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Cytosolic phospholipase A2 is a key regulator of blood–brain barrier function in epilepsy

Anika M. S. Hartz,[*,†] Ralf G. Rempe,[‡] Emma L. B. Soldner,[§] Anton Pekcec,[§] Juli Schlichtiger,[§] Richard Kryscio,[*,¶] and Bjoern Bauer[‡,‖,1]

[*]Sanders-Brown Center on Aging, [†]Department of Pharmacology and Nutritional Sciences, [‡]Department of Pharmaceutical Sciences, College of Pharmacy, [¶]Department of Statistics, and [‖]Epilepsy Center, University of Kentucky, Lexington, Kentucky, USA; and [§]Department of Pharmacy Practice and Pharmaceutical Sciences, College of Pharmacy, University of Minnesota, Duluth, Minnesota, USA

ABSTRACT: Blood–brain barrier dysfunction in epilepsy contributes to seizures and resistance to antiseizure drugs. Reports show that seizures increase brain glutamate levels, leading to barrier dysfunction. One component of barrier dysfunction is overexpression of the drug efflux transporters P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP). Based on our previous studies, we hypothesized that glutamate released during seizures activates cytosolic phospholipase A2 (cPLA2), resulting in P-gp and BCRP overexpression. We exposed isolated rat brain capillaries to glutamate *ex vivo* and used an *in vivo–ex vivo* approach of isolating brain capillaries from rats after status epilepticus (SE) and in chronic epileptic (CE) rats. Glutamate increased cPLA2, P-gp, and BCRP protein and activity levels in isolated brain capillaries. We confirmed the role of cPLA2 in the signaling pathway in brain capillaries from male and female mice lacking cPLA2. We also demonstrated, *in vivo*, that cPLA2 inhibition prevents overexpression of P-gp and BCRP at the blood–brain barrier in rats after status epilepticus and in CE rats. Our data support the hypothesis that glutamate signals cPLA2 activation, resulting in overexpression of blood–brain barrier P-gp and BCRP.—Hartz, A. M. S., Rempe, R. G., Soldner, E. L. B., Pekcec, A., Schlichtiger, J., Kryscio, R., Bauer, B. Cytosolic phospholipase A2 is a key regulator of blood–brain barrier function in epilepsy. FASEB J. 33, 000–000 (2019). www.fasebj.org

KEY WORDS: P-glycoprotein · seizures · BCRP · neurovascular unit

Epilepsy is a CNS disorder affecting over 70 million individuals worldwide (1). Approximately 30–40% of patients with epilepsy do not fully respond to antiseizure drugs (ASDs) (2). ASD resistance is complex, multifactorial, and a major obstacle in the clinical management of epilepsy. One factor contributing to ASD resistance is that ASDs do not reach their targets in the brain (3). To enter the brain, ASDs have to cross the capillary endothelium that constitutes the blood–brain barrier and controls what goes into and comes out of the brain. In this regard, the drug efflux transporters P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) in the luminal membrane of the capillary endothelium limit drug entry into the brain (4–7). Several ASDs have been shown to be substrates for P-gp and BCRP, which impair the ability of those ASDs to cross the blood–brain barrier and reach their targets within the brain (8–11). For example, P-gp transports phenobarbital, phenytoin, felbamate, lamotrigine, topiramate, and levetiracetam (10, 12–14). BCRP is involved in transporting phenobarbital, gabapentin, lamotrigine, levetiracetam, and zonisamide (6, 15). Furthermore, studies including our own show that seizures increase expression and activity levels of blood–brain barrier transporters such as P-gp and BCRP, which are thought to reduce ASD brain uptake and contribute to ASD resistance. P-gp protein expression is up-regulated in brain capillaries from ASD-resistant rats and patients with epilepsy, and this phenomenon correlates with reduced ASD brain levels and increased seizure frequency (16–18). Inhibiting P-gp, on the other hand, increases phenytoin brain levels in chronic epileptic (CE) rats, prevents seizure-induced transporter overexpression, and improves ASD efficacy in ASD-resistant animals (19, 20). Limited information is available for BCRP in epilepsy. Immunohistochemical data from brain slices indicate BCRP up-regulation in brain capillaries shortly after status epilepticus (SE), during the latent period after SE, and in CE rats. Additional

ABBREVIATIONS: AA, arachidonic acid; arachidonoyl thio-PC, 1-*O*-hexadecyl-2-deoxy-2-thio-R-(5Z,8Z,11Z,14Z-eicosatetraenoyl)-sn-glyceryl-3-phosphorylcholine; ATK, arachidonyl trifluoromethyl ketone; ASD, antiseizure drug; BCRP, breast cancer resistance protein; BSA, bovine serum albumin; CE, epileptic/epileptic; COX-2, cyclooxygenase-2; cPLA2, cytosolic phospholipase A2; DPBS, Dulbecco's PBS; KO, knockout; NBD-CSA, [*N*-ε (4-nitrobenzofurazan-7-yl)-D-Lys[8]]-cyclosporine A; P-gp, P-glycoprotein; PG, prostaglandin; Pilo, pilocarpine; SE, status epilepticus; WT, wild type

[1] Correspondence: Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, 333 Sanders-Brown Center on Aging, 800 S Limestone St., Lexington, KY 40536-0230, USA. E-mail: bjoern.bauer@uky.edu

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data from experiments with P-gp and BCRP knockout (KO) mice suggest that BCRP contributes to ASD efflux (4, 6, 20). We have demonstrated that seizure-induced P-gp up-regulation is mediated through cyclooxygenase-2 (COX-2) signaling (21–23). Upstream from COX-2 is the enzyme cytosolic phospholipase A2 (cPLA2) that cleaves phospholipids, thereby generating the COX-2 substrate arachidonic acid (AA). We linked cPLA2 to COX-2 signaling in brain capillaries by showing that glutamate, a neurotransmitter released during seizures, signals through the NMDA receptor, which activates cPLA2 and leads to increased matrix metalloproteinase protein expression and activity levels, resulting in blood–brain barrier leakage (24).

Here, we focused on cPLA2 and its role in the up-regulation of P-gp and BCRP at the blood–brain barrier in epilepsy. We exposed brain capillaries to glutamate *ex vivo* to discern the signaling steps and confirmed our findings in cPLA2 KO mice. We also used a cPLA2 inhibitor for *in vivo* experiments using an SE model and a rat CE model. Our data demonstrate that seizure-induced P-gp and BCRP up-regulation is mediated through cPLA2, indicating that cPLA2 could be a target to improve ASD brain uptake.

MATERIALS AND METHODS

Data and statistical analysis

Data and statistical analyses follow published guidelines for experimental design and analysis (25). Sample sizes (*e.g.*, animal numbers and number of brain capillaries) for individual experiments were based on power analyses of preliminary data and previously published data and are given in the figure legends (21–23, 26). The number of repetitions is stated in Results and the figure legends. Animals were randomly assigned to each group (simple randomization); data analysis was performed in a double-blinded fashion.

Statistical analyses focused on comparisons between experimental groups and did not include covariates. Mean response was compared using a 2-tailed, unpaired Student's t test for 2 groups and ANOVA for more than 2 groups. Analyses were performed using Microsoft Excel (Microsoft, Redmond, WA, USA) and Prism (v.7.00; GraphPad, La Jolla, CA, USA). Because animals in each experiment were independent, observed differences were considered to be statistically significant when $P < 0.05$ (*i.e.*, there were no multiple comparisons based on data from the same animals). For the Western blot data, the mean for each treatment was compared with the control using Dunnett's many-to-one t test with statistical significance determined when $P \leq 0.05$.

Chemicals

Dulbecco's PBS (DPBS) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Antibodies against cPLA2, phosphorylated (p)-cPLA2, BCRP (BXP-53), and β-actin were obtained from Abcam (Cambridge, MA, USA). The antibody against COX-2 was obtained from Cayman Chemicals (Ann Arbor, MI, USA); the antibody against P-gp (C219) was purchased from Thermo Fisher Scientific. The cPLA2 inhibitor arachidonyl trifluoromethyl ketone (ATK) and the cPLA2 substrate 1-*O*-hexadecyl-2-deoxy-2-thio-*R*-(5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoyl)-*sn*-glyceryl-3-phosphorylcholine (arachidonoyl thio-PC) were purchased from Cayman Chemicals, Bodipy-Prazosin was purchased from Thermo Fisher Scientific, and [*N*-ε (4-nitrobenzofurazan-7-yl)-D-Lys^8]-cyclosporine A (NBD-CSA) was custom-synthesized by R. Wenger [Sandoz Pharma AG, Basel, Switzerland (27)]. L-Glutamate, pilocarpine (Pilo), AA, NMDA, prostaglandin (PG)-E2, and all other chemicals and reagents were obtained from MilliporeSigma (Burlington, MA, USA). All supplies were purchased from Thermo Fisher Scientific.

Animals

Animal protocols were approved by the University of Minnesota (UMN) and University of Kentucky Institutional Animal Care and Use Committee (IACUC; 1012A93932 and 2014-1234; Principal Investigator, B.B.) and were in accordance with Assessment and Accreditation of Laboratory Animal Care (regulations, the U.S. Department of Agriculture Animal Welfare Act, and National Institutes of Health (NIH; Bethesda, MD, USA) animal guidelines.

