Electrochemical Failure of the Brain Cortex Is More Deleterious When it Is Accompanied by Low Perfusion

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Background and Purpose—Clinical and experimental evidence suggests that spreading depolarization facilitates neuronal injury when its duration exceeds a certain time point, termed commitment point. We here investigated whether this commitment point is shifted to an earlier period, when spreading depolarization is accompanied by a perfusion deficit.

Methods—Electrophysiological and cerebral blood flow changes were studied in a rat cranial window model followed by histological and immunohistochemical analyses of cortical damage.

Results—In group 1, brain topical application of artificial cerebrospinal fluid (ACSF) with high K+ concentration ([K+]ACSF) for 1 hour allowed us to induce a depolarizing event of fixed duration with cerebral blood flow fluctuations around the baseline (short-lasting initial hypoperfusions followed by hyperemia). In group 2, coapplication of the NO-scavenger hemoglobin ([Hb]ACSF) with high [K+]ACSF caused a depolarizing event of similar duration, to which a severe perfusion deficit was coupled (=spreading ischemia). In group 3, intravenous coadministration of the L-type calcium channel antagonist nimodipine with brain topical application of high [K+]ACSF/[Hb]ACSF caused spreading ischemia to revert to spreading hyperemia. Whereas scattered neuronal injury occurred in the superficial cortical layers in the window areas of groups 1 and 3, necrosis of all layers with partial loss of the tissue texture and microglial activation were observed in group 2.

Conclusions—The results suggest that electrochemical failure of the cortex is more deleterious when it is accompanied by low perfusion. Thus, the commitment point of the cortex is not a universal value but depends on additional factors, such as the level of perfusion. (Stroke. 2013;44:XXX-XXX.)

Key Words: neuroprotection ■ spreading depression ■ spreading ischemia ■ subarachnoid hemorrhage ■ vasospasm

Back in 1947, Aristides Leão had coined the idea that electrically induced short-lasting spreading depolarization (SD) in healthy, naïve tissue and the terminal SD succeeding arrest of the circulation are of the same nature.1 Later, experimental work confirmed that essential biophysical features of short-lasting SD correspond with those observed during the initial stage of terminal SD.2,4 Possibly, this commitment point is not universal but depends on neuroanatomical structure, neuron population, intracellular signals and proteases, leading to cell death if the depolarization outlasts a certain time point, termed commitment point.2,4 Consequently, spreading depolarization is associated with neuronal death in contrast to short-lasting depolarizations in healthy, naïve tissue.4,5 However, marked differences exist within this spectrum of near-complete sustained depolarizations.6,7 This applies, for example, to the sensitivity of the depolarizations to N–methyl–d–aspartate receptor antagonists,8–10 or the observation that terminal depolarization is associated with neuronal death in contrast to short-lasting depolarizations in healthy, naïve tissue.11,15

Since the end of the 1970s, experimental evidence has been accumulating that events with features between SD in healthy naïve tissue and terminal depolarization occur in the ischemic penumbra after middle cerebral artery occlusion, under hypoxia, hypoglycemia or in the presence of chemical factors, such as potassium.16–20 It has been increasingly recognized that those depolarizations of intermediate duration are associated with lesion progression, although the neurons seem to recover transiently from the ionic imbalance.21–25 Hence, it has been hypothesized that they entail triggers activating intracellular signals and proteases, leading to cell death if the depolarization outlasts a certain time point, termed commitment point.24 Possibly, this commitment point is not universal but depends on neuroanatomical structure, neuron population, developmental stage, and noxious condition.4 SD is observed as a large negative direct current (DC) change. The duration of the DC negativity is an extracellular
index for the duration of the cellular depolarization. Novel technology for invasive full-band DC signals has recently revealed that a spatio-temporally diverse spectrum from short-lasting to very prolonged large negative DC changes also occurs in the injured human brain. It is, therefore, interesting to study the commitment point under different conditions in animals. This might answer the questions whether the commitment point is a universal value or not, and whether it is necessary to combine DC recordings with recordings of other measures, such as cerebral blood flow (CBF), tissue partial pressure of oxygen, or parameters of cerebral metabolism to estimate the commitment point in patients.

