Impaired Glymphatic Perfusion After Strokes Revealed by Contrast-Enhanced MRI
A New Target for Fibrinolysis?

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Background and Purpose—The aim of the present study was to investigate the impact of different stroke subtypes on the glymphatic system using MRI.

Methods—We first improved and characterized an in vivo protocol to measure the perfusion of the glymphatic system using MRI after minimally invasive injection of a gadolinium chelate within the cisterna magna. Then, the integrity of the glymphatic system was evaluated in 4 stroke models in mice including subarachnoid hemorrhage (SAH), intracerebral hemorrhage, carotid ligature, and embolic ischemic stroke.

Results—We were able to reliably evaluate the glymphatic system function using MRI. Moreover, we provided evidence that the glymphatic system was severely impaired after SAH and in the acute phase of ischemic stroke, but was not altered after carotid ligature or in case of intracerebral hemorrhage. Notably, this alteration in glymphatic perfusion reduced brain clearance rate of low-molecular-weight compounds. Interestingly, glymphatic perfusion after SAH can be improved by intracerebroventricular injection of tissue-type plasminogen activator. Moreover, spontaneous arterial recanalization was associated with restoration of the glymphatic function after embolic ischemic stroke.

Conclusions—SAH and acute ischemic stroke significantly impair the glymphatic system perfusion. In these contexts, injection of tissue-type plasminogen activator either intracerebroventricularly to clear perivascular spaces (for SAH) or intravenously to restore arterial patency (for ischemic stroke) may improve glymphatic function. (Stroke. 2014;45:00-00.)

Key Words: magnetic resonance imaging ▪ subarachnoid hemorrhage ▪ thrombolytic therapy

The glymphatic system allows clearance of the brain interstitial fluid through para-arterial influx and para-venular efflux of cerebrospinal fluid (CSF).1 Because the rate of metabolic byproduct generation increases dramatically after neuronal injury, waste clearance through the glymphatic system may play a central role after stroke. Here, we hypothesized that stroke affects the glymphatic system function. To test this hypothesis, we developed a minimally invasive injection method of gadolinium chelate in the cisterna magna to follow spontaneous CSF flows using MRI in mice. Subsequently, we studied the function of the glymphatic system after subarachnoid hemorrhage (SAH), intracerebral hemorrhage, common carotid artery occlusion, and embolic ischemic stroke.

Methods

For the complete details of the methods used, see the online-only Data Supplement.

Intracisternal Injection

The function of the glymphatic system was evaluated by T1-weighted MRI after intracisternal DOTA-Gd (Dotarem, Guerbet, France) microinjection (1 μL for 1 minute). We modified a previously reported method by using a pulled hematological glass micropipette (Figure I in the online-only Data Supplement). Regions of interest for subsequent signal analyses were selected as shown in Figure II in the online-only Data Supplement. Common carotid artery occlusion,3 SAH,4 and collage- nase-induced intracerebral hemorrhage5 were performed as previously described. Embolic ischemic stroke was performed as described in the online-only Data Supplement. For immunohistological studies, sodium fluorescein (5 μg per mouse; Sigma-Aldrich, France) or FITC-Dextran (5 μg per mouse; Sigma-Aldrich) were injected in the cisterna magna.

Results

MRI Allows In Vivo Assessment of the Glymphatic System Function

We first investigated the efficiency of a modified contrast-enhanced MRI protocol to image intraparenchymal CSF flow in vivo. Five minutes after intracisternal DOTA-Gd

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Glymphatic System Is Impaired After SAH

Then, we looked for potential effects of SAH on the glymphatic system. We induced SAH by injection of 50 μL fresh arterial blood in the prechiasmatic cistern of mice. Interestingly, 24 hours after SAH induction, the glymphatic system appeared severely impaired as compared with sham animals. Indeed, 60 minutes after intracisternal microinjection of DOTA-Gd, enhancement was present in the cerebellum but spared the forebrain (Figure 2A), suggesting occlusion of para-arterial influx routes. We confirmed these results by immunohistology after injection of sodium fluorescein in the cisterna magna (Figure 2B). Importantly, this glymphatic blockade persisted after bilateral craniectomy (Figure III in the online-only Data Supplement), suggesting that it is independent of SAH-induced intracranial hypertension.

