Supplementary Figures and Data:

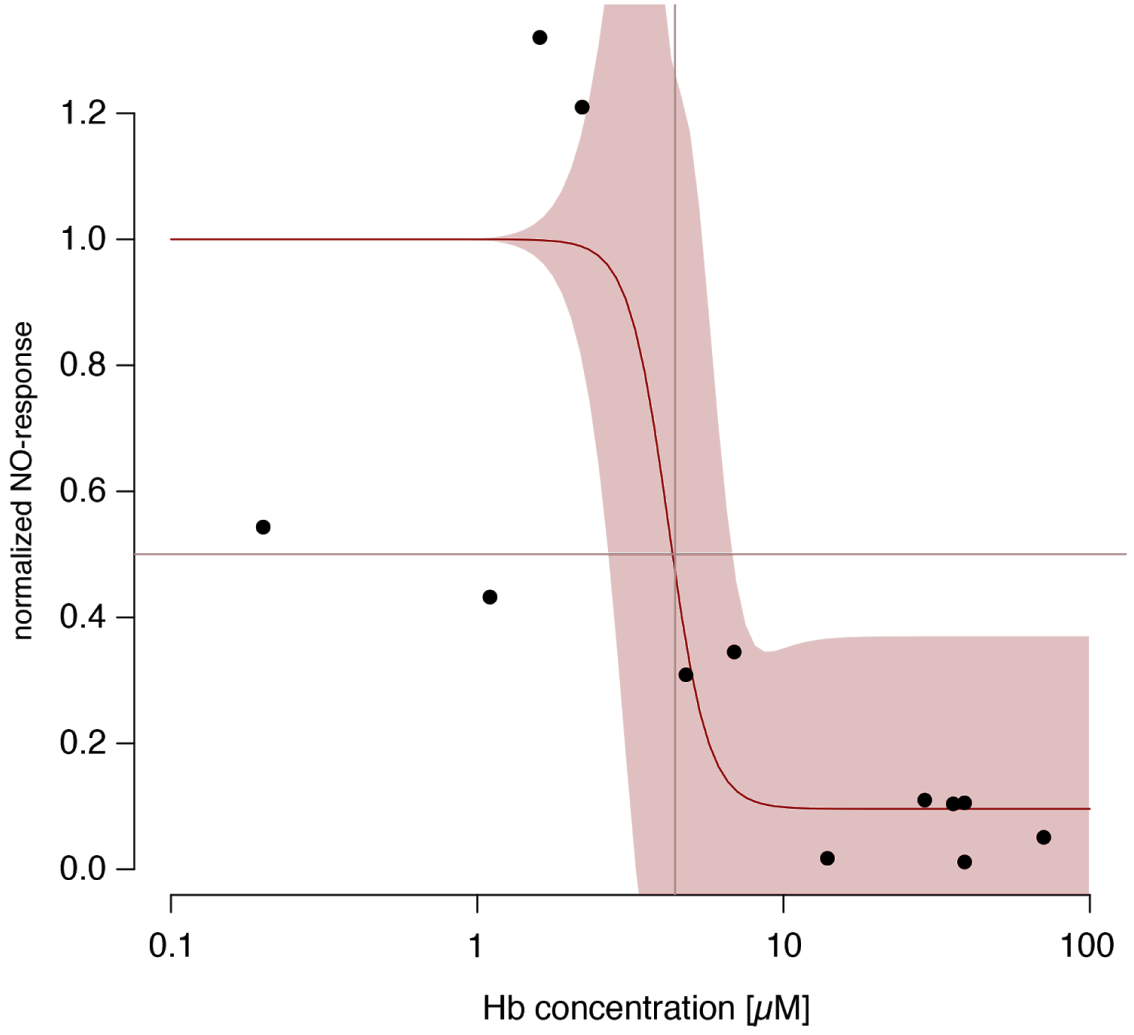

Figure S1: CSF-Hb concentration dependent suppression of NO-mediated arterial dilation

Vascular function experiments were performed as shown in Figure 1 with 12 porcine basilar artery segments immersed in 12 CSF samples collected during the at risk period for DIND (days 4 - 10) covering a range of Hb concentrations. A log-logistic function was fitted to the data to estimate the concentration by which dilation was suppressed by 50% (ED50 = 4.42 μM).

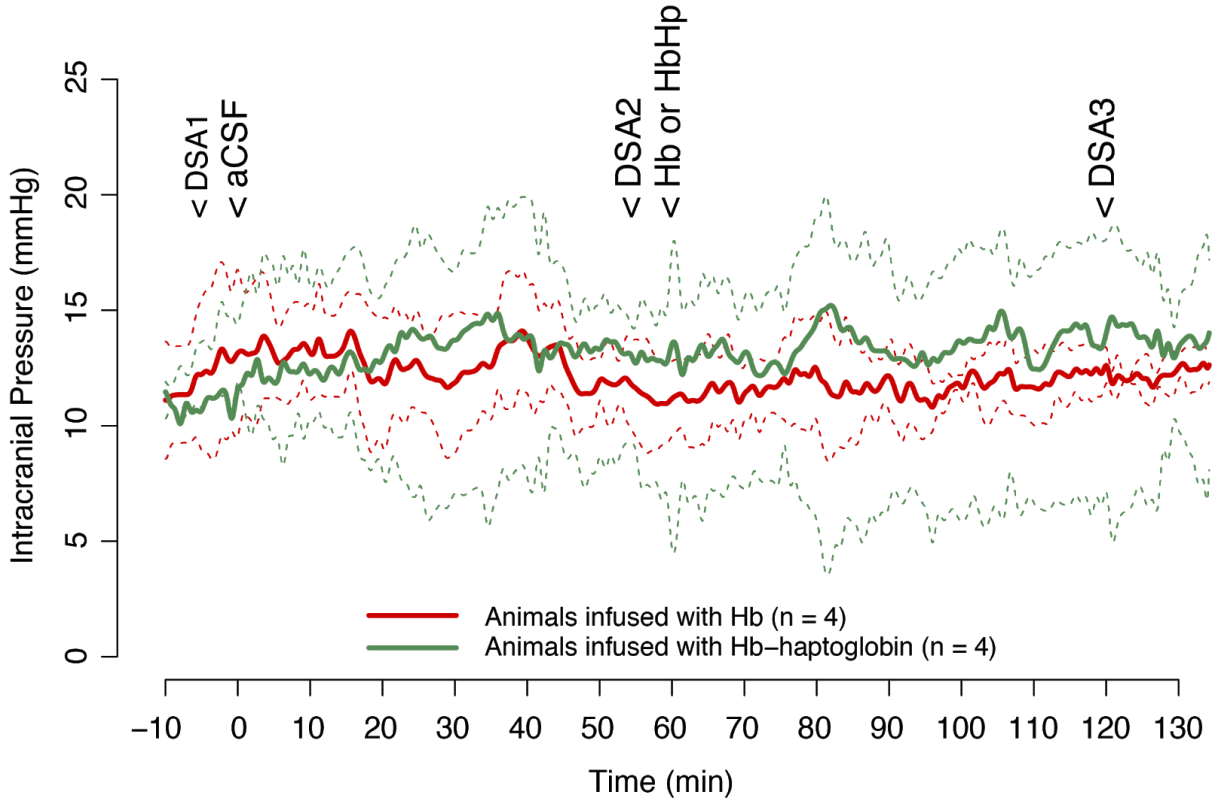

Figure S2: Continuous monitoring of intracranial pressure during the sheep experiments

