Published in final edited form as:

Neurobiol Dis. 2012 December ; 48(3): 495–506. doi:10.1016/j.nbd.2012.06.024.

Peri-infarct Blood-Brain Barrier Dysfunction Facilitates Induction of Spreading Depolarization Associated with Epileptiform Discharges

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Abstract

Recent studies showed that spreading depolarizations (SDs) occur abundantly in patients following ischemic stroke and experimental evidence suggests that SDs recruit tissue at risk into necrosis. We hypothesized that BBB opening with consequent alterations of the extracellular electrolyte composition and extravasation of albumin facilitates generation of SDs since albumin mediates an astrocyte transcriptional response with consequent disturbance of potassium and glutamate homeostasis. Here we show extravasation of Evans blue-albumin complex into the hippocampus following cortical photothrombotic stroke in the neighbouring neocortex. Using extracellular field potential recordings and exposure to serum electrolytes we observed spontaneous SDs in 80 % of hippocampal slices obtained from rats 24 h after cortical photothrombosis. Hippocampal exposure to albumin for 24 h through intraventricular application together with serum electrolytes lowered the threshold for the induction of SDs in most slices irrespective of the pathway of stimulation. Exposing acute slices from naive animals to albumin led also to a reduced SD threshold. In albumin-exposed slices the onset of SDs was usually associated with larger stimulus-induced accumulation of extracellular potassium, and preceded by epileptiform activity, which was also observed during the recovery phase of SDs. Application of ifenprodil (3μM), an NMDA-receptor type 2 B antagonist, blocked stimulus dependent epileptiform discharges and generation of SDs in slices from animals treated with albumin *in-vivo*. We suggest that BBB opening facilitates the induction of peri-infarct SDs through impaired homeostasis of K⁺.

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Keywords

astrocytes; blood-brain barrier; photothrombosis; potassium homeostasis; spreading depolarization, stroke

Introduction

Recent evidence shows that spreading depolarizations (SDs) occur frequently in patients with malignant stroke, subarachnoid or intracranial hemorrhage and traumatic brain injury (Dohmen et al. 2008; Dreier et al. 2009, 2011, 2012; Hartings et al. 2009, 2011; Lauritzen et al. 2011; Nakamura et al. 2010; Oliveira-Ferreira et al. 2010). SDs are characterized by a rapid and strong neuronal and glial depolarization and near-complete breakdown of the ionic gradients between the extracellular and the intracellular compartment due to a net influx of sodium and calcium into the cells, releasing of potassium and cellular edema (Dreier 2011; Kraig and Nicholson 1978; Hansen and Olson 1980; Somjen 2001). SDs propagate as a wave through gray matter at a rate of 2-6 mm/min (Kraig and Nicholson 1978; Somjen 2001) and can be detected as large, slow negative field potential waves representing the summation of depolarization in neurons and glia at the recording site (Canals et al. 2008). Spontaneous neuronal activity is typically suppressed during spreading depolarization, although epileptiform discharges evoked by electrical stimulation have been recorded during the late phase of SDs, where there is usually silencing of activity (Leão 1944; Pomper et al. 2006; Van Harrevald and Stamm 1953). Epileptiform discharges and seizures were recently also described in stroke patients following subarachnoid hemorrhage (Dreier et al. 2012).

In animal models of stroke, the incidence and duration of SDs around the ischemic lesions are correlated with worsening in prognosis and expansion of the infarct volume (Dreier 2011; Hartings et al. 2011; Iijima, Mies and Hossmann 1992; Nakamura et al. 2010). Recent human studies using subdural electrodes confirmed that SDs are recorded in the vicinity of the damaged brain area from the day of the insult up to two weeks later (Dohmen et al. 2008; Hartings et al. 2009; Oliveira-Ferreira et al. 2010). It is generally assumed that SD generation after ischemia is due to diffusion of potassium, glutamate and other toxic agents from the necrotic core tissue into its surround (Dirnagl, Iadecola and Moskowitz 1999; Woitzik, Back and Thome 2008). However, the detailed mechanisms underlying the generation of SDs in the peri-ischemic brain are not yet known. Since dysfunction of the blood-brain barrier (BBB) is a common hallmark of peri-lesional brain regions (Shlosberg et al. 2010), and since it has been recently suggested to promote the generation of seizures (Marchi et al., 2011), we hypothesized that it might also facilitate the generation of SDs. We have chosen the Rose bengal photothrombotic ischemia model, as it is associated with a robust BBB opening surrounding the ischemic core including the hippocampus (Stoll et al., 2009).

In the present study we tested the hypothesis that BBB opening contributes to a reduced threshold for the generation of SDs in a structure remote from the primary cortical lesion, like the hippocampus. We here studied the temporal development of BBB dysfunction in the peri-infarct hippocampus and recorded the incidence of SDs in BBB-affected hippocampus. We further focused on the hypothesis that exposure to serum- albumin is sufficient to lower the threshold for SDs due to disturbed potassium homeostasis.

Material and Methods

Experimental approaches and ethical approval

All experimental procedures were approved by LaGeSo Berlin (T0228/04, G0378/10, G0114/09) and the ethical committee at Ben-Gurion University of the Negev, Beer-Sheva. Male Wistar rats (200–280 g) were used for all experiments. Three experimental approaches were used to model hippocampal BBB dysfunction: (1) photothrombotic stroke model; (2) intraventricular application of serum-albumin; and (3) exposure of acute cortico-hippocampal slices to serum electrolyte solution containing albumin. For brevity we named that solution ASERUM (ASERUM), although it did not contain other serum proteins. We also compared effects of serum electrolyte solution devoid of any proteins (named albumin free serum or in short afSERUM) with that of artificial cerebrospinal fluid (ACSF).

Induction of cortical ischemia by photothrombosis

The well characterized Rose bengal method was used (Stoll et al. 2009; Watson et al. 1985). Anesthesia was induced with intraperitoneal injection of ketamine-xylazine (1.6 and 0.6 mg per Kg body weight, respectively) and a tail intravenous catheter was inserted. Xylocain (1 %) was injected subcutaneously over the skull before opening of the skin. Body temperature was monitored throughout and maintained at 37 ± 0.5 °C using a heating pad. For induction of photothrombosis the calvarium was exposed to a light beam that was vertically centered 1 mm posterior and 1 mm lateral from bregma using a stereotactic frame. Rose bengal was next administered intravenously (i.v. 20 mg per Kg body weight). The halogen light beam (diameter 3.5 mm) was directed for 15 minutes onto the intact skull. Following the procedure, the wound was sutured and treated with xylocain gel (2 %). Buprenorphin (0.05 mg per Kg body weight) was applied subcutaneously immediately after the surgical procedure. Sham operation consisted in the application of either a light beam or Rose bengal (total 9 animals, 4 and 5 animals, respectively).

Evaluation of blood–brain barrier integrity and ischemic region

To estimate BBB integrity after photothrombosis we used the Evans blue method as previously reported (Friedman et al. 1996; Seiffert et al. 2004). Evans blue containing solution (2 % in 154 mM NaCl solution) was injected i.v. (2.5 ml per Kg body weight) at different time points after the induction of photothrombosis; two hours (h) after injection animals were killed and the extravasation of Evans blue was measured using established methods (Seiffert et al., 2004) with slight modifications: Briefly, brains were removed and the hippocampi were dissected and dissolved in 0.1 M phosphate buffer solution (2 µl/mg tissue) containing 1 % sodium dodecyl sulfate (SDS, Carl Roth GmbH, Karlsruhe, Germany), homogenized and centrifuged ($12,000 \times g$; 30 min) with an Eppendorf scientific centrifuge (5417R, Westbury, NY) to precipitate confounding cell debris. Albumin–Evans blue complex concentration was spectrophotometrically measured in the supernatant at 595 nm wavelength using a µQuant spectrophotometer (µQuant, Bio-Tek Instruments Inc., Winooski, VT, USA). Data values (optical density) of the treated areas were normalized to the contra-lateral hippocampus. The borders of the necrotic regions were estimated using the well established TTC staining method (1% triphenyltetrazolium chloride in PBS, phosphate buffer solution in highly purified water, 1:5) for 30min, 37°C, Sigma-Aldrich (Barth & Mody, 2011; Preston & Webster, 2000). Images of brain sections were taken using a digital camera.

In-vivo intraventricular application of albumin

For intraventricular application of albumin, animals were subjected to a ketamine-xylazine anesthesia and placed in a stereotactic frame. Xylocain (1 %) was injected subcutaneously

over the skull before opening of the skin. After exposing the calvarium a burr hole (1.5 mm diameter) was drilled into the bone at 0.9 mm posterior, 1.6 mm lateral to bregma. Bovine serum albumin (0.4 mM, BSA purity 96 %, Sigma-Aldrich, A7906, Steinheim Germany) dissolved in 10 μ l of artificial cerebrospinal fluid (ACSF, 10 μ l) was injected at a depth of 3.5 mm from the surface of the skull **with a speed of about 1 – 2 μ l per minute**. Sham operated animals were treated with intraventricular injection of ACSF. Following the procedure, the wound was sutured and treated with xylocain gel (2 %). Buprenorphin (0.05 mg per Kg body weight) was applied subcutaneously immediately after the surgical procedure. To localize the distribution of albumin in the tissue, fluorescein isothiocyanate conjugated bovine albumin was used in a few experiments (0.4 mM, Sigma-Aldrich, ordering number A9771, Steinheim Germany). ACSF contained (in mM): 129 NaCl, 21 NaHCO₃, 1.25 NaH₂PO₄, 1.8 MgSO₄, 1.6 CaCl₂, 3 KCl, 10 glucose, pH 7.4 at 34–36°C, osmolality 300 $\pm$ 5 mOsmol/l **and was carbogenated with 95% O₂ and 5 % CO₂**.

Slice preparation

Horizontal hippocampal slices were prepared as previously described (Dreier and Heinemann 1991, Wöhrle et al. 2007a, 2007b). In some experiments sagittal slices were used to preserve the lamellar organization of the dorsal hippocampus and to allow for comparison to cortical peri - infarct tissue when required. Before decapitation, animals were anesthetized with isoflurane (1 % dissolved in 70 % N₂O, 30 % O₂) to prevent opening of the blood brain barrier observed with isoflurane alone (Tétrault et al. 2008). Brains were rapidly removed, washed in ice-cold ACSF and 400 μ m hippocampal slices were prepared using a Leica VT1200 vibratome (Wetzlar, Germany). Slices were immediately transferred to a home-made recording chamber, perfused with carbogenated ACSF at 1.5 – 2 ml/min, maintained at 36°C and left for recovery between 60–120 minutes before recordings were commenced.

In-vitro albumin exposure

Brain slices obtained from naive rats were left to recover for 60–120 minutes after the slicing procedure with **carbogenated** ACSF, then incubated for one h in carbogenated **albumin-containing serum electrolyte solution** (ASERUM) that contained (in mM): 0.4 BSA (bovine serum albumin, Sigma-Aldrich, Germany, 96% purity) dissolved in 129 NaCl, 21 NaHCO₃, 1.25 NaH₂PO₄, 0.8 MgSO₄, 1.3 CaCl₂, 5.7 KCl, 10 glucose, 1 glutamine, pH 7.4 at 34–36°C osmolality 305 – 312 mOsmol/l (Seiffert et al., 2004). Recordings were performed in the presence of carbogenated albumin-free serum electrolyte solution (afSERUM), which was prepared with the aforementioned electrolytic concentrations but without BSA, pH 7.4 at 34–36°C, osmolality 295–300 mOsmol/l.

Electrophysiology

In-vitro recordings were performed in stratum pyramidale of area CA1 and occasionally also in area CA3 using an interface chamber and extracellular glass electrodes (filled with 154 mM NaCl, tip diameter 2–3 μ m, resistance 2 to 4 M Ω) as previously reported (Hamon, Stanton, and Heinemann 1987; Ul haq et al. 2011). Brief (100 μ s) electrical pulses were applied using 25 μ m bipolar platinum electrodes (ca. 100 μ m distance between poles, exposed wire 50 – 100 μ m) placed in the stratum radiatum to activate Schaffer-collaterals and commissural fibers or in the stratum lacunosum moleculare to activate the temporoammonic pathway (also called direct cortical input). To avoid retrograde co-activation of Schaffer-collaterals during stratum lacunosum moleculare stimulation, a cut was made between CA1 and subiculum (Empson and Heinemann 1995; Wöhrle et al. 2007a, 2007b). Evoked signals were obtained using 50 % of the intensity required to induce maximal responses. Two protocols were used for recurrent repetitive stimulation: 10 trains

of 10 stimuli each with an inter-train interval of 1 s, or 3 trains of 40 stimuli each with an inter-train interval of 40 s (Wöhrl et al. 2007a; Ul haq et al. 2011). Stimulation frequencies were between 1 and 100 Hz (to stratum radiatum) or 20 or 200 Hz (stratum lacunosum moleculare). For low-frequency stimulation of stratum radiatum, 900 pulses were applied at 1, 5 or 8 Hz (Wöhrl, et al. 2007b). $[K^+]_O$ was measured using double barreled ion-sensitive microelectrodes (ISME, Lux & Neher 1973). The ion-sensitive barrel was filled with potassium chloride (100 mM) and the tip (2-3 μM) was filled with a K^+ -sensitive valinomycin based resin (Fluka 60031 ionophore, Sigma-Aldrich, Switzerland). The reference barrel was filled with sodium chloride (154 mM, 10 M Ω). Signals from both channels were fed into a high input impedance amplifier equipped with negative capacitance compensation. Due to different resistance in both channels, impedance was not optimally matched for fast signals. Therefore, K^+ signals were low pass filtered (< 30 Hz) and additionally their alternating current (AC) component was subtracted from the direct current (DC) component, permitting analysis of rise and decay kinetics of paired-pulse evoked K^+ signals (interval 50 ms).

Immunohistochemistry

For histological analysis standard methods were used (Seiffert et al., 2004). Tissue was fixed with 4% paraformaldehyde in 0.1 M phosphate buffered saline overnight, followed by cryoprotection in 30 % saccharose in 0.1 M phosphate buffer solution (PBS) overnight and sliced on a cryostat. 25 μm thick sections were used for immunofluorescence staining and incubated with rabbit anti-NeuN (1:500, Sigma-Aldrich, Germany). Signal detection was achieved by incubation with Alexa Fluor 568 goat anti-rabbit secondary antibody (1:200, MoBiTec, Göttingen, Germany) for two h at room temperature. Sections were mounted on gelatine-coated slides and cover-slipped using Fluoromount (Sigma-Aldrich, F4680).

Drugs

Ifenprodil (3 μM) was purchased from Tocris (Sigma-Aldrich, ordering number I2892, Germany) and was added to afSERUM solution for *in-vitro* perfusion of acute slices prepared from animals 'treated' *in-vivo* with bovine serum albumin (BSA) through intraventricular application.

Data acquisition and analysis

Signals were amplified (SEC-10L, NPI, Tamm, Germany), filtered at 3 and 0.03 KHz (field potential, $[K^+]_O$ signal, respectively), displayed on an oscilloscope, digitized on-line (CED-1401, Cambridge, UK) and stored for off-line analysis using Spike 2 v6.09 (CED-1401, Cambridge, UK) and home-made scripts (MATLAB R2010a for Windows). Data are represented as mean values $\pm$ SEM. Differences between control and treated slices were determined by Mann Whitney Rank Sum Test between two independent samples. SD incidence between independent samples was compared using the chi-square test, unless the total number of each group was < 10 with an assumed degree of freedom of one, in which case Fisher's exact test was employed. SD duration was measured at half of the maximal direct current (DC) voltage amplitude. Field excitatory postsynaptic potential was analyzed by measuring the slope between 20 and 80 % of its maximal amplitude (fEPSP₂₀₋₈₀). To compare mean fEPSP₂₀₋₈₀, population spike responses, maximal amplitude, half-time of rise and decay calculated from evoked $[K^+]_O$ signals in 4 independent samples with similar variance and normal distribution (ACSF, 24 h albumin *in-vivo*, afSERUM and ASERUM) one-way ANOVA was applied. For time-frequency analysis of epileptiform activity, the signal was high-pass filtered (> 500 Hz) using a Butterworth filter of 2nd order. Frequency analysis included short-time Fourier transform for frequencies ranging from 1.5 to 250 Hz. $[K^+]_O$ shown in all figures represents the time-course of the concentration change as

calculated from the original voltage traces using the Nernst equation according to the calibration obtained for each electrode (59 ± 1 mV per $[K^+]_O$ change from 3 to 30 mM). $[K^+]_O$ level at initiation of SD was obtained in reference to the start of local large negative deflection of the field potentials. P values < 0.05 were taken as statistically significant.

Results

Hippocampal blood-brain barrier dysfunction after cortical photothrombotic stroke is associated with frequent spreading depolarizations

We first recorded in brain slices from animals exposed to photothrombosis. As previously described (Stoll et al., 2009), exposing the skull to halogen light immediately after i.v. injection of Rose bengal results in a focal neocortical infarct and a wide brain region (including the cortical peri-infarct zone and subcortical regions) displaying dysfunctional BBB (Fig. 1A). While the subcortical hippocampus showed a clear extravasation of Evans blue, indicating increased vessels' permeability to blood proteins, triphenyltetrazolium chloride (TTC) staining confirmed that mitochondrial metabolic damage was restricted to the neocortex and did not involve the hippocampus. Injection of Evans blue at different time points after photothrombosis showed maximal hippocampal signal at 12 h with a gradual normalization to baseline at 7 d after treatment ($n=11$ animals, Fig. 1B). To immitate the disturbed extracellular electrolyte environment during BBB dysfunction, we recorded from hippocampal slices in-vitro in the presence of afSERUM. Stratum radiatum stimulation between 24 h (Fig. 1C2) and 7 d after *photothrombosis* confirmed apparently normal field potential responses in the CA1 field with apparent normal input-output curve and no evidence for hyperexcitability, excluding massive neuronal damage (data not shown). At 24h after photothrombosis, 8 out of 10 slices ($n=4$ animals) showed spontaneous SDs. As expected, stratum radiatum stimulation evoked responses were depressed during SDs (Fig. 1C2). In the remaining two slices, SDs were evoked only following recurrent repetitive stimulation (0.05 and 100 Hz respectively, stimulus intensity at 50 % of maximal response) applied to the stratum radiatum. Spontaneous SDs showed a maximal peak amplitude of 13.9 ± 2.5 mV and a duration at half-maximal amplitude of 67.5 ± 18.6 s ($n=8$). In 5 slices ($n=4$ animals), recurrent SDs (two to three SDs per slice, interval of recurrence 757 ± 159 s) were recorded within the first hour of recording, all with similar duration (60.5 ± 21 s) and amplitude (12.15 ± 0.2 mV). Recordings never showed spontaneous SDs under the same conditions (afSERUM) in slices from naive animals ($n=16$) or in slices obtained 7 days after photothrombosis ($n=9$), at a time when vascular permeability for Evans blue had returned to control levels (Fisher's exact test $p < 0.01$).

Spontaneous SDs were never recorded when slices obtained from animals 24 h post-photothrombosis were perfused with ACSF ($n=9$, ACSF vs. afSERUM 24 h post-stroke Fisher's exact test $p < 0.01$), suggesting that equilibration of electrolytes concentration between blood and brain due to dysfunctional BBB may contribute to the generation of SDs. However, in presence of ACSF where no spontaneous SDs occurred, SDs could be readily induced by recurrent repetitive electrical stimulation in 7 slices (78 %), suggesting a lower threshold for induction of SDs in the peri-ischemic tissue, even in the presence of normal brain electrolyte concentration. Peak amplitude of stimulus-induced SDs was 20.5 ± 1.6 mV and duration 54.0 ± 17 s (at half-maximal amplitude). Interestingly, in spite of BBB normalization 7 days after photothrombosis, SDs were still inducible in presence of afSERUM in 30 % of the slices ($n=9$ slices, 5 animals).

In-vivo exposure of the hippocampus to albumin facilitates induction of spreading depolarizations by recurrent repetitive stimulation in hippocampal slices

To simulate albumin extravasation following BBB opening in the peri-infarct hippocampus and to test the hypothesis that hippocampal exposure to serum albumin in-vivo is sufficient to induce SDs, we injected *bovine-serum-albumin (BSA)* into the right lateral ventricle to allow its diffusion into the adjacent hippocampus. The presence of albumin within the hippocampus was confirmed using intraventricular injection of fluorescein isothiocyanate conjugate (FITC) bovine-albumin, demonstrating extracellular and intracellular staining with FITC-albumin in both hippocampi 24 h after injection (Fig. 2A1). Consistent with previous data from the neocortex (Ivens et al., 2007), the morphology of FITC-albumin positive cells, their distribution within the hippocampus (majority outside the dense pyramidal cell layer) and their negative staining with anti-NeuN antibody (Fig. 2A2) suggest that the uptake of albumin is mostly into non-neuronal glial cells. To test SD susceptibility after BSA exposure, extracellular recordings were obtained from slices 24 h after intraventricular application of BSA under perfusion with either ACSF (n=29 slices) or afSERUM (n=10 slices). Under both recording conditions, spontaneous SDs were never observed. However, SDs could be induced by recurrent repetitive stimulation of stratum radiatum (consisting in pair-pulse stimulation at 0.05 Hz or 10 stimulation periods with 10 stimuli each, separated by 1 s intervals with frequencies ranging from 10 to 100 Hz Fig. 2B). The incidence of SDs was significantly higher (80 %, n=10 slices/8 animals) in slices obtained 24 hours after intraventricular application of albumin and exposed to afSERUM compared to those perfused with ACSF (10 %, n=29 slices/11 animals; chi-square test, $p < 0.01$). Likewise, the incidence of SDs in in-vivo albumin pretreated slices was significantly higher when compared to sham operated animals, where recurrent repetitive stimulation could not induce SDs either under afSERUM (n=6 slices/3 animals) or ACSF (n=15 slices/ 6 animals) (Fisher's exact test $p < 0.01$, chi-square test $p < 0.01$, respectively). SDs elicited in slices after *in-vivo* exposure to albumin showed a peak amplitude of 18.4 ± 3 mV (n=10) and were similar to the spontaneous (14.2 ± 1.5 mV n=8) and stimulus-induced SDs (20 ± 1.7 mV n=7) obtained in photothrombosis-treated animals ($p > 0.05$, Mann Whitney Rank Sum Test). Duration at half-maximal amplitude (33.8 ± 7.2 s, n=10), although shorter, did not show a significant difference to those spontaneous (67 ± 17 s, n=8) and stimulus-induced SDs (54.0 ± 17 s, n=7) recorded in slices from photothrombosis-treated animals ($p > 0.05$ Mann Whitney Rank Sum Test). These results confirm that hippocampal exposure to serum albumin is sufficient to reduce the threshold for stimulus induced SDs and that serum levels of electrolytes further enhance SD induction.

Induction of SDs in the CA1 region is independent of the origin of synaptic input

Area CA1 receives input from area CA3, the contralateral hippocampus and the entorhinal cortex. To test whether SDs initiation after *in-vivo* albumin treatment is pathway specific, we stimulated the direct cortical pathway whose axons run through stratum lacunosum moleculare (Empson and Heinemann 1995; Remondes and Schuman 2002; Wöhrle et al. 2007a), which transmits information from peri- and postrhinal cortex, and from lateral and medial entorhinal cortex to the hippocampus (Empson and Heinemann 1995). Coactivation of other pathways was prevented by a cut through stratum oriens, pyramidale and radiatum between the subiculum and area CA1 with the stimulation of the stratum lacunosum on the subicular side of the cut (Fig. 3A). Induction of SDs using recurrent repetitive stimulation was observed under afSERUM perfusion in all slices (Fig. 3C, n=10) obtained from *in-vivo* albumin treated animals (n=4), but never in slices perfused with ACSF (n=10 slices, 7 animals) nor in slices from sham-operated animals (Fig. 3B, under afSERUM n=5 slices, 4 animals). The amplitude of SDs was 18.6 ± 1.6 mV and duration at half maximal amplitude was 32.7 ± 12.6 s (n=6 slices/3 animals), comparable to SDs induced by stratum radiatum stimulation (Fig. 2B, $p > 0.05$ Mann Whitney Rank Sum Test). Notably, onset of SDs in

response to temporoammonic pathway stimulation could be delayed by up to 17 s with respect to termination of the stimulus train, in contrast to SDs induced by stratum radiatum stimulation, where there was usually no delay between stimulation and SD induction.

In-vitro exposure to artificial serum results in frequent SDs

It has been previously suggested that induction of SDs is dependent on the accumulation of extracellular K^+ ($[K^+]_O$), which critically depends on stimulation frequency (David et al. 2009; Heinemann and Lux 1977; Nixdorf-Bergweiler, Albrecht and Heinemann 1994). To study the threshold frequency for SD induction we exposed naive slices to a serum-like solution containing 0.4 mM albumin (ASERUM) for 1 h to allow albumin uptake into cellular compartments (Ivens et al. 2007) and subsequently 1 h to afSERUM, which contains only the electrolyte composition of serum. Applying increasing frequencies of stimulation to stratum radiatum while recording in CA1 resulted in the generation of SDs in 29 out of 65 tested slices (Fig. 4A, n=30 animals, table 1), with a peak amplitude of 21.2 ± 1.6 mV, duration of 30.0 ± 3.2 s (determined at half-maximal amplitude) and a propagation velocity of 2.5 ± 0.5 mm/min as determined in 4 experiments where simultaneous recordings in area CA3 and CA1 were performed (Fig. 4B). Under these experimental conditions (afSERUM perfusion after BSA exposure), SDs were induced in 12.5 % of slices when stimulation frequency was < 10 Hz (n=24 slices, 8 animals). SDs were significantly more likely to be induced (53 % of slices) when stimulation frequency was between 10 -100 Hz (n=32 slices, chi-square test, $p < 0.01$; Table 1). No significant differences were found between stimulation frequencies at the range of 10-100 Hz. Following the first SD, continuous recordings in the presence of low frequency stimulation (0.05 Hz) revealed recurrent SDs in 9 out of 29 slices (Fig. 4D, 7 animals). The median interval of recurrence was 802 seconds (range 469-2335 seconds). The first and second SD were similar in both amplitude and duration (21.2 ± 1.6 vs. 22.8 ± 0.9 mV and 30 ± 3.19 s vs. 35.3 ± 3.5 s for the first and second SDs, respectively, Mann Whitney Rank Sum Test, $p > 0.1$).

To distinguish between the effects of albumin and afSERUM on the inducibility of SDs, recordings were also performed in slices from naive animals exposed to 0.4 mM BSA dissolved in ACSF and perfused subsequently with ACSF. Under this recording condition, SDs were never observed irrespective of stimulation frequency (n=43 slices, 21 animals Table 1). However, in slices perfused with afSERUM without prior exposure to BSA, stimulus-induced SDs occurred in 12.8 % (6 out of 47 slices, 16 animals). In contrast, SDs could be induced overall in 44.6 % of the slices (n=65) that were pretreated with ASERUM (BSA *plus* afSERUM) and studied in the presence of afSERUM, showing a significant higher incidence of stimulus-induced SDs than in naive slices exposed only to afSERUM (chi-square test, $p < 0.01$). These results suggest an additive effect of serum electrolytes and albumin on the susceptibility for the induction of SDs.

Exposure to serum albumin is associated with alterations in the clearance of extracellular potassium

Since application of KCl triggers SDs (Dreier 2011; *Grafstein 1956*; Petzold et. al. 2008; Somjen 2001), and since BBB dysfunction can result in strong down-regulation of the glial inward rectifier potassium channel (Kir 4.1), reduced $[K^+]_O$ buffering and stimulus frequency-dependent excess accumulation of $[K^+]_O$ (David et al. 2009; Ivens et al 2007), we measured $[K^+]_O$ in slices obtained from animals injected with BSA or exposed to ASERUM *in-vitro*. Once SDs were induced, maximal rise in $[K^+]_O$ was similar regardless of treatment (66.9 ± 5.8 mM n=8 and 57.5 ± 4.5 mM n=10 for *in-vivo* BSA and *ex-vivo* ASERUM exposure, stratum radiatum stimulation) and stimulation site (61.0 ± 5.4 mM for *in-vivo* BSA, n=6, stratum lacunosum stimulation).

Since recurrent repetitive stimulation (between 10 - 100 Hz) evoked SDs in only 53 % of the slices exposed *ex-vivo* to ASERUM, we tested whether stimulus-induced $[K^+]_O$ accumulation was larger in those slices showing SDs compared to those slices that did not show SDs, and whether these values were higher than the normal potassium ceiling level (Heinemann and Lux 1977). In slices showing SDs, $[K^+]_O$ was measured at the initiation of SD during recurrent repetitive stimulation (defined as the sharp onset of the field potential deflection), while in slices that did not express SDs maximal rise in $[K^+]_O$ was determined during repetitive stimulation. Slices exposed to ASERUM showed larger $[K^+]_O$ rises than the assumed potassium ceiling level (12 mM, Fig. 4A2). Remarkably, $[K^+]_O$ at onset of SD was significantly larger compared to the stimulus-induced maximal rise in $[K^+]_O$ observed in non-SD slices (17.4 ± 0.8 mM, $n=8$ vs. 14.1 ± 1 mM, $n=9$, Mann Whitney Rank Sum Test, $p < 0.05$). Notably, both values were significantly higher compared to those measured in slices from naive animals perfused with afSERUM (11.8 ± 0.5 mM; $n=10$ slices / 7 animals). In slices from albumin-treated animals and exposed to ACSF (in which stimulus-induced SD were observed in only 10 % of slices), maximal rise in $[K^+]_O$ reached maximal levels of 10.2 ± 0.8 mM ($n=15$ slices / 7 animals) at 10-100 Hz stimulation frequency and 5.3 ± 0.2 mM ($n=5$ slices / 3 animals) when stimulation frequency was at 5 and 8 Hz.

To study potassium clearance, $[K^+]_O$ signals evoked by stratum radiatum paired-pulse stimulation (**50 ms inter-pulse interval**) were recorded after exposure to albumin: treated slices included those pre-exposed to ASERUM *ex-vivo* ($n=5$ slices / 5 animals) or slices from albumin-treated animals *in-vivo* ($n=5$ slices / 5 animals); recordings were obtained under *afSERUM* solution. Control slices were from naive animals perfused with ACSF ($n=5$ slices / 4 animals) or afSERUM ($n=4$ slices / 3 animals). Stimulation parameters were adjusted to yield similar field potential responses in all experimental conditions (Fig. 5). Changes in amplitude, half time of rise and half time of decay of $[K^+]_O$ signal were measured as well as the slope of the fEPSP₂₀₋₈₀ recorded in the stratum radiatum and the population spike amplitude in CA1 stratum pyramidale. Comparing the epsp-coupling (slope of the fEPSP₂₀₋₈₀ versus the amplitude of the population spike) between experimental conditions indicated a similar neuronal activation in all groups (Fig. 5B, ACSF $n=5$ slices / 4 animals; afSERUM $n=4/3$; ASERUM $n=5/5$; 24 h BSA *in-vivo* $n=5/4$). No differences were found between treatments in the mean rise and half time of rise in $[K^+]_O$ (in mM/ms: ACSF $0.37 / 41 \pm 4$; afSERUM $0.52 / 35 \pm 5$; ASERUM $0.35 / 52 \pm 5$, BSA *in-vivo* $0.42 / 52 \pm 5$; one-way ANOVA, $p > 0.05$). In contrast, the half time of decay in $[K^+]_O$ was significantly longer in slices exposed to ASERUM *in-vitro* ($1.6 \pm s$) or 'pre-treated' with BSA *in-vivo* (1.75 ± 0.32 s) compared to control slices treated with either ACSF or with afSERUM (0.95 ± 0.3 s and 0.55 ± 0.1 s, respectively, one-way ANOVA, $p = 0.0037$).

SDs in slices exposed to albumin are associated with epileptiform activity

A clear observation during this study was the appearance of fast epileptiform activity at the front of spontaneous SDs in most (Fig. 1C; Fig. 6A) hippocampal slices from animals exposed to focal neocortical photothrombotic stroke. In slices from animals 24 h after photothrombosis epileptiform activity preceded spontaneous SDs in 6 out of 8 slices. In the other cases we noted stimulus induced epileptiform discharges preceding onset of SDs. Similar activity was also noted in most slices under the experimental conditions described above, in which SDs were evoked by repetitive stimulation, regardless of stimulation frequency or pathway (Fig. 3C & D). Epileptiform activity appeared during the recovery phase of the SDs as well (Dreier et al. 2012; Leão 1944; Pomper et al. 2006; Van harrevald and Stamm 1953), when $[K^+]_O$ declined to 19.5 ± 1.6 mM, and it ceased when $[K^+]_O$ declined to less than 5.7 mM. This seizure-like activity during SD recovery lasted 38.7 ± 8.6 s and was composed of clonic like after discharges which lasted between 170 and 320 ms at

a frequency of 1 - 2 Hz (Fig. 6B2). The frequency content of the field potential fluctuations during a single clonic like after-discharges was usually not higher than 60 Hz (Fig. 6B2).

Blockage of the specific N-methyl-D-aspartate receptor type 2 B prevents stimulus induced epileptiform discharges and SDs

SD induction was proposed to be mediated by glutamate (Somjen 2002; Van Harreveld 1978). *In-vitro* recordings and computer-based modeling predicted that albumin-induced glial activation and the impaired buffering of $[K^+]_o$ underlie frequency-dependent facilitation of excitatory synaptic potentials, which is dependent on the activation of NMDA receptors (David et al. 2009; Seiffert et al. 2004). Contributing to this effect may also be the concomitant down-regulation of the astrocytic-specific excitatory aminoacid transporter 2 and impaired clearance of glutamate from the synaptic cleft (Cacheaux et al., 2009; David et al. 2009). To test whether a similar mechanism may apply to the initiation of SDs we tested the effect of ifenprodil (3 μ M) - an NMDA-2B receptor antagonist - on SD occurrence in slices from *in-vivo* BSA-treated rats, as it was shown to abolish the occurrence of SD in the mouse entorhinal cortex and in hippocampal area CA1 (Coutinho Faria and Mody 2004; Mody, Lambert and Heinemann 1987). In the presence of ifenprodil in afSERUM, recurrent repetitive stimulation of stratum radiatum or stratum lacunosum moleculare only rarely (1 out of 6 slices and 1 out of 5 slices, respectively) resulted in generation of SD after *in-vivo* albumin exposure for 24 h (stratum radiatum and lacunosum moleculare without ifenprodil: 90 %; with ifenprodil: 16.7 %, $p < 0.05$ Fischer's exact test). Ifenprodil also prevented generation of epileptiform discharges which were usually induced during recurrent repetitive stimulation ($n=6$, Fig. 6C), and prevented induction of SDs. Only in the slice in which ifenprodil did not prevent induction of SDs, ifenprodil also failed to prevent induction of epileptiform discharges.

Discussion

In this study we focused on the potential role of BBB dysfunction in the susceptibility for SDs and found that (1) the incidence of spontaneous and stimulus-induced SDs is increased in the BBB-dysfunctional hippocampal slices recorded 24 h after cortical photothrombosis; (2) exposure to serum albumin *in-vivo* or *in-vitro* is sufficient to reduce the threshold for induction of stimulus-induced SDs; (3) exposure to serum levels of electrolytes further increases the likelihood for the generation of SDs; (4) albumin-exposed hippocampus show reduced clearance of $[K^+]_o$ following repetitive stimulation; (5) SDs are usually triggered when $[K^+]_o$ rises above 17 mM, potentially due to disturbed $[K^+]_o$ homeostasis; (6) epileptiform activity is often preceding SDs and re-appearing during recovery from SDs. These findings are in contrast to a previous study in which susceptibility for generation of SDs was found to be reduced (Tomkins et. al. 2007). However, in that study, experiments were performed one month after blood brain barrier damage, at a time when epileptogenesis had already occurred. Moreover, the tissue was not exposed to afSERUM. This suggests that the alterations we describe apply to an early situation after induction of cortical photothrombosis.

BBB dysfunction is a common finding in the peri-ischemic brain in both animal studies (Stoll et al., 2009) and human patients (Bang et al., 2007). The increased permeability of blood vessels is associated with diffusion of serum electrolytes and proteins into the brain interstitial space with a consequent shift in water molecules which produce vasogenic edema and increased intracranial pressure. Here we show that even under well perfused conditions *in-vitro*, prior BBB dysfunction or intraventricular exposure to albumin are sufficient to induce SDs, either spontaneously or in response to physiological stimulation conditions (50% maximal intensity, >10hz). The detailed molecular mechanisms underlying BBB

dysfunction within the peri-ischemic brain tissue (despite an initial intact blood flow) are not known, although several mechanisms have been proposed (for review see Shlosberg et al. 2010). To study the role of primary BBB dysfunction in the remote peri-ischemic brain we used the Rose bengal phothrombosis model which has the advantage that (1) the location and size of infarction is highly reproducible and spatially restricted (Dietrich et al. 1994; Watson et al. 1985), and (2) that BBB dysfunction is prominent and consistent, even in remote, sub-cortical regions, allowing us to study the effects of vascular dysfunction in the well characterized hippocampal formation. While we cannot rule out that blood flow in the hippocampus is compromised in our model due to intravascular spread of thrombosis, this is unlikely since in experiments with an open cranial window, hippocampal opening of the BBB was milder or absent (unpublished data). Moreover, the hippocampus does not share blood supply or drainage with the somatosensory cortex, our experimental ischemic target (Anterior parts of the hippocampus are supplied from the anterior choroidal artery, of which terminal branches supply the dorsal hippocampus (Barth and Mody 2011; Dorr, Sled, and Kabani 2007).

Our recordings from peri-infarct hippocampus demonstrated increased incidence of SDs, consistent with a previous study showing SDs in remote cortical regions that were not part of the infarct core (Dietrich et al. 1994). Importantly, and consistent with the time window in which BBB opening is observed, human studies in traumatic brain injury, subarachnoid hemorrhage and stroke patients confirm high incidence of SDs in peri-lesional brain tissue (for review Dreier 2011).

Induction of SDs in the perilesional area may be related to diffusion of toxic substances (e.g. potassium, glutamate, free radicals) from the necrotic area into the surrounding tissue (Woitzik, Back and Thome 2008). The relatively large distance between the infarct core and the hippocampus, the separation of sub-arachnoid space between the cortex and hippocampus and the constant perfusion – washout – of slices with pre-determined extracellular solutions in our study make this explanation less likely for our model. Yet, spontaneous SDs were observed in the peri-infarct hippocampus when exposed to an extracellular solution containing serum levels of electrolytes (afSERUM), mimicking an *in-vivo* condition in which the BBB is dysfunctional. Dysfunction of the BBB results in equilibration of electrolytes between the extracellular space and serum, thus expected to lower extracellular concentrations of Mg^{2+} and Ca^{2+} and elevate K^{+} concentration. Such alterations affect neuronal excitability, synaptic transmission and glial function (Rausche, Igelmund and Heinemann 1990). The main differences between afSERUM and ACSF are higher K^{+} (5.7 vs. 3.0 mM), lower Mg^{2+} (0.8 vs. 1.8) and slightly lower Ca^{2+} (1.3 vs. 1.6). The lower Mg^{2+} and Ca^{2+} reduce surface charge screening, thereby increasing neuronal excitability (Frankenhäuser & Hodgkin, 1955). Mg^{2+} also serves as an antagonist to Ca^{2+} entry into presynaptic terminals and therefore lowering its concentrations results in increased Ca^{2+} dependent transmitter release (Hamon, Stanton, and Heinemann 1987). Lower $[Mg^{2+}]_o$ further enhances Na^{+} and Ca^{2+} influx through NMDA receptors and contributes to selective enhancement of glutamate-mediated neuronal depolarization. Lowering $[Ca^{2+}]_o$ was also shown to interfere with synaptic inhibition more than with excitation (Jones and Heinemann 1987), thereby increasing overall tissue excitability. Elevation of $[K^{+}]_o$ has multiple effects: it causes depolarization of neurons and astrocytes (Seifert & Steinhäuser, 2011), therefore increasing transmitter release (Liotta et al., 2011) and activation of NMDA-dependant currents (David et al., 2009). Depolarization further reduces the driving force for Na^{+} dependent glutamate transport (Wallraff et al. 2006, Matyash and Kettenmann 2010). Nevertheless, these ionic changes *per se* are not sufficient to induce SDs in slices from naïve animals, although they could lower the threshold for induction of seizure like events and stimulation-induced after discharges (Leschinger et al. 1993; Liotta et al. 2011).

We therefore investigated the potential role of brain exposure to physiological serum levels of albumin in the induction of SDs. Albumin is the most abundant serum protein and it has been recently shown to activate astrocytes by specific activation of transforming growth factor beta (TGF- β) signaling (Cacheaux et al. 2009; David et al. 2009; Ivens et al. 2007). Indeed, we report that exposure of hippocampus to serum-derived albumin, either *in-vivo* (for 24 h) or *in-vitro* (2 h) reduced the threshold for SD generation. The effect of serum albumin levels on the colloid osmotic pressure was mild and was always below 15 mOSM (5%). Furthermore, electrophysiological recordings of SDs were always in the presence of an iso-osmotic solution (afSERUM), free of BSA, excluding a direct, immediate effect of the increased osmotic pressure. The lower SD threshold following exposure to albumin was more pronounced in the presence of serum levels of electrolytes (afSERUM). Importantly, prior exposure to albumin was sufficient to induce the effect, suggesting that the binding of albumin to the extracellular matrix or its uptake into cellular elements, were sufficient to induce the SD promoting effect. We further presented evidence to indicate that increasing accumulation of potassium trigger SD under BBB opening.

Exposing control slices to serum electrolyte levels without albumin indeed resulted in augmented potassium accumulation during repetitive stimulation, suggesting enhanced neuronal excitability. However, $[K^+]_O$ under these conditions never reached levels beyond ~ 12 mM, which is below the threshold for ignition of SDs (Somjen 2001). Also in slices 'treated' with albumin *in-vivo* and recorded during ACSF perfusion, $[K^+]_O$ levels reached around 10 mM and SDs were usually not observed. Thus, our data indicate that the induction of SDs requires an abnormally large accumulation of $[K^+]_O$ to over 17 mM, exceeding the previously published ceiling levels (10-12 mM) of $[K^+]_O$ reported for the healthy brain (Heinemann and Lux 1977, Nixdorf-Bergweiler, Albrecht and Heinemann 1994). Our findings are thus consistent with *in-vitro* studies showing that continuous *in-vitro* exposure of normal cerebrospinal fluid with high K^+ concentration resulted in a SD threshold of ~ 17.8 mM $[K^+]_O$ in control neocortical slices (Maslarova et al. 2011). A lower threshold for SD generation in presence of abnormal potassium accumulation has also been demonstrated in young cortical and hippocampal tissue (Hablitz and Heinemann 1989), which may be correlated with postnatal development of astrocytes and reduced buffering of $[K^+]_O$ (Nixdorf-Bergweiler, Albrecht and Heinemann 1994).

Facilitated induction of stimulus-induced SDs has also been reported in slice cultures, presumably due to the presence of activated astrocytes and exposure to serum in cultured medium (Pomper et al. 2006). Interestingly, in that study repetitive stimulation induced epileptiform discharges which preceded SDs and re-occurred in the falling phase of $[K^+]_O$. Epileptiform discharges during the recovery phase of SD were described already by Leão and Van Harrevald and Stamm (Van Harrevald and Stamm 1953; Leão 1944) and were obtained in our recordings as well, in slices from albumin-treated animals as well as following *in-vitro* exposure to serum-like conditions. The clinical relevance of these findings has been confirmed recently in recordings from patients after subarachnoid hemorrhage stroke (Dreier et al. 2012). To what extent, and under what conditions recurrent SDs induce neuronal damage is still under investigation. Recurrent SDs can induce neuronal damage when oxygen levels declined to very low levels during an SD (Pomper et al. 2006). Measurements of changes in evoked field potentials after SDs did not indicate reduced neuronal response in our well-perfused slices. However, we rarely evoked more than two SDs, which might become damaging when recurrence is frequent or under conditions of reduced metabolic supply (Dreier 2011). In our experiments albumin exposure was never sufficient to induce spontaneous spreading depolarization and seizures as observed in slices exposed to afSERUM 24 h after photothrombosis. This suggests that other serum or brain proteins may contribute to the reduced threshold for spreading depolarization and seizures. Interesting candidates belong to the immune system including, for example TNF-alpha, as it

was recently suggested to be a critical factor in a model of eclampsia in hippocampal cultures (Cipolla et al. 2012). Indeed, albumin itself may promote a brain immune response, and the upregulation of cytokines (Cacheaux et al., 2009). The uptake of albumin may also vary dependent on species and disease model (Alonso et al. 2011). In a parallel study it was shown that intraventricular albumin is taken up by hippocampal NG2 positive cells, astrocytes and to some extent also by perivascular cells and neurons (Steinhäuser et al, personal communication).

Abnormal permeability of the BBB is frequent in human diseases and has been reported for status epilepticus, stroke, inflammation, infections and in peri-tumoral regions, but may also occur focally in Alzheimer's disease and other neurodegenerative and neuropsychiatric disorders (Abbott et al. 2006; Neuwelt 2004; Tomkins et al. 2001; Zlokovic 2008). It seems thus possible that part of the neurodegenerative processes occurring under these conditions are related to impaired extracellular ion homeostasis. This would indicate that preventing the activation of astrocytes might be a potential strategy as for the development of new therapeutical targets to prevent further insult to the brain (Friedman, Kaufer and Heinemann 2009; Shlosberg et al. 2010). The use of NMDA-antagonists in a sub-set of patients with BBB dysfunction may turn to be useful in the prevention of SDs and progression of damage. Certainly, BBB damage may expose the brain tissue to other blood- or brain-derived factors and further reduce the threshold for SDs generation. The strong inflammatory response which follows brain insults including alterations induced by albumin (Cacheaux et al. 2009; Vezzani et al. 2011; Vezzani and Friedman 2011) may further contribute to enhanced excitability of neurons. Other processes may also be involved, including for example the breakdown of extracellular matrix proteins which can reduce the mechanical force limiting shrinkage of the extracellular space. Reduction in the extracellular space would facilitate increased concentrations of potassium and glutamate, and thereby, facilitate the induction of SDs (Grafstein 1956; Van Harrevald and Stamm 1953).

In conclusion, our work suggest a key role for vascular integrity, and specifically for BBB dysfunction in lowering SD threshold following cortical insult. We suggest that BBB dysfunction, through alterations in extracellular concentrations of electrolytes and impaired extracellular homeostasis results in activity-dependent increase in $[K^+]_O$ reaching a threshold for epileptiform discharges which further accelerate $[K^+]_O$ increase, leading to SDs. These findings predict that patients with brain lesions associated with BBB dysfunction are more prone to seizures and SDs and associated complications, thus, highlighting the importance of diagnosis and potential targeting of BBB dysfunction in the clinical setting.

Acknowledgments

This work was supported by the *Bundesministerium für Bildung und Forschung* (BMBF CSB 01 EO 0801; BCCN 01 GQ1001C), the *Sonderforschungsbereich* TR3 (A.F, J.P.D, U.H), DFG 323/3 -1 (J.P.D), the German Israeli Foundation (GIF No. 124/2008, A.F., J.P.D) as well as by Epicure. Alon Friedman was a recipient of a Mercator professorship from the German Science Foundation (*Deutsche Forschung Gemeinschaft*). We are grateful for helpful discussions to Dr. Sebastian Ivens and to Dr. Hans Jürgen Gabriel and Bernd Schacht for skillful technical support.

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Highlights

1. Neocortical stroke in rats opens the BBB in the adjacent hippocampus (12- 24h)
2. BBB opening in the hippocampus is associated with SDs and epileptiform activity (EA)
3. Hippocampal exposure to serum & albumin in-vivo/vitro is also related to SDs and EA
4. The facilitated induction of SDs is related to excessive potassium accumulation
5. The induction of SDs and EA seems to depend on NMDA receptor activation

Figure 1. Blood-brain-barrier (BBB) opening *in-vivo* and spreading depolarization (SD) in hippocampus following Rose bengal induced cortical stroke

(A) Evans Blue extravasation (upper image) and TTC staining (lower image) shown in coronal slices obtained from animals 12 h after Rose bengal-induced photothrombosis. Note that while the hippocampus is stained with Evans blue, indicating BBB opening, the triphenyltetrazolium chloride (TTC) staining indicate that the ischemic core (in white) is limited to the neocortex while the hippocampus is red (indicating functional mitochondria). (B) Quantification of Evans blue extravasation in extracted hippocampus after photothrombosis compared with controls at different times following treatment. (C1) Hippocampal field potential recording 24 h post-stroke showing an SD during very low

frequency (0.05 Hz) stimulation of the stratum radiatum in presence of afSERUM. **(C2)** Evoked field potentials recorded at time points *a* and *b* (marked in C1) - before the SD and during recovery from its peak – showing depression of neuronal activity. **(C3)** Epileptiform activity preceding the SD (at time point marked by #). **(D)** Percent of slices in which SD was recorded spontaneously (black bars) or in response to 0.05 - 100 Hz stimulation (gray bars) in the presence of afSERUM. Recordings were made in slices from photothrombosis-treated animals (24 h and 7 d after treatment) and in slices from naive animals. * $p < 0.05$ (Fisher's exact test). *In all figures positive potential is upwards.*

