylation supplies cells with energy necessary for survival and produces a small percentage of electrons that become disassociated from the electron transport chain to form ROS, such as superoxides ($\bullet\text{O}_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\bullet\text{OH}$), where naturally occurring antioxidants assist in oxidase reduction [107]. However, electrode insertion induces a rampant foreign body response that activates NADPH oxidase (NOX) (Figure 7B) in multiple cell types acting as phagocytes, especially microglia, to produce reactive oxygen and nitrogen species [108–115], which can be referred to as a respiratory burst [116]. In fact, Potter *et al* [117] and Ereifej *et al* [118] demonstrated the presence of superoxide anions and the oxidation of nucleic acids, lipids, and proteins, respectively, surrounding electrode sites, confirming local oxidative stress at electrode sites.

Multiple homologues of the NADPH oxidase complex exist: NOX1–5 and DUOX1/2 [110, 119]. In this study, genes that regulate NOX1, NOX2, NOX4, and DUOX1 remained upregulated 7-day after implantation (Figure 1). Once activated through NOXA1, NOX1 produces ROS and indirectly activates the NF- κ B-mediated innate inflammatory response [120]. NOX2 formation, represented by cytochrome b beta's (CYBB) upregulation [121], suggests its activation within the cells (Figure 1). Additionally, previous reports indicate the upregulation of neutrophil cytosol factor 1 (NCF1) increases phagocytic NOX2's production of ROS [122]. Both CYBB and NCF1 simultaneously upregulated (Figure 1), illustrating the

ongoing NADPH oxidase activation and superoxide production [109, 119]. In the DFX-administered groups, a reduction in the upregulation of CYBB and NCF1 was seen at 72-hr, potentially decreasing the activation or assembly of NOX2 at this earlier time-point [109, 111, 119]. However, no significant differences in CYBB and NCF1 gene regulation at 7-day suggests DFX administration did not result in an overall reduction of NOX2 activity at that time-point. NOX4 can create an extremely cytotoxic environment due to its production of ROS to help phagocytose invading substances [111, 112, 119]. In the DFX group, NOX4 values were initially significantly higher at 48-hr (Figure 1), however these values reduced significantly at subsequent time-points. Dual oxidase 1 (DUOX1) is not only induced following NADPH oxidase production of ROS [123] but also itself produces ROS, specifically hydrogen peroxide [112, 114] and prolongs oxidative damage [114]. Macrophages also activate the synthesis of reactive nitrogen species, such as nitric oxide (NO), through neuronal nitric-oxide synthase (nNOS or NOS1) [113, 124–126] and inducible nitric-oxide synthase (iNOS or NOS2) [113, 124]. In the presence of ROS such as superoxides, nitric oxide can then form an additional potent pro-oxidant, peroxynitrite (ONOO⁻) [127]. Together, the NADPH oxidase complex homologues' and NOS2's significant upregulations in this study imply the widespread production of ROS and RNS in response to electrode insertion. While DFX administration did modulate pathways that would lead to a decrease in the production of ROS and RNS, its administration did not substantially affect all of the transcripts at multiple time-points, suggesting the complexity of ROS production and subsequently, iron-mediated oxidative stress mechanisms.

4.1.3 Antioxidant activity: Common antioxidants were also studied to determine if the cells responded to the increased production of pro-oxidant species. In this study, from 48-hr to 7-days after implantation, heme oxygenase 1 (HMOX1)'s transcript levels were consistently high for both groups (Figure 2). HMOX1, most known for its ability to cleave heme, has been shown to be present at the site of endothelial cells [128], microglia [129], neurons, and astrocytes [57, 130, 131] to protect cells from initial oxidative stressors [132, 133], and hyperthermia [131, 134]. Its chronic expression, however, could produce excess free iron from degraded heme available for chronic iron-mediated oxidative stress [57, 130]. The storage of free iron especially becomes cytoprotectant when increased levels of heme degradation from vascular disruption are present. Ferritin (FTH1) is a protein whose function is to oxidize and store free iron as a protectant against oxidative stress [103, 128, 135] and can be influenced by cellular oxygen and inflammatory secreted cytokine levels [136]. FTH1 significantly upregulated with DFX treatment at the 48-hr (Figure 2) as compared to the un-treated (control) group, suggesting DFX could have influenced intracellular storage of free iron rather than its complete sequester and removal. In relation to ferritin, AOX1's gene expression, which increased relative to time (Figure 1), has been documented to activate the release of iron from ferritin and act as an electron donor, similar to NADPH oxidase substrates, producing delayed free radicals [137–139]. This delayed release of iron could potentiate iron-mediated oxidative stress. Superoxide dismutases (SODs), glutathione peroxidases (GPX1, GPX4) and peroxiredoxins (PRDX1, PRDX2, PRDX3) role in the cell are mainly in relation to their degradation of reactive species (Figure 2) [107, 127, 140]. Superoxide dismutases are classified in relation to their location: cytoplasmic (SOD1), mitochondrial (SOD2), and extracellular (SOD3) [127], and they help