During the whole experiment, the intracranial pressure (ICP) of the sheep was continuously monitored with a probe implanted in the right frontal lobe. Timepoints of the infusions and digital subtraction angiograms are indicated in the plot. With our protocol, we observed no changes of the ICP after an infusion volume of 2.5 ml. The ICP (mean ±SD) of the continuous monitoring data is plotted separately for the sheep infused with Hb (red, n=4) and Hb-haptoglobin (green, n=4) respectively.

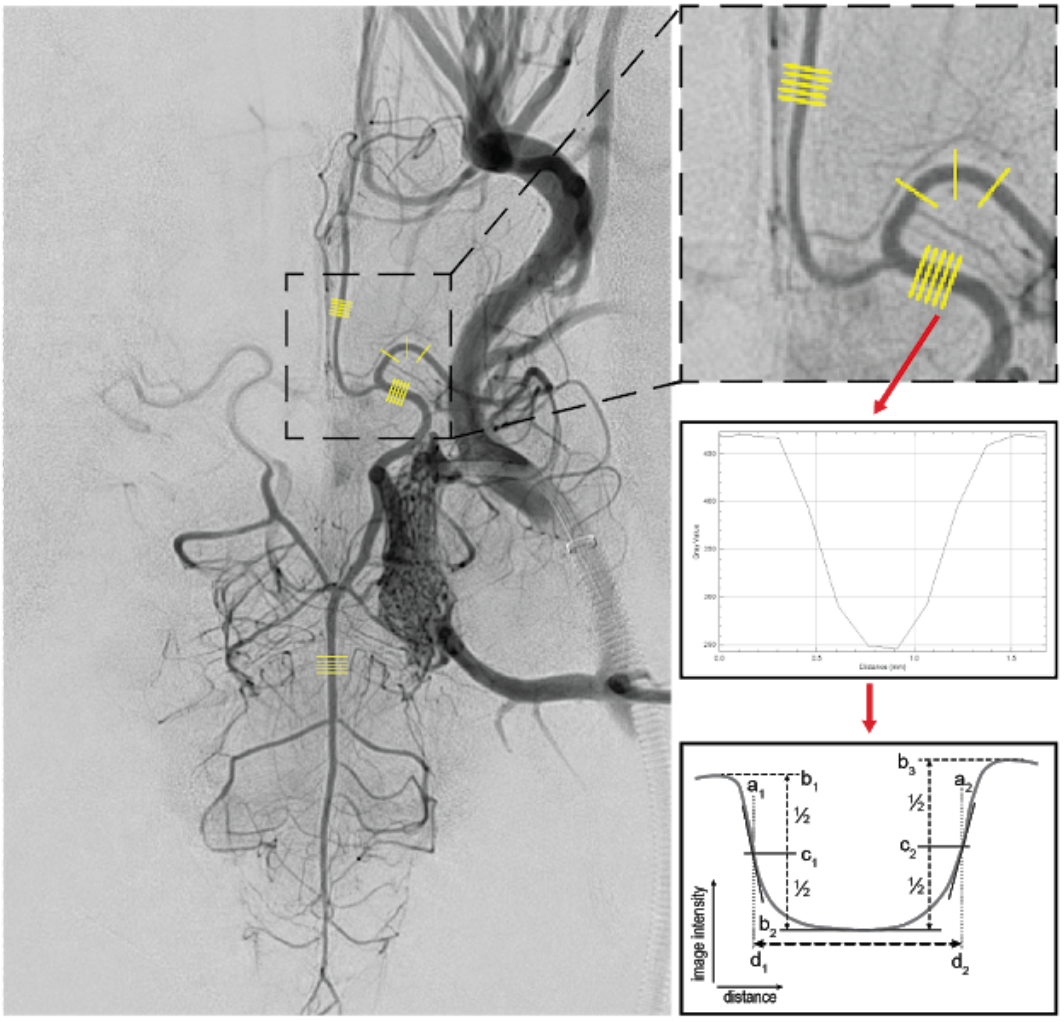

Figure S3: Semi-automatic quantification of cerebral blood vessel diameter

Semi-automated quantification of vessel diameters from DSA. The left figure and the inlay on the upper right display exemplary selections of linear ROIs (yellow) in the ACA, MCA, ICA, and BA. These were set automatically in the straight segment of the ACA, ICA, and BA with an interval of 0.5 mm (5 in number) and manually in the curvilinear segment of the MCA (3 in number). For all vessels, the diameter of the vessel from each ROI was calculated from the intensity profile of the cross section (middle right) as described previously by Fischer et al. 2010 (bottom right) and averaged over all ROIs of the respective vessel. The persons performing the data analysis were blinded to the experimental conditions.

