Innovative 3D-image analysis of cerebellar vascularization highlights transcriptomic changes in a murine model of apnea of prematurity.

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Abstract

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Introduction

Apnea of prematurity (AOP) consists of an abnormal respiratory rhythm where breathing is interrupted at least every 5 minutes and for at least 20 seconds, thus inducing a state of intermittent hypoxia (IH). Premature newborns are particularly prone to AOP due to their immature respiratory system (Moriette et al., 2010). This condition affects 50% of all preterm infants and nearly 100% of neonates born under 28 gestational weeks. The neonates will typically resume a normal breathing pattern by the corrected term, but several metadata provide evidence of a correlation between the duration of AOP and the incidence of developmental abnormalities that lead to long term behavioral deficits (Henderson-Smart, 1981; Janvier et al., 2004; Pergolizzi et al., 2022). Among these deficits, children suffer of motor coordination, language, and spatial orientation impairments, suggesting that a cerebellar alteration could be involved. According to this hypothesis, we have recently demonstrated, thanks to an established mouse model of AOP (Cai et al., 2012), that IH induces a delay of cerebellar cortex maturation and an alteration of the dendritic arborization of Purkinje cells, with long lasting transcriptional alterations (Leroux et al., 2022; Rodriguez-Duboc et al., 2023). The strong effect of AOP on the cerebellum is due to the developmental window of this nervous structure which mainly occurs after birth.

Indeed, in mice as in humans, cerebellar cortical development starts during embryogenesis from two germinative regions, the rhombic lip and the ventricular zone. Glutamatergic granule cells come from the rhombic lip and migrate tangentially to form the transitory external granular layer at the surface of the cerebellum. GABAergic Purkinje cells (and various interneurons) arise from the ventricular zone and migrate transversally to the surface to create a monolayer of Purkinje cells just below the external granular layer (EGL). Then, at birth, the cerebellum is composed of 3 layers, the EGL, containing granule cell precursors (GCP), and the Purkinje cell layer (PCL) separated by the molecular layer (ML). However, at this point the structure is still immature and has yet to undergo postnatal maturation. Firstly, the GCPs proliferate intensively and cross the ML and the PCL to form the definitive granule cell layer (GCL) until the total disappearance of the EGL. There, the GCPs finish their maturation and send their axons, called parallel fibers, to the ML. Meanwhile, Purkinje cells develop their dendritic trees into the ML to contact parallel fibers. This neurohistogenetic process is mirrored by angiogenesis within the cerebellar cortex. Much like in the rest of the brain, cerebellar vascularization initiates in the perineural vascular plexus that covers the neural tube. Then, collaterals from pial vessels enter the cerebellar cortex and irrigate the EGL. At first sparse in the EGL during the proliferative phase of GCPs, blood vessels then continue branching out and the number of capillaries increases in the ML and the GCL (Mecha et al., 2010). Therefore, as hypoxia is known to remodel the vascular organization and enhance the density of blood vessels in the brain (Guan et al., 2022; LaManna et al., 2004), we can hypothesize that AOP could disrupt vascularization during the postnatal development of the cerebellum (as it does with neuronal cells).

In line with this hypothesis, our previous transcriptomic results, showed that the expression of the hypoxia inducible factor 1 alpha (HIF1α) is affected in response to our IH protocol (Leroux et al., 2022). HIF1α is considered as the main regulator of angiogenesis since this factor responds to low levels of dioxygen (O_2) by promoting the expression of pro-

angiogenic actors, including VEGF, PDGFβ as well as miRNA (Monaci et al., 2024).Another angiogenic process could also occur in the brain via the Ang2/Tie2 pathway. Indeed, under hypoxic conditions, endothelial cells increase Ang2 production which acts in an autocrine manner on Tie2 receptor to destabilize capillaries and promote angiogenesis (Augustin et al., 2009). Interestingly, Tie2 has been recently described in Purkinje cells and it has been demonstrated that the neuro-vascular signalling Ang2/Tie2 controls dendritic morphogenesis of Purkinje cells during cerebellar postnatal development (Luck et al., 2021).

All these data led us to consider that the alteration of the Purkinje dendritic trees observed after IH in our AOP model could be linked to a perturbation of cerebellar vascularization. To provide elements in understanding this mechanism, we performed a transcriptomic study of the main factors involved in cerebellar angiogenesis at different postnatal ages. Moreover, to correlate putative modification of gene expression with defaults in vessel morphology, we developed an image analysis workflow using both IMARIS and VesselVio software to visualize the cerebellar vascularization in 3D and obtain comparable quantitative parameters between normoxic and IH conditions.

