SUPPLEMENTARY METHODS

Global forebrain ischemia/anoxia in anesthetized rats

Surgical procedures

Standard rodent chow and tap water were supplied ad libitum. The animals were housed under constant temperature, humidity, and lighting conditions (23 °C, 12:12 h light/dark cycle, lights on at 7 a.m.). Animals were anesthetized with 1.5-2 % isoflurane in N₂O:O₂ (70 % : 30 %) and allowed to breathe spontaneously through a head mask throughout the experiment. Body (core) temperature was kept at 37 °C with a feedback-controlled heating pad. Atropine (0.1 %, 0.1 ml) was administered intramuscularly shortly before surgical procedures to avoid the production of airway mucus.

A midline incision was made on the neck and both common carotid arteries were gently separated from the surrounding tissue and the vagal nerves. Lidocaine (1%) was applied topically before opening the tissue layers during preparation. A silicone coated fishing line used as occluder was looped around the common carotid arteries for the later induction of cerebral ischemia.

For electrophysiological data acquisition and imaging, a large craniotomy was prepared in the right parietal bone using a dental drill (ProLab Basic, Bien Air 810, Switzerland). The dura in the craniotomy was carefully removed, and the exposed brain surface was regularly rinsed with aCSF (mM concentrations: 126.6 NaCl, 3 KCl, 1.5 CaCl₂, 1.2 MgCl₂, 24.5 NaHCO₃, 6.7 urea, 3.7 glucose bubbled with 95 % O₂ and 5 % CO₂ to achieve a constant pH of 7.4).

Experimental protocols

In the electrophysiology series of experiments, incomplete global forebrain ischemia was induced by the permanent occlusion of both common carotid arteries (“2-vessel occlusion”, 2VO), which readily gave rise to a single spontaneous SD. In one rat, no SD emerged in response to 2VO, therefore that particular experiment was not taken for further analysis.

Electrophysiology

Two saline-filled (120 mM NaCl) glass capillary microelectrodes (20 µm outer tip diameter) were inserted 700 µm deep into the right somatosensory cortex. An Ag/AgCl electrode placed under the skin of the animal's neck was used as common ground. The microelectrode was connected to custom-made dual-channel electrometer (including AD549LH, Analog Devices, Norwood, MA, USA) via Ag/AgCl leads. The recorded LFP signal was further amplified and filtered by dedicated differential amplifiers and associated filter modules (NL106 and NL125, NeuroLog System, Digitimer Ltd., United Kingdom). The recorded analogue signal was converted and displayed live using a dedicated analog-to-digital converter (MP 150, Biopac Systems, Inc.) at a sampling frequency of 1 kHz. Local CBF changes were monitored with two laser-Doppler needle probes (Probe 403 connected to PeriFlux 5000; Perimed AB, Sweden) positioned next to the penetration site of the LFP microelectrodes. The LDF signal was digitalized and acquired, together with the DC potential, essentially as described above.

Construction of glutamate biosensors

Biosensors were constructed of Pt/Ir wires (Goodfellow, Huntington, UK), inserted into a pulled glass capillary (Harvard Apparatus, Edenbridge, UK). A poly-m-phenylenediamine (PPD) screening
layer was deposited to the Pt/Ir surface by electropolymerization (20 min) with 700 mV constant potential. An encapsulated bio-layer containing glutamate oxidase was then deposited and fixed on the Pt/Ir surface.

**Intrinsic optical signal (IOS) imaging in anesthetized rats**

For live imaging, the cortex was illuminated in stroboscopic mode (100 ms/s) with a high-power light emitting diode (LED) (530 nm peak wavelength; SLS-0304-A, Mightex Systems, Pleasanton, CA, USA) equipped with a bandpass filter (3RD 540–570 nm, Omega Optical Inc. Brattleboro, VT, USA). Green IOS was captured at 100 ms exposure each second by a CCD camera. Background images were captured at 1 frame per second frequency, to be used for the correction of raw images. A dedicated program written in LabVIEW environment synchronized the illumination and camera exposures.

