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Neuroinflammation after Subarachnoid Hemorrhage: The Role of Microglia

Project outline

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Background

Devastating and life-threatening aneurysmal **subarachnoid hemorrhages** (aSAH) occur in 7 out of 100,000 people. Although considerable advances in diagnostics and treatment strategies have reduced the morbidity and mortality after aSAH[1], secondary stroke syndrome, i.e. delayed cerebral ischemia (DCI), remains an only partially understood issue. Classically, cerebral vasospasm (CVS) has been associated with DCI, but current evidence suggests that the causes for DCI are multifactorial[2, 3].

In this context, compartmental inflammation is an emerging concept and claimed to be associated with the occurrence of CVS, DCI, delayed ischemic neurological deficits (DIND) and worse outcome in patients with aSAH[4-6]. Moreover, several cytokines, e.g. TNF-α, interleukin-6 and endothelin-1, have been linked with neurological complications in these patients[7]. Additionally, several animal studies have elucidated the contribution of proinflammatory mediators to the pathogenesis of CVS and DCI[8, 9].

These proinflammatory mediators are mainly produced by activated microglia, astrocytes and endothelial cells in response to acute central nervous system injury[7, 10]. Interestingly, specific inhibition of microglia activation has been shown to be efficient in reduction of secondary tissue injury after intracerebral hemorrhage and traumatic brain injury in rodents[11]. Conversely, depletion of microglia with a inducible genetic construct, was shown to deregulate neuroinflammation after ischemic stroke with excessive levels of IL-1β, IL-6 and TNFα[12, 13]. Yet, the specific contribution of microglia to the compartmental inflammatory response after SAH has not been further characterized.

Cortical spreading depression (CSD) is another pathophysiological phenomenon, which is reported to be linked to delayed neurological damage after aSAH[14, 15]. CSD may be defined

as electrical silence of brain electrical activity as a consequence or epiphenomenon of spreading depolarization due to a depolarization block of neuronal activity[16]. In animal models for aSAH, CSD could be linked to spreading ischemia[14]. Furthermore, a recent clinical trial could reproduce the link of CSD and progressive neurological damage in patients with aSAH[17]. CSD is known to be triggered by states of high extracellular potassium and low nitric oxide (NO) levels in the brain, which both commonly occur after aSAH[14, 15, 18]. More interestingly, also endothelin-1 (ET-1) was shown to trigger CSD in a rat model of SAH[19, 20]. ET-1 was found to be synthesized and released by activated mononuclear leukocytes in the CSF of SAH patients together with known acute-phase reactants (IL-1β, IL-6 and TNFα)[21]. However, neuroinflammatory response with activation of microglia and the typical burst of inflammatory mediators was never taken into account as a possible factor causing or facilitating CSD.

Brain edema formation is another detrimental process after acute cerebral lesions[22]. Interestingly, recent evidence suggests a close interaction of neuroinflammation and cerebral edema formation with Aquaporin 4 (AQP 4) as crucial common denominator, as nicely reviewed by Fukuda and Badaut[23]. AQP 4 is the most prominent water channel of the brain and is mainly expressed on the perivascular astrocyte endfeet. There is a close correlation between the expression and localization of AQP 4 and the extent of cerebral edema formation in diverse models of acute cerebral injury[24-27]. Moreover, AQP 4 expression was also shown to be altered after SAH in human brain tissue[28]. However, the mechanisms causing changes in AQP 4 function after acute cerebral injury remain widely unknown.

Neuroinflammation, brain edema formation and CSD are known to influence DIND and neurological outcome after aSAH. However, their complex interplay remains to be elucidated to develop targeted therapies for patients after aSAH.

Aims of the research project

The major focus of the proposed project is the investigation of the role of microglia in compartmental inflammatory response of the CNS and the development of early as well as delayed cerebral injury after SAH.

Hypothesis 1:

SAH in microglia depleted mice leads to deregulation of the neuroinflammatory response with excessive brain edema and impaired cortical perfusion. Moreover, microglia depletion aggravates cerebral vasospasms.

