SUPPLEMENTAL MATERIAL

DETAILED METHODS

CELL CULTURE: Commercially available bEND3 cells (ATCC® CRL-2299™, Molsheim, France) were cultured in high glucose DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1% penstrep (100 IU/ml penicillin and 100 µg/ml streptomycin), unless indicated otherwise. Human retinal microvascular endothelial cells (HRMV ECs) (Cell Systems, Kirkland, WA) were cultured in Endothelial Cell Growth Medium 2 (EGM2) (C-22011, Lonza, Braine-l'Alleud, Belgium). HUVECs were freshly isolated as previously described[5] and cultured in Endothelial Cell Growth Medium-2 (EGM2) (CC-3162, Lonza, Basel, Switzerland). Human primary brain endothelial cells were isolated from clinical brain specimens, obtained from the Department of Neurosurgery, University Hospital Zurich, from anonymous glioblastoma patients and from the temporal lobe of anonymous pharmacoresistant epilepsy patients. All procedures were conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Canton Zurich. Isolated human brain endothelial cells, commercially available human brain microvascular (HBMV) ECs (#1000, ScienCell, Basel, Switzerland) were cultured in Endothelial Cell Growth Medium-2 (EGM2) (CC-3162, Lonza, Basel, Switzerland). Isolated primary mouse endothelial cells from skeletal muscle, lung and brain were cultured in 50% EGM2 (CC-3162, Lonza, Basel, Switzerland) + 50% full M199 medium (31150030, Invitrogen, Zurich, Switzerland) containing endothelial cell growth factor supplements (ECGS; 30 mg/l) (E2759, Sigma-Aldrich, Darmstadt, Germany), 10 units/ml heparin (H3149, Sigma-Aldrich, Darmstadt, Germany), 1% penstrep (15140122, Thermo Fisher Scientific, Zurich, Switzerland), 20% FBS. Inhibitor experiments were performed by incubating cells the day

after seeding with BAY-876 (SML1774, Sigma-Aldrich, Overijse, Belgium), cytochalasin B (CYT B) (C6762, Sigma-Aldrich, Overijse, Belgium) or their vehicle (i.e. DMSO), for 24 hrs. Mitomycin C (M0503, Sigma-Aldrich, Overijse, Belgium) was used at 1 µg/ml for all proliferation blocking conditions.

MOUSE MODELS: To obtain inducible EC-specific GLUT1 knock out mice, we crossed GLUT1$^{lox/lox}$ mice[23] with Pdgfb-Cre^{ERT2} mice, an EC-selective inducible Cre-driver line[24] to generate GLUT1$^{EC-/-}$ mice upon tamoxifen treatment. Correct Cre-mediated excision of the floxed *Glut1* segment in tamoxifen-treated GLUT1$^{EC-/-}$ mice was confirmed via PCR analysis of genomic DNA using standard procedures. EC-specific GLUT1 deletion was obtained by intraperitoneal administration of 10 mg/kg tamoxifen (T5648, Sigma-Aldrich, Overijse, Belgium) dissolved in 1:10 EtOH:corn oil solution, once daily from P1 to P3 in GLUT1$^{EC-/-}$ neonates, or daily for 4 consecutive days in adult GLUT1$^{EC-/-}$ mice. GLUT1$^{EC-/-}$ mice were compared to tamoxifen-treated Cre negative (-/-) GLUT1$^{lox/lox}$ littermates (denoted as WT). Both males as well as females were included in the experiments, and no animals were excluded from the study. For all in vivo experiments, the investigator was blinded to the genotype.

ANIMAL HOUSING AND ETHICAL APPROVAL: Mice were housed at the KU Leuven Mouse Facility under a 12h/12h light/dark cycle. Animals were allowed access to rodent chow and water ad libitum. All experiments and procedures were approved by the local Ethical Committee of the KU Leuven (P127/2014), except for MRI experiments, which were carried out in the Animal Imaging Center in the Institute of Biomedical Engineering of ETH Zurich in compliance with the Swiss law of animal protection under license ZH014/16 of the Cantonal

Veterinary Office of the Canton of Zurich.

ISOLATION OF PRIMARY HUMAN BRAIN ENDOTHELIAL CELLS: For isolation of human primary brain endothelial cells, clinical brain specimens from glioblastoma patients and from the temporal lobe of pharmacoresistant epilepsy patients, were digested in 10 mg/ml collagenase/dispase (#11097113001, Roche, Basel, Switzerland) and dissociated with gentleMACS™ C Tubes (#130-093-237, Miltenyi Biotec, Cologne, Germany). Red blood cell lysis was performed by incubating the cell suspension in ACK buffer (A1049201, ThermoFisher Scientific, Zurich, Switzerland) for 3 min at room temperature. Cell concentration was determined and 20 µl of FcR blocking reagent (#130-059-901, Miltenyi Biotec, Cologne, Germany) and 20 µl of CD31 microbeads (#130-091-935, Miltenyi Biotec, Cologne, Germany) were added per 10^7 cells. The suspension was incubated at 4°C for 20 min; the cell aggregates were removed upon passing the suspension through a 40 µm cell strainer (#431750, Corning, New York, USA). Cells were then passed through a LS column (#130-042-401, Miltenyi Biotec, Cologne, Germany) placed in a magnetic field of a MACS separator (#130-042-301, Miltenyi Biotec, Cologne, Germany). The CD31-positive cells which remained bound to the column were washed out and cultured in collagen type I-coated dishes in EGM2.

ISOLATION OF PRIMARY MOUSE ENDOTHELIAL CELLS FROM BRAIN, SKELETAL MUSCLE AND LUNG: Primary ECs from brain, skeletal muscle and lung were isolated from adult GLUT1$^{EC-/-}$ mice and WT littermates 7 days after the first tamoxifen injection or from untreated adult WT mice when used for inhibitor experiments. Mice were euthanized and tissues were quickly dissected. For mouse brain ECs (mBrain ECs), whole cerebrum was dissected and

meninges associated vessels on the surface were removed, gray matters of cerebrum were separated and minced in EGM2 medium (CC-3162, Lonza, Basel, Switzerland) on ice. This mixture was passed through 230mm glass Pasteur pipets (Fischer scientific, Zurich, Switzerland) for several times until it looked homogenous. A similar volume of 30% dextran (#31390, Sigma-Aldrich, Darmstadt, Germany) solution (w/v) was then mixed with the homogenized tissue in a 15ml centrifuge tube (CLS430791, Corning, Darmstadt, Germany) and centrifuged at 4500g, 4℃ for 20 min. The pellets were resuspended and enzymatically dissociated with 1ml digestion buffer containing 0.5μg/ml DNaseI (D-4263, Sigma-Aldrich, Steinheim, Germany), 100μg/ml Collagenase IV (17104019, Thermo Fisher Scientific, Zurich, Switzerland) and 50μg/ml Dispase II (D4693, Sigma-Aldrich, Steinheim, Germany) at 37℃ for 25-30min. The reaction was stopped by adding 4ml cold PBS and centrifuged at 300g, 4℃ for 5min. Then the pellet was resuspended with growth medium (50% EGM2 + 50% fullM199) and seeded onto a collagen I coated cell culture dish. Due to the higher expression of P-glycoprotein in endothelial cells compared to contaminating cells, mBrain ECs were selected by adding 4 μg/ml puromycin (P8833, Sigma-Aldrich, Steinheim, Germany) immediately in the medium for 24 hours in order to obtain a pure EC population.

For mouse skeletal muscle ECs (mMuscle ECs) isolation, muscle tissue was dissected from the whole hind limb and minced in a petri dish on ice using two surgical blades. Next, the muscle tissue was enzymatically digested in digestion buffer containing 2 mg/ml Dispase II (D4693, Sigma-Aldrich, Steinheim, Germany), 2 mg/ml Collagenase IV (17104019, ThermoFisher Scientific, Zurich, Switzerland) and 2 mM $CaCl_2$ in PBS at 37°C for 40 min, with gentle shaking every 10 min. The reaction was stopped by adding an equal volume of 20% FBS in HBSS and the suspension was passed through a series of 100 μm cell strainers

(#352360, Corning, New York, USA) and 70 µm cell strainers (#352350, Corning, New York, USA) to remove any tissue debris. After a series of centrifugation and washing steps, the pellet was resuspended in growth medium and seeded onto collagen type I coated plates. mMuscle ECs were selected by adding 4 µg/ml puromycin (P8833, Sigma-Aldrich, Steinheim, Germany) in the medium overnight in order to obtain a pure population of ECs. After 7 days in culture, the purity of mBrain and mMuscle ECs were determined by CD31 fluorescence staining and cells were only used for further experiments in case of > 85% CD31 positive cells. For mouse lung ECs (mLung ECs) isolation, in addition to the procedures described in mMuscle ECs isolation, an additional CD31 pluribeads (29-03100-10, PluriSelect, Leipzig, Germany) purification was performed according to the manufacturer's instructions, 3 days after puromycin selection. To achieve maximal recombination efficiency, mBrain ECs, mMuscle ECs and mLung ECs from GLUT1$^{EC-/-}$ mice and WT littermates were treated *in vitro* for 96 hours with 5 µM 4-hydroxytamoxifen (T176, Sigma-Aldrich, Steinheim, Germany) before the start of the experiments.

FACS/MACS ISOLATION OF MOUSE BRAIN ENDOTHELIAL CELLS FOR RNASEQ: Brain endothelial cells were isolated from GLUT1$^{EC-/-}$ mice and WT littermates using either FACS sorting from neonates (P6) or using MACS sorting from 8 week old adult mice. Briefly, mice were euthanized and brains were quickly dissected and minced in a petri dish on ice, using two surgical blades. For FACS sorting a cell suspension was obtained upon digesting the tissue in 2 mg/ml Dispase II (D4693, Sigma-Aldrich, Steinheim, Germany), 2 mg/ml Collagenase IV (#1710401, ThermoFisher Scientific, Zurich, Switzerland) and 2 mM $CaCl_2$ PBS solution for 40 min at 37 °C with occasional shaking. Endothelial cells were FACS-sorted using a CD31-PE conjugated antibody (#12-0311-82, ThermoFisher Scientific,

Zurich, Switzerland), while negatively selecting for the brain microglia and macrophages using a CD45-APC conjugated antibody (#557235, BD Biosciences, Basel, Switzerland). ECs were sorted directly into RLT plus RNeasy plus lysis buffer and RNA was extracted using RNeasy Plus Micro Kit (Cat No. 74034, Qiagen, Hilden, Germany).

