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Supplementary Materials and Methods

Animals. Animal work was carried out following UK Home Office and local ethical guidelines. To obtain embryos of defined developmental stages, mice were paired overnight and the day of finding a copulation plug was designated embryonic day (e) 0.5. To stage-match embryos within a litter and between litters, we compared the number of somite pairs (**Supplemental Table 1**). CD1 and C57BL/6 mice were obtained from Charles River Laboratories, UK. *Nrp1*-null mice on a CD1 background (1) and conditional endothelial- and NPC-specific NRP1-null mice on a C57/Bl6 background have been described previously (2). In some experiments, pregnant females were injected intraperitoneally with 10 mg/kg BrdU either 1 or 24 h before embryos were removed. *Sox1-iCreER^{T2}*mice were generated by pronuclear injection of a modified 197 kb mouse genomic PAC clone (RPCI21 502 P07) spanning 94 kb upstream and 103 kb downstream of *Sox1* and in which the *Sox1* open reading frame was replaced with an iCreER^{T2}-SV40-polyA cassette (3) using a previously published method (4). For mosaic labelling of hindbrain NPCs, pregnant *Rosa^{tdTomato}* females, following mating to *Sox1-iCreER^{T2}* males, received 20 mg/kg tamoxifen diluted in peanut oil by oral gavage 24 h before embryos were removed.

Fluorescence staining and imaging. Hindbrains were fixed for 2 h in 4% formaldehyde in PBS. In some experiments, hindbrains were embedded in 3% agarose and sectioned using a vibratome (70 μm) or embedded in OCT (Agar Scientific) and sectioned on a cryostat (10 μm). Hindbrains, floating sections and cryosections were incubated for 1 h at 4ºC in PBS containing 0.1% Triton X-100 (PBT) and 10% serum-free block (DAKO). Samples were incubated with primary antibodies in PBT at 4ºC overnight, washed in PBT and incubated in secondary antibodies in PBT at room temperature (RT) in the dark for 2 h. Labelled samples were counterstained with DAPI for 1 min, postfixed in 4% formaldehyde for 20 min and mounted on glass slides using SlowFade (Life Technologies) or Mowiol (Sigma). For BrdU staining, samples were incubated for 10 min each in 1N HCl at RT, followed by 2N HCl at RT and then 2N HCl at 37ºC. Samples were neutralised by washing twice in 0.1 M sodium tetraborate and three times in PBT for 5 min each. The following antibodies were used: rabbit α-phospho-histone H3 (pHH3; 1:400, Millipore, cat. no. 06-570), rat anti-BrdU (1:250, Abcam, cat no. ab6326), mouse anti-RC2 (1:10, Developmental Studies Hybridoma Bank, DSHB), rabbit anti-LAMA1 (1:30, Sigma, cat. no. L9393), rabbit anti-red fluorescent protein (RFP; 1:500, MBL, cat. no. PM005), rabbit anti-cleaved caspase 3 (1:200, Cell Signalling, cat. no. 9661), rabbit TUJ1 (1:250, BioLegend, cat. no. PRB-435), mouse anti-Ki67 (1:100, BD Pharmingen, cat. no. 550609) and rabbit anti-GLUT1 (1:200, Millipore, cat. no. 07-1401). We used AlexaFluor-conjugated secondary antibodies appropriate for the species of the primary antibody (1:200; Thermo Fisher). For blood vessel staining, we used biotinylated IB4

(1:200, Sigma) followed by AlexaFluor-conjugated streptavidin (1:200, Thermo Fisher). Labelled samples were imaged with an LSM710 confocal microscope (Zeiss, Jena) and processed with Photoshop CS6 (Adobe, Inc.). 3D reconstructions of z-stacks were performed with Imaris (Bitplane).

Quantification of immunolabelling. Quantification of phenotypes in confocal z-scans was performed with the count tool in Photoshop CS6. The number of $pHH3^+$ NPCs and $IB4^+$ endothelial tip cells was determined in two representative areas of 0.25 and 0.2 mm^2, respectively, in the anterior portion of each hindbrain hemisegment, and the 4 readings were averaged to obtain the value for that hindbrain. The number of $Ki67^+$ NPCs, $BrdU^+$ NPCs, ratio of $Ki67^+/BrdU^+$ NPCs and the area of TUJ1 labelling were determined in 3 cryosections (10 μm) for each hindbrain, and the 3 readings for each embryo were averaged to obtain the value for that hindbrain. Quantification of cleaved caspase 3^+ cells was performed similarly, but using 5 sections per hindbrain due to the overall low number of apoptotic cells at the developmental stages examined. To quantify the level of GLUT1 staining in the neural parenchyme, the pixel area of GLUT1 staining that was also positive for IB4 was subtracted from the pixel area of total GLUT1 staining to exclude the endothelial contribution to GLUT1 staining; the resulting value was then normalised to the overall area of the hindbrain cross-section. GLUT1 staining was determined in 3 cryosections for each hindbrain. Data shown in each graph are the average of 3 or more hindbrains per genotype and time point (see **Supplemental Table 2** for a complete list of the number of embryos analysed).

