

Tissue clearing and quantitative confocal microscopy reveal significant impairment of cerebrovascular structure and function following moderate prenatal alcohol exposure

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Abstract

Fetal alcohol spectrum disorders (FASD) occur after prenatal alcohol exposure (PAE) and include a wide range of neurocognitive deficits potentially resulting from abnormal cerebrovascular development and the expression of factors regulating vessel formation and function. We previously determined that upregulation of miR-150-5p within brain microvascular endothelial cells (BMVECs) during PAE affects pathways required for angiogenesis, vascular development, and blood-brain barrier (BBB) integrity. Using a moderate PAE mouse model, we examined the structure and integrity of the cortical microvasculature at embryonic day 18 (E18). We used CLARITY, a tissue clearing and imaging technique allowing for high-quality quantitative analysis of the microvasculature. We also used fluorescent tracers to examine BBB integrity *in vivo*. In addition, we assessed microvascular structure and gene expression in embryonic cortices following miR-150-5p inhibition. We found that E18 PAE cortices displayed significant differences in microvascular structure compared to control cortices. We also found that E18 PAE cortices were significantly more permeable to dextran tracers compared to controls. miR-150-5p inhibition rescued specific PAE-induced microvascular defects. MiR-150-5p inhibition also resulted in elevated expression of genes involved in angiogenesis, BBB maintenance, and vascular development. Overall, we show that moderate PAE adversely affects cerebrovascular structure and BBB function in the developing brain.

Background

The most significant and disabling symptoms affecting children diagnosed with fetal alcohol spectrum disorders (FASD) are related to neurocognitive deficits that arise as a result of structural and functional abnormalities in the brain [1]; these features primarily include learning and intellectual disability [2–5], behavioral issues [6, 7], and mental health struggles [8]. Other research has described the contributions of vascular dysfunction to neurocognitive impairment [9], and brain vasculature has been shown to be impacted by prenatal alcohol exposure (PAE) [10, 11]. Thus, the neurocognitive deficits observed in FASD may result, in part, from abnormal vascular development in the brain and through altered pathways that regulate vessel formation and function.

We previously demonstrated that in a mouse model of moderate PAE, brain microvascular endothelial cells (BMVECs) had greater levels of miR-150-5p [12], which downregulates targets involved in angiogenesis and blood-brain barrier (BBB) function. miRNAs are regulatory non-coding RNA molecules that suppress gene expression by inducing the degradation of mRNA targets [13]. MicroRNAs are abundantly expressed in the brain and during development as they fine-tune gene expression in a developmental time-specific manner [14]. MiR-150-5p has been implicated as an anti-angiogenic factor resulting in decreased vascular density in animal models of stroke [15, 16]. Our previous research demonstrated the effects of miR-150-5p overexpression and inhibition on downstream targets and the angiogenic behavior of BMVECs [12], but work demonstrating the *in vivo* effects of miR-150-5p expression levels on the cerebrovasculature during PAE have not yet been reported.

Previous studies on postmortem human fetuses that were diagnosed with fetal alcohol syndrome (FAS), which is the most severe form of FASD, or partial FAS (pFAS) observed an abnormal vascular structure within the cerebral cortex at gestational week (GW) 30 to 38 [10]. In particular, the radial organization of blood vessels within the cortical layers was altered in the FAS/pFAS group compared to the control group [10]. Animal models of PAE have also demonstrated an abnormal vascular structure in the brain with abnormal radial orientation of vessels and decreased vessel density [10, 11]. In addition to structural effects, recent evidence suggests PAE-induced impairment of blood-brain barrier (BBB) integrity [17]. Tight-junction scaffold proteins that maintain BBB integrity are expressed more sporadically in the cerebral blood vessels of PAE mice compared to control animals [18], and PAE rats are more prone to brain micro-hemorrhage than control animals [19].

Here, we used CLARITY, a tissue clearing and 3D imaging method, to quantify structural parameters of the microvasculature in PAE cortices at embryonic day 18 (E18) compared to cortices from saccharin-exposed (SAC) control animals. We also examined the effect of miR-150-5p inhibition on the structure of the microvasculature during PAE. Additionally, using RNA-seq, we assessed the impact of miR-150-5p inhibition on gene expression profiles in the PAE mouse cortex, and using Ingenuity Pathway Analysis, characterized biological pathways associated with miR-150-5p inhibition. Finally, using fluorescent dextran tracers, we examined the effect of PAE on cortical microvessel permeability *in vivo*.

Methods

Moderate PAE Mouse Model

We utilized a previously established moderate PAE paradigm [20]. Adult C57BL/6J mice were purchased from The Jackson Laboratory. They were allowed to acclimate for two weeks before females were singly housed and randomly assigned to groups receiving either a 10% (w/v) ethanol (EtOH) in 0.066% (w/v) saccharin (SAC) solution or a 0.066% (w/v) SAC only solution as a control, for 4 hours daily, before breeding and throughout gestation. Alcohol consumption during gestation averaged 6.02 ± 0.46 g ethanol/kg body weight. Dams were sacrificed using isoflurane administered in a precision vaporizer followed by decapitation. Embryos were then harvested and sacrificed by decapitation. The embryonic brains were isolated for downstream experiments. All methods were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and approved by University of New Mexico Institutional Animal Care and Use Committee, approval number 22-201231-HSC.

Embryonic miR-150-5p Inhibitor Injections

At E12, pregnant mice were deeply anesthetized. Under sterile conditions, a laparotomy was performed to expose the embryos. Using a Hamilton syringe, intracerebroventricular (ICV) injections were performed using 0.1 μ L (20 pmol/ μ L) of miRCURY LNA™ miR-150-5p Power Inhibitors or controls

(Qiagen) into the embryonic brain ventricles. Embryos were placed back into the abdomen and the laparotomy was closed. Bupivacaine was administered at the site of incision. At E18, the dams were sacrificed and the embryonic brains were isolated, as above, for gene expression analysis and microvascular structural assessment (Supplementary Fig. 1a).