Thereafter, we studied the permeability of the paravascular CSF circulation pathways 24 hours after SAH. To this aim, we injected a high-molecular-weight fluorescent dye (FITC-Dextran) in the cisterna magna. We first confirmed by histological analyses that this dye accumulates in the perivascular space after intracisternal injection (Figure IV in the online-only Data Supplement). Interestingly, in SAH animals, intracisternally injected FITC-Dextran failed to reach the perivascular spaces in the olfactory bulbs, although it accumulated in the cerebellar perivascular spaces (Figure VA in the online-only Data Supplement). Moreover, immunohistological studies revealed the presence of fibrinogen in the perivascular spaces after SAH (Figure VB in the online-only Data Supplement). Altogether, these results suggested that perivascular spaces are occluded by fibrin clots after SAH.

To test this hypothesis, we performed intraventricular injection of a fibrolytic agent (tissue-type plasminogen activator) 15 minutes after hemorrhage onset and evaluated the glymphatic perfusion at 24 hours. According to our hypothesis, intraventricular injection of tissue-type plasminogen activator improved glymphatic perfusion in SAH mice as compared with control saline-treated animals (Figure 2C). Finally, to investigate the effect of glymphatic blockade on the brain clearance rate of small-molecular-weight compounds, we measured the clearance of DOTA-Gd from sham and SAH mice by serial T1-weighted MRI. As shown in Figure VI in the online-only Data Supplement, DOTA-Gd clearance from the striatum of SAH animals was slower than in control animals. This result suggested that glymphatic blockade by fibrin after SAH reduces waste clearance from the brain parenchyma. Interestingly, the clearance rate was diminished in SAH animals but was not completely abolished, suggesting the existence of other mechanisms driving DOTA-Gd clearance.

Ischemic Stroke Decreased Paravascular CSF Circulation

To study the impact of ischemic stroke on the glymphatic function, we induced embolic ischemic stroke in mice and studied the glymphatic system function by MRI and histofluorescence 3 and 24 hours after right middle cerebral artery occlusion. Interestingly, we noticed a blockade of the right glymphatic perfusion 3 hours after middle cerebral artery occlusion, as revealed by MRI (Figure 3A and 3B) and histology (Figure 3C and 3D). Again, glymphatic perfusion at 3 hours poststroke remained impaired after bilateral craniectomy (Figure VII in the online-only Data Supplement). At 24 hours after stroke onset, the glymphatic system function appeared normal. Notably, angiographies performed at the time of glymphatic imaging revealed that the middle cerebral artery were repermeabilized in all mice 24 hours after stroke onset (not shown). Thus, these
results suggested that glymphatic perfusion depends on arterial patency after embolic ischemic stroke.

**Glymphatic Imaging in Other Experimental Models of Cerebrovascular Diseases**

In another set of experiments, we performed glymphatic imaging 24 hours after common carotid artery occlusion or intracerebral hemorrhage. Neither common carotid artery occlusion nor intracerebral hemorrhage influenced paravascular circulation of the CSF (Figure VIII in the online-only Data Supplement).

**Discussion**

The current study demonstrates that the glymphatic system function can be evaluated in living mice using MRI coupled
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Figure 3. Ipsilateral paravascular cerebrospinal fluid circulation is impaired in the acute phase of ischemic stroke. A, Top: Embolic ischemic stroke was induced by topical application of ferric chloride on the right common carotid artery followed by embolization of the thrombotic material by mechanical pressure on the arterial wall. Only the mice presenting proximal middle cerebral artery (MCA) occlusion on a MR angiography (MRA) performed at +1 h were included for subsequent analyses. The white arrow indicates MCA occlusion site on MRA.

Bottom: Representative T1-weighted images demonstrating that the glymphatic system is impaired in the ipsilateral hemisphere (R) of mice with ischemic stroke at +3 h. B, Corresponding quantification (n=4 per group). C, Representative histofluorescence images of ischemic stroke mice 30 min after intracisternal injection of sodium fluorescein (NaF). Ipsilateral fluorescence (R) was reduced in ischemic stroke animals as compared with the contralateral side (L). White arrows indicate MCA location. D, Corresponding quantification (n=4 per group).

to a minimally invasive injection of DOTA-Gd in the cisterna magna. Our key finding is that the glymphatic system is severely impaired after SAH and in the acute phase of ischemic stroke, independently of intracranial hypertension.

