Glutamate-Mediated Upregulation of the Multidrug Resistance Protein 2 in Porcine and Human Brain Capillaries

Hiram Luna-Munguia, Josephine D. Salvamoser, Bettina Pascher, Tom Pieper, Thekla Getzinger, Manfred Kudernatsch, Gerhard Kluger, and Heidrun Potschka

Institute of Pharmacology, Toxicology, and Pharmacy, Ludwig-Maximilians-University, Munich, Germany (H.L.M., J.D.S., H.P.); Neuropediatric Clinic and Clinic for Neurorehabilitation, Epilepsy Center for Children and Adolescents (B.P., T.P., T.G., G.K.) and Clinic for Neurosurgery and Epilepsy Surgery (M.K.), Schön Klinik Vogtareuth, Vogtareuth, Germany; and Paracelsus Medical University, Salzburg, Austria (G.K.)

Received August 18, 2014; accepted December 10, 2014

ABSTRACT

As a member of the multidrug-resistance associated protein (MRP) family, MRP2 affects the brain entry of different endogenous and exogenous compounds. Considering the role of this transporter at the blood-brain barrier, the regulation is of particular interest. However, there is limited knowledge regarding the factors that regulate MRP2 in neurologic disease states. Thus, we addressed the hypothesis that MRP2 might be affected by a glutamate-induced signaling pathway that we previously identified as one key mechanism in the regulation of P-glycoprotein. Studies in isolated porcine brain capillaries confirmed that glutamate and N-methyl-d-aspartic acid (NMDA) exposure upregulates expression and function of MRP2. The involvement of the NMDA receptor was further suggested by the fact that the NMDA receptor antagonist MK-801 ([6S,10R]-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine), as well as the NMDA receptor glycine binding site antagonist L-701,324 [7-chloro-4-hydroxy-3-(3-phenoxyphenyl)-2(1H)-quinolinone], prevented the impact of glutamate. A role of cyclooxygenase-2 was indicated by coinubcation with the cyclooxygenase-2 inhibitor celecoxib and the cyclooxygenase-1/2 inhibitor indomethacin, which both efficaciously abolished a glutamate-induced upregulation of MRP2. Translational studies in human capillaries from surgical specimen demonstrated a relevant MRP2 efflux function and indicated an effect of glutamate exposure as well as its prevention by cyclooxygenase-2 inhibition. Taken together, the findings provide first evidence for a role of a glutamate-induced NMDA receptor/cyclooxygenase-2 signaling pathway in the regulation of MRP2 expression and function. The response to excessive glutamate concentrations might contribute to overexpression of MRP2, which has been reported in neurologic diseases including epilepsy. The overexpression might have implications for brain access of various compounds including therapeutic drugs.

Introduction

The expression of multidrug efflux transporters in brain capillary endothelial cells significantly affects the brain access of a large number of molecules (Cordon-Cardo et al., 1989; Schinkel, 1999; Miller et al., 2000; Cisternino et al., 2004). The physiologic function of these transporters is to regulate the entrance rates of endogenous substrates and to protect the brain against potentially toxic compounds (Fromm, 2004; Löscher and Potschka, 2005a). However, based on experimental and clinical data, several authors have proposed that these transporters can also have a significant influence on the success of pharmacotherapy (DeGorter et al., 2012; Potschka, 2012). Resistance mechanisms that involve cerebral drug efflux transporter overexpression have been intensely studied. Strong efforts are made to use this knowledge for the development of approaches counteracting the poor absorption of several central nervous system drugs and improve therapeutic extracellular concentrations at the brain target sites (Löscher and Potschka, 2005b; Aronica et al., 2012; Chen et al., 2012; Potschka, 2012).

Considering that the dynamic regulation of blood-brain barrier efflux transporters can have significant implications for toxicology and pharmacology, the elucidation of regulatory mechanisms is of major interest. In the field of epileptology, studies in rodent and human brain capillaries have revealed that glutamate can upregulate P-glycoprotein via an N-methyl-d-aspartic acid (NMDA) receptor/cyclooxygenase-2 signaling pathway (Bauer et al., 2008b; Zibell et al., 2009; van Vliet et al., 2010; Avemary et al., 2013). Considering the excessive extracellular glutamate concentrations reached during epileptic seizures, this signaling pathway explains the overexpression of P-glycoprotein, which has been repeatedly found in the epileptic brain (Tishler et al., 1995; Thomas et al., 2003, 2004, 2005; Aronica et al., 2004; Kubota et al., 2006; Ak et al., 2007).