breakdown superoxide into hydrogen peroxide, which can be further decomposed to molecular oxygen and water. Simultaneously, several antioxidant genes (GPX1, GPX4, PRDX1–3, SOD1, SOD2) followed the same trend, where they were upregulated in DFX groups at the 48-hr and quickly significantly downregulated (Figure 2), suggesting the possible effect of DFX in inducing antioxidant activity right after implant-induced injury.

A less documented oxidative stress response gene studied was mitochondrial inner membrane protein (MPV17). MPV17 is present within the inner membrane of mitochondria, where it has been documented to oversee mitochondrial oxidative phosphorylation, mitochondrial DNA functionality, ROS production, and cell survival [141–143]. MPV17's colocalization with neurons has also been shown to decrease likelihood of neuronal apoptosis [141]. MPV17, like other antioxidants, was significantly upregulated for the DFX-treated group at 48-hr, however the levels were close to baseline (Figure 2). In the peripheral nervous system tissue, SCARA3 becomes upregulated upon oxidative stressors to scavenge ROS, acting as a potent antioxidant [144, 145]. SCARA3 is also expressed in brain microvascular endothelial cells [146], where its modest upregulation could be compensating for ROS-mediated disruption to endothelial cells (Figure 2).

The presence of an imbalance in the levels of naturally occurring pro-oxidants and antioxidants creates a cellular environment prone to oxidative stress, where ROS can disrupt and oxidize cells via lipid peroxidation and DNA fragmentation [107], leading to the degradation of cells surrounding the electrode-tissue interface. Therefore, an overexpression of pro-oxidants (Figure 1) as compared to the lack of upregulation and even suppression of key antioxidants (Figure 2) at later time-points in this study suggests the presence of oxidative stress. DFX initially had varying effects on pro-oxidant and antioxidant genes in this study. Although DFX most likely sequestered excess available free iron and did upregulate the antioxidant activity at the earliest time-point, it was not able to decrease the overall activation of ROS producing species by 7-day. This may have been due to an abundance of reactive oxygen and nitrogen species at later time-points as well as unavailability of DFX due to its short half-life (~6-hours) [76], which may warrant a larger, more frequent administration of the drug.

4.2 Inflammatory response

Significant evidence demonstrates the profound activation and recruitment of immune cells to the injury site following intracortical electrode at acute [147] and chronic time-points [117, 148]. While they contribute towards the production of pro-oxidant species to phagocytose foreign objects, immune cells also produce various chemokines and cytokines (Figure 3) following their activation. An initial increase in expression of cytokines and chemokines from 6 to 24-hr following microelectrode insertion displayed their activation due to the localized immune response [82]. Initially, CCL3 and CXCL1 had higher gene regulation values for the DFX group and potentially affected the cells by inducing cytokine synthesis and attracting more neutrophils to the injury site [149] as compared to the control-group. By 7-day, DFX reduced CXCL2, IL1A, and IL1B transcripts; however, when chemokine and cytokine transcripts were upregulated, there was large inter-animal variability in the expression values, providing no significant differences between the groups

(Figure 3A). The vast chemokine and cytokine signaling cascade certainly extended to 7-day after implantation but at lower transcript levels (Figure 3A) than previously reported before 72-hr post-implantation [82]. This continuation of microglial and astroglial response to insertion is expected, as microglial and astroglial localization to implant site persists several weeks to months following probe insertion [15, 24, 150].