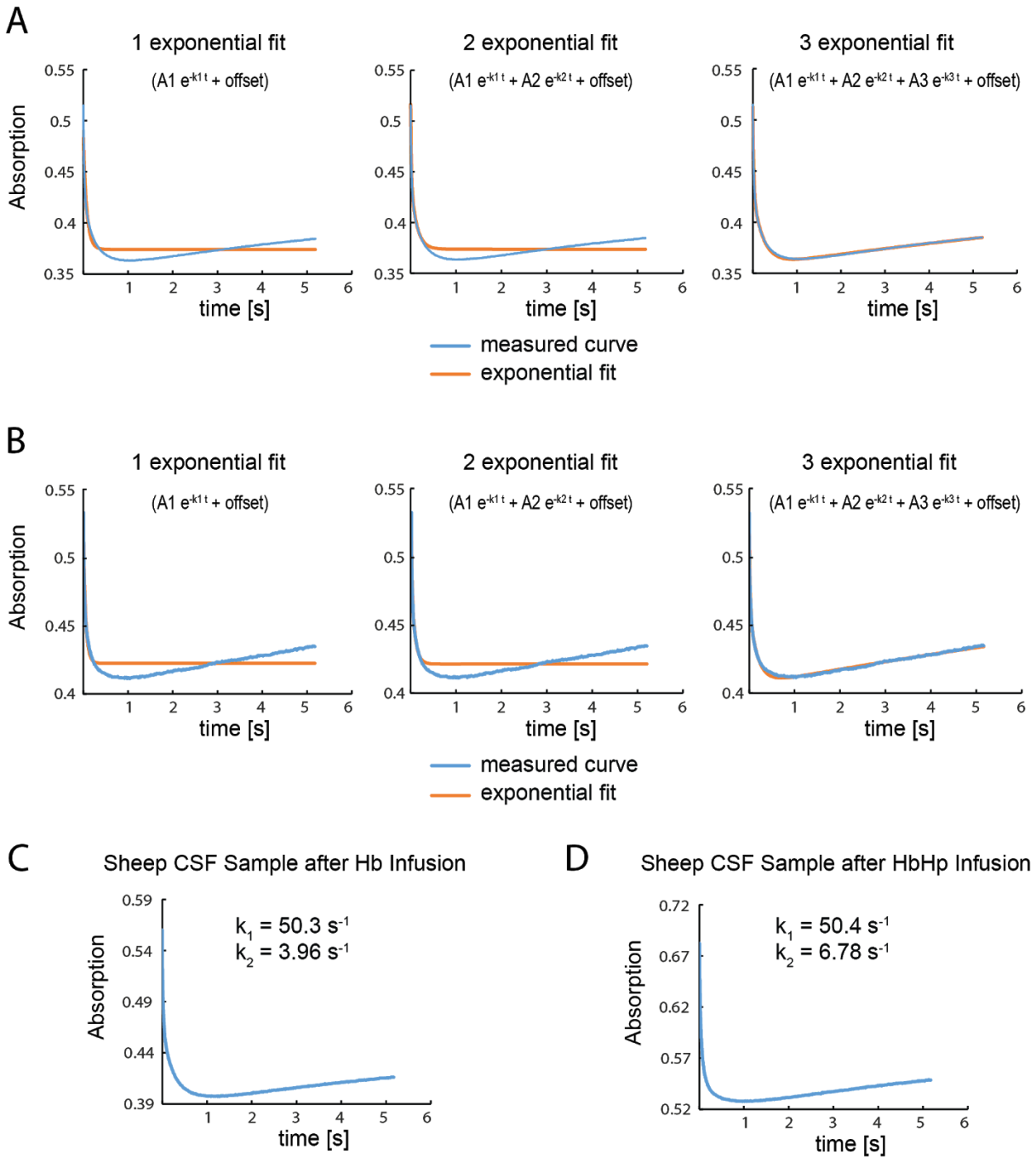

Figure S4: Stopped Flow Spectrophotometry of the NO reaction with oxyHb

Stopped-flow spectrophotometry experiments of NO with oxyHb **(A)** or oxyHb-haptoglobin complex **(B)** under conditions of NO-excess. The fit of the kinetic traces to a one-, two and three-exponential curve, illustrates and confirms the three phases of the reaction. The reaction rates of oxyHb from sheep CSF samples with NO **(C)** are comparable to the reaction rates of oxyHb-haptoglobin complexes in sheep CSF samples **(D)**. Absorbance was recorded at 405 nm.

Fitting equations:

- A (absorbance amplitude) is the absorbance change of a certain species during the reaction course (between the starting material and the product). When the reaction completes, it is representative of the total concentration converted. We expect this behavior for standard samples, unaffected by competition or inhibition. A decrease of A in samples compared to the standard means a diminished capability of the samples to react with NO.
- k is the pseudo-first-order or true first-order reaction rate constant. Dissociation reactions are usually true first-order, and the bimolecular reactions in our setup are pseudo-first-order because NO is always kept in excess, such that

$$\frac{d[P]}{dt} = k_{bi}[NO][S] \approx k_{pseudo-1st}[S] \quad with \quad k_{pseudo-1st} = k_{bi}[NO]$$

P is the product, S the substrate. [NO] does not change substantially during the reactions.