Figure 2. Intraventricular albumin *in-vivo* and susceptibility for SDs in *in-vitro* hippocampal slices

(A1) A coronal brain section showing the distribution of fluorescein isothiocyanate conjugate bovine (FITC)-albumin (green) 24 h after intraventricular injection. Cells were stained with DAPI (blue). (A2) Confocal image from FITC-albumin-injected animal immune-stained against NeuN (red). Note that albumin was excluded from neurons. White bar represents scale. (B) Stimulus induced changes in $[K^+]_o$ and field potential recording in slices obtained from a sham-operated animal (control) and a 24 h albumin-treated animal. Repetitive train stimulation at 10 Hz (10 stimuli, 10 times with train interval of 1 s) was applied at the stratum radiatum (see Methods). Note that $[K^+]_o$ at onset of sharp negative excursion of the SD was 21 mM as indicated by dotted line. Horizontal lines below traces indicate stimulation period. Line within trace indicates duration of SD at half-maximal amplitude. (C) Incidence of stimulus-induced SD in controls ($n=15$ in ACSF and $n=6$ in afSERUM) and 24 h after *in-vivo* albumin treatment in the presence of ACSF ($n=29$) or afSERUM ($n=10$). * $p < 0.05$, Fisher's exact test.

Figure 3. Induction of spreading depolarization (SD) by stimulation of the temporoammonic pathway

(A) Image of a slice showing stimulation electrode placed in the stratum lacunosum moleculare (temporoammonic pathway) and a cut separating the subiculum from area CA1. Recording electrode was placed in the stratum pyramidale of area CA1. (B) Repetitive stimulation of temporoammonic pathway with recurrent repetitive stimulation at 10 Hz (10 stimuli each, repeated 10 times with train interval 1 s) in a slice from a sham operated animal (control) showing the associated mild increase in $[K^+]_o$. Horizontal line below trace indicates stimulation period. (C) Repetitive recurrent stimulation-induced elevated $[K^+]_o$ and SD in a slice perfused with afSERUM from an animal treated with intraventricularly albumin 24 h earlier. Horizontal line below trace indicates stimulation period. Line inside the trace represents the duration of SD at half-maximal amplitude. (D) Expanded time course of field potential activity before onset of SD showing a fast “seizure like” activity. Discontinuous line below trace indicates 10TH stimulation period.

Figure 4. *In-vitro* exposure to albumin and spreading depolarization in hippocampal slices

(A) Representative traces showing simultaneous $[K^+]_O$ and field potential recorded during stratum radiatum repetitive recurrent stimulation (10 Hz, 10 stimuli each, repeated 10 times with train interval 1 s, indicated by horizontal bar) in slices exposed to ACSF (A1), to ASERUM for 1 h and subsequently to afSERUM (A2) and to afSERUM only (A3). Dotted lines (A2) indicate onset of SD and $[K^+]_O$ levels at SD initiation. Line inside the trace represents duration of SD at half-maximal amplitude. (B) Simultaneous recordings in area CA1 and CA3 during stimulation in the stratum radiatum showing the spreading of an SD from CA1. Stimulation is indicated by the horizontal bar below. Dots indicate delay between onset of SD between CA1 and CA3. (C) Field potential response to single stimulation showing depression of activity following SD; *a* and *b* correspond to the corresponding time points depicted in (B). (D) Recurring multiple-SDs can occur once a single SD had been induced by recurrent repetitive stimulation. This was independent whether low frequency stimulation was used to determine recovery of field potentials after an SD or not. In this example continuous stimulation was performed with 0.05 Hz. Discontinuous boxes indicate period of recording during SD (D1) and after SD (D2). (E1) Indicates depression of signal during SD (from D1), while (E2) represents the recovery of the evoked-activity (from D2).

Figure 5. Albumin exposure is associated with delayed recovery of stimulus-induced $[K^+]_O$ elevation

(A) Representative samples of paired-pulse evoked field potentials simultaneously recorded in stratum pyramidale and in stratum radiatum CA1 (B) Amplitudes of population spikes recorded in stratum pyramidale against the 20 – 80 % slope of fEPSPs recorded in stratum radiatum. All measurements were in signals evoked by 50 % of the stimulation intensity required for the induction of maximal responses under control conditions (ACSF, black filled circles), under ACSF (red) and afSERUM following albumin exposure *in-vivo* (red and gray circles). (C) Representative $[K^+]_O$ signals in response to a paired-pulse stimulus under the different experimental conditions. (D) Averaged and standard error of the mean of the half time of $[K^+]_O$ decay in control slices (under ACSF or afSERUM) and in slices from animals treated with intraventricular albumin 24 h earlier and perfused with afSERUM (red bar). * $p < 0.01$, one-way ANOVA.

Figure 6. Spreading depolarization is associated with epileptiform activity

(A) Representative traces of two consecutive SDs showing simultaneous $[K^+]_o$ and field recordings in the stratum pyramidale of area CA1. First and second SDs were induced by recurrent repetitive stimulation of stratum radiatum at 10 and 100 Hz, respectively (indicated by horizontal bars). Slices were obtained from animals treated with intraventricularly albumin 24 h earlier and perfused with afSERUM. (B) Slower time scale show that SDs are preceded (B1) and followed (B2) by fast epileptiform neuronal activity. Below is the spectral analysis of discharges recorded in B1 and B2 with superimposed levels of $[K^+]_o$ (white line). (C) Representative trace showing simultaneous $[K^+]_o$ and field recording in

stratum pyramidale of area CA1 during repetitive recurrent stimulation at 20 Hz (indicated by horizontal bar) of stratum radiatum in the presence of afSERUM and ifenprodil. The epileptiform discharges and the induction of SD were prevented during stimulation.

Table 1

Percentage of slices with stimulus-induced SD according to the frequency of stimulation

Frequencies Hz	ACSF	ACSF + BSA in-vitro	ASERUM in-vitro	afSERUM
0.05	0% (0/48)	0% (0/43)	13.8 % (9/65) ^a	2.1 % (1/47) ^b
1-8	0% (0/24)	0% (0/8)	12.5% (3/24)	8.3 % (2/24)
10-19	0% (0/5)	0% (0/8)	57.1% (4/7)	0 % (0/6)
20-40	0% (0/9)	0% (0/18)	53.8% (7/13)	18.2 % (2/11)
60-100	0% (0/10)	0 % (0/9)	50 % (6/12)	20 % (1/5)

(number of slices showing SD / total slices tested)

^a After induction of SD with stimulation at 0.05 Hz in 9 slices, no stimulation protocol was tested to induce another SD.^b On the only slice that showed SD during stimulation at 0.05 Hz, no stimulation protocol was tested to induce another SD