Male CD IGS Sprague-Dawley rats (8 wk old, 275–300 g; retired breeders, 500–750 g) were obtained from Charles River Laboratories (Wilmington, MA, USA). Male COX-2 KO mice (129P2-Ptgs2tm1UNC; 12 wk old; 30–50 g), and male wild-type (WT) mice (C57BL/6; 12 wk old; 30–50 g) were purchased from Taconic Biosciences (Rensselaer, NY, USA). Heterozygous cPLA2 breeding pairs were a kind gift from Dr. Joseph V. Bonventre (Brigham and Women's Hospital, Boston, MA, USA) (28). cPLA2 WT, heterozygous, and KO mice were bred at the University of Kentucky Animal Facility. All animals were housed under controlled conditions (23°C; 35% relative humidity; 12-h dark/light cycle) with free access to tap water and rodent chow and were given 1 wk to acclimate to the new environment prior to experiments.

Genotyping of cPLA2 mice

Genotyping was performed for cPLA2 WT, heterozygous, and KO mice using ear-punch samples. DNA was isolated with Phire Tissue Direct PCR Master Mix (F170S; Thermo Fisher Scientific). Each ear-punch sample (2-mm diameter) was placed in a PCR tube containing 20 μl dilution buffer and 0.4 μl DNA-release additive. Samples were centrifuged, incubated at room temperature for 5 min, and incubated at 98°C for 2 min using a SimpliAmp Thermal Cycler (Thermo Fisher Scientific). Isolated DNA samples were stored at −20°C until use. The PCR reaction was carried out with Phire Tissue Direct PCR Master Mix (2X) using the following 3 specific primers: 5′-CGACTCATACAGTGCCTTCATCAC-3′ (CPLA604), 5′-GGGAACTTCCTGACTAGGGG-3′ (PGKNEO), and 5′-TGTGTACAATCTTTGTGTTGTTTCA-3′ (CPLA3F) at a final concentration of 10 μM with 1 μl isolated DNA. PCR was carried out using a SimpliAmp Thermal Cycler with an initial denaturation at 98°C for 5 min; 40 cycles of a 3-step amplification including 98°C denaturation for 5 s, 62°C annealing for 5 s, and 72°C extension for 20 s; and a final extension at 72°C for 1 min. PCR products were resolved on E-Gel EX 4% agarose gels using an E-Gel Precast Agarose Electrophoresis System (Thermo Fisher Scientific). Gels were imaged using a ChemiDoc XRS+ System with accompanying Image Lab v.5.0 software (Bio-Rad, Hercules, CA, USA).

Brain capillary isolation

Animals were euthanized by CO_2 inhalation and subsequent decapitation. Brains were collected in ice-cold DPBS [2.7 mM KCl, 1.47 mM KH_2PO_4, 136.9 mM NaCl, 8.1 mM Na_2HPO_4, 0.9 mM $CaCl_2$, and 0.49 mM $MgCl_2$, supplemented with 5 mM

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D-glucose and 1 mM sodium pyruvate (pH 7.4)] and dissected; brain stem, meninges, and white matter were removed. Brain cortex tissue was collected, minced, and homogenized in DPBS using a Potter-Elvehjem homogenizer (Thomas Scientific, Swedesboro, NJ, USA) followed by homogenization using a Dounce homogenizer (VWR, Radnor PA, USA). The brain homogenate was mixed with Ficoll PM400 (final concentration, 15%; MilliporeSigma) and centrifuged (5800 *g*; 15 min; 4°C). The pellet containing brain capillaries was resuspended in DPBS with 1% bovine serum albumin (BSA), filtered through a 300 μm mesh, and passed over a glass-bead column using 1% BSA. Capillaries adhering to the glass beads were washed off and collected, followed by filtration through a 100-μm cell strainer (Falcon; Corning, Corning, NY, USA). After centrifugation (1,500 *g*; 3 min; 4°C), the capillary pellet was washed 3 times with DPBS. Capillaries from *in vivo* experiments were used immediately for endpoint measurements.

For *ex vivo* experiments, freshly isolated brain capillaries were exposed to 100 μM glutamate for 30 min, washed with DPBS, and incubated in glutamate-free DPBS buffer for an additional 0.5, 2.5, or 5.5 h. Capillaries were also exposed to 1 μM NMDA, 1–10 μM AA, or 0.5 nM PGE2 (with and without 25 μM of the cPLA2 inhibitor ATK) for 6 h. Afterward, brain capillaries were used for the endpoints described below. Note that glutamate exposure during the capillary isolation procedure is considered to be minimal because of: *1*) the early removal of cell types containing glutamate (*e.g.*, astrocytes and neurons), *2*) larger buffer volumes, and *3*) frequent buffer exchanges during the procedure.

Membrane sample preparation

Tissue samples were lysed in CelLytic M buffer (MilliporeSigma) containing Complete Protease Inhibitor (Roche, Basel, Switzerland) using a Polytron 2500E homogenizer (Kinematica, Bohemia, NY, USA). The cell lysate was centrifuged (10,000 *g*; 30 min; 4°C) and the resulting supernatant was centrifuged again (100,000 *g*; 90 min; 4°C). The final pellet (crude membrane fraction) was resuspended; capillary membrane samples were frozen for later use.

Western blotting

Sample protein concentrations were determined with the Bradford assay. Western blots were performed using the NuPAGE electrophoresis and blotting system (Thermo Fisher Scientific). After protein transfer, the blotting membranes were incubated overnight with primary antibody [BCRP (1 μg/ml; ab24115; Abcam), cPLA2 (1 μg/ml; ab58375; Abcam), p-cPLA2 (1 μg/ml; ab53105; Abcam), COX-2 (1 μg/ml; 160,126; Cayman Chemicals), P-gp [1 μg/ml; C219 (MA126528); Thermo Fisher Scientific], or β-actin (1 μg/μl; ab8226; Abcam)]. Afterward, the blotting membranes were washed and incubated with the corresponding horseradish peroxidase–conjugated secondary antibody (1:10000; Pierce, Rockford, IL, USA). Proteins were detected using SuperSignal West Pico chemiluminescent substrate (Pierce) and visualized with a Gel Doc XRS Imaging System (Bio-Rad). Bands were analyzed with Image Lab v.5.0 software.

Immunohistochemistry

Freshly isolated brain capillaries were resuspended in PBS and transferred to confocal imaging chambers with coverslip bottoms and allowed to attach for 30 min. Capillaries were fixed for 30 min with fixative (3% paraformaldehyde and 0.25% glutaraldehyde in PBS), permeabilized for 45 min with Triton X-100 Surfact-Amps (0.5% in PBS; Thermo Fisher Scientific), and blocked for at least 2 h with 1% BSA dissolved in PBS. Brain capillaries were incubated overnight at 4°C with cPLA2 primary antibody (20 μg/ml; ab58375; Abcam) in 1% BSA. After washing with 1% BSA, capillaries were incubated with the corresponding Alexa Fluor 488 secondary antibody (diluted in 1% BSA) for 1 h at 37°C in the dark. Cell nuclei were counterstained with DAPI (1 μg/ml in 1% BSA). Capillaries were imaged using a Leica TCS SP5 Acousto Optical Bream Splitter inverted confocal microscope (Leica Microsystems, Buffalo Grove, IL, USA) equipped with an HCX PL APO Lambda Blue Leica ×63 water objective (numerical aperture, 1.2; Leica Microsystems) using the 488-nM line of an argon laser and the 405-nM line of a laser diode (Leica Microsystems).

P-gp and BCRP transport activity assays

Freshly isolated brain capillaries were incubated for 1 h at 25°C with the fluorescent P-gp–specific substrate NBD-CSA (2 μM in DPBS) or the fluorescent BCRP-specific substrate Bodipy-Prazosin (2 μM in DPBS) (29, 30). Per treatment, images of 10 capillaries were acquired using a Zeiss LSM 710 inverted confocal microscope (C-Apochromat ×40/1.2 W Corr objective; 488-nM line of an argon laser; Carl Zeiss GmbH, Oberkochen, Germany). Images were analyzed by quantitating luminal fluorescence with Zen software (Carl Zeiss GmbH) and ImageJ v.1.41 software (NIH). As previously described, specific, luminal NBD-CSA fluorescence was taken as the difference between total luminal fluorescence and fluorescence in the presence of the P-gp–specific inhibitor PSC833 (5 μM); Bodipy-Prazosin fluorescence was taken as the difference between total luminal fluorescence and fluorescence in the presence of the BCRP-specific inhibitor fumitremorgin C [10 μM (29, 31)].

cPLA2 activity assay

cPLA2 activity in isolated brain capillaries was determined using a cPLA2 activity assay (Cayman Chemicals). In this assay, arachidonoyl thio-PC is the cPLA2 substrate (32). cPLA2-mediated hydrolysis of the arachidonoyl thioester releases a free thiol, which is detected with 5,5′-dithiobis(2-nitrobenzoic acid) in a colorimetric reaction that results in colored 5-mercapto-2-nitrobenzoate. Specifically, freshly isolated brain capillaries were suspended in DPBS containing Complete Protease Inhibitor and homogenized using a Polytron 2500E homogenizer (Kinematica). Samples were centrifuged (10,000 *g*; 15 min; 4°C) and the resulting supernatants (cytosol) were collected. Samples were transferred into 96-well microplates, arachidonoyl thio-PC was added, the plate was shaken, and samples were incubated for 120 min at 25°C. 5,5′-dithiobis(2-nitrobenzoic acid) and EGTA was added to stop cPLA2 activity and to develop the color reaction. Absorbance was measured at 414 nm using a SynergyH1 Multimode Reader (BioTek Instruments, Winooski, VT, USA). Raw data were analyzed, and results were expressed as nanomoles per minute per milliliter.