Along with monitoring cytokine and chemokine secretion, toll-like receptors (TLRs) play a key role in the innate inflammatory response and heavily contribute towards cytotoxicity and BBB dysfunction, colocalizing with both microglia and astrocytes [85, 86, 151–156]. In relation to TLRs, DUSP1 serves as an enzyme that provides negative feedback to TLR activity, which could in turn lessen inflammation [157]. In this study, TLR2 and TLR4 both remained upregulated following injury, while DUSP1's regulation remained suppressed, suggesting the ongoing traffic of inflammatory factors post-implantation through TLRs (Figure 3B). Of the two TLRs studied, TLR4 was recently shown to affect intracortical electrode functionality, decreasing its performance [158]. Both inflammatory secretion and TLR activation imply a continuation of inflammatory signaling at low but persistently upregulated values by 7-day, which may result in chronic inflammation deteriorative to the local brain parenchyma. Additionally, TLRs help initiate and perhaps prolong the NADPH oxidase complex activity [116] as seen in Figure 1. In fact, a summarization of NOX1, NOX2, NOX4, and DUOX1's ability to interplay with TLRs describes the ROS from NOX complexes mitigates TLR inflammatory pathways and vice versa [120]. This chronically activated microglial state along with NOX expression, especially NOX2, could deteriorate surrounding glial and neuronal cells [115]. The overall array of pro-inflammatory and pro-oxidant genes upregulated in this study (Figure 3) represents a cytotoxic environment unfit for cell survival.

Following high cellular stress, such as chemokine and cytokine secretion and TLR activation, the programmed cell death signaling of caspases (CASPs) can occur. CASP1 acts as a pro-inflammatory mediator, where its presence is necessary for the maturation of IL1B [159], while CASP3 and CASP7 contribute to the activation of microglia [87]. Caspases, specifically CASP3 has been previously reported to decrease following DFX treatment and enhance differentiation of microglia to the anti-inflammatory M2 phenotype [160]. However, in this study, all three CASPs were significantly upregulated at 48-hr in the DFX-treated group; DFX did, however, quickly return to near baseline values for CASP3 and CASP7 by 72-hr (Figure 3C). CASP1 values were consistent between the groups for the remainder of the study, which could potentially allow the maturation of interleukins (Figure 3A) present at those times and affect the recording quality of functional electrodes as demonstrated in a CASP1 knockout model [161]. While in general, DFX did not affect the inflammatory pathways suggested by similar expression between controls and DFX-treated groups, it is unclear why DFX caused an increased expression of caspases at 48-hr, which needs further evaluation.

4.3 Neurovascular unit dysfunction

Studying the expression of tight and adherens junctions provides insight into the paracellular activity of endothelial cells. However, disruption of the BBB can occur at both paracellular

and transcellular levels (Figure 7) as seen in various injury models such as traumatic brain injury and ischemic stroke where the BBB opens through both paracellular and transcellular-mediated pathways [162, 163]. BBB disruption has been reported for various electrode types through the expression of IgG and *in vivo* two-photon microscopy [31, 117, 164]. IgG expression provides visualization of increased permeability around electrode sites, while two-photon microscopy can decrease the likelihood of neurovasculature disruption during electrode implantation [165], potentially reducing neuronal and glial disruption and increasing electrode performance longevity. Both methods have provided enormous insight into the disruption that does occur to the BBB following electrode implantation.

The unique vasculature of the CNS, known as the BBB, limits peripheral blood-borne pathogens and components from internalizing and thus interacting with the brain tissue through its paracellular barriers (tight and adherens junctions) [34, 35, 42, 166]. Additional intercellular proteins of endothelial cells, such as junction adhesion molecules (JAMs) and platelet/endothelial cell adhesion molecules (PECAMs), actively suppress transendothelial migration of monocytes and neutrophils [167, 168], where a loss of these proteins increases paracellular permeability. The vasculature of the CNS and its transcellular activity is also unique as compared to peripheral nervous system blood vessels [169], where communication between endothelial cells and both astrocytes and pericytes actively maintains BBB integrity [35, 170], thus mitigating transcellular-mediated communication. Here, further insight into the acute molecular mechanisms of BBB disruption is provided at a paracellular and transcellular level following UMEA implantation (Figure 7A).