RNA extraction and qRT-PCR. Hindbrains were dissected from e10.5 embryos and total RNA was isolated using the RNeasy Micro Kit (Qiagen). cDNA was synthesised from isolated RNA by reverse transcription using Superscript IV (Thermo Fisher). qRT-PCR was performed with 50 ng cDNA on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems) and 0.45 μg of the following oligonucleotide pairs (Sigma-Aldrich): *Hif1a* 5'-AAACCACCCATGACGTGCTT-3' and 5'-GAGCGGCCCAAAAGTTCTTC-3', amplicon 182 bp; *Vegfa* 5'-CAGATCATGCGGATCAA-3' and 5'-TTGTTCTGTCTTTCTTTG-3', amplicon 100 bp; *Actb* 5'-AAGGCCAACCGTGAAAAGAT-3' and 5'-GTGGTACGACCAGAGGCATAC-3', amplicon 110 bp. For each gene, every sample was analysed in triplicate, and a no-template control was also included for each oligonucleotide pair. Data were collected using Sequence Detector Software (SDS version 2.2; Applied Biosystems), and expression levels were extrapolated using DART-PCR software (5) after normalisation to *Actb* expression levels.

Statistical analysis. To determine whether two datasets were significantly different, we determined

the *P* value with a two-tailed, unpaired *t* test using Excel (Microsoft). Statistical significance is reported in the figures (ns, $p \geq 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Supplemental References

1. Kawasaki T, *et al*. (1999) A requirement for neuropilin-1 in embryonic vessel formation. *Development* 126(21):4895-4902.
2. Fantin A, *et al*. (2013) NRP1 acts cell autonomously in endothelium to promote tip cell function during sprouting angiogenesis. *Blood* 121(12):2352-2362.
3. Claxton S, *et al*. (2008) Efficient, inducible Cre-recombinase activation in vascular endothelium. *Genesis* 46(2):74-80.
4. Lee EC, *et al*. (2001) A highly efficient Escherichia coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. *Genomics* 73(1):56-65.
5. Peirson SN, *et al*. (2003) Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis. *Nucleic Acids Res* 31(14):e73.

Supplementary Tables

Supplementary Table 1: Stage matching of mouse embryos. Embryos were stage matched by comparing the number of their somite pairs. The corresponding embryonic day post-conception (E) and average number of somite pairs (s) are shown for wildtype CD1 and C57Bl/6 mice.

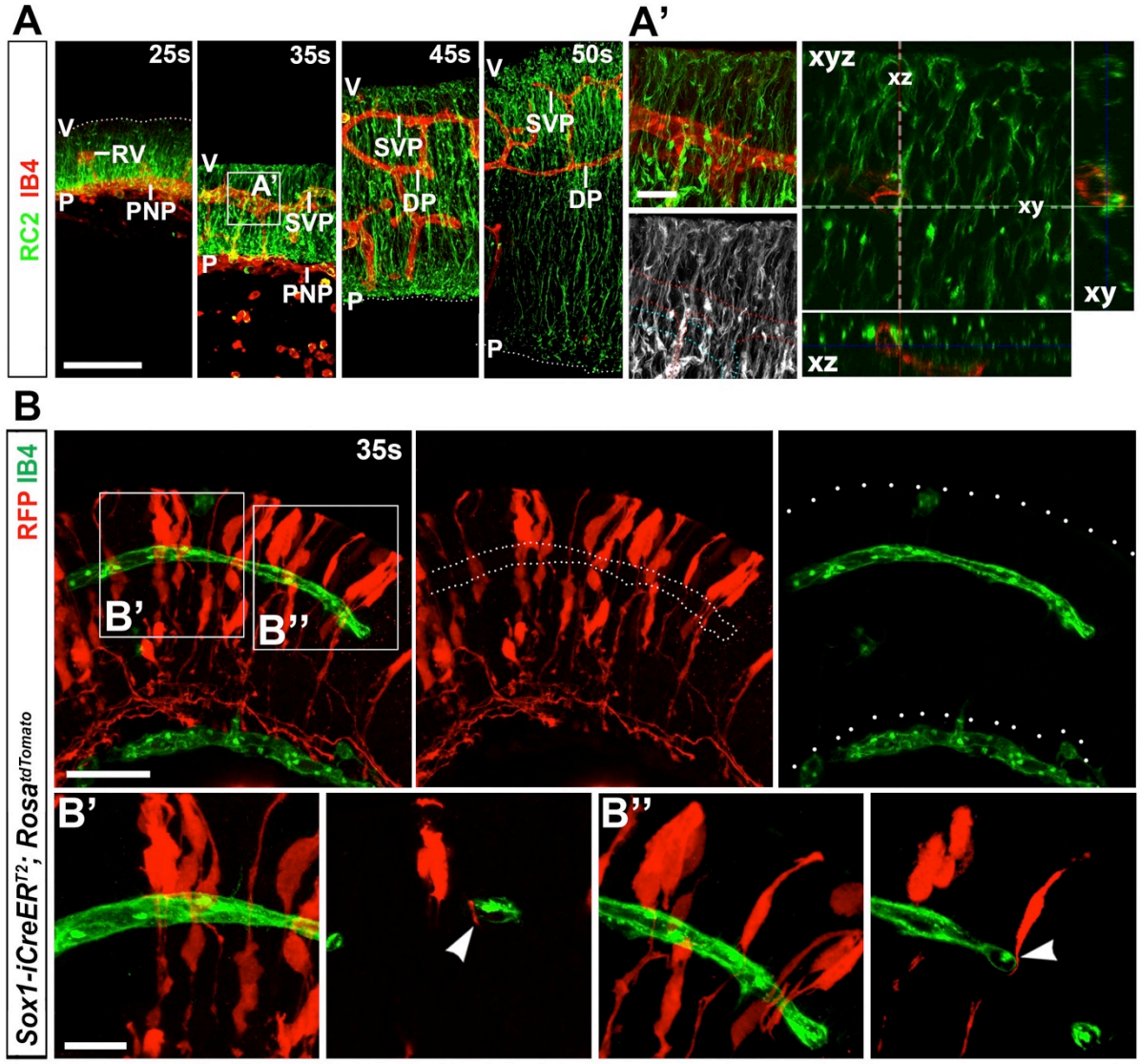
Wildtype embryonic day (dpc)	9.5	10.5	11.5	12.5
Average number of somite pairs (s)	25	35	45	50