For MACS sorting, digestion was done for 30 min at 37°C in a mix of 0.5 mg/ml collagenase type I (17100-017, Life Technologies, Ghent, Belgium), 0.25U/ml Dispase II (17105-041, Life Technologies, Ghent, Belgium) and 50 µl of 1mg/ml DNaseI (D4527, Sigma-Aldrich, Overijse, Belgium) while shaking with gentleMACS C-tubes (130-093-237, Miltenyi Biotech, Cologne, Germany). Red blood cell lysis was performed by incubating the cell suspension in Red Blood Cell Lysing Buffer Hybri-Max™ (R7757, Sigma-Aldrich, Overijse, Belgium) for three minutes at room temperature after which cold DPBS was added. After centrifugation, cells were resuspended in 90 µL of ice-cold MACS buffer and 10 µl of CD31 microbeads (130-097-418, Miltenyi Biotec, Leiden, The Netherlands) per 10^7 cells. This mixture was incubated for 30 min at 4°C, after which cells were washed with MACS buffer. Cell aggregates were removed upon passing the suspension through a 40 µm cell strainer. Cells were then passed through an MS column placed in a magnetic field of a MiniMACS™ Separator (130-042-201, Miltenyi Biotec, Leiden, The Netherlands). The CD31-positive cells which remained bound to the column were washed out and centrifuged at 300g for 10 minutes. The cell pellet was resuspended in RNA lysis buffer, RNA was extracted immediately using PureLink™ RNA Mini Kit PureLink™ RNA Mini Kit (#12183020, Invitrogen, Merelbeke, Belgium).

IMMUNOBLOTTING: Sorted endothelial cells or cultured HUVEC and bEnd3 were lysed using Laemmli buffer. After denaturing (1 hr at 37°C for GLUT1 and 5 min at 95°C for all other proteins), 15 µg of protein was loaded on 4-20% Mini-PROTEAN® TGX Stain-Free™ Protein Gels (#4568094, Bio-Rad, Cressier, Switzerland) and transferred using the Trans-Blot® Turbo™ Transfer System (#1704150EDU, Bio-Rad, Cressier, Switzerland). The PVDF membranes were incubated overnight in 5% milk solution in TBST with antibodies specific for GLUT1 (ab14683, Abcam, Cambridge, UK), CDH5 (ab33168, Abcam, Cambridge, UK), OCLN (ab31721, Abcam, Cambridge, UK), ZO1 (#61-7300, ThermoFisher Scientific, Basel, Switzerland), CLDN5 (ab15106, Abcam, Cambridge, UK), Phospho-AMPKα (Thr172) (#2531, Cell signaling, Leiden, The Netherlands), AMPKα (#2532, Cell signaling, Leiden, The Netherlands), p53 (1C12) (#2524, Cell signaling, Leiden, The Netherlands), p21 (ab109520, Abcam, Cambridge, UK), activated Notch1 (NICD) (ab8925, Abcam, Cambridge, UK), PFKFB3 (ab181861, Abcam, Cambridge, UK), β-Actin (8H10D10) (#3700, Cell signaling, Leiden, The Netherlands). Antibody specificity was determined using molecular weight markers as well as appropriate control samples (f.i. ECs isolated from GLUT1$^{EC-/-}$ or PFKFB3 $^{EC-/-}$ mice to determine specificity of anti-GLUT1/PFKFB3; Dll4 stimulated ECs for anti-NICD; serum starved cells for anti-AMPK and autophagy markers). Signal was detected with an HRP-conjugated secondary antibody (HRP linked anti-mouse IgG #7076 and HRP linked anti-rabbit IgG, Cell signaling, Leiden, The Netherlands). Image lab™ software version 6.0.0 (Bio-Rad Laboratories, California, USA) was used to quantify the band intensity and the gel's protein loading.

RNA SEQUENCING AND BIOINFORMATICS: RNA sequencing of endothelial cells from mouse brains of P6 pups and adult mice was performed by the Functional Genomics Center Zurich.

The libraries were prepared following Illumina TruSeq stranded mRNA protocol. The quality of the RNA and final libraries was determined using an Agilent 4200 TapeStation System. The libraries were pooled equimolecularly and sequenced in an Illumina NovaSeq sequencer (single-end 100 bp) with a depth of around 20 Mio reads per sample. 'Mapping and trimming of FASTQ format sequences was performed using Trimmomatic v0.3.3, and sequence quality control was assessed using FastQC. In a separate Excel file (RNAseq QC filtering and mapping QC), we report the number of input reads and the number of reads that survived QC filtering as well as the parameters used. The number of reads that were assigned to genes and those that were discarded because of mapping quality and because of mapping to intergenic regions ("Unassigned_NoFeatures") are also reported. Alignment to the Ensembl reference genome mm10 (Release 81) was performed using the STAR aligner. Gene expression values were computed with the function featureCounts from the R package Rsubread. Differential expression was computed using the generalized linear model implemented in the Bioconductor package edgeR. FDR values were calculated using the Benjamini–Hochberg method.

Pathway analysis was performed using the Gene Set Enrichment Analysis (GSEA) software from the Broad Institute (software.broadinstitute.org/GSEA)(version 4.0.1)[48,49]. A permutation-based p-value was computed and corrected for multiple testing to produce a permutation based Benjamini – Hochberg correction false-discovery rate (FDR) q-value that ranges from 0 (highly significant) to 1 (not significant). The same analysis was performed starting from the bottom of the ranked gene list to identify pathways enriched in the bottom of the list. The resulting pathways are ranked using NES, and FDR q-value, p-values are reported in the GSEA output reports.

Mouse_GOBP_AllPathways_no_GO_iea_August_01_2019.gmt from [http://baderlab.org/GeneSets] was used to identify enriched pathways in GSEA analysis.

GLUCOSE TRANSPORT ASSAY IN VITRO: ^{14}C-3MG: Zero-trans sugar uptake measurements, as previously described[50], were performed with minor modifications. Briefly, bEND3 cells were placed in serum-free DMEM for 2 hrs at 37°C in the presence of inhibitors or vehicle. Subsequently, cells were washed with 1 ml of DPBS. Next, wash medium was drained and cells were treated with 500 µl of 20 mM 3-*O*-methylglucose (3MG) containing 1 µCi/ml 3-*O*-[methyl-^{14}C]-methyl-D-glucose (^{14}C-3MG) (NEC377, PerkinElmer, Zaventem, Belgium) in DPBS in the presence of the inhibitors or vehicle; uptake proceeded for 30 min. Eventually, uptake was stopped by adding 1 ml of stop solution (50 mM glucose in ice-cold PBS) and each well was washed two more times with 1 ml of stop solution and treated with 0.5 ml of lysis buffer (0.1 N NaOH). Samples were counted by liquid scintillation counting (LSC). Each measurement was performed in triplicate.

GLYCOLYSIS IN VITRO: Glycolysis assays were performed as described[51]. Briefly, cells were incubated for 2 hrs in culture medium containing D-[5-^{3}H(N)]-glucose (NET53100, PerkinElmer, Zaventem, Belgium) at a final concentration of 0.4 µCi/ml medium. Glycolytic flux was measured by the rate of $^{3}H_2O$ production. After 2 hrs incubation, the supernatant was transferred into glass vials sealed with rubber stoppers. $^{3}H_2O$ was captured in hanging wells using a H_2O-soaked Whatman paper over a period of 48 h at 37°C to reach saturation. Radioactivity in ^{3}H-labeled papers was determined by LSC.

METABOLITE MEASUREMENTS USING LC-MS: Metabolite extraction was performed using 80% methanol and 0.2% of myristic-d27 acid (internal standard). After 5 min of incubation

cells were scraped and collected in a new tube. Following centrifugation at 20.000 x g for 10 min at 4°C, the supernatant was transferred to a new vial for MS analysis. The pellet was used for protein quantification. 10 µl of each sample was loaded into a Dionex UltiMate 3000 LC System (Thermo Scientific, Bremen, Germany) equipped with a C-18 column (Acquity UPLC -HSS T3 1. 8 µm; 2.1 x 150 mm, Waters) coupled to a Q Exactive Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) operating in negative ion mode. A step gradient was carried out using solvent A (10 mM TBA and 15 mM acetic acid) and solvent B (100% methanol). The gradient started with 0% of solvent B and 100% solvent A and remained at 0% B until 2 min post injection. A linear gradient to 37% B was carried out until 7 min and increased to 41% until 14 min. Between 14 and 26 minutes the gradient increased to 100% of B and remained at 100% B for 4 minutes. At 30 min the gradient returned to 0% B. The chromatography was stopped at 40 min. The flow was kept constant at 250 µl/min and the column was placed at 25°C throughout the analysis. The MS operated in full scan mode (m/z range: [70-1050] [300-800]) using a spray voltage of 3.2 kV, capillary temperature of 320°C, sheath gas at 10.0, auxiliary gas at 5.0. The AGC target was set at 3e6 using a resolution of 70.000, with a maximum IT fill time of 256 ms. Data collection and data analyses were performed by integrating the peak areas using the Xcalibur software (Thermo XCalibur Quan Browser software – Thermo Scientific, Bremen, Germany).