4.3.1 Paracellular disruption of brain endothelial cells: The interplay of chemokine and cytokines and the tight and adherens junctions of the BBB for up to 72-hr after implant injury have previously been reported [82]. Overall, an initial downregulation of major tight and adherens junctions of the BBB (Figure 4A) coincided with a sharp increase in inflammation (Figure 3A), where chemokine and cytokines such as TNF and IL1B potentially suppress tight and adherens junction expression [171], creating a permeabilizing effect on brain endothelial cells at a paracellular level [172–174]. A BBB breach results in the infiltration of neurotoxic factors and proinflammatory myeloid cells [175, 176] and reactive oxygen and nitrogen species leading to oxidative stress in the local tissue around the electrode sites [30]. An upregulation of NADPH oxidase complexes (Figure 1) could result in the presence of various ROS that can affect BBB permeability and integrity directly by altering the expression of tight junction [177–180] and adherens junction proteins [181–183]. NOX complexes can also exacerbate IL17A-mediated ROS production and BBB disruption through increased paracellular and transcellular BBB disruption [184]. Nitric oxide production, represented by profound NOS2 upregulation (Figure 1), also has permeabilizing effects on the BBB [185]. While DFX did not directly decrease the pro-oxidant species available at 7-day, its treatment could have still helped alleviate ROS-mediated BBB damage [183] as seen through increased expression of genes regulating the tight junctions, OCLN and ZO1 (Figure 4A). Adherens and tight junction downregulation indicates a degraded, or more permeable, BBB, whereas an upregulation of transcripts that regulate junction proteins indicates repair of tight junctions and a restoration of paracellular barriers of brain endothelial cells.

ROS is directly capable of acting on brain endothelial cells, causing oxidative damage, tight junction modification, and matrix metalloproteinase (MMP) activation, all of which can alter cell permeability and result in BBB disruption and dysfunction [50, 140, 186–193]. MMPs secreted from microglia and astrocytes can interact with endothelial cells by cleaving tight junctions, such as CLDN5, OCLN, and ZO1, and degrading extracellular matrix compartments [194–202], affecting paracellular integrity. The transcription of MMPs could have been influenced by the production of ROS (Figure 1), where ROS leads to an increase in the activity of MMP2 and MMP9 [180, 186–190]. An increase in MMPs occurred concurrently with decreased tight junction expression, which could have further led to BBB disruption (Figure 4). However, by 7-day MMP2 levels (Figure 4B) coincide with a significant increase in OCLN and ZO1 (Figure 4A) in the DFX group, perhaps suggesting MMP2's role in wound healing and paracellular homeostasis [203]. DFX administration influenced MMP regulation, decreasing the MMP9 transcripts seen 48-hr after implant (Figure 4B), potentially decreasing MMP's availability to degrade both tight junction and extracellular matrices. MMP2's time dependent regulation peaked at 7-days, where DFX treatment significantly increased the expression of MMP2 (Figure 4B). This may be attributed to the role of DFX in supporting wound healing through increased expression of MMP-2 at 7-day compared to controls [92, 93, 203], by mechanisms still unknown. Despite a highly acute reactive environment, delayed significant upregulation of junction proteins and MMP2 (Figure 4) indicates decreased permeability across the BBB, suggesting DFX's potential to restore paracellular barriers of the BBB (Figure 4A).