Supplementary Table 2: Sample sizes.

Number of embryos of the indicated genotypes analysed for each experiment.

Figure	Genotype	Developmental stage (sample size)
4B	*Nrp1*$^{+/+}$	32s (6), 36s (3), 40s (4)
	Nrp1$^{-/-}$	32s (4), 36s (4) 40s (5)
4C	*Nrp1*$^{+/+}$	32s (4), 36s (3), 40s (4)
	Nrp1$^{-/-}$	32s (3), 36s (4), 40s (5)
4E	*Nrp1*$^{+/+}$	25s (4), 32s (3), 36s (3), 40s (3), 42s (3) 46s (5)
	Nrp1$^{-/-}$	25s (3), 32s (6), 36s (3), 40s (4), 42s (6), 46s (3)
4G	*Nrp1*$^{c/+}$	36s (3), 42s (5), 45s (6), 49s (3)
	Nrp1$^{c/-}$ *Tie2-Cre*	36s (5), 42s (3), 45s (7), 49s (7)
4H	*Nrp1*$^{c/+}$	36s (3), 42s (6), 51s (3)
	Nrp1$^{c/-}$ *Nes-Cre*	36s (3), 42s (4), 51s (3)
5C	*Nrp1*$^{+/+}$	32s (3)
	Nrp1$^{-/-}$	32s (3)
	Nrp1$^{c/-}$ *Tie2-Cre*	32s (4)
S3	*Nrp1*$^{+/+}$	32s (3), 40s (4)
	Nrp1$^{-/-}$	32s (3), 40s (3)
S5	*Nrp1*$^{+/+}$	36s (3)
	Nrp1$^{-/-}$	36s (3)
S8	*Nrp1*$^{+/+}$	46s (3)
	Nrp1$^{-/-}$	46s (3)
S9A	*Nrp1*$^{+/+}$	32s (4)
	Nrp1$^{-/-}$	32s (3)
S9D	*Nrp1*$^{+/+}$ 20% O_2	32s (3)
	Nrp1$^{-/-}$ 20% O_2	32s (3)
	Nrp1$^{-/-}$ 80% O_2	32s (3)

Supplementary Figures and Figure legends

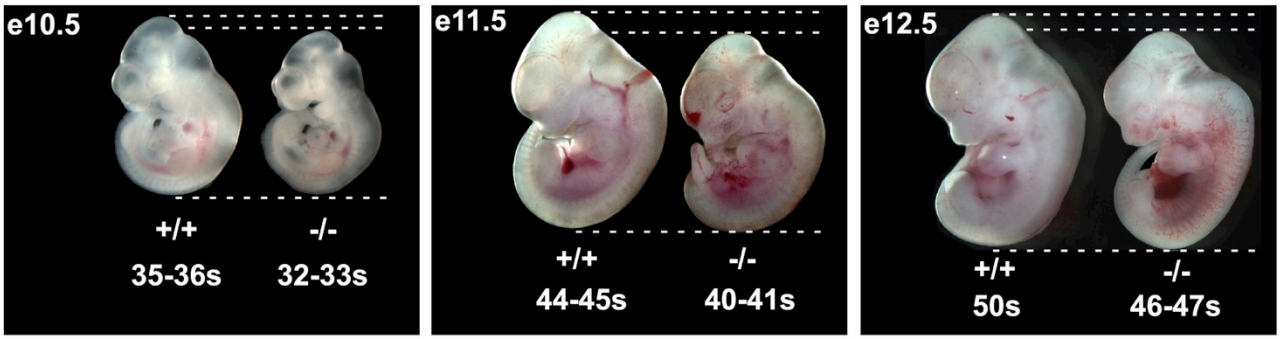


Supplementary Figure 1: NPC processes contact hindbrain vasculature.