Hypothesis 2:

Deregulated neuroinflammation through microglia depletion facilitates the occurrence of CSD and exacerbates neurotoxicity after SAH.

In detail, the following experiments will be performed:

1. Overall influence of microglia on course of disease after SAH

    1.1. Magnet Resonance Imaging and Confocal Laser-Scanning Microscopy

    To gain baseline values, animals will undergo MRI scans one day before lesion induction. MRI imaging will consist of T2 sequences and TOF sequences for anatomical pictures and angiography. Additionally, FAIR and DWI sequences for quantitative measurement of perfusion and diffusion will be acquired (see Fig. 1).

    CD11b-HSVTK mice will be treated with GCV intraperitoneally starting two days before endovascular filament perforation on day 0. MRI with in vivo assessment of angiographic vasospasm, perfusion changes and edema formation will be repeated on day 1 and day 3.

On day 4 animals will be sacrificed by a lethal dose of anesthesia and brain tissue will be collected upon trans-cardiac perfusion with PBS, followed by 4% formalin. Brains will be cut into 50 µm vibrotome sections and immunostained with antibodies against Iba-1 (microglia), GFAP (reactive astrocytes), aquaporin 4, CD45 (leukocytes) and podocalyxin (intraluminal vessel marker). Control animals will consist of CD11b-HSVTK (lesion, no GCV), CD11b-HSVTK (sham operation, GCV), CD11b-HSVTK (sham operation, no GCV) and the corresponding groups of transgene negative littermates.

1.2. Assessment of intracranial inflammatory response with biochemical methods

To biochemically quantify the contribution of microglia to the intracranial inflammatory response, CD11b-HSVTK mice treated with GCV will be sacrificed on day 1, day 3, day 4 after lesion induction. Brain tissue will be collected upon trans-cardiac perfusion with PBS. Subsequently brain tissue will be homogenized in PBS and Il-6, ET-1 and TNF-α levels determined by the use of enzyme-linked immunosorbent assay (ELISA). Additionally, expression levels of COX-2, HO-1 and NOS-2 as overall markers of induction of inflammatory response in the CNS will be measured in the brain homogenate.

Furthermore, microvasculature will be isolated by a mechanical microvessel purification protocol to determine expression levels of aquaporin 4 in perivascular astrocyte endfeet.

The control groups will be the same as in the first experiment.

2. Two-Photon Laser-Scanning Microscopy after SAH

2.1 Characterization of microglia after SAH

Two-Photon Laser-Scanning Microscopy (TPLSM) will be used to characterize the activation of microglia after SAH in CX3CR1-EGFP mice. These mice express enhanced green fluorescence protein (EGFP) under the control of the promoter for the fractalkine receptor (CX3CR1). Microglia are the only cells expressing this receptor in the CNS, which allows for direct visualization of dynamic changes in microglia cell morphology in the neocortex of mice in vivo[29, 30]. CX3CR1-EGFP mice will be repeatedly scanned after induction of SAH to determine the earliest time point and the peak of microglia activation. As controls sham-operated CX3CR1-EGFP mice will be used in this experiment.

To characterize microglial paralysis in CD11b-HSVTK mice in vivo, double-transgenic animals (CD11b-HSVTK/CX3CR1-EGFP) will be assessed after induction of SAH with and without GCV treatment. The number of microglial cells and their dynamic morphological changes will be analyzed by TPLSM.

2.3 In vivo imaging of CSD with TPLSM after SAH

The occurrence of CSD after SAH in wt mice and in CD11b-HSVTK mice will be compared using in vivo TPLSM NADH imaging as described in Takano et al. 2007[31].

The TPLSM experiments are planned as collaboration with the research group of Pharmacology, University Hospital of Zurich, Prof. Bruno Weber. This group has extensive experience with in vivo TPLSM in mice.