SE model

Each rat was handled for 2 min daily for 3 d prior to SE induction to minimize handling stress. Fourteen hours prior to Pilo dosing, all rats were given lithium chloride (127 mg/kg) by intraperitoneal injection; 30 min prior to Pilo dosing, all rats ($n = 30$) were given methylscopolamine (1 mg/kg) by intraperitoneal injection. To induce SE, rats ($n = 24$) received Pilo (10 mg/kg) by repeated intraperitoneal injections every 30 min until the onset of

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generalized convulsive seizures and continuous seizure activity; control rats (n = 6) received vehicle injections. The maximum number of Pilo injections was 12 per animal (maximum dose per animal: 120 mg/kg). SE was continuously monitored and seizure intensity was evaluated using the Racine scale. Seven of the 24 rats that received Pilo died during SE induction. All rats that developed SE (n = 9) showed continuous seizure activity (Racine score of 4–5) for 90 min. Rats that received Pilo but did not develop SE (n = 8) were utilized as Pilo control rats. Seizures were terminated after 90 min by intraperitoneal injection of diazepam (10 mg/kg). Animals were euthanized 48 h after SE induction using CO_2 and subsequent decapitation.

ATK *in vivo* study

To test the effect of cPLA2 inhibition in an SE model *in vivo*, naive rats (n = 6) were dosed with the selective cPLA2 inhibitor ATK (10 mg/kg, itraperitoneally) every 12 h for 3.5 d (total of 7 intraperitonal injections). After 4 treatments with ATK, rats underwent SE induction with Pilo as described above and then received 3 more ATK treatments every 12 h post-SE. Untreated rats (n = 10; vehicle injections instead of ATK) underwent SE induction with Pilo as previously described. Four of these 10 animals did not develop SE and served as Pilo control group (n = 4). All rats (n = 10) undergoing SE induction survived. Control rats (n = 6) received vehicle injections throughout the experiment. Rats [control rats (n = 6), Pilo control rats (n = 4), SE control rats (n = 6), and ATK-treated SE rats (n = 6)] were euthanized 48 h after SE induction using CO_2 followed by decapitation.

CE model and video monitoring

To generate rats with CE (spontaneous, recurrent seizures), 31 animals treated with and without ATK (10 mg/kg, intraperitoneally, every 12 h for 3.5 d; total of 7 intraperitoneal injections) underwent SE induction with Pilo as described; control animals (n = 10) received vehicle injections. Five animals died during SE induction; 4 animals did not develop SE and were excluded from the study. Twelve months after SE, the remaining 22 rats were continuously (24 h/d) video monitored for 2 wk to identify spontaneous recurrent seizures. Video data were collected using Bosch WZ16N408-0 compact integrated day-night cameras (Gerlingen, Germany) in infrared mode that were connected to an Exacq 1608-12-1000 16-camera digital video recorder (Exacq, Fishers IN, USA). Recorded video data were analyzed in a double-blind fashion using ExacqVision Pro Video Management System Software v.4 (Exacq) to determine seizure occurrence, frequency, duration, and severity. Seizure severity was ranked using the Racine scale as follows: class 3, forearm clonus either unilateral or bilateral; class 4, rearing; and class 5, rearing and loss of postural control (33). One animal of the 22 animals did not develop spontaneous recurrent seizures and was excluded from the study. The remaining 21 rats displayed spontaneous, recurrent seizures over the course of 12 mo and were then used to test the effect of the cPLA2 inhibitor ATK. CE rats were dosed again with ATK (10 mg/kg intraperitoneally, once daily for 2 wk); control animals received vehicle (intraperitoneal, once daily for 2 wk). During the CE phase, 2 animals of the group of CE rats treated with ATK for 2 wk (CE-ATK) and 1 animal of the CE group had to be euthanized because of seizure-unrelated causes (mammary tumor; liver infection; intestinal obstruction; total n = 18). After the second ATK treatment phase, the control rats (n = 10) as well as the CE rats (n = 5), CE-ATK rats (n = 8), and CE rats pretreated with ATK before SE induction and then ATK again for 2 wk during the CE phase (ATK-CE-ATK; n = 5) were euthanized using CO_2 followed by decapitation.

RESULTS

cPLA2 expression in rat brain tissues

To determine cPLA2 protein-expression levels, we isolated brain capillaries from male CD IGS Sprague-Dawley rats. **Figure 1*A*** shows a representative Western blot demonstrating cPLA2 protein expression in total brain lysate, brain capillary lysate, and brain capillary membranes. We also detected cPLA2 in isolated rat brain capillaries by immunohistochemistry (Fig. 1*B*). We further examined brain tissue (whole brain, parahippocampal cortex, and hippocampus) and brain capillaries isolated from untreated rats [control rats (n = 6)], from rats that received Pilo but did not develop SE (Pilo control rats: n = 8), and from rats that received Pilo and developed SE (SE rats; n = 9). We detected signals for functionally inactive, unphosphorylated cPLA2 as well as functionally active p-cPLA2 in all samples (Fig. 1*C*). Whereas protein levels of inactive cPLA2 were similar in all 3 groups, levels of active p-cPLA2 were increased in SE rats (**Table 1**). In brain, hippocampal, and capillary lysates from Pilo control rats (no SE), p-cPLA2 levels were slightly increased compared with control samples from untreated rats, which is likely caused by Pilo control rats experiencing a few seizures without developing a full SE.

These data show that cPLA2 is expressed in rat brain capillaries and that, after SE, protein levels of functionally active p-cPLA2 are increased in whole brain tissue, parahippocampal cortex tissue, hippocampus, and brain capillaries.

Glutamate increases phospholipase A2 protein and activity levels

Based on our published findings, we postulated that seizure-induced changes at the blood–brain barrier are caused by a release of the excitatory neurotransmitter glutamate (21, 24, 26). To test if glutamate changes cPLA2 protein expression and activity levels, we exposed isolated rat brain capillaries to 100 μM glutamate, removed glutamate after 30 min, and incubated capillaries in glutamate-free buffer for an additional 0.5, 2.5, or 5.5 h. After a total incubation time of 1, 3, or 6 h, brain capillaries were analyzed by Western blotting. Figure 1*D* shows that levels of unphosphorylated cPLA2 are slightly decreased after glutamate exposure compared with controls but that protein levels of p-cPLA2 were increased. When comparing the 3 time points, the effect seems to be most pronounced at 3 h (**Table 2**). Based on these results, we measured cPLA2 activity in brain capillary lysate at 3 h. Figure 1*E* shows that cPLA2 activity levels in brain capillaries after glutamate exposure were increased by 93% compared with controls (control: 0.61 ± 0.01 nmol/min/ml; glutamate: 1.18 ± 0.03 nmol/min/ml; $P < 0.01$; n = 3 independent experiments).

These findings demonstrate that glutamate triggers a time-dependent increase in p-cPLA2 protein-expression levels that is consistent with an increase in cPLA2 activity levels.

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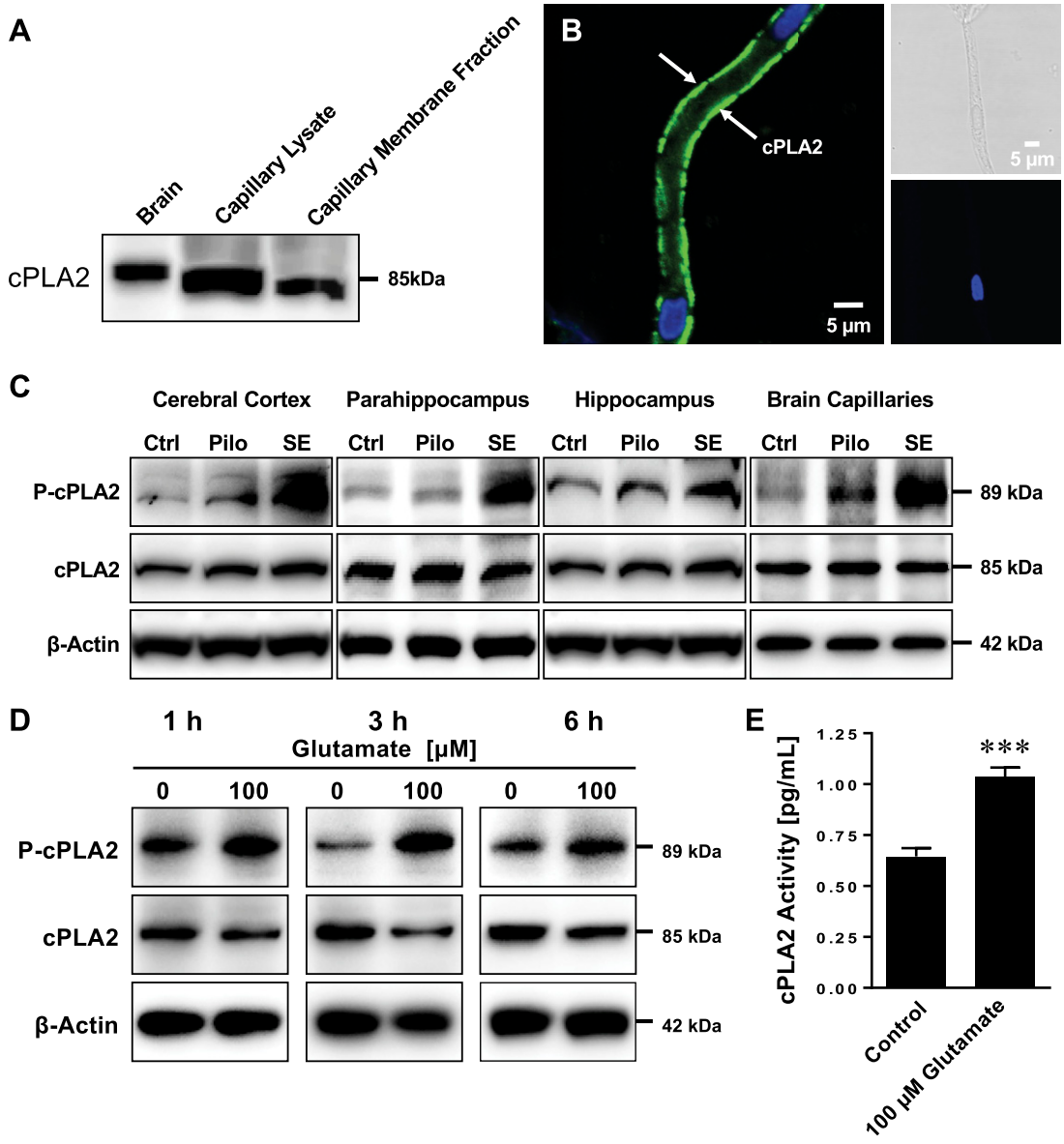

