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BCRP at the Blood−Brain Barrier: Genomic Regulation by 17β-Estradiol

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Abstract: At the blood−brain barrier (BBB), the ABC transporter breast cancer resistance protein (BCRP) actively extrudes a variety of therapeutic drugs, including cytostatics, and diminishes their pharmacological efficacy in the brain. Consequently, new strategies to circumvent BCRP-mediated multidrug resistance in the CNS are required. One major approach to increase brain drug levels is to manipulate signaling mechanisms that control transporter expression and function. In the present study, we investigated the long-term effect of 17β-estradiol on BCRP in an ex vivo model of isolated rat brain capillaries. BCRP function and protein expression were decreased after 6 h of incubation with nanomolar concentrations of 17β-estradiol in capillaries from male and female rats. Concomitantly, levels of BCRP mRNA were also reduced by 17β-estradiol suggesting that the transporter is down-regulated via a genomic pathway. Additionally, we identified the presence of both estrogen receptor (ER) subtypes α and β at the rat BBB. Experiments using selective ER agonists and antagonists revealed that ER subtype β is responsible for the hormone-induced reduction of BCRP function and protein expression. These findings were confirmed by the use of ERKO mice. Blocking the proteasome-dependent degradation by lactacystin reversed the 17β-estradiol-mediated decrease of BCRP supposing that transcriptional down-regulation of the efflux transporter is paralleled by protein degradation. This study demonstrates that 17β-estradiol induces the down-regulation of BCRP on transcriptional and translational levels via the activation of ERβ in rat brain capillaries after 6 h. These results could help to improve brain targeting of BCRP substrates in the treatment of CNS diseases such as brain tumors and also contribute to an enlarged understanding of BCRP−drug interactions at a chronic intake of phytoestrogens and oral contraceptives.

Keywords: BCRP; blood−brain barrier; 17β-estradiol; estrogen receptor; genomic regulation; transport

Introduction

Today, the blood−brain barrier still means a major impediment toward the successful treatment of many CNS diseases such as Alzheimer's, epilepsy, or brain tumors. One basic structural element of that barrier contributing to insufficient drug penetration into the brain is an efflux transporter such as P-glycoprotein or the breast cancer resistance protein (BCRP). Together with tight junctions, they are essential for the maintenance of brain homeostasis and proper neuronal function, but they also impede drug delivery into the brain resulting in pharmacoresistance.[1−4] Likewise, BCRP efflux activity has been shown to protect the brain from the carcinogen PhiP (2-amino-1-methyl-6-phenylimi-

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dazo[4,5-*b*]pyridine) or neurotoxic Amyloid β accumulation by increasing its cerebral clearance.[5−7] However, BCRP is partially the reason for the limited transport of cytostatics such as doxorubicin, imatinib, sorafenib, topotecan, and flavopiridol or CNS drugs such as riluzole into the brain thereby reducing the clinical outcome of the therapeutic regimen.[8−12] Moreover, the ABC transporter has been reported to be overexpressed under pathophysiological conditions such as inflammation as well as in stroke and glioblastoma multiforme, which renders successful treatment of those CNS diseases even more difficult.[13−15] In order to overcome BCRP-mediated multidrug resistance at the BBB and thus to improve brain drug targeting in patients suffering from CNS diseases, several strategies have already been considered: direct inhibition of BCRP efflux activity by GF120918 (elacridar) or KO143 resulted in an increased brain accumulation of tyrosine kinase inhibitors and mitoxantrone in Mdr1a/b (−/−) knockout mice.[11,16,17] However, those selective inhibitors bear neurotoxic side effects that limit their clinical use as comedication together with BCRP substrates.[18] Apart from the reversal of BCRP-mediated multidrug resistance by chemical compounds, the interference with its signaling mechanisms under physiological and in particular pathophysiological conditions provides another intriguing approach to manipulate BCRP function and expression at the blood−brain barrier.