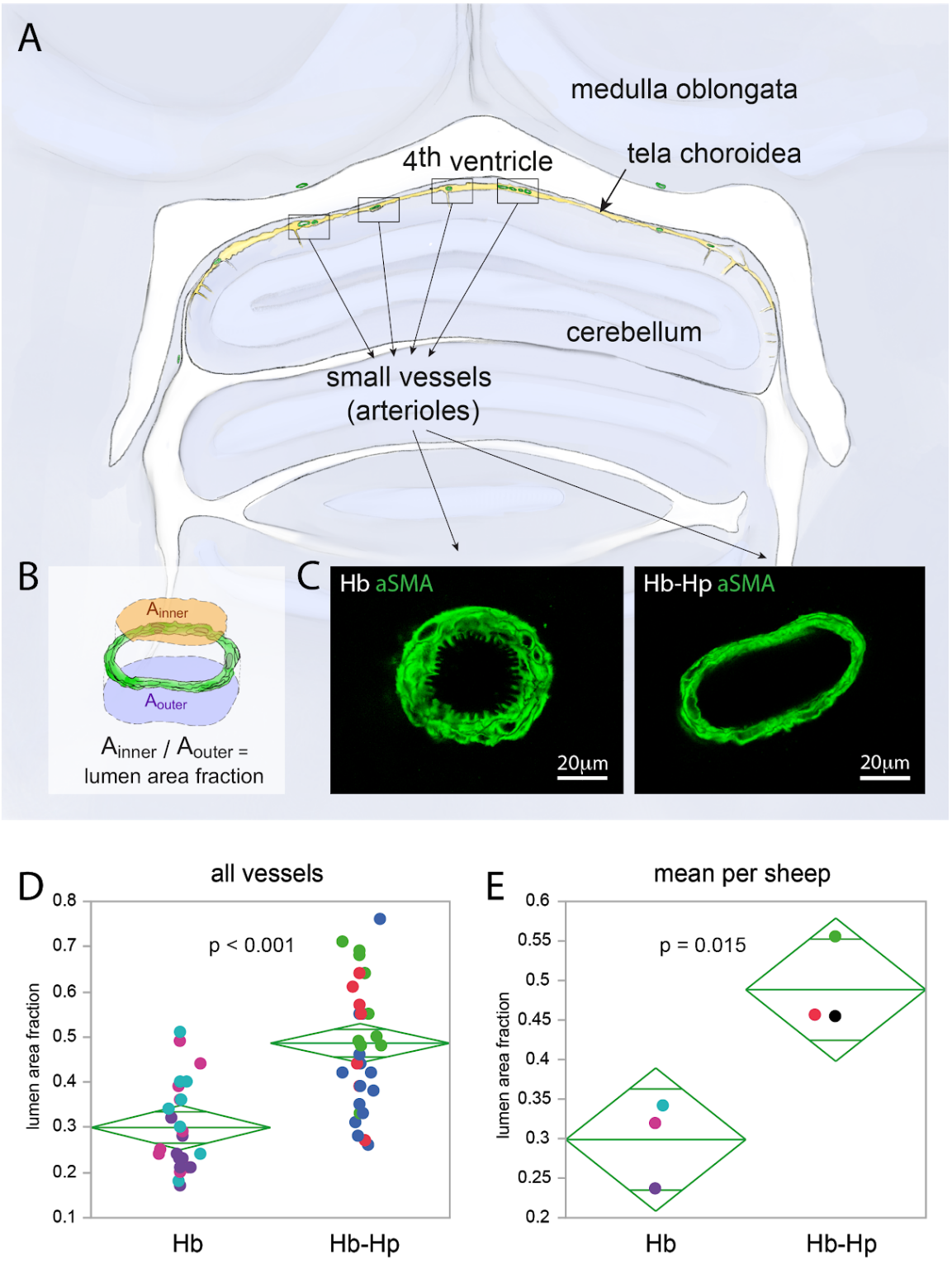

Figure S5: Histomorphometric analysis of arterioles in the tela choroidea of the fourth ventricle

120 µm sections of sheep brain were stained for alpha-smooth muscle actin (αSMA). **(A)** Illustration of the anatomical situation of the studied brain sections. A researcher blinded to the treatments recorded confocal images of the vessels in the tela choroidea. **(B)** Based on the

manual delineation of the inner and outer circumference of the αSMA positive structures with Image J software, the lumen area (A_{inner}), and total sectional vessel area (A_{outer}) were quantified for each vessel, and the *lumen area fraction* was calculated. These measurements were performed by three blinded researchers for all images, and the mean values were further analyzed. **(C)** Representative images of arterioles in the tela choroidea of a Hb and a Hb-haptoglobin treated sheep. **(D)** Plotted data and statistical analysis for all analyzed vessels (n=57). **(E)** Plotted data and statistical analysis for the mean lumen area fraction per sheep (n=3 sheep per treatment group). Colors indicate individual animals. The diamonds represent the mean and 95% CI. The overlap marks (horizontal lines above and below the mean-line) define statistical significant difference between groups if not overlapping ($p < 0.05$).

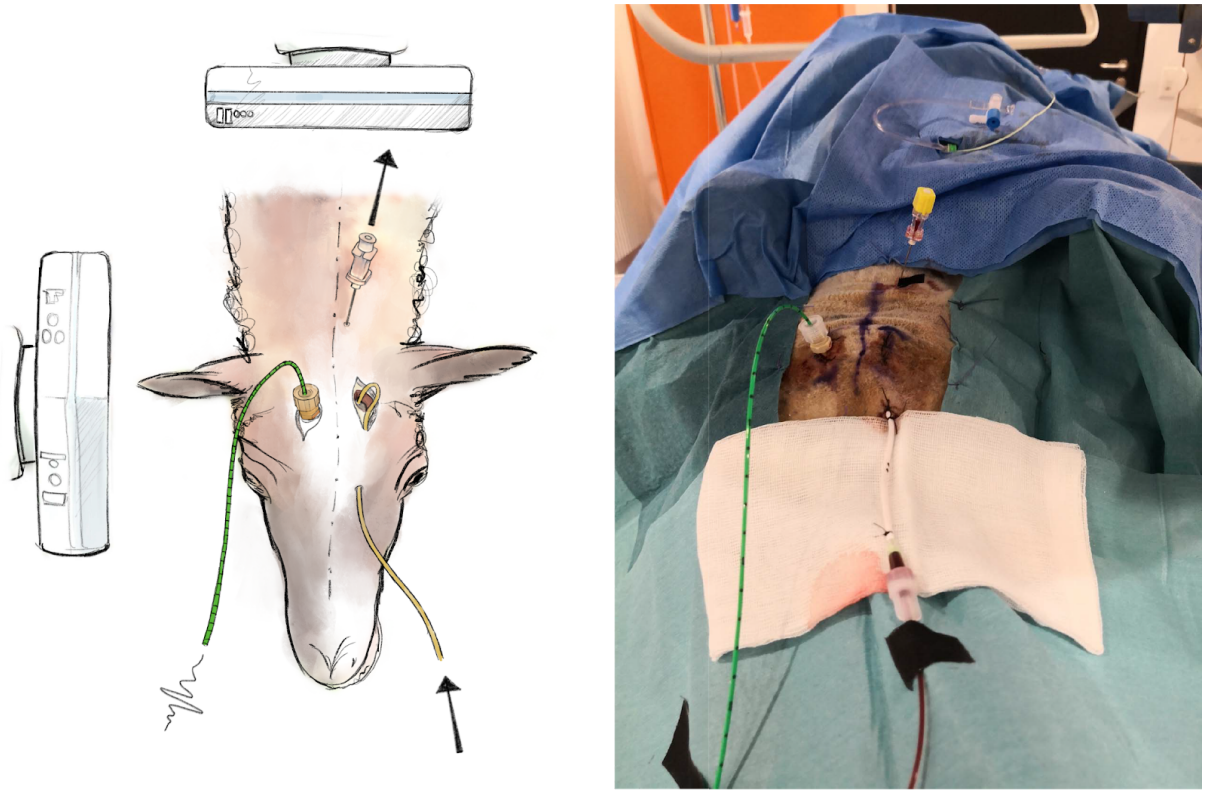

Figure S6: Sheep model - Intraoperative Setup

Scheme (left) and intraoperative photography (right) of the experimental setup with an external ventricular drain entering into the frontal horn of the left lateral ventricle, implanted neuromonitoring probe (green) in the right frontal white matter and a suboccipital spinal needle.

Supplemental Data:

Proteomics Data (Supplemental Data 1):

Table with the cluster-assignment of the protein identified in the LC-MS/MS proteome analysis of sequential CSF samples from patients with aSAH.

Supplemental Movies:

Movie S1:

3D reconstructions of the specific Mn-signal 20 minutes after infusion of MnHb or MnHb-haptoglobin respectively. The reconstructions obtained by subtraction of the baseline T1-weighted image from the T1-weighted image 20 minutes after infusion, display the rapid and equal distribution of the injected compounds through the ventricular system into the cisterna magna and the basal subarachnoid space.