Microvascular Structural Assessment

Cortical tissue was rendered optically transparent to improve quantitative analysis using a technique called CLARITY. The process involves removing the lipid content of a tissue leaving all of the original protein and nucleic acids in place. After isolation, embryonic brains were fixed in 4% paraformaldehyde (PFA), and 1mm coronal sections were acquired (Supplementary Fig. 1a-b). In the first part of CLARITY, the tissue was embedded in a hydrogel mesh using 4% acrylamide and 0.25% VA-044, a thermally triggered initiator, in 1X PBS for an overnight incubation at 4°C, followed by degassing with nitrogen and incubation under mineral oil for 3–7 hours at 37°C to polymerize biomolecules and conjugate monomers into a hydrogel. This covalently links the hydrogel monomers to biomolecules including proteins and nucleic acids while leaving the lipids loosely attached. Then, samples were washed with PBS, and unconjugated lipids were passively removed using 2–14-day incubation periods in 8% SDS in PBS at 37°C while shaking. Finally, the lipid-free hydrogel tissue-hybrid was incubated in a high refractive index matching solution comprised of 57.14% Histodenz (Sigma D2158) and 0.01% sodium azide in PBS with a pH of 7.5 until the tissue became optically transparent. The cleared tissue was then stained with Isolectin GS-IB4, AlexaFluor™ 488 Conjugate (ThermoFisher Scientific) to visualize blood vessels (Supplementary Fig. 1c). 3D confocal images were acquired as detailed below.

Structural Image Acquisition

The Leica TCS SP5 II laser scanning confocal microscope was used for imaging the blood vessels (Isolectin GS-IB4, Alexa Fluor™ 488 Conjugate). The Leica TCS SP8 Confocal microscope, fitted with a Leica HC FLUOTAR long working distance CLARITY objective designed for imaging cleared thick tissue sections, was used for Z-stack acquisition to image the 3D vascular structures (Supplementary Fig. 1d). An example of cortical regions selected for analysis is depicted in Supplementary Fig. 2a-b.

Quantification of Vascular Parameters

Imaris is an analysis software used for the visualization and quantification of complex 3D images. When paired with optical clearing techniques, it can provide a powerful analysis of the architecture of the brain microvasculature. The Imaris FilamentTracer module, which is often used for neuron morphology, can be used for surface rendering of the microvasculature to obtain parameters of vascular structure (Supplementary Fig. 1e-f). 3D confocal images were uploaded to the program and a surface mask was created over the 3D vessels to eliminate the background and non-specific staining (Supplementary Fig. 3a-b). The surface mask was then traced by the filament tracer tool using the automated threshold

algorithm settings. Lastly, detailed vascular metrics such as vessel length, vessel area, vessel volume, vessel mean diameter, vessel branch points, and total vessel count were extracted (Supplementary Fig. 1f).

Embryonic Blood-Brain Barrier Assay

At E18, pregnant mice were deeply anesthetized. A laparotomy was performed to expose the uterine horn and the embryos [21–25]. While still attached to the maternal circulation, embryos were injected using a Hamilton syringe with 5 μ L of high-fluorescence-intensity fixable dyes, 10kDa dextran-tetramethylrhodamine (TRITC), lysine fixable, 4 mg/mL (D3312 Invitrogen) or 3kDa TRITC, lysine fixable, 4 mg/mL (Invitrogen), into the fenestrated liver circulation. After 5 minutes to allow for dye circulation throughout the vasculature, dams and embryos were sacrificed, and embryonic brains were isolated and prepared for immunohistochemistry.

Immunohistochemistry

Embryonic brain cortices were isolated, fixed by immersion in 4% PFA overnight at 4°C, cryopreserved in 30% sucrose, and frozen in TissueTek OCT (Sakura). Sections of 12 μ m were then generated using a cryostat. Sections were blocked with 5% goat serum, permeabilized with 0.5% Triton X-100, and stained with Isolectin GS-IB4 (1:500; I21411, Molecular Probes) to visualize the vasculature. Slides were mounted in Fluoromount G (EMS) and imaged by confocal microscopy.

Quantification of Vessel Permeability

We developed a python-based program to analyze the BBB permeability assays and calculate a permeability index. Briefly, the program reads the dual-channel TIF image with OpenCV (opencv.org) and splits them into separate image NumPy arrays (numpy.org) where each value at an x,y location corresponds to the pixel intensity in the red channel (dextran: TRITC 3KDa (D3308), 10KDa (D1817)) and the green channel (vessel: Isolectin GS-IB4, Alexa Fluor™ 488 Conjugate (I21411)). From here, the user defines a pixel threshold corresponding to a positive dextran pixel (15/255) or a positive vessel pixel (5/255). The program then iterates through the image array determining positive dextran and vessel pixel counts and comparing if positive dextran pixels correspond to a positive vessel pixel in the same x,y location. Finally, the program outputs a ratio of the number of dextran pixels found within the corresponding vessel pixel. A total number of dextran positive and vessel positive pixels are calculated and stored to determine if the imaged slice contained a sufficient signal for both. All measurements are outputted to a Pandas dataframe (<https://pandas.pydata.org/>) and exported to a Microsoft Excel file for storage.

RNA-Seq

RNA sequencing was carried out by ArrayStar using paired-end sequencing of RNA extracted from the cortices of E18 PAE embryos that received either ICV injection of miR-150-5p LNA™ inhibitors or controls at E12. RNA libraries were prepared as follows: 1) Total RNA was enriched by oligo (dT) magnetic beads (rRNA removed) and 2) The KAPA Stranded RNA-Seq Library Prep Kit (Illumina) was used, which incorporates dUTP into the second cDNA strand and renders the RNA-seq library strand-specific. The completed libraries were qualified with the Agilent 2100 Bioanalyzer and quantified by absolute quantification qPCR. To sequence the libraries on the Illumina NovaSeq 6000 instrument, the barcoded libraries were mixed, denatured to single stranded DNA in NaOH, captured on an Illumina flow cell, amplified *in situ*, and subsequently sequenced for 150 cycles for both ends on the Illumina NovaSeq 6000 instrument.

Image analysis and base calling were performed using Solexa pipeline v1.8 (Off-Line Base Caller software, v1.8). Sequence quality was examined using FastQC software. The trimmed reads (trimmed 5', 3'-adaptor bases using cutadapt) were aligned to reference genome (GRCm38) using Hisat2 software. The transcript abundances for each sample were estimated with StringTie, and the FPKM value for gene and transcript levels were calculated with the R package Ballgown. Differentially expressed genes and transcripts were filtered using Ballgown. Novel genes and transcripts were predicted from assembled results by comparing to the reference annotation using StringTie and Ballgown, and then using CPAT to assess the coding potential of those sequences. Hierarchical clustering, pathway analysis, and volcano plots were performed with the differentially expressed genes in R, Python or shell environment for statistical computing and graphics.