Beyond their classical reproductive function in the brain, estrogens have been reported to modulate the development of neurodegenerative diseases based on neuroprotective, neurotrophic, and antiapoptotic effects and to attenuate BBB disruption after ischemic stroke.[19,20] Estrogens act via estrogen receptors (ERs) that are located in the nucleus and are associated with genomic effects.[21,22] Two nuclear hormone receptor subtypes, ERα and ERβ, can be distinguished, whereas membrane-associated estrogen G-protein-coupled receptors are also discussed as candidates to mediate rapid, nongenomic hormone activity.[23,24] Because of the local synthesis of 17β-estradiol by aromatase and the presence of ERs in the cerebral microvasculature, the brain endothelium is suggested to be another target tissue for sex hormone

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effects.[25] Also, the exposure toward environmental estrogens such as phytoestrogens in nutrition or endocrine disrupters in plasticizers or herbicides, which either mimic or antagonize endocrine functions of endogeneous hormones, provides another source of estrogenic activity in the brain.[26] Evidence has already been shown that efflux and uptake transporters such as Mrp3 and 4, Oats, and Oatps are regulated by sex hormones resulting in varying pharmacokinetics and adverse side effects of pharmaceutic drugs.[27−30] Considering BCRP, only few data exist about its hormone-dependent regulation, and they are controversial as yet: Ee et al. discovered an ERE in the promoter region of BCRP in a human placenta cell line and determined an up-regulation of BCRP protein upon stimulation with 17β-estradiol.[31] A down-regulation of BCRP expression and function, though, was detected by Imai et al. in a breast cancer cell line.[32] On the basis of these data, we addressed the question whether 17β-estradiol affects BCRP expression and function at the rat and mouse blood−brain barrier in the long term. In the present study, we show that BCRP is down-regulated on a transcriptional and translational level by 17β-estradiol at the blood−brain barrier after 6 h. Experiments using selective ERα and β agonists and antagonists as well as ERKO mice revealed ER subtype β to be involved in the genomic signaling pathway of BCRP in isolated brain capillaries. Moreover, the decline of BCRP function and expression is suggested to be accompanied by a proteasome-dependent degradation of BCRP on the protein level. These data provide a new regulatory pathway of BCRP that could be targeted in CNS diseases in order to prevent BCRP-induced multidrug resistance and thus to enhance or restore drug efficacy in the clinic.

Experimental Section

Chemicals. BODIPY FL Prazosin was supplied by Invitrogen (Karlsruhe, Germany). 17β-Estradiol was purchased from Sigma-Aldrich (Taufkirchen, Germany) and 1, 3-bis(4-hydroxyphenyl)-4-methyl-5-(4-(2-piperidinylethoxy)phenol)-1*H*-pyrazole dihydrochloride (MPP), diarylproprionitrile (DPN), 7α,17β-(9((4,4,5,5,5-pentafluoro-pentyl) sulfinyl)-nonyl)estra-1,3,5(10)-triene-3,17-diol (ICI182.780), 4,4′,4″-(4-propyl-(^{1}*H*)-pyrazole-1,3,5-triyl)trisphenol (PPT), and lactacystin were obtained from Biozol (Eching, Germany). Fumitremorgin C (FTC), the monoclonal antibodies to BCRP (BXP53), and β-actin (BV3598) were purchased from Alexis Biochemicals (Lörrach, Germany). Estrogen receptor α- and estrogen receptor β-antibodies were provided by Abcam (Cambridge, UK). Peroxidase-labeled secondary antibodies to mouse, rabbit, and rat IgG were supplied by KPL (Wedel, Germany), Alexa Fluor 488 labeled antibodies against mouse (goat), rabbit (goat), and rat (donkey) were purchased from Invitrogen (Karlsruhe, Germany).