Movie S2:

Representative sagittal reconstruction of the dynamic T1-weighted MR images acquired over 10 minutes during the ventricular infusion of MnHb or MnHb-haptoglobin complexes. The sequences show no difference between the macroscopic distribution of Hb and Hb-haptoglobin complexes.

Movie S3:

Representative sagittal reconstruction of the T1-weighted MR images acquired at an interval of 10 minutes over a time period from 10 minutes until 90 minutes after the ventricular infusion of MnHb or MnHb-haptoglobin complexes. The sequences confirm the rapid distribution of the substances into the outer, subarachnoid CSF space after ventricular infusion and showed no difference between the macroscopic distributions of Hb and Hb-haptoglobin complexes. The MnHb-haptoglobin complex is more stable providing higher signal intensity in MRI.

Supplementary Material and Methods

Preparation of CSF samples from aSAH patients

Immediately after collection CSF was centrifuged at 3000 g for 15 min to remove blood cells. CSF supernatants were stored frozen at -80° until the day of experiment. For wire myography experiments, samples of consecutive days were pooled if needed due to limited available CSF volumes from single days.

HPLC measurements

For qualitative and quantitative size-exclusion chromatography (HPLC), samples containing Hb and/or Hb-haptoglobin were separated on an analytical BioSep-SEC-s3000 (600 × 7.8 mm) LC column coupled with a BioSep-SEC-s3000 (75 × 7.8 mm) Guard Column (Phenomenex, Torrance, CA) and attached to an LKB 2150 HPLC Pump (LKB-Produkter AB, Bromma, Sweden). Potassium phosphate 20 mM pH 6.8 was used as the mobile phase. The absorption was measured at 414 nm with a Jasco UV-970 Intelligent UV/VIS Detector (JASCO International Co., Ltd., Tokyo, Japan) and recorded using Lab Chart software version 7.2.1 (AD Instruments, Hastings, UK). HPLC curves were analyzed and visualized with R statistical software version 4.2.3 (www.r-project.org).

Preparation of artificial CSF (aCSF)

The final composition of aCSF was 127 mM NaCl, 1.0 mM KCl, 1.2 mM KH_2PO_4, 26 mM NaHCO, 1.3 mM $MgCl_2*6H_2O$, 2.4 mM $CaCl_2*2H_2O$ and 6.7 mM Glucose.

Vascular function experiments - Isolation of basilar arteries

Basilar arteries were isolated from fresh heads of slaughtered pigs from the local slaughterhouse (SBZ, Zurich, Switzerland). The brainstem was carefully removed from the head by a sharp dissection and the level of the pontomesencephalic junction and the cerebellar peduncles after a bilateral paramedian osteotomy of the clivus. Using a dissection microscope (Olympus SZX9 Stereo Microscope, Olympus, Tokyo, Japan), the vertebral arteries were identified as an anatomical landmark and cut 2 mm proximal to the vertebrobasilar junction. Careful arachnoidal preparation allowed stepwise mobilisation of the basilar artery avoiding excessive mechanical manipulation of the vessel. The basilar artery segment between the vertebrobasilar junction and the caudal cerebral artery were used to prepare up to 6 vascular rings (length of 2 mm per ring). During all surgical preparations special attention was paid to avoid any compression or distension of the arteries. The time

between the death of the animal and the transfer of the dissected vessels into the buffer was kept below 90 minutes.

Cerebrospinal fluid proteomics - Protein extraction and proteomics sample preparation

For proteomics, the proteins in CSF samples were extracted using an MTBE-based extraction procedure with an MTBE/methanol/water solvent system at a ratio of 10:3:2.5 (v/v/v). The upper, lipid-containing organic phase was discarded, whereas the precipitated proteins were resuspended in the lower aqueous phase, dried in a speed vac and stored at -80 °C until further analysis. For mass spectrometry, the protein concentration was estimated using the Qubit® Protein Assay Kit (Life Technologies, Zurich, Switzerland). The samples were then prepared by using a commercial iST Kit (PreOmics Phoenix, Germany) with an updated version of the protocol. Briefly, 50 µg of protein was solubilized in 'Lyse' buffer, boiled at 95 °C for 10 minutes and processed with HIFU for 30 s, setting the ultrasonic amplitude to 85%. Afterwards, the samples were transferred to the cartridge and digested by adding 50 µl of the 'Digest' solution. After 60 minutes of incubation at 37 °C, the digestion was stopped with 100 µl of Stop solution. The solutions in the cartridge were removed by centrifugation at 3800 *g*, while the peptides were retained by the iST-filter. Finally, the peptides were washed, eluted, dried and resolubilized in 20 µL of buffer (3% acetonitrile, 0.1% formic acid) buffer for LC-MS analysis.

Ex vivo vascular function experiments:

Using a dissection microscope, porcine basilar arteries were isolated, and up to 6 vascular rings (length of 2 mm per ring) were prepared (details in the supplement*).* The vascular rings were mounted on two 0.2 mm diameter pins of a Multi-Channel Myograph System 620 M (Danish Myo Technology, Aarhus, Denmark) immersed in temperature-controlled (37 °C) and continuously aerated (95% O_2 and 5% CO_2 gas mixture) organ baths containing 5 ml of Krebs-Henseleit-Buffer (KHB). Then, the vessels were gradually stretched to the optimal IC1/IC100 ratio as determined in previous experiments. As a pre contracting agent, 10 µM prostaglandin F2α (PGF2α; Sigma, Buchs, Switzerland) was used. The NO-mediated vasodilatory responses were induced by the addition of MAHMA-NONOate (ENZO Life Sciences, Lausen, Switzerland) to the immersion buffer. All data were recorded using Lab Chart software version 7.2.1 (AD Instruments, Hastings, UK). If not otherwise stated, the recorded hemoglobin-induced vascular function responses were normalized relative to maximum NO dilatation without Hb exposure (= 100%) and the level of tonic contraction before addition of MAHMA-NONOate (= 0%), respectively, during a single experiment. Therefore, the responses in the plots are indicated as relative relaxation. For experiments with CSF, organ baths were equipped with 3D-printed customized inlays (volume of 2.5 ml) and CSF samples diluted 1:1 with artificial CSF to reduce the needed volumes of patient samples

for single experiments. Patient CSF samples were defined as preerythrolysed (day 1 - 3) and erythrolysed (day 4 - 14). Due to limited available CSF volumes from single days, samples of consecutive days were pooled if needed.