Figure 1. Expression and activity of cPLA2 in isolated rat brain capillaries. *A*) Western blot showing cPLA2 protein expression in whole brain lysate, capillary lysate, and capillary membrane fraction. *B*) Left: representative image of an immunostaining for cPLA2 in an isolated rat brain capillary. cPLA2 is shown in green; nuclei were counterstained with DAPI (blue). Right: negative control (no primary antibody). *C*) Western blot for cPLA2 and p-cPLA2 in brain, parahippocampal cortex, hippocampus, and isolated capillaries from control rats (Ctrl; $n = 6$), rats that received Pilo but did not develop an SE (Pilo; $n = 8$), and from rats after an SE induced with Pilo (SE; $n = 9$). β-Actin was used as protein-loading control. *D*) Western blot showing cPLA2 and p-cPLA2 in isolated rat brain capillaries exposed to 100 µM glutamate (Glu) for 1, 3 and 6 h; β-actin was used as protein-loading control. *E*) Glutamate increased cPLA2 activity in isolated rat brain capillaries. Data are means ± SD ($n = 3$ independent experiments; pooled tissue from $n = 10$ rats/experiment). ***$P < 0.001$, significantly higher than control.

Glutamate mediates P-gp up-regulation through cPLA2

We then focused on cPLA2 and its potential role in the signaling pathway underlying glutamate-mediated up-regulation of P-gp. We exposed isolated rat brain capillaries to 100 µM glutamate with or without the selective cPLA2 inhibitor ATK and assessed P-gp protein expression and transport activity levels. We found that ATK completely blocked the glutamate-mediated increase in P-gp protein-expression levels (**Fig. 2*A***). P-gp transport activity was determined with an assay we developed (21, 26, 31, 34, 35). This assay is based on measuring P-gp–specific accumulation of the fluorescent cyclosporin A derivative, NBD-CSA, in the brain capillary lumen using confocal microscopy and digital image analysis. Incubating isolated rat brain capillaries with 100 µM glutamate increased specific luminal accumulation of NBD-CSA more than 2-fold compared with control capillaries. Importantly, ATK blocked the glutamate-mediated increase in P-gp transport activity levels (Fig. 2*A*).

Studies including our own have shown that brain capillaries express NMDA receptor subunits and that

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TABLE 1. *Western blot analysis of cPLA2 protein-expression levels in brain, parahippocampus, hippocampus, and brain capillaries isolated from rats after SE*

Protein	Control	Pilo	SE
Brain			
p-cPLA2	100 ± 9.1	108 ± 11.8	133 ± 10.1[a]
cPLA2	100 ± 24.9	105 ± 24.3	122 ± 24
Parahippocampus			
p-cPLA2	100 ± 6.4	105 ± 5.8	133 ± 6.9[a]
cPLA2	100 ± 23.5	105 ± 23.4	101 ± 18.7
Hippocampus			
p-cPLA2	100 ± 26.6	98 ± 30.6	159 ± 20.8[a]
PLA2	100 ± 21.3	98 ± 21.4	95 ± 24.8
Brain capillaries			
p-cPLA2	100 ± 12	121 ± 11.2	139 ± 11.5[a]
cPLA2	100 ± 15.6	106 ± 18.1	96 ± 16.6

Data were normalized to β-actin levels; values are given as percentage of control ± SD (n = 3 technical replicates). [a]Statistical significance determined when $P \leq 0.05$ for each endpoint compared with control using Dunnett's many-to-one t test.

NMDA receptor agonists and antagonists affect capillary function (21, 36, 37). We also showed that glutamate mediates P-gp up-regulation by signaling through the NMDA receptor (21). Based on these studies, we exposed isolated rat brain capillaries to NMDA, an NMDA receptor agonist. We observed an increase in P-gp protein expression and transport activity levels in brain capillaries that were exposed to NMDA but did not observe such an effect in brain capillaries exposed to both NMDA and the cPLA2 inhibitor ATK (Fig. 2*B*).

AA is an inflammatory signaling molecule that cPLA2 cleaves from membrane phospholipids. Exposing isolated rat brain capillaries to 1 and 10 μM AA increased P-gp protein expression and transport activity levels (Fig. 2*C*). In contrast with glutamate and NMDA, ATK did not block the AA-mediated increase in P-gp protein and activity levels, indicating that AA signaling is downstream from cPLA2 (Fig. 2*D*).

These data show that cPLA2 is part of the signaling pathway responsible for glutamate-mediated up-regulation of P-gp protein expression and transport activity levels in isolated rat brain capillaries.

Glutamate mediates P-gp up-regulation through COX-2

We previously unraveled that COX-2 is also part of the mechanism underlying glutamate-mediated changes at the blood–brain barrier by converting AA into PGE2 (21). To test involvement of COX-2, we incubated isolated brain capillaries from WT and COX-2 KO mice with 10 μM AA or 0.5 nM PGE2 and determined P-gp protein expression and transport activity levels. Figure 2*E* shows that both AA and PGE2 increased P-gp protein expression and transport activity levels in brain capillaries from WT mice, whereas in brain capillaries from COX-2 KO mice, only PGE2 increased P-gp expression and transport activity levels compared with the control (Fig. 2*F*). This finding demonstrates that COX-2 is part of the signaling pathway that mediates glutamate-induced increases in P-gp expression and transport activity levels.

Glutamate mediates P-gp and BCRP up-regulation through cPLA2

The data shown in Figs. 1 and 2 suggest that cPLA2 is critical in glutamate-mediated up-regulation of brain capillary P-gp. We expanded our experiments to BCRP (ABCG2), which is another drug efflux transporter that has been implicated in ASD resistance. We used brain capillaries isolated from male and female WT and cPLA2 KO mice and exposed them to glutamate or the cPLA2 substrate AA with and without the cPLA2 inhibitor ATK. Isolated brain capillaries from male WT mice exposed to glutamate showed increased P-gp and BCRP protein expression and transport activity levels (**Fig. 3*A***). Glutamate-mediated up-regulation of both P-gp and BCRP was abolished by inhibiting cPLA2 with ATK. In cPLA2 KO mice, neither glutamate nor ATK had an effect on P-gp and BCRP protein expression and transport activity levels, indicating that cPLA2 is critical for glutamate-mediated up-regulation of both transporters (Fig. 3*B*; note that different band intensities for control capillaries from WT and cPLA2 KO mice result from different exposure times and not from different expression levels). In contrast, the cPLA2 product AA up-regulated P-gp and BCRP protein expression and transport activity levels in capillaries isolated from both WT and cPLA2 KO mice (Fig. 3*C, D*). We repeated the experiments with brain capillaries isolated from female WT and cPLA2 KO mice. Similar to the effects observed in capillaries from male mice, we found that glutamate increased P-gp and BCRP protein expression and transport activity levels in capillaries from female WT mice but not in capillaries from female cPLA2 KO mice (**Fig. 4*A, B***). Consistent with the findings in capillaries from male mice, AA increased P-gp and BCRP protein expression and transport activity levels in capillaries from both female WT and cPLA2 KO mice (Fig. 4*C, D*).

These data confirm that cPLA2 is critical for glutamate-mediated up-regulation of both P-gp and BCRP. These data also indicate that there is no male-female difference in glutamate signaling at the blood–brain barrier and that brain capillary protein expression and transport activity levels of P-gp and BCRP are similar between male and female mice.

TABLE 2. *Western blot analysis of cPLA2 and p-cPLA2 protein-expression levels in brain capillaries exposed to glutamate*

Protein	1 h	3 h	6 h
p-cPLA2			
Control	100 ± 6.8	100 ± 5.8	100 ± 9.8
100 μM glutamate	102 ± 7.1	140 ± 9.4[a]	112 ± 7.9
cPLA2			
Control	100 ± 10.0	100 ± 18.8	100 ± 24.8
100 μM glutamate	94 ± 11.1	100 ± 16.5	85 ± 20.6

Data were normalized to β-actin levels; values are given as percentage of control ± SD (n = 3 technical replicates). [a]Statistical significance determined at the 0.001 level for each endpoint compared with control using 2-sample Student's t tests.

