

17- β -Estradiol: a powerful modulator of blood–brain barrier BCRP activity

Anika MS Hartz¹, Anne Mahringer², David S Miller³ and Björn Bauer⁴

¹Department of Biochemistry and Molecular Biology, Medical School, University of Minnesota, Duluth, Minnesota, USA; ²Division of Pharmaceutical Technology, Institute of Pharmacy and Molecular Biotechnology, University of Heidelberg, Heidelberg, Germany; ³Laboratory of Toxicology and Pharmacology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina, USA; ⁴Department of Pharmaceutical Sciences, College of Pharmacy, University of Minnesota, Duluth, Minnesota, USA

The ATP-driven efflux transporter, breast cancer resistance protein (BCRP), handles many therapeutic drugs, including chemotherapeutics, limiting their ability to cross the blood–brain barrier. This study provides new insight into rapid, nongenomic regulation of BCRP transport activity at the blood–brain barrier. Using isolated brain capillaries from rats and mice as an *ex vivo* blood–brain barrier model, we show that BCRP protein is highly expressed in brain capillary membranes and functionally active in intact capillaries. We show that nanomolar concentrations of 17- β -estradiol (E2) rapidly reduced BCRP transport activity in the brain capillaries. This E2-mediated effect occurred within minutes and did not involve transcription, translation, or proteasomal degradation, indicating a nongenomic mechanism. Removing E2 after 1 h fully reversed the loss of BCRP activity. Experiments using agonists and antagonists for estrogen receptor (ER) α and ER β and brain capillaries from ER α and ER β knockout mice demonstrated that E2 could signal through either receptor to reduce BCRP transport function. We speculate that this nongenomic E2-signaling pathway could potentially be used for targeting BCRP at the blood–brain barrier, in brain tumors, and in brain tumor stem cells to improve chemotherapy of the central nervous system.

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Introduction

Despite extensive research, early diagnosis, state-of-the-art neurosurgery, radiation, and chemotherapy, effective treatments for brain tumors are limited, and the diagnosis of brain cancer often remains a death sentence (Buckner *et al*, 2007). Indeed, for most small molecule chemotherapeutics, failure to eradicate brain tumors as well as brain cancer stem cell remnants is due in large part to the blood–brain barrier and its ATP-driven drug efflux transporters. These transporters reside on the luminal membrane of brain capillaries that make up the barrier provid-

ing an obstacle to drug entry. One such transporter is breast cancer resistance protein (BCRP, *ABCG2*; Eisenblätter and Galla, 2002). Studies show that BCRP restricts brain penetration of the tyrosine kinase inhibitor, imatinib, and of topotecan, another chemotherapeutic drug. Thus, BCRP likely reduces the drugs' effectiveness (Breedveld *et al*, 2005; de Vries *et al*, 2007). Furthermore, recent reports indicate that BCRP works in concert with another ATP-driven drug efflux transporter, P-glycoprotein, to prevent brain penetration of two other tyrosine kinase inhibitors, dasatinib and lapatinib (Chen *et al*, 2009; Lagas *et al*, 2009; Polli *et al*, 2009). As a consequence, anticancer drugs that are substrates for transporters do not reach tumor sites in the brain, which limits successful pharmacotherapy, or they only reach tumors at subtherapeutic levels, which can cause drug resistance, and thus, complicate and aggravate the disease (Pal and Mitra, 2006). One strategy for increasing brain levels of certain chemotherapeutics is to manipulate BCRP activity in the brain capillary endothelium. However, mechanisms regulating BCRP at the blood–brain barrier have not been defined.

Correspondence: Dr B Bauer, Department of Pharmaceutical Sciences, College of Pharmacy, University of Minnesota, 1110 Kirby Drive, 232 Life Science, Duluth, MN 55812, USA. E-mail: bjbauer@d.umn.edu

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Ee *et al* (2004) recently used a human ovarian cancer cell line to identify an estrogen-response element in the promoter region of *ABCG2*, the gene-encoding BCRP. Other studies show that estrogens regulate BCRP in human breast cancer and placenta cell lines as well as in rat kidney and mouse liver *in vivo* (Imai *et al*, 2005; Tanaka *et al*, 2005; Wang *et al*, 2008, 2006; Zhang *et al*, 2006). On the basis of these reports, we hypothesized that estrogens could affect BCRP at the blood–brain barrier. We show here that the estrogen, 17- β -estradiol (E2), signals through both ER α and ER β to decrease BCRP transport activity in brain capillaries from rat and mouse. These effects were independent of transcription, translation, and proteasomal degradation. Our findings suggest one strategy to bypass BCRP at the blood–brain barrier, which could help improve CNS chemotherapy of brain tumors and brain tumor stem cells.

Materials and methods

Chemicals

The BCRP antibody (BXP-53, rat monoclonal IgG2a, raised against amino-acid residues 221 to 394 of mouse BCRP) and fumitremorgin C (FTC) were obtained from Alexis-Axxora (San Diego, CA, USA). β -Actin antibody was from Abcam (Cambridge, MA, USA; mouse monoclonal IgG1, raised against amino-acid residues 1 to 100 of human β -actin). BODIPY FL prazosin was from Molecular Probes (Eugene, OR, USA); MK571 was from Cayman (Ann Arbor, MI, USA). Lactacystin was obtained from Calbiochem-Novabiochem (La Jolla, CA, USA). Methylpiperidinopyrazole (MPP) and ICI182,780 were from Tocris (Ellisville, MO, USA). All other chemicals were purchased from Sigma (St Louis, MO, USA). Ko143 was a kind gift from Dr Alfred Schinkel (Netherlands Cancer Institute, Amsterdam, The Netherlands; Allen *et al*, 2002); GF120918 was a kind gift from GlaxoSmithKline (London, UK), and PSC833 was a kind gift from Novartis (Basel, Switzerland).

Animals

All experiments involving animals were conducted in accordance with the AAALAC regulations and the Guides to Animal Use of the University of Minnesota and NIH animal guidelines. Animal protocols were approved by the Institutional Animal Care and Use Committees of the University of Minnesota (IACUC protocol # 0710A17842; PI: Björn Bauer) and NIEHS/NIH (IACUC protocol #LPC99-14; PI: David Miller). Male and female Sprague-Dawley rats (male and female retired breeders: 6 months old, 300 to 500 g average body weight) were purchased from Taconic Farms (Germantown, NY, USA) and from Charles River (Portage, MI, USA). Male and female ERKO- α (estrogen receptor α -deficient, B6.129-*Esr1*^{tm1Ksk} N10), ERKO- β (estrogen receptor β -deficient, B6.129-*Esr2*^{tm1Unc} N9), and wild-type mice (C57BL/6 background) were a generous gift from Dr Kenneth Korach (NIEHS, RTP, NC, USA) (Lubahn *et al*, 1993). Mice were 10 weeks old with an average body

weight of female mice of 20 g and an average body weight of male mice of 25 g.

Isolation of Brain Capillaries

Capillaries were isolated from rat and mouse brains as described earlier (Hartz *et al*, 2008). For each preparation, 10 rats or 20 mice were killed by CO₂ inhalation and decapitated. Brains were removed, dissected, and homogenized in ice-cold PBS buffer (2.7 mmol/L KCl, 1.46 mmol/L KH₂PO₄, 136.9 mmol/L NaCl, 8.1 mmol/L Na₂HPO₄, 0.9 mmol/L CaCl₂, and 0.5 mmol/L MgCl₂ supplemented with 5 mmol/L D-glucose, 1 mmol/L sodium pyruvate, pH 7.4). The homogenate was mixed with Ficoll (final concentration 15%) and centrifuged at 5800 g for 20 min at 4°C. After resuspending the pellet in 1% BSA, the capillary suspension was passed over a glass bead column. Capillaries adhering to the glass beads were collected by gentle agitation in 1% BSA. Capillaries were washed with PBS and used for transport experiments, western blotting, immunostaining, or RNA isolation.

Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was isolated from rat brain capillaries, brain, choroid plexus, placenta, intestine, kidney, and liver using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and further purified using the RNeasy mini kit (QIAGEN, Valencia, CA, USA). Reverse transcription was performed using the GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA, USA). Polymerase chain reaction (PCR) of reverse transcriptase (RT) products was performed with *Taq* DNA polymerase from Promega (Madison, WI, USA) using primers for rat BCRP (forward, 5'-CATTCACCAGCCTCGGT ATTCCATC-3'; reverse, 5'-CTGGCAATCCATCTGAGCTGA TGAC-3'). Primers were custom synthesized by QIAGEN Operon (Alameda, CA, USA).

Western Blotting

Protein expression in tissues was analyzed by western blotting as described earlier (Hartz *et al*, 2008). Liver, kidney, brain, choroid plexus, and capillaries were homogenized in lysis buffer (Sigma) containing complete protease inhibitor (Roche, Mannheim, Germany). Homogenized samples were centrifuged at 10,000 g for 15 mins; denucleated supernatants were used as brain, choroid plexus, and capillary lysates. Crude membrane fractions from liver, kidney, and brain capillaries were obtained by centrifugation of denucleated supernatants at 100,000 g for 90 mins. Pellets of crude plasma membranes were resuspended in buffer and protein concentrations were determined.

Western blots were performed using the Invitrogen NuPage Bis-Tris electrophoresis and blotting system (Invitrogen, Carlsbad, CA, USA). After protein transfer, blotting membranes were blocked and incubated with primary antibody to BCRP (1:50, 5 μ g/mL) or β -actin (1:1000, 1 μ g/mL). Membranes were washed and incubated

with horseradish peroxidase-conjugated ImmunoPure secondary IgG (1:15,000; Pierce, Rockford, IL, USA) for 1 h. Proteins were detected using SuperSignal West Pico Chemoluminescent Substrate (Pierce). Bands were visualized and recorded using a BioRad Gel Doc 2000 gel documentation system (BioRad, Hercules, CA, USA). Densitometric analysis of BCRP band intensities and digital analysis of the molecular weights for BCRP monomer and dimer were performed with QuantityOne 1-D v4.6.5 software (BioRad). Molecular weight marker RPN800 used for analyses was from GE Healthcare (Piscataway, NJ, USA). In brain capillary membranes, BCRP monomer was identified at 78 kDa and BCRP dimer was identified at 156 kDa. In crude membranes from kidney and liver, BCRP monomer was identified at 72 to 73 kDa and BCRP dimer was identified at 144 to 148 kDa. The difference in BCRP monomer and dimer molecular weight between peripheral tissues (kidney, liver) and brain capillaries is likely due to differences in glycosylation.

Breast Cancer Resistance Protein Transport Assay

The BCRP transport activity was measured as described earlier (Shukla *et al*, 2009). Freshly isolated capillaries were transferred to glass cover slips and incubated for 1 h at room temperature with modulators in 2 μ mol/L BODIPY FL prazosin, a fluorescent BCRP substrate. For each treatment, images of at least 10 capillaries were acquired by confocal microscopy (Zeiss 410 invert laser scanning confocal microscope, $\times 40$ oil immersion objective, numerical aperture $\times 1.2$, 488 nm line of argon laser; Carl Zeiss Inc, Thornwood, NY, USA or Nikon C1 laser scanning confocal microscope unit, Nikon TE2000 inverted microscope, $\times 40$ oil immersion objective, 1.3 numerical aperture, 488 nm line of a Spectra Physics argon laser, model 163C, 515/30 nm band pass filter; Nikon Instruments Inc, Melville, NY, USA). Images were analyzed by measuring luminal BODIPY FL prazosin fluorescence using Scion Image software (Scion Corp., Frederick, MD, USA) and Image J software (NIH, Bethesda, MD, USA) as described earlier (Hartz *et al*, 2008; Shukla *et al*, 2009). Specific luminal BODIPY FL prazosin accumulation was determined by taking the difference between total luminal fluorescence and fluorescence in the presence of the metabolic inhibitor, NaCN (1 mmol/L) (Hartz *et al*, 2008). Sigmoidal dose–response curves and EC₅₀ values were calculated after nonlinear regression analysis (Hill slope fixed to 1) of specific luminal BODIPY FL prazosin fluorescence using GraphPad Prism Software Version 4.01 (GraphPad Software, Inc, La Jolla, CA, USA).

Immunostaining

Freshly isolated rat brain capillaries adhering to glass cover slips were fixed for 15 mins with 3% paraformaldehyde/0.2% glutaraldehyde at room temperature. After washing with PBS, capillaries were permeabilized for 30 mins with 0.1% (v/v) Triton X-100 in PBS and subsequently blocked with 1% BSA in PBS. Capillaries were incubated for 1 h at 37°C with the primary antibody to BCRP (1:20, 12.5 μ g/mL).

After washing with 1% BSA, capillaries were incubated for 1 h at 37°C with the corresponding Alexa Fluor 488-conjugated secondary IgG (1:1000, 2 μ g/mL; Invitrogen, Eugene, OR, USA); negative controls were incubated with secondary antibody only. Nuclei were counterstained with 5 μ g/mL propidium iodide for 15 mins. Staining of BCRP was visualized using a Zeiss 510 metalaser scanning confocal microscope (Zeiss 510 NLO laser scanning confocal microscope, $\times 40$ water immersion objective, numerical aperture $\times 1.2$, 488 nm line of argon laser; Carl Zeiss Inc, Thornwood, NY, USA).

Statistical Analysis

Data are presented as mean $\pm$ s.e.m. One- or two-tailed unpaired Student's *t*-test was used to evaluate the differences between controls and treated groups; differences were considered to be statistically significant when *P* < 0.05.

Results

Breast Cancer Resistance Protein Expression and Transport Activity in Brain Capillaries

We analyzed BCRP expression in isolated rat brain capillaries using RT-PCR, western blotting, and immunohistochemistry. By RT-PCR, we detected a signal for BCRP mRNA (426 bp amplicon) in all tissues examined, including brain capillaries (Figure 1A). Western blots showed a strong signal for BCRP monomer expression at 72 to 73 kDa in crude kidney membranes and kidney brush border membranes (positive controls, Figure 1B). In agreement with BCRP being a membrane protein, the BCRP protein signal was weak in total brain capillary lysate, but at the same protein loading level capillary plasma membranes showed a strong signal at 78 kDa, indicating localization and enrichment of BCRP protein in the brain capillary endothelial cell membrane. Immunostaining localized BCRP to the luminal membrane of brain capillaries (Figure 1C), which is consistent with earlier work in human, pig, rat, and mouse (Hori *et al*, 2004; Cooray *et al*, 2002; Aronica *et al*, 2005; Tachikawa *et al*, 2005; Eisenblaetter *et al*, 2003).

The BCRP functional activity was determined using an assay we recently developed for freshly isolated rat brain capillaries using live tissue confocal microscopy with quantitative, digital image analysis (Shukla *et al*, 2009). This assay allows real-time monitoring of functionally intact brain capillaries by measuring luminal accumulation of the fluorescent BCRP substrate, BODIPY FL prazosin (BP). Figure 2A (left image) shows a representative confocal image of a capillary incubated in buffer containing 2 μ mol/L BODIPY FL prazosin for 60 mins. Fluorescence intensity of BODIPY FL prazosin is low in the incubation medium, moderate in the endothelium, and highest in the capillary