IN VITRO ANALYSIS OF EC FUNCTION: Proliferation: Cells were incubated for 2 hrs in culture medium containing [6-^{3}H]-thymidine (NET355, PerkinElmer, Zaventem, Belgium) at a final concentration of 1 µCi/ml medium. Thereafter, cells were fixed with 100% ethanol for 15 min at 4°C and subsequently precipitated with 10% TCA. Finally, cells were lysed in 0.1 N NaOH and the resulting suspension was measured by LSC to determine the amount of [^{3}H]-

thymidine incorporated into DNA. Scratch wound assay: A scratch wound was applied on confluent EC monolayers (pre-treated with inhibitors or 1 µg/ml mitomycin C for 24 hrs where indicated) using a 200 µl tip. After scratch wounding (T0) and photography using a Leica DMI6000 B inverted microscope (Leica Microsystems, Mannheim, Germany), the cultures were further incubated in EGM2 and fixed with 4% PFA when closure was nearly reached (Tx) in the control condition. Cells were photographed again (Tx) and gap areas at both time points were measured using the Fiji software package (https://fiji.sc) in order to calculate the percentage of wound closure using the following expression: $(1 - (Tx_{gap\ area}/T0_{gap\ area})) \times 100$. LDH cytotoxicity assay: Cell death was measured using the Cytotoxicity Detection Kit (#11-644-793-001, Roche, Basel, Switzerland) according to the manufacturer's instructions. The release of lactate dehydrogenase (LDH) in the medium was used as a readout for cell death. Briefly, cells were seeded in a 48-well plate and 50 µl of medium, in which the cells were incubated for 24 hrs with or without inhibitors, was transferred to a 96-well plate. Next, cells were lysed in order to release all intracellular LDH into the medium and again 50 µl of medium was transferred to a 96-well plate. Spontaneous and maximal LDH release was measured in the 96-well plate after incubation of the reaction mixture; the absorbance from this coupled colorimetric assay was measured at 490 nm to calculate cytotoxicity. Spheroid capillary sprouting assay: Spheroids were prepared as previously described [5] with minor modifications. Briefly, spheroids containing 1000 HUVECs, HBMV or HRMV ECs per 25 µl droplet were plated overnight as hanging drops in a 20% methylcellulose (Sigma-Aldrich, Bornem, Belgium) in EGM2 mixture. The next day, spheroids were collected in 10% FBS in PBS, concentrated using several centrifugation steps and embedded in a Fibrinogen gel (5 mg/ml) in EGM2 medium with or without inhibitors and/or mitomycin C. Spheroids were cultured with medium containing the same concentrations of inhibitors with or without

mitomycin C on top of the gel to induce sprouting. 24 hrs later, spheroids were fixed with 4% PFA at room temperature and photographed using a Leica DM IL LED microscope (Leica Microsystems GmbH, Wetzlar, Germany). Models of EC quiescence: To generate contact inhibition, ECs were seeded in 50% EGM2 / 50% full M199 medium at a density of 15,000 cells/cm^2. To generate the corresponding proliferative control, contact inhibited cells were trypsinized and cultured for 24 hr to re-initiate proliferation. To induce quiescence using Dll4 stimulation, culture plates were coated with 1 µg/mL recombinant human Delta-like ligand 4 (rhDll4, R&D Systems) with 0.1% gelatin. The control plates were coated with 0.1% gelatin supplemented with 0.02% BSA. Prior to EC seeding, excessive coating solution was removed by aspiration and ECs were seeded at a density of 30.000 cells/cm^2. Cells were harvested 24 hr after seeding and either or not after treatment with the γ-secretase inhibitor N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT; 20 µM). NICD overexpression (NICD^{OE}) and KLF2 overexpression (KLF2^{OE}) strategies were performed as described previously[19].

EdU INCORPORATION ASSAY IN ISOLATED CULTURED MOUSE ECs: Primary mouse muscle ECs were isolated 7 days after the first tamoxifen injection from GLUT1$^{EC-/-}$ mice and WT littermates and cultured no longer than 8 days before the start of the experiment. As a measure of proliferation, incorporation of 5-ethynyl-2'-deoxyuridine (EdU) was assessed using the Click-iT™ Cell Reaction Buffer Kit (C10269, ThermoFisher Scientific, Zurich, Switzerland), according to the manufacturer's instructions. Briefly, ECs were incubated under standard growth conditions with 10 µM EdU for 15 hours. After EdU incorporation, cells were fixed with 4% paraformaldehyde for 10 min at room temperature and then washed twice with 3% BSA in PBS. Cells were permeabilized for 20 min at room temperature in

0.5% Triton X-100 with 3% BSA in PBS, then washed twice with 3% BSA in PBS and reacted with the Click-iT reaction cocktail for 45 min in the dark at room temperature. Thereafter, cells were washed briefly and counterstained with Hoechst (#62249, ThermoFisher Scientific, Zurich, Switzerland) and antibodies against CD31 (AF3628, R&D Systems, Minneapolis, USA) and Erg (#97249, Cell Signaling, Leiden, The Netherlands). Cells were imaged using an AxioObserver.Z1 fluorescence microscope (Carl Zeiss, Oberkochen, Germany). EdU positive cells and Erg positive endothelial cells were counted in at least 5 random fields and the percentage of EdU positive cells in ECs was calculated.

RT-qPCR: Total RNA was extracted using the PureLink™ RNA Mini Kit (#12183020, Invitrogen, Zurich, Switzerland) according to the manufacturer's instructions; quality and quantity were measured on a NanoDrop™ 2000 spectrophotometer (ND-2000, ThermoFisher Scientific, Zurich, Switzerland). cDNA synthesis was performed with the iScript™ cDNA Synthesis Kit (#1708891,Bio Rad, Cressier, Switzerland). RNA expression analysis was performed by Taqman quantitative RT-PCR using software designed (PerlPrimer) primer sets synthetised by microsynth (Balgach, Switzerland). (human Slc2a1 forward: GACCCTGCACCTCATAGGC; human Slc2a1 reverse: GATGCTCAGATAGGACATCCAGG; mouse Slc2a1 forward: GACCCTGCACCTCATTGG, mouse Slc2a1 reverse: GATGCTCAGATAGGACATCCAAG; human and mouse Pfkfb3 forward: TATGAAGCCAGCTACCAGCC, human and mouse Pfkfb3 reverse: TCTGGATGTGGTCCTGCAC; human Hey1 forward: TGGATCACCTGAAAATGCTGC; human Hey1 reverse: CGAAATCCCAAACTCCGATAGT; human Hes1 forward: TGAAGAAAGATAGCTCGCGGC, human Hes1 reverse: GGTACTTCCCCAGCACACTT; human 18S rRNA forward: AGTCCCTGCCCTTTGTACACA, human 18S rRNA reverse

CGATCCGAGGGCCTCACTA.

All subsequent qRT-PCR reactions were performed using POWRUP SYBR master mix (A25742, Applied Biosystems) on a CFX96 Touch Real-Time PCR Detection System- (BioRad). Thermocycler conditions: hold: 50°C, 2:00min; hold: 95°C, 10:00min; cycles: 40, 95°C, 0:15min, 60°C, 1:00min; hold:95°C, 0:15min; hold: 60°C, 1:00min. For normalization threshold cycles (Ct-values) of all replicate analyses were normalized to 18S ribosomal RNA (18S rRNA) within each sample to obtain sample-specific ΔCt values (= Ct gene of interest - Ct Hprt). 2-ΔΔCt values were calculated to obtain fold expression levels, where ΔΔCt = (ΔCt treatment - ΔCtcontrol).

HISTOLOGY: All methods for histology and immunostainings have been previously described [5]. Briefly, neonatal mice were euthanized and brain and both eyes were quickly dissected and fixed in 4% PFA overnight at 4°C. Eyes were washed 3 x 5 min in PBS before retinas were dissected with or without the removal of the hyaloid vessels. Adult mice were anesthetized using ketamine/xylazine (intraperitoneal injection of 100 mg/kg body weight ketamine (Eurovet Animal Health BV, Bladel, The Netherlands) and 10 mg/kg body weight xylazin (V.M.D., Arendonk, Belgium)) and transcardially perfused with 0.9% saline for 7 min and 4% PFA for 7 min. Brains were dissected and post-fixed in 4% PFA overnight at 4°C. Perfusion-fixed brains were either embedded in 5% low-melting SeaPlaque™ Agarose (Lonza, Verviers, Belgium) and coronally sectioned at 40 to 65 µm using a HM 650 V vibratome (VWR, Leuven, Belgium) or using a Vibratome Leica VT1000S or treated in 30% sucrose before embedding in 2-methylbutane on liquid nitrogen in Tissue-Tek® O.C.T.™ Compound (Sakura, Alphen aan den Rijn, The Netherlands) and coronally sectioned at 12 µm or 40 µm using a Microm HM 560 cryostat (ThermoFisher Scientific, Merelbeke,

Belgium). Immunostainings on retinas and brain sections were performed using the following primary antibodies: mouse anti-NeuN (MAB377, Chemicon, Wortegem-Petegem, Belgium) rabbit anti-Erg (ab92513, Abcam, Cambridge, UK) goat anti-GLUT1 (sc1605, Santa Cruz, Heidelberg, Germany) goat anti-CD105 (AF1320, R&D Systems, Minneapolis, USA) rabbit anti-ZO1 (#40-2200, ThermoFisher Scientific, Merelbeke, Belgium) mouse anti-CLDN5 (#35-2500, ThermoFisher Scientific, Merelbeke, Belgium) rabbit anti-Iba1 (#019-19741, Wako, Neuss, Germany), mouse anti-GFAP (G3893, Sigma-Aldrich, Overijse, Belgium), rat anti-CD206 (GTX-42263, GeneTex), goat anti-podocalyxin (AF1556, R&D), . Retinas and brain sections were then incubated with the appropriate fluorescently conjugated secondary antibodies (Alexa Fluor™ 488, 546, 568, 633 or 647, Molecular Probes or Jackson ImmunoResearch), or followed by amplification with the proper tyramide signal amplification systems when needed (Perkin Elmer, Life Sciences, Zaventem, Belgium). The vasculature was fluorescently labeled with Alexa Fluor™ 488 or 568 conjugated Isolectin GS-IB4 from Griffonia simplicifolia (IB4) (I21411 or I21412, respectively, Molecular Probes, Invitrogen, Life Technologies, Ghent, Belgium) where indicated. Vascular area quantification: Coronal cryosections (12 µm) from perfusion-fixed brains were immunostained for CD105 and imaged using a Leica DMI6000 B inverted microscope (Leica Microsystems, Mannheim, Germany). The vascular area was determined by thresholding the $CD105^{+}$ area using the Fiji software package (https://fiji.sc) and was expressed as a percentage of the total image field area in both cortical as well as the hippocampal regions CA1, CA3, DG. Vascular tight junction colocalization analysis: Coronal cryosections (40 µm) from perfusion-fixed brains were immunostained for ZO1 or CLDN5, in combination with IB4 labeling and imaged using a 63x (NA 1.4) objective on a confocal Zeiss LSM780 microscope (Carl Zeiss, Munich, Germany). Maximal intensity projections of

