

1 **Nimodipine reduces microvasospasms after experimental subarachnoid hemorrhage**

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11 Running title: Nimodipine reduces microvasospasms after exp. SAH

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19 **ABSTRACT**

20 **Background:**

21 The only established pharmacological treatment option improving outcome for patients
22 suffering from subarachnoid hemorrhage (SAH) is the L-type-calcium channel inhibitor
23 nimodipine. However, the exact mechanism of action of nimodipine conferring
24 neuroprotection after SAH has not been determined yet. More recently, spasms of the
25 cerebral microcirculation were suggested to play an important role in reduced cerebral
26 perfusion after SAH and, ultimately, outcome. It is unclear whether nimodipine may
27 influence microvasospasms and, thus, microcirculatory dysfunction. The aim of the
28 current study was therefore to assess the effect of nimodipine on microvasospasms
29 (MVS) after experimental SAH.

30 **Methods:**

31 Male C57Bl/6N mice (n=8/group) were subjected to SAH using the middle cerebral
32 artery perforation model. Six hours after SAH induction, a cranial window was prepared
33 and the diameter of pial microvessels was assessed *in vivo* by 2-photon-microscopy
34 before, during, and after nimodipine application.

35 **Results:**

36 Nimodipine significantly reduced the number of posthemorrhagic MVS. The diameter of
37 non-spastic vessels was not affected.

38 **Conclusions:**

39 Our results show that nimodipine reduces the formation of MVS thereby shedding new
40 light on the mode of action of a drug routinely used for the treatment of SAH for more

41 than three decades. Furthermore, our data suggest that L-type Ca^{2+} channels may be
42 involved in the pathophysiology of MVS formation.

43

44 **Keywords:** subarachnoid hemorrhage, microvasospasm, nimodipine, mice,
45 experimental

46 **INTRODUCTION**

47 Despite significant advances in aneurysm occlusion techniques and neurointensive care,
48 mortality and morbidity after aneurysmal subarachnoid hemorrhage (aSAH) remain
49 high.¹ The only drug approved for treatment of patients with aSAH in the United States
50 and Europe is the L-type dihydropyridine calcium channel antagonist Nimodipine; its
51 (prophylactic) use is recommended by current guidelines.^{2, 3} Starting in the 1980ies,
52 several clinical trials demonstrated a reduction of ischemic complications and improved
53 neurological outcome in SAH patients after Nimodipine treatment.⁴ The exact
54 mechanisms of Nimodipine-mediated neuroprotection, however, are still unclear.
55 Recently, it is increasingly recognized that microcirculatory dysfunction which occurs
56 early (first 72h) after SAH can contribute to posthemorrhagic brain damage and adverse
57 outcome.⁵ Prominent morphological features observed in cerebral microvessels during
58 “early brain injury” (EBI) are microarteriolar constriction and microvasospasms (MVS).⁶
59 While it is known that Nimodipine preferably dilates smaller arteriolar vessels,⁷ it is
60 unclear whether and how it affects posthemorrhagic microcirculatory dysfunction and
61 whether Nimodipine exerts its neuroprotective function by reducing MVS. The aim of
62 the current study was, therefore, to directly assess the effect of Nimodipine application
63 on post-SAH microcirculatory dysfunction with special focus on microvasospasm
64 formation using a murine endovascular middle cerebral artery (MCA)-perforation
65 model.

66 **METHODS**

67 **Animals and experimental groups**

68 8–10-week-old male C57Bl/6N mice (Jackson Laboratory, Bar Harbor, USA; weight 21-
69 22g) were used for the current study. All experiments were performed in a randomized
70 and blinded manner. To achieve maximum comparability with previous results, the
71 current study was performed in male mice only. Procedures, group size calculations, and
72 statistical methods were reviewed and approved by the Government of Upper Bavaria.

