Neurobiology of Disease

Lasting Blood–Brain Barrier Disruption Induces Epileptic Focus in the Rat Somatosensory Cortex

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Perturbations in the integrity of the blood–brain barrier have been reported in both humans and animals under numerous pathological conditions. Although the blood–brain barrier prevents the penetration of many blood constituents into the brain extracellular space, the effect of such perturbations on the brain function and their roles in the pathogenesis of cortical diseases are unknown.

In this study we established a model for focal disruption of the blood–brain barrier in the rat cortex by direct application of bile salts. Exposure of the cerebral cortex *in vivo* to bile salts resulted in long-lasting extravasation of serum albumin to the brain extracellular space and was associated with a prominent activation of astrocytes with no inflammatory response or marked cell loss. Using electrophysiological recordings in brain slices we found that a focus of epileptiform discharges developed within 4–7 d after treatment and could be recorded up to 49 d postoperatively in >60% of slices from treated animals but only rarely (10%) in sham-operated controls. Epileptiform activity involved both glutamatergic and GABAergic neurotransmission. Epileptiform activity was also induced by direct cortical application of native serum, denatured serum, or albumin-containing solution. In contrast, perfusion with serum-adapted electrolyte solution did not induce abnormal activity, thereby suggesting that the exposure of the serum-devoid brain environment to serum proteins underlies epileptogenesis in the blood–brain barrier-disrupted cortex. Although many neuropathologies entail a compromised blood–brain barrier, this is the first direct evidence that it may have a role in the pathogenesis of focal cortical epilepsy, a common neurological disease.

Key words: **blood–brain; cortex; epilepsy; epileptiform; slice; somatosensory**

Introduction

The blood–brain barrier (BBB) is a complex structure designed to maintain a constant neuronal extracellular environment by limiting the penetration of a wide range of hydrophilic molecules, proteins, and cellular elements into the brain. It is composed of the endothelial cells lining brain microvessels that are interconnected by tight junctions and are surrounded by pericytes and astroglial foot processes (Pardridge, 1998; Soreq et al., 2002). Several common brain pathologies such as stroke, trauma, brain tumors, and epilepsy are known to be associated with BBB disruption (for review, see Ballabh et al., 2004; Neuwelt, 2004). However, although abnormal BBB permeability has been documented in some cases for prolonged periods of time (Skoog et al., 1998; Tomkins et al., 2001), it is not known whether this increased permeability is a result from the disease, or is itself a contributor to the pathogenesis of the disease.

The aim of this study was to investigate the potential role of focal BBB opening in the development of cortical neuronal dysfunction. We established a model for focal BBB disruption in the rat somatosensory cortex by local application of low concentrations of the bile salts dihydrocholic acid or deoxycholic acid. These bile salts were chosen in accordance with previous studies showing their potency in opening tight junctions in brain capillaries (Greenwood et al., 1991) and nerve perineurium (Todd et al., 1997). Importantly, at high concentrations (>3 mM) these bile salts showed a detergent effect on the cell membranes, at lower concentrations a subtle modification of the BBB occurs, with no evidence of cellular injury in an electron microscopy study (Greenwood et al., 1991). This study shows that focal long-lasting BBB disruption leads to extravasation of serum proteins into the brain extracellular space, and early activation of astrocytes followed by a lasting neuronal hypersynchrony limited to the treated cortex. Our data suggest a new mechanism for the pathogenesis of focal epilepsy.

Materials and Methods

All experimental procedures were approved by the Berlin and Beer-Sheva animal ethics committees.

In vivo *animal preparation.* Adult male Wistar rats (180–250 gm) were deeply anesthetized with thiopental (40 mg/kg body weight) and placed into a stereotactic frame. The skin was disinfected and a 15 mm sagittal incision was made. A 4 mm diameter bone window was drilled over the somatosensory cortex (3 mm caudal, 5 mm lateral to bregma). The dura was opened and the underlying brain was perfused with artificial CSF

Received Jan. 8, 2004; revised July 15, 2004; accepted July 21, 2004.

This work was supported by the Sonderforschungsbereich 507 and TR3. A.F. is an Alexander-von-Humboldt fellow.

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DOI:10.1523/JNEUROSCI.1751-04.2004

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(aCSF) for 30–60 min. The composition of the aCSF was (in mM): 129 NaCl, 21 $NaHCO_3$, 1.25 NaH_2PO_4, 1.8 $MgSO_4$, 1.6 $CaCl_2$, 3 KCl, and 10 glucose. In treated rats, the disrupting agents dehydrocholic acid (DHC) or deoxycholic acid (DOC) were added to the aCSF (2 and 1 mM, respectively). In control experiments aCSF was applied either to the contralateral hemisphere of the same rat or to the right hemisphere of a sham-operated one. In some experiments the brain was perfused with aCSF solution adapted to serum levels of electrolytes (as determined by the laboratory of clinical chemistry from 10 rats) (in mM): 140 NaCl, 0.8 $MgSO_4$, 1.3 $CaCl_2$, and 5.7 KCl (aSERUM). Albumin was added to the aSERUM in concentrations of 0.04–0.4 mM (corresponding to 10–100% of serum albumin, osmolarity = 300–310 mOsmol/l). After perfusion, the bone window was carefully closed and the skin was sutured. Only rats with no apparent injury to the cortical surface or bleeding from cortical vessels as seen under the surgical microscope at the end of the procedure were included in this study.