z-stacks comprising cortical vessels were analyzed by measuring the area of the thresholded IB4 and ZO1 or CLDN5 positive structures using the Fiji software package (https://fiji.sc). The percentage of colocalization was calculated as the overlapping area relative to the total vascular (IB4 positive) area. Neuronal cell number quantification: Coronal cryosections (12 µm) from perfusion-fixed brains were immunostained for NeuN, counterstained using DAPI and imaged using a Leica DMI6000 B inverted microscope (Leica Microsystems, Mannheim, Germany). The number of $NeuN^+DAPI^+$ cells per field in randomly selected cortical regions was counted using the CellCounter plugin in the Fiji software package (https://fiji.sc). The same software was used to count the number of $NeuN^+DAPI^+$ cells in the hippocampal regions CA1, CA3 and DG, and to measure the length of these regions as a correcting factor. CD206 coverage: 65 µm free floating vibratome sections were immunostained for CD206 and Podocalyxin and imaged using a Leica SP5 confocal microscope (Leica Microsystems, Mannheim, Germany). Quantification of CD206 coverage was performed using FIJI software area fraction measurement tool. Binary images of Podocalyxin and CD206 signal were measured per field of view in cortex (both hemispheres) and hippocampus (both hemispheres). CD206 fraction per vessel area was calculated by dividing the CD206 fraction by the vessel area fraction.

RETINA AND BRAIN VASCULAR ANALYSES: Neonatal postnatal day 5 (P5) mice were injected with 10 µl of 5-ethynyl-2'-deoxyuridine (EdU) from the Click-iT™ EdU Cell Proliferation Kit for Imaging (C10269, ThermoFisher Scientific, Massachusetts, USA) 2 hours before dissection of the eyes and brains of these pups in order to evaluate *in vivo* proliferation of the retinal primary plexus or cortical brain vasculature. Retinal whole-mounts were prepared as described previously [5] and coronal vibratome sections (50 µm) were cut

from the brains of these same mice. Briefly, IB4 labeled retinas and brain sections were immunostained for the endothelial nuclear marker Erg (or GLUT1) and EdU was visualized using the Click-iT™ EdU Cell Proliferation Kit for Imaging (C10269, ThermoFisher Scientific, Massachusetts, USA). Imaging was performed using a confocal Zeiss LSM780 microscope (Carl Zeiss, Munich, Germany) and maximal intensity projections of z-stacks comprising retinal primary plexus or cortical brain vessels were analyzed for the number of EdU^+Erg^+ cells. IB4 positive blood vessels were thresholded using the Fiji software package (https://fiji.sc) and this area was measured in order to correct for vascular area. IB4-stained retinas from P6 pups were analyzed for radial outgrowth of the vascular plexus, branching point density, number of filopodia per mm vascular perimeter and number of filopodia per tip cell [5]. Tile scans of the retinas were acquired using a Leica DMI6000 B inverted microscope (Leica Microsystems, Mannheim, Germany) and filopodia were imaged using a 63x (NA 1.4) objective on a confocal Zeiss LSM780 microscope (Carl Zeiss, Munich, Germany). IB4-labeled coronal vibratome sections (40 µm) were analyzed for vascular length, number of tip cells per mm vascular length, number of filopodia per tip cell and length per filopodium as previously described[25], but with minor modifications. A large cortical area was imaged at high-resolution using a 63x (NA 1.4) objective on a confocal Zeiss LSM780 microscope (Carl Zeiss, Munich, Germany) and 40 µm z-stacks with 1 µm intervals were stitched and eventually converted to one large maximum intensity projection.

WATER CONTENT DETERMINATION: Brain wet weight and dry weight measurements were recorded and water content was determined as a percentage using the following formula: (1 – (dry weight/wet weight)) x 100.

EVANS BLUE LEAKINESS ASSAY: Adult GLUT1$^{EC-/-}$ mice and WT littermates were intravenously injected with 200 µl of 0.5% Evans Blue via the tail vein and euthanized by cervical dislocation after 30 min of circulation. Immediately after Evans Blue injections, several WT littermates were intraperitoneally injected with 1.4 M mannitol and served as a BBB-breaching positive control. Brains were dissected and homogenized in 500 µl formamide to extract Evans Blue for 48 hrs at 56°C. After centrifugation for 30 min at 12.000 x g, the Evans Blue absorbance in the supernatant was spectrophotometrically determined at 620 nm and Evans Blue content was calculated as ng Evans Blue/mg tissue using a standard curve of Evans Blue in formamide.

MRI MEASUREMENTS: The MRI experiments per animal comprised dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) and functional MRI (fMRI) measurements for assessment of baseline CBF, baseline CBV measurement and for the assessment of vascular reactivity, i.e. dynamic CBV in response to the injection of the pharmacological vasodilator acetazolamide. Mice were anesthetized using 4% isoflurane in a 20% O_2 / 80% air mixture, kept at 2% isoflurane during endotracheal intubation for mechanical ventilation until completion of all animal preparation steps. The head was positioned with the animal's incisors secured over a bite bar and fixed with stereotactic ear bars. Ophthalmic ointment was applied to the eyes and a rectal temperature probe inserted to monitor the animal's body temperature and maintain it at 36.0 °C by means of a warm-water circuit integrated into the animal support (Bruker Biospin GmbH, Ettlingen, Germany). The tail vein was cannulated using a 30 G cannula needle for intravenous administration of the neuromuscular blocking agent pancuronium bromide (Sigma-Aldrich, Steinheim, Germany), administered as a bolus at a dose of 1 mg/kg during animal preparation, as well

as for the administration of contrast agents (Gd-DOTA and Endorem) and acetazolamide for characterization of the cerebrovascular system. In order to minimize the variability of physiological state of the mice, animal preparation did not exceed 20 min in duration. The infusion line was connected to an infusion pump (Harvard Apparatus, Hollistan, USA) with specific volume and infusion rate parameters for each substance. Tubing segments of volume-adjusted length were separated by a small air bubble to prevent mixing of Gd-DOTA, Endorem and acetazolamide injections, which were each flanked by a 10 µl injection of saline before and a 20 µl injection afterwards in order to properly clear the tubing. Throughout the course of the experiment the animals were ventilated with 1.5% isoflurane in a 20% O_2 / 80% air mixture at a rate of 80 breaths/min, with a respiration cycle of 25% inhalation, 75% exhalation and an inspiration volume of 1.8 ml/min. MRI measurements were conducted using a BioSpec 94/30 (Bruker BioSpin MRI GmbH, Ettlingen, Germany) small animal MR system operating at 400 MHz. A four-element receive-only cryogenic phased array surface coil (2 x 2 geometry, overall coil size 20 x 27 mm^2) with the coil system operating at 30 K (Bruker BioSpin AG, Fällanden, Switzerland) was used in combination with a circularly polarized 86 mm volume resonator for transmission. For anatomical orientation, images in the sagittal, and coronal direction allowed exact positioning of the field of view and slices for the DCE-MRI and fMRI measurements (baseline CBF, CBV and dynamic CBV-fMRI), respectively. Prior to data acquisition the local field homogeneity has been optimized in the area of interest (spanning the brain regions from olfactory bulb to up to cerebellum) using previously acquired field maps.

DCE-MRI: For evaluating the blood-brain barrier permeability, a fast low angle shot (FLASH) sequence with TE/TR: 4.6/12 ms, $\alpha = 15°$ and 3 averages was repeated 300 times. While

scanning, Gd-DOTA (Dotarem, Guerbet, Paris, France) was injected intravenously as a 50 µl bolus (2 ml/min) after 2.5 min of acquisition. A single axial slice of 2 mm with a FOV of 2 cm x 2 cm and a matrix size of 80 x 80 was acquired, resulting in an in-plane resolution of 250 µm x 250 µm. The acquisition time was 14 min 24 s.

fMRI: To characterize baseline CBF and CBV as well as the response of the vascular system to a pharmacological vasodilator, fMRI data were acquired using gradient-echo echo-planar imaging (GE-EPI) sequences: FOV = 16 x 7 mm2, MD = 80 x 35, yielding an in-plane voxel dimension of 200 x 200 µm, flip angle = 60°, TR = 400 ms (baseline CBF, CBV) / 1000 ms (dynamic CBV), TE = 16 ms (BOLD, baseline CBF, CBV) / 8 ms (CBV), NA = 1, and 16 slices of 0.5 mm slice thickness, yielding a temporal resolution of 400 ms or 1 s, respectively, per image data set. For baseline CBF and CBV measurements, after acquisition of 1 repetition of a pre-contrast scan at TR = 400 ms and TE = 16 ms, superparamagnetic iron oxide nanoparticles (Endorem®, Laboratoire Guerbet SA, Roissy, France) were injected intravenously as a 60 µl bolus (2 ml/min) after 3 min of acquisition while scanning. The dynamics of Endorem entry and instant washout were captured for 5 min. Data acquisition for the evaluation of dynamic CBV response was started after the previously injected contrast agent Endorem was allowed to reach its plasma steady-state level. Total duration of the GE-EPI sequence with TR = 1000 ms and TE = 8 ms was 25 min, i.e. 1500 repetitions. At 5 min after start of data acquisition the carboanhydrase inhibitor acetazolamide (Diamox® parenteral, Goldshield Pharmaceuticals Ltd, Croydon, UK) was infused at a dose of 20 mg/kg in a volume of 1.5 µl saline / g body weight and at rate of 0.5 ml/min. Immediately after the MR experiment, the isoflurane dose was increased to 5% for minimum 5 min, and the animal was then sacrificed by decapitation.