Animals. Male and female Sprague−Dawley rats (average weight of 230−280 g, 5−8 weeks old) were purchased by Charles River Laboratories (Wilmington, MA, USA) and were distributed by IBF (Interfakultäre Biomedizinische Forschungseinrichtung, University of Heidelberg, Heidelberg, Germany). Male and female ERKO and wild-type mice (5−8 weeks old) were obtained from Taconic Europe (Laven, Denmark). Animals were housed according to the German animal protection law (section 2) and maintained on a 12 h/12 h light/dark schedule. Water and standard rat and mouse diet in pellets were available ad libitum. Rats and mice were anesthetized by isoflurane (2%) and decapitated. Brains and the choroid plexus were removed immediately and transferred to ice-cold aCSF (artificial cerebrospinal fluid, 118.0 mM NaCl, 3.0 mM KCl, 0.7 mM $Na_3PO_4 \times 12\ H_2O$, 18.0 mM $NaHCO_3$, 0.8 mM $MgCl_2 \times 6\ H_2O$, and 1.4 mM $CaCl_2 \times 2\ H_2O$, supplemented with 2.0 mM Urea and 12.0 mM glucose at pH 7.4).

Capillary Isolation. Brain capillaries were isolated as described previously with minor modifications.[33] In summary, the brains were cleaned from the meninges, choroid plexus, and white matter. Subsequently, the brains were minced thoroughly with scalpels, suspended in a total volume of 75 mL aCSF (10 brains), and homogenized with a pestle tissue grinder (clearance 150 μm) by 6 up- and down-lifts. Afterward, brain capillaries were separated by density gradient centrifugation: the homogenate was diluted with

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30% dextran solution (Sigma, Taufkirchen, Germany) at a ratio of 1:1 and centrifuged at 5800*g* for 20 min at 4 °C. The pellet was resuspended in 20 mL of aCSF containing 1% BSA and filtered through a 150 μm nylon mesh (neolab, Heidelberg, Germany). Capillaries were run over a glas bead column and washed with aCSF (1% BSA) to remove erythrocytes. Adherent capillaries were detached by gentle shaking with 1% BSA containing aCSF, filtered (80 μm), and washed three times with aCSF (1000*g*, 10 min, 4 °C). Isolated mouse and rat brain capillaries were used directly for transport experiments, immunostainings, or Western blotting.

BCRP Transport Activity. BCRP-specific transport function in isolated brain capillaries was studied using the BCRP-specific fluorescent substrate BODIPY-Prazosin.[34] After the freshly isolated brain capillaries had been exposed to the respective agonists, antagonists, and inhibitors for 6 h, they were transferred to confocal microscope incubation chambers and incubated with 1 μM BODIPY-Prazosin in aCSF for 1 h at room temperature. In studies focusing on the reversal of estrogenic effects, ER- or proteasome-inhibitors were preincubated for 15 min and 17β-estradiol was added for another 6 h since the active transport function of isolated brain capillaries from rodents is limited up to 6−8 h.[33] BCRP transport activity was monitored by confocal microscopy as described previously:[33] for each treatment group, confocal images of 7−12 capillaries were taken (Nikon C1Si-CLEM spectral imaging confocal laser scanning system, Nikon Imaging Center, Heidelberg; 60× water immersion objective, numerical aperture, 1.2, 488 nm line of argon laser; Nikon, Tokyo, Japan), and luminal BODIPY-Prazosin accumulation was quantified using Image J 1.40 software (Soft Imaging System Corp., Lakewood, CO, USA). Specific luminal BODIPY-Prazosin fluorescence was defined as the difference between the amount of luminal fluorescence in the abscence and presence of the BCRP inhibitor fumitremorgin C.

Immunohistochemistry. Isolated capillaries were pipetted onto glass coverslides and fixed with 3% paraformaldehyde and 0.25% glutaraldehyde in PBS (phosphate-buffered saline consisting of 137.0 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4 \times H_2O$, and 1.5 mM KH_2PO_4, pH 7.4) for 20 min. Subsequently, capillaries were washed and permeabilized for 15 min with 1% (v/v) Triton X-100 in PBS. To remove superfluent Triton, capillaries were washed twice with cold PBS. Before staining, unspecific binding was blocked for 30 min with 1% BSA and 5% serum (PBS) of the very host, in which the secondary antibody was generated (goat and donkey). Capillaries were incubated for 1 h at RT or overnight at 4 °C with the primary antibodies BXP53 (1:50, 5 μg/mL), ERα (1:100, 5 μg/mL), ERβ (1:50, 20 μg/mL), and β-actin (1:500, 1 μg/mL). After washing two times with 0.05% Triton X in PBS, capillaries were incubated for 45 min with the corresponding fluorochrome-conjugated secondary antibody at a 1:100 dilution (2% BSA in PBS) in a humid chamber in the dark. Nuclei were counterstained with DAPI (1 μg/mL). For each experiment, negative controls were included by omission of the primary antibody. Stained capillaries were viewed using confocal microscopy (Nikon C1Si-CLEM spectral imaging confocal laser scanning system, Nikon Imaging Center, Heidelberg, Germany).