4.3.2 Transcellular disruption of brain endothelial cells: To provide further molecular insight into the pathology of BBB transcellular disruption, the mRNA expression of AP2A1, TFRC, and MFSD2A was included. Endothelial cells of the CNS generally have lower rates of cellular uptake than the peripheral endothelial cell types such as cardiac and skeletal muscles [169]. Therefore, changes to transcellular uptake could drastically alter brain homeostasis. Brain endothelial cells have three major routes for transcellular endocytosis, where the two most robust are (1) clathrin-coated and (2) caveolae-mediated [204]. Transferrin receptor (TfR), assessed via TFRC mRNA expression, initiates receptor-mediated transcytosis through clathrin-coated pits and maintains iron homeostasis, where cells can acquire iron after transferrin binds to the TfR, leading to its uptake and thus dispersal [205, 206]. TFRC being significantly suppressed by 7-day (Figure 5) implies iron is present at high concentrations following electrode implantation, since the receptor's expression has been shown to correlate proportionally with the cell's need of iron or lack thereof [207–209]. Majority of receptors such as TfR utilize clathrin-coated vesicles for receptor-mediated transport, where clathrin-coated pits use a specific transporter called adaptor protein complex-2 (AP2) [204]. The alpha subunit of the AP2 complex, AP2A1, guides clathrin-coated vesicles to the cell membrane, potentially altering the rate of clathrin-mediated transport and the proteins assembly [210, 211]. A modest downward trend of AP2A1's regulation (Figure 5) suggests AP2's regulation could have initially altered transcytosis following electrode insertion but is not the causation of transcellular-mediated disruption. To include brain endothelial cell's second main route of transport, caveolae-mediated transport was assessed indirectly through major facilitator super family domain containing 2a's (MFSD2A) gene regulation (Figure 5). MFSD2A is selectively expressed in

the brain microvascular endothelial cells and functions to suppress vesicular transport (transcytosis) into brain endothelial cells [212]. Specifically, MFSD2A is shown to suppress transcytosis by creating a lipid environment that inhibits caveolae vesicle formation [213]. Initially, the suppression of MFSD2A's gene expression in the electrode implanted group could represent negative feedback, where caveolae-mediated transport is allowed, and thus higher rates of transcellular trafficking could be present (Figure 5). With DFX administration, MFSD2A significantly upregulated with at 72-hr (Figure 5), suggesting the suppression of caveolae-mediated transcytosis across endothelial cells, which could have been a possible route for endocytosis of DFX itself since the mechanism by which DFX transports into the CNS and across the BBB is still unclear. The suppression of caveolae-mediated transcytosis coupled with reduced paracellular trafficking (upregulation of tight junctions, Figure 4A) in DFX-treated animals suggests that DFX, through mechanism yet unclear, was able to restore the properties of the BBB. Out of the three transcellular markers of brain endothelial cells, it seems acute disruption of the BBB from electrode insertion occurs primarily at the paracellular level versus transcellularly. Transcellular BBB dysregulation needs to be further elucidated through additional mechanistic routes of transcellular transport.

4.3.3 Pericyte and astrocyte disruption following the foreign body

response: The neurovascular unit (Figure 7A) consists of neurons, endothelial cells, pericytes, and astrocytes and provides the brain parenchyma with a homeostatic environment [170, 195]. The complete role of pericytes in the CNS is still not fully understood. Pericytes secrete several angiogenic factors and pro-regenerative molecules [214, 215] and maintain the BBB. They are extremely sensitive to inflammatory factors such as MMP9, TNF, IL1B, and IL6 [216, 217] and assist in phagocytosis, cytokine secretion, transmigration of neutrophils, and blood vessel remodeling [216, 218–220]. Higher levels of MMP9 relate to lesser adhesion of neutrophils to pericytes, making neutrophil infiltration simpler [216]. They even propagate differentiation into microglial phenotypes following hypoxia [218]. Therefore, a pericyte specific gene involved in these processes, the regulator of G-protein signaling 5 (RGS5), was monitored [218, 219, 221]. In this study, RGS5 showed no changes in expression (Figure 6), suggesting its role in parenchymal homeostasis following the foreign body response is not significant up to 7-days post-implant, which is interesting since changes in RGS5 values have been characterized following blood vessel insult [219, 222]. Pericytes can also be identified through PDGFRb, where another study saw an insignificant regulation of PDGFRb at both 7-day and 14-day after intracortical electrode implantation [223]. Pericytes also mitigate MFSD2A-endothelial expression, where a lack of pericytes decreased MFSD2A, resulting in higher rates of transcytosis [212]. As previously discussed, there was decreased expression at 48-hr of MFSD2A without DFX administration, which could be the result of either endothelial or pericyte reactivity and increase transcytosis, whereas comparatively, an increase in MFSD2A expression could also be the result of either endothelial or pericyte dependence and decrease transcytosis.