Current evidence suggests that the glymphatic system plays an important role in cerebral waste clearance.6 Once impaired, the accumulation of metabolites within the parenchyma might lead to brain injury. This event could be particularly relevant during delayed cerebral ischemia occurring after SAH.7 Moreover, whether prolonged glymphatic dysfunction may promote vasospasm or microcirculatory impairment deserves further investigations.8 If true, restoring glymphatic perfusion by intraventricular fibrinolysis may be effective to improve patient outcome.

During the acute phase of ischemic stroke, the observed reduction of glymphatic perfusion may prevent adequate clearance of excitatory neurotransmitters (and other deleterious molecules) and promote neuronal death. The mechanisms responsible for this blockade remain elusive but may include: (1) a decrease of the arterial pulsation because of vessel occlusion9 or (2) the occlusion of the perivascular space because of compression by the intravascular thrombus. According to both hypotheses, the glymphatic perfusion appeared normal 24 hours after ischemic stroke, when the middle cerebral artery was repermeabilized. Consequently, beside restoration of arterial patency, intravenous thrombolysis may improve glymphatic perfusion in ischemic stroke patients. Our results support the findings from a recent study showing reduced interstitial fluid drainage after microstroke using 2-photon microscopy.10 In contrast, we failed to confirm the reduction of the ipsilateral glymphatic flow after carotid occlusion. Differences in the delay between arterial ligation and tracer administration (0.5 versus 24 hours) may explain this discrepancy.

Conclusions

In conclusion, we demonstrate that the glymphatic system is severely impaired after SAH and in the acute phase of ischemic stroke. Further studies are warranted to investigate the impact of such dysfunctions on neurological outcome. Notably, tissue-type plasminogen activator administration, either by removing perivascular fibrin after SAH or by restoring arterial patency after ischemic stroke, may improve glymphatic perfusion.

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

Impaired glympathic perfusion after strokes revealed by contrast-enhanced MRI: a new target for fibrinolysis?

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Supplementary Methods

Animals
Experiments complied with the European Directives and the French Legislation on Animal Experimentation. All the experiments were performed in male swiss mice (35-40g; CURB, Caen, France) anesthetized with isoflurane (2%) in 70%/30% NO2/O2. Body temperature was maintained at 37°C with a rectal temperature probe and homoeothermic heating pad. Mice received subcutaneous buprenorphine (0.05 mg/kg) at the time of the first anesthesia and during the imaging procedure at +24 hours, when appropriate.

Intracisternal injection
The function of the glymphatic system was evaluated either by T1-weighted MRI after intracisternal DOTA-Gd (Dotarem) microinjection (1 µL over 1 minute) or by histofluorescence after intracisternal sodium fluorescein (NaF) or FITC-Dextran microinjection (1 µL over 1 minute). To inject the tracer intracisternaly, we modified a previously reported method by using a pulled haematological glass micropipette (diameter= ~80 µm) instead of a 30 gauge needle (diameter= 311 µm) (Supplementary Fig. 1). This allowed us to perform a single small volume injection of contrast material without significant damage to the dura mater and thus, without CSF leakage. The micropipette was left in place for 1 additional minute and then the wound was closed.

Embolic ischemic stroke
To investigate the effect of ischemic stroke on paravascular CSF circulation, we wanted a model in which no intracranial vascular lesion was present. Moreover, the occlusive material should not exert excessive pressure on the middle cerebral artery (MCA) to avoid irrelevant blockade of para-arterial influx. Thus, we selected a model in which the occlusive material was endogenous and physiologically advanced from the right common carotid artery (CCA) to the MCA. Therefore, embolic ischemic stroke was induced in mice by mechanically promoting embolization of Ferric chloride (FeCl3) triggered thrombi (30% m/v, 1 minute application) from the right CCA to the MCA. Mice were included in the study if magnetic resonance angiogram (MRA) performed 60 minutes after embolization showed proximal MCA occlusion (~60% of the mice performed were included for subsequent analyses). Glymphatic imaging was performed 3 hours and 24 hours (in two separate groups of mice) after ischemia onset. Notably, all the mice showed spontaneous recanalization of the MCA at +24 hours. To perform sham animals, the right CCA was exposed but only saline was applied on the artery. All the performed mice had two angiographies: one was performed at +1 hour and constituted an inclusion criteria and another one at the time of glymphatic imaging (+3 or +24 hours). According to our findings, all the mice that presented proximal MCA occlusion at +1 hour, also presented proximal MCA occlusion at +3 hours. Therefore, there was no spontaneous recanalization between +1 and +3 hours. In contrast, all the mice performed presented recanalization at +24 hours, suggesting that spontaneous recanalization occured between +3 and +24 hours after ischemic stroke onset.