Here, we used an animal model in which artificial cerebrospinal fluid (ACSF) containing high K+ concentration (\([K^+]_{\text{ACSF}}\)) was applied topically to the brain. We specifically tested whether; (1) high \([K^+]_{\text{ACSF}}\) can induce a prolonged depolarizing event in vivo that is reversible on wash-out; and (2) the depolarizing event is not associated with a marked persistent CBF change. The high \([K^+]_{\text{ACSF}}\) model is interesting because it shares the drug resistance to N–methyl-D–aspartate receptor antagonists with terminal depolarization under anoxia, and it allows us to modify the hemodynamic response to the depolarizing event. Thus, coapplication of high \([K^+]_{\text{ACSF}}\) with an NO-synthase inhibitor, such as N–nitroarginine, converts the hemodynamic response from spreading hyperemia to ischemia (=inverse neurovascular coupling), whereas intravenous coapplication of the L-type calcium antagonist nimodipine causes the spreading ischemia to revert to the normal hyperemic response. The same inverse neurovascular response can also be produced by the coapplication of high \([K^+]_{\text{ACSF}}\) with an NO-synthase inhibitor, such as N–nitro-L–arginine, but not by the coapplication of high \([K^+]_{\text{ACSF}}\) with other vasoconstrictors, such as endothelin-1 (ET-1). The antagonistic effect of nimodipine on the inverse neurovascular response is attributable to its antagonistic action on the vasoconstrictors, which are released during the SD process, as previously shown in an in vitro model for spreading ischemia in the isolated middle cerebral artery. This antagonistic effect of nimodipine has been of particular clinical interest because, using subdural opto-electrodes for DC electrocorticography and laser-Doppler flowmetry, it was observed in patients that the inverse neurovascular response to SD is a mechanism involved in delayed ischemic stroke after subarachnoid hemorrhage. In previous clinical trials, prophylactic treatment with nimodipine was found to reduce the frequency and severity of delayed ischemic stroke. This beneficial effect of nimodipine was possibly because of its antagonistic effect on the inverse neurovascular response, a hypothesis further supported by the observation that nimodipine had no antagonistic effect on the proximal vasospasm after subarachnoid hemorrhage, another candidate pathogenesis underlying delayed ischemic stroke. For a more comprehensive account of the mechanisms involved in the inverse neurovascular response, including the possible role of astrocytes, we would like to refer the reader to a previous review. Notably, because of the markedly enhanced energy demand, SD can induce pockets of tissue hypoxia in most distant supply territories of cortical capillaries, even when the CBF response is hyperemic. Importantly, the tissue hypoxia is dramatically augmented when, in addition to the increased energy demand, the CBF response is inverted.

The features of the high \([K^+]_{\text{ACSF}}\) model as described above enabled us to investigate in vivo whether electrochemical neuronal failure of similar duration, induced by identical conditions in the subarachnoid space, has a more deleterious effect at a reduced level of perfusion or not. In an analogous fashion, we could have reduced the partial pressure of oxygen under high \([K^+]_{\text{ACSF}}\) to tackle our question, but this would have led to global side effects, and it would not have been possible to induce an energy compromise of similar severity because the animals would have died from cardiac arrest.