Ingenuity Pathway Analysis

Ingenuity Pathway Analysis software (IPA, Qiagen) was used to identify biological processes and molecular networks associated with miR-150-5p inhibition and to characterize their biological relevance. A Core Analysis was performed using differentially expressed genes from E18 PAE cortices with miR-150-5p inhibitors vs control inhibitors, identifying significantly enriched biological pathways, top molecular interactions, and predicted relationships based on the IPA Knowledge Base. Significance was determined using right-tailed Fisher's exact test ($p < 0.05$).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 11 (California, United States). Comparison between E18 SAC and PAE permeability indexes and vascular structure parameters were performed using the unpaired Student's t-test after determining normal distribution of the data. All graphs are represented as mean $\pm$ standard error of the mean (SEM). Statistical significance was designated by $p < 0.05$. Each embryo was considered as a sample.

Results

PAE alters the structure of the developing cerebrovasculature

To analyze the structure of the cortical microvasculature, we used a tissue clearing technique called CLARITY that entails removing the lipid content of tissue, leaving all of the original protein and nucleic acids in place to improve quantitative analysis of thick tissue sections [26–29]. We obtained 1mm brain sections, and after tissue clearing, stained the vasculature with Isolectin GS-IB4. 3D confocal images were taken and uploaded into the Imaris FilamentTracer program for quantification (Fig. 1a-b, Supplementary Videos 1 and 2). We found that E18 PAE cortices displayed a significant reduction in vessel area (μm^2), vessel volume (μm^3), and mean vessel diameter (μm) compared to SAC controls (Fig. 1c-e). Vessel length (μm), vessel branch points, and vessel count were not significantly altered between groups (Fig. 1f-h). All vascular parameters for E18 SAC and PAE cortices are listed in Supplementary Table 1.

miR-150-5p inhibition rescues specific vascular parameters during PAE

To determine whether miR-150-5p is involved in shaping microvascular structure *in vivo*, we performed ICV injections of LNA™ miR-150-5p inhibitors or controls into embryonic brain ventricles at E12 (Supplementary Fig. 1a). At E18, the dams were sacrificed, and the fetal cortices were isolated and cut down the middle. One hemisphere was used for gene expression analysis, and the other hemisphere was used for microvascular structural assessment using CLARITY and Imaris FilamentTracer (Supplementary Fig. 1b-f). ICV injections of miR-150-5p inhibitors decreased miR-150-5p expression in the cortices of both SAC and PAE embryos (Supplementary Fig. 4a-b). There were no changes in vascular parameters between miR-150-5p inhibition and control inhibition in the SAC group (Fig. 2a-h). All vascular parameters for SAC cortices with miR-150-5p inhibition are listed in Supplementary Table 2. Additionally, miR-150-5p expression in each cortex had no significant correlation to vascular parameters in each cortex (Supplementary Fig. 5a-f). In the PAE brains (Fig. 3a-b), however, miR-150-5p inhibition significantly increased mean vessel diameter compared to the negative control inhibition (Fig. 3e). Non-significant changes were observed for the other structural parameters (Fig. 3c-d,f-h). All vascular parameters for PAE cortices with miR-150-5p inhibition are listed in Supplementary Table 3. Notably, significant correlations were found between the levels of miR-150-5p expression and vessel volume, mean vessel diameter, and vessel count for individual cortices (Supplementary Fig. 6b-c,f). Significant correlations were not observed for vessel area, length, or branch points (Supplementary Fig. 6a,d-e).

miR-150-5p inhibition alters cortical gene expression and related biological processes during PAE

To assess the impact of miR-150-5p inhibition during PAE on global gene expression, we performed RNA-seq using cortices from PAE embryos subjected to miR-150-5p inhibition or negative control

inhibition. We found 490 genes to be differentially expressed between the two groups (FC > 1.2, FDR-adjusted $p < 0.05$). Hierarchical clustering of differentially expressed genes segregated control and miR-150-5p inhibitor samples (Fig. 4a). Top upregulated genes included Dcaf17, Fam107b, Kcnc2, Pigq, Wnt8b, and Zfp335, and downregulated genes included Acat3, Cbx1, Ccdc115, Dsel, Pcdhb21, and Pelo (Fig. 4b). Genes upregulated in the cortex after miR-150-5p inhibition could represent regulatory targets of miR-150-5p. Using TargetScanMouse 8.0, we identified 35 upregulated genes from our miR-150-5p inhibition data that are predicted to be targets of miR-150-5p, including Aplnr, Cacng8, Ephb3, Slc38a3, Tbl1xr1, Wnt8b, and Zfp335 (Table 1). All differentially expressed genes are shown in Supplementary Table 4.

Table 1
Potential miR-150-5p targets upregulated in E18 PAE cortices following miR-150-5p Inhibition vs Control Inhibition

Gene Name	Fold Change	P Value	Gene Name	Fold Change	P Value
AMDHD2	1.255720	0.010579	INPP5A	1.161916	0.017922
ANKRD24	1.210615	0.014554	MCAT	1.150013	0.011972
APLNR	1.158672	0.022323	MGA	1.608713	0.000615
AW011738	1.240482	0.000071	PFKFB2	1.214086	0.015140
BACH2	1.404184	0.013615	PGM2L1	1.251128	0.003572
CACNG8	1.196704	0.013432	RTN4RL1	1.170750	0.023641
DCAF17	2.224971	0.000102	S100A8	2.125024	0.023958
DIO2	1.161341	0.008377	SENP8	1.377377	0.033599
DNALI1	1.173617	0.045929	SLC38A3	1.281989	0.004187
DYRK1B	1.428456	0.009528	SPR	1.309086	0.003243
EBF2	1.280220	0.048780	TAZ	1.226664	0.009984
ELOVL1	1.279216	0.034912	TBL1XR1	1.330069	0.024625
EPHB3	1.164982	0.023105	TTLL7	1.268397	0.036983
FIGN	1.350729	0.049198	WNT8B	1.296676	0.039531
GLIS2	1.240554	0.004894	ZFP280B	1.477636	0.003600
GOLIM4	1.432384	0.000833	ZFP335	1.327364	0.025008
H2-EB1	1.226450	0.000776	ZFP687	1.192361	0.041683
HIST1H2BK	1.821686	0.007172			

We performed Ingenuity Pathway Analysis (IPA) using the differentially expressed genes in the PAE cortex following miR-150-5p inhibition. Several Physiological System Development and Function categories were identified with IPA. The most significant of these were nervous system development and function, organ morphology, embryonic development, organ development, and organismal development (Fig. 4c). Additionally, biological processes associated with Molecular and Cellular Functions were identified. Among these were cellular compromise, cell morphology, cellular function and maintenance, amino acid metabolism, and cellular development (Fig. 4d).