**Histology**

For fluorescent immunocytochemical labeling, antigens were retrieved with citrate buffer for 10 min at 95°C. The slices were permeabilized with 0.2 % TritonX-100 in PBS for 30 min, then blocked with 3 % BSA in PBS for 1 h at room temperature and incubated with primary antibodies (mouse anti-GFAP, Sigma, 1:500; mouse anti-NeuN, Millipore, 1:500; rabbit anti-cleaved caspase-3, Cell Signaling, 1:500) overnight in 3 % BSA in PBS at 4°C. The slices were incubated with secondary antibodies (goat anti-mouse Alexa-488, Jackson Laboratories, 1:500; goat anti-rabbit Cy3, Jackson Laboratories, 1:500; goat anti-mouse Star Red, Abberior, 1:500 for STED) in 1 % BSA in PBS for 1 h at room temperature. Finally, cell nuclei were labelled with Hoechst staining (1:1000) for 10 min. The slices were mounted with Fluormount-G and stored at 4°C. Photomicrographs were taken (3 per animal per each selected brain region of the hemisphere ipsilateral to the craniotomy) with a Nikon Eclipse TE2000U microscope at 20x magnification. Regions of interest, expected to be most vulnerable to ischemia/anoxia were: superficial and deeper layers of cortex, hippocampal CA1 and striatum. Representative confocal images were taken with Leica SP5 inverted laser scanning confocal microscope at 20x magnification (Z-stacks with steps of 0.4 μm). STED images were acquired with a Stedycon STED instrument (Abberior Instruments, Göttingen, Germany) attached to a Zeiss Axio Observer Z1 inverted microscope. Confocal and STED images were pieced together and filtered in ImageJ. The distinctive tissue autofluorescence of brain samples was corrected by subtracting images captured in the non-labelled far-red spectrum from the respective confocal photomicrographs. NeuN and GFAP immunopositive cells, alone and co-localized with cleaved caspase-3 were counted manually with Cell Counter plugin of the Image J software by two independent observers.

**Hypo-osmotic cerebral edema model in ex vivo rat brain slice preparations**

**Brain slice preparation, incubation media and experimental protocols**

Briefly, coronal brain slices (350 μm) anterior to bregma were cut with a vibrating blade microtome (Leica VT1000S) and collected in ice-cold aCSF (composition of aCSF in mM concentrations: 130 NaCl, 3.5 KCl, 1 NaH2PO4, 24 NaHCO3, 1 CaCl2, 3 MgSO4 and 10 D-glucose). Six to eight slices were transferred to an incubation chamber filled with carbogenated aCSF (difference in composition in mM concentrations: 3 CaCl2 and 1.5 MgSO4) and kept at room temperature (~20°C). Selected slices were placed into an interface type recording tissue chamber (Brain Slice Chamber BSC1, Scientific Systems Design Inc., Ontario, Canada) continuously perfused with carbogenated aCSF at a rate of 2 ml/min and
kept at 32°C using a dedicated proportional temperature controller unit (PTC03, Scientific Systems Design Inc., Ontario, Canada).

Preparation of fluorocitrate solution

Fluorocitrate (0.5 mM) was prepared from its barium salt (DL-Fluorocitric acid barium salt, Sigma F9634). Barium was first removed in a solution of 6 ml 0.1 M HCl, 18 drops of Na₂SO₄, and 12 ml Na₂HPO₄. pH was adjusted to 7.3-7.4. The resultant solution was aliquoted in Eppendorf tubes and centrifuged at 1000 g for 5 min. Then the supernatant was removed, and 7.8 ml fluorocitrate was added to 50 ml aCSF. The solution was prepared on the day of the experiment and was stored at 2-8°C.