Quantitative Real-Time RT-PCR. Total RNA from capillaries was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA concentrations were determined from UV absorbance at 260 nm with a Nanodrop1000 device (Pequlab, Erlangen, Germany), and purity was checked by the absorbance ratio $A_{260\ nm}/A_{280\ nm}$. RNA was reverse-transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). cDNA samples were diluted 1:5 and applied as a template. Porphobilinogen deaminase (*Pbgd*) was used as a housekeeping gene. Real-time PCR was performed on a LightCycler 1.5 instrument (Roche Diagnostics, Mannheim, Germany) using the LightCycler FastStart DNA Master plus SYBR Green I kit (Roche Diagnostics) and the following primers: *Abcg2* (GenBank accession no. NM_181381, 383bp), 5′-AAGACCATG AAGCAAACAAG-3′ (forward primer, bases 1013−1032) and 5′-AGGACTGA TGGTTGGTCACA-3′ (reverse primer, bases 1376−1395); and *Pbgd* (GenBank accession no. NM_013168, 203bp), 5′-TGATGCTGT-TGTTTTTCACC-3′ (forward primer, bases 490−509) and 5′-CCTCAAGTCACGGTAATAGG-3′ (reverse primer, bases 673−692). The amplification profile consisted of the following steps: (1) denaturation (10 min, 95 °C), (2) amplification (95 °C/10 s, 60°C/9 s, 72 °C/15 s), (3) melting curve program (95 °C/0 s, 65 °C/60 s, 95 °C/0 s; heating rate, 0.1 °C/s), and (4) cooling program (40 °C). Amplified products were tested by electrophoresis in a 1.5% agarose gel and visualized by ethidium bromide staining. Standard curves from diluted, pooled cDNA samples of all animals were generated in order to calculate PCR amplification efficiency. Crossing points (C_p) were determined by the second derivative maximum method using Light Cycler software 3.5.2. The relative expression of Abcg2 in relation to Pbgd was calculated on the basis of the $2^{(-\Delta\Delta Ct)}$ method according to Pfaffl et al. using the relative expression software tool REST.[35]

Membrane Isolation and Western Blot Analysis. To isolate the membrane fractions from rat and mouse capillaries, the tissue was washed with 4 °C cold aCSF after treatment. Ice-cold CelLytic-MT Mammalian Tissue Lysis/Extraction Reagent and Complete EDTA-free Protease Inhibitor (Roche, Mannheim, Germany) were added (25:1, 20 mL for 1 g tissue). Capillaries were homogenized by 15 strokes in a glass homogenizer and left on ice for 1 h. Afterward, the lysed capillaries were centrifuged at 10.000*g* for 30 min at 4 °C to remove cell nuclei and organelles. The

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supernatant was used and centrifuged at 100.000*g* at 4 °C to isolate the membrane fraction. Proteins from the brain, capillaries, choroid plexus, kidney, and liver were isolated in a similar manner after 1 h of homogenization in lysis buffer on a magnetic stirrer. Pellets were restored in lysis buffer until protein content was determined by the Biuret method.[36]

Protein samples were mixed with 2× Laemmli's sample buffer, and fresh DDT was added to a final concentration of 50 mM. Samples were boiled at 70 °C for 10 min. Membrane proteins were subjected to electrophoresis (150 V, 1.5 h) on a 7.5% sodium dodecyl sulfate−polyacrylamide gel and transferred electrophoretically onto polyvinylidene difluoride membranes (300 mA, 1.5 h). The blots were then blocked overnight at 4 °C with PBS containing 1% nonfat dry milk powder and 1% bovine serum albumin. After three washing steps with PBS, the proteins were hybridized for 75 min at room temperature with the monoclonal antibody against BCRP, ERα, ERβ, or β-actin, respectively. Subsequently, the blots were incubated with the secondary horseradish peroxidase-conjugated antibody (1:10000) for 1 h at room temperature, followed by enhanced chemoluminescence detection (Western Lightning Western blot Chemiluminescence Reagent Plus, ChemiDocXRS software).