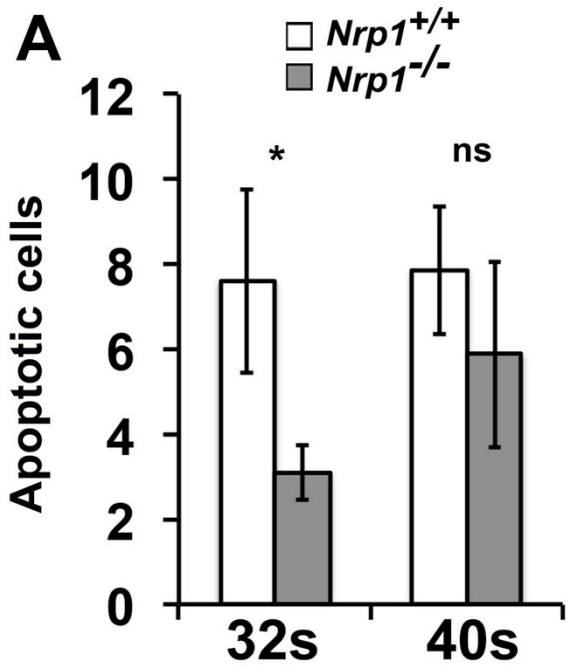
(**A**) Maximal projections (xy) of confocal z stacks of 70µm transverse wildtype hindbrain sections at the indicated stages, labelled with IB4 for blood vessels and antibodies for the radial glia marker RC2. Scale bar: 100 µm. The boxed area in (**A**) is shown at higher magnification in (**A'**), with RC2 labelling only shown in grey scale in the lower left panel; outlines indicate position of blood vessel. Single (1.25 µm) optical section of the z stack shown in right panel. Flanking boxes display orthogonal xy and xz projections of single optical section; note how RC2^{+} densities directly contact IB4^{+} vessels. Scale bar: 20 µm.

(**B**) Maximal projections (xy) of confocal z stacks of a 70µm transverse sections of a *Sox1-iCreER^{T2};Rosa^{tdTomato}* 35s hindbrain after induction with 20mg/kg of tamoxifen for 24 hours, labelled with IB4 and antibodies for RFP to detect the tomato reporter. Dotted lines in the middle panel demarcate position of an SVP vessel, dotted lines in the right-hand panel demarcate the hindbrain surfaces. The boxed areas in (**B**) are shown at higher magnification in (**B, B''**), as a confocal z-stack (left panel) or single (1.25µm) optical section of each z stack (right panel). Arrowheads indicate RFP^{+} NPC processes contacting an SVP vessel. Scale bar: 50 µm (**B**), 20 µm (**B', B''**).

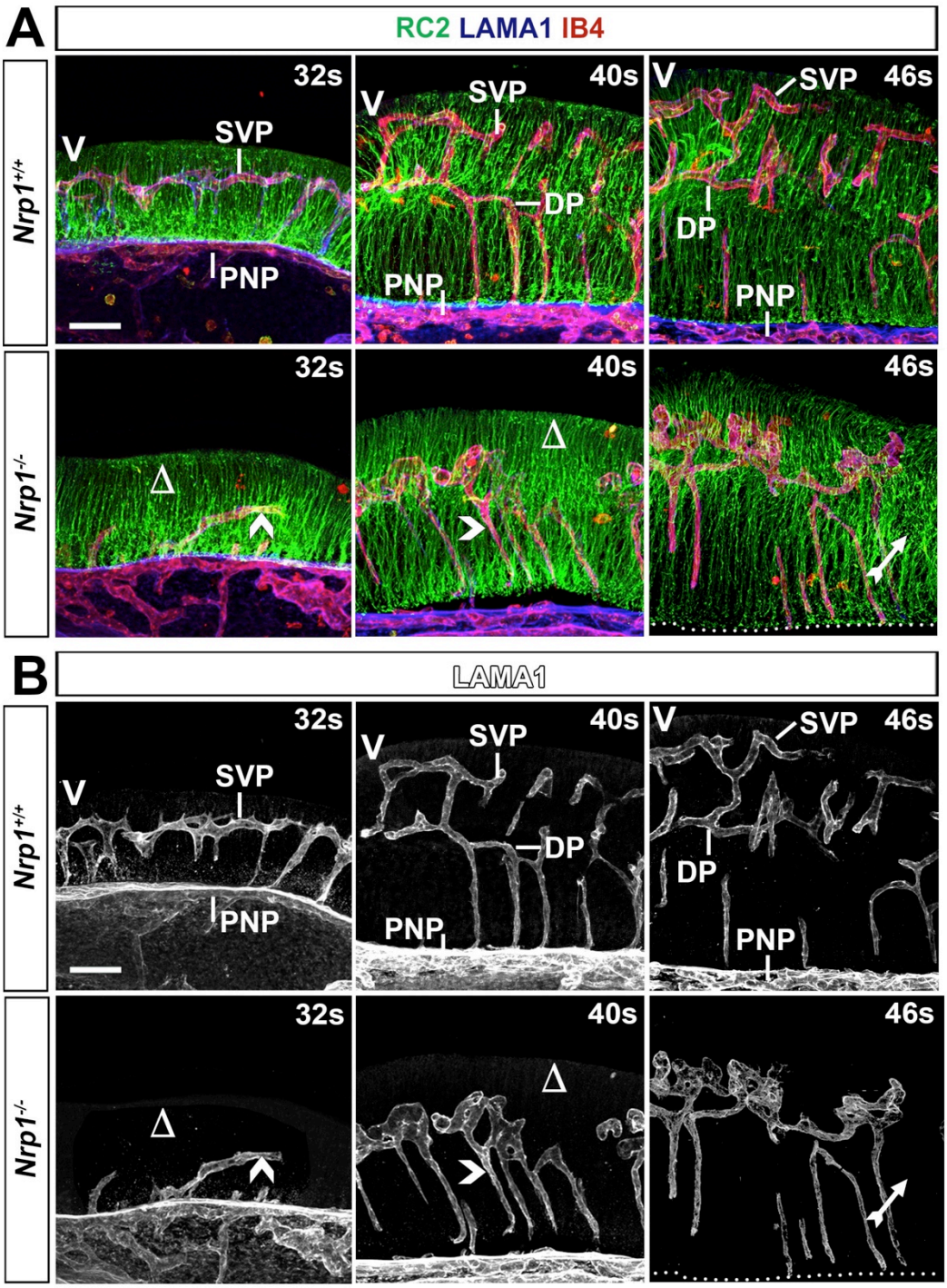
Abbreviations: P, pial surface; V, ventricular surface; RV, radial vessels; SVP, subventricular vascular plexus; DP, deep plexus; PNP, perineural vascular plexus; GZ, germinal zone.