Common carotid artery occlusion
Left common carotid artery occlusion (CCAo) was performed as previously described. Briefly, the left CCA was exposed and double-ligated with 6-0 silk sutures. All the mice were included in the final analyses.

Intracerebral hemorrhage
Intracerebral hemorrhage (ICH) was induced by collagenase injection as previously described\(^3\). Briefly, using a stereotactic frame a pulled hematological micropipette was inserted into the right striatum at bregma: 0.5 mm; mediolateral: 2.5 mm; dorsoventral: 3 mm. Then, 0.5 μL of saline containing 0.04 IU of collagenase VII-S (Sigma-Aldrich, L'Isle-d'Abeau, France) was injected. All the mice were included in the final analyses.

**Subarachnoid Hemorrhage**

Subarachnoid hemorrhage (SAH) was induced by injection of fresh arterial blood into the prechiasmatic cistern\(^4\). The mouse was placed within a stereotactic frame. A hole was drilled in the skull 4.5 mm anterior to the bregma, after we had observed and avoided the superior sagittal sinus. A 30-gauge needle was advanced at a 40° angle for 5 mm until the skull base was touched. It was withdrawn from 0.5-1 mm, so that the tip was in the prechiasmatic cistern. Arterial blood was withdrawn from the left ventricle of another anesthetized mouse using a 25 gauge needle. 50 μL of blood were manually injected through the 30 gauge needle in 15 seconds. We chose the volume of 50 μL instead of 100 μL\(^4\) in order to induce mild subarachnoid hemorrhages with fast recovery of the mice and no mortality. For sham animals, the same procedure was followed, except that the intracisternal injection solution was made of saline. All the mice were included in the final analyses.

**Intraventricular fibrinolysis**

To induce intraventricular fibrinolysis, tissue-type plasminogen activator (tPA, 3 μg in 1 μL) was injected in the right lateral ventricle (coordinate: AP +0mm; LR +1.75mm ; DV = -3mm) 15 minutes after SAH. Control animals received intraventricular saline (1 μL).

**DOTA-Gd clearance**

SAH was induced in mice and 24 hours thereafter, we performed an intrastriatal injection of 1 μL of a diluted solution of DOTA-Gd. Then, mice underwent two MRI sessions at +1 minute and +60 minutes after in situ DOTA-Gd injection to measure DOTA-Gd clearance from the brain. Data were quantified as a signal intensity ratio between the ipsilateral (injected) and the contralateral (control) striatum.

**Craniectomy**

Mice were anesthetized at +1 hour after ischemic stroke onset or at +22 hours post SAH with isoflurane (2%) and placed in a stereotaxic frame to immobilize the head. An injection of buprenorphine (0.05 mg/kg) was performed subcutaneously in the neck. Then, a skin incision was performed between the two ears to expose the skull. A large craniectomy was performed (diameter =~1cm) in the center of the skull by drilling. Animals that presented a damaged dura matter after the procedure were excluded. After craniectomy, the wound was stitched using 5.0 silk sutures and the mice were allowed to recover.