Astrocytes vary in phenotype and function in the CNS but play a major role maintaining the BBB by communicating through its astrocytic endfeet. Astrocytes can signal through various pro-inflammatory factors such as IL1B, LCN2, TNF, and TLRs [224–226] as well as

astrocyte specific markers such as AQP4 and SHH [95, 96, 224]. Aquaporin-4 (AQP4) is one of the main water channels in the CNS for transporting water through the cell membrane [95] and thus responsible for the causation or resolution of cellular edema. A BBB breakdown results in paracellular transport of Na^+ , Cl^- , and water across the permeable cell membrane, which results in brain edema and opening of more water channels to cause further inflammation, edema, and BBB breakdown [227, 228]. AQP4's upregulation did not occur until 7-day, suggesting delayed swelling, or edema, and BBB dysfunction. AQP4 has been previously reported to decrease in expression following DFX administration 3 and 14-days following ischemia [228]. However, no significant differences between the control and DFX groups (Figure 6) were found. Lipocalin-2 (LCN2) was monitored to determine the degree of astrocytes' reactivity, as LCN2 is induced during reactive astrocytic activity, involved in pro-inflammatory cytokine and chemokine induction [225], and acts as a powerful neurotoxic element [229]. LCN2 was significantly upregulated at 48-hr and at subsequent time-points (Figure 6). Zamanian et al [225] delves into how reactive astrocytes can promote both positive and negative effects on the environment due to an astrocyte's heterogenic response, which is unique to its injury type. Astroglial scarring presents a challenge to the functional recording of neurons; therefore, documenting at what times and how significantly astrocytes are active remains important yet hardly documented for its distinct phenotype. Sonic hedgehog (SHH), which is expressed along astrocyte-endothelial cell junctions [96], slightly upregulated at 48-hr (Figure 6). SHH's role within the gliovascular unit has been shown to support tight junction maintenance within endothelial cells as well as protect the CNS by decreasing leukocyte migration across endothelial cells and therefore, levels of pro-inflammatory markers [96, 170, 224]. SHH only initially upregulated for both groups (Figure 6), suggesting astrocyte-mediated repair of tight junctions through SHH upregulation resolves before 7-day and that tight junction dysregulation primarily occurs acutely following electrode-induced trauma.

In general, mixed regulation values were observed in several key genes mediating oxidative stress and inflammation in the DFX-treated group compared to the untreated group. This could be due to the different cell types that secrete these pro-oxidant or pro-inflammatory factors. For instance, at 48-hr LCN2 and NOX4 was significantly upregulated in DFX-treated groups (Figures 6 and 1, respectively). Reactive astrocytes secrete LCN2 and also have the ability to mitigate an inflammatory response [225, 229]. Even NADPH oxidase complexes are expressed in multiple cell types such as neurons, glial cells (astrocytes and microglia), endothelial cells, and various phagocytes [111, 230, 231]. By 7-day, DFX and control groups shared similar pro-oxidant and pro-inflammatory gene regulation values, where the tissue could experience similar cellular stressors, resulting in inflammation and oxidative stress in chronically implanted electrodes.

5. Conclusions

This study provides further mechanistic insights into oxidative stress, inflammation, and BBB disruption following microelectrode insertion. The use of gene expression provides a detailed analysis that allows the vast pathophysiological events that occurs following microelectrode insertion to be simultaneously monitored. The results demonstrate the extent of BBB disruption and degradation of junction proteins that are indicative of increased

