# Microcirculation deficits in the early phase after subarachnoid hemorrhage are independent from pericyte constrictions

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## ABSTRACT

**Background:** The early phase after subarachnoid hemorrhage (SAH) is characterized by microcirculatory dysfunction. Arteriolar constrictions and spasms of the cerebral microcirculation have previously been demonstrated in patients and after experimental SAH. Pericytes regulate the cerebral perfusion as part of the neurovascular unit and were observed causing capillary constrictions after ischemic stroke, their role after SAH is unclear. We therefor investigated the extend of pericyte constrictions and the compression of cerebral microvessels after experimental SAH using in-vivo 2-photon microscopy.

**Methods:** Neural/glial antigen 2 reporter mice were imaged with 2-photon microscopy before and 3h after SHAM surgery or induction of SAH through an MCA filament perforation model. The cerebral microcirculation was visualized by intraarterial Fluorescein isothiocyanate dextrane injection. $NG2^+$ pericytes were then assessed regarding the location of microarteriolar constriction. Pericytes were also quantified 24h after sham/SAH immunohistochemically in C57Bl6 animals.

**Results:** Microarteriolar diameters were reduced, and constrictions occurred in all investigated vessel categories down to the capillary level. No pericyte migration or loss was detected in the acute phase of subarachnoid hemorrhage. Pericytes did not colocalize with microarteriolar spasms or constrictions after experimental SAH.

**Conclusion:** Our results suggest that microcirculatory dysfunction after SAH is not relevantly mediated by pericyte constriction. The pathophysiology of early posthemorrhagic microcirculatory disturbances therefore seem to differ from changes observed after ischemic stroke.

## INTRODUCTION

Spontaneous subarachnoid hemorrhage (SAH) results most commonly from the rupture of intradural aneurysms and is associated with a 35% mortality and permanent disabilities.[1] An early reduction of the cortical blood perfusion was observed patients[2] and animal models[3] as part of early brain injury. The underlying pathophysiology is not fully understood, but constrictions of pial and penetrating arteries[3, 4] and consecutive microthromboses[5] were observed in animal models within hours after hemorrhage induction. Microvascular impairment does also occur after ischemic stroke. Here, pericytes constrictions reduce the capillary blood flow already minutes after vessel occlusion.[6] Also, a link between pericyte constriction and occurrence of microthromboses during EBI after SAH was discovered.[7]

In this study we investigated in Neural/glial antigen 2 $(NG2)^+$ DsRed[8] mice by 2-photon microscopy in vivo whether pericytes impair capillary blood flow during early brain injury after SAH.

## METHODOLOGY

### Animals and experimental groups

All animal procedures, group size calculations, and statistical methods used were approved by the Government of Upper Bavaria. The results of the study are reported in accordance with the ARRIVE guidelines. 8–10-week-old male $NG2^+$ DsRed mice (Jackson Laboratory, Bar Harbor, USA) were used for in-vivo imaging to visualize pericytes.[8] Immunhistochemistry (IHC) was performed in 8-10-week-old male C57Bl6/n mice (Jackson Laboratory, Bar Harbor, USA)

### SAH/SHAM surgery

SAH and sham surgery were performed as published previously[3, 9], under anesthesia with a mixture of 0.05 mg/kg fentanyl (Janssen-Cilac, Neuss, Germany), 0.5 mg/kg medetomidine (Pfizer, USA) and 5 mg/kg midazolam (Braun, Germany) under continuous ventilation. Temperature, $pO_2$, $pCO_2$, blood pressure, cerebral blood flow (CBF) and intracranial pressure (ICP) were monitored during surgery. The Circle of Willis was perforated with a prolene 5-0 filament. ICP peaked >50 mmHg in all animals. For sham surgery, the same filament was inserted intravascularly, but the Circle of Willis was not perforated. Monitoring was continued for 20 minutes after SAH induction. Anaesthesia was then antagonized by subcutaneous injection of 1.2 mg/kg naloxone (Actavis, Ireland, USA), 0.5 mg/kg flumazenil (Inresa, Germany) and 2.5 mg/kg atipamezol (Pfizer, USA).

## Two-photon microscopy

In-Vivo-Imaging was performed with a LSM 7 microscope (Zeiss, Germany), equipped with a Li:Ti laser (Chameleon, Coherent, USA), before and 3h after SAH/sham surgery in anaesthetized animals. A thinned skull window was prepared above the ipsilateral hemisphere of the filament perforation.