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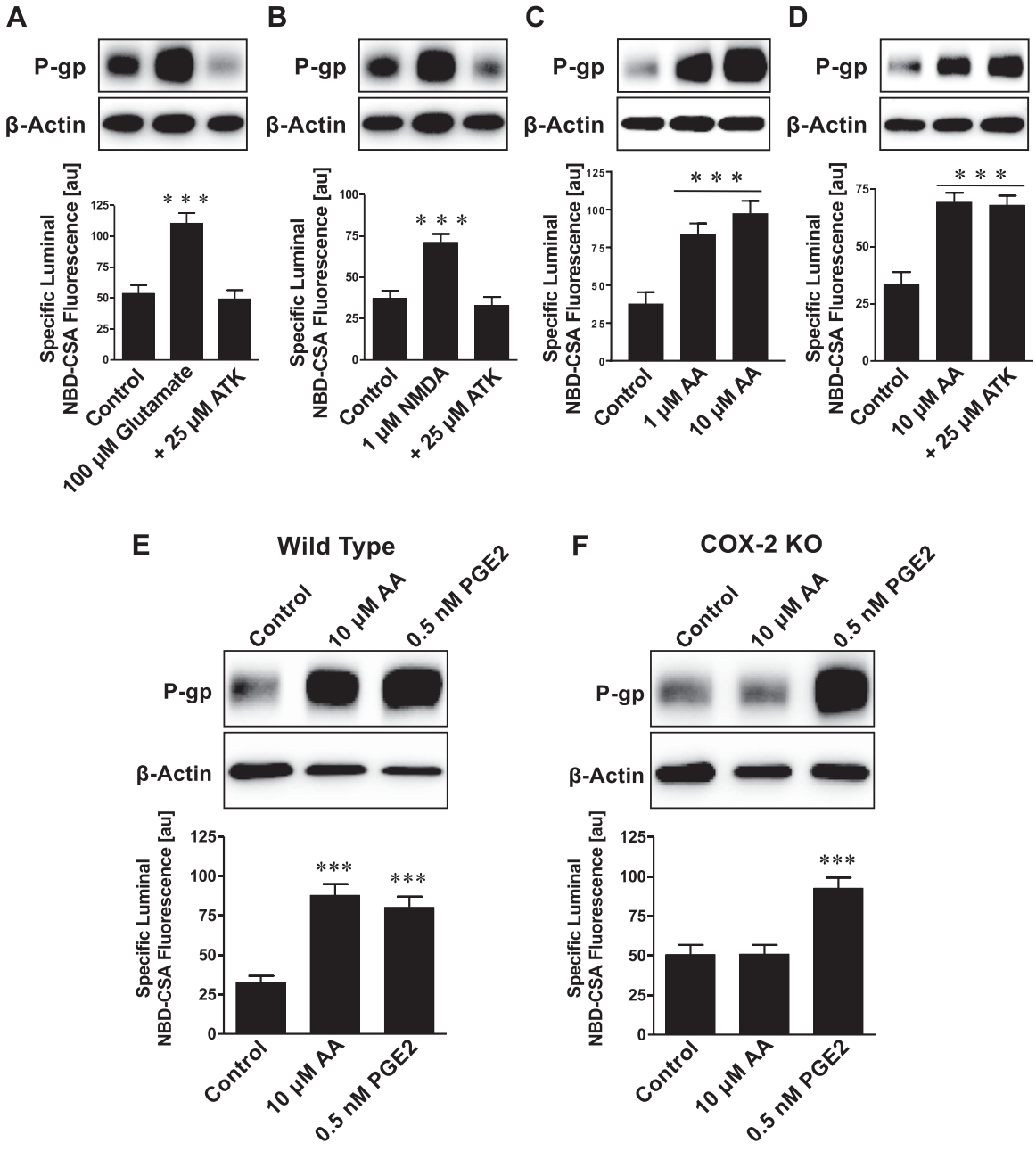

Figure 2. Glutamate increases P-gp protein expression by acting through cPLA2 and COX-2. Isolated rat brain capillaries were exposed to indicated concentrations of glutamate, NMDA, and AA with or without ATK. Inhibition of cPLA2 with ATK fully abolished the action of glutamate (*A*) and NMDA (*B*). *C*) AA increases P-gp protein expression and transport function. *D*) Inhibition of ATK did not block the action of AA. P-gp protein expression was assessed by Western blotting; β-actin was used as protein-loading control. P-gp transport activity was measured as specific luminal NBD-CSA accumulation in capillary lumens. *E*) Exposing isolated brain capillaries from WT mice to the cPLA2 substrate AA and the COX-2 product PGE2 increased both P-gp protein expression and transport activity levels. *F*) Exposing isolated brain capillaries from COX-2 KO mice to AA had no effect on P-gp, whereas PGE2 increased P-gp protein expression and transport activity levels. P-gp and COX-2 protein expression were assessed by Western blotting; β-actin was used as protein-loading control. P-gp transport activity was measured as specific luminal NBD-CSA accumulation in capillary lumens. Statistics: For specific luminal NBD-CSA fluorescence, data represent the means ± SD for 10 capillaries from a single preparation (pooled tissue from 10 rats). Units are arbitrary fluorescence units (au; scale: 0–255). ***$P < 0.001$, significantly higher than controls.

cPLA2 inhibition prevents P-gp and BCRP up-regulation in a rat SE model

Our data in brain capillaries from SE rats indicate that cPLA2 is involved in glutamate-induced up-regulation of P-gp and BCRP expression and transport activity levels and that levels of active p-cPLA2 are increased as well. To provide additional *in vivo* evidence that cPLA2 is involved in seizure-induced changes in blood–brain barrier P-gp and BCRP, we tested the effect of the cPLA2 inhibitor ATK in rats that experienced SE (**Fig. 5*A***). Rats were dosed with ATK (10 mg/kg, i.p.) every 12 h for 3.5 d (total of 7

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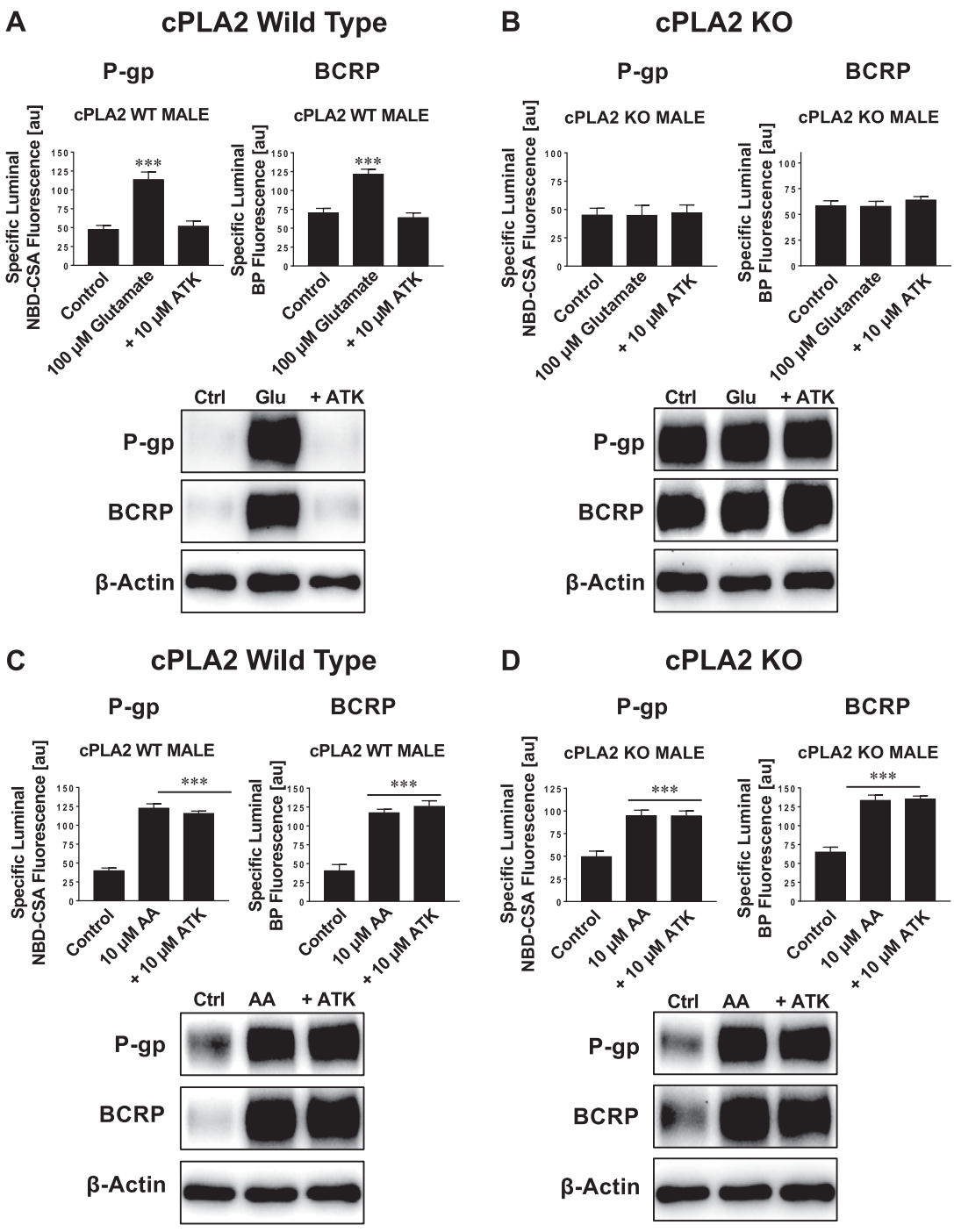