The results of the present study may have relevance for aneurismal subarachnoid hemorrhage or the ischemic penumbra after cerebral vessel occlusion because SDs can induce perfusion deficits under these conditions both in animals and humans.
and 3 mildly hyperventilated (Table 1 in the online-only Data Supplement). In group 1 (n=9), [K+]ACSF (250 mmol/L) was applied alone. Before the cluster of recurrent SDs, [K+] rose from 3.0 to 4.5±0.8 mmol/L in a cortical depth of 400 µm, and CBF increased mildly from 100 to 123±23%. The first SD in the cluster showed a negative intracortical DC shift of −19.1±4.0 mV, which lasted for 100±34 seconds. The delay between the 2 microelectrodes was 48±29 seconds, indicating propagation of SD. In a depth of 400 µm, the isolated depolarizations occurred superimposed on an ultraslow positive potential of 10.4±1.6 mV, and then melted progressively with each other, so that the negative intracortical DC amplitude of individual depolarizations decreased to −5.5±5.2 mV as shown in the representative traces of 1 animal in Figure 1. Subdurally, the ultraslow potential was negative during the cluster (−25.2±6.1 mV), [K+] rose transiently to 40.8±4.4 mmol/L during the first SD. It did not return fully to baseline before the next depolarization started. As a consequence, baseline [K+] rose slowly and steadily between the recurrent depolarizations, until it reached a plateau of 52.9±31.7 mmol/L. From this plateau, [K+] rose transiently to 70.2±21.6 mmol/L with each depolarization (Figure 1). Coupled to each SD, short-lasting initial hypoperfusion to 73±20% for 49±16 seconds was followed by short-lasting spreading hyperperfusion to 142±39%. In Figure 1, the CBF fluctuations are not much larger than the low-frequency vascular fluctuations that preceded the SD cluster. The cluster ended within 19.3±14.7 minutes after wash-out of high [K+]ACSF.

In group 2 (n=5), brain topical application of [K+]ACSF (250 mmol/L)/HbACSF (2 mmol/L) without nimodipine did not change CBF before the first SD started (102±20%). The SDs were characterized by a negative subdural DC-shift of −26.2±4.0 mV, to which spreading ischemia was coupled lasting for 72±21 minutes (Figure 2A). During spreading ischemia, CBF dropped to 21±7% (Figure 2A).

In group 3 (n=5), combined administration of nimodipine intravenously (2 µg/kg per minute) with topical [K+]ACSF (250 mmol/L)/HbACSF (2 mmol/L) increased CBF to 164±11% before the first SD started. This CBF level before the first SD was significantly higher than in the other 2 groups (1-way ANOVA with Bonferroni post hoc test; P<0.05). The cluster of SDs was characterized by a negative subdural DC shift of −19.9±4.0 mV. Coupled to single SDs, short-lasting initial hyperperfusion with a drop from 164 to 119±16% for 61±30 seconds was followed by short-lasting spreading hyperperfusion rising to 198±26% (Figure 2B). During the depolarizing event, the mean CBF level was 97±39% in group 1, 42±7% in group 2, and 161±12% in group 3. All groups differed significantly from each other (1-way ANOVA with Bonferroni post hoc test; P<0.05; Figure 2C).

Animals of group 2 showed hemiparesis at 48 hours after spreading ischemia (average grade: 2.6±1.1 on a scale from 0 [no deficit] to 4 [severe paresis]), whereas no significant neurological deficit was observed in animals of groups 1 and 3, and the 3 control animals, in which single SDs had occurred in response to 80 mmol/L [K+]ACSF (0.2±0.4 in each group).

Histological Changes
Histological analysis revealed brain herniation in the window area (Figure 2Aa and 2Ba) and a meningeal reaction with local accumulation of neutrophils in all experimental animals. Single neurons in the first cortical layer were surrounded by edema in the control group. Brains derived from groups 1 (high [K+]ACSF) and 3 (high [K+]ACSF/HbACSF/nimodipine) showed tissue necrosis in the first cortical layer with single shrunken acidophiles cells. In the second cortical layer, the pathological changes mainly consisted of neuronal shrinkage as well as perineuronal and perivascular swelling (edema; Figure 3Ab). In these layers, 2 main types of damaged neurons were detected: shrunken nonscalloped and shrunken scalloped cells. The shrunken neurons contained nuclei of normal size and form without signs of chromatin condensation. The cytoplasm of these neurons was somewhat denser than normal and contained dispersed Nissl substance. Around the cell body, there was a rim of pericellular swelling. Shrunken scalloped neurons had highly compressed triangular bodies, sometimes with marked proximal dendrite stems. These cells were surrounded by a wide pericellular space. Deeper layers were mostly free of cellular injury (Figures 2Ba and 3Ab).