Slice electrophysiology

Microelectrodes were connected to a custom-made dual-channel electrometer (including AD549LH, Analog Devices, Norwood, MA, USA) and dedicated differential amplifiers and associated filter modules (NL106 and NL125, NeuroLog System, Digitimer Ltd., United Kingdom). The recorded analogue signal was converted and displayed live using an Acknowledge environment (MP 150, Biopac Systems, Inc.) at a sampling frequency of 1 kHz (65). The DC potential traces confirmed the occurrence of depolarization events. Further, the DC potential recordings were used off-line to characterize the spreading or simultaneous nature of depolarization events, and to determine event latency and duration at half amplitude. Slices, in which spontaneous SD1 did not occur, were not taken for quantitative analysis.

Evoked field potentials (EFPs) were recorded as described in Materials and Methods section in the main text. LFP was filtered between 1-200 Hz, and the recorded analogue signal was converted and displayed live using an Acknowledge environment. Recordings were used off-line to determine the amplitude of the evoked potentials.

Measurement of extra-synaptic glutamate concentration in vitro

Glutamate biosensors were used and calibrated as described in Materials and Methods section in the main text. Biosensors were implanted adjacent to the LFP microelectrodes together with glutamate null sensors. The magnitude of changes in extra-synaptic glutamate concentrations with depolarization events were expressed as area under the curve (AUC; µM × s).

Brain slice histology

The brain slices (n=12) were incubated in a 2 % solution of TTC in 0.1 M PBS for 20 minutes at room temperature. The sections were subsequently immersed and stored in 4 % PFA for 24 h. The stained sections were then mounted on microscope slides, coverslipped with glycerol, and the number of particles per 1000 µm² was calculated after calibration and manual thresholding by using the automated inbuilt function “analyze particles” in FIJI.

To examine the morphology and swelling of astrocytes, a modified Golgi-Cox staining was used (27). Some brain slices were stained with FD Rapid GolgiStainTM Kit (FD Neurotechnologies, Inc., USA). The slices were postfixed in 4 % PFA containing 8 % glutaraldehyde for 24 h at room temperature. The sections were incubated in Golgi impregnation solution (equal volumes of solution A and solution B). Following impregnation, the brain slices were transferred into solution C and incubated at 4 °C for 24 h in dark. After the incubation in solution C, stained slices were dehydrated in solutions of increasing ethanol concentration (50 %, 70 % and 95 % and absolute ethanol), then mounted on
microscope slides in 0.2 % polyvinyl-alcohol, and coverslipped with glycerol. Representative images were taken from the cortical layer 2-3 with Nikon-DS Fi3 camera attached to a Leica DM 2000 Led light microscope (Leica Microsystems GmbH, Germany). Images of Golgi-Cox-stained astrocytes were processed after auto leveling and background correction in FIJI. Cellular swelling was measured after binary conversion and manual thresholding using the embedded Otsu filter in FIJI. Astrocyte soma areas were outlined, then the outlined area was measured automatically using the “analyze particles” function in FIJI. Finally, the swelling was expressed as area change relative to the mean area in the ACSF control group.

The samples for electron microscopy were postfixed in 3 % glutaraldehyde and 2.25 % dextran in 0.1 M phosphate buffer (pH=7.4) for a week. Semi-thin sections were cut plane on an ultramicrotome (Ultracut E, Reichert-Jung) and stained on object glasses with toluidine blue. Ultrathin sections were cut from the same blocks and collected on copper slot grids. The preparations were contrasted with 5 % uranyl acetate and Reynolds lead citrate solution. The samples were analyzed with a Jeol JEM-1400 Plus transmission electron microscope, and photographs were taken with a charge-coupled device camera.