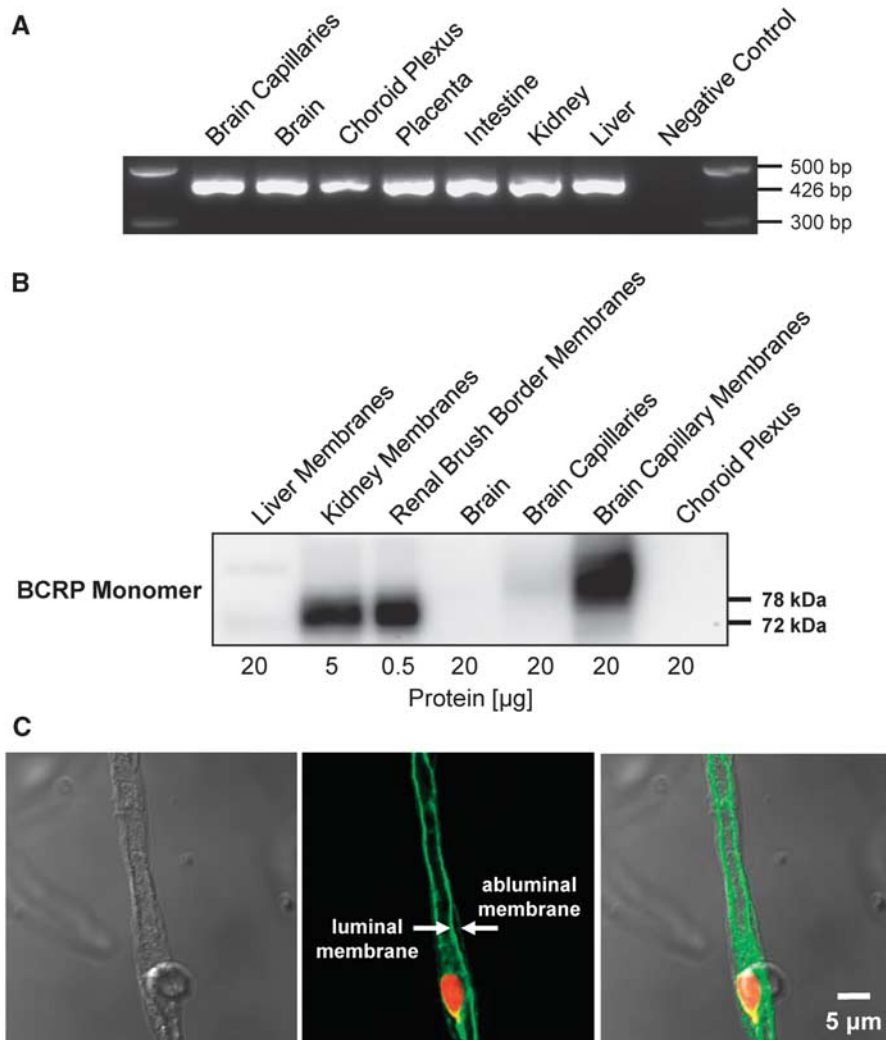


Figure 1 The BCRP expression in isolated rat brain capillaries. **(A)** RT-PCR of liver, kidney, intestine, placenta, choroid plexus, brain, and brain capillaries for rat *Abcg2*. The RT-PCR shows a signal for *Abcg2* mRNA at 426 bp in all tissues that were examined. **(B)** Western blot of liver crude membrane, kidney crude membrane, renal brush border membrane, brain homogenate, brain capillary lysate, brain capillary membrane, and choroid plexus for BCRP. The western blot shows protein expression of BCRP monomer (72 to 73 kDa) in kidney membrane, renal brush border membrane (positive control), and brain capillary membrane (78 kDa). **(C)** Left: Transmitted light image of an isolated rat brain capillary. Middle: Representative image of an isolated brain capillary immunostained for BCRP (green) and counterstained for nuclei (red); the arrows indicate BCRP staining in the luminal membrane and lack of staining in the abluminal membrane. Right: Overlay of fluorescence channel and transmitted light channel. BCRP, breast cancer resistance protein; RT-PCR, reverse transcriptase-polymerase chain reaction.

lumen, indicating concentrative transport of BODIPY FL prazosin from bath to lumen. Exposing capillaries to 5 $\mu\text{mol/L}$ of the BCRP-specific inhibitor, FTC, substantially blocked luminal BODIPY FL prazosin accumulation (Figure 2B). Figure 2C shows the time course of BODIPY FL prazosin accumulation in brain capillary lumens. Luminal fluorescence increased rapidly and reached steady-state levels after about 60 mins. For the experiments reported here, BODIPY FL prazosin transport was measured at steady state, that is, after 60 mins of incubation with 2 $\mu\text{mol/L}$ BODIPY FL prazosin. Figure 3A shows that luminal fluorescence was reduced by about 60% when metabolism was inhibited by NaCN and when capillaries were exposed to the BCRP inhibitors,

FTC, Ko143, and GF120918. A P-glycoprotein inhibitor (PSC833) and Mrp substrates/inhibitors (LTC₄, MK571, probenecid) were without effect. Note that roughly 40% of steady-state luminal BODIPY FL prazosin was not affected by BCRP inhibitors, unlabeled prazosin, or NaCN. This component of BODIPY FL prazosin accumulation in brain capillary lumens is likely due to nonspecific binding of the dye to capillaries plus simple diffusion into the luminal space, which is consistent with our findings from transport assays for P-glycoprotein and Mrp2 (Bauer *et al*, 2008; Hartz *et al*, 2008).

Detailed dose-response studies showed that prazosin reduced luminal BODIPY FL prazosin fluorescence in a concentration-dependent manner