Evaluation of BBB integrity. To estimate BBB integrity rats were intraperitoneally injected with the albumin-binding dye Evans blue (5 ml of 2% solution in 0.9% NaCl, Sigma, St. Louis, MO). One or two hours after injection the anesthetized rat was decapitated, and the brain was quickly removed. Evans blue–albumin complex in the brain parenchyma was quantified by two approaches. (1) Coronal slices (~0.5-mm-thick) were prepared and incubated in 4% paraformaldehyde solution for 24 hr, followed by incubation in 30% sucrose (in 0.1 M phosphate buffer) for 24 hr. Fifty micrometer sections were then cut on a cryostat. Tissue concentrations of the fluorescent albumin–Evans blue complex were measured by reading color intensity in digitized low power-field cortical imaging (10×; microscope from Zeiss, Oberkochen, Germany; 580 nm wavelength) using Mathlab 6.5 software. To validate albumin penetration to the cortex when locally perfused, the same procedure was applied, but Evans blue (0.1 mM) was added to the albumin-containing aCSF. (2) In the second approach, the treated or control neocortical slices were dissected from the underlying brain, dissolved in 0.1 M phosphate buffer solution (10 μl/mg tissue) containing 1% SDS (Carl Roth GmbH, Karlsruhe, Germany), homogenized, and centrifuged (12,000 × *g*; 5 min) with an Eppendorf Scientific (Westbury, NY) centrifuge (5417R) to precipitate confounding cell debris. Albumin–Evans blue complex concentrations were spectrophotometrically measured in the supernatant at 595 nm wavelength using an Eppendorf Biophotometer.

Histology. For histological experiments, rat brains were fixed after the BBB opening by transcardial perfusion with modified Lillie fixative (Dreier et al., 2000). After perfusion, brains were kept in the same fixative at 4°C overnight. Brains were then removed from the skull, dissected, and treated with 96% alcohol overnight and subsequently paraffin-embedded in accordance with routine procedures. Eight to ten micrometer coronal sections were mounted and stained using cresyl violet, hematoxylin, and the eosin and vanadium acid fuchsin-toluidine blue method for the detection of neuronal injury (Victorov et al., 2000). Immunocytochemistry was performed on 10 μm paraffin sections. Sections were incubated with primary antibodies at 4°C overnight. Microglial cells were stained with FITC–Griffonia simplicifolia isolectin-B4 (GFS-IB4; 1:20; Sigma), and CD40 with rat anti-mouse mAb (1:100; PharMingen, San Diego, CA). Signal detection was achieved applying biotinylated secondary antibodies for 2 hr at room temperature followed by a standard ABC–DAB development (Bechmann et al., 2000; Gimsa et al., 2001).

Electrophysiological recordings. For all electrophysiological experiments, rats were deeply anesthetized with dimethyl ether and decapitated. Brains were quickly removed, and coronal slices (400-μm-thick) were prepared from the somatosensory cortex using a vibratome (Campden Instruments, Loughborough, UK). Slices were maintained in a humidified, carbogenated (5% CO_2 and 95% O_2) gas atmosphere at 36 ± 1°C and perfused with aCSF in a standard interface chamber. Electrophysiological recordings were performed after at least 1 hr of incubation (Pavlovsky et al., 2003). Field potentials were recorded using extracellular glass microelectrodes (~3 MΩ) filled with 154 mM NaCl. Intracellular recordings were performed with sharp microelectrodes as previously reported (Fleidervish et al., 1996). Slices were stimulated with brief (100 μsec) pulses using bipolar stimulation electrodes placed at the border of white and gray matter.

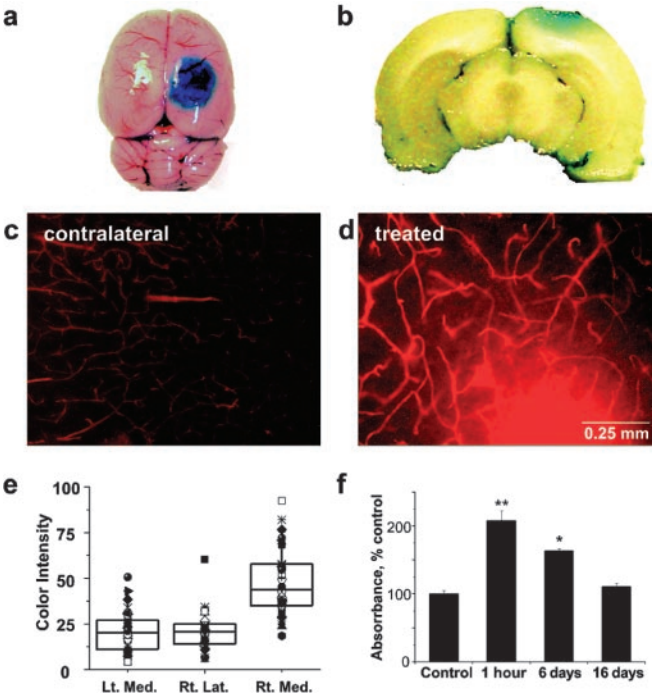

Figure 1. Bile salt-induced long-lasting BBB opening. *a*, *b*, Rats were injected intraperitoneally with the albumin-binding dye Evans blue; pictures show brains 1 hr (*a*) and 7 d (*b*) after focal cortical perfusion with DHC. At both time intervals, extravasation of Evans blue–albumin complexes into the parenchyma within the treated area indicates an open BBB. *c*, *d*, Under fluorescence, microscopic extravasation of the Evans blue–albumin complex is seen (red) in the treated cortex, but not in the cortex from the sham-operated contralateral window. *e*, Quantitative image analyses of sections from the left medial (sham-operated), right lateral (not-treated), and right medial (treated) somatosensory cortex ($n = 50$ sections from each region), show enhanced staining in the treated region. *f*, Photometric analysis of Evans blue–albumin content in control and treated tissue. Enhanced absorbance was measured at 1 hr and 6 d after treatment. $*p < 0.05$; $**p < 0.001$ (in comparison to control values).

Drug application. Drugs were applied through addition to the bathing solution. NMDA, AMPA—KA, or GABA receptors were blocked by 50 μM 2-amino-5-phosphovaleric acid (APV), 10 μM 6-cyano-7-nitroquinoxyline-2, 3-dione (CNQX), or 5 μM bicuculline, respectively. All drugs were obtained from Sigma-Aldrich (Deisenhofen, Germany).

Data acquisition and analysis. Signals were amplified (SEC-10L; NPI Electronics, Tamm, Germany), filtered at 2 kHz, displayed on an oscilloscope, digitized on-line (CED-1401; Cambridge Electronics Design, Cambridge, UK), and stored for off-line analysis. Data are expressed throughout as means ± SEM. Differences between treated and control slices were determined by ANOVA, whereas the effect of pharmacological agents was tested using the Wilcoxon signed rank nonparametric tests for related variables. All statistical tests were performed using SPSS 10.1 for Windows. $p < 0.05$ was taken as the level of statistical significance.