Four random 609 x 609µm regions of interest (ROI) were imaged to a depth of 400µm before and 3h after SAH induction at 20x magnification. Animals were continuously monitored for endexpiratory $pCO_2$, body temperature, heartrate and peripheral oxygen saturation during imaging.

For baseline imaging, 100µl Fluoreszeinisothiocyanate (FITC) dextran (0.5% in saline, Sigma Aldrich, USA) were injected intravenously in the tail vein. After SAH/SHAM the injection was repeated through a femoral artery catheter.

## Histological analysis and Quantification

Free floating sections were collected of C57Bl6/n mice perfused transcardially 24 hours after SAH or sham surgery. The vasculature was stained with FITC lectin and pericytes with Platelet Derived Growth Factor Receptor β (PDGF Rβ, 3169 NEB, USA). and DAPI (Vector Labs, USA). Cortex, hippocampus and striatum were imaged by confocal microscopy at a magnification of 20% and analyzed in blinded fashion.

## Data analysis

Image analysis was done with Fiji Image J, Version 2.3. Arteries and arterioles were defined as vessels with continuous $NG2^+$ cell coverage and a diameter of 15-40 µm. Capillaries were defined as vessels with discontinuous coverage with $DsRed^+$ cells

and a diameter of <15µm. Vessel diameters were measured randomly at nine locations /ROI before and after SHAM/SAH. Capillary diameters were measured randomly at height of pericytes and directly adjacent thereof.

**Statistical analysis**

Data are presented mean ± standard deviation, if parametric, otherwise in median ± IQR. Normal distribution was tested with D'Agostino & Pearson test. Significancy was tested with t tests (parametric data) of Mann-Whitney test (non parametric data) in Prism 8 (Graphpad Software LLC.)

## RESULTS

After SAH induction, regional cerebral bloodflow decreased to a median minimum of 13% IQR 14% of baseline (**Fig. 1A**). Within 20 minutes, the cortical perfusion approximates baseline at 46%, significantly less in comparison to SHAM (p=.003).

In direct comparison of the same volume of interest before and after SAH, we detected an 8 % reduction of vessel diameters in arterioles with an initial width of 30-40µm and 20-30µm, significantly different to sham operated animals (p <.001). Smaller arterioles with a width of 10-15µm were 6% narrower after sham and SAH surgery, still significantly different (p=.008). Perfused capillary volume is rarified after SAH but not after SHAM surgery in direct comparison of the microcirculation before and after SHAM/SAH (**Fig. 1B, Suppl. Fig. 1).** Pial and penetrating arteries (Fig. **1C**) as well as perfused capillaries (Fig. **1D**) are narrower after SAH in comparison with SHAM operated animals.

Immunohistochemistry shows a colocalization of $NG2^+$ cells with PDGF Rβ (**Suppl. Fig. 2**). Pericyte density does not significantly differ between cortex, hippocampus and striatum (**Fig. 2D**). Quantity and location of PDGF $R\beta^+$ pericytes was not significantly different in the cortex, the hippocampus or striatum of mice 24 hours after sham or SAH surgery (**Fig. 2D**).

In vivo imaging revealed an almost complete coverage of arterioles with $NG2^+$ pericytes, while capillaries are covered discontinuously (**Fig. 3A**).

In perfused vessels, the comparison of vessel diameters after SAH at the height of the pericyte did not significantly differ in comparison to the adjacent vessel segments (p=.579, **Fig. 3E**).

## DISCUSSION

Microvascular impairment is an essential part of early brain injury after subarachnoid hemorrhage.[10] Although pericytes can constrict capillaries and are discussed to impair cerebral blood flow after ischemic stroke[6], they do not constrict or proliferate in the early phase after SAH according to our results.

The impairment of the microcirculation after SAH is therefore likely caused by a constriction of pial and penetrating arteries and the occurrence of vessel spasticity.[3] This could be caused by mechanisms such as direct interaction with blood degradation products[9] or inflammation[11] and needs to be further investigated. Limitingly, we only investigated small ROIs 3h after SAH. Chronic constrictions could therefor occur at a later timepoint and impact longterm outcome after SAH.