**MRI experiments**

MRI was performed on a 7T Pharmascan MRI system (Bruker, Germany) equipped with surface coils. High-resolution 3D T1-weighted imaging was performed with a Fast Low Angle Shot (FLASH) sequence with parameters set as follow: TR/TE 15/3.57 ms, Angle= 25°, Field of View= 20*17*14.4 mm, Matrix= 256*218*96 leading to a resolution of 78*78*0.15 μm, acquisition time= 5m 14s. T2-weighted imaging and Diffusion weighted imaging were performed as previously described\(^5\). Quantification of the images was performed using ImageJ (1.48, NIH) in a blinded manner and regions of interest were set as described on supplementary figure 2.
Immunohistochemistry
Deeply anesthetized mice were transcardially perfused with cold heparinized saline (15 mL) followed by 150 mL of fixative (PBS 0.1 M, pH 7.4 containing 2% paraformaldehyde and 0.2% picric acid). Brains were post-fixed (18 hours; 4°C) and cryoprotected (sucrose 20% in veronal buffer; 24 hours; 4°C) before freezing in Tissue-Tek (Miles Scientific, Naperville, IL, USA). Cryomicrotome-cut transversal sections (8-10 µm) were collected on poly-lysine slides and stored at −80°C before processing. Sections were co-incubated overnight with goat anti-collagen-type IV (1:1500, Col IV; Southern Biotech), chicken anti-GFAP (1:8000; Abcam) or sheep anti-fibrinogen (1:4000, purified Serum from sheep immunized with fibrinogen) in veronal buffer (pH 7.4). Primary antibodies were revealed using Fab’2 fragments of Donkey anti-goat, sheep or chicken IgG linked to FITC or TRITC (1:500, Jackson ImmunoResearch, West Grove, USA). Washed sections were coverslipped with antifade medium containing DAPI and images were digitally captured using a Leica DM6000 microscope-coupled coolsnap camera and visualized with Metavue 5.0 software (Molecular Devices, USA) and further processed using ImageJ 1.48 software (NIH). Measurement of fluorescence intensity was performed using ImageJ using unprocessed images (mean fluorescence of the whole image). Three different slices per mouse were selected randomly in the target area and the mean fluorescence was computed. Image acquisition parameters were set at the same values for all the animals (Acquisition time = 1000 ms).