MRI data analysis*:* Analysis was performed using functions of the AFNI software package (http://afni.nimh.nih.gov/) and custom-written Python code. Preprocessing of DCE-MRI data and data sets for assessment of CBF, CBV and vascular reactivity consisted of the following steps: The first 50 volumes of each data set were discarded to account for the T1 relaxation. Data were slice-time corrected and co-registered to a strain-specific template. Further, a weak Gaussian blur (FWHM = 0.3mm) was applied. For estimating Gd-DOTA tracer leakage by DCE-MRI, k_{trans} was derived on a voxel-by-voxel basis from the initial slope of the signal enhancement curve $E(t) = \big(S(t) - S(0)\big)/S(0) = A \cdot \{1 - \exp\,(-k_{trans} \cdot t)\}$. The asymptotic value of $E(t \to \infty)$ was used as a measure of the extracellular leakage volume. For estimating CBF from the dynamic Endorem-induced signal change we first estimated the concentration time curve (CTC) from the expression $\mathrm{CTC(t)} = -\ln\big(S_{pre}/S(0)\big)\,/\,\mathrm{TE}$. The first pass of the CTC(t) was fitted using a gamma-variate function $y(t) = a\,(t - t_0\,)^r \cdot \exp$ (-k (t $-t_0$)), with t_0 being the contrast agent (CA) arrival time and a, r, k the fitting parameters. The ratio of the first moment divided by the zeroeth moment of the filled curve yielded the mean transit time (MTT), $\mathrm{MTT} = \int_0^\infty t \cdot y(t)dt \,/\, \int_0^\infty y(t)dt$, while the zeroeth moment is a measure for the blood volume. Relative CBF was derived by dividing CBV by the MTT, $\mathrm{CBF} = \mathrm{CBV}/\,\mathrm{MTT}$. For CBV analysis, values were derived for CA-induced changes in R_2^* [s^{-1}] according to $\Delta\mathrm{R}^*_{2,\mathrm{CA}} = \mathrm{r}^*_{2,\mathrm{CA}} \cdot \mathrm{c}_{\mathrm{ss,CA}} \cdot \mathrm{CBV}$, with $\mathrm{r}^*_{2,\mathrm{CA}}$ being the molar relaxivity, and $\mathrm{c}_{\mathrm{ss,CA}}$ being the steady-state concentration of CA in plasma. Relative $\mathrm{CBV_{bsl}}$ was estimated for each voxel according to $\mathrm{CBV_{bsl}} \propto \Delta\mathrm{R}^*_{2,\mathrm{CA}}(\mathrm{bsl}) = \ln\,(\mathrm{S_{pre}}/\mathrm{S}(0))/\mathrm{TE}$, with S_{pre} and S(0) being the steady-state signal intensities prior to and 10 min after injection of Endorem (Kim et al., 2013). For estimating induced CBV changes $(\Delta\mathrm{CBV})$ upon administration of acetazolamide, the first 50 volumes of each data set were discarded to account for the T1 relaxation.

$\Delta R^*_{2,CA}(t)$ values were computed according to $\Delta R^*_{2,CA}(t) = \ln(S(0)/S(t))/TE$, with S(t) being the signal intensity at time t. Values for ΔCBV relative to baseline values were then derived according to $\Delta CBV(t)/CBV_{bsl}[\%] = \Delta R^*_{2,CA}(t)/\Delta R^*_{2,CA}(bsl) \cdot 100$. The signal change as result of CA injection was modeled according to first order kinetics by fitting $\Delta CBV(t)/CBV_{bsl}[\%] = B(1 - e^{-k_{trans}\,t} \cdot e^{-wt})$, with B as a scaling factor, k_{trans} and w being the parameters of the exponential terms describing tracer influx and tracer washout, respectively. The slope of the induced CBV response (slope ΔCBV/Δt), obtained from the derivative of the fitted curve, served as a measure for vascular reactivity. The individual parameters were averaged within pre-defined regions-of-interest placed over the cortex. The mean parameter values were tested across genotypes using Student's *t*-tests.

CSF AND PLASMA GLUCOSE DETERMINATION: GLUT1$^{EC-/-}$ mice were anesthetized using ketamine/xylazine (intraperitoneal injection of 100 mg/kg body weight ketamine (Eurovet Animal Health BV, Bladel, The Netherlands) and 10 mg/kg body weight xylazin (V.M.D., Arendonk, Belgium)) and cerebrospinal fluid (CSF) was sampled by direct extraction from the cisterna magna. Next, plasma was prepared from a terminal blood withdrawal from the posterior vena cava. CSF and plasma samples were frozen until determination of the glucose concentration the Amplex™ Red Glucose/Glucose Oxidase Assay Kit (A22189, Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions.

ENZYME-LINKED IMMUNOSORBENT ASSAYS (ELISAS): VEGF in mouse plasma from GLUT1$^{EC-/-}$ mice and WT littermates was assayed using the murine VEGF Quantikine ELISA kit (VEGFA, #MMV00; R&D Systems, MN, USA) according to the manufacturer's instructions. All absorbance measurements were done at a wavelength of 450 nm using a

microplate reader (Spark10M; Tecan, Switzerland) with wavelength correction to 540 nm. Readings at 540 nm were subtracted from those at 450 nm for correction purposes. A standard curve from recombinant mouse protein was constructed according to the manufacturer's instructions to determine protein levels from the corrected optical densities. VEGF level in each sample was determined according to the best-fit curve.

SPONTANEOUS MOVEMENT: Mice were video-recorded while exploring a new cage for 2 min. Spontaneous movement was quantified by manually tracing the total distance (in cm) of its trajectory.

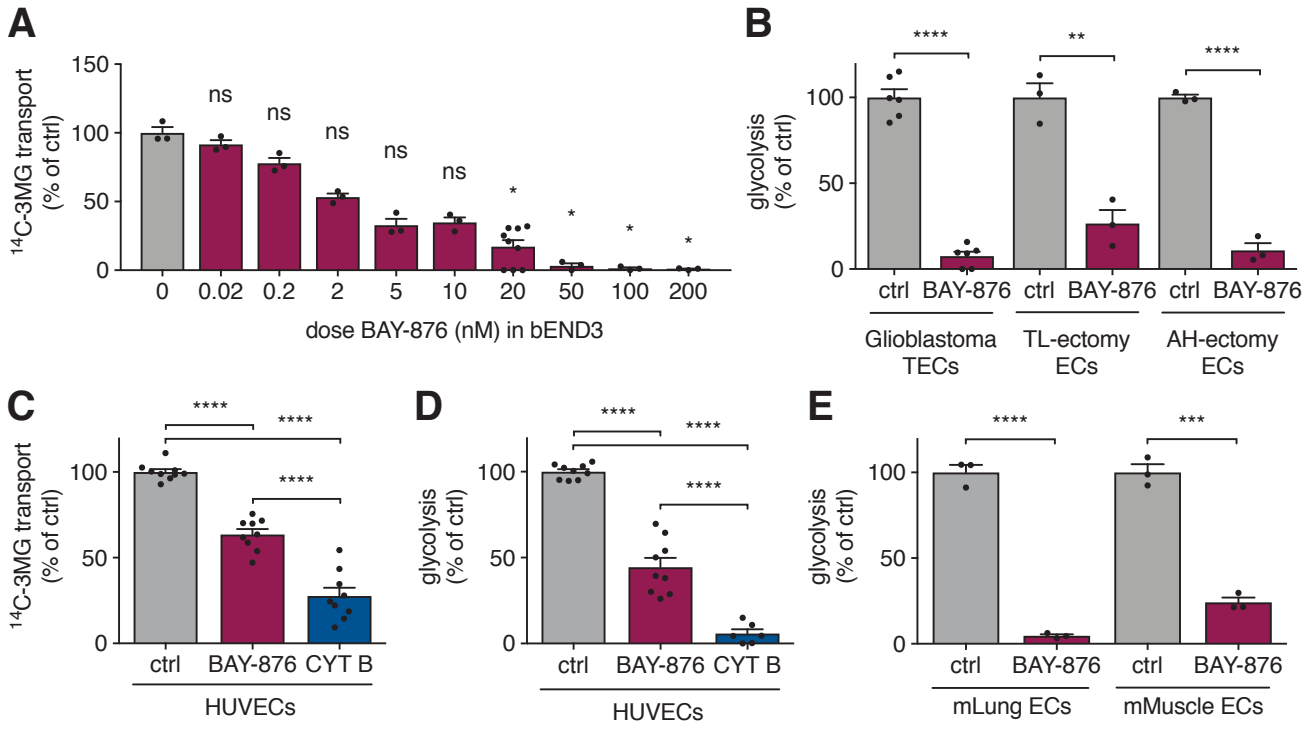
ECOG MEASUREMENTS: To monitor the electrocorticographic (ECoG) changes in adult mice, we implanted a sterilized radiotelemetric mouse transmitter (EA F20, Data Sciences International®, Tilburg, The Netherlands). Mice were anesthetized with 3.5% isoflurane in 100% oxygen for 2 min. After this induction, anesthesia was maintained during the entire duration of the surgery by 2.5% isoflurane in 100% oxygen via a facemask. Their skin on the skull and neck was disinfected with iso-Betadine® Dermicum solution (Meda Pharma, Hoeilaart, Belgium), before being placed in the stereotaxic frame. An incision was made at the level of the skull and 20 mg/ml lidocaine hydrochloride solution (Roxane Laboratories, Hoorn, The Netherlands) was used for local anesthesia. The transmitter was placed subcutaneously and two small bore holes were drilled in the skull, one for the cortical recording electrode (red lead fixed at a screw) and one for the reference electrode (white lead fixed at a screw at the height of the cerebellum). After receiving a subcutaneous injection of 5 mg/kg ketoprofen (Merial, Victoriaville, QC, Canada), mice were gently removed from the stereotaxic fame and gained consciousness in their home cage on a

heating pad. Next, mice were allowed to recover from surgery for 2 weeks. The day before the first tamoxifen injection, mice were placed in a monitoring unit, equipped with a radiotelemetric receiver (PhysioTel™ Receiver Model RPC-1; Data Sciences International®, Tilburg, The Netherlands) coupled to the Notocord-hem Evolution acquisition software (Notocord, Croissy-sur-Seine, France). The ECoG signal was continuously recorded 24hrs/day at a frequency of 100 Hz, both before the first tamoxifen injection for a baseline ECoG and after the start of the tamoxifen treatment regimen until the end of the experiment.