Results

Focal cortical BBB disruption

To study the consequences of a lasting focal BBB lesion, we established a model for unilateral opening of the BBB in the medial (trunk) region of the rat somatosensory cortex. The BBB lesion was induced by direct perfusion of the exposed cortex with aCSF containing either DHC ($n = 19$ animals) or DOC ($n = 27$) (Greenwood et al., 1991). Injection of the dye Evans blue, which quickly binds to serum albumin, confirmed the presence of BBB opening in all cortical layers of the perfused region (Fig. 1*a*,*b*). In most cases Evans blue was confined to the cortical region under

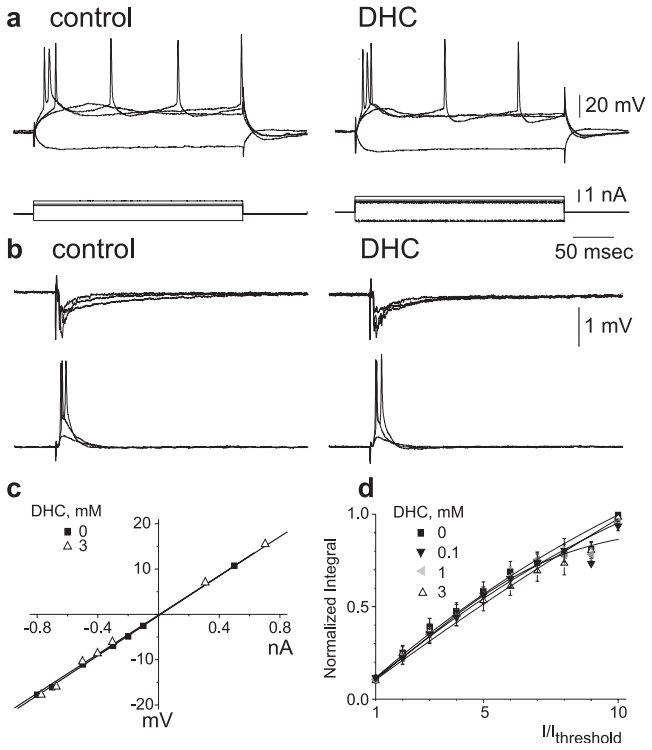

Figure 2. Bile salts do not induce neuronal toxicity or epileptiform activity *in vitro*. *a,* Intracellular recording from a layer V neuron in response to injected current pulses under aCSF (control) and 40 min under 3 mM DHC. *b,* Simultaneous extracellular (top trace) and intracellular (bottom trace) recordings from the same neuron as in *a*, in response to white matter stimulation at increasing intensities. *c, I–V* curve before and after wash-in of DHC. The input resistance was unaltered. *d,* Quantitative analysis of the field potential integral in seven slices at different stimulus intensities and different concentrations of DHC. Responses were normalized to control values for each slice.

the bone window, although in few cases a more extended region showed BBB disruption probably caused by diffusion of the bile salt. Evans blue fluorescence was analyzed microscopically in histological sections as well as in brain homogenates to determine albumin extravasation from capillaries to the brain parenchyma (Fig. 1*c–e*). Quantitative analysis confirmed a significantly increased staining of treated cortices relative either to nontreated regions of the same hemisphere or to a sham-operated contralateral cortex under a similar bone window perfused with aCSF (Fig. 1*e*) ($n = 5$ animals). Significant BBB opening was demonstrated 60 min after bile salt perfusion ($n = 5$ animals), and was still evident 6, but not 16 d after treatment (Fig. 1*b,f*) ($n = 6$). To further exclude direct neuronal toxicity of the bile salts, we recorded from cortical slices maintained *in vitro* in the presence of similar bile salt concentrations. Intracellular recordings were obtained from 4 layer II and 4 layer V regular spiking neurons 30–60 min during wash-in of 3 mM DHC. Stable resting potential (-73.3 ± 1.4), input resistance (37.8 ± 4.2 MΩ in aCSF vs 38.1 ± 4.6 MΩ after DHC), firing properties to threshold and suprathreshold stimulation and evoked synaptic responses to extracellular stimulation were noted (Fig. 2). Extracellular recordings further revealed no change in evoked neuronal activity under a wide range of bile salt concentrations, even when neurons were exposed to higher concentrations (4–6 mM) of bile salts than those used in this study to open the BBB (Fig. 2*b,d* results for DOC not shown) ($n = 15$ slices in 11 animals). In higher concentrations (>10 mM), bile salts caused a reduction in the amplitude of evoked responses but never induced epileptiform activity. Because no differences were found between the effects of DHC and DOC on the BBB opening or on cortical electrophysiological responses, data from both experimental procedures were combined in the remainder of the study.

Electrophysiological activity in the BBB-disrupted cortex

To test the long-term effects of BBB disruption, we recorded electrophysiological activity from 102 neocortical slices (41 animals) at different time intervals (2 hr to 49 d) after salt application. In a control neocortical slice, a brief stimulation of the white matter evoked a typically fast (<50 msec) synaptic response. This response represents the averaged fast excitatory and inhibitory postsynaptic potentials sequence, which characterizes the neocortical responses to intracortical stimulation in the *in vitro* slice preparation (Connors et al., 1982). Calculation of the amplitude and the integral of the early evoked potentials (<50 msec after stimulus) showed a linear increase with increasing stimulus intensity in both treated and sham-operated slices ($n = 32$ and 19 slices, respectively). In treated slices, the maximal slope of the response–intensity curve was significantly larger ($p < 0.05$) than that in slices from sham-operated rats (Fig. 3*d*) and was associated with a decreased time-to-peak (1.10 ± 0.27 msec in DOC-treated slices vs 2.33 msec in sham-operated; $p < 0.01$), suggesting increased local excitability. From the fourth day after opening the BBB, the early evoked response was followed by a paroxysmal all-or-none, long-duration (100–500 msec) field potential (Fig. 3*b*). Such abnormal activity was observed in 64 of 93 slices from treated rats (68.8% of the slices; 33 of 36 animals) and was always limited to the treated cortical region. In contrast, paroxysmal events were observed in only 2 of 19 slices from sham-operated rats (10.5% of the slices; one of six animals; Pearson χ^2; $p < 0.001$). Simultaneous recordings from extracellular and intracellular electrodes separated by ~300 μm showed paroxysmal responses to be generated synchronously in an extended region of the treated cortex (Fig. 3*c*). The integral of the late paroxysmal responses (50–500 msec after stimulus artifact) was significantly larger in treated slices under all stimulus intensities but was most pronounced under low intensity stimulation ($<5\times I_{threshold}$) (Fig. 3*e*). Paroxysmal activity was synchronously evoked in all cortical layers of the same vertical axis (i.e., cortical column, data not shown), and although it could be triggered by stimulation of neighboring nontreated cortical columns and propagated along the treated region (3–5 mm), it never propagated to the healthy cortex (Fig. 3*a*). Spontaneous interictal-like activity was recorded in slices from 4 of 13 treated rats, but never in sham-operated ones (see below and see Fig. 5*e*).