This work was sponsored by the National Council for Sciences and Technology of Mexico [Grant 203881] (to H.L.M.). H.L.M. and J.D.S. contributed equally to this work.

ABBREVIATIONS: Glut-1, glucose transporter 1; L-701,324, 7-chloro-4-hydroxy-3-(3-phenoxy)phenyl-2(1H)-quinolinone; MK-571, 5-{3-[2-(7-chloroquinolin-2-yl)ethenyl]phenyl}-8-dimethylcarbamyl-4,6-dithiaoctanoic acid sodium salt hydrate; MK-801, (6S,10R)-(+)5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine; MRP2, multidrug-resistance protein 2; NMDA, N-methyl-d-aspartic acid; PBS, phosphate-buffered saline; SC-560, 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole; TBST, Tris-buffered saline/Tween 20.
Based on the fact that efflux at the blood-brain barrier is based on the concerted action of different multidrug transporters, it is of major interest to determine whether the NMDA receptor/cyclooxygenase-2 pathway also affects other transporters. Among the multidrug resistance-associated protein (MRP, ABCC) family, the members MRP1, -2, -4, and -5 (ABCC1, -2, -4, and -5) seem to contribute to blood-brain barrier efflux function (Löschner and Potschka, 2005a,b; Dallas et al., 2006). The mechanisms of their expression regulation have not been intensely studied yet. Preliminary findings from a recent pilot study give first evidence that the NMDA receptor might contribute to seizure-associated induction of MRP2 (Hao et al., 2012), which has been described in rodent models and human tissue (Aronica et al., 2004, 2012; van Vliet et al., 2005; Hoffman and Löschner, 2007). However, the study does not allow conclusions regarding cellular and molecular mechanisms of MRP2 regulation as the authors systemically administered the NMDA receptor blocker MK-801 [(5R,10R)–(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine] before status epilepticus without controlling for a putative impact on behavioral and electrographic seizure activity. Therefore, it is impossible to rule out an indirect effect via neuronal NMDA receptors.

The present study was conducted to thoroughly address the question whether the glutamate-triggered signaling pathway described for P-glycoprotein also affects function and expression of MRP transporters. Considering the findings from expression analysis in epileptic tissue and transport data for antiepileptic drugs, the experiments were focused on MRP2. The role of the candidate signaling factors was confirmed in isolated porcine brain capillaries. A translational study using capillaries from surgical specimens argued against relevant species differences and substantiated that findings from porcine capillaries can be extrapolated to human capillaries.

Materials and Methods

Chemicals and Antibodies. Glutamate, MK-571 [5-(3-(2-(7-chloroquinolin-2-yl)ethenyl)phényl)-8-dimethylcarbamyl-4,6-dithiaoctanoic acid sodium salt hydrate], Texas Red, celecoxib, indomethacin, SC-560 [5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole], cycloheximide, actinomycin D, LH-701,324 [7-chloro-4-hydroxy-3-(3-phenoxy)phenyl-2(1H)-quinoxalinone], and the mouse monoclonal β-actin antibody were purchased from Sigma-Aldrich (Tauffkirchen/Schnelldorf, Germany). NMDA, dizocilpine (MK-801), and the monoclonal antibody for MRP2 were obtained from Abcam (Cambridge, UK). The polyclonal antibody for the glucose transporter 1 (Glut-1) was provided by Millipore (Schwalbach, Germany).

Tissue. Brain samples from male and female pigs between 5 and 6 months old were kindly provided by the local Munich slaughterhouse. Human brain tissue was from surgical resections from two female patients (5 and 15 years old) and one male patient (2 years old) with a history of chronic intractable epilepsy suffering from focal cortical dysplasia (Neuropediatric Department of the Schön Clinic, Vogtareuth, Germany). The use of surgical specimen was approved by the ethic committees of the institutions involved according to the Declaration of Helsinki, and informed consent was obtained from the parents of each patient.

Porcine brains were washed with cold running water for 5–7 minutes before further processing. Porcine and human brain tissue was kept in a freshly prepared ice-cold buffer referred to as isolation solution [105 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 10 mM HEPES, 1.2 mM MgSO₄ × 7 H₂O, and 2.5 mM CaCl₂ × 2 H₂O, pH 7.4].