## CONCLUSION

Early microcirculatory deficits after SAH do most likely not underly pericyte constrictions, but mechanisms of artiolar constrictions. Further studies are needed to investigate the underlying mechanisms to find therapeutic targets.

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## Figure Legends

### Figure 1

Transcranial laserdoppler measurements during SAH induction shows an immediate drop of cerebral blood flow after SAH induction (**A**). 3D reconstruction of the cerebral microcirculation imaged in vivo by 2-photon microscopy in an individual NG2-ds red mouse before (left) and after (right) SHAM surgery (top) or experimental subarachnoid hemorrhage through MCA filament perforation (bottom) to a depth of 400µm (**B**). Vessels were labelled with FITC (green), NG2+ pericytes are labelled in red. The capillary network is rarified after hemorrhage (bottom right). Vessel Diameters of arterioles (**C**) and of perfused capillaries (**D**) are significantly reduced after SAH induction in comparison to SHAM. Scale bar 50µm, Magnification 20x.

### Figure 2

Representative confocal microscopy of the cortical microcirculation with immunohistochemical staining of PDGF Rβ, FITC lectin and DAPI 24 hours after sham surgery (**A**) or SAH induction (**B**). PDGF $R\beta^+$ and $DAPI^+$ cells were regarded as pericytes, closely associated with FITC $lectin^+$ microvessels. Primarily arteries > capillaries > venules are covered by $NG2^+$ perivascular cells (**C**). Quantification of pericytes in the cortex, hippocampus and striatum 24 h after surgery (**D**) revealed no significant differences between quantity and distribution of pericytes between the SAH and sham group. Data points represent mean numbers of PDGF $R\beta^+$, $NG2^+$ and $DAPI^+$ cells per ROI (n=8-10, t test).

**Figure 3**

High resolution axial (**A**) and coronar (**B**) In-vivo 2 photon microscopy 3h after SAH in NG2 ds-Red mice reveals continuous pericyte coverage of arterioles and discontinuous coverage in capillaries and veins **(A, B)**. Pericytes are located at spastic (C) and non spastic vessels. Vessel diameters are not punctually constricted by pericytes after SAH, as shown by the comparison of vessel diameters at height of pericytes with the directly proximal and distal vessel diameters 3h after sham surgery or SAH (**D**). n=8-10, Mann-Whitney test

## Conflicts of Interest

None.