Statistics. Data are presented as the mean ± SEM. Differences between control and treated samples were determined using One-Way ANOVA, which was corrected by a Bonferroni posthoc test (GraphPad Prism 4.00 software). Values of $p^* < 0.05$ were considered statistically significant ($p^{**} < 0.01$, $p^{***} < 0.001$).

Results

BCRP Protein Expression in Brain Capillaries. The Western Blot in Figure 1A represents the expression of BCRP in the brain homogenate, brain capillaries, and the membrane fraction of brain capillaries in rats. The ABC transporter is enriched in the membrane fraction of rat brain capillaries and scarcely expressed in the choroid plexus and brain tissue including neurons, astrocytes, and pericytes. These results are supported by previous data having determined BCRP expression in brain capillaries and microglia cells.[8,37−40] Moreover, the immunostaining in Figure 1B demonstrates the luminal localization of BCRP in the plasma membrane of brain capillaries, which is consistent with its function as an efflux transporter.

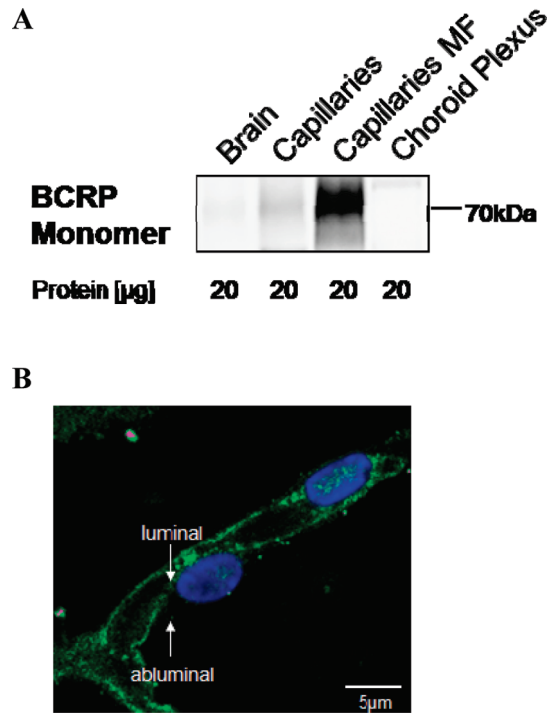

Figure 1. (A) BCRP protein expression in rat brain, capillaries, capillary membrane fraction, and choroid plexus. The BCRP protein was prepared under reducing conditions and detected as a monomer at a molecular weight of 72 kDa. BCRP is highly enriched in the membrane fraction of brain capillaries and shows only a minor signal in the brain, capillaries, and choroid plexus. Samples were pooled from 10 male SD rats. (B) The immunostaining for BCRP in a rat brain capillary shows transporter localization at the luminal membrane (green). Nuclei were counterstained with DAPI (blue).

Down-Regulation of BCRP Function and Expression after 6 h of Incubation with 17β-Estradiol. In order to determine changes in BCRP efflux activity, a fluorescence-based assay using BODIPY-Prazosin as a selective BCRP substrate was used.[41] Figure 2A demonstrates the extrusion of BODIPY-Prazosin in isolated brain capillaries. The fluorescence dye accumulates inside the capillary lumens, and this accumulation could be inhibited by 10 μM fumitremorgin C, a selective BCRP inhibitor (Figure 2B). When capillaries from male rats were incubated with 1 and 10 nM