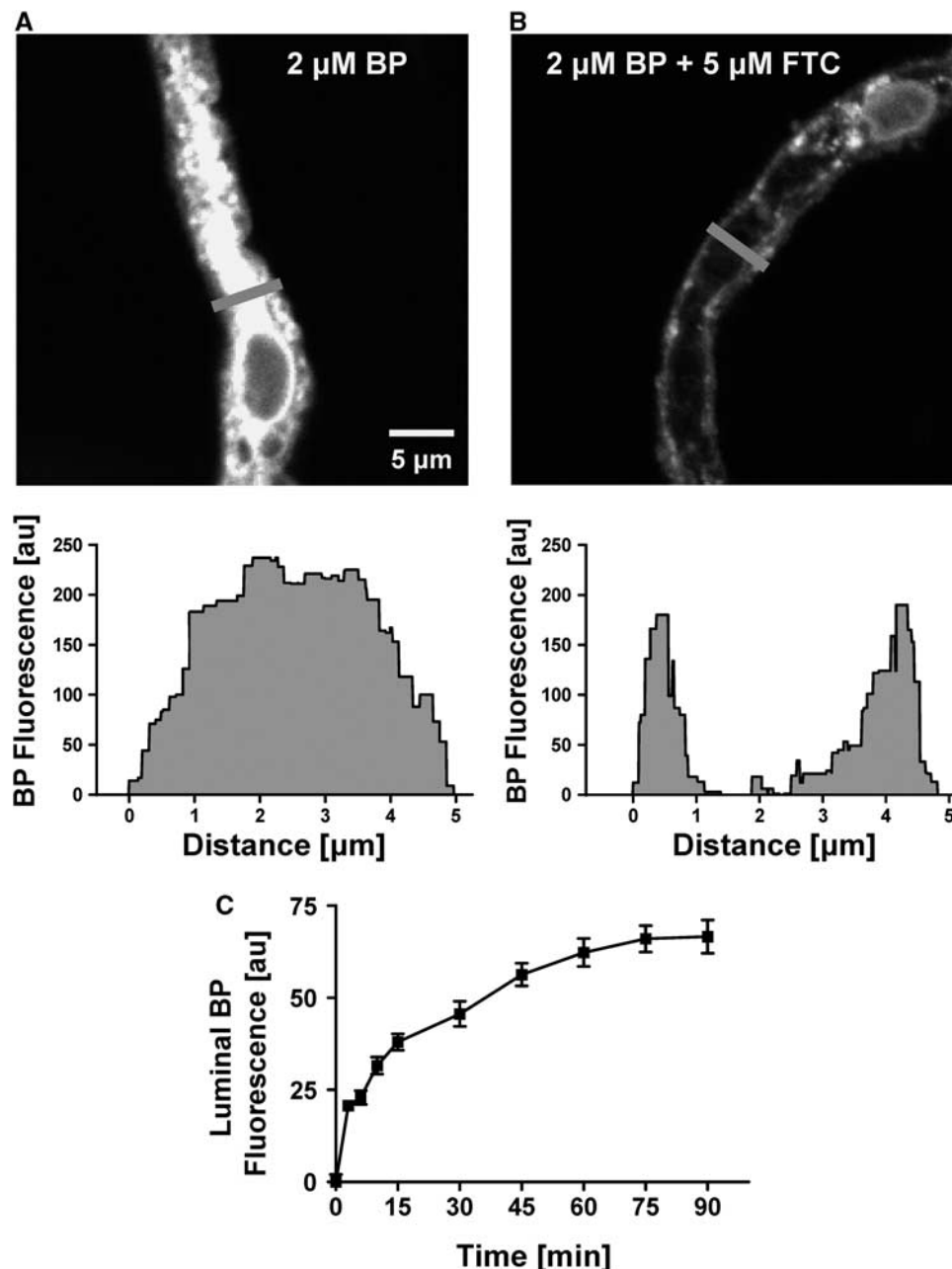


Figure 2 The BCRP-mediated BODIPY FL prazosin transport in isolated brain capillaries. **(A)** Representative image of an isolated rat brain capillary at steady state after 1 h exposure to 2 $\mu\text{mol/L}$ BODIPY FL prazosin. The image analysis of the cross-section (gray) is shown below the image. Note that BODIPY FL prazosin (BP) transport is concentrative from bath (no visible fluorescence) to endothelium (low fluorescence) to capillary lumen (high fluorescence). **(B)** The BCRP inhibitor, FTC, blocks concentrative BODIPY FL prazosin transport. Analysis of the cross-section (gray) shows fluorescence in the endothelial cells but no accumulation of BODIPY FL prazosin in the capillary lumen. **(C)** Time course of BODIPY FL prazosin accumulation in capillary lumens. Each data point represents the mean $\pm$ s.e.m. for 10 to 15 capillaries from a single preparation (pooled tissue from 10 rats). Units are arbitrary fluorescence units (scale 0 to 255). BCRP, breast cancer resistance protein; FTC, fumitremorgin C.

and that luminal fluorescence was maximally blocked by inhibitor concentrations as low as 0.25 $\mu\text{mol/L}$ Ko143, 1 $\mu\text{mol/L}$ FTC, and 5 $\mu\text{mol/L}$ GF120918 (data not shown). Figures 3B–3E show sigmoid dose–response curves for all four compounds; nonlinear regression analyses (Hill coefficient=1) yielded EC₅₀ values of 93.7 nmol/L for

prazosin, 94.1 nmol/L for GF120918, 49.8 nmol/L for FTC, and 3.1 nmol/L for Ko143 (Figure 3). The same ranking of EC₅₀ values for GF120918, FTC, and Ko143 have been reported earlier in BCRP-over-expressing cell cultures (Henrich *et al*, 2007; Pick *et al*, 2008). However, due to the lower BCRP expression levels in brain capillaries compared with

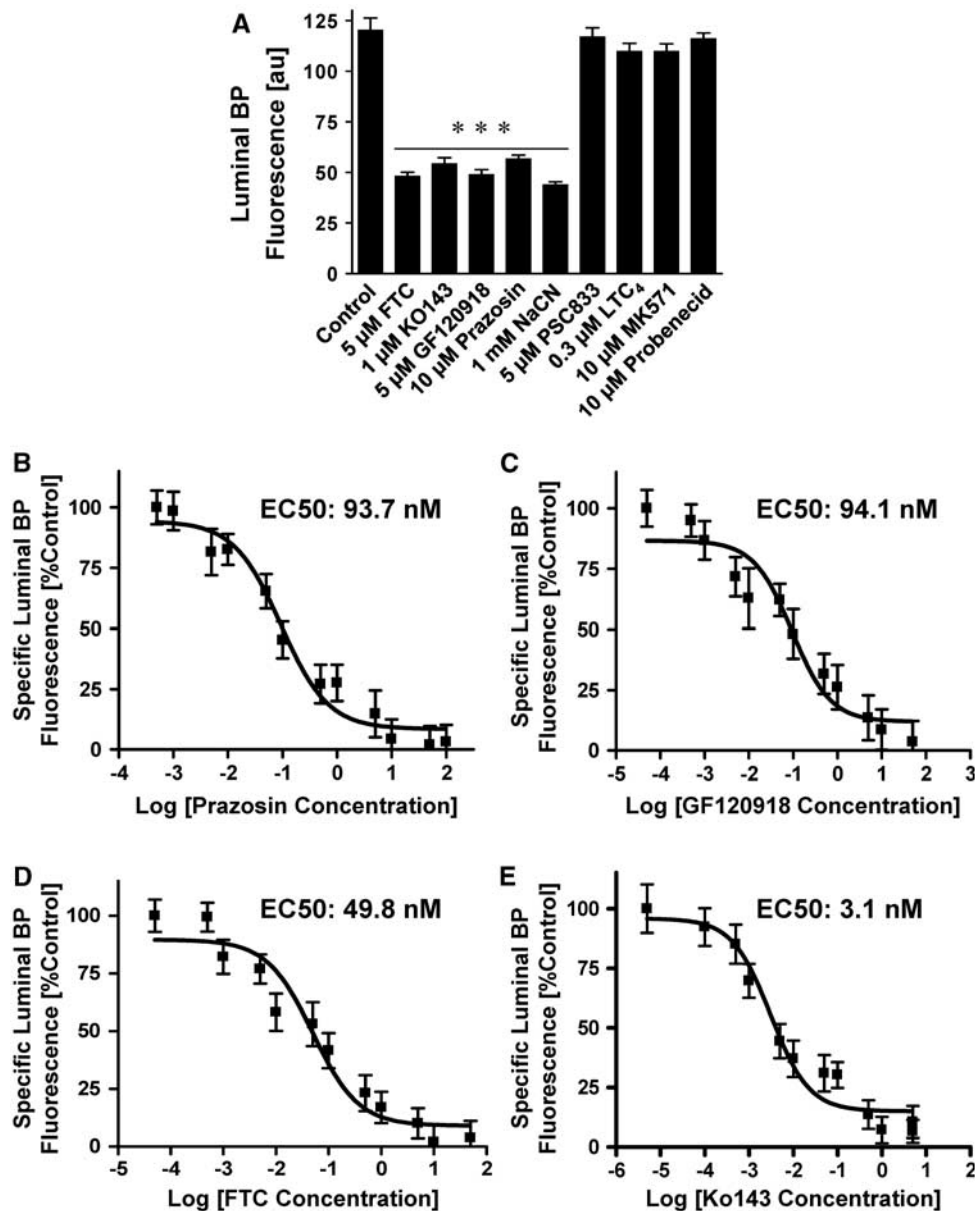


Figure 3 The BCRP-specific BODIPY FL prazosin transport in isolated brain capillaries. **(A)** Accumulation of BODIPY FL prazosin (BP) fluorescence in capillary lumens is sensitive to unlabeled prazosin, to the BCRP inhibitors, FTC, Ko143, GF120918, and to the metabolic inhibitor, NaCN. In contrast, the P-glycoprotein-specific inhibitor, PSC833, and the Mrp substrates/inhibitors, LTC₄, probenecid and MK571, had no effect on luminal BODIPY FL prazosin accumulation. These data indicate that BODIPY FL prazosin transport in brain capillaries is BCRP specific. **(B–E)** Nonlinear regression curves (Hill coefficient = 1) for prazosin **(B)**; EC₅₀: 93.7 nmol/L, GF120918 **(C)**; EC₅₀: 94.1 nmol/L, FTC **(D)**; EC₅₀: 49.8 nmol/L, and Ko143 **(E)**; EC₅₀: 3.1 nmol/L. Each data point represents the mean $\pm$ s.e.m. for 10 to 15 capillaries from a single preparation (pooled tissue from 3 to 10 rats). Units are arbitrary fluorescence units (scale 0 to 255). Statistical comparison: ***significantly lower than controls, $P < 0.001$. BCRP, breast cancer resistance protein; FTC, fumitremorgin C.

relatively high BCRP levels in these engineered overexpressing systems, the EC₅₀ values calculated in this study are about 20- to 80-fold lower than those reported earlier. Together, these data show that concentrative, specific, and ATP-driven bath-to-lumen transport of BODIPY FL prazosin in brain capillaries was mediated by BCRP.