Neurotransmission underlying epileptiform activity

To further investigate the transmitter systems that underlie paroxysmal events, we exposed treated slices to antagonists of the main synaptic receptors. A blocker of the AMPA–KA-mediated receptor, CNQX (10 μM), significantly reduced the amplitude of both the early nonparoxysmal and late paroxysmal responses (reduction to 51 and 20% of control values respectively, within 20 min perfusion; $p < 0.05$) (Fig. 4*a,b*). The NMDA antagonist APV (50 μM), reversibly reduced the amplitude of paroxysmal responses to 35% of their original values (Fig. 4*c,d*). However, this effect was not complete because in most slices small paroxysmal events could still be evoked, even after 60 min of perfusion with the antagonist, by increasing the stimulus intensity or by paired stimulation (data not shown). Unlike CNQX, APV had no effect on the early evoked response, emphasizing the different nature of each response.

In several dual intracellular and extracellular recordings in the BBB-treated cortex, the population paroxysmal activity was presented as a depolarizing potential with an apparent reversal below the spike threshold (Fig. 5*a*), suggesting the participation of GABA-mediated Cl^- potentials in cortical hypersynchrony. Indeed, the addition of the GABA-A antagonist bicuculline to the perfusion solution, resulted in large, long-duration field potentials as expected (Gutnick et al., 1982). Importantly, simultaneous recordings in treated and nontreated regions of the same slice showed that the effect of bicuculline (5 μM) is more pronounced in the treated region (Fig. 5*b*). This result suggests that enhanced excitatory transmission is efficiently suppressed by GABA-mediated inhibition. The involvement of GABA-mediated inhibition in epileptiform activity was further supported by the effect of bicuculline. Both spontaneous (Fig. 5*c*) and evoked activity (Fig. 5*d*,*e*) were initially suppressed by the addition of bicuculline to the aCSF. Figure 5*e* shows quantitative analyses of the area under late paroxysmal events, demonstrating a decrease in all slices to a mean of 47% of their original values 10 min after the addition of bicuculline ($n = 4$). With increasing perfusion time (corresponding to increasing bicuculline concentrations), paroxysmal responses were replaced by high-amplitude, long-duration potentials (Fig. 5*b*,*d*).

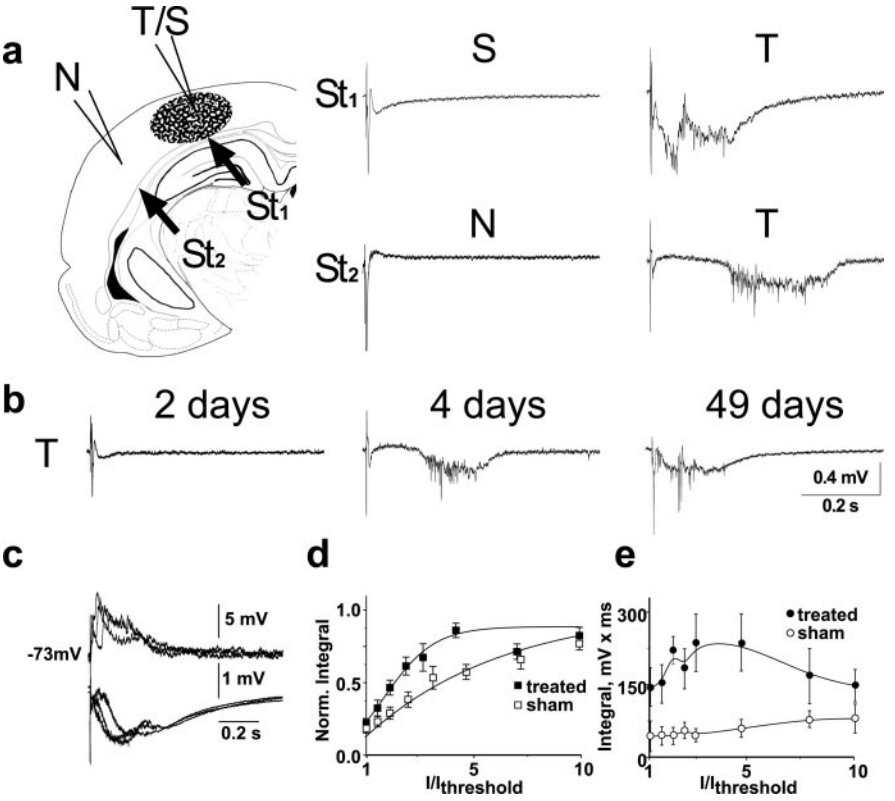

Figure 3. Electrophysiological responses in BBB-disrupted cortex. *a,* Electrophysiological recordings were performed in slices from BBB-treated as well as nontreated cortical regions from operated and sham-operated animals. In the BBB-treated region the typical potential evoked in response to white matter stimulation (St_1) showed an early, short-duration potential followed by a late, long-duration, event with a variable latency. Stimulation in the white matter outside the treated region (St_2) often elicited delayed paroxysmal activity limited to the treated region. *b,* Paroxysmal events were first recorded in slices 4 d after treatment. Scaling refers to *a* and *b*. *c,* Overlapping traces in response to three consecutive extracellular stimuli (0.1 Hz); extracellular (bottom) and intracellular recordings (top) show that synaptic paroxysmal responses are evoked synchronously. Interelectrode distance was ~300 μm. *d, e,* The integral of the early (*d*, <50 msec after stimulus artifact, values for each slice were normalized to the maximal response) and late (*e*, 50–500 msec) evoked responses in slices from treated (filled symbols) and sham-operated (open symbols) cortices under increasing stimulus intensity (normalized to threshold intensity: $I_{threshold}$). *T*, Treated; *S*, sham-operated; *N*, nonoperated.

Mechanisms underlying the generation of an epileptic focus

We hypothesized that cortical dysfunction after BBB opening results from exposure of the brain to serum components. To exclude the possibility that this was a specific bile salt effect, direct perfusion of the rat cortex with rat serum was performed. One week after treatment 67% of the slices ($n = 18$ slices; four animals) showed paroxysmal activity. Similar results (77%; $n = 13$; four animals) were obtained after cortical exposure to denatured serum (incubated at 70°C for 1 hr), or to 0.4, 0.2, or 0.1 mM albumin in aCSF (100, 50, and 25%, respectively, of serum albumin levels). Decreasing albumin concentrations to 10% of serum levels resulted in small paroxysmal events in 4 of the 25 (16%; $n =$ 5 animals) slices tested, not significantly different from sham-operated controls (Fig. 6*b*). Albumin penetrance to the cortex was verified by microscopically examining cortical sections treated with albumin–Evans blue containing aCSF (Fig. 6*c*). A solution containing serum levels of electrolytes (aSERUM) revealed normal responses in all treated slices (Fig. 6*a*,*b*). To test the possibility that increased albumin concentrations at the brain extracellular space may open the BBB, we measured Evans blue–albumin penetration into the brain after focal perfusion of an aCSF-containing albumin (0.1 mM) solution. No significant increase in the penetrance of Evans blue–albumin complexes to the brain was evident in all but one animal ($n = 6$) locally perfused with albumin-containing (0.1 mM) solution as detected by quantitative analyses of photomicrographic images and quantitative fluorometry evaluation. Thus, we can exclude a direct effect of albumin on BBB permeability (Fig. 6*c*,d).

Because our data suggest that the most abundant serum protein, albumin, induces epileptogenesis, we examined the direct effect of albumin on extracellular and intracellular activity. Perfusion of cortical slices *in vitro* with albumin for up to 2 hr did not induce apparent changes in evoked activity. Intracellular recordings for up to 1 hr in the presence of 0.1 mM serum albumin showed a stable resting potential (-74.9 ± 1.2 mV; $n = 8$) with no change in input resistance (45.5 ± 10.5 vs 45.7 ± 10.2 MΩ; $p > 0.1$), firing properties or evoked synaptic potentials ($n = 8$). Our data thus suggest that epileptogenesis after BBB opening is associated with slow processes regulated at the transcriptional–translational level, triggered by the increased concentration of proteins in the brain extracellular fluid (see Discussion).

We further used histological stainings to detect the penetration of cellular elements and/or cell loss after bile salts or albumin treatments. Histological sections were made from animals 1–14 d after either treatment (1–2 d, $n = 6$; 4–6 d, $n = 4$; 9–14 d $n = 3$). Standard hematoxylin–eosin or the vanadium acid fuchsin-toluidine blue method–sensitive for acidophilic injured neurons (Victorov et al., 2000)–showed slight brain edema, mainly in the first 48 hr after treatment but did not reveal any loss of the normal

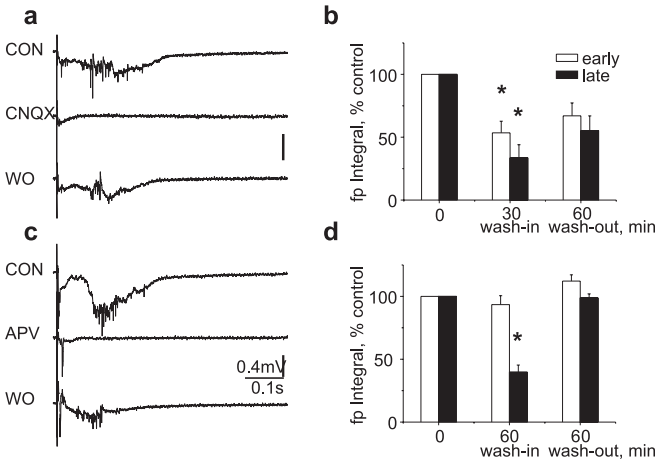

Figure 4. AMPA–KA or NMDA receptor antagonists blocked paroxysmal responses. *a, b,* Slices showing paroxysmal activity under aCSF (CON) were perfused with 30 μM of CNQX to block the AMPA–KA receptors. The abolition of late paroxysmal events (middle trace and black bars) and the significant reduction of early potentials (white bars) are shown by measuring the integral of the maximal response. This effect was partially reversible. *c, d,* NMDA receptors were blocked by APV (30 μM). Although early PSPs were not affected, late paroxysmal activity was significantly reduced. This effect was fully reversible. *$p < 0.05$ (in comparison to control values).

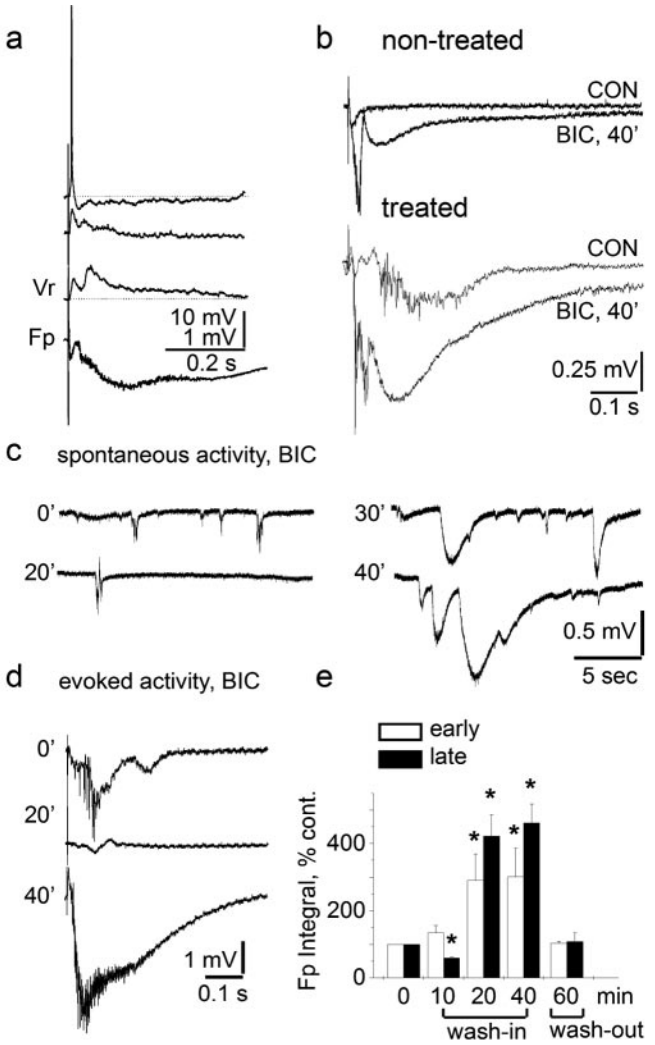

Figure 5. Paroxysmal activity is associated with GABA-mediated inhibition: *a,* Simultaneous intracellular (top traces) and extracellular recordings (Fp, bottom traces) are shown in response to extracellular stimulation. Responses to consecutive stimulation are shown at different membrane voltages in relation to the resting potential ($V_r = -70$ mV). *b,* Simultaneous extracellular recordings from the nontreated and treated somatosensory cortex of the same slice. Superimposed traces are shown under aCSF (CON) and 40 min after the addition of bicuculline (BIC). Note the enhanced negative deflection in the field potential recorded in the treated region of the slice. *c,* Recordings from a slice with spontaneous interictal-like epileptiform activity during perfusion of bicuculline-containing aCSF. Note the initial suppression followed by increase in frequency of appearance and amplitude of spontaneous activity. *d,* Extracellular recordings during bicuculline perfusion were associated with an initial reduction in duration and amplitude of paroxysmal activity (20′) followed by the appearance of large field potential (40′). *e,* Quantitative analyses of the integral of early and late evoked responses under bicuculline ($n = 4$; see Results). *$p < 0.05$ (in comparison to control values).

cortical structure or evidence for neuronal injury (Fig. 7*a,b*). Furthermore, no decrease in cell number or penetration of red or white blood cells could be detected in the treated region (Fig. 7*e*). Inflammatory cells were not detected in the treated cortex at a level significantly different from the nontreated contralateral hemisphere by immunostainings with antibodies directed against CD40 and GFS-IB4. In contrast, a prominent enhanced GFAP staining, suggestive of activated astrocytes, was observed in both DHC and albumin-treated cortices from day 1 after treatment. A significant increase in the number of counted GFAP cells was found 2 and 14 d after treatment (11 and 6.02% of all counted cells respectively, compared with 2.05% in the contralateral nontreated region; $p < 0.05$) (Fig. 7*b,c*). These data suggest that opening the BBB resulted in the penetration of proteins into the brains extracellular space followed by a rapid astrocytic response and delayed epileptogenesis (see Discussion).

Discussion

Our data have shown that: (1) prolonged BBB disruption induces delayed cortical dysfunction characterized by epileptiform paroxysmal hypersynchronous activity; (2) abnormal paroxysmal activity is focal and restricted to the treated region; (3) AMPA–KA as well as NMDA and GABA receptors are involved in the generation of the epileptiform activity observed in the BBB-disrupted cortex; and (4) cortex exposure to low levels of serum albumin is sufficient to induce early and partially reversible activation of astrocytes followed by delayed onset and long lasting epileptiform activity.

The abundance of clinical and pathological data suggests that BBB disruption under various neurological conditions may underlie or contribute to the disease processes. Furthermore, most if not all common etiologies for acquired epilepsy (e.g., trauma, neurosurgery, infection and tumors; Nashef, 1996) are frequently associated with BBB opening (for review, see Ballabh et al., 2004). However, to the present there are no data available concerning if and to what extent or duration BBB disruption precedes the development of acquired epilepsy in human patients. Concern about the possible role of BBB dysregulation in the different epilepsies has been raised previously (Janigro, 1999) on the basis of ultrastructural studies. In these studies they observed increased micropinocytosis, a reduced number of mitochondria in endothelial cells, a thickening of the basal membrane, and abnormal tight junctions in human epileptic tissue when compared with controls (Kasantikul et al., 1983; Cornford and Oldendorf, 1986). In our study we found interictal-like epileptiform activity in the majority of cortical slices 4–49 d after the BBB opening, including spontaneous epileptiform discharges in a subset of slices. This suggests that BBB dysfunction may trigger cortical reorganization and focal epilepsy. Although others previously reported (Zappulla et al., 1985a,b) that either focal or intracarotid bile salt

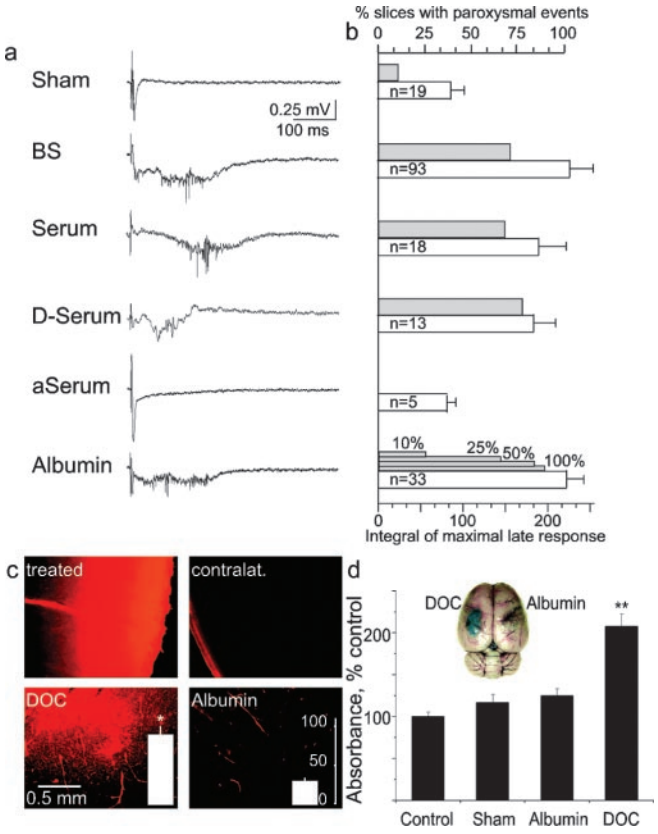

Figure 6. Mechanisms underlying BBB-induced epileptiform activity. *a,* Somatosensory cortex was perfused with aCSF (sham), bile salts (BS), serum, denatured serum (D-Serum), electrolytic solution with serum concentrations (aSerum) and serum albumin in increasing concentrations (Albumin). Representative traces from extracellular recordings 7 d after treatment are displayed. *b,* Quantification of the averaged integral of the late evoked responses in all slices (white bars) and percentage of slices in which paroxysmal activity was observed (gray bars). Albumin concentrations (100% represents serum levels) are noted. *n* refers to the number of slices examined under each experimental condition. *c,* Focal perfusion of albumin–Evans blue solution resulted in penetration of the albumin into the cortex of the ipsilateral (treated) hemisphere. In a different experiment, after intraperitoneal injection of Evans blue, extravasation of fluorescent albumin was more prominent in the DOC-treated compared with the albumin-treated hemisphere. The white bars represent averaged color intensity values ($n = 20$ sections in each experiment). *d,* Photometric quantification of Evans blue–albumin brain concentrations in nontreated (control), sham-operated, albumin and DOC-treated brains. Note that only DOC treatment caused a significant increase in dye penetration. Inset, Brain after bilateral cranial window operation and intraperitoneal Evans blue injection. The right hemisphere was perfused with albumin and the left with DOC. $^*p < 0.05$; $^{**}p < 0.01$.

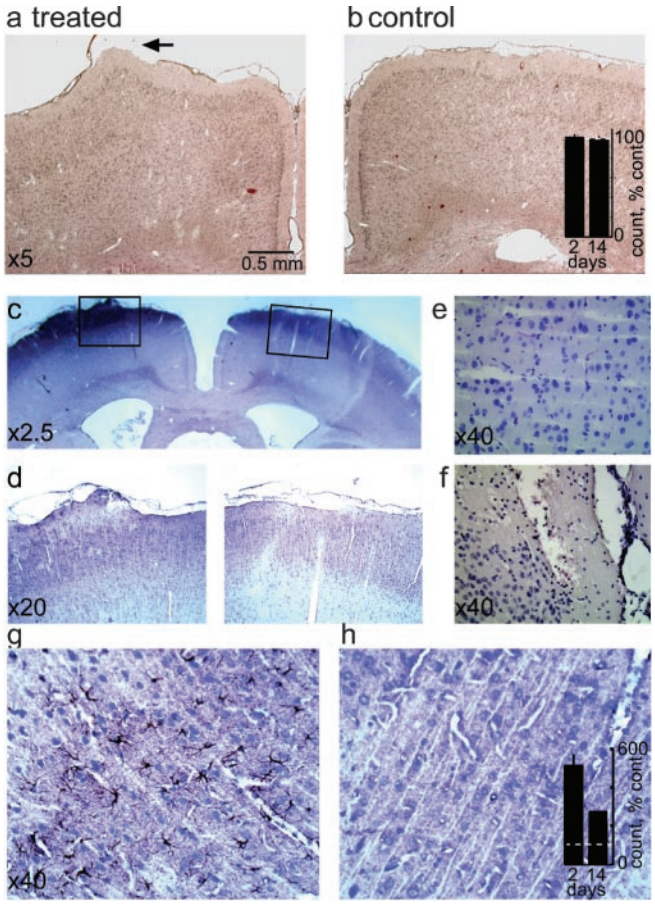

Figure 7. Focal cortical perfusion with DHC or albumin caused astrocytic activation with no neuronal loss. *a, b*, Whereas histological sections 24 hr after treatment show slight extracellular edema in the treated hemisphere, cortical structure is otherwise normal. Arrow points to the treated, exposed cortical region. Bar graphs represent cell count from histological sections 2 and 14 d ($n = 6$ each) after treatment in percentages compared with the contralateral nontreated region. *c, d*, Coronal sections 48 hr after perfusion with albumin, showing normal cortical structure under low magnification. The black boxes represent the cortical regions seen under a larger magnification in *d*. *e,* In a large magnification normal cortical structure is observed. Note a typical cortical capillary showing blood cells limited to the vessel lumen. *f,* For comparison, 24 hr after a traumatic cortical injury extravasation of blood cells is observed in the cortical tissue (for methodological details, see Eyupoglu et al., 2003). *g, h*, A prominent increase in the number of GFAP-stained astrocytes is seen in an albumin-treated cortex compared with the nontreated contralateral cortex. Bar graphs represent counted GFAP-positive cells (in percentage to the contralateral hemisphere) from histological sections 2 and 14 d ($n = 6$ each) after treatment.

application induces immediate epileptiform activity *in vivo*, those studies used a 100-fold higher concentration of the salts, which has been shown to induce direct damage to cell membranes because of their detergent effect (Greenwood et al., 1991). To eliminate and control for such effects, we used considerably lower bile salt concentrations (<3 mM), which opened the BBB with no concurrent effect on cortical structures or electrophysiological responses including passive membrane properties, firing characteristics, and synaptic responses in cortical slices maintained *in vitro* (Fig. 2). Our finding that epileptogenesis was also induced by focal application of serum, denatured serum, or albumin-containing solution, confirms our hypothesis that epileptogenesis was not a specific response to the bile salts but indeed resulted from the exposure of the brain extracellular space to serum proteins.

Cortical responses after BBB opening were typically delayed, multiphasic in appearance, had a prolonged duration (100–500 msec), and were all-or-none, paroxysmal in nature. Under low stimulation intensities they appeared in full size, and when stimulus intensity was increased, their amplitude was reduced or blocked (Fig. 3*e*). Thus, paroxysmal events recorded in the BBB-treated region were similar to those described in cortical slices from chronic animal models of epilepsies (e.g., the chronically injured cortex: Prince and Tseng, 1993; Jacobs et al., 1996; chemical kindling: Barkai et al., 1994; or pilocarpine treatment: Sanabria et al., 2002). One important feature of epileptiform activity in the treated cortices is the prominence of GABA-mediated inhibition. Intracellular recordings often showed a depolarizing inhibitory postsynaptic potential (with a reversal potential of ~60 mV) that was synchronized with the extracellular paroxysmal event. Extracellular recordings further suggest that inhibitory transmission participates in the paroxysmal events, because these were reduced in amplitude and duration and, when spontaneous, the frequency of appearance was reduced at low concen-

trations of GABA-A receptor antagonists. The enhanced glutamatergic excitation in the cortices treated with higher concentrations of the GABA-A receptor antagonist suggests that inhibitory transmission was sufficient to partially suppress enhanced excitation. These findings concur with those of previous anatomical studies of the chronically injured cortex that show an increase in symmetrical synapses on dendritic shafts (Rutledge, 1978), and with physiological studies that show an increase in inhibitory synaptic currents in layer V pyramidal neurons (Prince et al., 1997). In contrast, a recent quantitative study on the chronically injured cortex showed an increase in the ratio between the AMPA–KA receptor-mediated excitation and GABA-A receptor-mediated inhibition (Li and Prince, 2002). Future studies are required to clarify these changes in the excitatory and inhibitory receptors and in synaptic connectivity in the BBB-treated cortex, and to compare these changes with other models of epilepsy.

Cortical dysfunction after BBB disruption was observed from day 4 after treatment. Although this delay concurs with the results of previous studies that used a model of focal epilepsy in the guinea pig partially deafferented cortex (Hoffman et al., 1994), in which 1–2 weeks passed before the development of abnormal activity, this delay differs from a recent report of an isolated cortex in cats, in which epileptiform activity was observed within a few hours after injury in a trauma model (Topolnik et al., 2003). Although this may reflect methodological differences or species-specificity, it is more likely that two different mechanisms underlie the paroxysmal activity appearing acutely and that appearing days or weeks after trauma. The latter is likely to be associated with changes in gene expression and in reorganization of the local circuit. This view is supported by anatomical data showing axonal sprouting and synaptic changes in both animal (Purpura and Houdepian, 1961) and human (Castejon et al., 2002) epileptic cortex. Furthermore, clinical data demonstrate that early and delayed post-insult epilepsies differ in characteristics and responses to antiepileptic medications (Herman, 2002). It is noteworthy that different insults resulting in delayed seizures (e.g., stroke, trauma, brain infections), are all associated with a BBB opening. In the absence of an efficient method allowing to close the BBB it is difficult to design an experimental protocol that could examine the contribution of other factors (neuronal damage, devascularization, and/or deafferentation) that may contribute to cortical reorganization. However, the present study shows that BBB disruption by itself may result in cortical dysfunction that persists after BBB has returned to normal.

Many important differences between the brain extracellular fluid and blood components may be considered in initiating cortical reorganization. However, our experiments show that BBB disruption, although being associated with Evans blue–albumin penetration to the extracellular space, did not lead to a detectable inflammatory response or the accumulation of red blood cells. The role of albumin in epileptogenesis was also supported by the experiments showing that serum albumin was sufficient to induce focal epileptiform activity, in a dose-dependent manner. Because denatured serum was similarly efficient in inducing cortical dysfunction, it is suggested that the effect of albumin was most likely attributable to the increased concentration of proteins in the brain extracellular space.

The mechanisms by which increased protein levels in the extracellular space cause delayed epileptic activity are not known. Exposure of brain slices to bile salts or albumin did not trigger epileptic activity, suggesting that BBB opening-induced reorganization is not triggered by recurrent seizures. This is also supported by EEG recordings during stress-induced BBB opening (Sharma, 2004) and by our own observations (J. P. Dreier and A. Friedman, unpublished data). Also, significant cell injury or death could not account for epileptogenesis (Fig. 7), although it has been previously shown that vasogenic edema may be associated with accumulation of serum proteins in the cytoplasm of injured neurons (Loberg et al., 1994), followed by cytochrome release and DNA fragmentation (Matz et al., 2001). Although cell loss was not found, we show here robust enhanced GFAP staining restricted to the BBB-disrupted cortex. Activation of astrocytes in cultures (Perillan et al., 1999) or in freeze lesion-induced dysplastic rat neocortex (Bordey et al., 2001) has been related to decreased inward rectifying K^+ current. Interestingly, in human epileptic foci, astrocytes are intensely stained for GFAP and express an increased Na^+ to K^+ conductance ratio compared with nonepileptic human or rat cortex (Bordey and Sontheimer, 1998). Such changes in astrocytic function were suggested to cause hyperexcitability in both acute (Janigro et al., 1997) and chronic (Heinemann et al., 2000) epileptic tissue. Importantly, astrocytic activation in the BBB-treated cortex was prominent 24 hr after treatment, when epileptic activity was not yet recorded. This is consistent with recent studies showing reactive astrocytes together with impaired K^+ buffering in the hippocampus of rats 2 d after fluid-percussion injury (D'Ambrosio et al., 1999). Future studies are needed to explore the role of early activation and dysfunction of astrocytes in epileptogenesis.

In conclusion, this study presents the first evidence of epileptiform activity being induced by endogenous, serum-derived components accessing the neuronal network because of BBB disruption. Furthermore, our data show that serum albumin is sufficient to induce a delayed epileptic focus and stress the importance of the BBB as a new target for research into the prevention and treatment of partial epilepsy.

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