barrier permeability and BBB dysfunction. The fact that this occurs even at acute time-scales warrants the need for addressing the acute and chronic damage, BBB disruption, oxidative stress, and neuroinflammation that occurs as a result of electrode implant-induced injury. The data also suggests the efficacy of deferoxamine mesylate (DFX) in stabilizing the disrupted BBB. By 7-days, DFX was able to significantly increase the expression of junction proteins that indicates decreased paracellular permeability across the BBB, suggesting DFX treated animals had a more stable BBB. Since DFX does not drastically attenuate the initial cellular response, its ability to reduce chronic inflammation and oxidative damage needs further and extended evaluation. DFX upregulated the expression of an iron storage protein, ferritin, as well as several antioxidants compared to control animals. The effect of DFX in reducing the oxidative stress via increasing the antioxidant expression and suppression of pro-oxidant activity at the acute time-points tested in this study suggests its role in the cellular response to oxidative stress. Overall, the foreign body response to UMEA promotes an environment with an overexpression of oxidants at acute time-points as compared to antioxidants, suggesting the presence and onset of oxidative stress. DFX therapy in the chronic period may be beneficial to minimize iron-mediated oxidative pathways and BBB dysfunction because of the presence of reactive oxygen species at chronically implanted electrode sites. As a result, the cells in the immediate vicinity of the electrode array could be subject to chronic lipid peroxidation and therefore, cellular degradation due to chronic oxidative stress.

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Figure 1: mRNA regulation for **pro-oxidant** transcripts that are involved in the generation of reactive oxygen and nitrogen species for control and DFX-treated animals. Fold change values shown are relative to unoperated control animals at 48-hr, 72-hr, and 7-day for UMEA implanted animals. Mean $\pm$ SD is shown for each group (n=5 rats/group). (* p<0.0332; ** p<0.0021; *** p<0.0002; **** p<0.0001)

Figure 2: mRNA regulation for **antioxidant** transcripts involved in heme degradation and iron storage for control and DFX-treated animals at 48-hr, 72-hr, and 7-day for UMEA implanted animals. Mean±SD is shown for each group (n=5 rats/group). (*p<0.0332; ** p<0.0021; *** p<0.0002; **** p<0.0001)

Figure 3: mRNA regulation of (A) inflammatory cytokines and chemokines, (B) other pro-inflammatory transcripts such as the dual specificity phosphatases (DUSP) and the toll-like receptors (TLRs) and (C) those from the caspase family that regulate cell fate, for control and DFX-treated animals. Fold change values shown are relative to unoperated control animals at 48-hr, 72-hr, and 7-day for UMEA implanted animals. Mean $\pm$ SD is shown for each group (n=5 rats/group). (* $p<0.0332$; ** $p<0.0021$; *** $p<0.0002$; **** $p<0.0001$)

A.

B.

Figure 4:

mRNA expression of the genes regulating paracellular activity through (A) the tight junction proteins CLDN5, OCLN, ZO1, and ZO2 and the adherens junction proteins CDH1 and CDH5 and (B) matrix metalloproteinases for control and DFX-treated animals. Fold change values shown are relative to unoperated control animals at 48-hr, 72-hr, and 7-day for UMEA implanted animals. Mean±SD is shown for each group (n=5 rats/group). (*p<0.0332; ** p<0.0021, *** p<0.0002; **** p<0.0001)

Figure 5: mRNA expression of the genes regulating transcellular activity of endothelial cells of the neurovascular unit (NVU). Fold change values shown are relative to unoperated control animals at 48-hr, 72-hr, and 7-day for UMEA implanted animals. Mean±SD is shown for each group (n=5 rats/group). (*p<0.0332; ** p<0.0021, *** p<0.0002; **** p<0.0001)

Figure 6: mRNA expression of the genes regulating astrocytes and pericytes of the neurovascular unit (NVU). Fold change values shown are relative to unoperated control animals at 48-hr, 72-hr, and 7-day for UMEA implanted animals. Mean±SD is shown for each group (n=5 rats/group). (*p<0.0332; ** p<0.0021, *** p<0.0002; **** p<0.0001)

Figure 7:

(A) The neurovascular unit consists of the neurons, endothelial cells, astrocytes, and pericytes. Highlighted in the endothelial cells are two routes of transport across the BBB. The tight and adherens junctions (cadherin (CDH1 and CDH5), claudin (CLDN5), occludin (OCLN), and zonula occludens (ZO1 and ZO2)) are located intracellularly, suppressing excess paracellular transport. Two routes of transcytosis are highlighted through clathrin-coated and caveolae-mediated vesicular transport, where we monitored AP2 (AP2A1), MFSD2A, and TFRC. Astrocytes and pericytes are included to visualize aquaporin 4 (AQP4), sonic hedgehog (SHH), and regulator of G-protein signaling 5 (RGS5) expression for endothelial cell interactions. (B) An overview of the various pathways (oxidative stress: green; inflammation: red) evaluated in this study and how they affect multiple targets (yellow) to cause widespread disruption of the neurovascular unit.