Breast Cancer Resistance Protein Expression and Transport Activity at the Blood–Brain Barrier is Gender Independent

The BCRP is a ‘half transporter’ that exists as monomer, dimer, and tetramer; however, it is the BCRP dimer that is functionally active (Xu *et al*,

2004). We therefore analyzed plasma membranes for BCRP dimer in addition to the monomer. Lower BCRP expression levels for females compared with

males have been reported in kidney, liver, and intestine for human, rat, and mouse (Merino *et al*, 2005; Tanaka *et al*, 2005). The western blots in

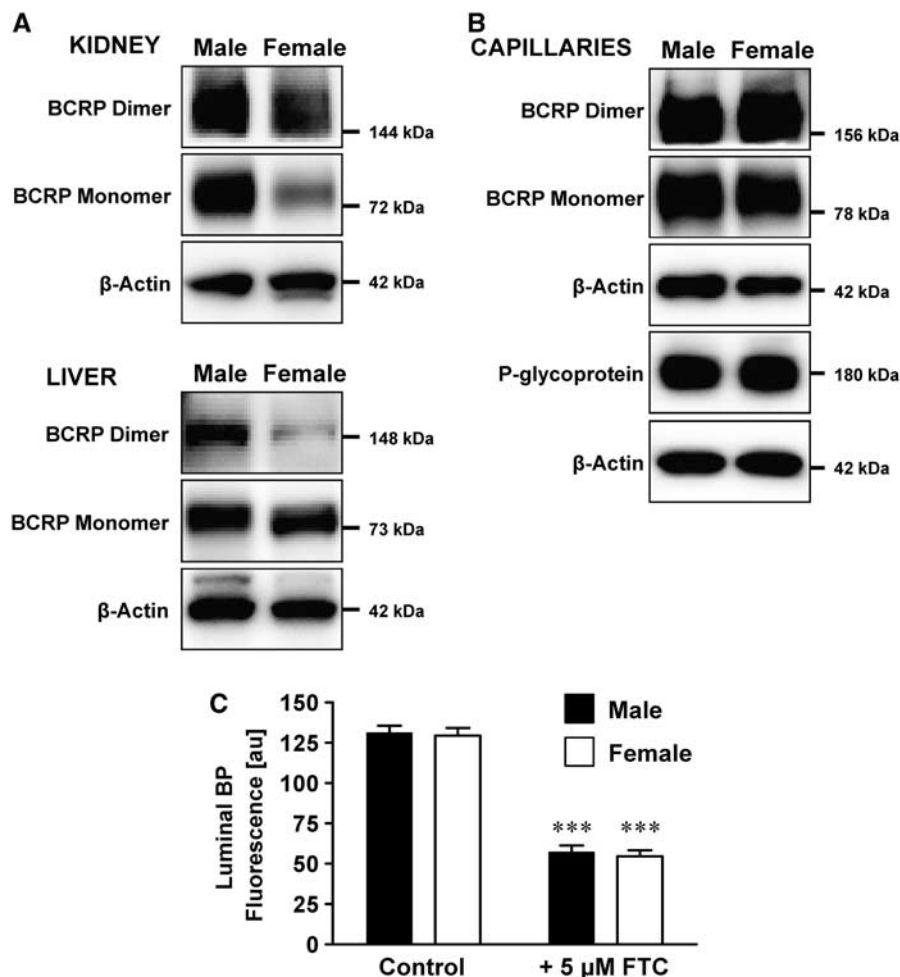
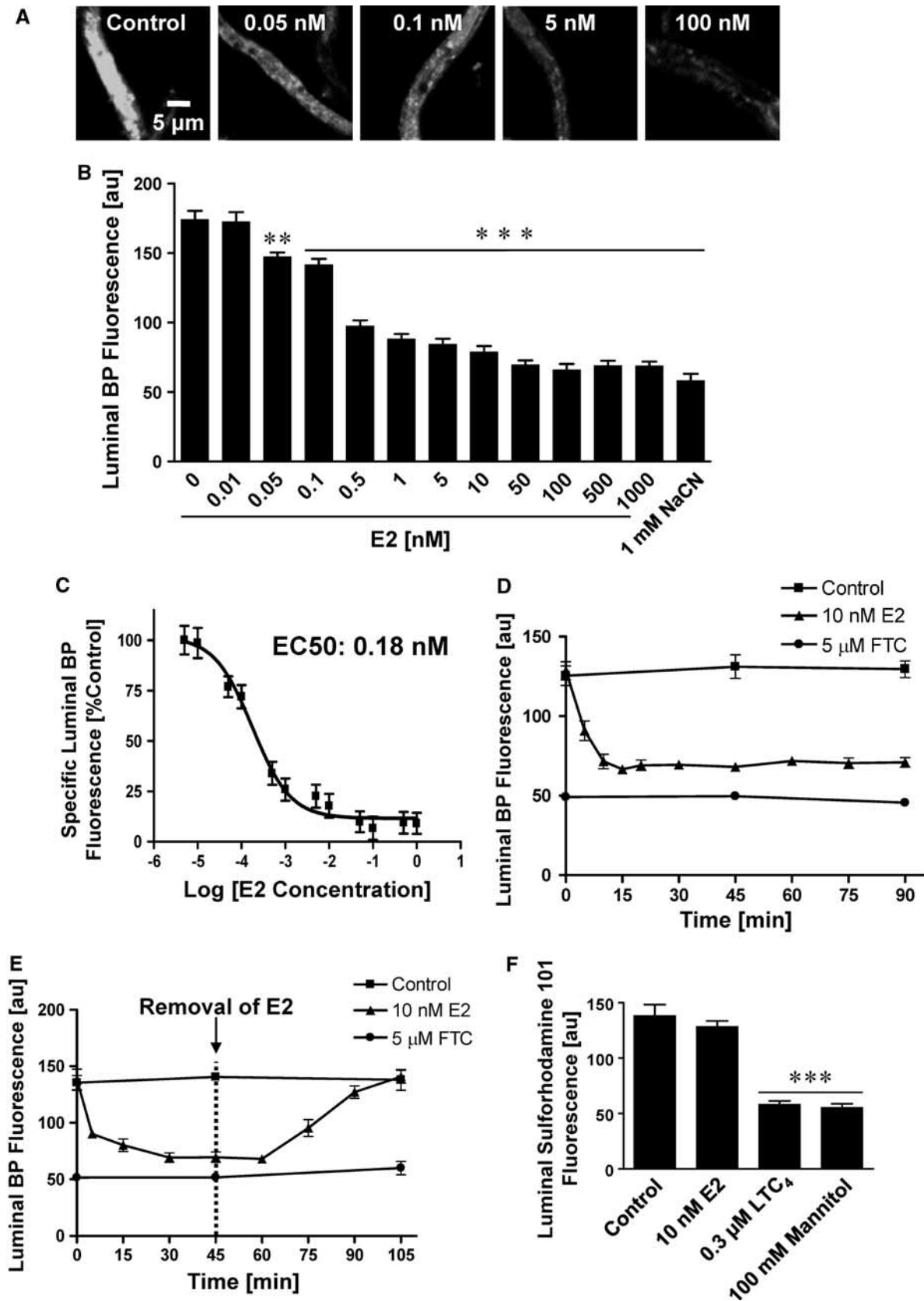


Figure 4 The BCRP expression and function in isolated rat brain capillaries is gender independent. **(A)** In female rats, BCRP monomer and dimer expression in kidney and BCRP dimer expression in liver are reduced compared with male rats. **(B)** BCRP and P-glycoprotein expression in brain capillaries from male and female rats is gender independent. For all western blots, β -actin was used as protein loading control. **(C)** BCRP-mediated transport of BODIPY FL prazosin (BP) into the lumens of capillaries from male and female rats is gender independent in untreated control capillaries and FTC-treated capillaries. For luminal BODIPY FL prazosin fluorescence, each data point represents the mean $\pm$ s.e.m. for 10 to 15 capillaries from a single preparation (pooled tissue from 10 rats). Units are arbitrary fluorescence units (scale 0 to 255). Statistical comparison: ***significantly lower than controls, $P < 0.001$. BCRP, breast cancer resistance protein; FTC, fumitremorgin C.

Figure 5 E2 decreases BCRP transport activity. **(A)** Representative images of isolated rat brain capillaries incubated for 1 h with 2 μ M BODIPY FL prazosin with or without E2. Luminal steady-state accumulation of BODIPY FL prazosin decreased with increasing concentrations of E2. **(B)** Analysis of confocal images reveals a concentration-dependent decrease of luminal BODIPY FL prazosin fluorescence in capillaries exposed to E2. **(C)** Nonlinear regression curve for E2 (Hill coefficient = 1; EC₅₀: 0.18 nmol/L. **(D)** Rapid decrease of BCRP transport activity in capillaries exposed to E2. Capillaries were loaded with 2 μ M BODIPY FL prazosin for 60 mins to steady state. After adding 10 nmol/L E2 (time 0 min), BCRP transport activity decreased within 15 mins. **(E)** E2 effects on BCRP transport activity are reversible. Capillaries were loaded for 60 mins to steady state with 2 μ M BODIPY FL prazosin. When 10 nmol/L E2 was added to the buffer (time 0 on graph), BCRP activity decreased rapidly; BCRP activity recovered completely when E2 was removed (time point 45 mins). **(F)** E2 had no effect on luminal accumulation of sulforhodamine 101, an Mrp2-specific substrate, whereas mannitol, a tight junction opener, and the Mrp2 inhibitor, LTC₄, both decreased Mrp2-mediated sulforhodamine 101 accumulation in brain capillaries. These data indicate that E2 did not alter tight junction permeability, but specifically reduced luminal BODIPY FL prazosin fluorescence through decreased BCRP transport activity. Each data point represents the mean $\pm$ s.e.m. for 7 to 10 capillaries from a single preparation (pooled tissue from 10 rats). Units are arbitrary fluorescence units (scale 0 to 255). Statistical comparison: **significantly lower than controls, $P < 0.01$, ***significantly lower than controls, $P < 0.001$.

Figure 4A confirm these findings. In kidney, we found that BCRP monomer was significantly reduced, and BCRP dimer was slightly reduced in

female rats compared with male rats ($48\% \pm 1\%$ ($n=2$ western blots) and $77\% \pm 2\%$ ($n=3$ western blots), respectively, compared with 100% male



controls; determined by densitometric analysis; BCRP levels were normalized to β -actin). In liver tissue from female rats, BCRP monomer was unchanged, but BCRP dimer was significantly reduced compared with male rats ($34\% \pm 12\%$ compared with 100% male controls ($n = 4$ western blots); determined by densitometric analysis; BCRP levels were normalized to β -actin). However, no data are available on gender-dependent expression of BCRP at the blood–brain barrier. Figure 4B shows that there was no difference in the expression of BCRP and P-glycoprotein in brain capillaries isolated from male and female rats and this was confirmed by densitometric analysis ($n = 4$ for BCRP monomer, $n = 3$ for BCRP dimer, $n = 3$ for P-glycoprotein). Consistent with this, we found no gender difference in BCRP transport activity in brain capillaries (Figure 4C).

E2 Decreases Breast Cancer Resistance Protein Transport Activity

To determine whether blood–brain barrier BCRP is modulated by estrogen, we exposed freshly isolated brain capillaries from male rats to 0.01 to 1000 nmol/L 17- β -estradiol (E2) for 1 h and then measured BCRP transport activity. Control capillaries (Figure 5A, left image) showed high luminal BODIPY FL prazosin fluorescence, but capillaries exposed to 17- β -estradiol showed a concentration-dependent decrease in luminal fluorescence (Figures 5A and 5B). With a calculated EC₅₀ value of 0.18 nmol/L (nonlinear regression analyses; Hill coefficient = 1), 17- β -estradiol proved to be more potent in reducing BCRP transport function than the specific BCRP inhibitors GF120918, FTC, and Ko143 (Figures 3B–3E and 5C). Additional experiments indicated that E2 had the same effect on BCRP-mediated transport in capillaries from female rats (data not shown).

To determine the time course of E2 action on BCRP, we preloaded capillaries to steady state with 2 μ mol/L BODIPY FL prazosin and then added 10 nmol/L E2. In control capillaries, luminal fluorescence was unchanged over the entire time course. In contrast, E2 rapidly decreased luminal BODIPY FL prazosin fluorescence. Within 15 mins, accumulation was reduced by 47%. Luminal fluorescence remained at this reduced level over the remaining 75 mins of the time course (Figure 5D). Removing E2 after 45 mins of exposure resulted in a rapid increase of luminal BODIPY FL prazosin to control levels (Figure 5E). Thus, E2 rapidly and reversibly reduced BCRP function.

The E2-mediated decrease in luminal BODIPY FL prazosin fluorescence could have resulted from reduced BCRP transport function or from opening of tight junctions leading to leakage of BODIPY FL prazosin out of capillary lumens. If E2 affected blood–brain barrier tight junction integrity, one would also expect decreased luminal fluorescence of substrates that are transported into the lumen by

other ATP-driven efflux pumps such as Mrp2. We previously showed that sulforhodamine 101, an organic anion, is transported into rat brain capillary lumens by Mrp2 (Bauer *et al*, 2008). Figure 5F shows that the Mrp inhibitor, LTC₄, and the osmotic tight junction disruptor, mannitol, both reduced luminal fluorescence of sulforhodamine 101. Importantly, E2 had no effect on Mrp2-mediated accumulation of sulforhodamine 101 in capillary lumens. Thus, E2 did not alter tight junction permeability, but specifically reduced luminal BODIPY FL prazosin fluorescence through decreased BCRP transport activity.

Mechanism of E2-Mediated Decrease of Breast Cancer Resistance Protein Transport Function

Despite well-documented, long-term, genomic effects of 17- β -estradiol involving regulation of gene expression, there is now substantial evidence for nongenomic E2 effects in several cell types (Meyer *et al*, 2009; Raz *et al*, 2008). Such nongenomic effects of steroid hormones are rapid, reversible, and independent of gene transcription, protein synthesis, or proteasomal degradation (Boonyaratanakornkit and Edwards, 2007; Simoncini and Genazzani, 2003). We used these criteria to determine whether E2 could signal through a nongenomic pathway in isolated rat brain capillaries. Capillaries exposed to E2 for 1 h showed no change in protein expression of BCRP monomer or dimer (Figure 6A). Inhibiting proteasomal degradation with lactacystine, a selective inhibitor of the 20S proteasome, did not reverse the E2-mediated decrease of BCRP transport function (Figure 6B). In addition, inhibitors of transcription (actinomycin D) and protein translation (cycloheximide) did not affect E2-mediated downregulation of BCRP function (Figures 6C and 6D). These findings are consistent with E2 acting on BCRP in brain capillaries through a nongenomic-signaling pathway (Simoncini and Genazzani, 2003).

E2 acts through two classical receptors: estrogen receptor α (ER α) and estrogen receptor β (ER β). We used two strategies, pharmacological tools and ER-null mice, to determine through which of these receptors E2 signaled rapid downregulation of BCRP function in brain capillaries. Exposing isolated rat brain capillaries for 1 h to the ER α agonist, propylpyrazoletriol, or the ER β agonist, diarylpropionitrile, significantly decreased BCRP transport activity (Figures 7A and 7B). Blocking ER β with ICI182,780 did not affect E2-mediated downregulation of BCRP activity and blocking ER α with MPP only partially reversed BCRP downregulation (Figure 7C). However, blocking both receptors with MPP and ICI182,780 in combination completely reversed the E2 effect on BCRP activity (Figure 7C). These data suggest that E2 can signal BCRP downregulation through either receptor.