## Acknowledgments

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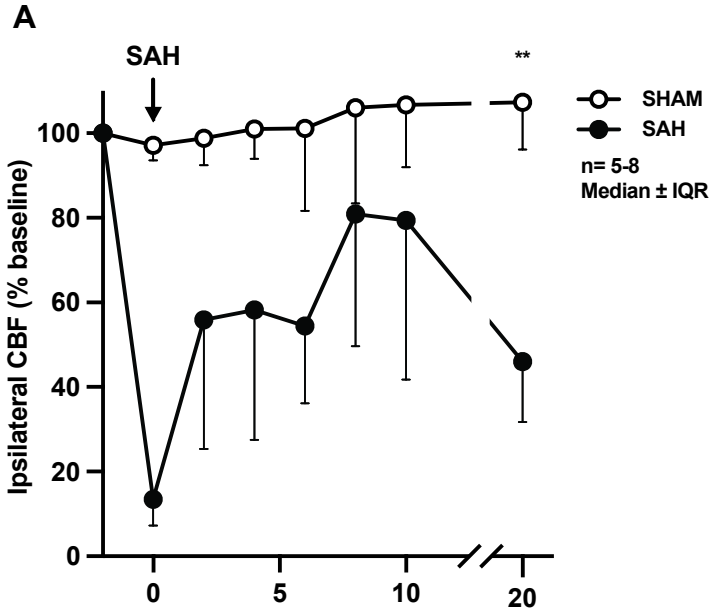
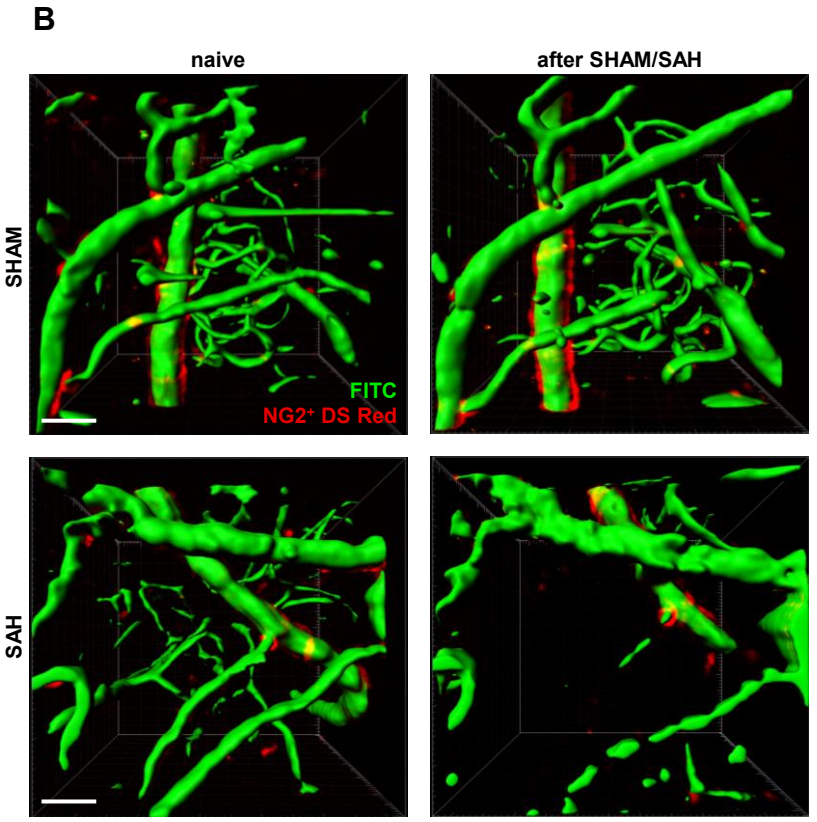
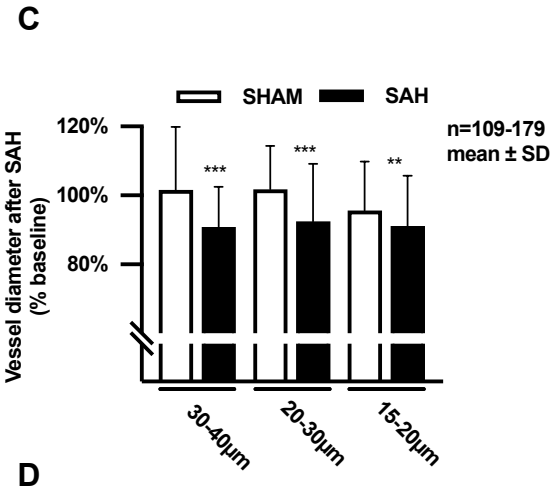
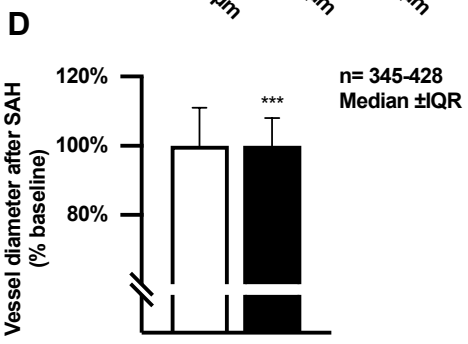
# Figure 1