Figure 3. Glutamate mediates P-gp and BCRP up-regulation in male WT mice but not in male cPLA2 KO mice. Brain capillaries were isolated from male WT and cPLA2 KO mice and exposed to glutamate or AA with or without the cPLA2 inhibitor ATK. *A*) Exposing capillaries from WT mice to glutamate resulted in increased P-gp and BCRP protein expression and transport activity. This effect was abolished by the cPLA2 inhibitor ATK. *B*) Glutamate had no effect on P-gp and BCRP protein expression or transport activity levels in capillaries isolated from cPLA2 KO mice. *C*) Exposing capillaries from WT mice to AA resulted in increased P-gp and BCRP protein expression and transport activity; this effect remained unchanged with ATK. *D*) Capillaries isolated from cPLA2 KO mice also showed increased P-gp and BCRP protein expression and transport activity compared with WT mice; this effect was not abolished by ATK. P-gp and BCRP protein expression were assessed by Western blotting; β-actin was used as protein-loading control. P-gp transport activity was measured as specific luminal NBD-CSA accumulation in capillary lumens; BCRP transport activity was measured as specific Bodipy-Prazosin (BP) accumulation in capillary lumens. Statistics: For specific luminal NBD-CSA and BP fluorescence, data represent the means ± SD for 10 capillaries from a single preparation (pooled tissue from 10 rats). Units are arbitrary fluorescence units (au; scale: 0–255). ***$P < 0.001$, significantly higher than controls.

intraperitoneal injections); control rats received vehicle injections. After 4 ATK treatments, rats underwent SE induction with Pilo; after SE induction, animals received 3 more ATK treatments. Note that ATK pretreatment had no effect on SE induction in animals. Forty-eight hours after SE, brain capillaries were isolated from control rats ($n = 6$),

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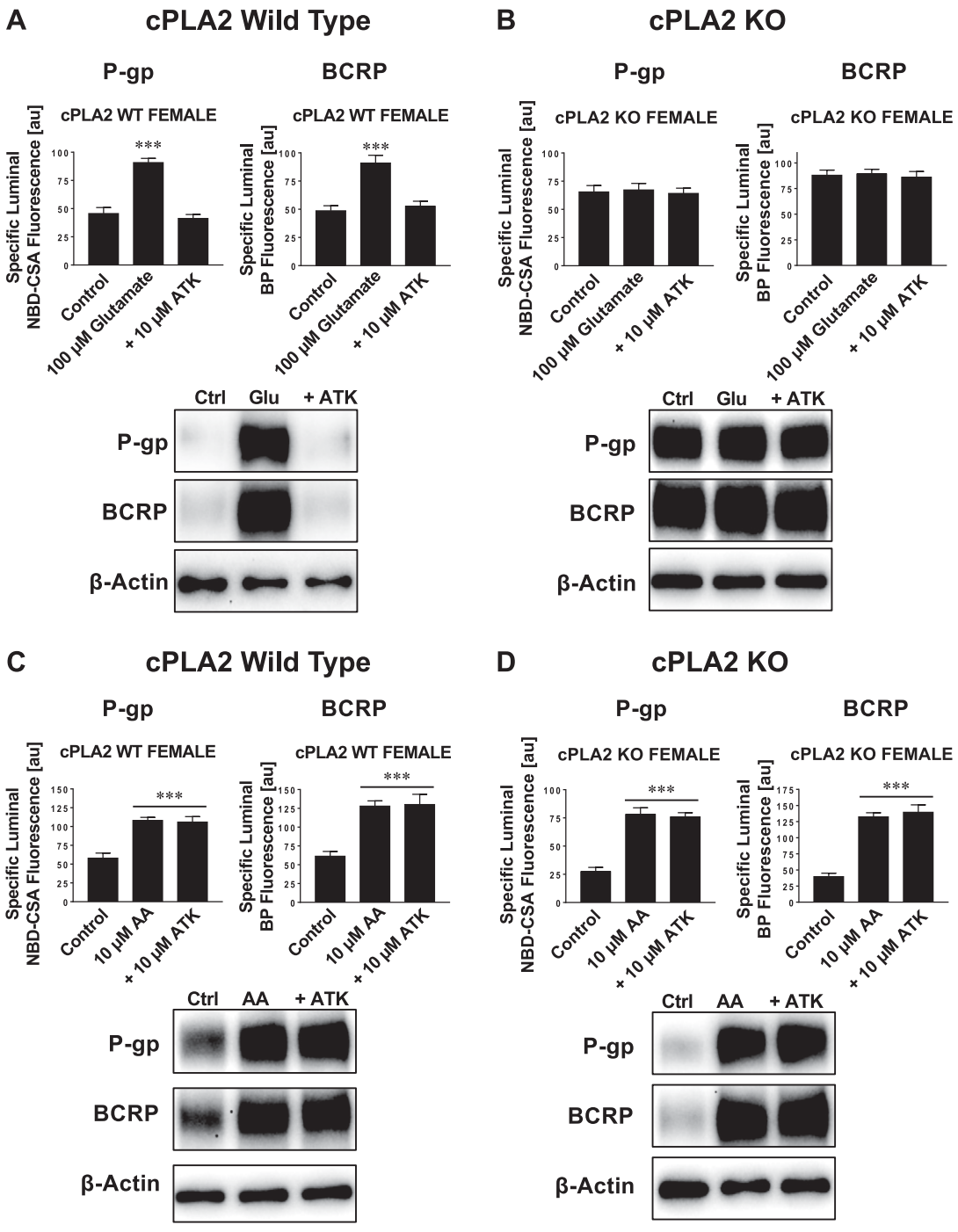

Figure 4. Glutamate mediates P-gp and BCRP up-regulation in female WT mice but not in female cPLA2 KO mice. Brain capillaries were isolated from female WT and cPLA2 KO mice and exposed to glutamate or AA with or without the cPLA2 inhibitor ATK. *A*) Exposing capillaries from WT mice to glutamate resulted in increased P-gp and BCRP protein expression and transport activity. This effect was abolished by the cPLA2 inhibitor ATK. *B*) Glutamate had no effect on P-gp and BCRP protein expression or transport activity levels in capillaries isolated from cPLA2 KO mice. *C*) Exposing capillaries from mice to AA resulted in increased P-gp and BCRP protein expression and transport activity; this effect remained unchanged with ATK. *D*) Capillaries isolated from cPLA2 KO mice also showed increased P-gp and BCRP protein expression and transport activity compared with WT mice; this effect was not abolished by ATK. P-gp and BCRP protein expression were assessed by Western blotting; β-actin was used as protein-loading control. P-gp transport activity was measured as specific luminal NBD-CSA accumulation in capillary lumens; BCRP transport activity was measured as specific Bodipy-Prazosin (BP) accumulation in capillary lumens. For specific luminal NBD-CSA and BP fluorescence, data represent the means ± SD for 10 capillaries from a single preparation (pooled tissue from 10 rats). Units are arbitrary fluorescence units (au; scale: 0–255). ***$P < 0.001$, significantly higher than controls.

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Pilo control rats ($n = 4$), SE rats ($n = 6$), and ATK-treated SE rats ($n = 6$). Using Western blotting, we detected increased protein-expression levels of P-gp and BCRP in capillaries isolated from rats that experienced SE compared with control and Pilo control rats (Fig. 5*B*). Although Pilo treatment did not change P-gp and BCRP transport activity compared with control capillaries, capillaries from SE animals had significantly higher P-gp and BCRP transport activity compared with untreated control capillaries. This is consistent with our previous findings showing that SE increases protein expression and transport activity levels of efflux transporters involved in ASD resistance in brain capillaries compared with brain capillaries from untreated rats and Pilo control rats that received Pilo but did not develop SE (21, 26). Importantly, P-gp and BCRP expression and transport activity levels in capillaries from SE rats treated with the cPLA2 inhibitor ATK were comparable with control levels, suggesting that cPLA2 is critical for glutamate-mediated transporter up-regulation (Fig. 5*C, D* and **Table 3**).

Thus, using an *in vivo* SE model, we show that cPLA2 inhibition blocks seizure-induced up-regulation of P-gp and BCRP protein expression and transport activity in brain capillaries. These data support the hypothesis that cPLA2 is involved in seizure-induced up-regulation of blood–brain barrier efflux transporters.

cPLA2 inhibition prevents P-gp and BCRP up-regulation in CE rats

In a final *in vivo* experiment, we determined if cPLA2 is involved in seizure-induced changes at the blood–brain barrier in a rat model of CE. We used vehicle-treated control rats, vehicle-treated CE rats, CE-ATK rats (once daily; 10 mg/kg), and ATK-CE-ATK rats (**Fig. 6*A***). Note that 12 mo were between the 2 ATK treatment phases. **Table 4** summarizes seizure burden (frequency, duration, and severity) for CE, CE-ATK, and ATK-CE-ATK rats. Seizures were classified according to the Racine scale (33).