PAE impairs BBB integrity during development

To assess BBB permeability *in vivo*, we performed *in utero* injections with 3kDa and 10kDa TRITC-Dextran tracers into the embryonic livers of exposed embryos at E18 and allowed them to circulate for 5 minutes [21]. The embryos were then sacrificed, and the brains were prepared for imaging of cerebral vessels and the TRITC-Dextran tracers (Supplementary Fig. 11a-b). A permeability index was calculated based on the percentage of TRITC-Dextran pixels found outside the vessel signal, stained with Isolectin GS-IB4, to total TRITC-Dextran pixels. There was no significant change in permeability between conditions for the 3kDa dextran tracer (Fig. 5a-b). E18 PAE embryos that received the 10kDa dextran tracer displayed a significantly higher permeability index compared to SAC controls (Fig. 5c-d).

Discussion

In accordance with previous literature [10, 11], we found defects in the structure of the cortical microvasculature during PAE. Specifically, PAE significantly decreased vessel area, vessel volume, and vessel mean diameter in the cortical microvasculature compared to SAC controls. We linked miR-150-5p expression levels to microvascular alterations by demonstrating that miR-150-5p inhibition rescued some of the deleterious effects of PAE. MiR-150-5p has been shown to alter angiogenesis and vascular integrity during many disease states. Its upregulation is associated with decreased angiogenesis and increased permeability in animal stroke models as well as the inhibition of cancer cell migration and invasion [15, 16, 30–33]. We previously found that miR-150-5p was upregulated in primary BMVECs isolated from E18 cortices during PAE [12]. We also identified vascular endothelial zinc finger 1 (Vezf1) as a novel target of miR-150-5p that may regulate angiogenesis during development by mediating BMVEC migration and tube formation [12].

We performed RNA-seq to characterize global gene expression in whole cortices following miR-150-5p inhibition in PAE animals. Among a number of differentially expressed genes, we identified putative targets of miR-150-5p, which were upregulated with the inhibition of the microRNA. Using IPA, we characterized the biological context of the differential gene expression induced by miR-150-5p inhibition in PAE cortices and linked miR-150-5p inhibition to biological processes related to development, morphology, and function of cells and organ systems, particularly in the nervous system. Predicted targets of miR-150-5p involved in critical biological pathways impacted by the inhibition of miR-150-5p in PAE cortices included *Aplnr*, *Cacng8*, *Ephb3*, *Slc38a3*, *Tbl1xr1*, *Wnt8b*, and *Zfp335*.

Our microvascular structure studies may have interesting implications for the contributions of aberrant cerebrovascular development to the symptoms of FASD. The *in vivo* cortical microvascular structural assessments were performed using E18 cortical sections corresponding to the primary somatosensory and motor cortex (Supplementary Fig. 2a-b). Previous research has indicated that PAE results in abnormal intraneocortical connections (INC) of the somatosensory and visual cortex of newborn mice; specifically, cell populations in the putative visual cortex and somatosensory cortex that do not overlap in control animals had overlapping connections in PAE animals [34]. These INCs of the somatosensory cortex are responsible for integrating sensorimotor information, and any alterations in the network have been shown to lead to cortical function and behavioral changes [34]. Other rodent models of PAE have also demonstrated alterations in the morphology and migration of pyramidal neurons in the somatosensory cortex [35, 36]. The sensorimotor system is responsible for transmitting information from the periphery (touch, temperature, pain) to the primary motor cortex to elicit fine motor movement [37]. Children with FASD have impairments in complex fine motor skills and visual-motor integration, affecting small hand muscles and leading to issues with pencil grasp, writing pressure, handwriting, tying shoelaces, doing up buttons, and threading beads [38]. Determining if the alterations in the cortical microvasculature that we have observed contribute to abnormal neuronal development in the primary somatosensory and motor cortex and the resulting alterations to sensorimotor function during PAE could be an interesting and impactful avenue of future research.

In addition to the effect of an aberrant vasculature on neuronal development, the effects of PAE on cortical gene expression, partially mediated by the upregulation of miR-150-5p in BMVECs and whole cortex, likely have significant effects on structural and functional brain development. Multiple gene interaction pathways associated with various biological processes including genes upregulated in PAE cortices following miR-150-5p inhibition were identified. One such network connecting CHD7, ZFP335, MXD3, S100A8, and FAM107B was associated with nervous system development and function, tissue development, and cellular development (Supplementary Fig. 7). In the developing brain, loss of CHD7 function is associated with reduced thickness of the neuroepithelium and the cerebral cortex [39, 40]. The role of CHD7 is not characterized in the brain vasculature; however, mutations to CHD7 cause CHARGE syndrome, which includes developmental anomalies in the great vessels ⁴¹. The involvement of ZFP335 in brain development is noted primarily in the association between variants of the gene and microcephaly [42]. MXD3 is most associated in the brain with granule neuron precursor proliferation [43]. Spatio-temporal expression of FAM107B in the brain suggests its involvement in corticogenesis [44]. The role of S100A8 in brain development is less obvious as it is typically associated with neuroinflammation in a variety of pathological states [45]. In regards to the vasculature, S100A8 is associated with vascular inflammation and increased immune cell adhesion to the endothelium [46]. Another network connected APLNR and KCNC2 and was associated with nervous system development and function, embryonic development, and organ development (Supplementary Fig. 8). APLNR is ubiquitously expressed in the brain, and the apelinergic system in which it functions is associated with a variety of brain-related diseases [47] as well as vascular development, barrier integrity, and endothelial homeostasis [48]. KCNC2 is a potassium channel that has largely been associated with developmental and epileptic

encephalopathy in certain genetic mutations [49, 50]. A network connecting PFKFB2, RTN4RL1, and EPHB3 was associated with nervous system development and function, cellular function and maintenance, and behavior (Supplementary Fig. 9). PFKFB2 has tissue-specific expression including in the brain and vasculature, and it is primarily a metabolic regulator [51–53]. In the brain, RTN4RL1 has significant function in synapse formation [54]. EPHB3 has been linked to synapse formation, cortical endothelial cell death and impaired BBB integrity following traumatic brain injury, and angiogenesis during nervous system development [55, 56]. Our final network connected CACNG8, TEAD2, RGS11, and BLOC1S6 and was associated with cellular function and maintenance, cellular assembly and maintenance, and cellular movement (Supplementary Fig. 10). Mutations in CACNG8 are associated with disruption to AMPA receptor signaling [57]. TEAD2 is influential in the maintenance and differentiation of neural stem cells [58], and its loss is associated with impaired endothelial cell proliferation and angiogenesis [59]. RGS11 is a GTPase accelerating protein with brain region-specific expression and is associated with light response in bipolar neurons [60, 61]. BLOC1S6, also called pallidin, maintains normal amino acid profiles and metabolism in the hippocampus and protects against lysosomal and autophagic defects that promote epileptic encephalopathy [62, 63]. Together, these molecular networks demonstrate the potential far-reaching effects of altered miR-150-5p expression in the developing cortex during PAE, in both the neuronal and vascular compartments. These effects could underly the previously-characterized PAE-mediated effect of miR-150-5p overexpression on the cortical microvasculature and endothelial cell behavior.