Brains derived from group 2 showed a prominent inflammatory response consisting of Iba1-immunoreactive microglia and macrophages in the window area (Figure 3Ba). In comparison, the inflammatory reaction was significantly reduced in brains derived from group 3 (Figure 3Bb). Quantification of Iba1-immunoreactive cells in the cortical area below the cranial window revealed significant differences between group 2 and all other groups (group 1: 1.1±0.4×10^4 cells/mm^2; group 2: 2.8±0.2×10^4 cells/mm^2; group 3: 0.8±0.3×10^4 cells/mm^2; controls: 1.7±0.1×10^4 cells/mm^2; 1-way ANOVA with Bonferroni post hoc tests; P<0.001; Figure 3Ba, 3Bb, and 3C).

The density of glial fibrillary acidic protein-immunoreactive cells was slightly higher in brains derived from group 2, but this...
difference did not reach statistical significance (1-way ANOVA with Bonferroni post hoc test; Figure 3Ba, 3Bb, and 3D). Few TUNEL-positive cells were found in the cortex below the cranial window in brains derived from group 3 (Figure 4Ab), whereas a high number of TUNEL-positive cells was detected across all cortical layers in brains derived from group 2 (Figure 4Aa). Quantification of TUNEL-positive cells in the cortical area below the cranial window demonstrated a significant difference between groups 2 and 3 (group 1: 0.8±0.2×10^4 cells/mm^3; group 2: 1.8±0.9×10^4 cells/mm^3; group 3: 0.4±0.2×10^4 cells/mm^3; controls: 1.0±0.3×10^4 cells/mm^3; Kruskal–Wallis 1-way ANOVA on Ranks with Dunn post hoc test; P<0.05; Figure 4B). In all experimental groups, astrocytes and microglia were mildly activated all over the brain (including the contralateral hemisphere) when compared with naive animals.

Discussion

In the present study, we induced a depolarizing event by brain topical application of high [K+]_{ACSF} for 1 hour. The depolarizing event consisted of recurrent SDs melting with each other as reported previously for SDs induced by high [K+]_{ACSF} in the rat hippocampus. 20 Subarachnoid K+ concentrations of 250 mmol/L do not occur in natural disease conditions. However, the model provides interesting information for the fundamental understanding of SD and cortical damage because it allows us to determine a commitment point for a prolonged depolarizing event at relatively normal perfusion level. In the present study, the duration of about 60 minutes was not sufficient to cause damage in deeper cortical layers, although the intracortical K+ concentration reached a level comparable with that in natural disease conditions. [K+]_{o} rose to about 70 mmol/L in a cortical depth of 400 µm similar to the level of [K+]_{o} reported previously for terminal SD induced by anoxia in vivo. 38 However, it remains possible that the harmful effect of SD is underestimated because it could be deleterious through loss of intracellular potassium, and high [K+]_{ACSF} may mitigate the K+ outward flux. 39

The classic experimental approach to demonstrate the harmful effect of SD has been to ignite it chemically outside of the ischemic penumbra after middle cerebral artery occlusion. Those SDs then propagated into the penumbra, and it was measured that they enlarged the ischemic core as evaluated...
by continuous apparent diffusion coefficient of water mapping.22,23 A similar conclusion was reached using brain topical application of ET-1 in rats.21 In this model, the vasoconstrictive effect of different ET-1 concentrations can be titrated. An ET-1 concentration was chosen, at which 50% of animals developed a local low-flow area giving rise to spontaneous SDs.

Figure 3. Impact of nimodipine treatment on neuronal cell loss and gliosis induced by high [K+]ACSF/ [Hb]ACSF. 

**Aa**, Hematoxylin and eosin staining of the cortex of an animal from group 2 (high [K+]ACSF/[Hb]ACSF). Note the extensive necrosis in superficial and deeper cortical layers. Neurons with condensed nuclei are indicated by triangles. 