STATISTICS

The images presented in the manuscript are representative of the data (quantification of image is approximately the group average) and the image/staining quality. All data represent mean ± SEM. GraphPad Prism software (version 7.0e) was used for statistical analyses. Investigators were always blinded to group allocation. Shapiro-Wilk test was performed to analyze data distribution. Non-normally distributed data were analyzed by non-parametric tests: Mann-Whitney U test to compare two groups. For more than two groups, we used a Kruskall-Wallis with Dunn's multiple comparisons test. Normally distributed data were analyzed using parametric tests: when comparing two group means, Student's *t*-test was used in an unpaired two-tailed fashion. For more than two groups, one-way ANOVA with Tukey's multiple comparisons test was used and for experimental set-ups with a second variable, two-way ANOVA with Tukey's multiple comparisons test was used. A separate file including all statistics has been added as a supplement to this manuscript. For time-course experiments GLUT1$^{EC-/-}$ mice were compared versus WT littermates using a two-way repeated measures ANOVA with Sidak's multiple comparisons test. No experiment-wide multiple test correction was applied. $P>0.05$ is considered non-significant (ns).

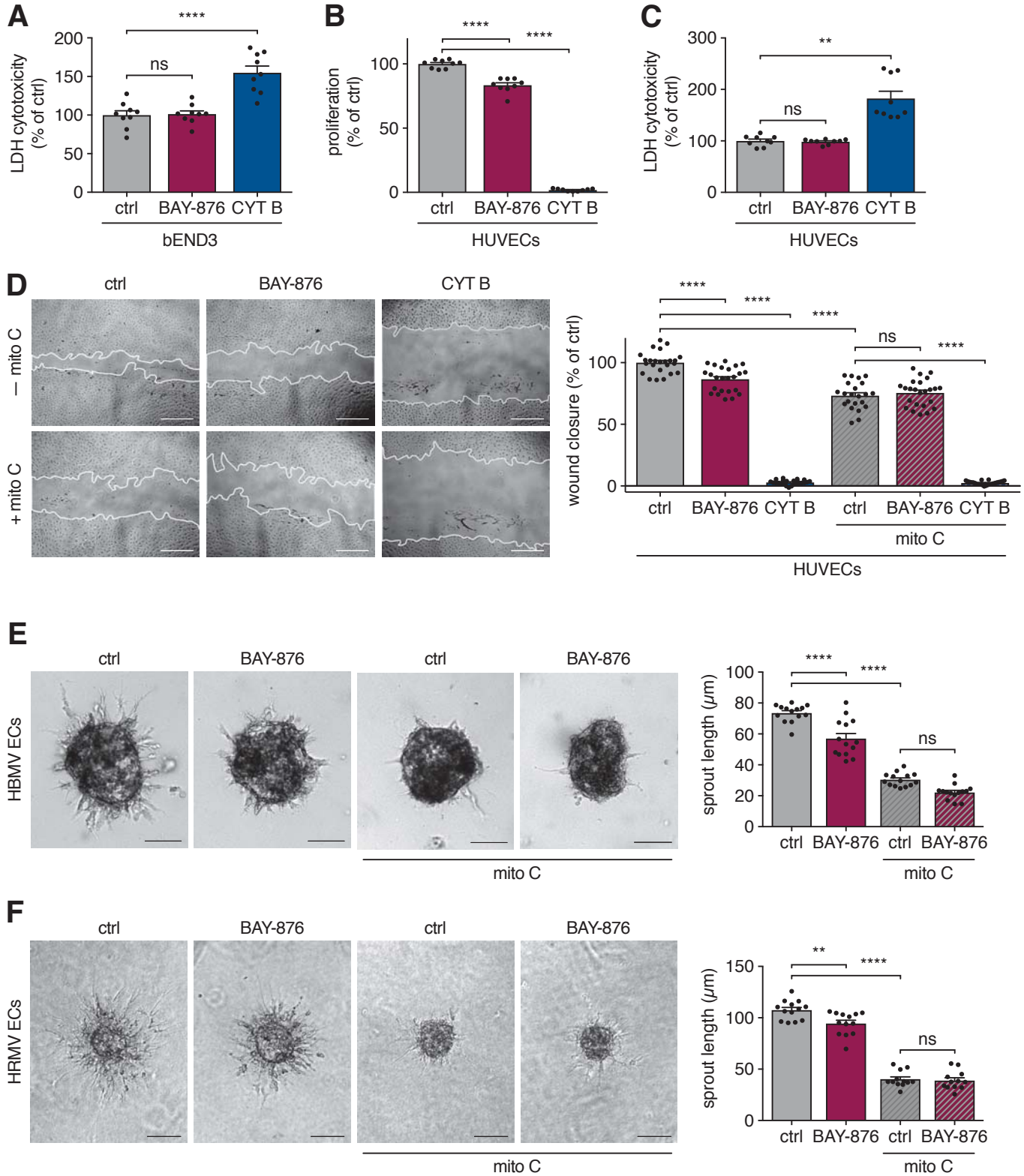
Online Figure I



ONLINE FIGURE I: GLUT1 controls glucose uptake and glycolysis in ECs *(related to Figure 1)*

A, ^{14}C-3MG transport measurements in bEND3 cells at different concentrations of BAY-876 (Kruskall-Wallis and Dunn's multiple comparison test). **B**, Glycolytic flux in glioblastoma tumor ECs, temporal lobectomy (TL-ectomy) and amygdalohippocampectomy ECs (AH-ectomy) incubated with 20 nM BAY-876 versus control (Student's *t*-test). **C** and **D**, ^{14}C-3MG transport (**C**) and glycolytic flux (**D**) in HUVECs incubated with 20 nM BAY-876 and 20 µM CYT B versus control (One-way ANOVA and Tukey's multiple comparisons test). **E**, Glycolytic flux in cultured primary isolated mouse lung and muscle ECs incubated with 20 nM BAY-876 versus control (Student's *t*-test). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

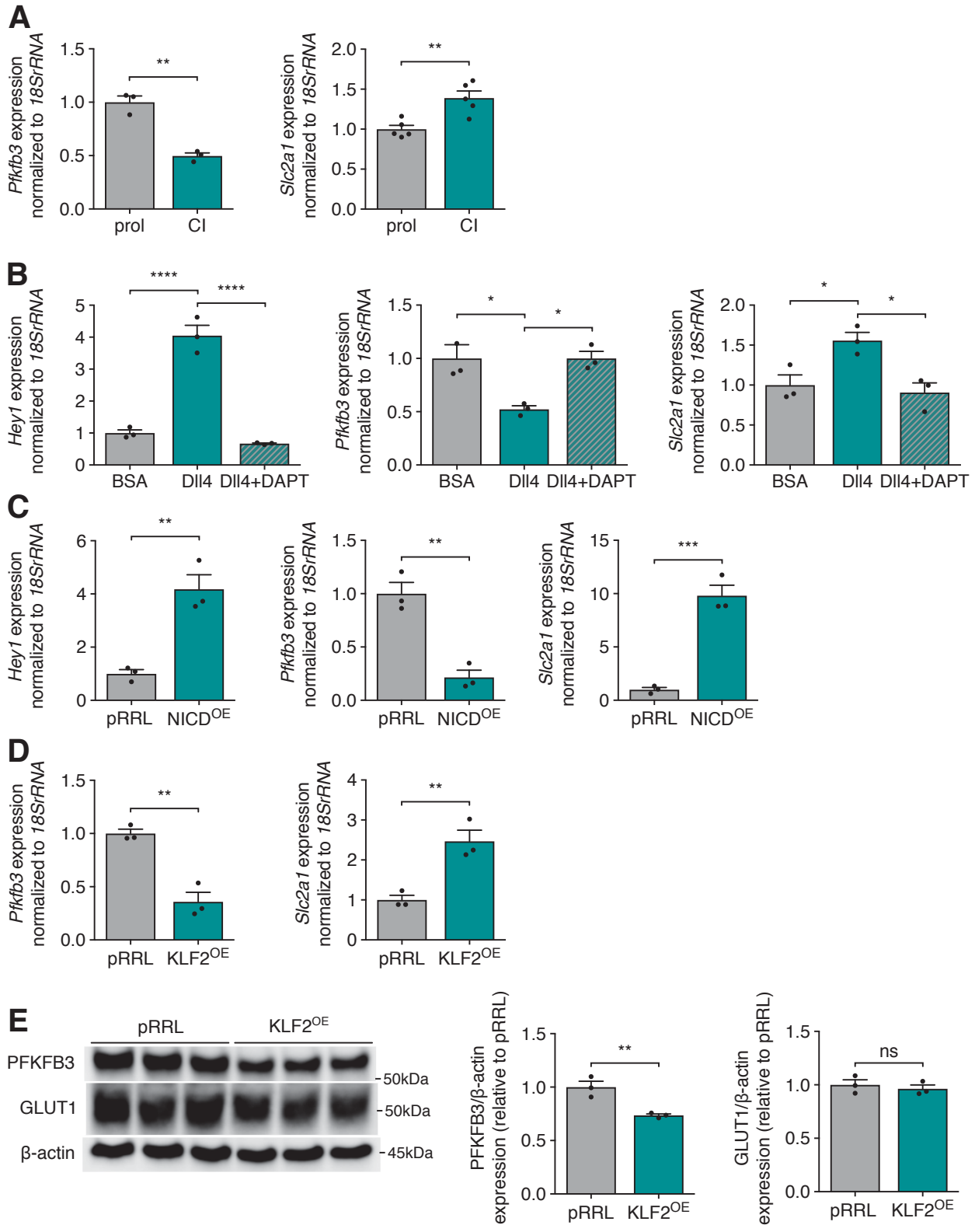
Online Figure II



ONLINE FIGURE II: GLUT1 inhibition reduces EC proliferation but not migration *(related to Figure 1)*

A, Cytotoxicity measurements using the LDH release assay in bEND3 cells incubated with 20 nM BAY-876 and 20 µM CYT B versus control (One-way ANOVA and Tukey's multiple comparisons test). **B**, Proliferation rate of HUVECs incubated with 20 nM BAY-876 and 20 µM CYT B versus control (One-way ANOVA and Tukey's multiple comparisons test). **C**, Cytotoxicity measurements using the LDH release assay in HUVECs incubated with 20 nM BAY-876 and 20 µM CYT B versus control (Kruskall-Wallis and Dunn's multiple comparison test). **D**, Representative pictures and quantifications of scratch wound closure in HUVECs incubated with 20 nM BAY-876 and 20 µM CYT B versus control in conditions with and without pre-treatment of mitomycin C (Two-way ANOVA and Tukey's multiple comparisons test). **E** and **F**, Representative pictures and quantifications of sprouting HBMV (**E**) and HRMV (**F**) EC spheroids incubated with 40 nM BAY-876 versus control in conditions with and without pre-treatment of the proliferation blocker mitomycin C (Two-way ANOVA and Tukey's multiple comparisons test). Scale bar = 500 µm (**D**) and 100 µm (**E-F**). **P<0.01, ****P<0.0001.