Supplementary Figure 2: *Nrp1*$^{-/-}$ embryos are developmentally delayed.
Representative brightfield images of wildtype (*Nrp1*$^{+/+}$) and *Nrp1*$^{-/-}$ littermates in the CD1 background at the indicated ages. The typical range of somite pairs at that age is shown for each genotype below the image of the embryo. Dotted lines highlight the reduced crown-rump length of mutants.



Supplementary Figure 3: Cell survival is not compromised in *Nrp1*$^{-/-}$ hindbrains.
Quantification of apoptotic (cleaved caspase 3^{+}) cells per 10 μm transverse hindbrain section at 32s and 40s. Data are the mean ± standard deviation of the mean; n≥3 for each genotype; ns, not significant, p≥0.05, * p<0.05.



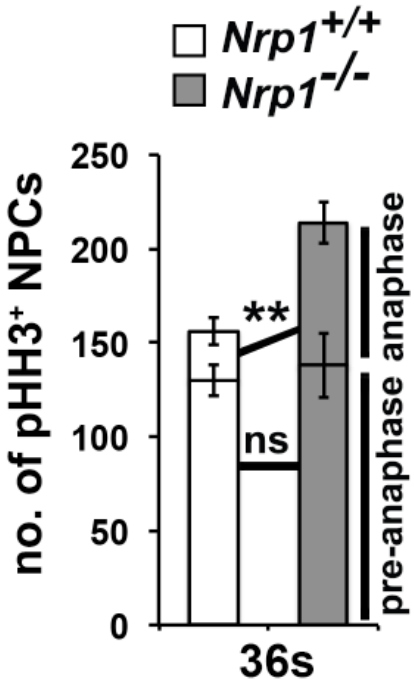
Supplementary Figure 4:

RC2^{+} processes and laminin expression in NRP1-deficient hindbrains.

(**A**) Maximal projections (xy) of confocal z stacks of 70µm transverse sections from stage-matched wildtype and *Nrp1*$^{-/-}$ hindbrains after labelling with IB4 and antibodies for RC2 (NPC processes) and LAMA1 (extracellular matrix).

(**B**) LAMA1 staining is preserved in the vasculature of NRP1-deficient hindbrains. **Δ** denotes avascular regions; chevrons indicate abnormally orientated vessels; the fletched arrow indicates a region of NPC process disorganisation.

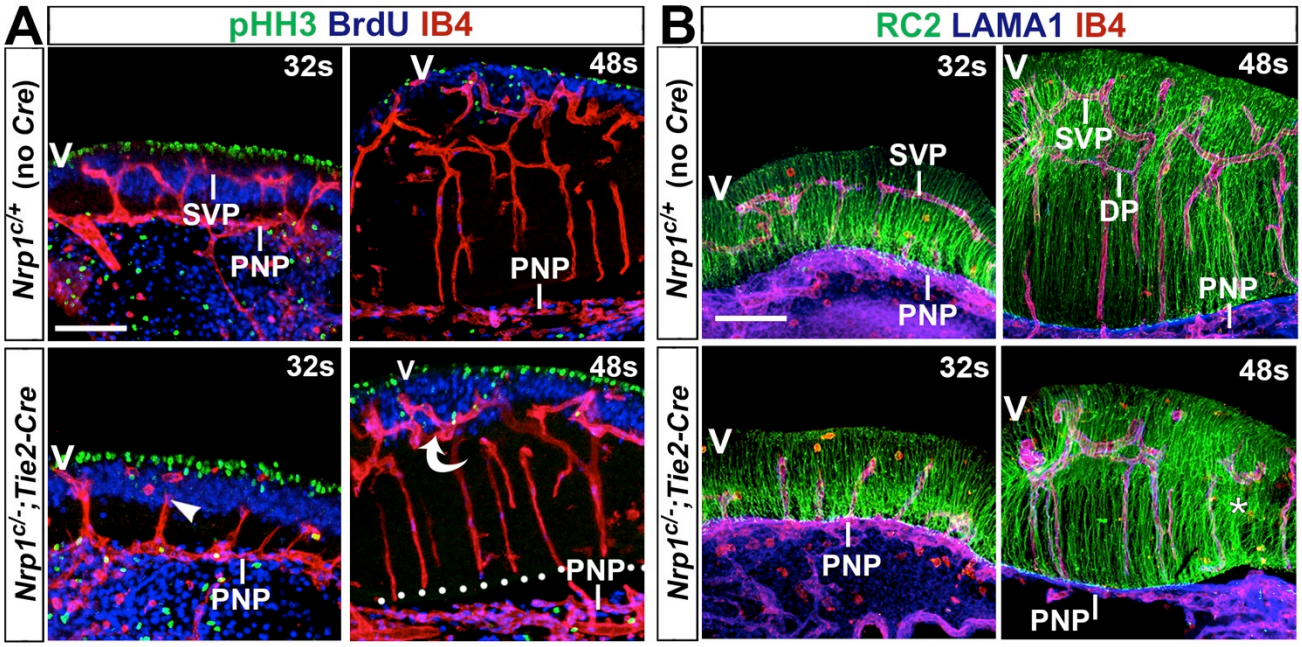
Abbreviations: P, pial surface; V, ventricular surface; SVP, subventricular vascular plexus; DP, deep plexus; PNP, perineural vascular plexus; GZ, germinal zone. Scale bar: 100µm.