Brain Capillary Isolation. Capillaries from human and porcine (10–12 animals per preparation) brain were isolated using a partially modified protocol based on previous studies (Miller et al., 2000; Hartz et al., 2004; Avemary et al., 2013). All steps in the isolation procedure were carried out in freshly prepared solutions maintained at 4°C. The first step was to remove meninges, blood vessels, and cerebellum from the brains. Then, the tissue was washed and gently homogenized with 30% dextran solution aliquots. The homogenate was centrifuged at 7500g for 12 minutes at 4°C. The resulting pellet was resuspended in isolation solution (supplemented with 0.5% bovine serum albumin, 25 mM NaHCO₃, 10 mM glucose, and 1 mM sodium pyruvate, final pH 7.4) and then filtered through a 210 μm nylon filter. The filtrate was passed through a glass column filled with glass beads and washed with 1 l of the isolation solution enriched with bovine serum albumin. Capillaries that bound to the glass beads were collected by gentle agitation, washed with isolation solution, and filtered through a new 210 μm nylon filter. The filtrate was centrifuged (7500g for 12 minutes at 4°C), and the resultant pellet was resuspended in a buffer referred to as incubation solution (isolation solution plus 10 mM glucose). MRP2 functional activity was evaluated by using an assay based on confocal microscopy and digital image analysis to measure luminal accumulation of Texas Red, whereas MRP2 expression was assessed by Western blot. Because of the limited amount of human tissue obtained from the surgical resection, human capillaries were used for punctal transport assay experiments.

Transport Assay. All transport experiments were conducted at room temperature (22–24°C). Freshly isolated porcine brain capillaries were exposed to glutamate (50, 75, and 100 μM) or NMDA (1, 3, and 5 μM) for 30 minutes, transferred to glutamate/NMDA-free medium, and used 5.5 hours later to evaluate transport activity. Actinomycin D (1 μM), cycloheximide (1 μM), MK-801 (100 nM), celecoxib (1 μM), indomethacin (5 μM), or SC-560 (50 nM) were added 5 minutes before glutamate exposure. The selective antagonist at the glycine binding site of the NMDA receptor, L-701,324 (1 μM) was added 15 minute before glutamate exposure. To rule out direct effects of the inhibitors on MRP2 regulation, celecoxib, indomethacin, SC-560, MK-801, and L-701,324 were additionally tested without glutamate coinubation.

Freshly isolated human brain capillaries from the three patients were exposed to glutamate (100 μM) for 30 minutes, transferred to glutamate-free medium, and used 4.5 hours later to evaluate transport activity. Surgical specimens from two patients were large enough to collect capillaries for additional assessment of the effects of celecoxib (1 μM), which was added 5 minutes before glutamate exposure. Controls were incubated in glutamate/NMDA-free medium for 5 or 6 hours depending on the origin of the tissue. To measure transport, 150 μl of isolated brain capillaries suspension was transfereed to a covered imaging chamber (Ibidi, Martinsried, Germany), and the fluorescent substrate Texas Red was added. Texas Red has been repeatedly used to analyze MRP2-mediated transport in previous studies (Miller et al., 2000; Bauer et al., 2008a; Reichel et al., 2008; Wang et al., 2010). The luminal substrate accumulation was measured 1 hour later. In some experiments, 50 μM MK-571, a transport inhibitor for MRP2, was added to the incubation medium 30 minutes before the fluorescent substrate. To acquire images of 10 capillaries, the chamber containing the isolated capillaries was mounted on the stage of a Zeiss LSM model 510 microscope (Carl Zeiss GmbH, Jena, Germany; using a 40 × 1.2 water immersion objective) for each exposure and incubation protocol. Images were saved, and luminal fluorescence was quantitated using Image J 1.4.3.67 software (Laucher Symmetry software; NIH, Bethesda, MD). MRP2-mediated transport was defined as the difference between the total luminal Texas Red accumulation and the total accumulation in the presence of MK-571. Data are presented as MRP2 specific luminal Texas Red fluorescence.