**Ab**, Hematoxylin and eosin staining of the cortex of an animal from group 3 (high [K+]ACSF/[Hb]ACSF/nimodipine). Pathological changes are mostly restricted to the superficial cortical layers with perivascular swelling and edema (arrows), shrunken nonscalloped neurons (open triangle), and shrunken scalloped neurons (closed triangle). 

**B**, Double immunohistochemical staining for glial fibrillary acidic protein (GFAP) and Iba1 reveals numerous Iba1-immunoreactive microglia and macrophages in brains derived from group 2 (Ba) and less Iba1-positive cells in brains obtained from group 3 (Bb). Reactive GFAP-positive astrocytes are detected in both groups (Ba and Bb). 

**C**, Quantification of Iba1-immunoreactive cells in the window area shows a significant increase in the density of Iba1-positive cells in brains derived from group 2 compared with groups 1 and 3 (*1-way ANOVA with Bonferroni post hoc tests; P<0.001). 

**D**, No significant differences in the densities of GFAP-positive astrocytes were detected among the 3 groups. Scale bars=100 µm (A) and 20 µm (B). Hb indicates hemoglobin.

Figure 4. Triphosphate nick-end labeling (TUNEL)-positive cells after spreading ischemia. 

**Aa**, Comparison of TUNEL-positive cells in the window area between an animal with spreading depolarizations after topical administration of high [K+]ACSF and [Hb]ACSF (group 2; Aa) and another animal treated with nimodipine intravenously (group 3; Ab). TUNEL-positive cell nuclei are visualized by the brown 3,3′-diaminobenzidine (DAB) stain. 

**Ab**, Quantification of TUNEL-positive cells in the window area reveals a significantly increased density of TUNEL-positive cells in brains derived from group 2 compared with group 3 (*Kruskal–Wallis 1-way ANOVA on Ranks with Dunn post hoc test; P<0.05). Scale bar=20 µm. Hb indicates hemoglobin.
Subsequent histological analysis revealed selective neuronal necrosis restricted to this area. In the remaining 50% of animals, the same ET-1 concentration induced a low-flow area, but neither SD nor focal necrosis (=nonresponders). However, when an SD was triggered chemically in nonresponders and propagated into ET-1–exposed cortex, those animals also developed focal necrosis. Surprisingly, local duration of this SD, as evaluated by the large negative DC shift, was only little longer in the ET-1–exposed cortex compared with the nonischemic cortex. However, the large negative DC shift was superimposed on a shallow ultraslow potential component with opposing polarity between the 2 regions, being positive in the nonischemic and negative in the ET-1–exposed cortex. It was suggested that the negative ultraslow component was related to cellular injury.20,26,40 In the present study, high [K+]ACSF alone produced recurrent SDs superimposed on a similar ultraslow potential component. Interestingly, the ultraslow potential was negative at the surface but positive deep in the cortex. Cortical damage was limited to the superficial cortical layers. This supports the idea that the polarity of the ultraslow potential may be related to the damage. The generators of the ultraslow negativity are unknown but probably of nonneuronal origin, which might include both astrocytic and noncellular mechanisms as reviewed recently.41 If the negative ultraslow potential is of noncellular origin, it should be a diffusion potential, that is, attributable to ion gradients in the extracellular space caused by spillover of the ion content of cells on membrane lysis.

Necrosis of all cortical layers only developed in group 2 (high [K+]ACSF/HbACSF), in which a severe perfusion deficit was coupled to the depolarizing event. In contrast, the cortical damage was limited to the surface when intravenous nimodipine caused the severe perfusion deficit to revert to hyperemic flow responses in group 3. Topical application of [K+]ACSF (3 mmol/L)/HbACSF (2 mmol/L) to the brain was previously studied, and neither inverted the neurovascular response to SD15 nor resulted in significant neuronal injury.42 These observations support the concept that decreased oxidative substrate supply shifts the commitment point to an earlier period. What could explain this?