The BBB is a protective, semi-permeable barrier that separates the circulation from the central nervous system (CNS), maintaining ion balance in the brain and preventing infiltration by inflammatory factors and toxins. Its function is achieved through intercellular junctions and the interactions between components of the neurovascular unit (NVU) including endothelial cells, neurons, astrocytes, and pericytes [64]. An excessively permeable BBB is associated with numerous disorders of the CNS including increased risk of stroke but also mood imbalance and cognitive impairments [64–66]. Increased permeability of the BBB as a result of PAE, as demonstrated in our findings, may partially underly the mood and cognitive effects of PAE that manifest in FASD [67, 68]. Because BBB integrity is predicated on interactions between NVU cells, impaired BBB integrity induced by PAE may stem partially from alterations to intercellular communication between NVU cells in PAE that we previously characterized [69].

Our studies involved some practical limitations. ICV injection is a technically challenging and invasive technique. In future studies, we will explore noninvasive techniques such as intranasal administration during early postnatal timepoints. Another limitation is that we did not have sufficient numbers of embryos to evaluate sex differences in our experiments. In future studies, we will incorporate analyses of biological sex.

In conclusion, we have characterized PAE-induced alterations to cerebrovascular structure and integrity, and we have identified a gene expression network that is altered following miR-150-5p inhibition during PAE. PAE-mediated upregulation of miR-150-5p in BMVECs likely alters structural parameters of the

cortical microvasculature as well as the expression of BBB integrity and angiogenic genes. This altered gene profile may promote BBB leakiness and contribute to the microvascular structural abnormalities found within the somatosensory and motor cortex. Future studies are required to determine if inhibition of miR-150-5p might prevent or reverse some of the neurocognitive and behavioral effects of PAE.

Declarations

Statement of Ethics

All methods were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and approved by the University of New Mexico Institutional Animal Care and Use Committee, approval number 22-201231-HSC.

Conflict of Interest Statement

The authors report no conflicts of interest, financial or otherwise.

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Author Contribution

GP performed the bulk of the experiments and was a major contributor to writing the manuscript. MRW assisted in performing experiments and was a major contributor to writing the manuscript. RJO developed the permeability index pipeline. ASG conceived the research, assisted in performing experiments, and was a major contributor in writing the manuscript.

Data Availability

Raw data will be made available upon reasonable request to the corresponding author. RNA-seq data is available in the Gene Expression Omnibus (GEO) repository at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE348250> with accession record number GSE348250.

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Figures

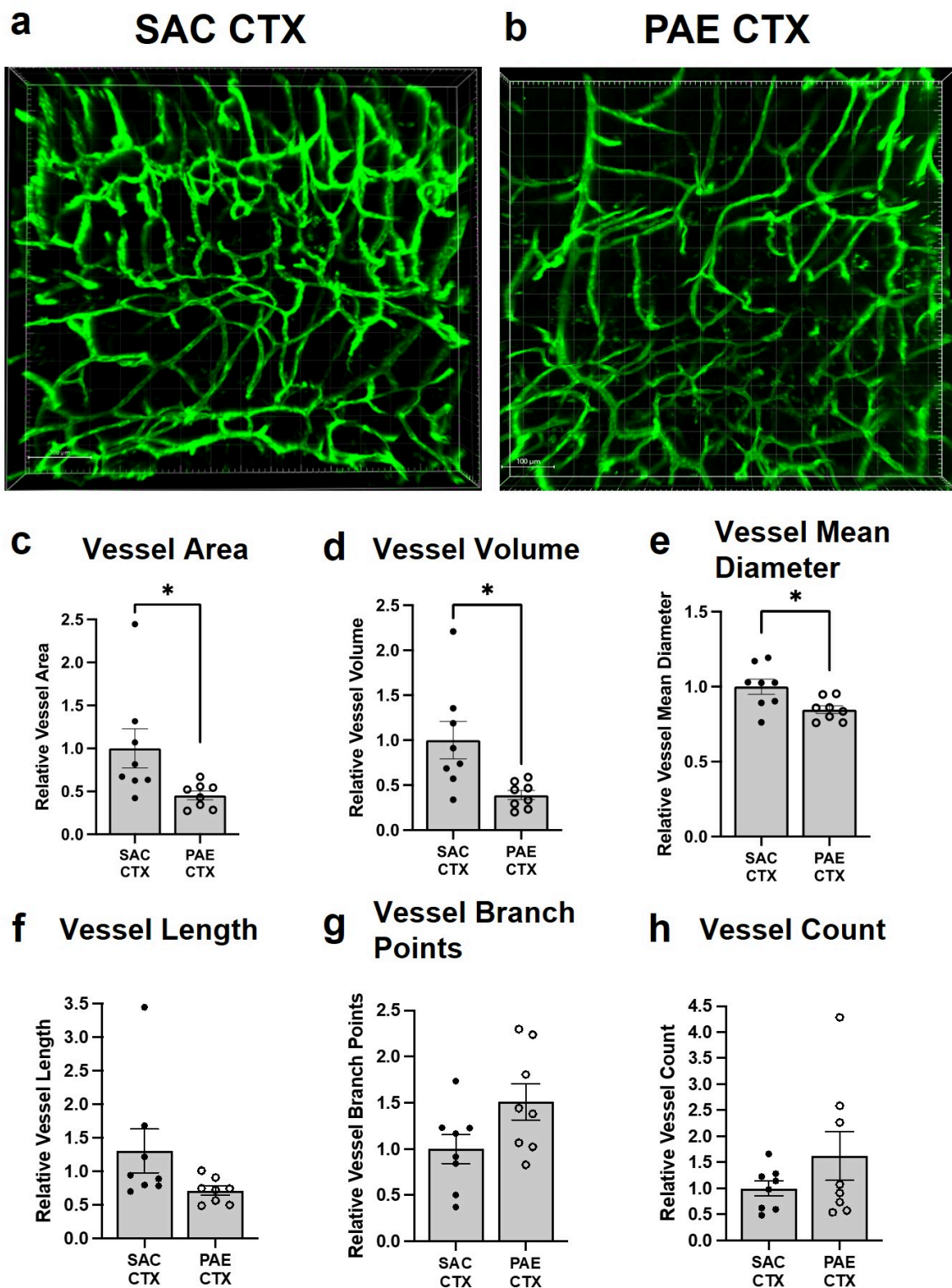


Figure 1

PAE alters the structure of the cortical microvasculature at E18

Representative images of the **a**, E18 SAC and **b**, E18 PAE cortical microvasculature (n=8). **c**, Analysis of mean vessel area (p=0.0354), **d**, vessel volume (p=0.0127), **e**, vessel mean diameter (p=0.0188), **f**, vessel

length ($p=0.0949$), **g**, vessel branch points ($p=0.0601$), and **h**, vessel count ($p=0.2248$) in E18 SAC vs PAE cortices. $*p<0.05$. Error bars represent standard error of the mean (SEM).