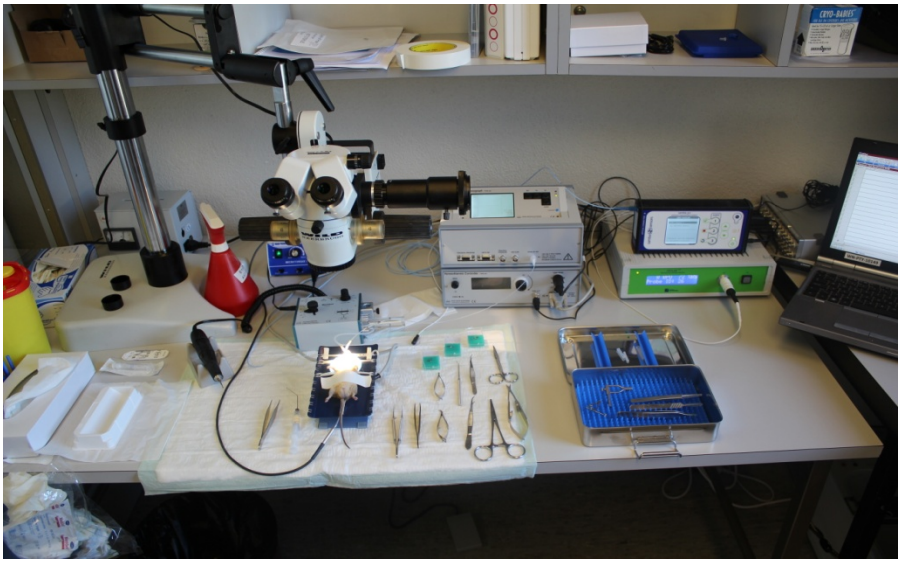
Methods

Mouse model of SAH using the filament perforation technique

To induce standardized SAH in mice, the filament perforation technique will be used with monitoring of intracranial pressure (ICP) first described by Feiler and co-workers[32]. In this model, animals are anesthetized and placed in a supine position. The neck is opened by a midline incision and the left carotid artery is exposed. A 5-0 monofilament is advanced via the external carotid artery (ECA) into the internal carotid artery (ICA). ICP is continuously monitored in the epidural space with an ICP micro-sensor. The filament is then pushed further until a sharp increase of ICP indicating the successful induction of SAH. Subsequently, the suture is withdrawn into the ECA to allow full perfusion of the ICA. In sham-operated animals the filament is inserted into the ICA without perforating the Circle of Willis.

This model is superior to others using injection of autologous blood into the cisterna magna[33] or perforating an intracisternal vein[34], because the internal perforation of an arterial vessel closely resembles aneurysmal hemorrhage with endothelial damage.

Figure 1:



Surgical setup at University Hospital of Zurich. Mice are fixed on a homeothermic regulated operation table. Ventilation is continuously controlled by capnometry. During filament perforation CBF and ICP are measured using a laser doppler flow probe and an epidurally implanted pressure micro-sensor, respectively.