Supplementary Figure 5:

A significant proportion of NPCs stalls in anaphase in 36s *Nrp1*$^{-/-}$ hindbrains.

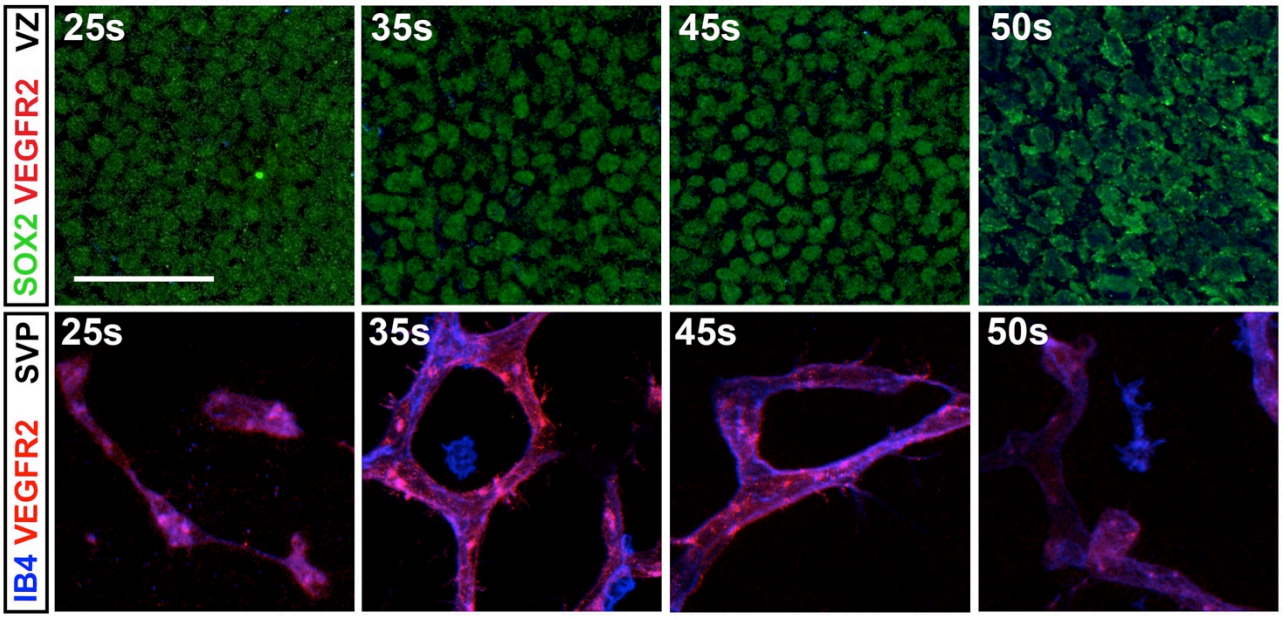
Quantification of the density of pre-anaphase and anaphase mitotic NPCs in stage-matched control and *Nrp1*$^{-/-}$ hindbrains at 36s, determined by wholemount pHH3 immunolabelling of the hindbrain VZ (see far-right panel in **Fig. 1E**). Data are expressed as mean ± standard deviation of the mean; n≥3 for each genotype; ns, not significant $p \geq 0.05$, ** $p < 0.01$.



Supplementary Figure 6:

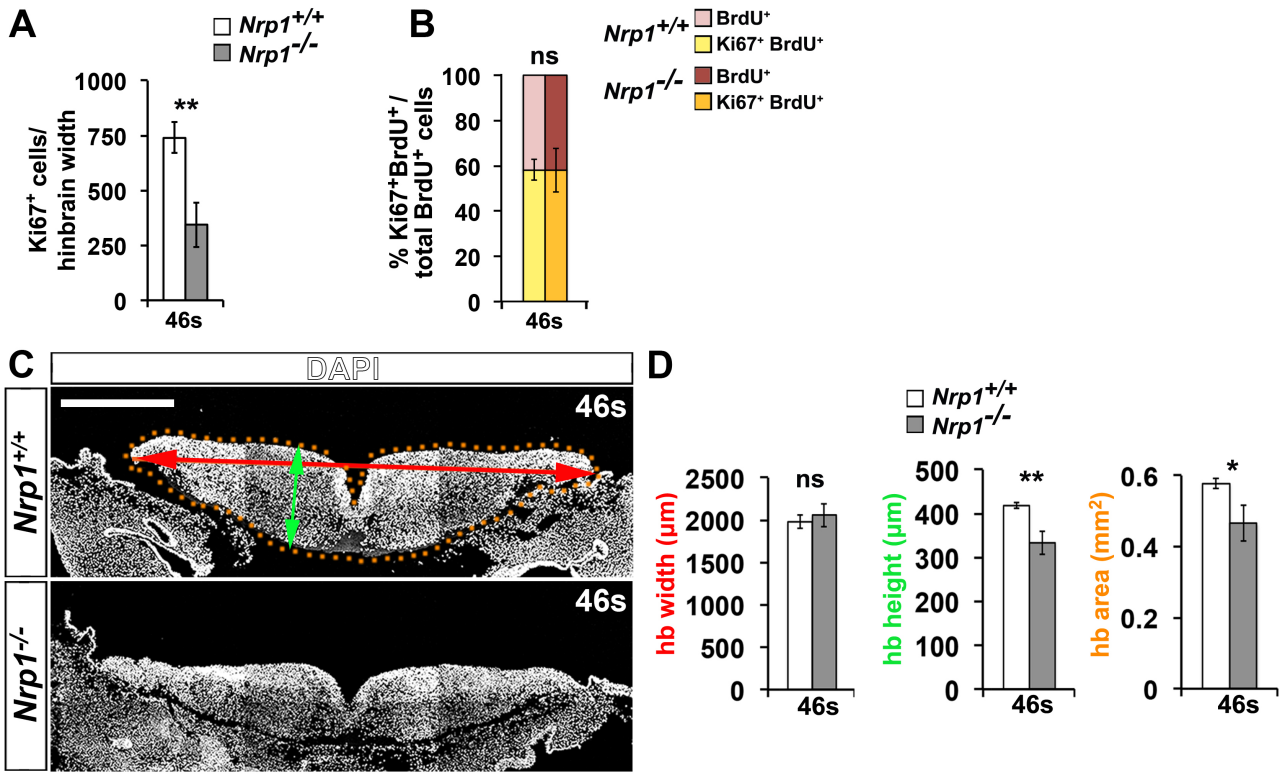
Endothelial NRP1 loss impairs hindbrain vascularisation, but not RC2 organisation.

Maximal projections (xy) of confocal z stacks of 70µm transverse sections of stage-matched control and *Tie2-Cre;Nrp1*$^{c/-}$ hindbrains at the indicated stages after labelling with IB4 and antibodies for pHH3 and BrdU (**A**) or antibodies for RC2 and LAMA1 (**B**). Arrowhead in (**A**) indicates blind-ended radial vessel; the asterisk in (**B**) indicates a region where NPC processes appear disorganised. The dotted line in (**A**) demarcates the pial hindbrain boundary at 48s; separation of the hindbrain parenchyme from the PNP in the perineural membrane is a sectioning artefact. Abbreviations: P, pial surface; V, ventricular surface; SVP, subventricular vascular plexus; DP, deep plexus; PNP, perineural vascular plexus. Scale bar: 100 µm.



Supplementary Figure 7: VEGFR2 is expressed by vessels, but not NPCs in the hindbrain.

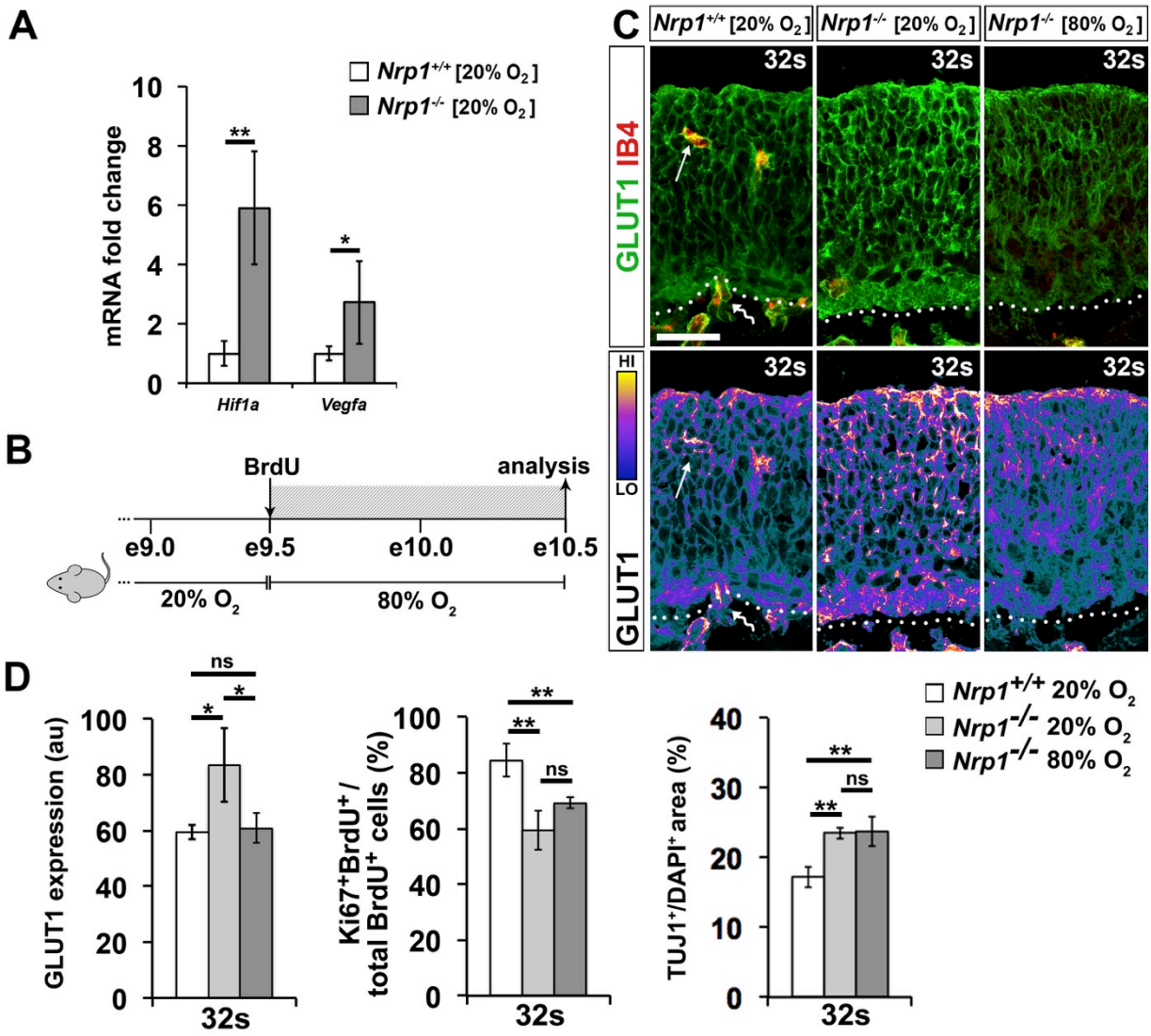
Maximal projection (xy) of confocal z stacks of mouse embryo hindbrains at the indicated stages after labelling with the dual vessel and microglia marker IB4 together with antibodies for SOX2 to detect NPC nuclei. Flatmounted hindbrains were, imaged at the level of the VZ (top panels) or SVP (bottom panels) for each hindbrain and at each stage. Abbreviations: VZ, ventricular zone; SVP, subventricular vascular plexus. Scale bar: 100μm.