Cerebrospinal fluid proteomics - Liquid chromatography-mass spectrometry analysis

Mass spectrometry analysis was performed on a Q Exactive HF mass spectrometer (Thermo Scientific) equipped with a Digital PicoView source (New Objective) and coupled to an M-Class UPLC (Waters). The solvent composition at the two channels was 0.1% formic acid for channel A and 0.1% formic acid, 99.9% acetonitrile for channel B. Each sample was diluted 5 times, and 3 µL of peptides was loaded on a commercial MZ Symmetry C18 Trap Column (100 Å, 5 µm, 180 µm x 20 mm, Waters) followed by a nanoEase MZ C18 HSS T3 Column (100 Å, 1.8 µm, 75 µm x 250 mm, Waters). The peptides were eluted at a flow rate of 300 nL/minutes by a gradient from 5% to 35% B in 120 minutes and 95% B in 5 min. Samples were obtained in a randomized order. The mass spectrometer was operated in data-dependent mode, acquiring a full-scan MS spectra (350−1,500 m/z) at a resolution of 120,000 at 200 m/z after accumulation to a target value of 3,000,000, followed by HCD (higher-energy collision dissociation) fragmentation on the twelve most intense signals per cycle. HCD spectra were acquired at a resolution of 30,000 using a normalized collision energy of 28 and a maximum injection time of 50 ms. The automatic gain control was set to 100,000 ions. Charge state screening was enabled. Singly-charged and unassigned ions and ions with charge states higher than eight were rejected. Only precursors with intensities above 90,000 were selected for MS/MS. Precursor masses previously selected for MS/MS measurement were excluded from further selection for 30 s, and the exclusion window was set to 10 ppm. The samples were acquired using internal lock mass calibration on m/z 371.1012 and 445.1200.

The mass spectrometry proteomics data were handled using the local laboratory information management system (LIMS) (1).

Measurement of Hb−NO reaction by stopped-flow spectrophotometry

The HbO_2 reaction with NO was measured with a SX18MV stopped-flow spectrophotometer (Applied Photophysics Ltd., Leatherhead, UK). In order to obtain sensitive detection of reactions at the heme, the monochromator with a resolution bandwidth of 2 nm was set to 405 nm, in the range of the Soret band (2).

The instrument was made completely anaerobic by purging the thermostat circuit and the syringe piston leads continuously with nitrogen. The temperature was always set to 25 °C. Solutions were loaded from gastight syringes (Hamilton Inc., Reno NV, USA) the PTFE parts

of which were deaerated beforehand in a glove box. NO gas was produced immediately before the experiment in a reaction of $NaNO_2$ with $FeSO_4$ under acidic and anoxic conditions. The final NO solution had a concentration of approximately 160 µM NO. HbO_2 was used at 1.5 - 6µM (heme).

The kinetic data obtained at 405 nm from the reaction of NO with the oxygenated hemoglobin species always showed a triphasic behavior with a rapid absorbance drop after initial combination with NO, followed by a slower absorbance decay, consistent with the observations made by Herold (3). In order to obtain a fit based on a sufficient number of supporting points, the entire curve was approximated with a function containing a sum of three exponentials. This can be justified by the assumption that the remaining parts of the trace represent subsequent reactions with NO, which are pseudo first-order. This condition is met because NO was always more than 10-fold in excess over heme. The initial phase is true first-order because it relates to the elimination of nitrate from the heme.

The fit procedure was carried out with MS Excel by means of the Solver module. Fit functions were directly entered into the spreadsheet, as well as the residuals calculation. The target function was the sum of the squared residuals, to be minimized by one of the optimization functions contained in the Solver add-on. Fits were monitored by displaying the experimental and the fitted curves together in a chart window. The pseudo first-order rate constants calculated by the fitting procedure were divided by the according heme concentrations in order to obtain the second-order rate constant.

Sheep model

Animals, housing and care

Female swiss alpine sheep (Staffelegghof, Küttigen bei Aarau, Switzerland) with an age of 2-4 years and a mean weight of 76.8 kg (weight range: 61.5 - 92.3 kg) were transferred to the experimental unit of the Veterinary Hospital of Zurich and allowed to acclimatize to the new environment for at least 7 days. Standardized screening with physical examination and blood testing was performed through a veterinarian during the acclimatization period. Animals were preoperatively fasted with free access to water for 18-24 hours.

Anesthesia

30 minutes before induction of anesthesia, the animals physical status was checked again by the attending veterinary anesthesiologist. Each animal was then premedicated with buprenorphine (0.01 mg/kg BW intramuscular) and medetomidine (0.07-0.1 mg/kg BW intramuscular). After 20 minutes, the sedated animal was transported to the angiography suite, the degree of sedation and the health status were again clinically assessed.

A 14 G, 3.5 inch intravenous catheter was inserted under sterile preconditioning into the left jugular vein and surgically fixed to the skin. Anesthetic state was intravenously induced by fixed dose injection of midazolam (0.1 mg/kg BW), ketamine (3 mg/kg BW) and variable dose injection of propofol (0.3-1.5 mg/kg BW) to effect.
Thereafter, the larynx of each animal was desensitized using lidocaine 10% sprayed directly and under visual control through a laryngoscope. After allowance of 20-30 seconds, the animals tracheas were then intubated using 11 or 12 mm ID sized appropriately long silicone endotracheal tubes. The animals were then positioned in left lateral recumbency onto a flexible vacuum mattress on the examination table of the Allura Clarity angiography suite. They were immediately connected to a coaxial circle breathing system of appropriate size and artificial ventilation was started at a rate of 6-8 breaths per minute and 10 mL/kg BW of tidal volume. Isoflurane was delivered in Oxygen at 0.8-2.0 Vol% throughout the whole procedure. A pulse oximeter probe was connected to the animal to assess and ensure adequate hemoglobin oxygenation status throughout the whole procedure. Ventilation was adjusted to maintain normocapnia as assessed by capnography (end-tidal CO_2) and by arterial blood gas measurement as well as by calculation the arterial-to-alveolar difference and using Bohr's alveolar gas equation and thereby estimating the relative functioning of the ventilation-perfusion match. Maintenance of normal and constant arterial partial pressure of CO_2 (2.5-5.5 kPa) was of crucial importance, as arterial CO_2 is known to represent a major factor of cerebrovascular reactivity, particularly in the range of 4-9 kPa.
A urinary catheter (Foley, Size 9), was inserted into the urinary bladder to allow urine to deflow during the procedure and for monitoring of urine production over time.
Under ultrasonographic control, an 8F intravascular cannula was inserted into the right external carotid artery as a port for later introduction of intravascular catheters. Once placed, 100 IU/kg BW of unfractionated heparin were administered intravenously to assure anticoagulation. This was repeated every 6 hours throughout the procedure.
The animals were then positioned in sternal recumbency with their front legs flexed under the neck and their hind legs flexed forward as a final body position that was maintained for the rest of the procedure unvaried.
Both auricular arteries were cannulated using 20G Surflo Terumo ® catheters for continuous direct arterial blood pressure monitoring and arterial blood sampling for intermittent blood gas analysis.
Animals were instrumented for continuous measurement (Datex- Ohmeda S3 compact life signs monitor) and recording (via a laptop computer) of heart rate, electrocardiogram, invasive direct arterial blood pressure, oxygen hemoglobin saturation, and inspiratory and expiratory concentrations of oxygen, carbon dioxide and isoflurane.
Animals were administered lactated Ringer's solution intravenously over the whole duration of the procedure at a standard rate of 3 ml/kg BW/hr, adjusted when needed to maintain normotension (60-100 mmHg mean arterial blood pressure) and normal urine production of 2 ml/kg BW/hr.