After 2 wk of ATK treatment, brain capillaries were isolated from all groups and analyzed by Western blotting. Figure 6*B* shows P-gp and BCRP protein-expression levels in capillaries from control rats as well as from treated and untreated CE rats. P-gp and BCRP protein-expression levels were increased in brain capillaries isolated from CE rats compared with capillaries from control rats. In contrast, P-gp and BCRP expression levels in capillaries from CE rats treated with ATK were similar to those observed in control animals. Treatment with ATK prior to SE induction and then again during the CE phase showed the strongest effect on seizure-induced up-regulation of both efflux transporters. Figure 6*C, D* shows that capillaries from CE animals had significantly higher P-gp and BCRP transport

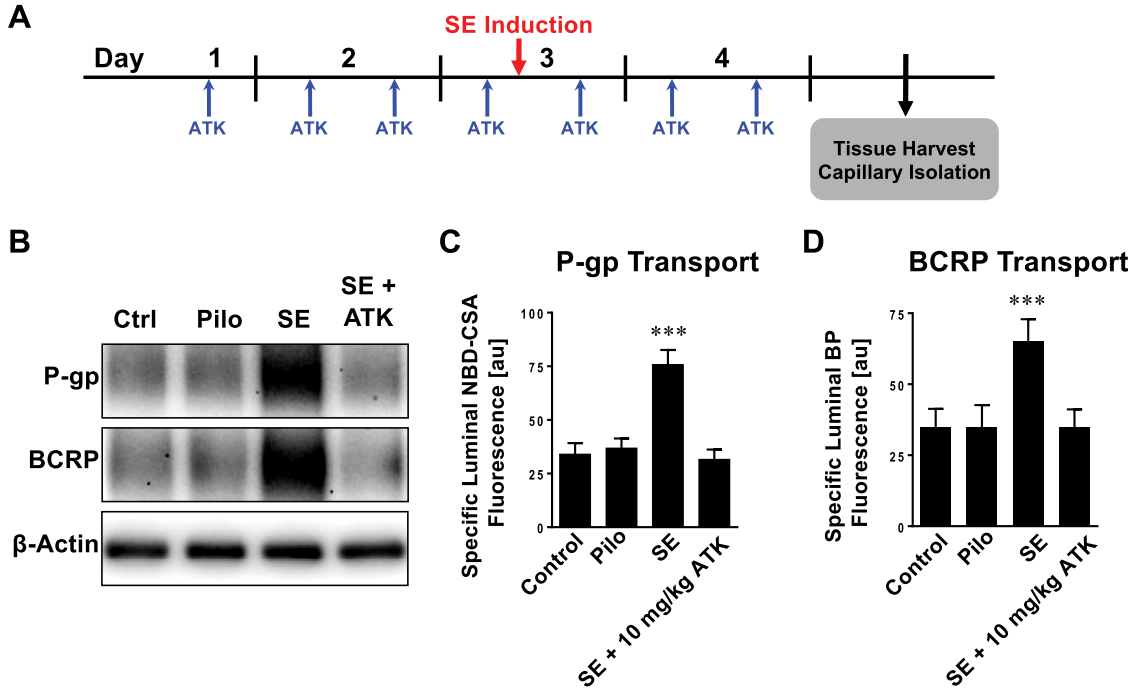

Figure 5. cPLA2 inhibition prevents P-gp and BCRP up-regulation in a rat SE model. *A*) ATK-dosing regimen and SE induction. *B*) Western blot showing P-gp and BCRP in brain capillaries isolated from control rats (Ctrl), rats that received Pilo but did not develop SE (Pilo), rats that experienced Pilo-induced SE, and rats that experienced and received the cPLA2 inhibitor ATK (SE+ATK). *C, D*) Inhibition of ATK reduced P-gp and BCRP protein expression in brain capillaries of ATK-treated SE mice to control levels. ATK reduced P-gp transport activity (*C*) and BCRP transport activity (*D*) in isolated capillaries from ATK-treated SE rats to levels detected in control rats, whereas P-gp and BCRP transport activity was increased in untreated SE rats. For Western blots, β-actin was used as protein-loading control. P-gp transport activity was measured as specific luminal NBD-CSA accumulation in capillary lumens; BCRP transport activity was measured as specific Bodipy-Prazosin (BP) accumulation in capillary lumens. For specific luminal NBD-CSA and BP fluorescence, data represent the means ± SD for 10 capillaries from a single preparation (pooled tissue from control rats ($n = 6$), Pilo control rats ($n = 4$), SE control rats ($n = 6$), and ATK-treated SE rats ($n = 6$). Units are arbitrary fluorescence units (au; scale: 0–255). ***$P < 0.001$, significantly higher than controls.

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TABLE 3. *Western blot analysis of P-gp and BCRP protein-expression levels in brain capillaries isolated from seizure rats treated with ATK*

Protein	Control	Pilo	SE	SE-ATK
P-gp	100 ± 12.1	125 ± 15.4	223 ± 10.8[a]	140 ± 11.5[a]
BCRP	100 ± 16.5	129 ± 16.2	231 ± 12[a]	131 ± 19.7

Data were normalized to β-actin levels; values are given as percentage of control ± SD ($n = 3$ technical replicates). [a] indicates statistical significance determined when $P \leq 0.05$ for each endpoint compared with control using Dunnett's many-to-one t test.

activity levels compared with capillaries from control rats. Both ATK treatment regimens (CE-ATK and ATK-CE-ATK) blocked seizure-induced up-regulation of P-gp and BCRP transport activity, resulting in comparable transport activity levels in capillaries from control animals (**Table 5**).

These findings demonstrate that P-gp and BCRP are up-regulated in capillaries from CE rats and that cPLA2 inhibition abolishes this seizure-induced transporter increase at the blood–brain barrier.

DISCUSSION

Here, we show that cPLA2 is expressed and functional in isolated rat brain capillaries (Figs. 1 and 2). We show that seizures increase levels of active, p-cPLA2 in cerebral cortex, parahippocampus, hippocampus, and brain capillaries (Fig. 1C). We demonstrate that glutamate up-regulates cPLA2 activity in isolated rat brain capillaries (Fig. 1D), which increases P-gp and BCRP protein expression and transport activity level (Fig. 2). We demonstrate in isolated capillaries from COX-2 KO and cPLA2 KO mice that COX-2 and cPLA2 mediate glutamate-induced increases in P-gp and BCRP expression and transport activity levels (Figs. 2–4). Our data also indicate that there is no male-female difference in glutamate signaling regulating P-gp and BCRP. We confirmed these findings *in vivo* by using the cPLA2 inhibitor ATK in a rat SE model and in CE rats (Figs. 5 and 6). In both models, we found that ATK treatment blocks the seizure-induced increase of P-gp and BCRP protein expression and transport activity. Based on these data, we propose that seizure-induced activation of cPLA2 increases P-gp and BCRP protein expression and activity levels (**Fig. 7**). Together, our data suggest that cPLA2 could be a potential target to normalize P-gp and BCRP levels at the blood–brain barrier, which could help increase ASD brain uptake and improve ASD efficacy. In the following paragraphs, we discuss this study in more detail.

We used a systematic *ex vivo–in vivo* approach to identify signaling steps that change efflux transporter expression and activity levels at the blood–brain barrier in epilepsy. Previous studies from us and others describe a role for COX-2 signaling in seizure-induced P-gp overexpression (19, 21–23). COX-2 involvement in P-gp overexpression and multidrug resistance has also been described in cancer including cell models for breast cancer, gastric cancer, hepatocarcinoma, bladder cancer, and colorectal cancer (38–41). Reports show that COX-2 also

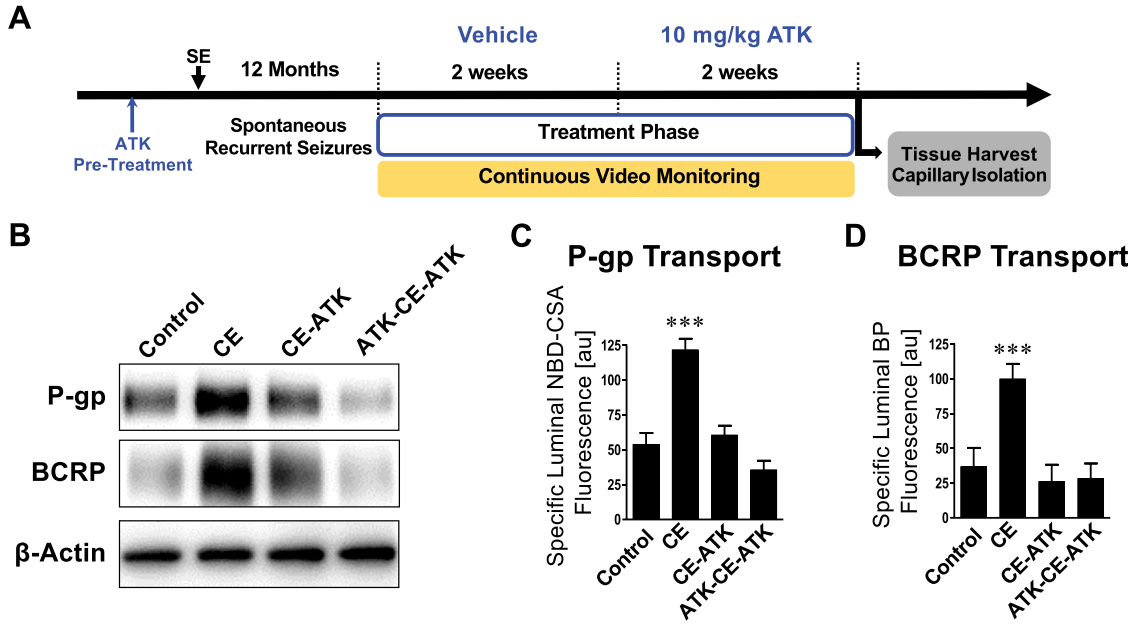

Figure 6. cPLA2 inhibition prevents P-gp and BCRP up-regulation in CE rats. *A*) Dosing scheme for CE rats. *B*) Western blot showing P-gp and BCRP in brain capillaries isolated from control rats (Ctrl), CE, CE-ATK rats, and ATK-CE-ATK rats. *C, D*) ATK treatment prevented up-regulation of P-gp and BCRP protein expression. ATK treatment also abolished up-regulation of P-gp (*C*) and BCRP (*D*) transport activity in CE-ATK rats and ATK-CE-ATK rats. For Western blots, β-actin was used as protein-loading control. P-gp transport activity was measured as specific luminal NBD-CSA accumulation in capillary lumens; BCRP transport activity was measured as specific Bodipy-Prazosin (BP) accumulation in capillary lumens. For specific luminal NBD-CSA and BP fluorescence, data represent the means ± SD for 10 capillaries from a single preparation (pooled tissue from control rats ($n = 10$), CE rats ($n = 5$), CE-ATK rats ($n = 8$), and ATK-CE-ATK rats ($n = 5$). Units are arbitrary fluorescence units (au; scale: 0–255). ***$P < 0.001$, significantly higher than controls.