73 **Induction of subarachnoid hemorrhage**

74 SAH was induced as previously described ⁸. Briefly, mice were anesthetized and
75 mechanically ventilated. Core body temperature, pO₂, pCO₂, oxygen saturation,
76 ipsilateral, and contralateral cerebral blood flow (CBF) were continuously monitored
77 during surgery. The Circle of Willis was perforated at the outlet of the left middle
78 cerebral artery with a 5-0 filament advanced intravascularly via the common carotid
79 artery. Multimodal monitoring was continued for 30 minutes after SAH induction. 5.5
80 hours later, mice were re-anesthetized for cranial window-preparation and intravital
81 microscopy under multimodal imaging, including invasive blood pressure measurement.

82 **Drug application**

83 Mice received 10 mg/kg Nimodipine (Merck, Darmstadt, Germany) in 5% ethanol, 5%
84 DMSO, 40% PEG 400, and PBS or vehicle solution six hours after SAH induction (**Fig. 1A**),
85 as previously described.⁹

86 **Two-photon microscopy**

87 *In-vivo* imaging was performed with a 2-photon microscope (LSM 7, Zeiss, Jena,
88 Germany) six hours after SAH (**Fig. 1A**). A thinned skull window was prepared over the

89 left MCA territory.¹⁰ To visualize the microcirculation, 100 μ l fluorescein-isothiocyanate
90 (FITC) dextran (0.5% in saline, Sigma Aldrich, Darmstadt, Germany) were injected
91 intraarterially. Before drug/vehicle application, the animals underwent baseline
92 scanning for spastic pial arteries and volumes of interest (VOIs, 500 x 500 x 250 μ m, **Fig.**
93 **1B**) to determine target vessels. These structures were subsequently scanned before
94 (therapy baseline), 5, 20, 35, and 50 min after treatment. Mean arterial blood pressure,
95 end-expiratory pCO₂, body temperature, heart rate, and peripheral oxygen saturation
96 were continuously monitored during imaging.

97 **Data analysis**

98 Vessels were selected in axial and tangential planes. Microvasospasms were identified
99 by comparisons of constriction grades of spastic vessel segments with non-spastic
100 segments.¹⁰ Microvasospasm was defined as a reduction of the vessel diameter \geq 15%.
101 Only vessels with MVS in pre-treatment scans were included for further analysis.
102 Detailed methods of image analysis and statistics are described in the supplementary
103 methods.

104

105 **RESULTS**

106 After SAH induction, brain perfusion was globally reduced in all mice. There was no
107 difference between groups before application of Nimodipine (**Fig. 2A**).

108 **Effect of drug application on physiological parameters during imaging**

109 After treatment with Nimodipine or vehicle, we observed a non-significant decrease of
110 systemic blood pressure which was equally observed in both groups. pCO₂, body
111 temperature, and heart rate were comparable between groups (**Suppl. Tab. 1**).

112 **Nimodipine reduces MVS after SAH**

113 SAH induced MVS (**Fig. 3A**) in pial arterioles as previously described.¹¹ We included 85
114 spastic vessel segments with an average diameter of 16±7 μm and a median of 2 IQR 1
115 microvasospasms (MVS)/segment from the vehicle group and 37 spastic vessel
116 segments with an average diameter of 18 ± 7 and a median of 2 IQR 1 MVS/segment
117 from the Nimodipine group into the analysis.

118 Nimodipine treatment significantly reduced the number of MVS after SAH compared to
119 the control group. Thirty-five minutes after application of Nimodipine, MVS were almost
120 completely abolished in the treatment group, while vehicle treated mice showed no
121 relevant changes of MVS over time (p=.02, **Fig. 3B**).

122 All investigated vessels slowly dilatated over time (p=.192, **Fig. 3C**), however, there was
123 no difference between groups.