1. During SD, electrochemical failure is only near-complete. Preserved function of the Na,K-ATPase seems to prevent the complete electrochemical failure. When energy deprivation causes failure of the Na,K-ATPase, complete electrochemical failure might ensue, resulting in neuronal death.

2. Absence of perfusion could halt subcellular energy-dependent protective processes downstream of the electrochemical failure.

3. Although astrocytes themselves are more resistant to energy deprivation than neurons because of their higher anaerobic capabilities, energy deprivation may cause astrocytes to lose important protective effects on neurons, and this may render neurons more vulnerable.40 This is further discussed in the Discussion in the online-only Data Supplement.

**Clinical Implications**

In patients with subarachnoid hemorrhage, prolonged SDs may be attributable to the rise of potassium to up to 50 mmol/L in the subarachnoid clot, endogenous ouabain-like factors, glutamate release, hypoglycemia, hypoxia, hyperperfusion, or other factors yet to be determined that interfere with excitability, extracellular clearance of metabolites, or Na,K-ATPase function.42 Moreover, SDs could be modified by drugs, such as sedatives, anticonvulsants, or the L-type calcium antagonist nimodipine, which inhibits calcium influx into neurons.43 Such factors may not only influence the duration of SD,23,31,41 but also modify subcellular mechanisms, and may thus shift the commitment point. The effect of attendant circumstances on the commitment point implies that a multimodal recording in patients, including measurements of DC electrocorticography, CBF, tissue oxygen, and glucose, is probably superior to DC electrocorticography alone to determine the tissue outcome.27,46

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**Disclosures**

None.

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SUPPLEMENTAL MATERIAL.

Supplemental Methods:

Animals

All animal experiments were performed in compliance with the Governmental Animal Care and Use Committee (Landesamt für Arbeitsschutz, Gesundheitsschutz und technische Sicherheit Berlin (LAGetSi)). The animals were housed in groups (2-4 animals per cage) under a 12h light/dark cycle with food and tap water available ad libitum. Twenty-two male Wistar rats (250 to 400g; Charles River Laboratories, Wilmington, MA, USA) were anesthetized with halothane (1.5% in 30% O₂ and 70% N₂O) and breathed spontaneously. Body temperature was maintained at 38.0±0.5°C. The tail artery was cannulated and continuously infused with saline solution. Systemic arterial pressure (RFT Biomonitor, Zwönitz, Germany), endexpiratory pCO₂ (Heyer CO₂ Monitor EGM I; Bad Ems, Germany) and arterial blood gases were monitored with a Compact 1 Blood Gas Analyzer (AVL Medizintechnik GmbH, Bad Homburg, Germany).

An open cranial window of 4x7mm was implanted over the frontoparietal cortex using a saline-cooled drill as reported previously14,28. The dura mater was removed, and ACSF continuously applied brain topically. The physiological composition of the ACSF in mM was: Na⁺ 152, K⁺ 3, Ca²⁺ 1.5, Mg²⁺ 1.25, HCO₃⁻ 24.5, Cl⁻ 136, glucose 3.7, and urea 6.7. The ACSF was equilibrated with a gas mixture containing 6.6% O₂, 5.9% CO₂, and 87.5% N₂.

Data Collection and Statistical Analysis

CBF was monitored by laser-Doppler flowmetry (Perimed AB, Järfälla, Sweden). The surface DC-electrocorticogram (ECoG) (bandpass: 0–45Hz) was measured with a subdural Ag-AgCl electrode. In 4 animals of group 1, changes of the extracellular K⁺ concentration ([K⁺]₀) and intracortical DC-ECoG were recorded with two K⁺-selective microelectrodes in a cortical depth of 400µm. K⁺-selective microelectrodes were prepared and tested as described previously from double-barrelled thetaglass capillaries (Kugelstätter, Garching, Germany)²². Potassium
ionophore I-cocktail A (Fluka/Sigma-Aldrich, Steinheim, Germany) was used as ion exchanger. Electrodes were connected to a custom-made differential amplifier. Analog-to-digital conversion was performed using a Power 1401 (Cambridge Electronic Design Limited, Cambridge, UK). Data were recorded continuously by using a personal computer and a chart recorder (DASH IV, Astro-Med, Inc., West Warwick, RI).