Supplementary Figure 8: Loss of hindbrain NPCs correlates with stunted hindbrain growth.

(**A,B**) NRP1-deficient hindbrains contain fewer cycling NPCs at 46s. Quantification of the total number of Ki67^{+} cells per section (**A**) and proportion of Ki67^{+}BrdU^{+} in all BrdU^{+} cells (**B**) in 46s control and *Nrp1*$^{-/-}$ hindbrains. For this analysis, embryos were injected with BrdU and analysed hindbrains 24 h later. Data are expressed as mean ± standard deviation of the mean; n=3 embryos for each genotype; ** $p<0.01$; ns, not significant $p\geq0.05$.

(**C,D**) Reduced growth of *Nrp1*-null hindbrains. Maximal projections of confocal tile scan z stacks of 10 µm transverse sections through 46s wildtype and *Nrp1*-null DAPI-stained hindbrains (**C**) including arrows to illustrate measurements taken to determine hindbrain lateral width (red line), radial height (green line) and cross-sectional area (orange dotted outline). (**D**) Quantification of lateral width, radial height and area of hindbrain (hb), expressed as mean ± standard deviation of the mean; n=3 for each genotype; ns, not significant, $p\geq0.05$, * $p<0.05$, ** $p<0.01$. Scale bar: 100µm.



Supplementary Figure 9: The SVP does not regulate NPCs by relieving tissue hypoxia.

(**A**) Increased expression of the hypoxia-regulated genes *Hif1a* and *Vegfa* in 32s *Nrp1*-null hindbrains. Expression levels were determined by qPCR analysis, normalised to *Actb* and expressed as fold change compared to stage-matched wildtype hindbrains. Data are expressed as mean ± standard deviation of the mean, n≥3 for both genotypes; * p<0.05, ** p<0.01.

(**B-D**) Rescue of hindbrain hypoxia, but not NPC self-renewal defects, in 32s *Nrp1*-null hindbrains by housing pregnant dams in a hyperoxic atmosphere. (**B**) Schematic representation of the hypoxia rescue experiment. Pregnant dams received an intraperitoneal BrdU injection when their embryos where at e9.5 and were then transferred from normoxia to an 80% oxygen atmosphere for 24 hours before analysis e10.5. (**C**) 10 µm transverse sections from hindbrains of 32s wildtype and *Nrp1*$^{-/-}$ embryos from dams housed in 20% oxygen and *Nrp1*-null embryos from dams in an 80% oxygen environment, labelled for GLUT1 (green) and IB4 (red). 'Heatmap' for GLUT1 staining shown in lower panels. Scale bar: 25 µm. Arrows and wavy arrows indicate the GLUT1^{+} SVP and PNP, respectively. (**D**) Hyperoxia rescues oxygenation of *Nrp1*-null hindbrains (left-hand graph; arbitrary units, au). NPC self-renewal (percentage of Ki67^{+}BrdU^{+} double-labelled NPCs) and neuronal differentiation (TUJ1^{+} hindbrain area), shown in middle and right-hand graphs, respectively). Data are expressed as mean ± standard deviation of the mean; n=3 for each genotype and oxygen level; ns, not significant, p≥0.05, * p<0.05, ** p<0.01.