The anesthetic state was maintained and when necessary anesthesia depths varied by adjusting isoflurane inspired concentration and/or a propofol variable rate infusion administered intravenously by means of a syringe pump (Perfusor®, BBraun; rate 0.5-2 mg/kg BW/hr).
Furthermore, to reduce movement artifacts and to facilitate artificial respiration, rocuronium was administered intermittently at a dose of 0.5 mg/kg BW intravenously every 2 hours or when clinical assessment of the degree of muscle relaxation indicated the latter to be insufficient throughout the duration of the procedure.

Monitoring

Continuous monitoring of intracranial pressure (ICP), brain temperature, heart rate, blood pressure, oxygen saturation and end-tidal CO_2 was performed in all animals. Additionally, arterial blood gas analyses were analyzed every 30 minutes during the whole experimental procedure and urinary output was monitored.

Surgical procedures

Anesthetized animals were initially positioned in left lateral recumbency onto a flexible vacuum mattress on the examination table in an angiography suite. Under ultrasonographic control, an 8F intravascular cannula was inserted into the right common carotid artery as a port for later introduction of intravascular catheters. Once placed, 100 IU/kg BW of unfractionated heparin was administered intravenously to assure anticoagulation. This administration was repeated every 6 hours throughout the procedure. Then, the animals were positioned in sternal recumbency with rigid fixation of the head in a customized holding device, with their front legs flexed under the neck and their hind legs flexed forward as final body position for the experiment. After clipping and disinfection of the skin, sterile draping was placed around the surgical field. A neuromonitoring probe (Luciole Medicale AG, Zürich, Switzerland) was inserted through a right frontal paramedian burr hole using a neurosurgical bolt kit (Raumedic, Helmbrechts, Germany). An external ventricular drain (EVD) (DePuys Synthes, Oberdorf, Switzerland) was inserted into the frontal horn of the left lateral ventricle through an 11 mm burr hole. A suboccipital cisternal puncture for CSF release and sampling with a standard 20G spinal needle (Dalhausen, Köln, Germany) was performed under fluoroscopic guidance. CSF samples were immediately centrifuged at 1500 *g* for 15 minutes. The supernatant was collected for measurement of Hb concentrations and vascular function experiments. For controlled ventricular injections, a PHD Ultra syringe pump (Harvard Apparatus, Holliston, USA) was connected to the EVD. Ventricular injections were performed with maximal flow rates of 30 ml/h. For illustration of the experimental setup, see Supplementary Figure S6.

At the completion of the study period, i.e., when all planned data acquisition was terminated, the animals were euthanized under anesthesia by intravenous administration of pentobarbital at 150 mg/kg BW. Death was confirmed by means of the attached monitoring instruments as well as by transthoracic auscultation.
After euthanasia, the CSF space was flushed with 10 mL PFA 4% through the EVD with an infusion rate of 30 ml/h before harvesting the brain. The whole brain was cut into 1 cm thick coronal slices and fixed in 4% PFA at 4 °C overnight. Then, the slices were cropped to a suitable size for further processing, embedded in 4% agarose in PBS and cut to 120 µm floating sections using a vibratome (Leica VT1000 S Vibrating blade microtome, Leica Biosystems, Wetzlar, Germany). The sections were either processed immediately or stored in anti-freeze solution (Propylene Glycol, Glycerin, PBS 0.1 M and ddH_2O in a 1:1:1:1 ratio) at -20 °C until further processing as described in section histology.

Digital subtraction angiography (DSA) data analysis

Digital subtraction angiography was performed in an Allura Clarity angiography suite (Philips, Hamburg, Germany). The largest anastomosis between the right maxillary artery and the extradural rete mirabile was selectively catheterized with an angiographic 5F catheter through the arterial port in the right common carotid artery. Biplanar oblique lateral and dorsoventral projections were acquired simultaneously. A contrast bolus of 11 ml ioversol 300 mg iodine/ml (Optiray 300, Guebert AG, Zurich, Switzerland) was injected with 2 ml/s through the microcatheter with a high-pressure contrast agent injector (Accutron MR, Medtron AG, Saarbrücken, Germany).

Angiograms were processed using ImageJ, and vessel diameters were measured with a plug-in for ImageJ (4). For each sheep, angiograms at the time points pre-aCSF, pretreatment and posttreatment (60 minutes after Hb or Hb-haptoglobin infusion) were compared. Within the image sequence of the single DSA at the mentioned time points, lateral projections served to define the beginning of the venous phase by contrast depiction of cortical vein influx to the superior sagittal sinus (“venous T sign”). For each time point, a stack of the last three images of the arterial phase was intensity averaged. All vessel measurements were performed on dorsoventral projections in a stack combining the intensity-averaged arterial phase of pre-aCSF, pretreatment and posttreatment angiograms (Supplementary Figure S3). Measurements were performed in the anterior cerebral artery (ACA), middle cerebral artery (MCA), cisternal part of internal carotid artery (ICA) and basilar artery (BA). For each vessel, the most proximal non-superimposed segment was selected on pre-aCSF images. In the vessels with an anatomically straight course (ACA, ICA and BA), five linear regions of interest (ROIs) were automatically generated at 0.5 mm intervals. In the curvilinear MCA, three ROIs were defined manually. For each linear ROI, the vessel diameter was determined using the ImageJ plug-in (4). The diameter of the vessel was calculated from the mean of the

measured ROIs. Vessel-specific mean diameter changes were compared between pre-aCSF, pretreatment and posttreatment angiography for each sheep and set in relation to the treatment modality (Hb vs. Hb-haptoglobin).