After the experiments, the wounds were treated with lidocaine HCl gel (2%) and sutured. Five rats per experimental group (groups 1-3) and three controls were allowed to awaken after disconnection of the cranial window from the syringe pump. The opioid agent, buprenorphine (0.5mg/kg body weight), was administered subcutaneously as postoperative analgesic. Animals recovered quickly. They drank and groomed on the first postoperative day. Rat brains were fixed by transcardial perfusion of the animals with modified Lillie fixative (absolute alcohol (70%), 37% formalin (20%), glacial acetic acid (10%)) at 48 hours after the experiment. Before perfusion fixation, rats were examined neurologically in a blinded fashion and graded according to the scale of Menzies and colleagues1.

Data were analyzed by comparing absolute changes of $[K^+]_o$ and intracortical and subdural DC potentials as well as relative changes of CBF calculated in relation to baseline (100%) at measurement onset. Data in text and figures are given as mean value ± standard deviation. Statistical tests are mentioned in the results section. A $P$-value of <0.05 was considered statistically significant.

**Hemoglobin preparation**

Hemoglobin was freshly prepared from heparinized arterial rat blood. Blood was centrifuged (2500G, 5min, 4°C) and the plasma discarded. Cells were washed five times with three to four volumes of cold 0.9%NaCl, and the buffy coat was removed. The cells were lysed by sonication. The suspension of lysed cells was subjected to centrifugation (15,000G, 10min, 4°C) and the pellet removed. The hemoglobin-containing supernatant was transferred by gel
chromatography to the ACSF (Bio-Gel P-6, BioRad, Hercules, CA). Both Hb concentration and composition were measured using a radiometer (total [Hb]_{ACSF}: 2.0 \pm 0.4\text{mM}; \text{oxy-Hb}_{ACSF}: 95.2\%) (radiometer, ABL System 625; Radiometer A/S, Copenhagen, Denmark). The hemoglobin concentration in our experiments was five times higher than that measured in human cerebral hematomas\(^2\). The necessity for relatively high hemoglobin concentrations was possibly related to the following factors: (i) small mammals exhibit higher ischemic thresholds and better collateralization, compared with human subjects - species influences are supported by the observation that the typical syndrome of delayed cerebral ischemia is not observed in subarachnoid hemorrhage models in small animals\(^3\); (ii) the time of incubation with hemoglobin was shorter in our experiments, compared with that after subarachnoid hemorrhage; (iii) the site of hemoglobin application spared the base of the brain, so that spasm of basal arteries would not contribute to the energy compromise. Moreover, the decreased NO availability after subarachnoid hemorrhage may not only be caused by the NO scavenger hemoglobin but also by the release of endogenous NO-synthase inhibitors, uncoupling of endothelial NO-synthase and the destruction of nitrergic perivascular nerves\(^4,5\).

**Histology, Immunohistochemistry and TUNEL Assay**

After perfusion each brain was refrigerated in situ in the same fixative overnight. Thereafter, the brain was removed from the skull. Dissected brains were treated with 96% alcohol overnight before being paraffin-embedded. Twelve-micrometer sagittal sections were mounted onto slides coated with albumin and stained using hematoxylin and eosin. Five to 10 sections were obtained every 200\(\mu\)m. Neighboring sections were stained using histochemical and immunohistochemical methods so that direct comparisons were possible. As reported previously\(^14,28\), sections for immunohistochemistry were deparaffinized and rehydrated. Antigen retrieval was performed by boiling sections in Tris-EDTA buffer (pH 9.0) for 30min. After blocking in Tris-buffered saline containing 20% normal donkey serum and 0.3% Triton X-100,
primary antibodies against GFAP (1:500, mouse-anti-rat; Sigma, Seelze, Germany) and Iba1 (1:200, rabbit-anti-rat; Abcam, Cambridge, UK) were added overnight at 4°C. Alexa594-conjugated donkey-anti-mouse and Alexa488-conjugated donkey-anti-rabbit antibodies (Invitrogen, Eugene, Oregon, USA) were added at room temperature for 4h to visualize the stainings, or an appropriate 3,3’-diaminobenzidine (DAB) staining kit was used (Vector Laboratories, Burlingame, California, USA). Omission of primary antibodies served as negative controls. A laser confocal scanning microscope (Leica, Solms, Germany) was used for image acquisition. The TUNEL assay was performed using the ApoTag Kit (Intergen, Oxford, UK) according to the manufacturer’s protocol. Omission of the terminal deoxynucleotidyl transferase reaction served as negative control. All brain sections were carefully evaluated by a neuropathologist (I.V.).