CD11b-HSVTK mice

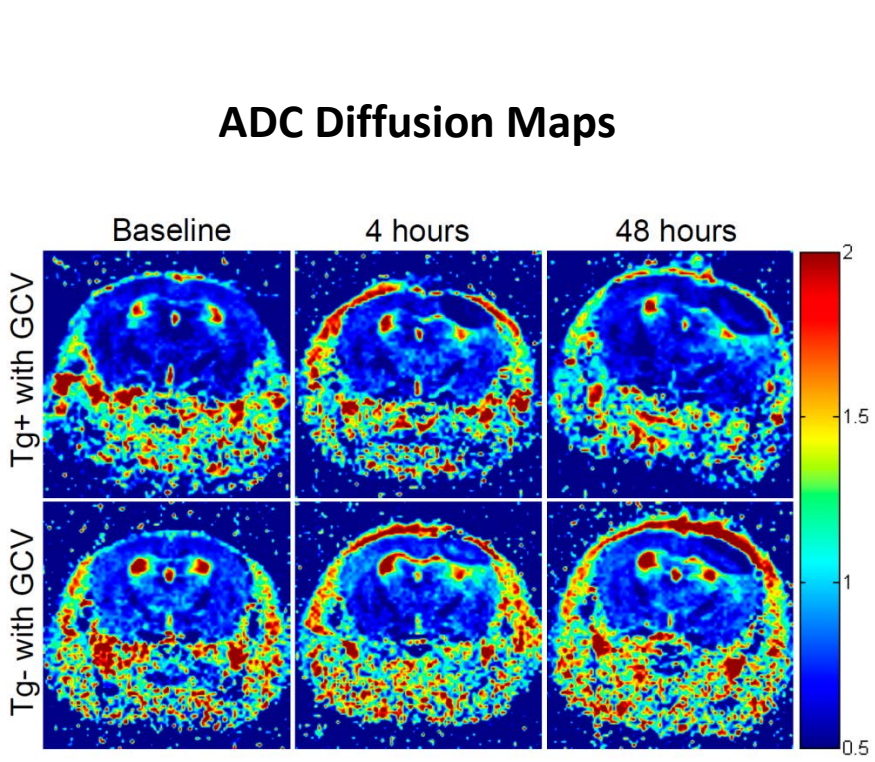
Microglia are the brain's resident immune cells. To investigate their role in a mouse model of multiple sclerosis, Heppner and co-workers have generated a genetic construct allowing for conditional depletion of these cells[35]. Briefly, in these CD11b-HSVTK mice, the thymidine kinase of Herpes Simplex virus is expressed under the control of a promoter, which is mainly active in microglia and macrophages. In these cells, the thymidine kinase processes the drug ganciclovir (GCV) into a toxic compound which leads to apoptosis. Therefore, in adult CD11b-HSVTK mice microglia can be conditionally depleted upon the administration of GCV.

Magnet Resonance Imaging

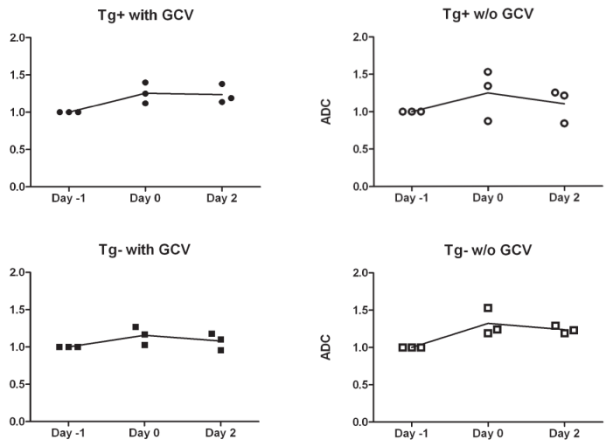
Magnet resonance imaging will be performed with a small animal scanner (Bruker 4.7 Tesla) at the University Hospital of Zürich. This scanner allows for sophisticated *in vivo* imaging of anatomical structures (T1/T2 sequences and TOF angiography) as well as quantitative measurement of functional parameters like perfusion and diffusion (FAIR and DWI) with the corresponding illustrations (Perfusion and ADC maps). All these sequences and data post processing have been established in our lab in the scope of other ongoing projects (see Figure 2).

Figure 2:

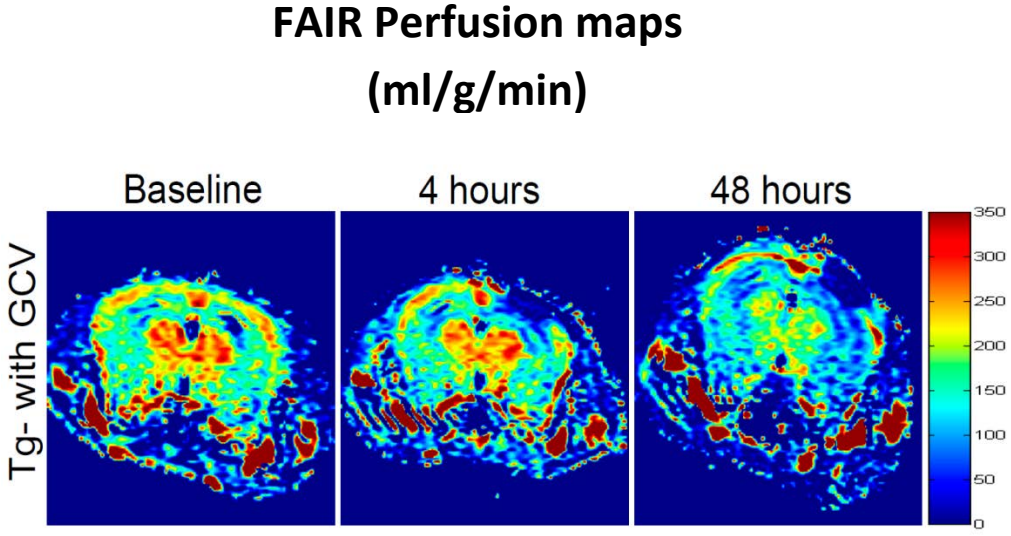
a.)


ADC Diffusion Maps
Baseline
4 hours
48 hours
Tg+ with GCV
Tg- with GCV
2
1.5
1
0.5

b.)

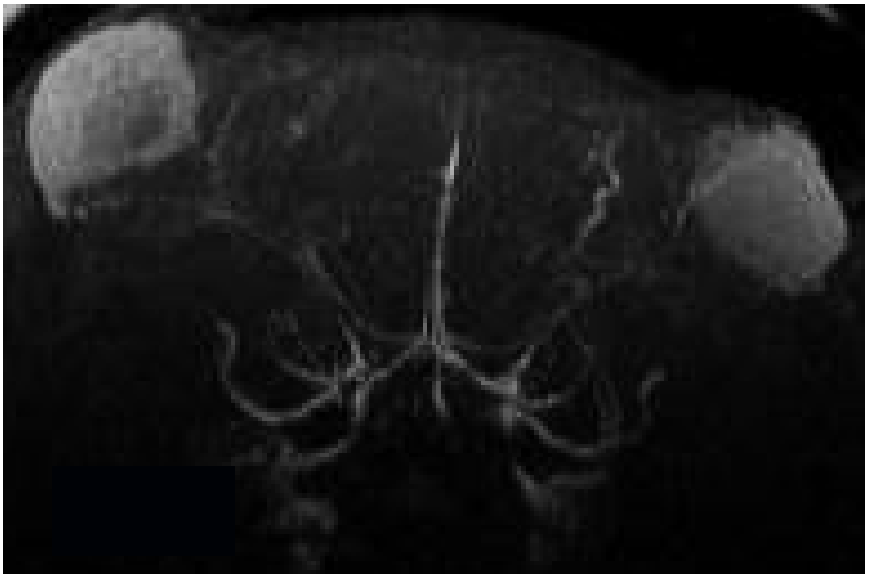

Tg+ with GCV
Tg+ w/o GCV
Tg- with GCV
Tg- w/o GCV
ADC
Day -1
Day 0
Day 2

c.)


FAIR Perfusion maps
(ml/g/min)
Baseline
4 hours
48 hours
Tg- with GCV
350
300
250
200
150
100
50
0

d.)