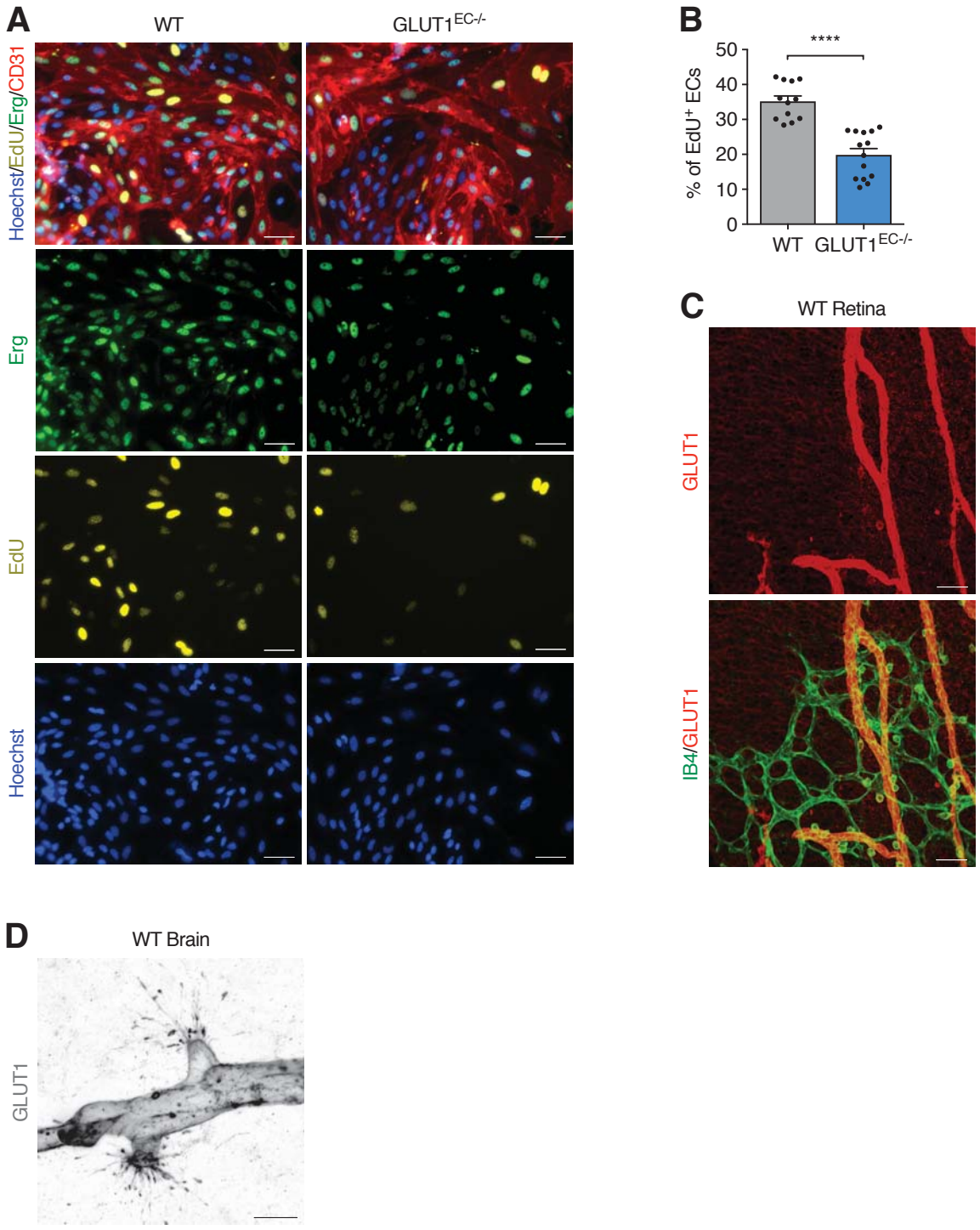
Online Figure III



ONLINE FIGURE III: GLUT1 expression is increased in quiescence and uncoupled from glycolysis *(related to Figure 2)*

A, *Pfkfb3* and *Slc2a1* mRNA levels in proliferating (prol) versus contact inhibited HUVECs (Student's *t*-test). **B**, *Hey1*, *Pfkfb3* and *Slc2a1* mRNA levels in HUVECs cultured on BSA or Dll4 coated plates with or without DAPT (20μM) treatment (One-way ANOVA and Tukey's multiple comparisons test). **C**, *Hey1*, *Pfkfb3* and *Slc2a1* mRNA levels in HUVECs with overexpression of NICD versus empty control overexpression vector (pRRL) (Student's *t*-test). **D**, *Pfkfb3* and *Glut1* mRNA levels in HUVECs with overexpression of KLF2 versus empty control overexpression vector (pRRL) (Student's *t*-test). **E**, Representative image and quantification of Western Blot of NICD, PFKFB3, and GLUT1 protein levels in HUVECs with overexpression of KLF2 versus empty control overexpression vector (pRRL) (Student's *t*-test). All values are normalized to the control condition (**A-D**). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

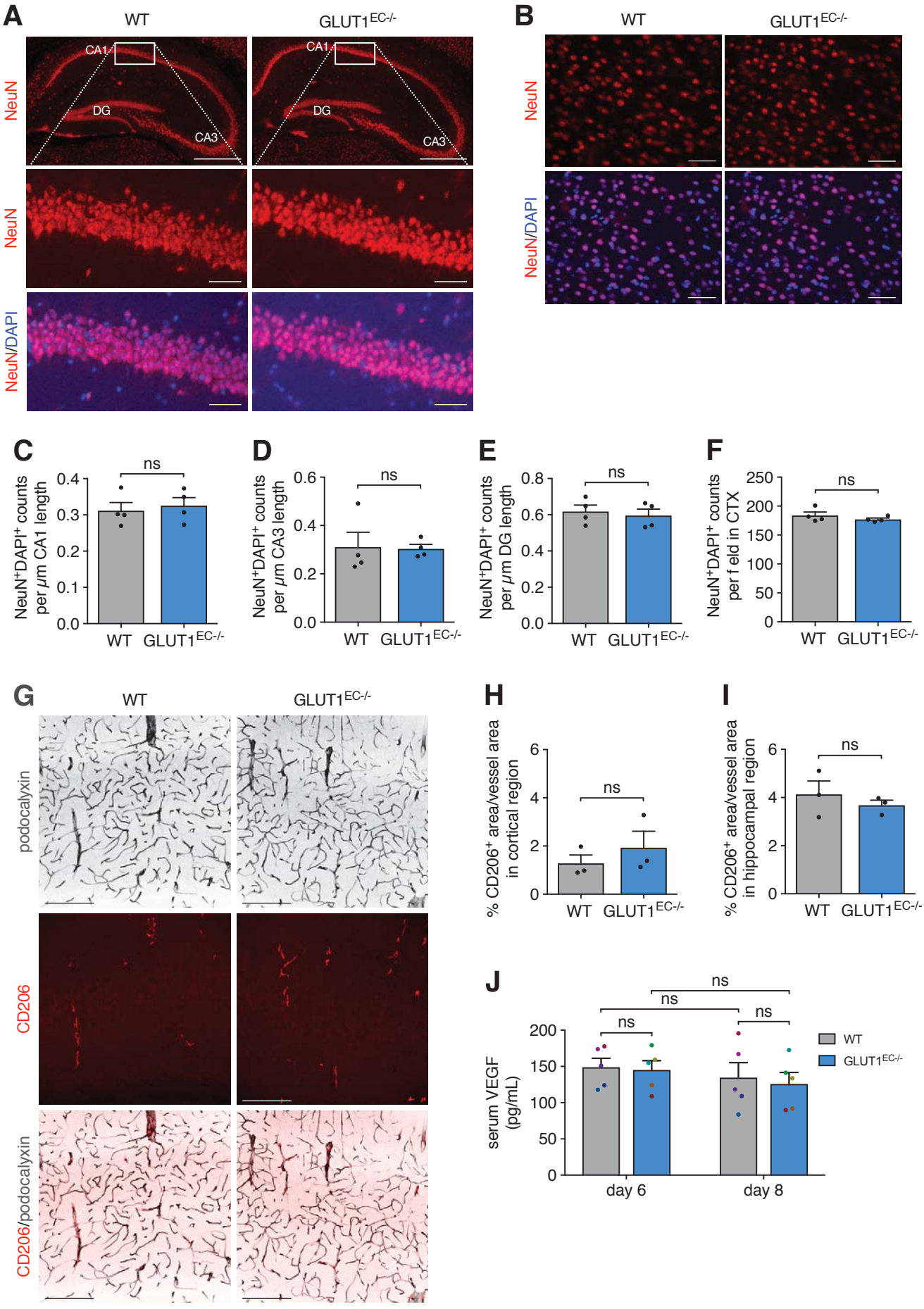
Online Figure IV



ONLINE FIGURE IV: Loss of EC-GLUT1 impairs neonatal retinal angiogenesis *(related to Figure 3)*

A and **B**, Representative pictures (**A**) and quantification (**B**) of EdU^+/Erg^+ proliferating cultured primary isolated mouse ECs from (Mann-Whitney U test). CD31 staining is used to verify purity of mouse EC cultures. **C**, Representative pictures of the primary retinal plexus with vessels visualized by IB4 and co-stained for GLUT1 in flat-mounted retinas of P6 WT pups. **D**, Representative picture of GLUT1-stained tip cells in the brain vasculature of P6 WT pups. Scale bar = 50 µm (**A,C**) and 10 µm (**D**). ****P<0.0001.