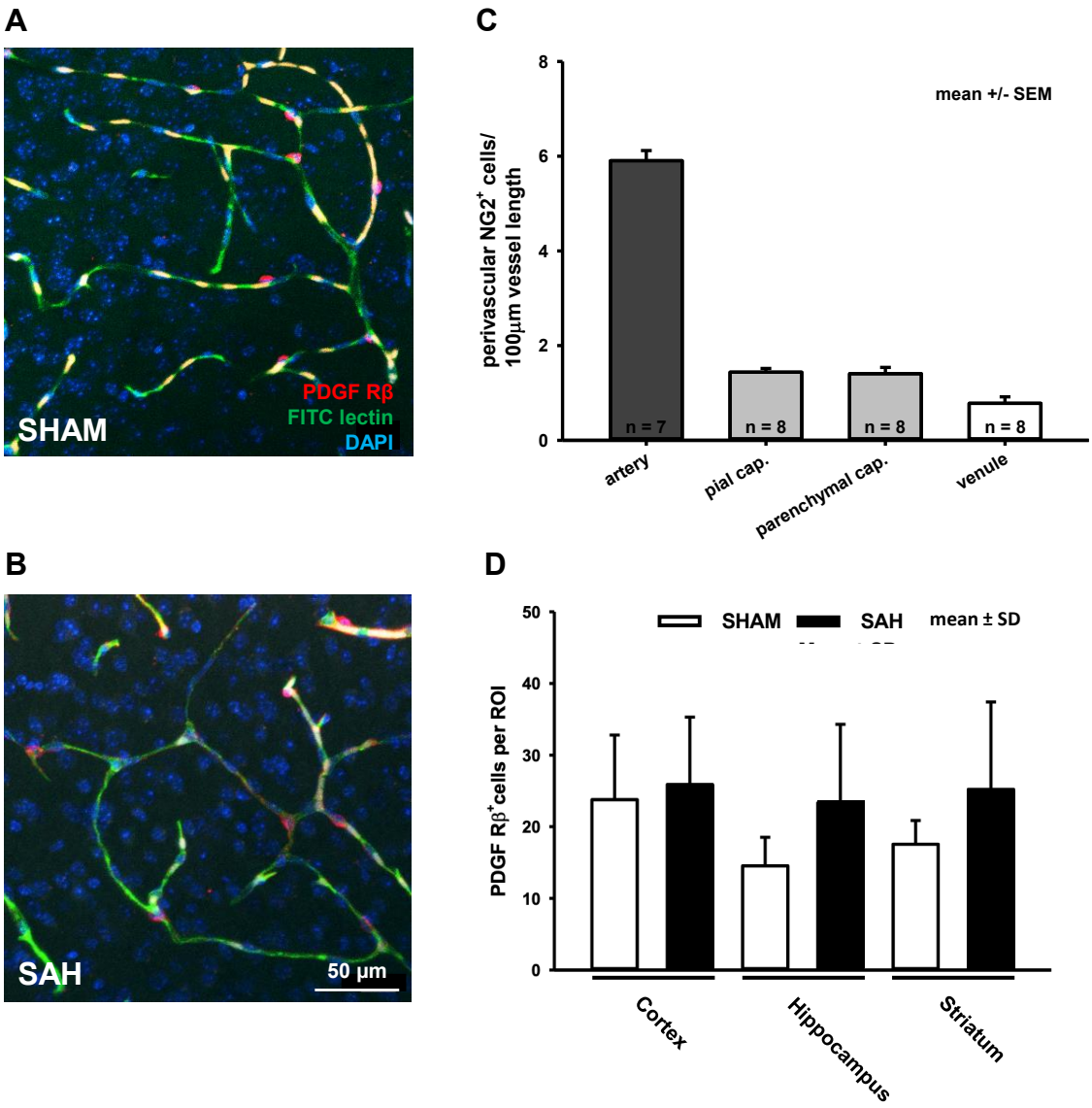

A
SAH
**
SHAM
SAH
n= 5-8
Median ± IQR
Ipsilateral CBF (% baseline)
100
80
60
40
20
0
0
5
10
20