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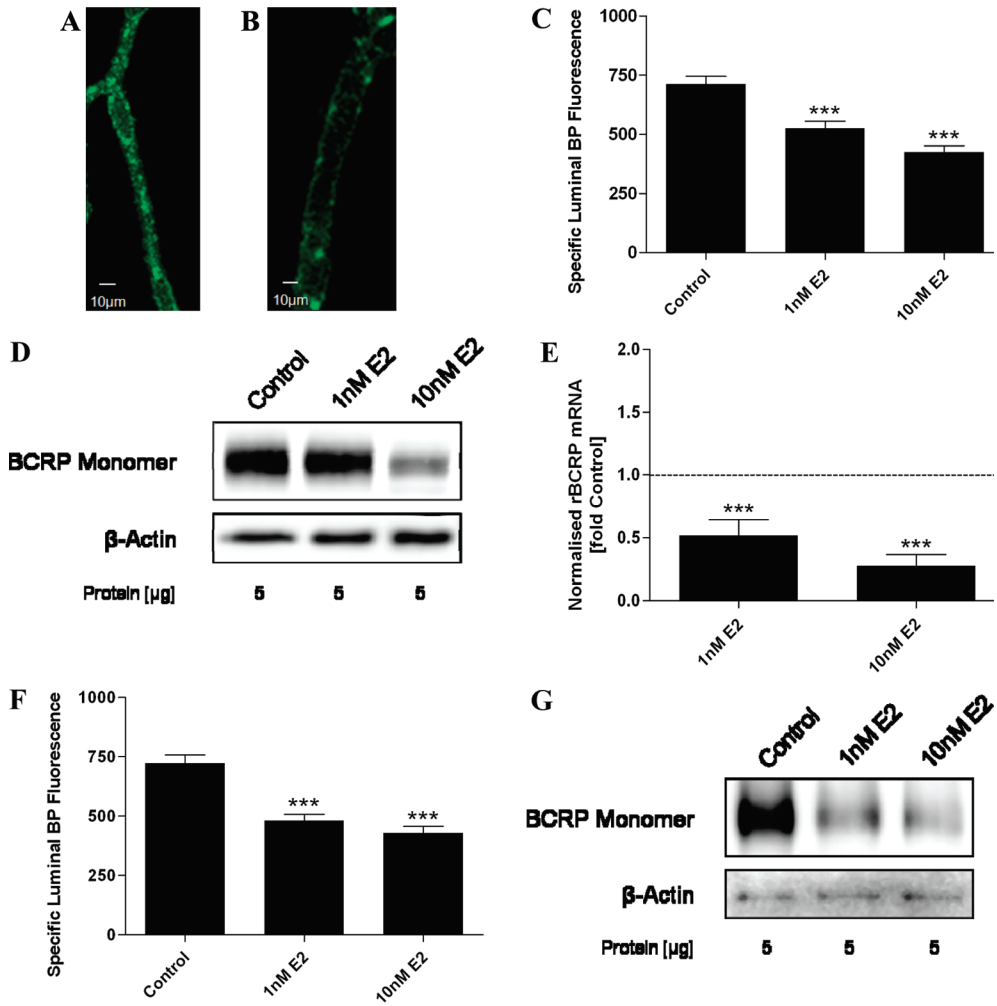

Figure 2. (A) Confocal picture of an isolated rat brain capillary that actively extruded the fluorescent BCRP substrate BODIPY-Prazosin from the surrounding incubation medium into the capillary lumen. (B) Inhibition of BCRP transport activity by 10 μM FTC, a selective BCRP inhibitor, decreased the amount of BODIPY-Prazosin in the capillary lumen. Instead, the fluorescent substrate accumulated in the endothelial cells of the capillary wall. The following transport studies display specific, luminally accumulated BODIPY-Prazosin fluorescence, which has been subtracted by the amount of fluorescence detectable in the vessel lumen after complete inhibition of BCRP efflux with FTC. The small amount of fluorescence, that still enters the lumen despite BCRP inhibition, may result from passive diffusion into capillary lumens. (C) BCRP-specific BODIPY-Prazosin efflux is significantly reduced by 17β-estradiol after isolated capillaries from male rats had been exposed to the hormone for 6 h (mean $\pm$ SEM, $n = 7-10$ capillaries from 6 pooled SD rats, $p^{***} < 0.001$). (D) In parallel, BCRP protein expression is decreased by 1 and 10nM 17β-estradiol in males. β-Actin was used for normalization purposes. The figure shows one representative Western blot. (E) Down-regulation of BCRP mRNA expression in male rat brain capillaries after 6 h of incubation with 1 and 10 nM 17β-estradiol (dashed line, control = 1). Data are expressed as the *rBCRP*/*rPbgd* ratio relative to controls (mean $\pm$ SEM; data are the results from 2 independent experiments with samples pooled from 10 male SD rats per experiment, $p^{***} < 0.001$). (F) BCRP efflux activity is decreased by 17β-estradiol in female rats (mean $\pm$ SEM, $n = 7-10$ capillaries from 6 pooled SD rats, $p^{***} < 0.001$). (G) Down-regulation of BCRP protein expression in females after 6 h of incubation with 1 and 10 nM 17β-estradiol.