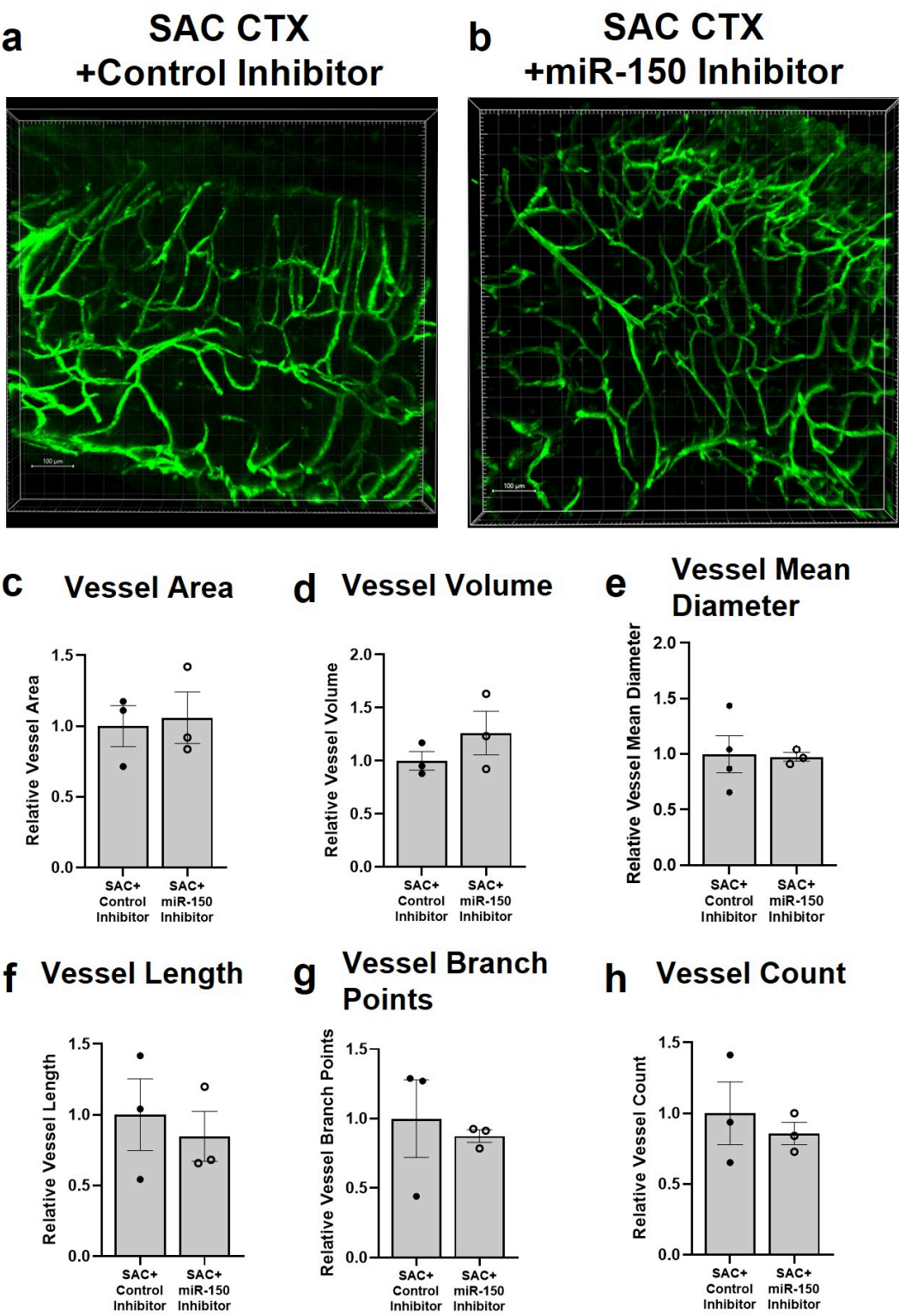


Figure 2

miR-150-5p inhibition has no effect on vascular parameters in cortices from SAC embryos

Representative images of the cortical microvascular structure in E18 SAC cortices with **a**, control inhibitors (n=3) or **b**, miR-150-5p inhibitors (n=3). **c**, Analysis of mean vessel area (p=0.8132), **d**, vessel volume (p=0.3057), **e**, vessel mean diameter (p=0.3717), **f**, vessel length (p=0.6450), **g**, vessel branch points (p=0.6812), and **h**, vessel count (p=0.5752) in SAC+control inhibitors vs SAC+miR-150-5p inhibitors. Error bars represent SEM.