Statistics
Results are the mean +/- SD. Statistical analyses were performed using the Kruskall-Wallis test followed by post hoc comparison with the Mann-Whitney U test.
References
Supplementary Figure I. Minimally invasive intracisterna magna injection. (A) We used pulled hematological glass micropipettes instead of 30 Gauge needles to perform intracisterna magna injection to avoid dura mater injury. (B) Macroscopic view of the injection method. Left: Initial position of the mouse. Middle: Neck skin incision revealing the muscular layer. Right: Final view after dissection of the muscular layer and micropipette placement. (C) Operator view of the injection method. Left: visualization of the dura mater and underlying cerebral structures. Middle: position of the micropipette (yellow arrowhead) immediately before insertion inside the cisterna magna. Right: final position of the micropipette and injection of DOTA-Gd. (D) Pre- and post-injection views showing the lack of apparent damage of the dura mater by the microinjection procedure.
Supplementary Figure II. Regions of Interest. The representative images are from a control mouse, 30 minutes after DOTA-Gd microinjection in the cisterna magna. (A) For the analyses performed in the subarachnoid hemorrhage (SAH) model, regions of interest were placed in the cerebellum and olfactory bulb at the center of the brain (medial Sagittal plane). (B) For the analyses performed in the embolic ischemic stroke model, the intracranial hemorrhage model (ICH) and after common carotid artery occlusion (CC Ao), the regions of interest were placed around the left and right middle cerebral arteries.
Supplementary Figure III. Glymphatic blockade persists in SAH animals despite bilateral craniectomy. To investigate whether reduced glymphatic perfusion in the forebrain after SAH may be explained by an increase in intracranial pressure, we performed glymphatic imaging at 24 hours after SAH in craniectomized animals. (A) Representative T2-weighted images of a craniectomized mouse 24 hours after SAH revealing herniation of the upper part of the brain. (B) Representative T1-weighted images of the glymphatic system after intracisternal DOTA-Gd injection in SAH and sham mice. (C) Corresponding quantification (n=4). The glymphatic system remained impaired despite craniectomy.
Supplementary Figure IV. FITC-Dextran (70 KDa) accumulates in the perivascular spaces 1 hour after intracisternal injection. To allow morphological microscopy of the perivascular spaces, we injected a high molecular weight compound (FITC-Dextran, FITC-Dx, 5µg) in the cisterna magna of control mice. One hour after injection, the brain were processed for immunohistofluorescence studies. (A) Representative images of a brain vessel revealed by collagen type IV staining (Col IV) after intracisternal FITC-Dextran injection. FITC-Dextran accumulated outside the basal lamina of the endothelial cells. (B) Representative images of the brain after intracisternal FITC-Dextran injection and GFAP immunostaining. FITC-Dextran accumulated on the luminal side of the astrocyte end-foot processes. Therefore, FITC-Dextran accumulated in the space between the basal lamina and the astrocyte end-foot processes, i.e. the perivascular space. (C) Schematic representation of the immunohistological findings.
Supplementary Figure V. Perivascular spaces are occluded by fibrin after SAH. To understand why glymphatic perfusion was impaired in SAH mice, we performed immunohistological analyses of the brain 24 hours after SAH onset (at a time when the glymphatic perfusion is diminished in the forebrain). (A) After intracisternal injection of FITC-Dextran that accumulates in perivascular spaces, immunohistofluorescence revealed the occlusion of the perivascular spaces after SAH in the anterior part of the brain. Indeed, no perivascular space was visible despite FITC-Dextran administration 24 hours after SAH in the olfactory bulbs, although perivascular spaces appeared unaffected in the cerebellum. (B) To investigate why perivascular spaces are occluded after SAH, we performed additional immunohistological studies. Upper panel: Left, representative images of the prechiasmatic cisterna after fluorescent staining of fibrinogen, revealing the presence of a large fibrin-rich clot surrounding the optic nerves, in the subarachnoid space. Right, higher magnification revealed the presence of fibrinogen in the perivascular spaces. Lower panel: this perivascular localisation of fibrinogen was confirmed after co-staining for collagen type IV (Col IV) by revealing fibrinogen around the basal lamina of some brain vessels. Altogether, these immunohistological results suggested that the glymphatic function is impaired after SAH because of perivascular space occlusion by fibrin clots.
Supplementary Figure VI. Brain clearance of DOTA-Gd is impaired after SAH. To investigate whether glymphatic impairment induced by SAH influences clearance of low molecular weight molecules from the brain, we performed serial MRI of mice after intrastrital injection of DOTA-Gd. This protocol allowed us to compare clearance rates in control and SAH animals. Two sets of T1-weighted images were acquired at +1 and +60 minutes after DOTA-Gd injection in animals subjected to SAH or sham operation 24 hours before DOTA-Gd injection. (A) Schematic representation of the performed experiment. (B) Representative T1-weighted images of mouse brains after in situ unilateral DOTA-Gd injection at 2 time points: +1 and +60 minutes. (C) Corresponding quantification (n=4 per group) revealing slower DOTA-Gd clearance in SAH animals as compared to sham animals. Interestingly, DOTA-Gd clearance was not abolished in SAH animals despite apparent complete blockade of glymphatic perfusion, suggesting that other mechanisms of DOTA-Gd clearance are involved. ns = not significant.
Supplementary Figure VII. Glymphatic impairment persists in ischemic stroke animals despite bilateral craniectomy. To investigate whether reduced glymphatic perfusion in the forebrain after ischemic stroke may be explained by an increase in intracranial pressure, we performed glymphatic imaging at 3 hours after stroke onset in craniectomized animals. (A) Top left: Representative DWI-weighted images of a craniectomized mouse 1 hour after stroke onset (and 2 hours before glymphatic imaging). The lack of brain herniation suggests that the intracranial pressure is not significantly increased in this model at this time point. Top right: Representative angiography after craniectomy showing proximal occlusion of the right MCA after thrombus embolization. Bottom: Representative T1-weighted images of the glymphatic system after intracisternal DOTA-Gd injection in a mouse 3 hours after stroke onset revealing an impaired glymphatic perfusion in the ischemic cortex. (C) Corresponding quantification (n=4). The glymphatic system remained impaired despite craniectomy.
Supplementary Figure VIII. Neither ICH nor CCAo influenced glymphatic perfusion of the brain. Glymphatic imaging was performed 24 hours after collagenase-induced ICH or CCAo. (A) Representative T1-weighted images in mice with unilateral CCAo and in sham mice 60 minutes after DOTA-Gd injection. No difference between ipsilateral and contralateral side was detected in term of contrast enhancement. (B) Corresponding quantification (n=5 per group). (C) Representative set of T1-weighted images in mice with ICH one hour after DOTA-Gd injection. There was no decrease in the influx of DOTA-Gd either globally or around the hematoma. (D) Corresponding quantification revealed no difference between ICH and sham mice (n=5 per group). ns = not significant.