Materials and methods

Animals

Animals used in this study were wild type C57Bl6/J mice born and bred in an accredited animal facility (approval number B.76-451-04), in accordance with the French Ministry of Agriculture and the European Community Council Directive 2010/63/UE of September 22nd, 2010, relative to the protection of animals used for scientific purposes. Mice lived on a 12-hour light/dark cycle and had free access to food and water. Sex was determined based on anogenital distance measurement as well as pigment-spot localization (Wolterink-Donselaar et al., 2009). From P2 onwards, mice were assigned a unique identifier before initializing the IH protocol. There was no blinding in this study, and sample size was chosen based on power determination from our preliminary studies.

Intermittent hypoxia protocol

To mimic AOP, our IH protocol relies on a custom hypoxia chamber, which is based on the protocol developed by Cai et al., and has been validated to mimic AOP (Cai et al., 2012; Leroux et al., 2022; Rodriguez-Duboc et al., 2023). IH is achieved by the repeated succession of 2-min cycles of hypoxia (5% O_2; 20 s/cycle) and reoxygenation, for 6 hours per day, while mice are in their sleep phase (10 am - 4 pm). The protocol was initiated on neonatal P2 pups (IH group) until the desired stage, or for 10 days maximum. Throughout the protocol, the following parameters were constantly monitored in the chamber: oxygen concentration, hygrometry, temperature, and atmospheric pressure. The control group (N) was placed in a chamber open to room air but mimicking the hypoxia chamber's environment to control for external stressors.

Sample gathering

For the transcriptomic study, mice were sacrificed at stages P4, P8, P12, P21, and P70 by decapitation after anesthesia by isoflurane inhalation (Iso-VET). Whole brains were immediately harvested and set in pure isopentane at -30°C. They were then stored in sterile

containers at -80°C until further use. Sample sizes for RT-qPCR were: 13 for P4 (7N + 6IH), 16 for P8 (6N + 10IH), 25 for P12 (15N + 10IH), 17 for P21 (9N + 8IH), and 8 for P70 (5N + 3IH).

For imaging studies, brains from P4 mice were directly harvested and fixed by immersion in 4% paraformaldehyde, given their diminutive size. For P8, P12, P21, P70 stages, mice were lethally anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg), and then sacrificed by intracardiac perfusion of NaCl 9‰ and paraformaldehyde 4%, before removing the brains. Brains were then postfixed overnight in 4% paraformaldehyde, and stored in phosphate buffer saline (PBS). Sample sizes were: 8 for P4 and P8 (4N + 4IH) and 7 for P12 and P21 (3N + 4IH).

Panels and primer design

The vascularization gene panel was built based on an analysis conducted with the Cytoscape software (v.3.9.1; Shannon et al., 2003) using protein interaction data retrieved from STRING-DB (Szklarczyk et al., 2021). The panel was further expanded by enrichment with the plugin stringApp (v 2.0.1; settings: maximum additional interactors = 10, confidence cutoff = 0.40; Doncheva et al., 2019) and then cross referenced with ClueGO pathway (v.2.5.9; Bindea et al., 2009) and functional data retrieved from the literature. For readability and ease of interpretation, these findings are summarized in Table 1, and the resulting functions are used to group the qPCR results.

Function abbreviation	**Functional pathway**	**Genes with a putative positive effect on the function**	**Genes with a putative negative effect on the function**
VSM	Vascular stabilization and maturation	Tgfb1; Flk1; Angpt1; Tek; Vegfa	Tie1
EEP	ECM and endothelial permeability	Cdh5; Vegfa; Mmp2; Mmp9; Angpt2	Angpt1
VIS	Vascular invasion and sprouting	Cdh5; Fgf2; Vegfa; Serpine1; Nrp1; Anpep; Mmp9; Ndp; F3	Flt1; Col1a1; Thbs1; Timp1
EPS	Endothelial proliferation and survival	Pgf; Tgfb1; Flk1; Nrp1; Angpt1; Tie1	Lect1; Angpt2; Thbs1; Serpinf1
VRP	Vascular remodelling and patterning	Flt1; Tie1	Vegfa
HIA	Hypoxia-induced angiogenesis	Mmp2; Mmp9; Tek; Tie1; Vegfa; Serpine1	Serpinf1