To confirm these findings, we conducted experiments with brain capillaries isolated from male and

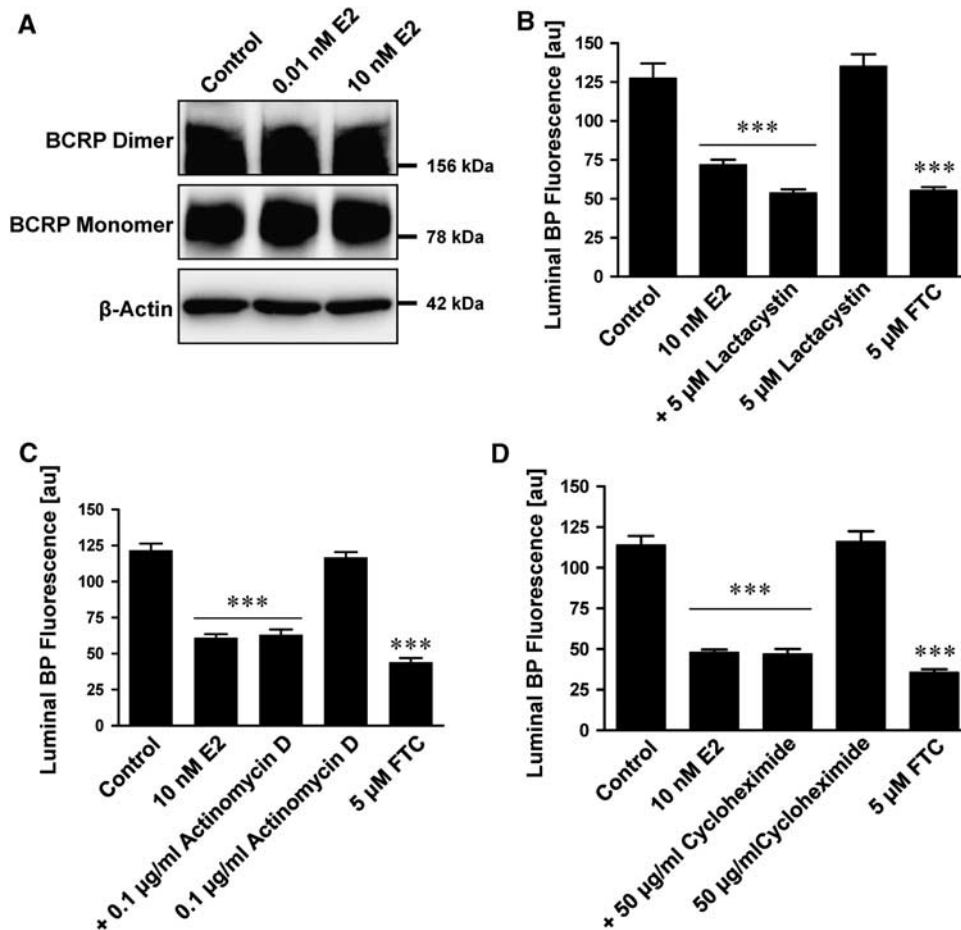


Figure 6 E2 decreases BCRP transport activity through nongenomic signaling. (A) One hour exposure of brain capillaries to E2 does not affect BCRP monomer or dimer protein expression. β -Actin was used as protein loading control. (B) Lactacystin, a proteasome inhibitor, does not block E2-mediated reduction of BODIPY FL prazosin fluorescence in capillary lumens, indicating that proteasomal degradation is not involved in BCRP downregulation caused by E2. Inhibition of transcription with actinomycin D (C) and inhibition of translation with cycloheximide (D) did not reverse BCRP downregulation by E2. This indicates that E2-mediated downregulation of BCRP is independent of transcription and translation. For B, C, and D, FTC was used as a positive control for BCRP inhibition. Each data point represents the mean $\pm$ s.e.m. for 10 to 15 brain capillaries from a single preparation (pooled tissue from 10 rats). Units are arbitrary fluorescence units (scale 0 to 255). Statistical comparison: ***significantly lower than controls, $P < 0.001$. BCRP, breast cancer resistance protein; FTC, fumitremorgin C.

female ER α and ER β knockout and wild-type C57BL/6 control mice (these KO mice were constructed on a C57BL/6 background; Lubahn *et al*, 1993). Figures 7D and 7E show that in capillaries from male wild-type mice, E2 reduced BCRP transport activity. In capillaries from male KO mice, E2 significantly decreased BCRP transport function despite the lack of receptor. We found similar effects in capillaries from female wild-type, ER α and ER β knockout mice (data not shown). Note that E2 had a partial inhibitory effect in brain capillaries from both male and female ER α knockout mice, which is in agreement with the data obtained from pharmacologically blocking ER α with MPP (Figure 7C). Thus, both ERs, ER α and ER β , can be involved in signaling E2-induced rapid downregulation of BCRP transport function.

Discussion

The BCRP is an ABC 'half transporter' that limits the efficacy of cancer chemotherapy and, therefore, has been the center of many studies investigating its regulation. These studies mainly focused on transcriptional regulation of the transporter, and it was discovered that activation of the nuclear receptor, aryl hydrocarbon receptor, induces BCRP expression (Ebert *et al*, 2005). Other groups analyzed the promoter of the human *ABCG2* gene-encoding BCRP and found response elements for hypoxic stimuli and peroxisome proliferator-activated receptor γ (Robey *et al*, 2009). Estrogen has also been shown to regulate BCRP in various tissues (Imai *et al*, 2005; Tanaka *et al*, 2005; Wang *et al*, 2008, 2006; Zhang *et al*, 2006) and Ee *et al* (2004) recently discovered an