TOF angiography



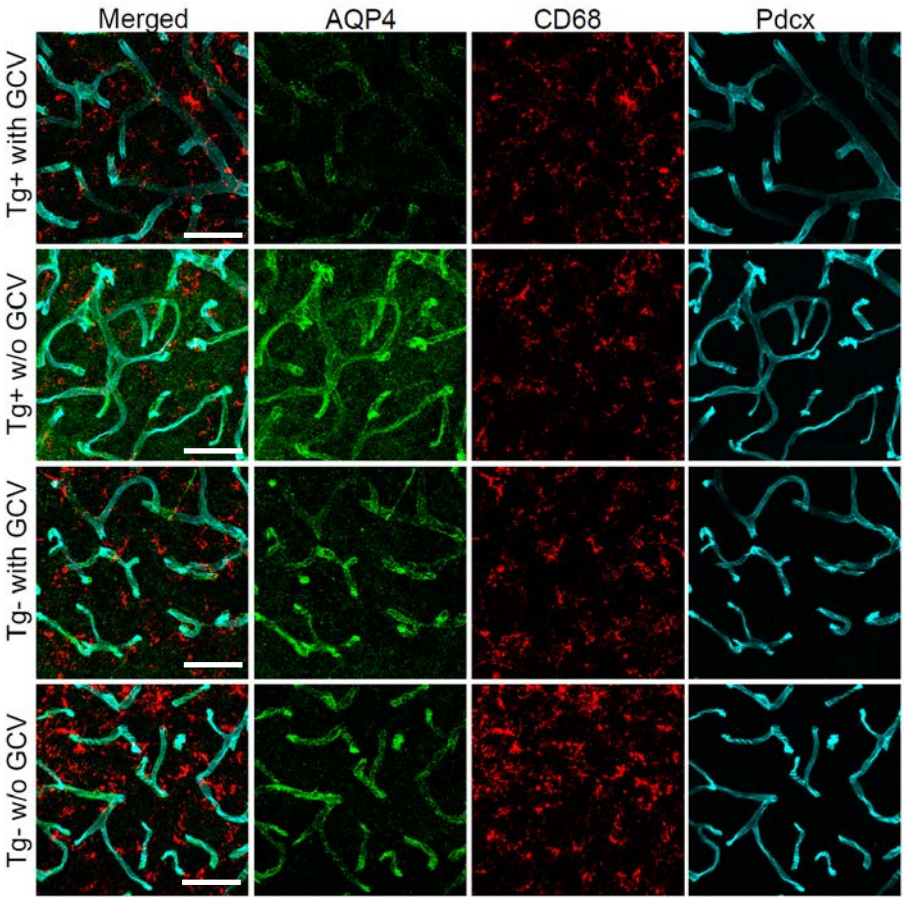
MRI analysis of CD11b-HSVTK mice after induction of photothrombotic lesion in the sensorimotor cortex. Aparent diffusion coefficient (ADC) maps for quantification of cytotoxic and angiogenic edema with restriction of diffusion in lesion center and perifocal edema **(a)**. Quantification of perifocal edema over time. Microglia-depleted mice show prolonged edema persistence **(b)**. Perfusion maps with physiologically high perfusion in cortex and basal ganglia. Early restriction of cortical perfusion after lesion induction **(c)**. TOF angiography of Tg+ mouse with detailed depiction of the arteries of the Circle of Willis **(d)**.

unpublished data, M. Hugelshofer & M. Wurnig 2013

Confocal Laser-Scanning Microscopy (CLSM)

This imaging modality allows for structural analysis of the neurovascular unit on a subcellular level. Combinations of antibodies derived from different species (rat, rabbit and goat) enable triple immunostainings to delineate the functional components of the blood brain barrier (BBB)[36]. The staining and CLSM imaging of pericytes (CD13), AQP 4, microglia (Iba-1, CD68), reactive astrocytes (GFAP), leukocytes (CD45) and vessels (podocalyxin) has already been optimized in the scope of another project in our lab (see Fig. 2).

Figure 3:



Confocal Laser-Scanning of immunostained brain cortex in CD11b-HSVTK mice with photochemical cortical lesion (Scale bar 50 µm). Reduction of perivascular AQP 4 staining in lesion border zone of microglia depleted mice.