Data analysis

LFP and extracellular glutamate concentration for both in vivo and in vitro electrophysiology were acquired, displayed live, and stored using a personal computer equipped with dedicated software (AcqKnowledge 4.2 for MP 150, Biopac Systems, Inc., USA for LFP; and eDAQ Chart, eDAQ Pty Ltd., Colorado Springs, CO, USA for glutamate concentration). Other physiological variables (CBF, MABP) of anesthetized rodents were recorded simultaneously. Data analysis was conducted offline and was assisted by the inbuilt tools of AcqKnowledge 4.2 software. Local CBF changes along the experimental protocol were calculated based on 100 % baseline taken shortly before ischemia induction, and the residual LDF signal after anesthetic overdose, considered as biological zero. CBF changes were expressed relative to baseline (%). For IOS imaging (anesthetized rats and ex vivo brain slice preparations), depolarization events were visualized after background subtraction, contrast enhancement and smoothing (3 point moving average) performed in Fiji. Traces of IOS intensity changes were extracted from raw image sequences at 4 ROIs positioned in the images offline, along the propagation of the first SD. Optical signal intensity changes were expressed as ΔF/F or ΔI/I, respectively.

The velocity of SD propagation in the LFP experiments was calculated by taking the distance between the two LFP electrodes (typically 4-5 mm in vivo and 1000-1500 μm in vitro), and the time between the occurrence of the signature of an SD at the two electrodes. In the imaging experiments, the calculation of the velocity was aided by the visual signal and was more accurate due to the perceptible direction of SD propagation.

The IOS image sequences, several spatial features of SD/SiD events were measured after background subtraction and inbuilt automatic or manual thresholding in Fiji and expressed relative to the full surface area of the cortex in the brain slice. First, the focal area of each event was estimated. Next, the area of sustained IOS intensity elevation following SD1 was expressed. Finally, the maximal cortical area covered by SD1 propagation or SiD were measured.

Brain slice swelling was shown in representative, contrasted IOS images as the 2D displacement of the contour of the parieto-temporal cortex. Slice swelling over the experimental protocol was quantitatively evaluated by the change in the surface area of the full slice relative to baseline, at a
temporal resolution of 1/100 s, and depicted in temporal correspondence with IOS variations at two ROIs. Slice area measurements were performed after contrast enhancement in Fiji.

The excitability of the nervous tissue was characterized with the amplitude of evoked field potentials, and the electric threshold of SD elicitation. For the latter, the charge delivered was calculated as $Q[\mu C]=I[\text{mA}] \times t[\text{ms}]$, and it was raised stepwise with an interstimulus interval of 2 min until SD was observed. The threshold of SD elicitation was illustrated on a logarithmic scale.

Multiphoton image stacks were processed offline. Image stacks were auto leveled, background subtracted and converted to RGB color in FIJI. Images of individual astrocytes were cropped, and cellular movement artefacts were corrected using the “Template Matching” plugin in FIJI. The SD associated intracellular calcium waves were measured on green fluorescent images ($\Delta F/F$) by placing a 15x15 µm ROI on the soma of selected astrocytes. Astrocyte swelling was determined on red fluorescent image sequences by the morphometric analysis of somatic area changes in the lateral dimensions. After binary conversion, images were manually thresholded, astrocyte somatic areas were outlined, the outlined area was measured automatically using the inbuilt “analyze particles” function of FIJI and expressed as % area of baseline.

**SUPPLEMENTARY RESULTS**

**Supplementary Video legends:**

**Supplementary Video 1.** A green intrinsic optical signal image sequence of the parietal cortical surface of an anesthetized rat. The image sequence was generated by background subtraction. Note the simultaneous depolarization (brightening of the signal) of a considerable bulk of tissue, arising instantaneously in the frontal part of the cranial window in response to anoxia.

**Supplementary Video 2.** A green intrinsic optical signal image sequence of the parietal cortical surface of an anesthetized rat. The image sequence was generated by background subtraction. Note the spreading depolarization (brightening of the signal) propagating over the cortical surface from a punctual focus, triggered by KCl.

**Supplementary Video 3.** A green intrinsic optical signal image sequence of the parietal cortical surface of an anesthetized rat. Note the macroscopic movement of the tissue, corresponding to cerebral edema, upon oxygen withdrawal. Simultaneous depolarization occurs at around 300 s.