Western Blot. After the capillary preparation procedure, the membrane fractions were isolated from the porcine brain capillaries. Briefly, the capillaries were homogenized in lysis buffer containing complete EDTA-free protease inhibitor (Sigma Fluka, Tauffkirchen, Germany). To remove cell nuclei and organelles, samples were first centrifuged at 10,000g for 30 minutes at 4°C. The resulting pellet was discarded, and the supernatant was then centrifuged at 100,000g for 60 minutes at 4°C.
Pellets were resuspended in lysis buffer containing complete EDTA-free protease inhibitor, and protein concentrations were determined by the Lowry method. Samples were mixed with 5× Laemmli sample buffer and heated at 95°C for 5 minutes. Then, the samples were kept at room temperature for 10–15 minutes, loaded onto an 8% SDS-polyacrylamide gel, and separated by electrophoresis (starting at 100 V for 25 minutes and then increasing to 200 V for 40 minutes). Afterward, proteins were transferred electrophoretically (1 hour at a constant amperage of 80 V) to a polyvinylidene difluoride membrane (Peqlab, Erlangen, Germany) using a transfer buffer containing 25 mM Tris and 96 mM glycine. The membranes were blocked for 1 hour at room temperature with freshly prepared blocking solution [Tris-buffered saline/Tween 20 (TBST) plus 5% bovine serum albumin]. After blocking, membranes were incubated overnight at 4°C with the adequate monoclonal antibody for MRP2 (1:50) and for β-actin (1:2,000). Twenty hours later, after three washing steps with TBST pH 7.7, the membranes were incubated for 1 hour at room temperature with horseradish peroxidase–conjugated goat anti-mouse secondary antibody (1:15,000). Membrons were triple washed again in TBST pH 7.7, and Western blots were developed for visualization of the immunoreaction using enhanced chemoluminescence detection and Fusion-SL Advance 4.2 MP (Peqlab). The protein levels were normalized against the expression level of β-actin as a loading control. For negative controls the same procedure was performed without adding the primary antibodies.

Immunohistochemistry. Freshly isolated porcine and human brain capillaries were fixed on microscope slides (HistoBond adhesion slides; Marienfeld, Lauda-Koenigshofen, Germany) with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 minutes at room temperature. After washing in PBS, capillaries were blocked for 2 hours with PBS containing 0.5% Triton X-100 (AppliChem GmbH, Darmstadt, Germany) and 10% bovine serum albumin (Sigma-Aldrich). Then, the capillaries were incubated overnight in a cover plate apparatus at 4°C with the monoclonal antibody against MRP2 (1:50; Alexis Biochemicals, Lörach, Germany) and the polyclonal antibody against Glut-1 (1:500). After washing with PBS, capillaries were incubated for 2 hours with the fluorochrome-conjugated secondary antibody (1:200). Immunostainings were evaluated using a confocal laser scanning microscope (Zeiss LSM 510 microscope; Carl Zeiss GmbH). For negative controls the same procedure was performed without adding primary antibodies.

Statistical Analysis. Data are presented as mean ± S.E.M. For comparisons between control and treated samples the Student’s t test was used. Concentration-dependent effects of glutamate and NMDA on transport activity were evaluated by analysis of variance and post hoc Dunnett’s test. In all statistical comparisons, *P < 0.05, **P < 0.01, and ***P < 0.001 were used as criterion for significance.

Results

Colocalization of MRP2 with the Endothelial Cell Marker Glut-1 in Freshly Isolated Porcine and Human Brain Capillaries. Before starting with studies focusing on the regulation of transport function and expression, we aimed to confirm MRP2 expression and its colocalization with the endothelial marker Glut-1 in our capillary preparations. The immunohistochemical staining of porcine brain capillaries substantiated the colocalization of MRP2 with the endothelial cell marker Glut-1 (Fig. 1, A–C). As expected, the colocalization of MRP2 with Glut-1 was also confirmed in human brain capillaries (Fig. 1, D–F).

Glutamate Upregulates Texas Red Luminal Accumulation and MRP2 Expression in Porcine Brain Capillaries. Accumulation of the fluorescent MRP2 substrate Texas Red was used to assess the impact of glutamate on transport function (Fig. 2, A–C). Exposing capillaries to 100 μM glutamate for 30 minutes resulted in significantly increased...
luminal fluorescence (Fig. 2B). The concentration of glutamate for this and further experiments was chosen based on previous experimental and clinical studies demonstrating a respective extracellular concentration of glutamate during epileptic seizures and confirming an impact of this concentration on P-glycoprotein expression and function (Thomas et al., 2003, 2004, 2005; Bauer et al., 2008b; Avemary et al., 2013). As expected, MK-571 exerted a relevant inhibitory effect on MRP2-mediated luminal Texas Red accumulation (Fig. 2C). The quantification of Texas Red accumulation in control capillaries suggested that glutamate increased the luminal fluorescence by 30%, whereas MK-571 reduced it by 18% (Fig. 2D). The difference between total luminal Texas Red fluorescence and fluorescence in the presence of the inhibitor MK-571 represents MRP2-mediated transport (Fig. 2E).