B
naive
after SHAM/SAH
SHAM
SAH
FITC
NG2+ DS Red

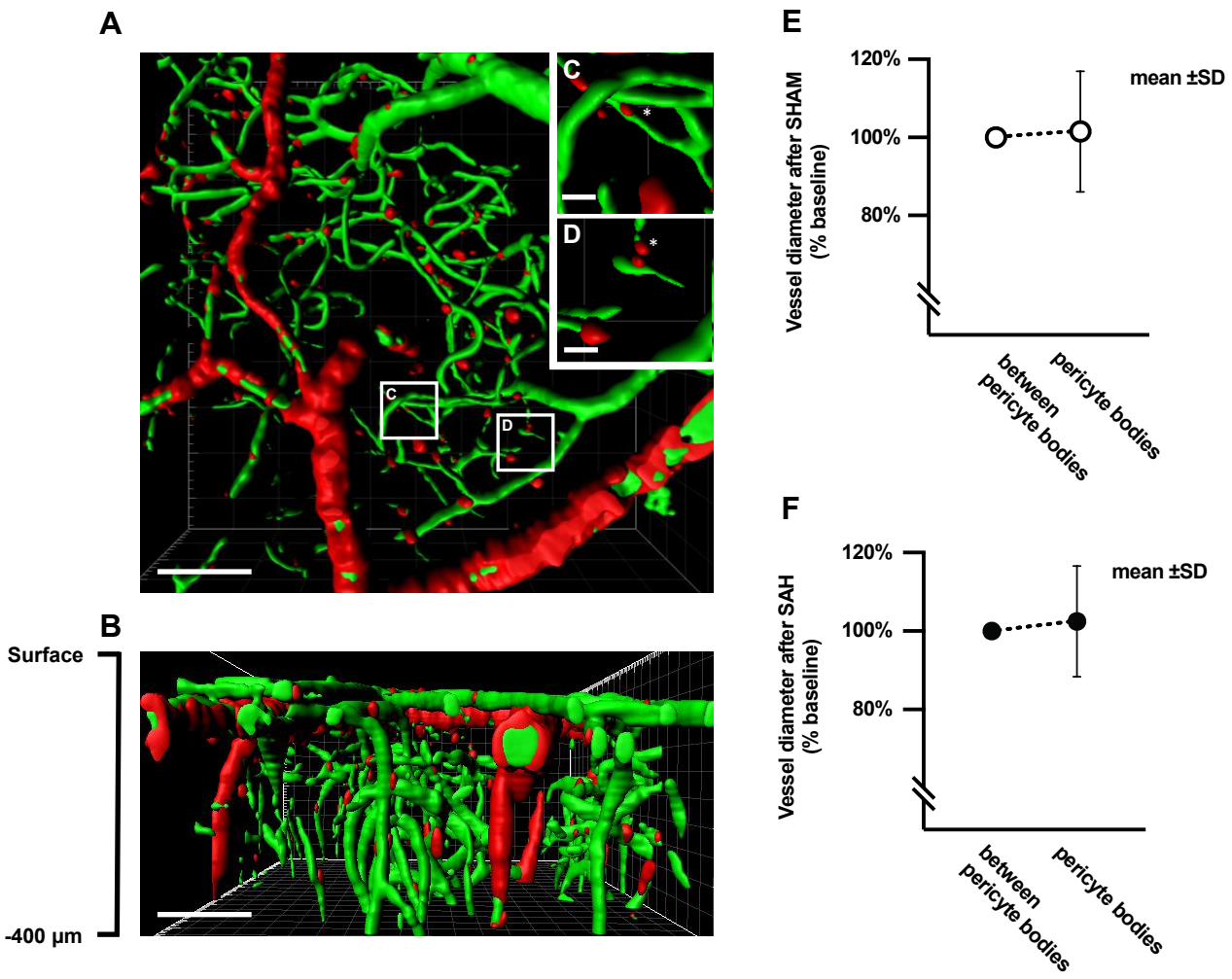

C
SHAM
SAH
n=109-179
mean ± SD
Vessel diameter after SAH (% baseline)
120%
100%
80%
***
***
**
30-40µm
20-30µm
15-20µm


D
n= 345-428
Median ±IQR
Vessel diameter after SAH (% baseline)
120%
100%
80%
***

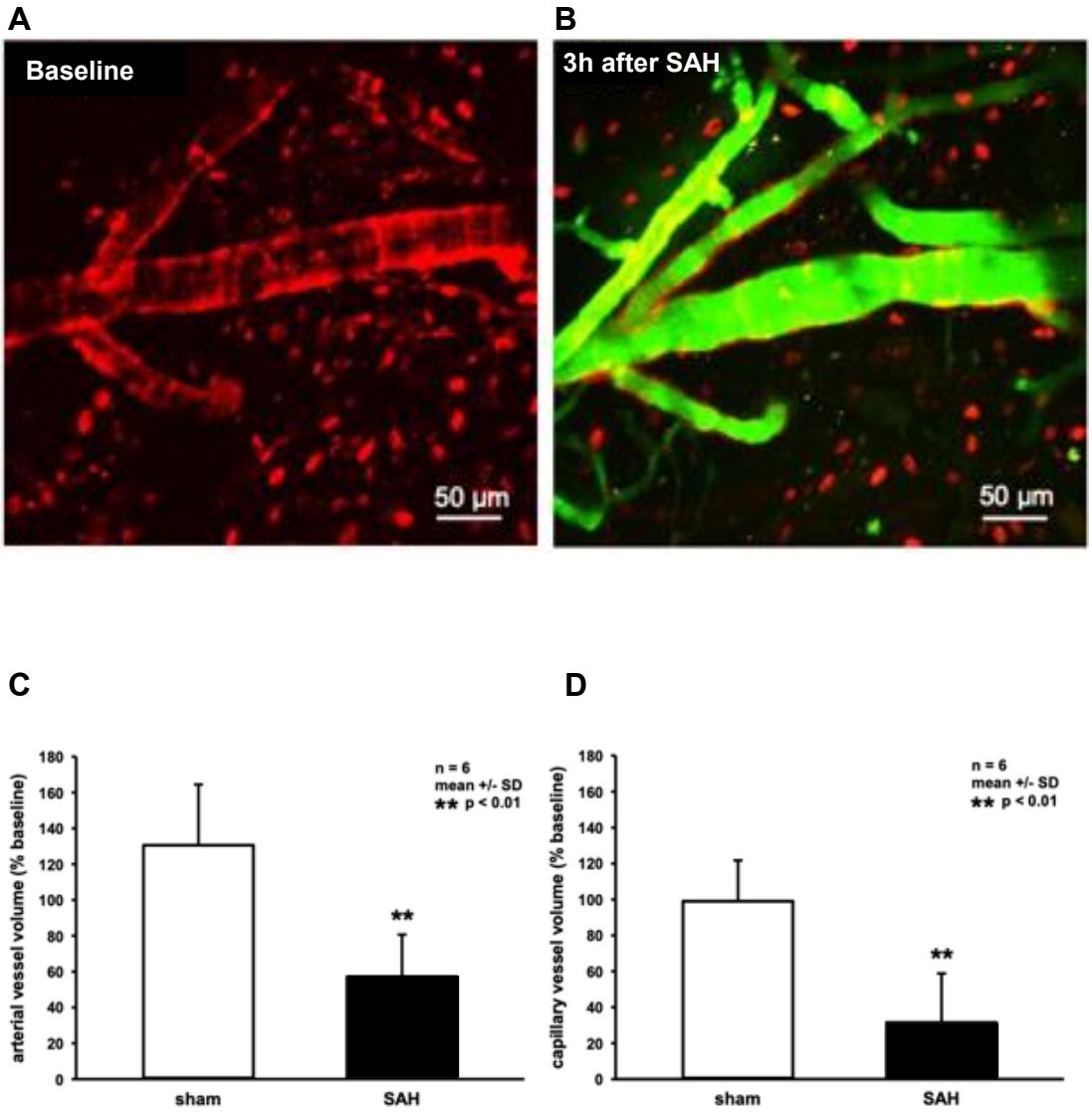
# Figure 2


A
SHAM
PDGF Rβ
FITC lectin
DAPI
B
SAH
50 µm
C
mean +/- SEM
perivascular NG2+ cells/
100µm vessel length
8
6
4
2
0
n = 7
n = 8
n = 8
n = 8
artery
pial cap.
parenchymal cap.
venule
D
SHAM
SAH
mean ± SD
PDGF Rβ+cells per ROI
50
40
30
20
10
0
Cortex
Hippocampus
Striatum

# Figure 3


A
C
D
B
Surface
-400 µm
E
Vessel diameter after SHAM (% baseline)
120%
100%
80%
mean ±SD
between pericyte bodies
pericyte bodies
F
Vessel diameter after SAH (% baseline)
120%
100%
80%
mean ±SD
between pericyte bodies
pericyte bodies

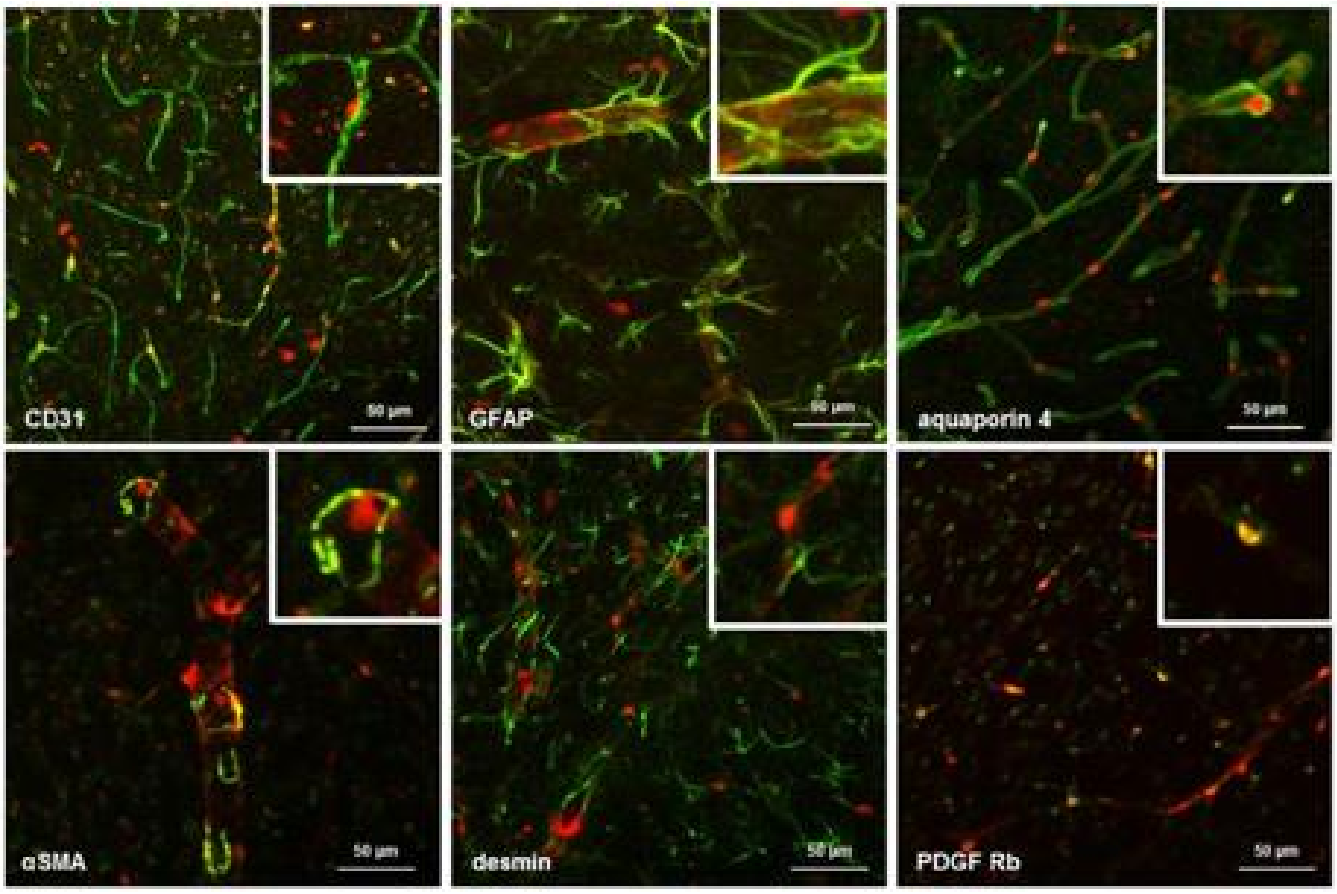
# Supplementary Figure 1




**SAH induces microcirculatory vasospasms and a reduction of perfused arterial and capillary volume**

(**A**) Representative projection of a two-photon microscopy z stack. Baseline imaging before SAH induction in a transgenically labelled NG2$^+$ animal. The diameter of the vessel is clearly outlined. Big solitary superficial NG2$^+$ cells were associated to remaining bone. (**B**) Repetition of the imaging 3h post SAH induction shows the same ROI after SAH with green plasma dye (FITC dextran). Pial vessel volume (**C**) and perfused capillary volume (**D**) are significantly reduced after SAH in comparison with sham operated animals.

# Supplementary Figure 2




**Microvessel adjacent NG2⁺ cells in the cortex represent typical characteristics of pericytes**

Transgene expression of NG2⁺ cells (red) and immunohistological characterization of different cell markers (green). Pericytes are located at the parenchymal side of CD31+ endothelial cells. No colocalization with NeuN (data not shown), desmin or GFAP was detected. Astrocytic endfeet (aquaporin 4) are covering NG2⁺ cells from the parenchymal side. NG2+ cells around arteries co-localize with αSMA. NG2⁺ cells in the microcirculation co-localize with PDGF Rβ.