**Morphometrical analysis**

The densities of Iba1-, GFAP- or TUNEL-positive cells in the window areas were determined for animals of groups 1-3 as well as controls by a blinded investigator (M.K.) using a stereological approach with the optical fractionator probe. Typically, more than 50 frames of 100 × 100µm in the cortical area under the cranial window were analyzed in 1-2 brain sections per animal.

**Supplemental Discussion:**

**The role of astrocytes for neuronal survival during spreading depolarization**

Notably, during spreading depolarization under normoxic and normoglycemic conditions, astrocytes in contrast to neurons are functional. Their depolarization is produced passively by the decline in the potassium transmembrane gradient following the release of potassium from neurons. The astrocytic depolarization leads to a flux of negatively charged chloride ions into the astrocytes. In turn, the chloride influx attracts potassium which follows chloride. In addition,
potassium enters astrocytes in exchange with sodium by activation of the Na,K-ATPase causing a decline in intra-astrocytic sodium. Hence, during spreading depolarization under normoxic and normoglycemic conditions, astrocytes buffer the changes produced by the neurons. But, this compensatory action of astrocytes is hindered under ischemic conditions when the astrocytic Na,K-ATPases lack ATP. Under such conditions, the intra-astrocytic sodium concentration appears to increase markedly, as found in primary astrocyte cultures under simulated ischemia, and potassium appears to be spilled out instead of taken up. Consistent with these notions, astrocytes do not show significant volume changes in normoxic and normoglycemic tissue during spreading depolarization but they swell substantially under ischemia. The loss of astrocytic function under ischemia may be detrimental for neurons. Even the failure of astrocytic function alone is in fact sufficient to cause neuronal death despite normoxic and normoglycemic conditions. This was previously found when the aerobic metabolism in astrocytes was selectively blocked by aconitase inhibitors. These compounds induced a cluster of spreading depolarizations with deleterious outcome after a few hours.
**Supplemental Table S1** Physiological variables

<table>
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<tr>
<th>Group</th>
<th>[K⁺]ACSF (250mM) topically</th>
<th>Mean arterial pressure (mmHg)</th>
<th>Arterial pO₂ (mmHg)</th>
<th>Arterial pCO₂ (mmHg)</th>
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<td>80.0 ± 7.3</td>
<td>119.9 ± 12.1</td>
<td>42.6 ± 2.2</td>
<td>7.40 ± 0.02</td>
</tr>
<tr>
<td>2</td>
<td>[K⁺]ACSF (250mM) / HbACSF (2mM) topically</td>
<td>77.7 ± 7.2</td>
<td>117.5 ± 25.8</td>
<td>47.5 ± 3.5</td>
<td>7.36 ± 0.07</td>
</tr>
<tr>
<td>3</td>
<td>[K⁺]ACSF (250mM) / HbACSF (2mM) / nimodipine (2µg/kg/min) i.v.</td>
<td>74.1 ± 6.9</td>
<td>139.4 ± 19.6</td>
<td>51.0 ± 3.4*</td>
<td>7.31 ± 0.04*</td>
</tr>
</tbody>
</table>

*P<0.05 versus group 1*
Supplemental References:


