Seizure-Induced Up-Regulation of P-Glycoprotein at the Blood-Brain Barrier through Glutamate and Cyclooxygenase-2 Signaling

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ABSTRACT

Increased expression of drug efflux transporters at the blood-brain barrier accompanies epileptic seizures and complicates therapy with antiepileptic drugs. This study is concerned with identifying mechanistic links that connect seizure activity to increased P-glycoprotein expression at the blood-brain barrier. In this regard, we tested the hypothesis that seizures increase brain extracellular glutamate, which signals through an N-methyl-D-aspartate (NMDA) receptor and cyclooxygenase-2 (COX-2) in brain capillaries to increase blood-brain barrier P-glycoprotein expression. Consistent with this hypothesis, exposing isolated rat or mouse brain capillaries to glutamate for 15 to 30 min increased P-glycoprotein expression and transport activity hours later. These increases were blocked by 5H-dibenzo[a,d]cyclohepten-5,10-imine (dizocilpine maleate) (MK-801), an NMDA receptor antagonist, and by celecoxib, a selective COX-2 inhibitor; no such glutamate-induced increases were seen in brain capillaries from COX-2-null mice. In rats, intracerebral microinjection of glutamate caused locally increased P-glycoprotein expression in brain capillaries. Moreover, using a pilocarpine status epilepticus rat model, we observed seizure-induced increases in capillary P-glycoprotein expression that were attenuated by administration of indomethacin, a COX inhibitor. Our findings suggest that brain uptake of some antiepileptic drugs can be enhanced through COX-2 inhibition. Moreover, they provide insight into one mechanism that underlies drug resistance in epilepsy and possibly other central nervous system disorders.

Up to 40% of epileptic patients respond poorly if at all to conventional pharmacotherapy, and impaired drug uptake into the brain is considered to be one important contributor to therapeutic failure (Loscher and Potschka, 2005; Kwan and Brodie, 2006). Seizures are known to increase the expression of drug efflux transporters at the blood-brain barrier, and recent experiments in animal models of epilepsy show that brain uptake of antiepileptic drugs can be significantly improved by coadministration of tariquidar, a selective and potent inhibitor of the ATP-driven drug efflux pump, P-glycoprotein (Brandt et al., 2006; van Vliet et al., 2006). Together, these findings point to increased P-glycoprotein expression as one consequence of seizure activity that limits pharmacotherapy with antiepileptic drugs.

The present study is concerned with mechanistic links that connect seizure activity to increased P-glycoprotein expression. Our goals are to identify therapeutic targets that can be manipulated to prevent seizure-induced transporter overexpression and to improve pharmacotherapy with antiepileptic drugs. The combined in vitro/in vivo experiments are focused

ABBREVIATIONS: COX, cyclooxygenase; aCSF, artificial cerebrospinal fluid; CNS, central nervous system; Glut-1, glucose transporter 1; NBD-CSA, [N-ε-(4-nitrobenzofurazan-7-yl)-D-Lys[8]]cyclosporin A; NMDA, N-methyl-D-aspartic acid; PBS, phosphate-buffered saline; TBS, tris-buffered saline; P-gp, P-glycoprotein; EEG, electroencephalogram; MK-801, 5H-dibenzo[a,d]cyclohepten-5,10-imine (dizocilpine maleate); NS-398, N-[2-(cyclohexyloxy)-4-nitrophenyl]-methane sulfonamide; SC-560, 5-(4-chloro-phenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole; PSC833, cyclo(((2S,4R,6E)-4-methyl-2-(methylamino)-3-oxo-6-octenoyl)-L-valyl-N-methylglycyl-N-methyl-L-leucyl-L-valyl-N-methyl-L-leucyl-L-alanyl-D-alanyl-N-methyl-L-leucyl-N-methyl-L-leucyl-N-methyl-L-valyl).

on glutamate and cyclooxygenase-2 (COX-2) signaling. Our hypothesis is that seizures increase extracellular glutamate, which signals through the NMDA receptor and COX-2 in brain capillaries to increase blood-brain barrier P-glycoprotein expression. The following observations led us to this hypothesis. First, glutamate released from neurons during an epileptic seizure accumulates in brain interstitial fluid (Holmes, 2002), and glutamate has been shown to increase P-glycoprotein expression in rat brain endothelial cells (Zhu and Liu, 2004). Second, enhanced glutamatergic signaling increases COX-2 (Strauss and Marini, 2002), which reaches its highest expression levels in the early phase after an epileptic seizure (Voutsinos-Porche et al., 2004; Lee et al., 2007). Third, in rat renal mesangial cells, COX-2 proved to be a potent inducer of P-glycoprotein (Patel et al., 2002).

The present in vitro results show that exposing isolated rat or mouse brain capillaries to glutamate increased both P-glycoprotein expression and transport activity. These increases were blocked by an NMDA receptor antagonist and by a selective COX-2 inhibitor; no such glutamate-induced increases were seen in brain capillaries from COX-2-null mice. Our in vivo results are consistent with these findings. In rats, intracerebral microinjection of glutamate caused locally increased brain capillary P-glycoprotein expression. Moreover, using a pilocarpine-induced status epilepticus rat model, we observed seizure-induced increases in brain capillary P-glycoprotein expression that could be prevented by the administration of a COX inhibitor.

Materials and Methods

Chemicals. Glutamate, NMDA, pilocarpine, actinomycin D, cycloheximide, indomethacin, and MK-801 were purchased from Sigma (St. Louis, MO). Celecoxib was from LKT Laboratories (St. Paul, MN), and NS-398 and SC-560 were from Cayman Chemical (Ann Arbor, MI). Mouse monoclonal C219 antibody to P-glycoprotein was purchased from Signet Laboratories (Dedham, MA), mouse monoclonal β-actin antibody was from Abcam (Cambridge, MA), rabbit polyclonal COX-2 antibody was from Cayman Chemical, and mouse monoclonal NMDA receptor antibody was from Upstate Biotechnology (Billerica, MA). NBD-cyclosporin A (NBD-CSA) was custom-synthesized by R. Wenger (Basel, Switzerland) (Schramm et al., 1995). PSC833 was a kind gift from Novartis (Basel, Switzerland). All other chemicals were of highest analytical grade and were obtained from commercial sources.

Animals. Animals used were male retired breeder Sprague-Dawley rats (500–600 g; Taconic, Germantown, NY), female Wistar Unilever rats (200–220 g; Harlan-Winkelmann, Borchen, Germany, and Harlan Netherlands, Horst, The Netherlands), male COX-2 knockout and wild-type mice (30–50 g; C57BL/6, 129P2-Ptgs2tm1UNC; Taconic). Animals were kept under controlled environmental conditions (24–25°C, 50–60% humidity, 12-h dark/light cycle) with free access to tap water and standard feed. Before using animals for experiments they were allowed to adapt to the new environment for at least 1 week. All housing and dosing protocols were approved by the Institutional Animal Care and Use Committees of the University of Munich and the National Institute of Environmental Health Sciences/National Institutes of Health and were in accordance with the German Animal Welfare Act and the National Institute of Environmental Health Sciences/National Institutes of Health guidelines.

Brain Capillary Isolation. Brain capillaries were freshly isolated according to a protocol described previously (Hartz et al., 2004, 2006; Bauer et al., 2007). In brief, animals were euthanized by CO_2 inhalation and decapitated; brains were immediately put in ice-cold PBS buffer (2.7 mM KCl, 1.46 mM KH_2PO_4, 136.9 mM NaCl, 8.1 mM

Na_2HPO_4, supplemented with 5 mM D-glucose, and 1 mM sodium pyruvate, pH 7.4). Brains were dissected by removing meninges, choroid plexus, and white matter and then homogenized in PBS. The homogenate was mixed with Ficoll (final concentration, 15%; Sigma) and centrifuged at 5800g for 20 min at 4°C. The capillary pellet was suspended in 1% bovine serum albumin-PBS, and the capillary suspension was passed over a glass-bead column. Capillaries were collected by gentle agitation in 1% bovine serum albumin-PBS and washed with PBS. For in vitro experiments, freshly isolated brain capillaries were exposed to glutamate for 30 min, washed, and left to incubate in glutamate-free buffer for 5.5 h. After a total of 6 h, capillaries were used for transport experiments, plasma membrane isolation followed by Western blotting, or immunohistochemistry.

P-Glycoprotein Transport Activity. P-glycoprotein transport activity in isolated brain capillaries was assessed as described previously (Hartz et al., 2004, 2006; Bauer et al., 2007). Capillaries were incubated in confocal imaging chambers for 1 h at room temperature with 2 μM concentration of the fluorescent P-glycoprotein substrate, NBD-CSA. For each treatment, confocal images of 10 to 15 capillaries were acquired (Zeiss LSM 510 META inverted confocal microscope or Zeiss LSM 410 inverted confocal microscope, 40× water immersion objective, numerical aperture = 1.2) and luminal NBD-CSA fluorescence intensity was quantitated using Zeiss Image Examiner (Carl Zeiss GmbH, Göttingen, Germany) software or Scion Image software (Scion Corporation, Frederick, MD). Specific luminal NBD-CSA fluorescence was taken as the difference between the total luminal fluorescence and fluorescence in the presence of the P-glycoprotein-specific inhibitor PSC833. This difference provides a measure of specific P-glycoprotein transport activity in the capillaries.

Capillary Membrane Isolation and Western Blotting. Capillaries were homogenized in mammalian tissue lysis buffer (Sigma) containing protease inhibitor cocktail (Roche, Mannheim, Germany). Samples were centrifuged at 10,000g for 15 min, and denucleated supernatants were centrifuged at 100,000g for 90 min. Pellets (crude plasma membranes) were resuspended, and protein concentrations were determined. Western blots were performed using the NuPage electrophoresis and blotting system (Invitrogen, Carlsbad, CA). Membranes were incubated overnight with antibody to P-glycoprotein (1:100, 1 μg/ml), β-actin (1:1000, 1 μg/ml), COX-2 (1:1000, 0.5 μg/ml), and NR-1 (1:750, 1 μg/ml). Membranes were then washed and incubated with the corresponding horseradish peroxidase-conjugated ImmunoPure secondary antibody (1:15,000; Pierce, Rockford, IL). Proteins were detected using SuperSignal West Pico Chemoluminescent Substrate (Pierce), and protein bands were visualized with a Gel Doc XRS imaging system (Bio-Rad Laboratories, Hercules, CA). In preliminary experiments, we measured immunoreactive P-glycoprotein signal (integrated band intensity) as a function of the amount of membrane protein applied and found an approximately linear relationship (data not shown).

Hippocampal Glutamate Microinjection and Behavioral Evaluation. Guide cannulae were implanted in 16 female Wistar Unilever rats that had been anesthetized with chloral hydrate (360 mg/kg, i.p.). All rats were unilaterally implanted into the right hippocampus with stainless steel cannulae (outer diameter, 640 μm; inner diameter, 400 μm) aimed at 1 mm above the intended injection site. The stereotaxic coordinates used in millimeters relative to Bregma were AP -3.0, L -1.6, DV -3.5. In two animals, a bipolar electrode was implanted directly adjacent to the guide cannula to allow EEG recordings. To secure the mounted material, cannulae were embedded in dental acrylic cement. Cannulae were closed full-length by removable mandrins before and after microinjection.

After a recovery period of at least 1 week after cannulae implantation, microinjections were performed as described previously (Pekcec et al., 2007) using a modified protocol in unanesthetized, freely moving rats. For microinfusion, an injection cannula (outer diameter, 350 μm; inner diameter, 150 μm) was attached to a 2-μl Hamilton syringe with a polyethylene tube, and the cannula was inserted into the hippocampus through the implanted guide. Sterile artificial cerebrospinal fluid (aCSF; Sigma, Taufkirchen, Germany)

(n = 7) or 5 nmol glutamate (in 300 nl of aCSF) (n = 9) was microinfused during a period of 3 min (100 nl every minute) to the right hippocampus. An infusion volume of 300 nl was ensured by monitoring the movement of a small air bubble in the tubing. One rat from the aCSF group was excluded at this point, because appropriate dosing was not ensured. In the remaining animals, the injection cannula was removed after a delay of 3 min to avoid fluid drainage from the injection site. The guide cannula was then closed by the mandrins, and rats were placed into an open field for evaluation of behavior.

Behavior was monitored continuously during the next 2 h and then at intervals of 15 min for up to 4 h. EEG recordings were obtained from two animals before microinjection, 5 min and 15 min after microinjection of glutamate, and then every 15 min for up to 2 h. After vehicle injection, no changes in the behavior of the rats were observed. Rats that received glutamate microinjections exhibited phases of hyperactivity with stereotypic grooming, sniffing, and phases of behavioral arrest. Wet dog shakes were observed in two animals, and myoclonus of the head-neck area was observed in one animal. None of the animals exhibited generalized seizure activity. Electrographic recordings in the two animals that received glutamate injections did not reveal any abnormal patterns compared with the basal EEG (i.e., no signs of hypersynchronicity and epileptiform discharges were detected).

One day after microinjection, rats were decapitated, and the brains were immediately removed, embedded in Tissue Freezing Medium (Jung, Nussloch, Germany), and frozen in liquid nitrogen. Frozen brains were stored at $-80°C$, cut at 14 μm using a cryostat (HM 560 M; Microm, Walldorf, Germany), and sections were mounted onto Histo-Bond adhesion slides (Marienfeld, Lauda-Koenigshofen, Germany).

Induction of Status Epilepticus with Pilocarpine. Female Wistar Unilever rats received either injections of indomethacin (2.5 mg/kg i.p. in 5% sodium bicarbonate) or of vehicle twice daily for 3 days. A status epilepticus was induced by repeated pilocarpine administration on the second day. For induction of status epilepticus lithium chloride (127 mg/kg i.p.; Sigma) was administered 12 h and methyl-scopolamine (1 mg/kg i.p.; Sigma) was administered 30 min before pilocarpine. As described previously (Glien et al., 2001), pilocarpine (Sigma) was given intraperitoneally (10 mg/kg) every 30 min until the onset of convulsive status epilepticus consisting of ongoing generalized convulsive seizures. The total number of pilocarpine injections was limited to 10 per animal, and seizure activity was monitored behaviorally. Status epilepticus was terminated after 90 min by injection of diazepam (10 mg/kg). If seizure activity continued, diazepam administration was repeated up to three times. Only rats displaying continuous convulsive seizure activity during status epilepticus were used for further analysis. Control rats were treated similarly, but saline was given instead of pilocarpine and methyl-scopolamine.

Eighty-nine percent of indomethacin-treated animals (n = 16 of 18) developed a status epilepticus in response to repeated injections of the cholinomimetic pilocarpine. In the vehicle-treated group, a status epilepticus was successfully induced in 67% of the animals (n = 8 of 12). No significant difference was observed in the amount of pilocarpine required to induce a status epilepticus. In indomethacin-treated rats, a mean pilocarpine dosage of 42.5 ± 6.5 mg/kg (mean ± S.E.M.) was administered before onset of status epilepticus. Vehicle-treated rats required a mean dosage of 52.5 ± 11.1 mg/kg pilocarpine (mean ± S.E.M.). The severity of the status epilepticus was comparable in both groups of rats. Once a second generalized seizure was observed, the rats exhibited ongoing generalized seizure activity continuing until administration of diazepam. Comparison of the mortality rate during status epilepticus revealed no significant differences between both groups (Fisher's exact, p = 0.6214).

Two days after termination of status epilepticus, animals were deeply anesthetized with chloral hydrate and were transcardially perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline, pH 7.4. Brains were removed, transferred to 30% sucrose, and stored at 4°C. Later, 40-μm coronal sections were cut on a dry ice-cooled block on a sliding microtome (Frigomobil, Leica, Germany). Sections were stored at $-20°C$ in cryoprotecting solution (glycerol and 0.1 M phosphate buffer, pH 7.4; 1:1 in volume). Cannulae localizations were verified in thionin-stained brain sections.

Immunohistochemistry of Brain Sections. Brain sections of all rats were processed simultaneously to obtain comparable staining intensity. Analysis of P-glycoprotein (P-gp)-expression was performed using a monoclonal mouse antibody (C219; 1:100; Calbiochem, Darmstadt, Germany). The immunoreaction was visualized by means of a nickel-intensified diaminobenzidine reaction using a protocol described previously (Volk et al., 2004). The brain-type glucose transporter (Glut-1) was immunostained using a polyclonal rabbit antibody against Glut-1 (1:500; Chemicon, Hofheim, Germany) as described recently (Volk et al., 2004). Evaluation of neurodegeneration was performed in NeuN-stained sections. NeuN immunohistochemistry was processed as follows (Pekcec et al., 2007). Sections were washed with 0.05 M Tris-buffered saline (TBS), pH 7.6, incubated in 0.5% TBS-buffered H_2O_2 for 30 min, washed with TBS, incubated for 1 h in blocking solution containing 2% bovine serum albumin, 0.3% Triton X-100, and 5% normal rabbit serum (Dako Deutschland GmbH, Hamburg, Germany)), and transferred into primary antiserum (mouse anti-NeuN; 1:500, Chemicon) and incubated overnight at 4°C. The next day, sections were washed with TBS, incubated for 1 h in secondary antiserum (1:500 biotinylated rabbit anti-mouse; Dako), rinsed again in TBS, incubated for 60 min in horseradish peroxidase-labeled streptavidin (1:375; Dako). After washing with TBS, the nickel-intensified diaminobenzidine reaction (0.05% 3,3-diaminobenzidine, 0.01%, nickel ammonium sulfate; both from Sigma, and 0.01% H_2O_2) was performed. Finally, all sections were washed, mounted onto glass slides, air-dried, dehydrated, and coverslipped with Entellan (Merck, Darmstadt, Germany).

Histological Evaluation and Image Analysis. The area labeled for P-glycoprotein (labeled surface area) was analyzed using a computer-assisted image analysis system, as described in detail previously (Volk et al., 2005). The hardware consisted of an Axioskop microscope with a Plan-Neofluar lens (Zeiss), a single chip charge-coupled device color camera (Axiocam; Zeiss), and a Pentium III-based computer equipped with an image capture interface card (V7-Mirage; Spea, Tyler, TX). For analysis of brain sections, a 400× magnification was used. The captured images were 1300 × 1030 pixels in dimension and were processed using KS400 image analysis software (Windows Release 3.0; Carl Zeiss Vision). Detailed image analysis methodology has been published previously and validated for P-glycoprotein using several seizure models (Volk et al., 2004, 2005). It is based on initially defining a signal threshold value before analysis and using that threshold for all sections within an experiment (Volk et al., 2005). Thus, the data reported reflect the density of pixels above the background/threshold. Given that capillaries comprise a small fraction of brain volume and that P-glycoprotein expression is highest in capillary endothelial cells, we expect only a small percentage of pixels to show staining above the threshold value. P-glycoprotein expression was investigated in different hippocampal subfields of six sections (two per section level: -2.3, -3.8, and -5.8 relative to bregma) per animal. The area labeled for P-glycoprotein was evaluated using 3 to 10 fields of 43,434 μm^2 per hippocampal subfield. In all experiments, image analysis was done in an observer-blinded fashion.

For evaluation of neurodegeneration, NeuN-stained sections of the following subregions of the hippocampus were visually scored for damage: CA1, CA2, CA3a, CA3c/CA4, dentate gyrus, and hilus. Severity of neuronal damage was semiquantitatively assessed by a grading system: score 0, no obvious damage; score 1, slight lesions involving one third of neurons; score 2, lesions involving two thirds of the neurons; score 3, lesions involving more than two thirds of the neurons. In this respect, it is noteworthy that neuronal loss must exceed 15 to 20% before it is reliably detected by visual inspection.

Visual assessment was conducted by a person not aware of the treatment of the animals. Prompted by the data from visual inspection of sections, neurons were then stereologically counted in the dentate hilus. The number of NeuN-positive cells was quantified as described recently (Brandt et al., 2003; Pekcec et al., 2007). The hilus was defined by the inner edge of the granule cell layer and lines connecting the tips of the two granule cell blades to the beginning of the CA3c/CA4 pyramidal cell layer of Ammon horn. Again, all measurements were conducted by a person not aware of treatment of the animals. Hilar NeuN-positive cells were counted in at least three sections (-3.14, -3.8, and -4.8 mm relative to bregma) according to Paxinos and Watson (2005) within the boundaries of the hilus described above.

Statistical Analysis. Data are expressed as mean $\pm$ S.E.M. Statistical differences between control and treated groups were analyzed using the Mann-Whitney U test or the appropriate Student's t test, depending on the existence of Gaussian distribution (tested by the Kolmogorow-Smirnov test). Differences between means were considered to be statistically significant when $P < 0.05$.

Results

Analysis of P-Glycoprotein Activity and Expression in Brain Capillaries. We exposed isolated brain capillaries from male Sprague-Dawley rats to glutamate for a short period of time (15–30 min) and measured P-glycoprotein transporter expression and functional activity after 6 h. This protocol was designed to mimic conditions in vivo in which glutamate is released during seizures, with the neurotransmitter transiently reaching interstitial concentrations of 10 to 100 μM (Ronne-Engström et al., 1992; Ueda and Tsuru, 1995). P-glycoprotein expression was measured by Western blotting of isolated brain capillary membranes. P-glycoprotein functional activity was determined using an assay we developed previously (Hartz et al., 2004, 2006; Bauer et al., 2007). This assay is based on measuring P-glycoprotein-specific accumulation of the fluorescent cyclosporin A derivative, NBD-CSA, in brain capillary lumens using confocal microscopy and digital image analysis. Figure 1A shows a control capillary that was incubated in buffer with 2 μM NBD-CSA for 1 h (steady state). Note that fluorescence is low in the bath containing 2 μM NBD-CSA, somewhat higher in the endothelial cells, and substantially higher in the capillary lumen, indicating concentrative transport into the luminal space. Capillaries that were first exposed to 100 μM glutamate for 30 min and then 6 h later incubated with NBD-CSA for 1 h showed much higher luminal fluorescence (Fig. 1B). Luminal NBD-CSA accumulation was greatly reduced in capillaries exposed to the P-glycoprotein-specific inhibitor PSC833 (Fig. 1C). Quantitation of steady-state luminal NBD-CSA accumulation showed that PSC833 reduced luminal fluorescence to approximately 50% of control capillaries (Fig. 1D). Fluorescence remaining after inhibition of transport reflects passive diffusion and nonspecific binding of the dye to the tissue (Bauer et al., 2006, 2007; Hartz et al., 2006). Thus, the difference between total luminal NBD-CSA fluorescence and fluorescence in the presence of the inhibitor PSC833 represents P-glycoprotein-mediated transport (portion above the dotted line in Fig. 1D). This provides a measure of transport specific to P-glycoprotein and is thus an indication of transporter activity in the intact capillary. Previous studies with untreated rat brain capillaries have shown that P-glycoprotein expression and transport activity do not change over a 6- to 8-h period in control medium. However,

they do increase in parallel after exposure of capillaries to chemicals that activate the nuclear transcription factors pregnane X receptor (drugs) and nuclear factor-κB (tumor necrosis factor-α) (Bauer et al., 2004, 2006, 2007).

Glutamate Increased P-Glycoprotein Expression in Rat Brain Capillaries. Figure 2A shows that exposing isolated rat brain capillaries for 30 min to 50 to 100 μM glutamate increased in a concentration-dependent manner P-glycoprotein expression and transport activity measured after 6 h. Increasing the glutamate concentration above 150 μM reduced both P-glycoprotein expression and transport activity, suggesting vascular toxicity (data not shown). In time course experiments, we determined that glutamate exposures as short as 15 min significantly increased transporter expression and activity after 6 h (Fig. 2B); 30-min exposure to 100 μM glutamate resulted in maximal stimulation of expression and transport activity. Extending glutamate exposures for more than 30 min also reduced P-glycoprotein expression and activity, suggesting toxicity (data not shown). From these first observations we designed a standard protocol to unravel the signals connecting glutamate exposure and increased P-glycoprotein expression and activity. In this protocol, brain capillaries were exposed to 100 μM glutamate for 30 min, removed to glutamate-free medium, and then assayed for P-glycoprotein expression and transport activity
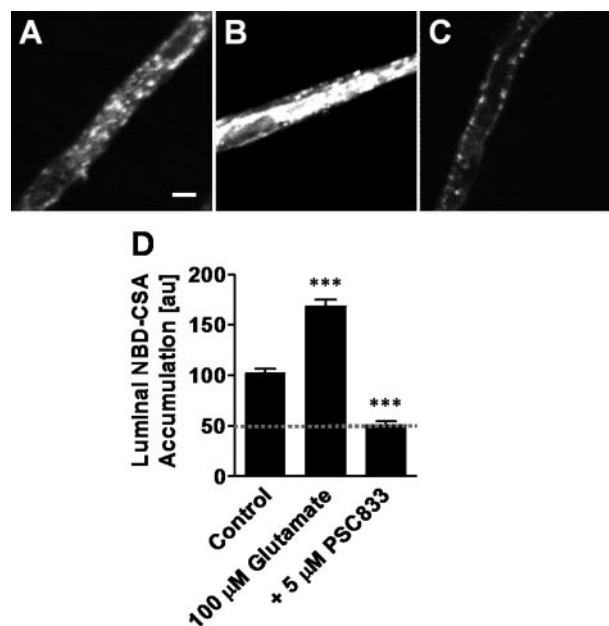

Fig. 1. P-glycoprotein transport in isolated brain capillaries. A, representative image showing steady-state NBD-CSA accumulation in the lumen of a control capillary after 1-h incubation with 2 μM NBD-CSA (scale bar, 5 μm). B, glutamate exposure significantly increases NBD-CSA accumulation in brain capillary lumens. C, inhibition of P-glycoprotein with 5 μM PSC833, a specific P-glycoprotein inhibitor, dramatically reduces luminal NBD-CSA fluorescence. D, digital image analysis of luminal fluorescence shows that P-glycoprotein inhibition with PSC833 reduces NBD-CSA accumulation to approximately 50% of control capillaries. Remaining fluorescence reflects passive diffusion and nonspecific binding of the dye to the tissue. The difference between total luminal NBD-CSA fluorescence and fluorescence in the presence of PSC833 (portion above the dotted line) represents P-glycoprotein-specific NBD-CSA transport. Data shown for total luminal NBD-CSA accumulation represents the mean value from 10 preparations (analysis of 10 capillaries per preparation, pooled tissue from 10 rats per preparation). Variability is given by S.E.M. bars. Units are arbitrary fluorescence units (scale, 0–255). Statistical comparison: ∗∗∗, significantly different from controls, $P < 0.001$.

5.5 h later (6-h experiment). Controls were incubated in glutamate-free medium for 6 h. When noted, capillaries were exposed continuously to specific antagonists starting 5 min before glutamate exposure. Preliminary experiments demonstrated that none of the pharmacological agents used alone had any significant effect on P-glycoprotein transporter expression or transport activity over a 6-h experiment (data not shown). In 10 separate experiments using this protocol, 100 μM glutamate increased specific luminal accumulation of NBD-CSA by $135 \pm 37\%$ and P-glycoprotein expression in Western blots by $101 \pm 23\%$.

NMDA Receptor Mediated the Induction of P-Glycoprotein. In neurons, glutamate exerts its effects through metabotropic and ionotropic receptors in the plasma membrane to signal profound changes in cell function (e.g., neurotransmission and altered gene expression patterns). Previous studies have shown that brain capillaries express NMDA receptor subunits and that NMDA receptor agonists and antagonists affect capillary function (Sharp et al., 2003; András et al., 2007). In agreement with this, we detected protein expression of the NMDA receptor subunit, NR1, in total brain and brain membranes that were used as positive controls (Fig. 3A). We also found enrichment of NMDA-NR1 in brain capillary membranes compared with total brain capillaries, suggesting that glutamate could signal through NMDA receptors present in capillary plasma membranes. Exposing isolated capillaries to 1 to 5 μM NMDA (30-min exposure), a specific NMDA receptor agonist, increased P-glycoprotein expression and transport function in a concentration-dependent manner after 6 h (Fig. 3B); NMDA concentrations greater than 5 μM reduced expression and activity, suggesting toxicity (data not shown). Blocking ionotropic NMDA receptors with MK-801, a noncompetitive NMDA receptor antagonist, abolished the effects of both glutamate and NMDA on P-glycoprotein expression and transport ac-
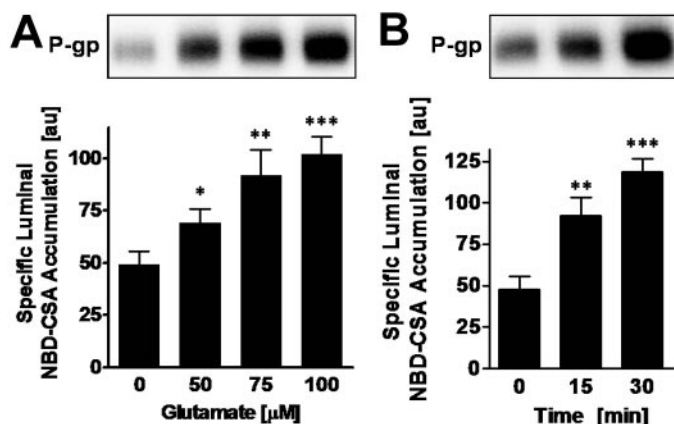
tivity (Fig. 3, C and D). The glutamate-induced increases in transporter expression and transport activity were abolished when transcription was inhibited with actinomycin D (Fig. 4A) or when protein synthesis was blocked with cycloheximide (Fig. 4B). Together, these results indicate that glutamate signaled P-glycoprotein up-regulation through NMDA receptors expressed in brain capillary plasma membranes and that this process involved transcription and translation.

Glutamate Signaled through COX-2. In epilepsy, brain levels of several proinflammatory factors (e.g., interleukins, tumor necrosis factor-α) are elevated after seizures. CNS inflammation after seizures is also reflected by increased brain COX-2 expression and by increased prostaglandin levels. COX-2, which converts arachidonic acid to prostaglandin H2, is a known downstream target of NMDA receptor activation (Pepicelli et al., 2005; Hewett et al., 2006). For example, in neurons, COX-2 is a therapeutic target in conditions in which excess glutamate release causes neurotoxicity (Iade-
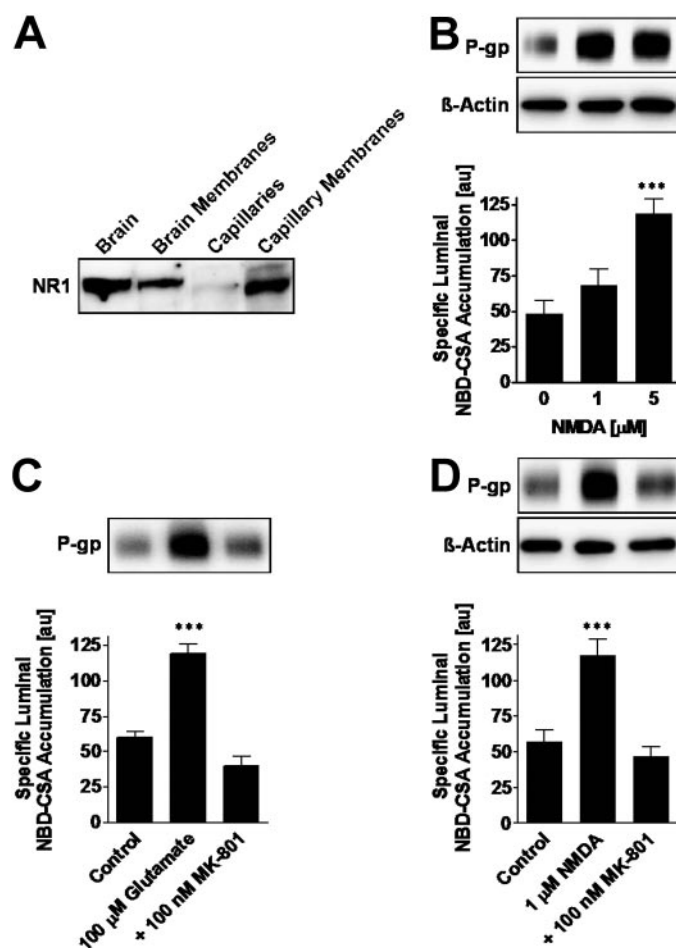

Fig. 2. Glutamate increases P-gp expression (Western blots) and transport activity (steady-state, specific luminal NBD-CSA accumulation) in rat brain capillaries. A, glutamate dose response. Capillaries were exposed to indicated concentration of glutamate for 30 min and then incubated in glutamate-free medium for 5.5 h. B, time course of glutamate action. Capillaries were exposed to 100 μM glutamate for the indicated time and then further incubated in glutamate-free medium (glutamate exposure time plus incubation time was 6 h). For specific luminal NBD-CSA fluorescence, data represent the mean value for 10 to 15 capillaries from a single preparation (pooled tissue from 10 rats); variability is given by S.E.M. bars. Units are arbitrary fluorescence units (scale, 0–255). Statistical comparison: ∗, significantly higher than controls, $P < 0.05$; ∗∗, significantly higher than controls, $P < 0.01$; ∗∗∗, significantly higher than controls, $P < 0.001$.

Fig. 3. Glutamate increases P-gp expression (Western blots) and transport activity (steady-state, specific luminal NBD-CSA accumulation) by acting through an NMDA receptor. A, Western blot showing expression of the NMDA-NR1 subunit in brain homogenate, brain membranes, and brain capillary membranes. B, NMDA dose response. Capillaries were exposed to indicated concentrations of NMDA for 30 min and then incubated in glutamate-free medium for 5.5 h. C, MK-801, an NMDA receptor antagonist, blocks the action of glutamate. D, MK-801 blocks the action of NMDA. For specific luminal NBD-CSA fluorescence, data represent the mean value for 10 to 15 capillaries from a single preparation (pooled tissue from 10 rats); variability is given by S.E.M. bars. Units are arbitrary fluorescence units (scale, 0–255). Statistical comparison: ∗∗∗, significantly higher than controls, $P < 0.001$.

cola et al., 2001). Furthermore, COX-2 has been reported to be involved in P-glycoprotein induction in tumor cells (Patel et al., 2002). We found the expression of COX-2 protein in both rat brain capillaries and brain capillary plasma membranes (Fig. 5A). Exposing rat brain capillaries to the specific COX-2 antagonist celecoxib or the COX-1/COX-2 antagonist indomethacin abolished the increases in P-glycoprotein expression and transport activity caused by glutamate (Fig. 5, B and C). The COX-1-specific antagonist SC-560 was without effect (Fig. 5D).

To further clarify the role of COX-2 in glutamate signaling, we carried out parallel experiments using brain capillaries isolated from wild-type and COX-2 knockout mice. In brain capillaries from wild-type mice, glutamate significantly increased P-glycoprotein expression and transport activity, and this increase was abolished by celecoxib (Fig. 6A); these results are similar to those found for rat brain capillaries (above). It is noteworthy that when we repeated this experiment with capillaries from COX-2 knockout mice, glutamate did not alter P-glycoprotein expression or transport activity, and celecoxib was without effect (Fig. 6B; note that different band intensities for control capillaries from wild-type and COX-2 knockout mice result from different exposure times and not from different expression levels). Together, our in vitro findings for brain capillaries indicate that 1) COX-2 plays a key role in glutamate/NMDA receptor signaling to P-glycoprotein, and 2) COX-2 is involved in P-glycoprotein up-regulation but not in maintenance of baseline P-glycoprotein expression.

In Vivo Studies. Two studies were conducted to provide initial in vivo proof of principle for the effects of glutamate and COX-2 inhibition on P-glycoprotein expression at the blood-brain barrier. Female Wistar Unilever rats were used for these studies. Preliminary experiments with isolated brain capillaries from these rats showed responses to glutamate and COX-2 inhibition that were identical with those seen in male Sprague-Dawley rats (data not shown).

In the first study, the effect of glutamate microinjections on P-glycoprotein expression was analyzed in the hilus and the CA3 region of the right hippocampus, brain regions located ventral to the injection site. Data were compared with the contralateral hippocampus that received no injection and with control rats that received vehicle injections. The method of immunostaining and analysis used in the present study was chosen based on the finding that it detects transporter expression in brain capillaries, not in parenchymal cells (Volk et al., 2005). As expected, analysis of P-glycoprotein immunolabeling in the hilus and the CA3 region of control

Fig. 5. Glutamate increases P-gp expression (Western blots) and transport activity (steady-state, specific luminal NBD-CSA accumulation) by acting through COX-2. A, Western blot showing COX-2 expression in brain capillaries and brain capillary plasma membranes. B, Celecoxib, a COX-2-selective inhibitor blocks the action of glutamate. C, indomethacin, a COX-1/-2 inhibitor blocks the action of glutamate. D, SC-560, a COX-1-selective inhibitor, does not block the action of glutamate. For specific luminal NBD-CSA fluorescence, data represent the mean value for 10 to 15 capillaries from a single preparation (pooled tissue from 10 rats); variability is given by S.E.M. bars. Units are arbitrary fluorescence units (scale, 0–255). Statistical comparison: ∗∗∗, significantly higher than controls, $P < 0.001$.
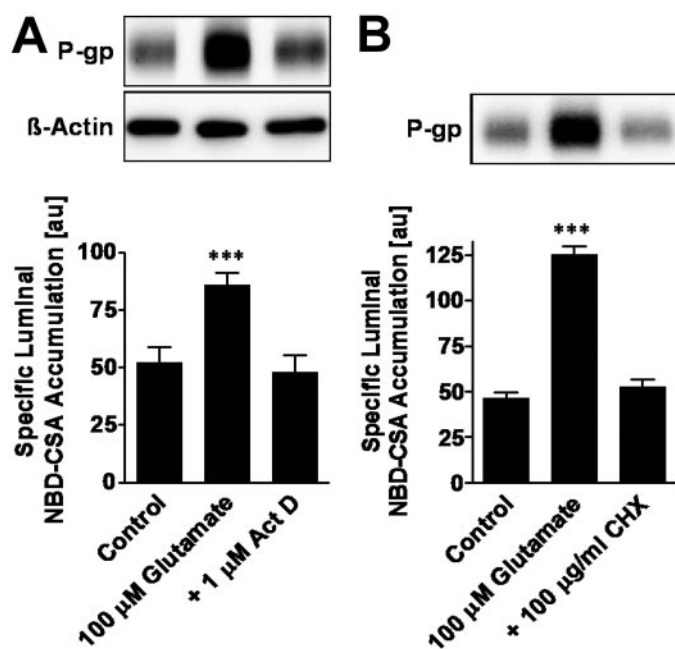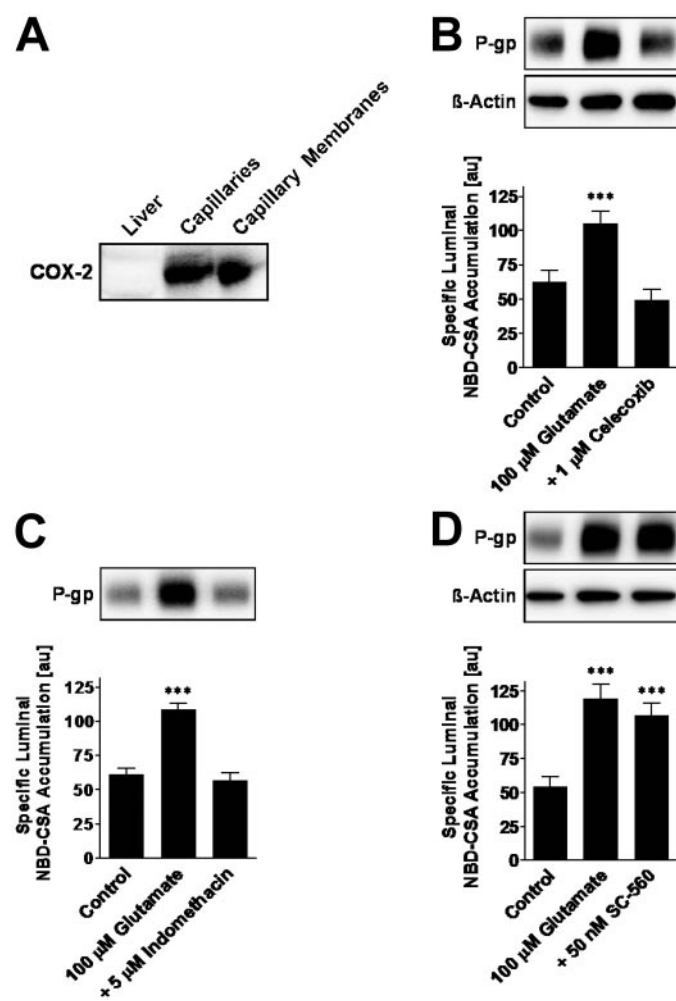

Fig. 4. Blocking transcription (A) or translation (B) abolishes the effects of glutamate on P-gp expression (Western blots) and transport activity (steady-state, specific luminal NBD-CSA accumulation). A, actinomycin D blocks the action of glutamate. B, cycloheximide blocks the action of glutamate. For specific luminal NBD-CSA fluorescence, data represent the mean value for 10 to 15 capillaries from a single preparation (pooled tissue from 10 rats); variability is given by S.E.M. bars. Units are arbitrary fluorescence units (scale, 0–255). Statistical comparison: ∗∗∗, significantly higher than controls, $P < 0.001$.

and glutamate-dosed rats indicated that the signal was restricted to endothelial cells. Figure 7 shows that glutamate microinjection significantly ($P < 0.05$) increased P-glycoprotein expression in the right hilus over vehicle-injected controls. No increase was evident in the left hilus, which was contralateral to the site of glutamate microinjection. Labeling in the CA3 region was not significantly increased, suggesting that effects of microinjected glutamate were indeed local, and capillaries in the right CA3 region were not exposed to a high enough concentration of glutamate for a long enough time to significantly increase regional P-glycoprotein expression.

In contrast to these findings for P-glycoprotein, immunohistological analysis of the expression of the endothelial cell marker, Glut-1, showed no differences between groups of rats (data not shown). Thus, increase in P-glycoprotein labeling found 1 day after glutamate injection was due to enhanced transporter expression in existing capillaries rather than induction of angiogenesis.

In the second in vivo experiment, rats were given pilocarpine to induce status epilepticus or pilocarpine plus the COX inhibitor indomethacin, and P-glycoprotein expression in brain capillaries was determined 2 days later by immunostaining. As above, analysis of P-glycoprotein labeling in sections from the hilus and the CA3 region indicated that staining in both groups of rats was restricted to endothelial cells (Figs. 8, A–C). Pilocarpine-induced status epilepticus increased P-glycoprotein labeling in the hilus and CA3 region significantly. It is noteworthy that in both regions,
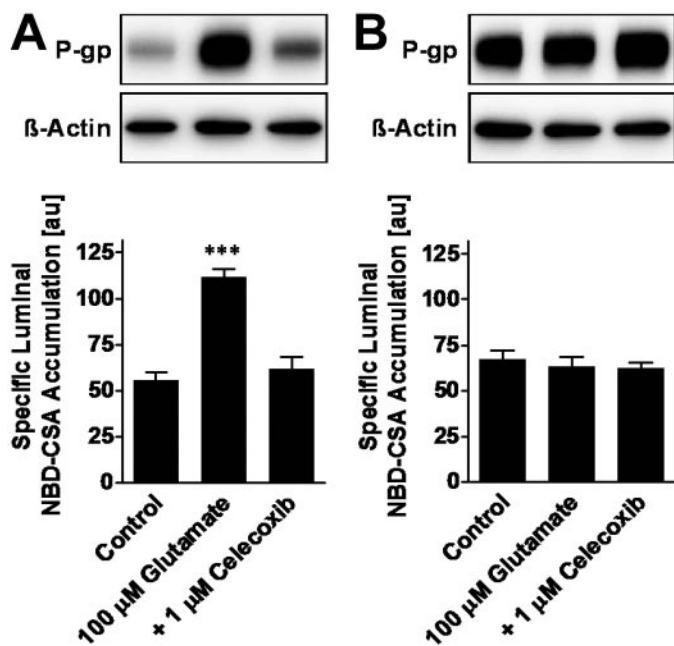
indomethacin reduced labeling to the extent that it was not significantly higher than controls (Fig. 7, D and E). Previous studies have shown that experimentally induced status epilepticus does not affect expression of the endothelial cell marker, Glut-1, indicating that changes in P-glycoprotein labeling in response to status epilepticus are not due to enhanced angiogenesis (Volk et al., 2004, 2005).

Finally, the extent of neurodegeneration was evaluated in the hilar region. As shown in Fig. 9, pilocarpine-induced status epilepticus significantly decreased hilar neuron counts. Indomethacin treatment significantly diminished cell loss, indicating that COX inhibition was to some extent neuroprotective.

Discussion

Excessive glutamate release during epileptic seizures and its signaling via NMDA receptors is a major contributor to the pathophysiology of epilepsy (Barnes and Slevin, 2003). In the epileptic brain, activation of NMDA receptors and subsequent downstream events contribute to excitotoxic damage and loss of neurons (Gardoni and Di Luca, 2006). Transcriptional activation of the gene encoding COX-2 has also been reported in a number of epilepsy models (Voutsinos-Porche et al., 2004; Kawaguchi et al., 2005; Takemiya et al., 2006; Lee et al., 2007) and in the human epileptic brain (Desjardins et
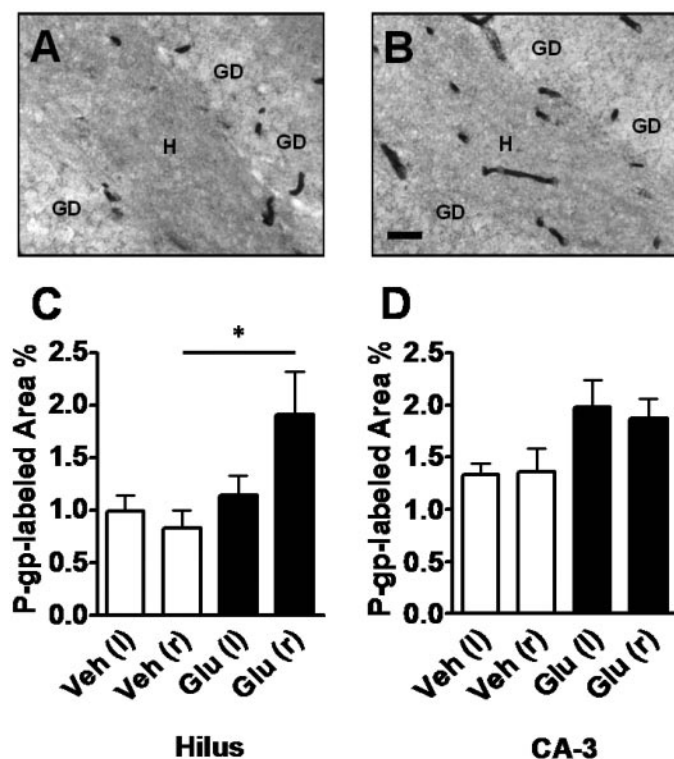

Fig. 6. Glutamate effects on P-gp expression (Western blots) and transport activity (steady-state, specific luminal NBD-CSA accumulation) in capillaries from wild-type (A) and COX-2 (B) knockout mice. A, as in the rat, celecoxib blocks the action of glutamate in wild-type mice. B, glutamate and celecoxib do not have an effect on P-glycoprotein in mice lacking COX-2. Please note that different band intensities for wild-type and COX-2 knockout controls result from different exposure times and not from different expression levels. For specific luminal NBD-CSA fluorescence, data represent the mean value for 10 to 15 capillaries from a single preparation (pooled tissue from 20 mice); variability is given by S.E.M. bars. Units are arbitrary fluorescence units (scale, 0–255). Statistical comparison: ∗∗∗, significantly higher than controls, $P < 0.001$.

Fig. 7. Glutamate injection into the hippocampus of rats increases P-glycoprotein expression in the hilus but not in the CA3 region. Right hippocampus was injected with either glutamate [Glu (r)] or vehicle [Veh (r)]. Left hippocampus was not injected and served as control for glutamate-injected rats [Glu (l)] or vehicle-injected rats [Veh (l)]. A, immunostaining of P-glycoprotein in a section of the hilus (H) of the right hippocampal dentate gyrus (GD) from a vehicle-injected rat. B, P-glycoprotein immunostaining of the corresponding section from a glutamate-injected rat. Note the striking increase in P-glycoprotein-immunolabeled capillaries with glutamate injection (scale bar, 25 μm). Analysis of P-glycoprotein immunostaining in the hilus (C) and the CA3 region (D). Data are given as mean ± S.E.M. Statistical comparisons: ∗, significantly higher than uninjected controls and contralateral region, $P < 0.05$.

al., 2003). Moreover, a link between neuronal NMDA receptor activation and enhanced COX-2 expression has been demonstrated repeatedly. That is, NMDA receptor-mediated neuronal excitotoxic damage can be abolished by COX-2 inhibition (Manabe et al., 2004; Hewett et al., 2006), and formation of the COX products prostaglandin E2 and 8-epi-PGF(2α) in neurons can be prevented by blockade of the NMDA receptor (Pepicelli et al., 2005).

Brain capillary endothelial cells also possess both NMDA receptors and COX-2 activity ((Mark et al., 2001; Sharp et al., 2003; András et al., 2007; present study), and endothelial COX-2 induction has already been described in an epilepsy model (Takemiya et al., 2006). Here we present for the first time data showing that a comparable glutamate/NMDA receptor/COX-2 signaling pathway is active at the blood-brain barrier and that one consequence of pathway activation by elevated extracellular glutamate is increased expression and transport activity of the drug efflux pump, P-glycoprotein. The present in vitro experiments with isolated brain capillaries from rat and mouse show increased P-glycoprotein expression and transport activity hours after capillaries were transiently exposed to 10 to 100 μM glutamate. Glutamate effects were mimicked by NMDA; the effects by both were blocked by an NMDA receptor antagonist (MK-801). Inhibiting transcription or translation also blocked glutamate-induced increases of P-glycoprotein. Signaling downstream of the NMDA receptor was through COX-2, because glutamate effects on transporter expression and activity were blocked by the COX-2-selective inhibitor celecoxib and by the nonselective COX inhibitor indomethacin; a COX-1-selective inhibitor was without effect. In capillaries from COX-2 knockout mice, glutamate did not stimulate P-glycoprotein expression or transport activity.

Parallel in vivo experiments with rats showed increased P-glycoprotein expression in capillaries in the hilus after glutamate microinjection into the hippocampus. Thus, gluta-mate alone in the absence of seizure activity was sufficient to locally increase P-glycoprotein expression. P-glycoprotein expression in capillaries in the hilus and CA3 regions of the hippocampus was also increased after induction of status epilepticus with pilocarpine. The effects of these seizures on transporter expression were attenuated when the rats were pretreated with indomethacin. This nonselective COX-1/COX-2 inhibitor was used in these in vivo experiments because it does not affect seizure severity in the pilocarpine model (Ikonomidou-Turski et al., 1988), whereas celecoxib has been reported to have both pro- and anticonvulsant effects in epilepsy models (Baik et al., 1999; Shafiq et al., 2003).

Taken together, our results indicate that seizure-induced elevation of brain glutamate levels signal increased P-glycoprotein expression through activation of COX-2. They have important implications for CNS pharmacotherapy. First, they partially define a signaling pathway through which excess extracellular glutamate increases P-glycoprotein expression and activity. How this pathway relates to other pathways recently disclosed to modulate P-glycoprotein expression in brain capillaries (e.g., one pathway activated by the proinflammatory cytokine tumor necrosis factor-α) (Hartz et al., 2006; Bauer et al., 2007) awaits a fuller characterization of the signaling events that connect NMDA receptors to COX-2, and COX-2 to increased transporter gene transcription. In this regard, it is likely that phospholipid metabolism and prostaglandin signaling are important links in the pathway. Experiments are currently underway to investigate these possibilities, because they could provide additional molecular targets for therapeutic intervention in epilepsy and in other CNS disorders (e.g., stroke).

Second, an association between seizure-induced P-glycoprotein overexpression and resistance to antiepileptic drugs has been suggested by numerous studies in rodent epilepsy models and in epileptic tissue of pharmacoresistant patients (Loscher and Potschka, 2005). Recent studies also show that
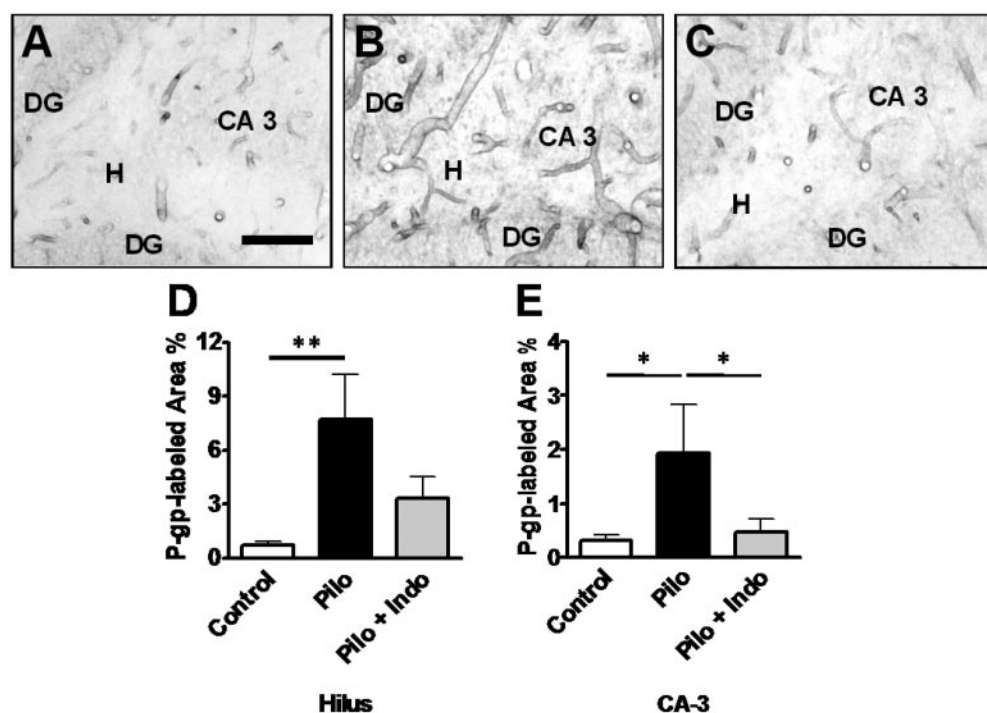

Fig. 8. Indomethacin pretreatment of rats inhibits P-glycoprotein up-regulation in the hippocampus after pilocarpine-induced seizures. A, representative image showing P-glycoprotein immunostaining of a brain section from a vehicle-treated control rat. The image shows the hippocampal CA 3 subregion (CA 3) and the hilus (H) of the dentate gyrus (DG) (scale bar, 50 μm). B, representative image showing P-glycoprotein immunostaining of a brain section from a rat after pilocarpine-induced status epilepticus. Note the striking increase in P-glycoprotein staining of the brain capillary endothelium. C, representative image showing P-glycoprotein immunostaining of a hippocampal section from a rat pretreated with indomethacin followed by pilocarpine-induced status epilepticus. Note the reduction in P-glycoprotein staining of capillaries compared with capillaries of nontreated rats after pilocarpine-induced seizures. Image analysis of P-glycoprotein immunostaining in the hippocampal hilus (D) and the CA3 region (E). Data are given as mean ± S.E.M. Statistical comparisons: ∗, significantly higher than controls, $P < 0.05$; ∗∗, significantly higher than controls, $P < 0.01$.

specific P-glycoprotein inhibitors can be used to overcome resistance in animal models of epilepsy (Brandt et al., 2006). Although there is controversy with regard to which antiepileptic drugs are transported by P-glycoprotein, several first-line antiepileptic drugs seem to be P-glycoprotein substrates (Loscher and Potschka, 2005; Brandt et al., 2006; van Vliet et al., 2006).

For antiepileptic drugs in which brain penetration is limited by P-glycoprotein, reducing transporter expression should have a beneficial effect. It could allow therapeutic brain levels to be achieved with lower drug doses, and it may help to prevent or overcome drug resistance in a subpopulation of patients. Whether this can be best done by targeting blood-brain barrier COX-2 or some other element of the signaling chain remains to be determined. Nevertheless, the strategy of targeting signals that up-regulate transporter expression has potential advantages over the use of specific P-glycoprotein inhibitors. It is noteworthy that it may leave basal P-glycoprotein expression and function at the blood-brain barrier and in other barrier and excretory tissues unaffected, thus preserving the protective role of the transporter in those tissues.

On the other hand, targeting a component of the brain's inflammatory response can have both expected and unintended consequences. In the majority of studies, nonselective COX-1/-2 or selective COX-2 inhibitors exhibited a beneficial neuroprotective effect (Takemiya et al., 2006). However, detrimental effects with aggravation of seizures and neuronal loss have also been described previously (Baik et al., 1999). In the present study, COX-1/-2 inhibition did not affect seizure severity during status epilepticus and produced a slight neuroprotective effect in the hilus of the hippocampus.

In addition, gastrointestinal, renal, cardiovascular, and cerebrovascular side effects of nonselective and selective COX inhibitors are also a concern. However, the inhibition of brain COX-2 is achieved with lower doses of celecoxib than those needed for anti-inflammatory activity in an arthritis model (Ciceri et al., 2002).

In conclusion, the present combined in vitro/in vivo study elucidates a signaling pathway through which seizures can up-regulate P-glycoprotein expression at the blood-brain barrier. Our findings provide insight into the mechanisms that can contribute to drug resistance in epilepsies and possibly other CNS disorders. The data suggest that COX-2 inhibition may be one way to improve the response to antiepileptic drugs. Further studies are needed to determine the extent to which these new insights into regulation of P-glycoprotein provide a basis for practical strategies to overcome or prevent pharmacoresistance.

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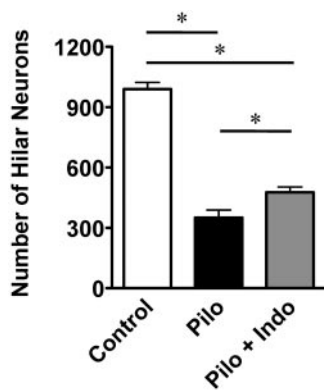
Fig. 9. Effect of pilocarpine or pilocarpine plus indomethacin on average numbers of polymorphic neurons in the hilus of the hippocampal dentate gyrus of rats. Data are given as mean ± S.E.M. Significant intergroup differences were obtained in both groups of rats with status epilepticus compared with the corresponding control group. Even though indomethacin treatment did not prevent seizure-induced damage of hilar neurons, the degree of damage was significantly lower compared with vehicle-treated rats with pilocarpine-induced status epilepticus. Statistical comparisons: *, significantly different from means, $P < 0.05$.

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