The impact of glutamate on MRP2 transport function was further confirmed in a concentration-response analysis. This experiment revealed a concentration-dependent effect with significant effects observed after exposure to 75 and 100 μM glutamate but not after exposure to a lower concentration (50 μM) (Fig. 2F).

These findings raised the question whether this increase in transport function is related to de novo protein biosynthesis increasing transporter expression rates. The consequences of glutamate exposure on MRP2 expression were studied by Western blot analysis. In support of our hypothesis, 100 μM glutamate significantly upregulated MRP2 expression by 46% (Fig. 2G).

Further confirming de novo protein biosynthesis, the inhibition of translation and transcription by actinomycin D or cycloheximide starting 5 minutes before glutamate exposure abolished the glutamate-induced increases in transport activity (Fig. 3, A–E) and protein expression (Fig. 3F). These results indicate that glutamate upregulates MRP2 through a process involving transcription and translation.

Role of the NMDA Receptor in the Regulation of MRP2 Function and Expression in Porcine Capillaries. Previous studies have reported a glutamate-induced signaling...
via NMDA receptors to activate a cascade of intracellular events that culminates with P-glycoprotein upregulation (Bauer et al., 2008b; Avemary et al., 2013). Thus, we hypothesized that the upregulation of MRP2 might also be related to signaling via NMDA receptors. In line with this hypothesis, a concentration-dependent increase of MRP2 transport activity was observed when isolated porcine brain capillaries were exposed to three different concentrations of NMDA for 30 minutes (Fig. 4, A–E). To further confirm the role of the NMDA receptor in MRP2 regulation, the impact of coincubation with a noncompetitive NMDA receptor antagonist was evaluated. Therefore, 100 nM MK-801 was added to the capillaries 5 minutes before the start of glutamate exposure.

The blockage of NMDA receptors efficaciously prevented the effects of glutamate, thereby keeping MRP2 transport activity at basal levels (Fig. 4, F–I). The role of the NMDA receptor was further substantiated by suggesting that MK-801 also blocks the glutamate-mediated upregulation of MRP2 expression (Fig. 4J). To further confirm the impact of the NMDA receptor, we studied whether the glutamate-induced upregulation of MRP2 can also be prevented by L-701,324, a selective antagonist at the glycine binding site of the NMDA receptor. L-701,324 efficaciously abolished the effect of glutamate on MRP2 transport activity (Fig. 4, K–N). Direct effects of the inhibitors MK-801 and L-701,324 on MRP2 regulation were ruled out in a separate experiment without glutamate exposure (see Fig. 6, H–K).

Role of Cyclooxygenase-2 in the Regulation of MRP2 Function and Expression in Porcine Capillaries. Considering that previous experiments confirmed that arachidonic acid signaling involving cyclooxygenase-2 activity can be involved in downstream signaling events after NMDA receptor activation, we hypothesized that cyclooxygenase-2 might contribute to MRP2 regulation. A 5-minute exposure of porcine brain capillaries to the specific cyclooxygenase-2 inhibitor celecoxib (1 μM) (Fig. 5, A–D) and the cyclooxygenase-1/-2 inhibitor indomethacin (5 μM) (Fig. 5, F–I) abolished the increases in MRP2 transport activity. Furthermore, supporting these data, celecoxib also kept MRP2 expression in glutamate-exposed capillaries at control levels (Fig. 5E). We also ruled out that cyclooxygenase-1 contributes to the effects of glutamate on MRP2 transport. The selective cyclooxygenase-1 inhibitor, SC-560 (50 nM), exerted no effects on the glutamate-mediated upregulation of MRP2 (Fig. 5, J–M). In addition, we ruled out direct effects of the inhibitors celecoxib, indomethacin, and SC-560 on MRP2 function in a separate experiment without glutamate exposure (Fig. 6, A–G).

Regulation of MRP2 Transport Activity in Human Capillaries. The data obtained with porcine capillaries raised the questions whether these findings can be translated to human capillaries or whether species differences exist in the glutamate-induced regulation of MRP2 transport. Analysis was limited to transport assays because of the limited amount of human capillaries available.

Fig. 3. Effects of actinomycin D (Act D) and cycloheximide (Chx) in freshly isolated porcine brain capillaries. (A–D) Representative images showing the luminal accumulation of Texas Red in a control capillary (A); an increased luminal Texas Red accumulation after 100 μM glutamate exposure (B); MRP2 transport inhibition with 1 μM Act D and 1 μM Chx, respectively, reducing the Texas Red luminal fluorescence (C and D); scale bars: 10 μm. Blocking transcription or protein synthesis with 1 μM Act D or 1 μM Chx, respectively, abolishes the effects of glutamate on transport activity (E) and protein expression (F). Data shown for specific luminal Texas Red accumulation are plotted as the mean value from the analysis of ten different capillaries from three different preparations. Data are shown as mean ± S.E.M. *P < 0.05; ***P < 0.001 significant difference versus control.
of surgical tissue available from each patient, not allowing Western blot-based protein expression analysis.

Isolated brain capillaries from three different surgical specimens at the age of 2 years (male), 5 years (female), and 15 years (female) were used to assess MRP2 transport activity under control conditions and with a 30-minute glutamate exposure, based on the luminal accumulation of Texas Red (Fig. 7, A–C). The exposure to 100 μM glutamate significantly increased the luminal fluorescence by an average of 101%, whereas MK-571 decreased it by 38% (Fig. 7E). The difference between total luminal Texas Red fluorescence and fluorescence in the presence of the inhibitor MK-571 represents MRP2-mediated transport (Fig. 7F). When data from the three specimen were compared, the glutamate-induced increase in MRP2 transport activity was comparable in all cases. Surgical specimens from two patients at the age of 2 years (male) and 5 years (female) were large enough to collect capillaries for additional assessment of the effects of cyclooxygenase-2 inhibitor coincubation (Fig. 7, A–D). In line with the findings from porcine capillaries, celecoxib efficaciously controlled MRP2 transport function and prevented glutamate-induced increases in accumulation of the MRP2 substrate (Fig. 7G). Figure 6H represents the MRP2-mediated upregulation of MRP2 transport activity. Data shown for specific luminal Texas Red accumulation are plotted as the mean value from the analysis of ten different capillaries from three different preparations. Data are shown as mean ± S.E.M. **P < 0.01; ***P < 0.001 significant difference versus control.

**Discussion**

Experiments using porcine brain capillaries suggest a role of a glutamate/NMDA receptor/cyclooxygenase-2 signaling pathway in the regulation of the blood-brain barrier efflux transporter MRP2. The impact of glutamate on transport function was further confirmed using human capillaries. To our knowledge, the latter experiment did not only render novel information about the regulation of human MRP2 but also represents the first ex vivo analysis showing MRP2 transport activity in intact human capillaries.

A series of previous studies using rodent, porcine, and human brain capillaries has elucidated a signaling pathway, which is
activated by elevated glutamate concentrations and which drives overexpression of the major multidrug transporter P-glycoprotein (Zhu and Liu, 2004; Bauer et al., 2008b; Avemary et al., 2013). The pathway involves the endothelial NMDA receptor and subsequent activation of arachidonic acid signaling mediated by cyclooxygenase-2 and the prostaglandin E2 EP1 receptor (Bauer et al., 2008b; Pekcec et al., 2009; Zibell et al., 2009; Potschka, 2010; van Vliet et al., 2010; Avemary et al., 2013). Our present findings indicate that glutamate does not only induce P-glycoprotein but also affects expression and function of MRP2. These findings might be of clinical relevance for central nervous system diseases characterized by excessive glutamate release such as epilepsy (Thomas et al., 2003, 2004, 2005; Potschka, 2010). Considering that glutamate is highly concentrated in the extracellular space during the ictal period (During and Spencer, 1993; Wilson et al., 1996; Cavus et al., 2005; Luna-Munguia et al., 2011), this might imply that signaling of glutamate via the endothelial NMDA receptor is a possible mechanism of MRP2 upregulation in the epileptic brain. Studies in both rodent epilepsy models and in human epileptic tissue previously revealed an overexpression of this blood-brain barrier efflux transporter (Aronica et al., 2004, 2012; van Vliet et al., 2005; Hoffmann and Löscher, 2007). These data are in line with our mechanistic findings explaining the mode of regulation of this transporter in response to epileptic seizures.

Transporter efflux function can be regulated by different cellular mechanisms. Trafficking between intracellular vesicles and the outer membrane as well as de novo protein biosynthesis can significantly contribute to an upregulation of transporter expression in the outer membrane associated with enhanced efflux function (McCaffrey and Davis, 2012; McCaffrey et al., 2012). In this context it is important to consider that Western
blot analysis confirmed a pronounced glutamate-induced MRP2 overexpression in the cell membrane fraction and that inhibition of transcription and translation blocked the effects of glutamate. Taken together these findings suggest that the consequences of excessive glutamate exposure on MRP2 efflux function are mediated by de novo protein biosynthesis.

Despite some earlier controversies in this area, cumulating evidence exists that different ionotropic and metabotropic glutamate receptors are expressed in brain capillary endothelial cells (Stacny et al., 2002; Zhu and Liu, 2004; Bauer et al., 2008b; Neuhaus et al., 2011; Avemary et al., 2013). Based on our previous findings demonstrating that the NMDA receptor mediates the effects of glutamate on P-glycoprotein expression (Bauer et al., 2008b; Avemary et al., 2013), we hypothesized that this glutamate receptor also contributes to regulation of MRP2. In support of this hypothesis, our data substantiate that the inhibition of the NMDA receptor by MK-801 efficaciously prevents glutamate’s effects on MRP2. The fact that MRP2 expression and function are kept at control levels by MK-801 indicates that the NMDA receptor might exclusively mediate glutamate-induced influences on MRP2. In addition, the glutamate-induced upregulation of MRP2 can also be prevented by L-701,324, the selective antagonist at the glycine binding site of the NMDA receptor. Thus, the data suggest the NMDA receptor as a major key player in the regulation of MRP2.

Activation of NMDA receptors can trigger arachidonic acid signaling in different brain cell types including neurons and endothelial cells (Bauer et al., 2008b; Shelat et al., 2008; Taylor et al., 2008; Avemary et al., 2013). The processing of arachidonic acid by cyclooxygenase-2 resulting in the generation of a prostanoid precursor and enhanced prostaglandin E2 signaling proved to be critically involved in glutamate-mediated increases of P-glycoprotein (Pekcec et al., 2009; Zibell et al., 2009; van Vliet et al., 2010). Using a pharmacological approach based on coincubation of capillaries with glutamate and the cyclooxygenase-2 inhibitor celecoxib and the cyclooxygenase-1/-2 inhibitor indomethacin, we now obtained evidence that cyclooxygenase-2 is involved in the downstream signaling events affecting MRP2 upon NMDA receptor activation.

**Fig. 6.** Effects of celecoxib, indomethacin, SC-560, MK-801, and L-701,324 themselves on MRP2 regulation in porcine brain capillaries. (A–C) Representative images showing the luminal accumulation of Texas Red in a control capillary (A), MRP2 transport activity with 1 μM celecoxib (B), the quantification of the luminal Texas Red accumulation from the previous images shows that celecoxib does not affect the regulation of MRP2 (C). (D–F) Representative images showing the luminal accumulation of Texas Red in a control capillary (D), MRP2 transport activity with 5 μM indomethacin (E), MRP2 transport activity with 50 nM SC-560 (F), the quantification of the luminal Texas Red accumulation from the previous images shows that indomethacin and SC-560 do not affect the regulation of MRP2 (G). (H–J) Representative images showing the luminal accumulation of Texas Red in a control capillary (H), MRP2 transport activity with 100 nM MK-801 (I), MRP2 transport activity with 1 μM L-701,324 (J); the quantification of the luminal Texas Red accumulation from the previous images shows that MK-801 and L-701,324 does not affect the regulation of MRP2 (K). Data shown for specific luminal Texas Red accumulation are plotted as the mean value from the analysis of ten different capillaries from three different preparations. Data are shown as mean ± S.E.M. Scale bar: 10 μm.
For further confirmation, future studies would be of interest analyzing whether the effect of glutamate on MRP2 function is also abolished by cyclooxygenase-2 genetic deficiency in capillaries obtained from knockout mice.