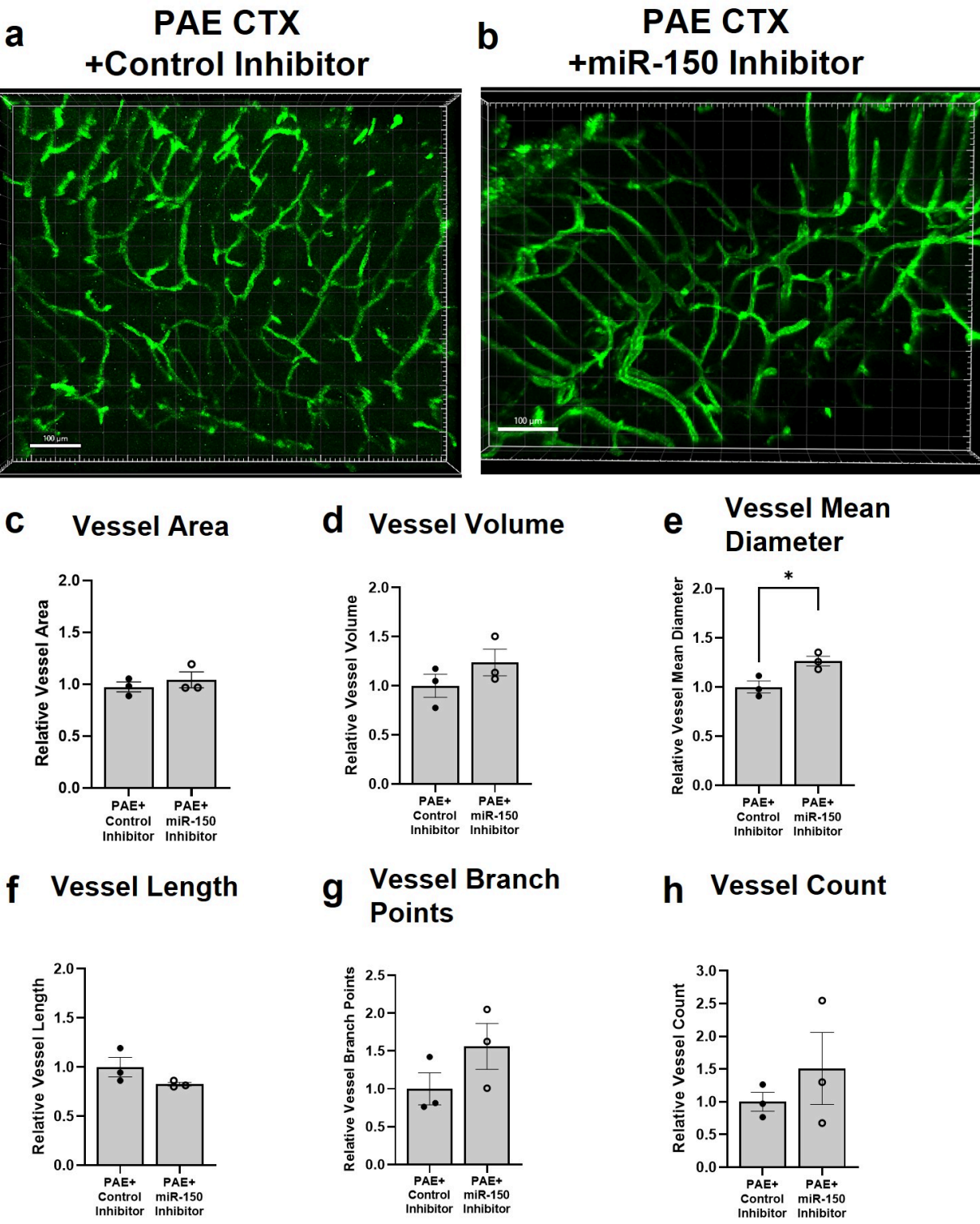


Figure 3

miR-150-5p inhibition rescues specific vascular effects of PAE in developing cortices

Representative images of the cortical microvascular structure in E18 PAE cortices with **a**, control inhibitors (n=3) or **b**, miR-150-5p inhibitors (n=3). **c**, Analysis of mean vessel area (p=0.2439), **d**, vessel volume (p=0.1281), **e**, vessel mean diameter (p=0.0142), **f**, vessel length (p=0.1607), **g**, vessel branch points (p=0.0547), and **h**, vessel count (p=0.4225) in PAE+control inhibitors vs PAE+miR-150-5p inhibitors. *p<0.05. Error bars represent SEM.

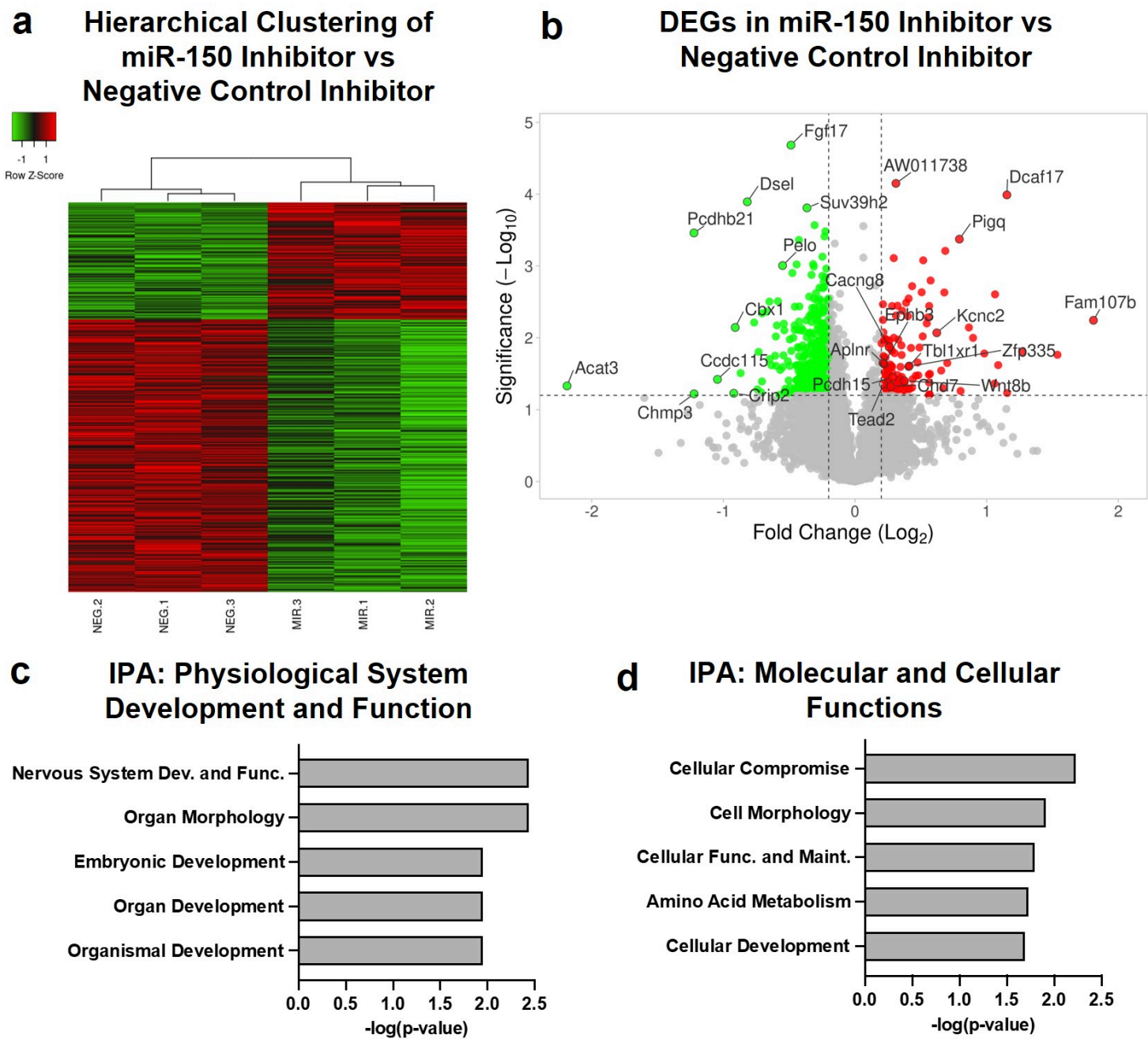
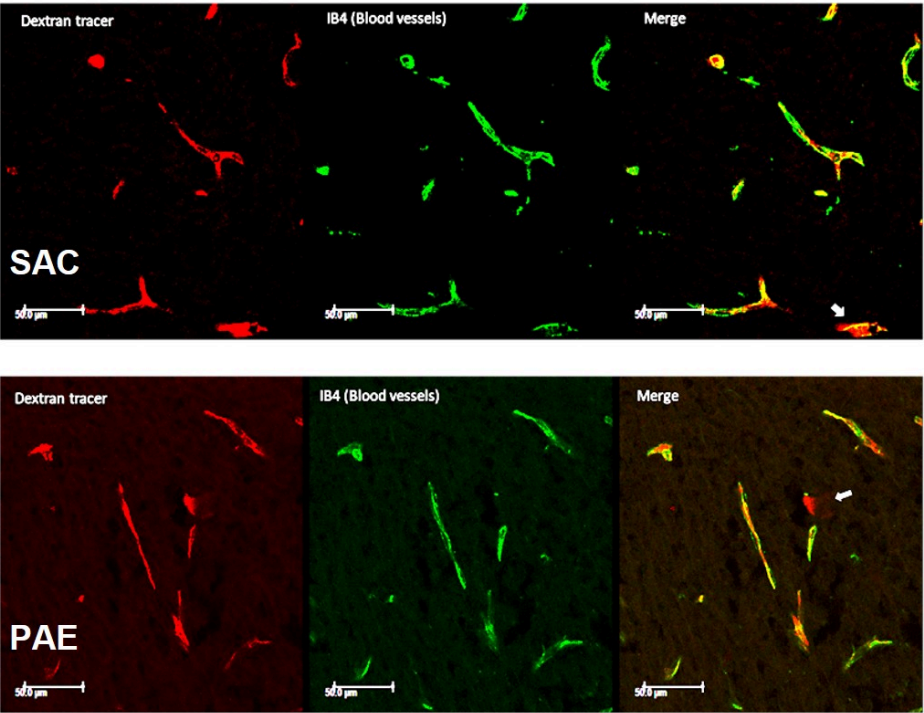