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TABLE 4. *Seizure burden in CE animals as determined by video analysis*

Parameter	Seizure frequency (seizures/d)	Seizure duration (s)	Seizure severity (scale 0–5)
Control (n = 10)	0	0	0
CE (n = 5)	5 ± 4.5	30 ± 9	5 ± 0.2
CE-ATK (n = 8)	4 ± 4.9	28 ± 7	5 ± 0.3
ATK-CE-ATK (n = 5)	5 ± 7.3	26 ± 15.7	4 ± 2.1

Seizure duration, frequency, and severity were analyzed. Each animal served as its own control.

regulates BCRP expression and transport activity in cancer cells (42, 43). These studies suggest that COX-2 is a regulator of both P-gp and BCRP. In the epilepsy field, much focus has been on P-gp, but van Vliet *et al.* (20) found that other efflux transporters like BCRP, multidrug resistance-associated protein (Mrp)1, and Mrp2 are also overexpressed at the rat blood–brain barrier shortly after SE, during the latent period, and in CE rats. Weidner *et al.* (44) showed in formalin-fixed paraffin-embedded brain tissue from patients with epilepsy (n = 33 patients with drug-resistant mesial temporal-lobe epilepsy) that BCRP staining correlates with COX-2 and translocator protein staining, suggesting a potential relationship between inflammatory markers and efflux transporters. Using COX-2 KO mice, we have shown that glutamate mediates up-regulation of efflux transporters in brain capillaries by activating COX-2 (21). Bankstahl *et al.* (45) confirmed these data. On the other hand, Salvamoser *et al.* (46) reported reduced BCRP transport activity in porcine brain capillaries and human brain capillaries from patients resistant to ASD. The effect in human capillaries, however, is most likely caused by glutamate-mediated toxicity in brain capillaries from resected human epileptic tissue rather than transporter down-regulation [see also Tang *et al.* (47)]. Because capillaries isolated from resected epileptic brain tissue have been exposed to excessive glutamate levels during seizures, exposing these capillaries to glutamate *ex vivo* most likely causes excitotoxicity, which could explain the observed decrease in BCRP. Moreover, the data by Salvamoser *et al.* have thus far not been independently confirmed, whereas BCRP up-regulation has been demonstrated by multiple groups (4, 44, 48–50).

Data indicate that COX-2 is key for seizure-induced changes in transporter expression and activity levels at the blood–brain barrier (21–23). In epilepsy, however, targeting COX-2 appears to be a double-edged sword. On the one hand, studies show that COX-2 inhibition significantly enhances brain uptake of phenytoin in CE rats and, therefore, targeting COX-2 could potentially help to increase ASD levels in the epileptic brain (19, 51). On the other hand, COX-2 inhibitors bear the risk of adverse effects including increased incidence for heart attack and stroke. Studies in a rat temporal-lobe epilepsy model have shown that COX-2 inhibition can lead to severe adverse effects and increased mortality (52). Specifically, inhibiting COX-2 with SC-58236 increased the number of seizures in more than 50% of CE rats. We therefore focused on the signaling pathway upstream from COX-2. Active p-cPLA2 releases AA from phospholipids, and COX-2 converts AA to PGG2. cPLA2 protein expression and activity has also been described in brain cortex in a rat ischemia-reperfusion injury model and a middle cerebral-artery occlusion model (53, 54). Anfuso *et al.* (55) showed in rat brain endothelial cells and a bovine retinal pericyte coculture model that activating PKC-α leads to phosphorylation of cPLA2 in endothelial cells. The authors proposed that the PKC-α–cPLA2 complex may be key in the early phase of endothelial cell–pericyte interactions regulating blood–retina or blood–brain barrier maturation.

We demonstrate that seizures and glutamate increase cPLA2 activity in brain capillaries. Our data indicate that there are no interspecies differences with regard to cPLA2 signaling between the KO mouse models and rat models utilized in the present study. Furthermore, our data suggest no difference in cPLA2 signaling between brain capillaries from male and female mice. However, we cannot exclude male-female differences in cPLA2 signaling in other organs. For example, sex-related differences in cPLA2 signaling have been shown in the periphery. Chen *et al.* (56) demonstrated that the female sex hormone E_2 has vascular protective properties resulting from estrogen-mediated activation of cPLA2 signaling, which leads to AA release and production of PGs. This mechanism is thought to contribute to the sex-related differences in vascular tone in peripheral microvessels, resulting in vascular protection and reduced cardiovascular diseases observed in women. Thus, the adverse effect profile for cPLA2 inhibitors could differ in male and female patients.

It is well-established that epileptic seizures stimulate cPLA2 expression and activity, which leads to an increase in AA levels in neurons, resulting in neuroinflammation (57, 58). Moreover, Zhou *et al.* (59) found in a mouse model of autosomal-dominant lateral temporal epilepsy that

TABLE 5. *Western blot analysis of P-gp and BCRP protein-expression levels in brain capillaries isolated from CE rats treated with ATK*

Protein	Control	CE	CE-ATK	ATK-CE-ATK
P-gp	100 ± 16.4	264[a] ± 12.2	137[a] ± 15.1	102 ± 16.7
BCRP	100 ± 12.1	184[a] ± 8.4	127[a] ± 12	95 ± 10.0

Data were normalized to β-actin levels; values are given as percentage control ± SD (n = 3 technical replicates). [a]Statistical significance determined when $P \leq 0.05$ for each endpoint compared with control using Dunnett's many-to-one t test.

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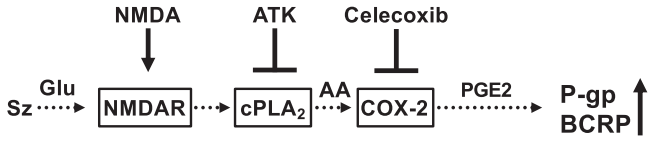

Figure 7. Proposed signaling pathway in brain capillaries. Based on our data presented here and our previously published data (21–23, 26), we propose that seizure (Sz)-induced release of glutamate signals through the NMDA receptor (NMDAR) which leads to activation of cPLA2. This, in turn, leads to release of AA, which COX-2 converts to PGE2, which in turn leads to increased P-gp and BCRP protein expression and transport activity levels at the blood–brain barrier.

dysregulation of cPLA2 and COX-2 signaling leads to hyperexcitability of cortical pyramidal neurons. We recently showed that seizures drive barrier leakage through seizure-induced cPLA2 activation, leading to more seizures, thereby likely promoting epilepsy progression (24). Consistent with this, inhibiting cPLA2 with an extract from Asiatic pennywort (*Centella asiatica*) showed anticonvulsant effects when given in a high dose (60, 61). We did not observe an effect of ATK on seizure burden (Table 4). However, statistical analysis of our video-monitoring data indicated a trend toward reduced seizure duration in ATK-pretreated animals ($P = 0.065$; Fisher's exact test); this trend was not statistically significant. Overall, these studies and our findings suggest that cPLA2 is key in seizure-mediated up-regulation of P-gp and BCRP and that cPLA2 could be a target to improve ASD brain uptake and reduce seizure burden in epilepsy.

Collectively, our findings suggest that cPLA2 is involved in up-regulating P-gp and BCRP protein expression and transport activity levels in epilepsy. Thus, preventing ASD-transporter overexpression through cPLA2 inhibition could potentially serve as a therapeutic strategy to increase ASD brain uptake and reduce seizure burden. Studies are ongoing to investigate the effect of cPLA2 inhibition on ASD resistance and seizure genesis. FJ

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AUTHOR CONTRIBUTIONS

A. M. S. Hartz contributed to data acquisition, data analysis, interpretation of data, and drafted and revised the article; R. G. Rempe contributed to data acquisition and analysis and revised the article; E. L. B. Soldner contributed to data acquisition and analysis and revised the article; A. Pekcec contributed to data acquisition, interpretation of data, and revised the article; J. Schlichtiger contributed to interpretation of data and revised the article; R. Kryscio contributed to data analysis (biostatistics) and drafted and revised the article; and B. Bauer contributed to data acquisition, data analysis, interpretation of data, and drafted and revised the article.

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