Whereas regulation of brain capillary P-glycoprotein by various signaling factors has been intensely studied, there are only a few studies analyzing the molecular mechanisms of MRP2 regulation at the blood-brain barrier. The majority of these studies focused on the mechanisms of xenobiotic-induced upregulation of MRP2, indicating that diesel exhaust particles can induce MRP2 expression and that the pregnane X receptor, the constitutive androstane receptor, the farnesyl X receptor, and the activation of the transcription factor NF-E2–related factor-2 (Nrf2) can contribute to the impact of xenobiotic exposure on MRP2 expression and transport function (Maher et al., 2005; Miller, 2010; Wang et al., 2014). However, to our knowledge no study has been conducted exploring the signaling mechanisms of disease-associated MRP2 regulation on a cellular level. Thus, our findings regarding the role of glutamate, NMDA receptor, and cyclooxygenase-2 seem to provide the first information for a MRP2 regulatory pathway, which might have relevance for neurologic diseases. The data indicate that this signaling occurs in endothelial cells. However, as we cannot completely exclude that the preparation contains parts of other cells, it cannot be ruled out that other cells, such as astrocytes, might also contribute to the signaling cascade.

Interestingly, the findings obtained with porcine capillaries were further confirmed by experiments in capillaries derived from human surgical specimen. In this context, it needs to be considered that fresh capillaries are only available from patients with neurologic disease and that the capillaries used here were from patients suffering from drug-refractory epilepsy. Both the seizure history and the treatment history might have already upregulated the expression rates of transporters in these specimens above physiologic levels. However, when suspecting a putative overexpression state in the isolated capillaries, it becomes even more impressive that the capillaries still exhibit a pronounced response to glutamate exposure.
The data raise the question about potential clinical implications. As already pointed out above, upregulation of MRP2 can occur in the epileptic brain (Aronica et al., 2004, 2012; van Vliet et al., 2005; Hoffmann and Löschner, 2007). However, it is rather questionable whether overexpression of this efflux transporter in brain capillaries has any implications for the brain penetration and efficacy of antiepileptic drugs. Earlier studies in rats lacking a functional MRP2 transporter gave evidence that it significantly affects brain access and efficacy of the antiepileptic drug phenytoin (Potschka et al., 2003). In contrast, several in vitro studies including an analysis in cell lines expressing the human MRP2 isoform argued against a relevant transport of several antiepileptic drugs including phenytoin (Luna-Tortos et al., 2010). Thus, there might be only a limited functional impact of MRP2 expression on antiepileptic drug responses.

Nevertheless, the regulation of efflux transporter regulation in disease states is of general interest for several reasons (Miller, 2010). First of all, in patients with seizures related to brain tumors, the brain penetration of MRP2 substrate cytostatic drugs like cisplatin, doxorubicin, epirubicin, and etoposide might be impaired as a consequence of MRP2 overexpression (Löschner and Potschka, 2005c). In addition, disease-associated regulation might have long-term implications for brain access of endogenous substrates and of harmful xenobiotics. Moreover, the gain in knowledge about the mechanisms of efflux transporter dynamics points to novel strategies allowing to upregulate transporters or to control transporters at basal expression levels in different disease states. In this context it is important to note that the efficacy of celecoxib with prevention of MRP2 upregulation was confirmed in translational experiments with human capillaries.

In the past there was an ongoing debate about the expression of MRP2 in human capillaries (Dombrowski et al., 2001; Aronica et al., 2004; Nies et al., 2004; Kubota et al., 2006). Our findings demonstrate that MRP2 can be expressed at functionally relevant levels in human brain capillaries. This obviously applies to capillaries from patients with drug-refractory epilepsy.

In conclusion, the study provides the first evidence that glutamate signaling via the endothelial NMDA receptor and cyclooxygenase-2 prostanooid generation affects MRP2 function and expression in porcine capillaries. The data suggest that this signaling pathway contributes to seizure-associated upregulation of MRP2. Comparative analysis of the impact of glutamate exposure in human capillaries argued against relevant species differences between regulation of the transporter in porcine and human specimen.

The findings might have general implications for neurologic disorders with excessive glutamate release with an impact on endogenous and exogenous MRP2 substrate brain access. Future studies are necessary to further explore the link between MRP2 expression regulation and functional consequences.

Acknowledgments
The authors thank Olga Cabezas-Montalegre and Barbara Kohler for excellent technical assistance.

Authorship Contributions
Participated in research design: Luna-Munguia, Salvamose, Pascher, Pieper, Getzinger, Kudernatsch, Kluger, Potschka.
Conducted experiments: Luna-Munguia, Salvamose, Pascher, Pieper, Getzinger, Kudernatsch.


Address correspondence to: Dr. Heidrun Potschka, Institute of Pharmacology, Toxicology, and Pharmacy, Ludwig-Maximilians-University, Koenigstr. 16, D-80539, Munich, Germany. E-mail: potschka@pharmtox.vetmed.uni-muenchen.de