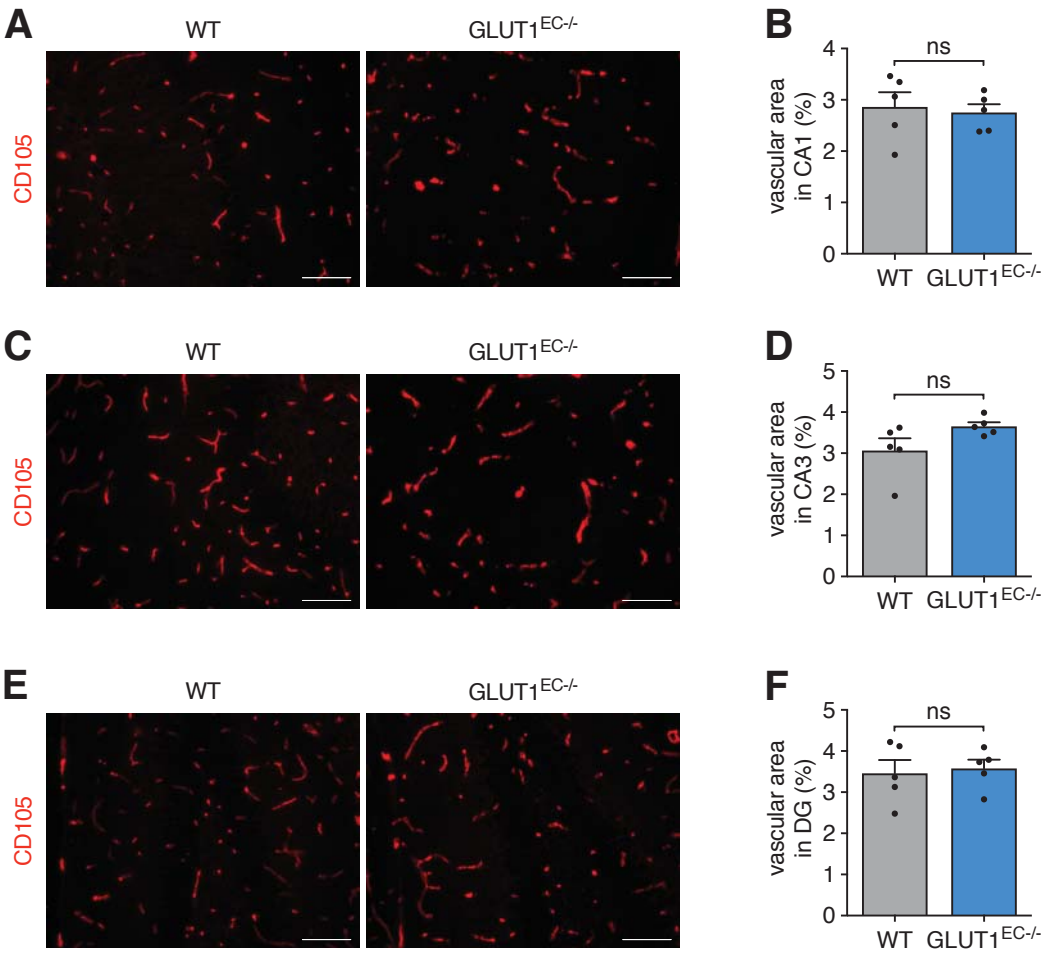
Online Figure V



ONLINE FIGURE V: Loss of EC-GLUT1 leads to progressive neuronal loss, CNS inflammation, and rapid lethality *(related to Figure 5)*

A and **B**, Representative pictures of hippocampal (**A**) and of cortical (**B**) $NeuN^+/DAPI^+$ neuronal nuclei in $GLUT1^{EC-/-}$ mice versus WT littermates at 7 to 8 days. **C** to **F**, Quantifications of the number of $NeuN^+/DAPI^+$ cells in the CA1 (**C**) CA3 (**D**) DG (**E**) and cortical region (**F**) in $GLUT1^{EC-/-}$ mice versus WT littermates (Student's *t*-test). Scale bar = 500 µm for upper panels, 50 µm for middle and lower panels (**A**) and 50 µm (**B**). **G** Representative pictures of cortical $CD206^+$ macrophages associated to $podocalyxin^+$ blood vessels in $GLUT1^{EC-/-}$ mice versus WT littermates at 8 days. **H** and **I**, Quantifications of the $CD206^+$ area per vessel (podocalyxin) area in (**H**) cortical and (**I**) hippocampal region in $GLUT1^{EC-/-}$ mice versus WT littermates (Student's *t*-test). Scale bar = 200 µm. **J**, Serum VEGF levels at 6 and 8 days in $GLUT1^{EC-/-}$ mice versus WT littermates (Repeated measures ANOVA and Sidak's multiple comparisons test). Dots with the same color represent one single mouse.

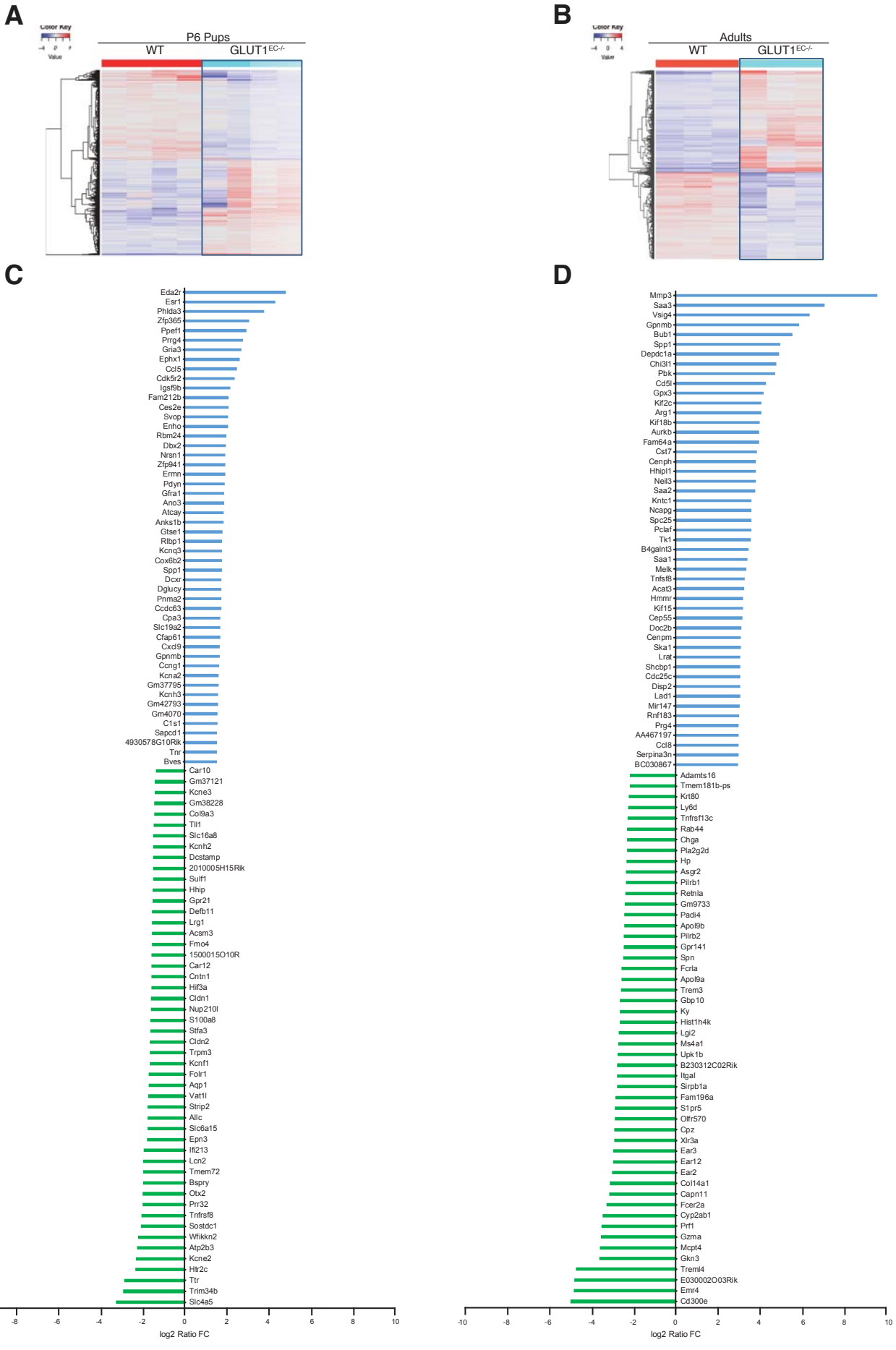
Online Figure VI



ONLINE FIGURE VI: Loss of EC-GLUT1 does not impair brain vascular function *(related to Figure 6)*

A, Representative pictures of the CD105-stained hippocampal vasculature in adult GLUT1$^{EC-/-}$ mice versus WT littermates in the CA1 (**A**) CA3 (**C**) and DG region (**E**) and quantifications of vascular area in the CA1 (**B**) CA3 (**D**) and DG region (**F**) (Student's *t*-test). Scale bar = 100 µm (**A,C,E**).

Online Figure VII



ONLINE FIGURE VII: Transcriptional alterations upon loss of GLUT1 in brain ECs *(related to Figure 8)*

Transcriptome analysis via RNA sequencing (RNAseq) of brain endothelial cells at P6 (left) or in adulthood (right). **A**, Heat map and hierarchical clustering of WT and GLUT1$^{EC-/-}$ brain ECs in P6 pups (n=4 per condition). **B**, Heat map and hierarchical clustering of WT and GLUT1$^{EC-/-}$ brain ECs in 8 weeks old adult mice (n=3 per condition). **C**, Top 50 significantly upregulated (blue) or downregulated (green) genes detected by RNAseq in GLUT1$^{EC-/-}$ brain ECs as compared to WT in P6 pups. **D**, Top 50 significantly upregulated (blue) or downregulated (green) genes detected by RNAseq in GLUT1$^{EC-/-}$ brain ECs as compared to WT in 8 weeks old adult mice. Differentially regulated genes are arranged according to fold change of gene expression.