Table 1. Selected angiogenesis-associated genes, the functions they are associated with, and their roles within these functions. *Angpt1: angiopoietin 1; Angpt2: angiopoietin 2; Anpep: alanyl aminopeptidase; Cdh5: vascular epithelium-cadherin; Col1a1: collagen, type I, alpha 1; F3: coagulation factor III; Fgf2: fibroblast growth factor 2; Flk1: vascular endothelial growth factor receptor 2; Flt1: vascular endothelial growth factor receptor 1; Lect1: chondromodulin; Mmp2: matrix metallopeptidase 2; Mmp9: matrix metallopeptidase 9; Ndp: Norrie disease protein; Nrp1: neuropilin 1; Pgf: placental growth factor; Serpine1: plasminogen activator inhibitor 1; Serpinf1: pigment epithelium-derived factor; Tek: endothelial-specific receptor tyrosine kinase; Tgfb1: transforming growth factor, beta 1; Thbs1: thrombospondin 1; Tie1: tyrosine kinase with immunoglobulin-like and EGF-like domains 1; Timp1: tissue inhibitor of metalloproteinase 1; Vegfa: vascular endothelial growth factor A.*

Gene primers were designed with the Primer Express software (v3.0.1; ThermoFischer Scientific) using nucleotide sequences from the NCBI Pubmed database. Primer pairs were ordered from Integrated DNA Technologies and their specificity was validated by linear regression of serial dilution data. Primer pairs were chosen preferentially to be on exon joining sites, with the least possible hairpin and dimer formation, and with similar size, GC percentage, and melting temperature for forward and reverse primers. Each sequence was blasted on Pubmed to ensure specificity. See Appendices A and B for primer pair sequences and specifications.

RNA Extraction

The samples were homogenized in 1 ml of Trizol (ThermoFisher), and mRNAs were purified on column using the Nucleospin RNA extract II from Macherey-Nagel (cat. 740 955 250) according to manufacturer recommendations. RNA quantity and purity were analyzed by UV spectrophotometry (Nanodrop Technologies). The optical density (OD) of RNA was read at 230, 260, and 280 nm. The ratios OD 260 nm/OD 280 nm and OD 260 nm/DO 230 nm are calculated as indicators of protein, and salt/ethanol contamination, respectively. Ratio values between 1.6 and 2.0 were considered acceptable. As per the MIQE guidelines (Bustin et al., 2009), mRNA quality assessment was performed by a bioanalyser gel electrophoresis on RNA 6000 Pico chips (cat. 5067-1513, Agilent), and samples with an RNA integrity number (RIN) between 7 and 10 were considered qualitative enough to be analyzed. The mRNAs were then stored at -80°C until the next step.

Real time qPCR

Messenger RNAs were converted to cDNA by reverse transcription using the Prime Script RT reagent kit (cat. RR037A, Takara). Relative gene expression level determination was done by real time PCR in 384-well plates. Total reaction volume was 5 µL including: 2.5 µL 2X Fast SYBR Green PCR Mastermix (cat. 4385612, Thermofisher), target gene-specific sense and antisense primers (0.15 µl of each, 100 nM final concentration), 1µL PCR-grade water, and 1.2 µL of sample solution. The cDNA samples and reaction mixes were distributed via the Bravo 1 liquid handling platform (Agilent). The real time PCR reaction took place in a QuantStudio Flex 12k thermal cycler (Applied Biosystems). For each GOI, sample measurements were conducted at least in duplicate and with a minimum of two housekeeping genes (HKG) from Table 2. Values were calculated via the $2^{(-\Delta\Delta Cq)}$ method where:

$$2^{-\Delta\Delta Cq} = 2^{(-((CqGOI_{IH} - CqHKG_{IH}) - (CqGOI_N - CqHKG_N)))}$$

Gene	Name	Function	NCBI reference	Primer pair sequence	
Gapdh	glyceraldehyde-3-phosphate dehydrogenase	glucose metabolism	NM_001289726.1	Fw	CATGGCCTTCCGTGTTCCTA
				Rv	CCTGCTTCACCACCTTCTTGA
Hsp90ab1	heat shock protein 90 alpha (cytosolic), class B member 1	signal transduction	NM_008302.3	Fw	CAGAAATTGCCCAGCTCATGT
				Rv	CCGTCAGGCTCTCATATCGAA
Ppia	peptidylprolyl isomerase A	protein folding	NM_008907.1	Fw	CCACTGTCGCTTTTCGCCGC
				Rv	TGCAAACAGCTCGAAGGAGACGC
Ywhaz	tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein zeta	signal transduction	NM_011740.3	Fw	AGGACCTAAAAGGGTCGGTCA
				Rv	CGGGGTTTCCTCCAATCACT

Table 2. Selected housekeeping genes for RT-qPCR experiments. *Table providing the list of housekeeping genes used, their full name and National Center for Biotechnology Information (NCBI) reference number. Primer sequences indicated were designed on the Primer Express software and ordered from Integrated DNA Technologies. Fw: forward; Rv: reverse.*