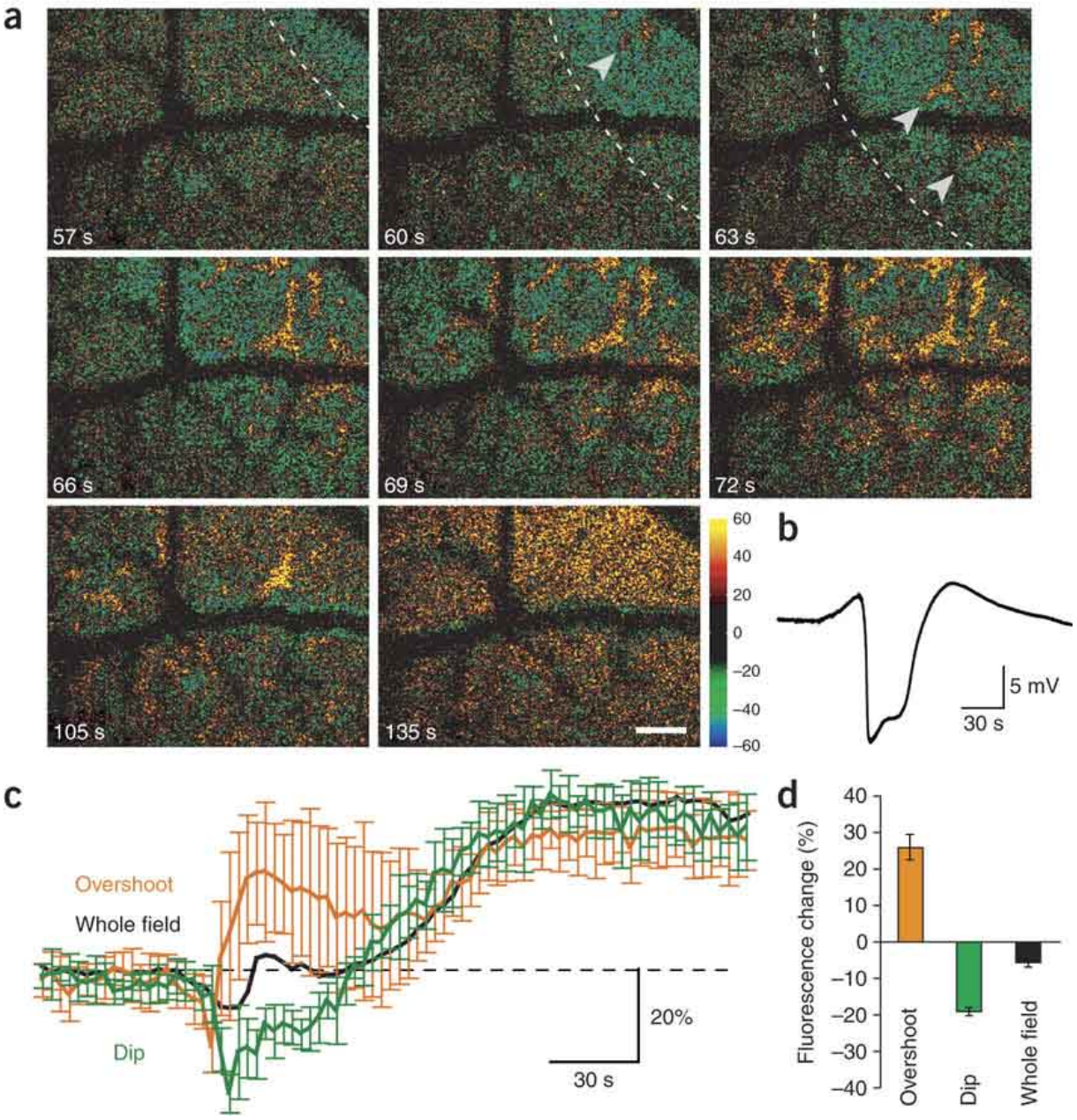
Antibodies: AQP 4: water channel on astrocyte endfeet, CD 68: microglia, podocalyxin: vessels

unpublished data, M. Hugelshofer 2013

Two-Photon Laser-Scanning Microscopy (TPLSM)