**Supplementary Table 1.** Physiological variables.

|                  | Baseline | Ischemia | Anoxia    |
|------------------|----------|----------|-----------|
| **MABP (mmHg)**  | 90±24    | 93±28    | 72±26     |
| **pH**           | 7.34±0.03| 7.31±0.05| 7.4±0.06*## |
| **pO$_2$ (mmHg)**| 107.7±23.1| 117.5±31.8| 34.4±10.5***## |
| **pCO$_2$ (mmHg)**| 37.7±4.0 | 41.3±4.05| 35.4±13.0 |
| **Lactate (mM/l)**| 0.47±0.14| 0.43±0.07| 0.85±0.72** |

Data are given as mean±stdev. A one-way analysis of variance (ANOVA) was used for statistical evaluation of the data, followed by a Sidak post hoc test ($p<0.05^*$ and $p<0.01^{**}$ vs. baseline, $p<0.05^*$ and $p<0.01^{**}$ vs. ischemia). Abbreviations: MABP, mean arterial blood pressure.
**Supplementary Figures:**

**Supplementary Figure 1.** The occurrence of SD and SiD in the cerebral cortex of anesthetized rats. A representative recording of a spontaneous SD in response to ischemia onset (SD1), and a subsequent SiD in response to anoxia. Abbreviations: CBF, cerebral blood flow; MABP, mean arterial blood pressure.

**Supplementary Figure 2.** Direct current potential traces obtained with two microelectrodes (Ch 1 and Ch 2, about 1.3 mm apart) from representative ex vivo brain slice preparations exposed to hypo-osmotic medium (HM). **A,** Both the first spreading depolarization (SD1), and the subsequent simultaneous depolarization (SiD) occurred spontaneously, cancelling the need for the anoxic initiation of SiD. Note that the evolution of spontaneous SiD was similar to that of SiD triggered with transient anoxia in other slices. **B,** The repolarization phase of the first spreading depolarization (SD1) was incomplete, and small amplitude, irregular field oscillations evolved in synchrony on the two channels. This pattern of the DC signal was typical in case SiD ensued.
Supplementary Figure 3. Intrinsic optical signal (IOS) signal imaging in ex vivo brain slice preparations. A, The correspondence between the intrinsic optical signal (IOS) intensity changes and the direct current potential recordings in a representative ex vivo brain slice preparation exposed to hypo-osmotic medium (HM). The evolution of a spreading depolarization event (SD1) that occurred spontaneously in HM is shown. The two regions of interest (ROI1-2) to be used for the extraction of the optical signal intensity changes were positioned adjacent to the two local field potential glass capillary microelectrodes (LFP1-2). The corresponding traces are given in the lower righthand corner. The temporal resolution of the image sequences is given in the upper right corner in seconds; time is displayed with respect to SD1 onset. B, Representative images of a recurrent spreading depolarization (rSD) triggered with transient anoxia in an ex vivo brain slice preparation exposed to hypo-osmotic medium (HM). Note that the event propagated from three foci, attesting a multi-focal origin. Intrinsic optical signal intensity changes at three regions of interest (ROI1-3) placed between the foci demonstrated the propagation of the rSD. Images were obtained by template matching (movement artefact correction), background subtraction, smoothing and contrast enhancement. The temporal resolution of the image sequences is given in the lower right corner in seconds.
**Supplementary Figure 4.** The degree of slice swelling relative to baseline at selected time points. Data are given as mean±stdev, the number of events analyzed is shown in the legend. Statistical evaluation relied on a repeated measures (p<0.05*p<0.01**), followed by a Sidak post hoc test (p<0.05*& p<0.01** vs. aCSF).

**Supplementary Figure 5.** The antagonisms of ionotropic glutamate receptors (MK-801 and CNQX) does not block tissue swelling, expressed relative to baseline at selected time points. Data are given as mean±stdev; the number of events analyzed is shown in the legend.