Imaging

Cerebella previously fixed with 4% paraformaldehyde underwent a clearing protocol consisting of the following steps. 1) Samples are dehydrated by being submerged in increasing concentrations of methanol (MeOH; 20%, 40%, 60%, 80% and 100%). 2) Dehydrated samples undergo bleaching in a solution of 5% H2O2 (hydrogen peroxide) and 95% MeOH for 24 hours in order to decrease tissue autofluorescence. 3) Samples are then rehydrated by being submerged in decreasing concentrations of MeOH (80%, 60%, 40% and 20%), then washed in solution PTx.2 (100 mL PBS 10X + 2 mL Triton-X100 + Q.S. distilled water). 4) Samples are then permeabilized for one day at 37 °C under agitation with a solution containing 1X PBS, 0.2% TritonX-100, 20% dimethyl sulfoxide (DMSO), glycine (23 mg/mL) and thimerosal at 0.1 g/L (antifungal). 5) Finally, non-specific binding sites are blocked with a 1X PBS solution containing 0.2% Triton-X100, 10% DMSO, 6% normal donkey serum (NDS) and thimerosal for one day at 37 °C under agitation.

Thereafter, the brains are incubated with the primary antibodies diluted in PTwH solution (100 mL PBS 10X + 200 µL Heparin (50 mg/mL) + Q.S. distilled water), containing 5% dimethyl sulfoxide (DMSO) and 3% NDS at 37 °C under agitation for 6 days. After 6 rinses with PTwH at room temperature, the samples are incubated for 5 days with the appropriate secondary antibodies (Table 3) diluted in PTwH containing 3% NDS at 37°C under agitation. The brains are then rinsed several times with PTwH at room temperature under agitation, followed by dehydration in MeOH baths of increasing concentration (20%, 40%, 60%, 80% and 100%). A delipidation of the brains is then performed by incubation in a solution containing 66% dichloromethane (DCM) and 33% MeOH for one night under agitation and then in DCM 100% for 30 minutes. These last two steps homogenize the refractive indexes of the cellular structures and induce their transparency once placed in dibenzylether (DBE).

The 3D acquisitions of the transparent cerebella were performed on the Ultramicroscope Blaze (Miltenyi Biotec) using the software ImspectorPro. Image analysis and vessel modelling were done using the Imaris software (Oxford Instruments) and then transferred to VesselVio 1.2 software to obtain quantitative and comparative parameters (Bumgarner et al., 2022).

Primary Antibodies	Target	Dilution	Species	Supplier	Secondary antibodies	Dilution
Podocalyxin	Capillaries	1:200	Goat	R&D Systems (#AF1556)	DAG-Alexa 594	1:200
α-SMA-Cy3	Arteries	1:500	Mouse	Sigma-Aldrich (#C6198)	N/A	N/A
PECAM1	Arterioles	1:200	Rat	Millipore (#CBL1337)	DARt-Alexa 594	1:400

Table 3. Antibodies used for the visualization of blood vessels. *DAG: donkey anti-goat; DARt: donkey anti-rat; α-SMA: smooth muscle actin alpha; PECAM1: platelet endothelial cell adhesion molecule 1. The secondary antibodies were purchased by Molecular Probes.*

Statistical analysis

Statistical analyses were performed within the R statistical computing environment (version 4.3). Both real time PCR and imaging data were modeled through the Generalized Linear Mixed Model (GLMM) framework, using the {glmmTMB} package (Brooks et al., 2017). For real time PCR data, a Gaussian likelihood with an identity link function was used to model the distribution of the DCq samples for each gene of interest. When DCq samples for one gene were split over multiple plates, a random intercept was added to account for intra-plate correlations.

Model diagnostics were done using the {DHARMa} (Hartig, 2022) and {performance} (Lüdecke et al., 2021) packages. The fitness of each model was assessed through both visual checks (e.g., posterior predictive checks, QQ plots, residuals vs predicted values) and quantitative indices of model fit (e.g., AIC: Aikake Information Criterion). When several competing models were possible a priori, we selected the most plausible one primarily based on our theoretical understanding of the response's properties, and, to a lesser extent, to minimize AIC and favor model parsimony.

Contrasts and p-values for relevant hypotheses were obtained using the {emmeans} package (Lenth, 2022). They were computed on the link scale, using Wald t-tests, without any multiplicity adjustments. For all analyses, $p < 0.05$ was considered significant and for each figure asterisks indicate the level of statistical significance: one for $p < 0.05$, two for $p < 0.01$, and three for $p < 0.001$.

Results

Effect of IH on the expression of angiogenesis-related factors

The transcriptomic study was done on whole cerebella at different stages of postnatal development (Figure 1). We tested a panel of 23 genes, of which 22 were differentially regulated in at least one stage. The exception being Col1a1 which did not vary across our experiments, as such, it is not represented in Figure 1.

At stage P4, we found 30.4% regulated genes (7/23 genes), all of which are upregulated in the IH condition. Most notably, the vascular endothelial growth factor A (Vegfa) and these two VEGF receptors Flk1 and Flt1 are overexpressed, indicating the importance of this pro-

angiogenic signaling pathway at this stage. Interestingly, some negative regulators of angiogenesis are also overexpressed, such as endothelial adhesion molecule thrombospondin 1 (Thsb1).

P8 is the most regulated stage overall with 73.9% (17/23) differentially expressed genes, but in contrast with P4, all were downregulated under IH. This includes the previously P4 upregulated growth factors but also nearly all genes we tested from the angiopoietin and VEGF signaling pathways. In parallel to this effect on pro-angiogenic molecules, IH also acts negatively on some anti-angiogenic factors such as Thbs1 and chondromodulin1 (Lect1). Moreover, despite being 6 days into the hypoxia protocol, genes involved in hypoxia induced angiogenesis, such as Vegfa and the angiopoietin 1 inhibitor Tie1, are also downregulated at P8.

Then, P12 had 60.9% (14/23) of regulated genes with a partial switch to upregulation compared to P8. This is the case of the growth factors Fgf2, Pgf and Tgfb1 but also the anti-angiogenic Thsb1. Interestingly, our results showed that IH upregulates the expression of matrix metalloproteases 2 and 9 involved in vessel invasion and sprouting.

The decrease in IH-regulated genes continues at P21 with only 39.1% (9/23) differentially expressed genes. All of them are under-expressed, including the vascular endothelial growth factor receptor 1 (Flt1), a negative regulator of neovascularization. Surprisingly, 43.5% (10/23) were still regulated at P70, suggesting that the vascular response to hypoxia is long lasting on a transcriptomic level. This long-term effect decreases angiogenic mechanisms, especially with the downregulation of hypoxia-induced angiogenic factors and positive regulators of vascular invasion and sprouting.

Effect of IH on the vascularization of the postnatal cerebellum

Workflow of the cerebellar vascularization analysis

Thanks to a triple labelling (α-SMA, PECAM and podocalyxin), we were able to visualize all the vascular networks, namely, the arteries, veins, and capillaries, at all stages of postnatal development (Figure 2). The labelling is present in all cerebellar structures. Signal intensity is stronger in the cortex than in the depth of the tissue at P12 and P21 because of the higher volume of the sample that impedes antibody penetration despite applying an increasing permeabilization protocol. Due to the variability of the cerebellum's morphology, no method currently exists to analyze the vasculature of postnatal mice cerebella. Therefore, we developed the following workflow by optimizing each step of analysis using Imaris and Vesselvio software.

The file resulting from the 3D lightsheet acquisition was converted to an *.ims* format, then the *Surface* function of the Imaris software was used to delineate de cerebellum from the whole hindbrain 3D acquisition (Figure 3A). Because of the complexity of the cerebellum, the delineation was performed manually, by drawing cerebellar outlines every 20 µm in the 2D-slices mode (Figure 3B). The resulting *Surface* was used to obtain a channel (mask) including only the cerebellum (Figure 3C), which allows the calculation of the total cerebellar volume as well as the total volume of the vasculature as follows. The vascularization modelling was built with the *Filament* modality and applied to the cerebellum channel. *The Automated Autopath Algorithm* mode was chosen among the six algorithms proposed by the software because this

process models dense networks with numerous branches. The algorithm is comprised of three steps and is primed on a representative region of interest.

The first step involves choosing the detection modalities. The observation of the vascular organization on a sagittal cerebellar section revealed a high heterogeneity of vessel diameter (Figure 3D). Large vessels are located on the surface of the cortex, and fine vessels are in the center of the cerebellum (Figure 3E). Thus, to optimize the quality of the modelling beforehand, we split the vascularization into two networks based on a threshold of voxel intensity. All the positive voxels were considered as the "superficial network" and all the negative voxels represented the "deep network", which includes all the vessels in the organ's core (Figure 3F).

The second step of the protocol involves defining the diameter range to be considered. These diameters constitute the detection threshold for branching points, defined as the junctions between each vascular segment. The network splitting allowed more homogeneity of the vessel diameters. However, we chose a *Multiscale* option to guarantee the detection of all vessels. Thus, a diameter range of [1 - 10µm] was chosen for the "deep network" and a [1 - 20µm] range for the "superficial network."

The third step of the algorithm requires training the artificial intelligence (AI) in three different phases. In the first phase, the aim is to define the threshold for detecting seedpoints. This step is crucial because it enables the subsequent training of the software to classify these points and then generate the segments. So the wider the range is, the more efficient the training will be (Figure 3G). In the second phase, based on the threshold, the AI predicts keeping or discarding seedpoints. The AI can be further trained by correcting potential choice errors until a satisfactory result is achieved, but the correction must not exceed 100 manual points to avoid conflicting information, which could disrupt the training (Figure 3H). With the same method, the third phase consists of classifying and selecting the resulting segments (Figure 3I). Finally, the process is applied to the entire 3D volume (Figure 4A). For each postnatal stage, the same AI parameters were saved and re-used on all normoxic and hypoxic cerebella.

Then, for each network (deep and superficial), a channel was created from the filament modelling via a Matlab macro linked to Imaris (*Image Processing → create channel from Filaments*) and then opened in the Fiji software via the *Image to Fiji* bridge. Thus, the files were converted in 8-bit images, binarized, and saved into *.tiff* and *.nii* formats. Each file is then opened in the VesselVio software and the vascular skeletons were visualized in *.tiff* files to check file conversion before analyzing (Figure 4B). Then, all segmented vasculature datasets were downloaded in *.nii* files for analysis. Finally, a single excel file is created with the specific parameters of each vasculature. In this study, the parameters of interest were i) the total volume, area, length and number of segments for the whole cerebellar network, ii) the mean volume, area, length and radius for all segments, and iii) some specific vessel characteristics such as the branchpoints, endpoints, tortuosity and segment partitioning (Figure 4C).

Effect of IH on the parameters of cerebellum vascularization

Because of the significant inter-individual variability, all the following results were normalized relative to the total volume of the cerebellum.

Concerning the general parameters of the entire vascular network, our IH protocol induced the major modifications at P4 (Figures 5 and 6). Thus, a strong increase in the volume, area and length was observed at P4 in IH condition, indicating a denser network from the very first days

(Figure 5). The higher number of segments, branchpoints and partitioning confirms the increased complexity of the vascularization after hypoxia. In contrast, the tortuosity is decreased at P4 after our protocol, indicating an inhibiting effect on vessel remodeling (Figure 6). Thanks to our analysis workflow (Figure 3F), that makes a differential analysis of the superficial and deep networks possible, we showed that the differences observed for the volume, the length and the partitioning are mainly due to an effect of IH on the superficial network, whereas the increase of the segments and branchpoints is attributable to the deep vessels (Figure 8A). From P8 onwards, all the general IH-induced modifications are attenuated and become mostly non-significant (Figures 5-6), suggesting that hypoxia no longer has an effect at later stages. However, our differential study revealed a specific increase of the deep vessel partitioning at P8, and of the length, area and partitioning of the superficial network at P12 (Figure 8A). At P21, only the number of endpoints is affected by IH (Figure 6) but, by looking at the two networks separately, we showed that this decrease is also present at P8 and mainly affects the deep vessels (Figure 8A).

Meanwhile, the effects of IH on segment characteristics are more persistent and variable between ages. At P4, all mean segment parameters are affected by IH with an increase of the mean volume but a decrease of the area, length and radius (Figure 7). All these variations are mainly due to the superficial network, whose tortuosity is also specifically altered (Figure 8B). At P8, we only observed a continuing higher mean volume of segments after IH in studying both networks together (Figure 7) but the differential analysis showed a decreased mean length of deep segments and an increased mean area of superficial segments, which are cancelled by a non-significant opposite effect of IH on the deep network (Figure 8B). At P12, hypoxia induced a decrease of the mean segment volume, area and length largely attributable to the superficial vessels (Figures 7, 8A, 9). Finally, at P21, only slight alterations were detected with a higher tortuosity of the superficial vascular segments (Figure 8B).

Discussion

An optimized method to visualize the cerebellar vascular network in 3D

Despite the advancements in the study of cerebellar neurogenesis, we still lack knowledge regarding its angiogenesis during development. This lack of information is mainly due to the difficulties in accessing the cerebellar depths with conventional imaging techniques. Fortunately, the advancement of clearing protocols and light sheet microscopy in the past decade has gradually enabled the 3D visualization of the vasculature of the cerebellum in its earliest stages as well as in later ages. However, the analysis of these 3D images is still difficult because only a few software programs can model and produce quantitative data from such 3D files.

One of the typically used software packages for 3D modelling is IMARIS from Oxford Instruments Company. However, due to the complexity and the extreme variability of cerebellar vasculature, the IA module is not efficient to model the whole network at once. Therefore, a threshold of intensity needs to first be applied to separate vessels into a superficial network containing the large vessels (>20-µm diameter) and a deep network consisting of the small deep capillaries. Then machine learning can be used to optimize modelling, but these learning steps

must be limited because errors quickly increase due to the accumulation of conflicting information. Furthermore, although this software is the most efficient for modelling, its statistical tools are not adapted to the study of vascular networks. It is therefore necessary to turn to other software solutions to obtain quantitative data. Vesselvio (Bumgarner and Nelson, 2022) proved to be a good solution, as each vascular parameter is well defined, it is free, and it has already been used in other vascular studies. Therefore, by numerous steps of optimization and software tests, we created here a practical, optimal, and user-friendly workflow for 3D vascularization analysis.

Early changes

Our innovative approach to 3D image analysis enabled us to assess the evolution of several vascular parameters in response to our hypoxia protocol. The most changes in the cerebellar vascular network features appear at the P4 stage, after only two days of hypoxia. Overall, the network of IH mice has a higher volume, length and surface area than the control animals mostly contributed to by the superficial network. This increase is consistent with the upregulation of several factors promoting vessel growth such as vascular endothelial growth factor a (Vegfa; Molina et al., 2013) and its Vegf receptor Flk1 (Huang et al., 2015; Wang et al., 2014), transforming growth factor beta 1 (Tgfb1; Molina et al., 2013) or F3 (Peña et al., 2017). This suggests that an acute response to hypoxia at this early stage involves grossly increasing vascularization to compensate for IH exposure. In contrast, the decreased tortuosity of vessels in IH mice, especially in the superficial network, could suggest less remodeling. This hypothesis can be supported by the overexpression of two negative regulators of vascular invasion and sprouting (VIS), namely Flt1 (Nesmith et al., 2017; Wild et al., 2017) and Thbs1 (Lawler and Lawler, 2012).

The P4 enlarged network is composed of more numerous segments with more branchpoints, especially in the deep network, accompanied by an increased segment partitioning, predominantly in the superficial network. However, the vascular segments of IH mice are shorter and thinner, predominantly in the superficial network. This observation supports the early emphasis on increasing the network rather than refining it after hypoxia, and suggests that hypoxia-induced angiogenesis (HIA) leads to a more complex but less mature vascular network. This hypothesis also correlates with the high upregulation of Vegfa which is a known actor of HIA (Nesmith et al., 2017) as well as of Angpt2 which promotes EEP in response to hypoxia (Trollmann et al., 2018; Vates et al., 2005) and of F3 which stimulates neovascularization (Heidari et al., 2024; Peña et al., 2017).

Vascular adaptation during IH

In contrast to P4, no significant difference in general network parameters was observed at P8, and only the increase in the mean segment volume is maintained after 4 days of IH. In parallel, all tested genes were downregulated in all functional groups, including the previously upregulated growth factors, but also a large panel of positive regulators of angiogenesis. Among them, we can notice the strong under-expression of the angiogenic receptor Tek and its regulator Tie1 (Lee et al., 2009; Savant et al., 2015), of metalloprotease 2 (MMP2; Quintero-Fabián et al., 2019; Sang, 1998), of Anpep (Rangel et al., 2007) and of Serpine 1 (Zhang et al., 2023), which are all known to be implicated in HIA. This result suggests that the initial acute

angiogenesis triggered at the beginning of the protocol in response to IH has ceased by P8. In accordance with this hypothesis, our differential study across networks reveals that the length and the surface area of the superficial vessels increase with a slight decrease of the partitioning. This indicates that the network is still growing but is less complex with fewer vessel interconnections. In contrast, a remodelling process is ongoing in the depth of the cerebellum as an increase of the vessel partitioning associated with a decrease of the number of endpoints and of the mean segment length are observed. This could be linked to the under-expression of some negative regulators of VIS, namely Flt1 and Thbs1 (Lawler and Lawler, 2012; Nesmith et al., 2017; Wild et al., 2017) or of endothelial cell mechanisms such as Serpinf1 (He et al., 2015; Tombran-Tink, 2005) or Angpt1 (Lee et al., 2009; Zacharek et al., 2007).

At P12, like at P8, general parameters are not altered but we see a decrease of the mean segment volume and area, suggesting that the overall shape of blood vessels stabilizes during the protocol. However, while the superficial vascular network was only slightly affected at P8, it shows several hypoxia-related alterations at P12, namely an increase in length, area and partitioning. This complexification of the network could be explained by a partial switch in the expression of several genes. Indeed, while some pro-angiogenic factors remain under-expressed, others become over-expressed such as the matrix metalloproteases MMP2, MMP9, and Angpt2, which make the ECM more permissive to vessel endothelial tip cells (Cai et al., 2017; Quintero-Fabián et al., 2019; Sang, 1998; Vates et al., 2005). In addition, norrin (Ndp), which was previously not affected by IH, becomes under-expressed, magnifying the effect on the matrix (Luhmann et al., 2008; Ohlmann et al., 2010; Ohlmann and Tamm, 2012; Zhang et al., 2017). Taken together with the upregulation of Fgf2, Pgf and Tgfb1, involved in EPS and VSM, this reveals the emergence of a second phase of IH response during the protocol (Lee et al., 2023; Luna et al., 2016; Molina et al., 2013).

Ten days after the end of the IH protocol, P21 IH mice present a lower number of vessel endpoints than their normoxic counterparts, largely attributable to the deep network. This coincides with the emergence of newly downregulated VIS factors such as the critical Angpt2 (Raybaud, 2010) but also F3 (Heidari et al., 2024; Peña et al., 2017), Fgf2 (Mori et al., 2017), as well as Cdh5 (Bentley et al., 2014; Nan et al., 2023; Wallez et al., 2006) at P70. At P21, the cerebellum is also characterized by a higher tortuosity of superficial vascular segments, indicating that, despite compensatory mechanisms occurring at the end of the IH protocol, minor alterations to the vascular network remain visible in the long term. According to this observation, the majority of genes under-expressed at P21 are still under-expressed at P70, including Flk1, Tek, and Vegfa (Nesmith et al., 2017; Zacharek et al., 2007).

Vasculogenesis and cerebellar development during IH

The brain, being so energy demanding, is particularly dependent on vascular function, thus its growth is hindered by poor blood supply (Donkelaar, 2014). Our results confirm that it is also the case for the cerebellum, as they demonstrate that IH induces an alteration of the cerebellar angiogenesis associated with a delay in cerebellar maturation (Leroux et al., 2022). Moreover, beyond the classical role of the vasculature as O_2 supplier, it is now well known that the crosstalk between blood vessels and nerve cells is also critical for various cell processes which occur during brain development such as migration, differentiation or precursor proliferation (Segarra et al., 2019). As cerebellar development takes place mainly during the

postnatal period in both mice and humans, the vascular abnormalities induced by our IH protocol could lead to the cellular disorganization observed at P12 in mice that underwent hypoxia (Leroux et al., 2022).

Moreover, across the literature, we can also observe that neurons and endothelial cells share a large array of cellular pathways (Larrivée et al., 2009; Weinstein, 2005). It follows that we cannot exclude that both neural and vascular systems are affected in parallel by IH. For example, NRP1 is known to be a guidance cue for both neurons and blood vessels (Fantin et al., 2009; Vieira et al., 2007) by either binding VEGFA (Erskine et al., 2011) or SEMA3A (Eichmann et al., 2005) and the expression of these three factors are significantly decreased during our IH protocol (Rodriguez-Duboc et al., 2023). We can also cite the matrix metalloproteases, which are involved, not only in brain angiogenesis (Girolamo et al., 2004) but also in the morphogenesis of the cerebellar cortex (Luo, 2005).

From a cellular standpoint, the parallels between vascularization and neurite development also confirm our previous findings on Purkinje cell arborization. Indeed, the angiopoietin 2 (Angpt2)/Tie1 pathway was highly regulated during our protocol. This system is well known to participate in vessel formation (Augustin et al., 2009) but has been more recently described as a regulator of dendritic tree development in Purkinje cells (Luck et al., 2021). Given our findings on the alterations of Purkinje cell morphogenesis after IH (Leroux et al., 2022; Rodriguez-Duboc et al., 2023), this cements the idea of a parallel alteration of neurogenesis and angiogenesis during IH. Finally, despite few observable differences in the vascular network of IH mice at P21, the expression of numerous genes implicated in this system is still dysregulated and may contribute to behavioral, histological and neuronal deficits we have shown in adults after our IH protocol (Leroux et al., 2022; Rodriguez-Duboc et al., 2023).

Conclusion

The present study confirms that neurogenesis and angiogenesis are extremely interlocked during cerebellar postnatal development as during brain embryogenesis. Moreover, both systems are affected by a perinatal IH with the alteration of both vascular network shape and cerebellar cortex histogenesis. Thus, our results indicate that, in pathologies such as apnea of prematurity mimicked by our protocol (Cai et al., 2012), the two components participate in short and long-term deficits observed in patients. More generally, when treating patients with neurodevelopmental disorders, it would be a mistake to focus therapeutic approaches solely on neurological damage, without taking into account the vascular aspect.

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