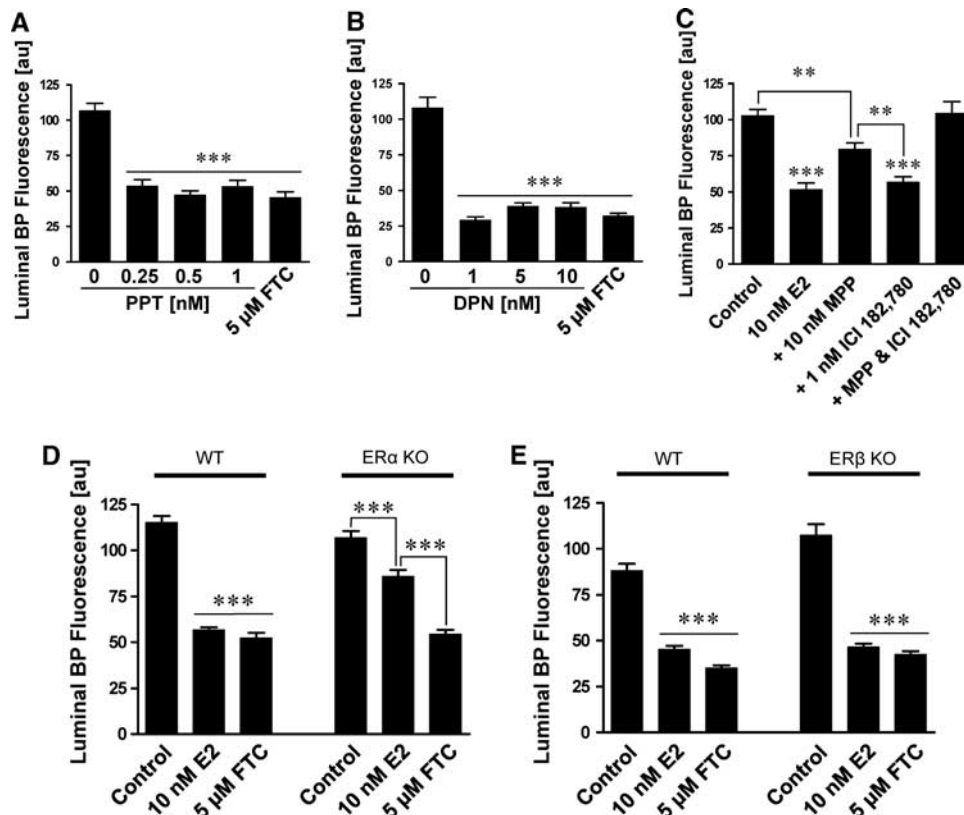


Figure 7 E2 signals through ER α and ER β in isolated brain capillaries. Propylpyrazoletriol, an ER α agonist (**A**), and diarylpropionitrile, an ER β agonist (**B**), decrease luminal BODIPY FL prazosin fluorescence. This indicates that activation of both ER α and ER β causes downregulation of BCRP transport function in rat brain capillaries. (**C**) ICI182,780, an ER β antagonist, does not reverse the E2 effect on luminal BODIPY FL prazosin fluorescence. Methylpiperidinopyrazole, an ER α antagonist, partially reverses the E2 effect. Brain capillary exposure to both methylpiperidinopyrazole (ER α antagonist) and ICI182,780 (ER β antagonist) completely blocks the E2 effect on luminal BODIPY FL prazosin fluorescence. These data indicate that E2 can signal BCRP downregulation through either ER α or ER β . (**D** and **E**) E2 downregulates BCRP transport activity in brain capillaries from wild-type and ER α (**D**) and ER β (**E**) knockout mice. Note that the E2 effect was only partial ER α knockout mice, which is in agreement with the data shown in (**C**). In **A**, **B**, **D**, and **E**, FTC was used as positive control for BCRP inhibition. Each data point represents the mean $\pm$ s.e.m. for 10 to 15 brain capillaries from a single preparation (pooled tissue from 3 to 10 rats or 20 mice). Units are arbitrary fluorescence units (scale 0 to 255). Statistical comparison: **significantly lower than controls, $P < 0.01$; ***significantly lower than controls, $P < 0.001$. BCRP, breast cancer resistance protein; ER, estrogen receptor; FTC, fumitremorgin C.

estrogen-response element in the human *ABCG2* promoter region. This study provides new insights into the regulation of BCRP. It shows E2-induced, rapid and reversible loss of BCRP transport activity at the blood–brain barrier with E2 signaling through both ER α and ER β to decrease BCRP transport function. This E2-driven effect did not involve transcription, translation, or proteasomal degradation, indicating E2 action through a nongenomic mechanism. Several aspects of our study require further discussion.

We show here that basal BCRP activity and expression levels were the same in brain capillaries from male and female rats. In contrast, in kidney and liver, we (present study) and others found that BCRP is expressed at much lower levels in female rats compared with male rats (Merino *et al*, 2005; Tanaka *et al*, 2005). This observation may be explained by the high expression levels of the enzyme, aromatase, which converts testosterone to E2 in male brain tissue, but that is at best poorly expressed in kidney and

liver. In addition, the gender-divergent BCRP expression pattern in peripheral tissues is thought to result from the suppressive effect of E2 in female animals.

We show that E2 downregulated BCRP transport activity in brain capillaries. This E2 effect on BCRP activity could result from direct E2–BCRP interaction, from E2 signaling through ERs, or from both. Regarding the first possibility, a steroid-binding element has been identified in one transmembrane domain of the BCRP protein (Ee *et al*, 2004). It has also been shown in cell culture that hormones including E2 altered ATPase activity upon binding to BCRP, but it was not tested whether BCRP still mediated transport (Velamakanni *et al*, 2008). Although we cannot fully exclude a direct effect of E2 on BCRP, the nanomolar concentrations of E2 that were effective in our experiments are too low to suggest competitive inhibition given that the fluorescent transporter substrate concentration was in the micromolar range. This is strong evidence for

E2 acting through signaling rather than directly interacting with the transporter.

Prior characterization of ER α and ER β knockout mice has revealed that both receptor subtypes have unique but also overlapping functions *in vivo* (Liu *et al*, 2008). In human breast cancer cell lines, ER α activation is associated with transcriptional upregulation and posttranscriptional downregulation of BCRP (Imai *et al*, 2005; Zhang *et al*, 2006). In human placenta cell lines, ER β activation is involved in transcriptional up- and downregulation of BCRP (Wang *et al*, 2008; Wang *et al*, 2006). Thus, both ER α and ER β are involved in E2 regulation of BCRP, but the signaling pathways (ER α and/or ER β) and the effects on BCRP (up- or downregulation) seem to be tissue specific. Consistent with this, our experiments with ER agonists and antagonists, and with ER knockout mice strongly suggest that both ERs are involved in the rapid (minutes) E2-mediated downregulation of BCRP transport activity in brain capillaries. However, the existence of several receptor subtypes for both ER α and ER β makes E2 signaling potentially more complex and further studies are needed to fully elucidate the range of receptors involved in E2-mediated BCRP downregulation at the blood-brain barrier.

Several studies show that E2 can signal through nongenomic mechanisms (Boonyaratanakornkit and Edwards, 2007; Simoncini and Genazzani, 2003). Compared with genomic signaling where cellular responses take hours due to the time required for protein synthesis of estrogen-regulated genes (O'Lone *et al*, 2004), nongenomic E2 signaling is rapid and occurs within minutes or even seconds. Nongenomic E2 signaling is independent of transcription and translation (Simoncini and Genazzani, 2003), but can be mediated through PI3K/AKT, ERK, PLC, p42/44 MAP kinase, or CREB (Honda *et al*, 2001; Migliaccio *et al*, 1996; Zhou *et al*, 1996). For example, in human neuroblastoma cells, nongenomic estrogen signaling increased ERK1/2 phosphorylation within 5 to 10 mins (Watters *et al*, 1997), and in cortical and hippocampal neurons, such signaling increased phosphorylation of PI3K/AKT and CREB (Honda *et al*, 2001). In agreement with these findings, our preliminary data (not shown) also suggest involvement of PI3K/AKT in E2 signaling to BCRP. Moreover, we show here that E2 rapidly reduced BCRP transport function within 15 mins and that this effect was fully reversible and independent from transcription, translation, and proteasomal degradation. Thus, our findings are consistent with an E2-mediated, nongenomic-signaling mechanism. They show for the first time that nongenomic E2 signaling involving both ER α and ER β occurs at the blood-brain barrier.

Our results showing an estrogen effect on BCRP in brain capillaries imply that estrogens could have a substantial effect on barrier function. The brain produces E2, controls E2 release, and responds to E2, and thus, is a major target of physiologic estrogen

action. In addition, xenoestrogens such as the pesticides, DDT, and bisphenol A, enter the body through air, drinking water, and food, or are ingested as therapeutic drugs such as oral contraceptives and antiestrogens used to treat breast cancer, menopausal symptoms, or osteoporosis. Clearly, blood-brain barrier BCRP can be exposed to both endogenous estrogens and xenoestrogens that could both alter tissue estrogen homeostasis and impact blood-brain barrier BCRP function. On the other hand, estrogens are used to reduce stroke damage and delay onset of Alzheimer's disease, and our data suggest that estrogens could potentially also be used therapeutically to reduce BCRP transport activity to improve brain delivery of chemotherapeutics to brain tumors. This is especially important given emerging evidence that BCRP and P-glycoprotein work in concert at the blood-brain barrier (Chen *et al*, 2009; de Vries *et al*, 2007; Lagas *et al*, 2009; Polli *et al*, 2009). Indeed, for certain drugs, clinically valid strategies that target both P-glycoprotein and BCRP may be required to dramatically improve CNS drug delivery. In this regard, drug-resistant brain cancer stem cell remnants pose a tremendous challenge to neurooncologists because they often regenerate into larger and more aggressive tumors after surgical removal of the primary tumor (Cheshier *et al*, 2009). It is now clear that BCRP is highly expressed in brain cancer stem cells, and increasing evidence indicates that BCRP contributes to drug resistance in these cells (Patrawala *et al*, 2005). However, it should be noted that E2 signaling to BCRP may be altered in the context of brain cancer or other CNS disorders involving inflammation. For example, von Wedel-Parlow *et al* (2009) reported that the inflammatory mediators, TNF- α and IL-1 β , affected BCRP protein and function in porcine brain capillary endothelial cells and we previously showed that endothelin-1, a peptide released in brain disease, significantly decreased BCRP protein expression in isolated rat brain capillaries (Bauer *et al*, 2007). Thus, it remains to be seen whether targeting the E2-signaling pathway described in this study will be a practical approach to increasing the efficacy of chemotherapeutics that are BCRP substrates through downregulation of BCRP at the blood-brain barrier and in brain cancer stem cells.

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Conflict of interest

The authors declare no conflict of interest.

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