Magnetic resonance imaging

In vivo magnetic resonance imaging was performed in a clinical 3 Tesla MRI unit (Philips Ingenia, Amsterdam, Netherlands). Axial and sagittal T2-weighted images were acquired for anatomical orientation and postsurgical control of catheter placement. Dynamic 3D T1-weighted imaging was performed with high temporal resolution over 10 minutes covering the time of MnHb infusion. Dynamic 3D T1-weighted imaging was continued for the following 80 minutes in 10-minute intervals with higher spatial resolution. DICOM data were processed using HOROS software (Nimble Co LLC d/b/a Purview, Annapolis, MD USA) for image reconstruction and analysis.

Preparation of MnPP-Hb and MnPP-Hb-haptoglobin complexes

A 40 mg/ml solution of apoHb or apoHb-haptoglobin was mixed with an equal volume of Mn(III) protoporphyrin IX chloride and incubated at 37 °C for 1 hour. The pH was adjusted slowly to 7.45 with 144 mM ortho-phosphoric acid (Sigma-Aldrich, St. Louis, MO, USA), and isotonicity was adjusted with 154 mM NaCl (Sigma-Aldrich, St. Louis, MO, USA). The solution was centrifuged at 4000 rpm for 10 minutes to remove any precipitate and sterile filtered (0.22-micron syringe filter, Pall, Port Washington, NY, USA). For the preparation of MnPP Hb-haptoglobin complexes, apoHb was mixed with human haptoglobin purified from plasma (CSL Behring, Kankakee, IL, USA) to approximate a 1:1 binding ratio. The mixture was assessed by SEC (BioSep-SEC-s3000 600x7.8 mm column, Phenomenex, Torrance, CA, USA) to confirm complete apoHb-haptoglobin complex formation. Monitoring of elution peaks was performed at a wavelength of 280 nm on a Waters 2998 Photodiode Array Detector (Waters Corporation, Milford, MA, USA). A 10 mM solution of Mn(III)).

Mouse Model

Wild-type male C57BL/6J mice (10-12 weeks, total n = 9) were obtained from Charles River (Sulzfeld, Germany) and maintained at the animal facility of the University of Zurich (LASC) and treated in accordance with the guidelines of the Swiss Federal Veterinary Office. The studies were conducted according to the Swiss legal requirements for animal protection and welfare (TschG 455) and received ethical approval from the cantonal veterinary authorities ‘Kantonale Tierversuchskommission Zürich’ (permission No ZH073/17).

Mice were anesthetized with isoflurane (4-5% for induction, 1-2% for maintenance; Baxter Healthcare Co. Deerfield, IL, USA) evaporated in 100% oxygen. Throughout the surgical

procedure, body temperature was monitored with a rectal probe and maintained constant with the use of an electronic thermostat-controlled warming blanket. Anesthetized mice were placed in a motorized stereotaxic frame (Stoelting, Dublin, Ireland) and a 1 cm long midline skin incision was made. After removal of the soft tissue covering the skull, the frame referenced to bregma and a small burrhole was drilled above the entry point for the target. Then a 33G needle was slowly advanced (0.1 mm/s) into the cisterna chiasmatica (bregma coordinates: 0 mm ML, 0 mm AP, 5 mm DV) followed by injection of 10µL TCO-labeled Hb, polymerizedHb or Hb-haptoglobin (each 1 mM) into the subarachnoid space with an infusion rate of 100 nl/s. The needle was left in place for 5 minutes, then removed slowly and the skin incision was closed with a suture. After the surgical procedure, the animals were placed in a heated wake up box and monitored until they were fully recovered and could be transferred back to their homecage. Two hours after subarachnoid injection, mice were deeply anesthetized with a lethal dose of an i.p. anaesthesia mix (Ketamine 100 mg/ml, Xylazine 16 mg/ml, Acepromazine 10 mg/ml) and transcardially perfused with 0.1 M PBS followed by 30 ml ice-cold PFA 4%. The brains were removed, followed by four hour post-fixation in 4% PFA at 4 °C. Using a vibratome (Leica VT1000 S Vibrating blade microtome, Leica Biosystems, Wetzlar, Germany), the brains were cut to 60 µm sections and stained as described in the section histology.

Histology

For histological analysis, purified protein solutions (Hb, haptoglobin) were labeled with TCO-NHS-ester (CLK-1016-1G, Jena Bioscience, Jena, Germany). In the first step, floating sections from mice (60 µm) or sheep brain (120 µm) were preconditioned in permeabilization buffer (2% BSA with 0.5% Triton-X-100 in PBS) for 4 hours at room temperature. Sheep sections were incubated with 2 ml blocking buffer (2% BSA in PBS) containing 40 nM tetrazine-Cy5 (CLK-015-05, Jena Bioscience, Jena, Germany), a FITC-coupled monoclonal antibody against α-smooth muscle actin (1:200, clone 1A4, F3777, Sigma), and a rabbit polyclonal antibody against recombinant rat Aqp4 (1:200, PAB28892, Abnova, Taiwan, China) at 4 °C for 48 hours, followed by the secondary antibody Alexa Fluor 555-conjugated goat anti-rat IgG (1:300, A-21434; Life Technologies, Gaithersburg, MD). Mice sections were only incubated with 40 nM tetrazine-5-TAMRA (CLK-017-05, Jena Bioscience, Jena, Germany). Then, nuclei were stained with Hoechst 33342 (1:2000 dilution, H3570, Invitrogen, Carlsbad, CA) for 40 minutes at room temperature. After three washes with PBS for 15 minutes, the sections were mounted with FluoroSafe (Merck Millipore, Burlington, MA). Whole-slide scans of the histological sections were produced by stitching together single images with a 10x magnification obtained with a Zeiss Observer. Z1 microscope coupled to a Colibri.2, an ApoTome.2 system and a motorized stage (Carl Zeiss AG,

Feldbach, Switzerland). Images with higher magnifications were obtained using a Leica SP8 confocal microscope (Leica Microsystems, Wetzlar, Deutschland).

Antibodies used for Immunohistology

The following antibodies were used in immunofluorescence experiments: FITC-coupled monoclonal antibody against α-smooth muscle actin (1:200, clone 1A4, F3777, Sigma), rabbit polyclonal antibody against recombinant rat Aqp4 (1:200, PAB28892, Abnova, Taiwan, China) and Alexa Fluor 555-conjugated goat anti-rat IgG (1:300, A-21434; Life Technologies, Gaithersburg, MD).

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