Advances in imaging technologies now provide means to investigate cellular morphology and even functional states in vivo with not only a micrometer spatial resolution in a penetration depths of 500-1000 µm in viable tissue, but also with the possibility of dynamic measurements over a wide range of time scales[37]. In Brief, TPLSM combines the technique of confocal laser scanning microscopy with the use of very short laser pulses with duration of approximately 100 femtoseconds to excite a fluorescent dye. Only the energy of two photons reaching the fluorescent dye simultaneously causes excitation. This allows deeper tissue penetration with less phototoxicity and excellent yield of fluorescents[38], which makes this technique suitable for in vivo imaging. Furthermore, with the development of various techniques of fluorescent labeling of tissue components in vivo, TPLSM has become the method of choice to investigate functional states of viable cellular structures under pathological conditions in animal models. Furthermore, TPLSM provides subcellular metabolic imaging by utilizing the intrinsic fluorescence of β-nicotinamide adenine dinucleotide (NADH) as a sensitive indicator of energy metabolism[39-42]. This technique allows for detection of CSD as a front of pathophysiological metabolic activity exceeding changes of NADH fluorescence evoked by normal physiological activity by more than one order of magnitude[31].

Figure 4:



(**a**) Pseudocolored images of NADH fluorescence changes during the passage of a wave of CSD 50 µm below the cortical surface. NADH decreases (dips) are displayed in green-blue and NADH increases (overshoot) in red-yellow. Dashed lines indicate the wave front moving across the field. Arrowheads indicate areas with NADH overshoot. Scale bar, 100 µm.

(**b**) Local field potential recording of the CSD wave in **a**.

(**c**) Time-course relative changes of NADH fluorescence in dip (green), overshoot (orange) and integration of the whole field (black) of the CSD wave shown in **a**. Means and s.d. were calculated from eight areas of dip and overshoot in the field.

(**d**) Summary histogram of initial peak NADH fluorescence changes (mean ±s.e.m.).

Figure 4: Takano T, Nat Neurosci 2007

Outlook

Despite considerable advances in the therapy of aSAH patients in modern neurointensive care medicine, DIND remain an only partially understood issue. There is a broad line of evidence supporting the hypothesis that compartmental inflammatory response is an important factor contributing to DIND, and microglia, as the brain's resident immune cells, have been reported to be highly activated in paradigms of acute and chronic neurodegeneration.

The pathophysiological mechanisms of neurotoxicity in development of DIND after aSAH are still speculative. Classically, secondary stroke syndrome has been associated with cerebral vasospasms (CVS). Based on clinical results, which showed insufficient correlation of CVS with occurrence of DIND and poor outcome in aSAH patients, this dogma has recently been challenged. Conversely, cortical spreading depression (CSD) associated with cortical spreading ischemia (CSI) attained growing attention in SAH research, and several animal models of CSD could show similar patterns of ischemic cortical damage closely resembling DCI after SAH in humans.

Our study will investigate the role of microglia in the compartmental inflammatory response and the course of disease after aSAH. The mechanisms of neurotoxicity after aSAH will be addressed combining transgenic and biochemical methods with modern imaging modalities, i.e. MRI, CLSM and TPLSM. The latter will not only provide insight into dynamic morphological changes on a subcellular level, but also visualize occurrence of CSD after aSAH. This will enable us to bring new insights about the dynamic interplay between microglia activation, brain edema formation and the occurrence of CSD which could lead to the development of therapeutics for treatment of DIND.

A profound understanding of the basic mechanisms underlying the development of DIND in patients with aSAH will be crucial for the development of new therapeutic strategies in neurointensive care medicine. In particular, pharmacological targeting of specific steps of neuroinflammatory response and cerebral edema formation, but also monitoring of inflammatory mediators involved in neurotoxic mechanisms, which allows early identification of patients at risk for DIND, could potentially improve outcome after aSAH significantly. Moreover, the role of compartmental inflammation-triggering neurotoxic processes, e.g. CSD, may also have implications for more chronic paradigms of neurodegeneration, such as multiple sclerosis, Alzheimer's and Parkinson's disease.

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