Table 1:

Summary of experimental animal groups

Animal Groups	Gene Expression			DFX-Safety
	48-hr	72-hr	7-days	7-days
DFX-treated	5	5	5	5
Controls (no treatment)	5	5	5	5
Unoperated controls	10			-
Total				40

Table 2:

Summary of gene transcripts used to target BBB, inflammation, and oxidative stress

Target	Transcripts Used
Oxidative Stress	AOX1, CYBB, DUOX1, FTH1, GPX1, GPX4, HMOX1, MPV17, NCF1, NOS1, NOS2, NOX4, NOXA1, PRDX1, PRDX2, PRDX3, SCARA3, SOD1, SOD2, SOD3
Inflammation	CASP1, CASP3, CASP7, CCL3, CXCL1, CXCL2, DUSP1, IL1A, IL1B, IL6, IL17A, TLR2, TLR4, TNF
Blood-brain barrier	AP2A1, AQP4, CDH1, CDH5, CLDN5, LCN2, MMP2, MMP9, OCLN, MFSD2A, RGS5, SHH, TFRC, ZO1, ZO2

Abbreviations: AP2A1 (adaptor related protein complex 2 alpha 1 subunit), AOX (aldehyde oxidase), AQP (aquaporin), CDH (cadherin), CASP (caspase), CLDN (claudin), CCL and CXCL (chemokine ligand), CYBB (cytochrome b beta chain), DUOX (dual oxidase), DUSP (dual specificity oxidase), FTH (ferritin heavy chain), GPX (glutathione peroxidase), HMOX (heme-oxygenase), IL (interleukin), LNC2 (lipocalin-2), MFSD2A (major facilitator superfamily domain containing 2A), MPV (mitochondrial inner membrane protein), MMP (matrix metalloproteinases), NCF (neutrophil cytosolic factor), NOS (nitric oxide synthase), NOX (NADPH oxidase), PRDX (peroxiredoxin), SCARA3 (scavenger receptor class A member 3), SHH (sonic hedgehog), SOD (superoxide dismutase), TFRC (transferrin receptor complex) TLR (toll-like receptor), TNF (tumor necrosis factor), ZO (zonula occludens)

Table 3.

Blood chemistry to assess safety of DFX-administration

Blood Chemistry	Control (untreated)	DFX-treated	Normal Range
Blood Urea Nitrogen (BUN)	24 ± 12.708	20.2 ± 4.919	11–23mg/dL
Creatinine	0.32 ± 0.130	0.28 ± 0.045	0.3–0.6mg/dL
BUN/Creatinine Ratio	65.54 ± 16.420	74.66 ± 25.446	35–38
Calcium	10.08 ± 0.228	9.78 ± 0.657	5.7–12.4mg/dL
Phosphorus	11.32 ± 0.798	10.42 ± 1.121	6.5–12.2mg/dL
Sodium	141.4 ± 2.408	140.6 ± 2.608	-
Chloride	107.2 ± 10.545	102 ± 1.225	-
CO ₂	23.2 ± 1.924	24 ± 3.742	-
Glucose	150 ± 12	130.2 ± 11.278	50–135 mg/dL
Total Protein	5.98 ± 0.683	5.96 ± 0.404	6.3–7.4g/dL
Albumin	3.46 ± 0.358	3.26 ± 0.230	2.8–5.3g/dL
A/G Ratio	1.378 ± 0.082	1.212 ± 0.087	1.39–2.25
AST	153.8 ± 29.828	149.4 ± 52.161	63–175U/L
ALT	51.8 ± 13.293	52 ± 8.062	36–80U/L
Total Bilirubin	1.34 ± 0.695	1.16 ± 0.598	0.2–0.7mg/dL