Figure 4

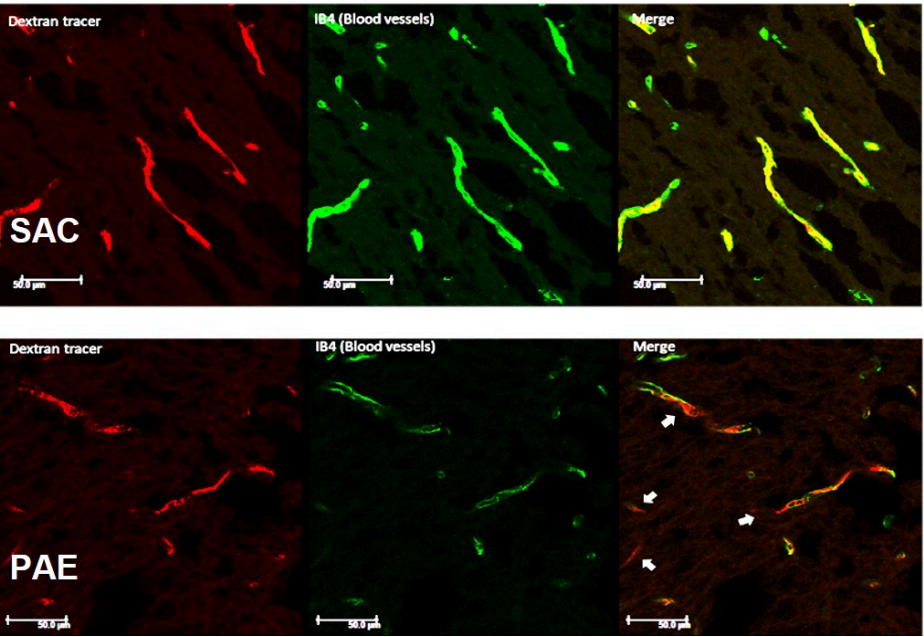
miR-150-5p inhibition alters gene expression in the cortices of PAE embryos

a, Heat map depicting the hierarchical clustering of differentially expressed genes (DEGs) in whole cortices from E18 PAE mice with miR-150-5p inhibitors or negative control inhibitors (n=3). **b**, Volcano plot of DEGs in E18 PAE cortices with miR-150-5p inhibitors or negative control inhibitors (n=3). **c**, Top physiological system development and function categories identified through IPA, associated with the DEGs following miR-150-5p inhibition in the PAE cortex. **d**, Top molecular and cellular functions identified through IPA, associated with the DEGs following miR-150-5p inhibition in the PAE cortex.

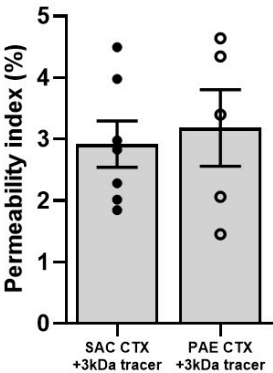
a E18 CTX + 3kDa-TRITC tracers



c E18 CTX + 10kDa-TRITC tracers



b Permeability of 3kDa tracers



d Permeability of 10kDa tracers

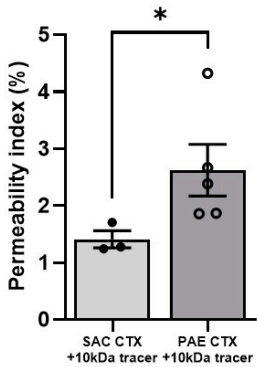


Figure 5

PAE increases the permeability of developing cortices at E18

a, Representative image of dextran tracer staining (red), blood vessel staining (isolectin, green) and merged images of E18 SAC (n=7) and PAE (n=5) cortical sections following injection with 3kDa TRITC-dextran tracers. **b**, Analysis of permeability in SAC vs PAE cortices following injection with 3Da TRITC-dextran tracers ($p=0.3550$). **c**, Representative image of dextran tracer staining (red), blood vessel staining (isolectin, green) and merged images of E18 SAC (n=3) and PAE (n=5) cortical sections following injection with 10kDa TRITC-dextran tracers. **d**, Analysis of permeability in SAC vs PAE cortices following injection with 10kDa TRITC-dextran tracers ($p=0.0450$). * $p<0.05$. Error bars represent SEM.

Supplementary Files

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