**Supplementary Figure 6.** The duration of depolarization events taken at half amplitude of the negative DC potential shift. Both the first spreading depolarization (SD1) and recurrent SD (rSD)/simultaneous depolarization (SiD) lasted significantly longer in hypo-osmotic medium (HM) than in artificial cerebrospinal fluid (aCSF). Data are given as mean±stdev, individual values are shown in a dot plot. After the application of a Shapiro-Wilk normality test (p<0.05*), a Kruskal-Wallis non-parametric test was used (p<0.01**, followed by a Dunn’s post hoc test (p<0.05* vs. SD1 in aCSF; p<0.05° vs. rSD in aCSF).
Supplementary Figure 7. Terminal SiD and terminal SD in the human brain. Both patients were enrolled at Charité – Universitätsmedizin Berlin in a research protocol of invasive neuromonitoring approved by the local ethics committee, as published previously.44 A-C Terminal depolarization with very short depolarization arrival differences between different electrodes during dying in the wake of circulatory arrest. Decompressive hemicraniectomy was performed on day 1 because of malignant hemispheric stroke. A subdural electrode strip was implanted at the border zone between infarct and viable tissue. On day 4, neuroimaging showed several newly developed infarcts in other vascular territories and a Do Not Resuscitate-Comfort Care (DNRCC) order was activated followed by terminal extubation on day 5. A, Recordings of the direct current (DC)/alternate current (AC) electrocorticogram (ECoG) (frequency band: 0 - 45 Hz, upper 6 traces), the power of the AC band from 0.5 – 45 Hz at the 6 different electrodes (traces 7 – 12), brain tissue partial pressure of oxygen (ptiO2, intracortical Licox, Integra, Plainsboro, USA, trace 13), regional CBF (opto-electrode strip for laser-Doppler flowmetry, Perimed, Järfälla, Sweden, trace 14), and mean arterial pressure (radial artery catheter, trace 15). The changes during the final 180 seconds of the patient’s life are displayed. Parallel decreases of mean arterial pressure, rCBF and ptiO2 first result in non-spreading depression of spontaneous activity (traces 7-12). The terminal depolarization follows the complete non-spreading depression after 13 seconds. Note the almost simultaneous start of terminal depolarization at the 6 different electrodes in traces 1-6 suggesting SiD. Note, however, that in the exceptional condition that an SD propagates vertical to the electrode strip, “simultaneous depolarization” would also be perceived. B, Computed tomography (CT) reveals the infarct in the right middle cerebral artery territory. An electrode of the subdural electrode strip above the posterior part of the infarct is visible in this sectional plane (arrow). This is located almost exactly on the boundary between necrotic and living tissue. Typical streak artefacts are seen around the electrode (arrow). C, T2-weighted fluid-attenuated inversion recovery (FLAIR) magnetic resonance imaging (MRI) shows the infarct with higher accuracy. Electrodes are invisible in MRI scans. D-F Terminal SD during dying in the wake of circulatory arrest in a patient with subarachnoid and intracerebral hemorrhage. On day 2, a DNRCC order was activated and the patient was terminally extubated. D, Traces are similar to A, but traces 7-12 display simple spontaneous activity (0.5 – 45 Hz). The neuromonitored changes during the final 15 minutes of the patient’s life are displayed. Non-spreading depression of activity occurs during the falling phase of ptiO2. A very slow, homogeneous DC positivity started simultaneously with the fall in ptiO2. This is assumed to result from interferences of ptiO2 and pH with the electrode material.67 Superimposed on the DC positivity, terminal SD then started at electrode 3 and spread to the other electrodes. E, CT shows the condition after surgical removal of the large intracerebral hemorrhage. An electrode of the subdural electrode strip above living tissue of the frontal lobe is visible in this sectional plane (arrow). F, FLAIR image showing lesion and living tissue with higher accuracy.