124 **DISCUSSION**

125 The neuroprotective effect of nimodipine treatment after SAH was thought to be due to
126 alleviation of delayed spasms of larger cerebral arteries, a process hypothesized for
127 decades to be the most important pathomechanism of delayed cerebral ischemia,
128 cerebral infarcts, and, ultimately, adverse outcome after SAH. This notion was derived
129 from experimental studies using Nimodipine in different animal SAH-models.⁷ Pivotal
130 clinical studies confirmed improved outcome in SAH-patients treated with Nimodipine,
131 however, failed to show a consistent effect of Nimodipine on (angiographic) large artery
132 spasms.⁴ Since then, other mechanisms of action of Nimodipine-mediated
133 neuroprotection were discovered, e.g. involving the anti-inflammatory effects, the
134 glymphatic system, or mitochondrial function.¹²⁻¹⁴

135 Microvascular changes occurring early (< 72h) after SAH are prominent features of Early
136 Brain injury and are thought to contribute to secondary cerebral ischemia and adverse
137 outcome. Microarteriolar perfusion deficits and microvasospasms (MVS) have been
138 detected in SAH-patients and after experimental SAH;^{15, 16} while it is not entirely clear
139 whether and how EBI and microvasospasm formation is involved in the development of
140 delayed cerebral ischemia, a reduction of MVS after experimental SAH was shown to be
141 associated with better outcome and lower mortality.⁸ In the present study, we
142 demonstrate that Nimodipine significantly reduces posthemorrhagic microvasospasm
143 formation without affecting global microarterial constriction. The mechanisms leading
144 to MVS formation and subsequent reduction of cortical perfusion therefore may involve
145 L-type Ca²⁺ channels or other pathways positively affected by Nimodipine. While the
146 sample size in this study is small, our results still point to a statistically significant and

- 147 previously unknown mode of action of Nimodipine and underscore the importance of
148 MVS-formation in the development of post-SAH brain damage.

149 **Conflicts of Interest**

150 The authors declare no conflict of interest.

151

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156

157 **Disclosures**

158 none

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205

206 **Figure Legends**

207 **Figure 1**

208 **Experimental setup**

209 (A) Experimental timeline. (B) Position of volumes of interest (VOI) investigated by in-
210 vivo microscopy.

211

212 **Figure 2**

213 **SAH induction and effect of Nimodipine on systolic blood pressure**

214 SAH (t = 0) led to significant drops in ipsilateral (A) and contralateral (B) cerebral blood
215 flow (CBF) without significant differences between groups. C. Mean arterial blood
216 pressure during in-vivo microscopy decreased over time in both groups without
217 differences between the Nimodipine or vehicle group. Multiple Mann-Whitney tests in
218 (A – C), $p < 0.21$

219

220 **Figure 3**

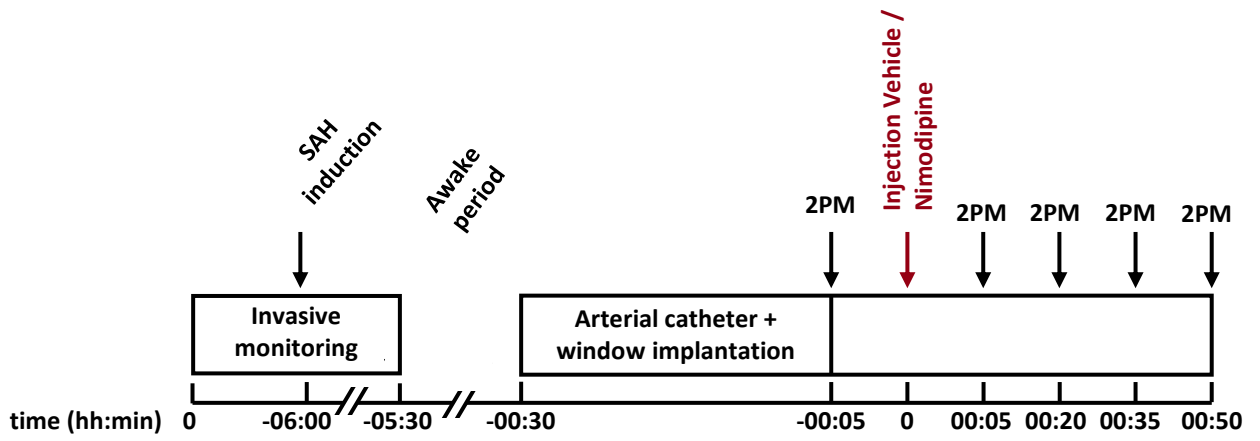
221 **Nimodipine reduces microvasospasms (MVS) after SAH**

222 (A) Exemplary intravital microscopy images after injection of Vehicle (upper row) or
223 Nimodipine (lower row). Arrows label pearl string-shaped spasms in pial arteries. (B)
224 Median number \pm IQR of microvasospasms. (C) Diameter of vessel segments \pm SEM in
225 comparison to baseline (BL).

226 Scalebars = 50 μ m. Mann-Whitney test.

Figure 1

A



B

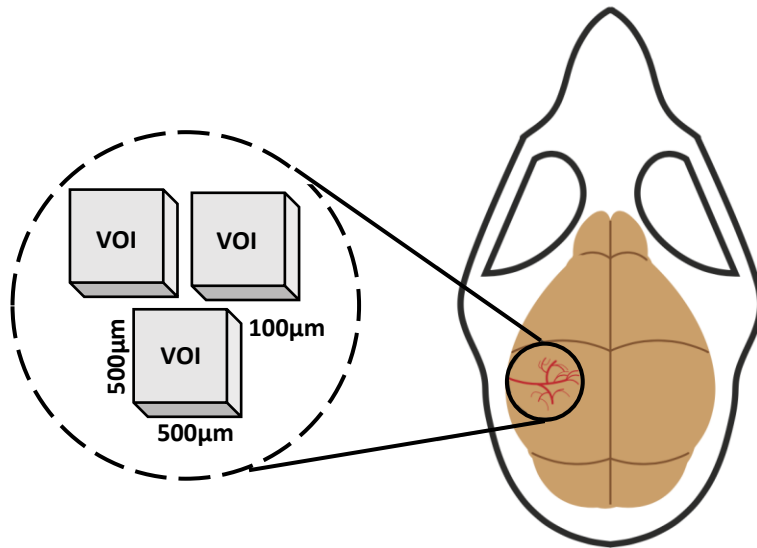


Figure 2

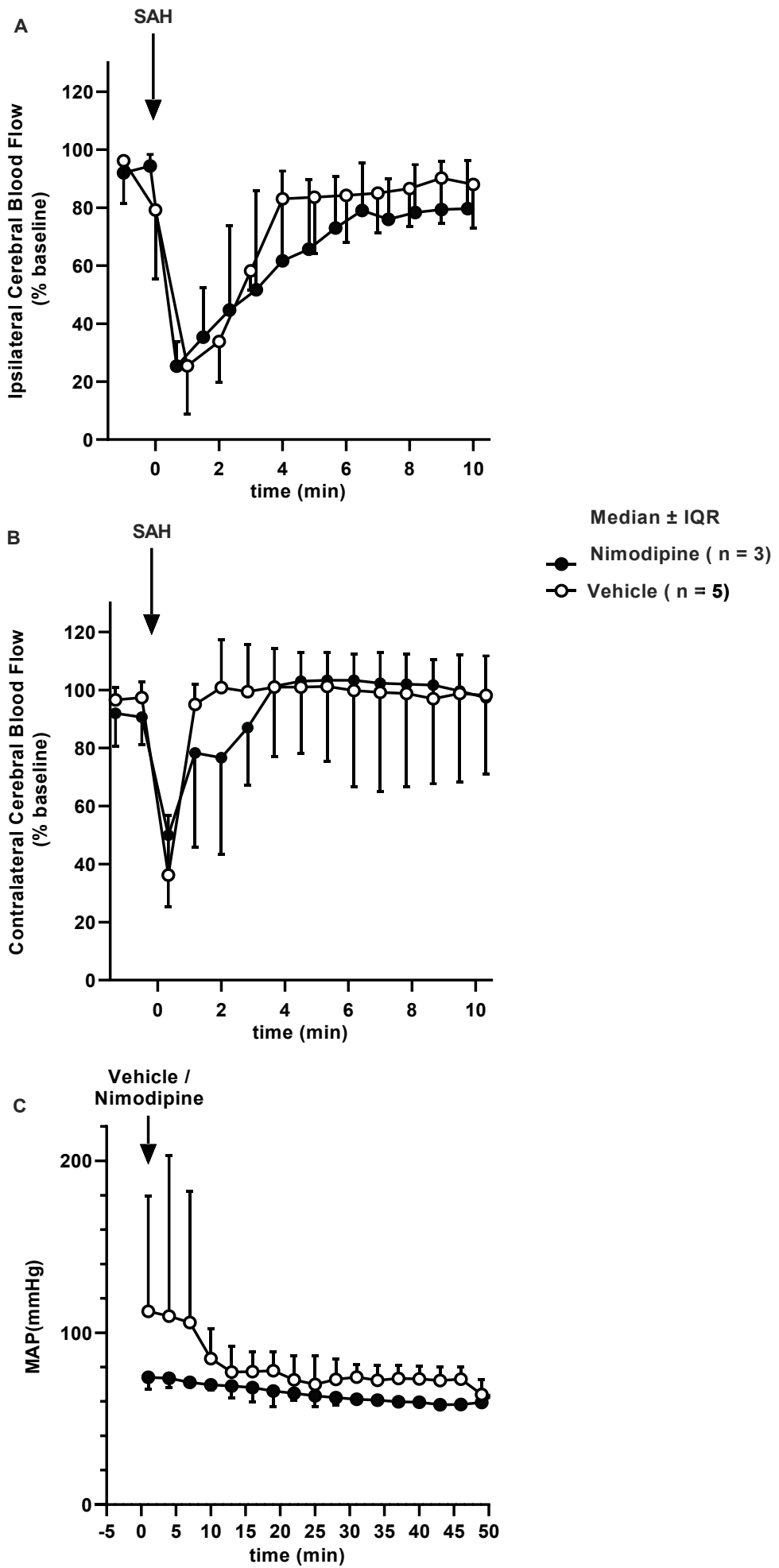
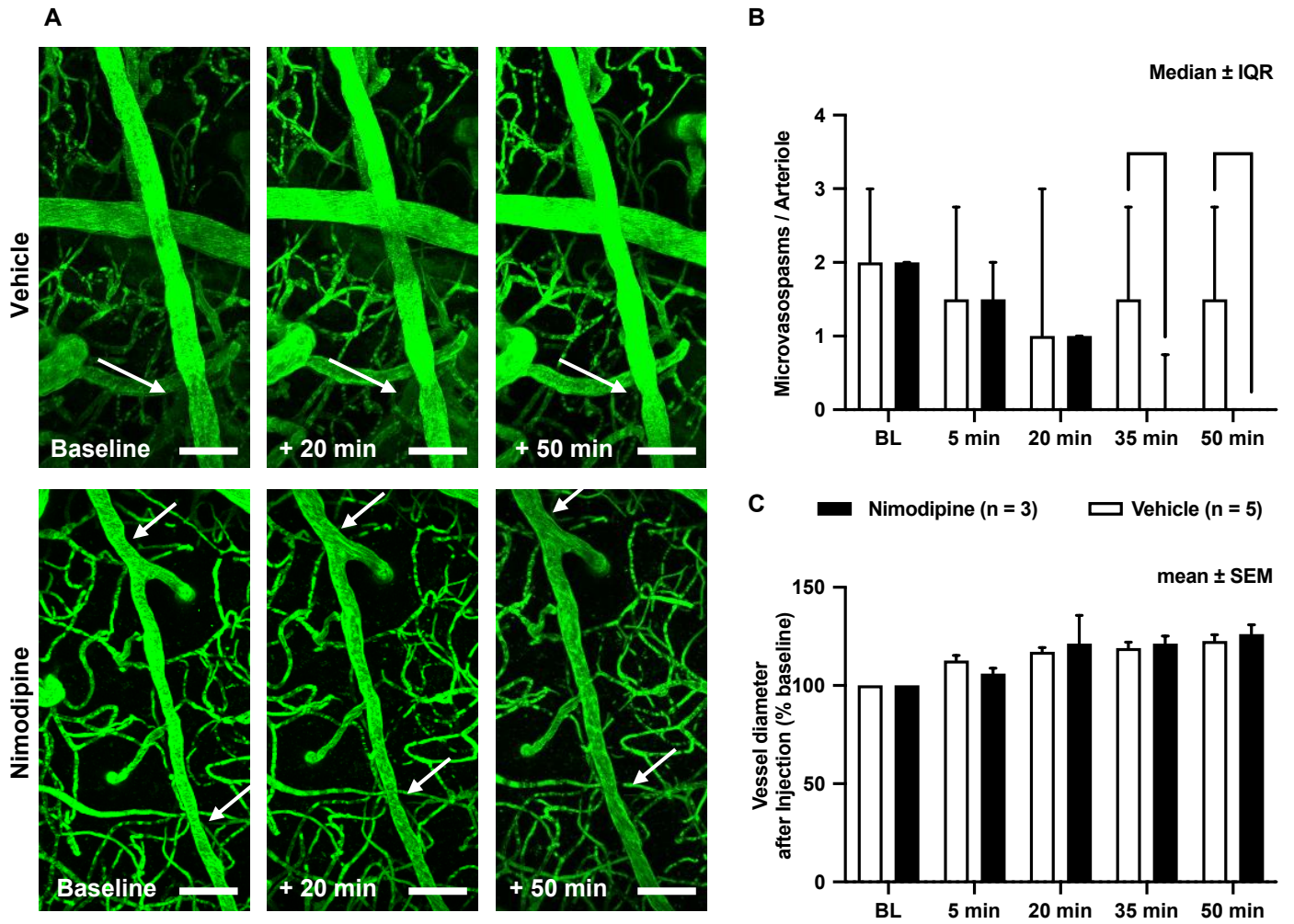


Figure 3



Supplementary Material

Nimodipine reduces microvasospasms after experimental subarachnoid

hemorrhage

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Content:

Supplementary Material and Methods

Supplementary Results

Supplementary References

Suppl. Tab. 1

Suppl. Fig. 1

Supplementary Material and Methods

All procedures performed on animals, group size calculation, and all statistical methods used to analyze in vivo data were reviewed and approved by the Government of Upper Bavaria. The results of the present study are reported in accordance with the ARRIVE guidelines.¹ Animal husbandry, health screens, and hygiene management checks were performed in accordance with Federation of European Laboratory Animal Science Associations guidelines and recommendations.²

Group size calculation

All group sizes were calculated with the Software G*Power (Version 3.1.9.6, available from <https://www.psychologie.hhu.de/arbeitsgruppen/allgemeine-psychologie-und-arbeitspsychologie/gpower>, University of Duesseldorf, Germany).

Alpha error was set for all groups at 5% and statistical power was set to 20%. Based on experience from previously published studies,²⁻⁴ the expected effect size d was set to 2 based on an expected difference of 20% with a SD of 10% for both groups. 2 animals died before randomization and were replaced to achieve the calculated target group size ($n=16$ total).

Overall, mortality was similar in comparison with previous experiments. However, as it is difficult to distinguish pial arteries and penetrating arterioles during in-vivo imaging, a total of $n = 8$ animals had to be excluded from the analysis because we could not analyze the targeted vessels. An overview of all included animals with dropout rates is shown in **Suppl. Fig. 1**.

Randomization and Blinding

All animals were randomly assigned to surgery, after preparation of the cranial window, group allocation was obtained by drawing a number allotted to a syringe filled with either nimodipine or vehicle. Syringe preparation was performed by a researcher not involved in experimental procedures, surgery, or data analysis; syringe content was not obvious or recognizable for the researcher performing the injection and two-photon microscopy after SAH. Subsequent data analysis was performed by a researcher blinded towards group allocation of the animals.

Experimental subarachnoid hemorrhage

Anesthesia & Monitoring

Animals were anesthetized by intraperitoneal injection of medetomidine, midazolam, and fentanyl, orotracheally intubated, and mechanically ventilated as previously described.³ Anesthesia was induced by intraperitoneal injection of fentanyl, midazolam, and medetomidine under continuous monitoring of end-tidal CO₂ partial pressure (by capnometry, Capnograph 340, Harvard Apparatus, March-Hungstetten, Germany), oxygen saturation, heart rate (by oximetry at the hindpaw, SpO₂-MSE, Kent Scientific Corporation, Torrington, CT, USA), and body temperature (by a feedback controlled heating pad, FHC Bowdoinham, Bowdoin, ME, USA). Cerebral blood flow was measured over the ipsi- as well as the contralateral MCA territory via Laser-Doppler-Flowmetry (LDF; Perimed, Järfälla, Sweden).^{4, 5} For LDF, glass fiber probes were glued perpendicular to the skull bi-temporally after partial removal of the insertion of the temporal muscle to allow for placement of the probe directly on the skull. All parameters were continuously registered using LabChart (AD Instruments, Oxford, UK) and post-processed with FlexPro software (Weisang, Mönchengladbach, Germany). Monitoring data was continuously recorded at a sample rate of 100 Hz and averaged every 30 seconds. Baseline recordings were obtained for a total of 10 minutes prior to SAH induction.

Induction of subarachnoid hemorrhage

Subarachnoid hemorrhage was induced by the endovascular filament perforation model.^{4, 6, 7} After establishment of multimodal monitoring, a paramedian incision was performed on the left cervical side in supine position. A 5-0 monofilament was inserted into the external carotid artery and advanced towards the circle of Willis under continuous control of cerebral perfusion until a sharp drop in cerebral perfusion below 20% of baseline on the ipsilateral side as well as a drop below 30% on the contralateral side was observed as a sign of successful vessel perforation. Animals not fulfilling these criteria were excluded from further analysis. After induction of SAH, multimodal monitoring was continued for 20 minutes. After surgery and monitoring the anesthesia was antagonized as previously described³ by injection of flumazenil, naloxone, and atipamezole; animals were then kept in a heating chamber at 34°C and 40% humidity for 12 hours in order to prevent hypothermia and dehydration. After completion of in-vivo microscopy, an arterial blood sample was obtained and analyzed for electrolytes, pO₂, pCO₂, glucose, and lactate.

In vivo 2-photon imaging

5.5 hours after SAH induction, anesthesia was re-induced and animals re-intubated. Multimodal monitoring was re-introduced; additionally, systemic blood pressure was continuously monitored via a catheter placed in the left femoral artery. A 3 x 3 mm open cranial window was prepared on the left parietal side over the MCA territory. In vivo 2-photon microscopy was performed six hours after induction of subarachnoid hemorrhage every 10 minutes for 90 min as previously described⁸⁻¹⁰ using a confocal microscope (LSM 7, Zeiss, Germany) equipped with a Li:Ti laser (Chameleon, Coherent, Santa Clara, CA, USA) and a water immersion objective (20x Plan Apochromat, NA 1.0, Zeiss, Germany). Vessels were visualized by i.v. injection 0.1 ml FITC dextran 0.5% in saline. Three-dimensional images/ Z-stacks (volume of interest, VOIs) with 500 x 500 x 250 μm were obtained. During baseline imaging, vessels were scanned; depending on direction of blood flow, they were classified into arteries and veins.

Data analysis

Intravital microscopy data was analyzed using ImageJ version 1.52p. Microvascular constriction and the number of microvasospasms were evaluated as previously described.^{6, 9, 11} A vessel segment was considered spastic when its diameter was 85% or less than non-constricted vessel segments; spasm severity is given as percent constriction of vessel diameter compared to non-constricted vessel segments. Only vessels with MVS in pre-treatment scans were included for further analysis. VOIs were localized using a micromanipulator table and scanned consecutively for each evaluation time-point. Arteriolar constriction was assessed in segments without visible MVS and compared over time.

Statistical analysis

Data is given as median \pm interquartile range for non-parametrical and mean \pm standard error of mean for parametrical data. Statistically significant differences between groups were tested with student's t tests for parametric data and with the Mann-Whitney test for non-parametric data. All calculations were done using using Prism 8 (Graphpad Software LLC, USA). All data was tested for normality using the Kolmogorov-Smirnov test. Differences between groups was assessed by Student's t tests for parametric data and Mann-Whitney test for non-parametric data. Differences between groups were considered to be significant at $p < 0.05$.

Supplementary results

Physiological Monitoring during SAH induction and *in-vivo* microscopy

During surgery, animals were closely monitored for physiological parameters shown in **Suppl.**

Tab. 1. No significant differences were detected between groups.

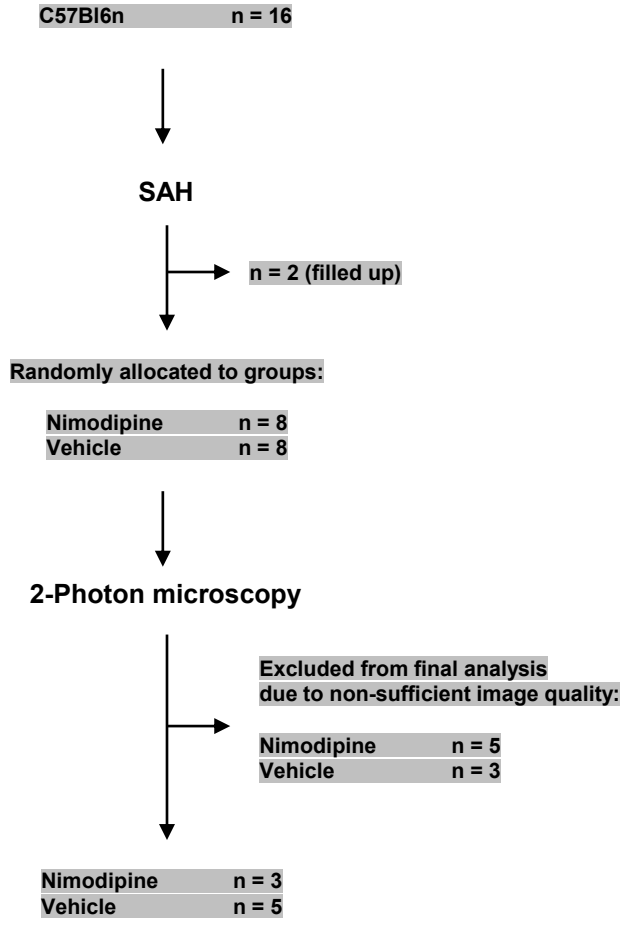
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Supplementary Table 1

	Vehicle	Nimodipine	p
pCO ₂	17.5 ± 2	16.2 ± 1	> 0.99
respiratory rate	152 ± 1	157 ± 1	0.39
oxygen sat	85 ± 4	89 ± 3	0.14
heart rate	266 ± 49	273 ± 34	0.85

Supplementary Figure 1



Experimental groups

Flowchart of experimental dropouts