17β-estradiol for 6 h followed by incubation with the efflux indicator BODIPY-Prazosin, BCRP transport activity was significantly reduced by approximately 30% (Figure 2C). With regard to protein expression levels, BCRP was down-regulated by nanomolar 17β-estradiol concentrations in the membrane fraction of isolated male rat brain capillaries as well (Figure 2D), whereas the expression level of the marker β-actin remained constant. Moreover, quantitative real-time PCR revealed that 17β-estradiol also diminishes BCRP mRNA expression corroborating the fact that estrogens might regulate BCRP by a genomic mechanism (Figure 2E). This means that not only BCRP function but also transcription and translation rates are affected by the incubation with 17β-estradiol for 6 h and that our previously introduced acute, nongenomic effect is probably maintained at the long-term and further developed on the genomic level.[42] However, BCRP efflux activity and

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expression are not fully abrogated, which can be either explained by the selected incubation interval of 6 h, which might evidence only the beginning of a proceeding regulatory pathway, or by the fact that estrogens only partly down-regulate BCRP at the blood−brain barrier.

In Figure 2F and G, brain capillaries from female rats were treated with physiologic concentrations of 17β-estradiol. BCRP-mediated BODIPY-Prazosin extrusion was significantly reduced in brain capillaries, which was paralleled by a decline in BCRP monomer expression. A comparison of the effect by 17β-estradiol after 6 h of incubation on BCRP transport and protein expression in male and female capillaries revealed no significant differences between genders. Hence, the ABC transporter is down-regulated by 17β-estradiol in male and female rat brain capillaries on both functional and expression levels after 6 h.

Estrogen Receptor (ER) Expression at the Blood−Brain Barrier in Rats. Since BCRP harbors an estrogen responsive element (ERE) in its promotor region and ERs are commmonly known as transcription factors that exert genomic actions within several hours, the expression of the two ER subtypes α and β was determined at the rat blood−brain barrier.[24,43] In Figure 3A, protein expression levels of ERα were compared among homogenates derived from the liver, kidney, choroid plexus, whole brain, brain capillaries, and capillary membranes. ERα is expressed in the liver, kidney, and choroid plexus, whereas no signal was detected in any of the brain fractions. To exclude the possibility that signals in the brain or capillaries were superimposed by the strong adjacent signals, the brain, capillaries, and capillary membranes were analyzed separately for the presence of ERα (Figure 3B). A slight band signal for the ER subtype α was determined in the rat brain, which increased in brain capillaries and the membrane fraction, respectively. Hence, ERα protein expression was observed in the kidney, liver, and choroid plexus and, to a lower extent, in the brain, capillaries, and capillary endothelial membranes. These results suggest that despite its weak presence in capillary endothelial cells, ERα might be a candidate to communicate the established effect of 17β-estradiol on BCRP at the blood−brain barrier. Moreover, ERα expression in the kidney, liver, and human choroid plexus was also described by others. [44−46]

Figure 3C shows an immunostaining for ERα in male rat brain capillaries. In spite of the weak band that was detectable in capillaries in the Western blot (Figure 3B), no staining could be observed for the very ER subtype. Apparently, the epxression of ERα at the blood−brain barrier is very low compared to that in other organs.

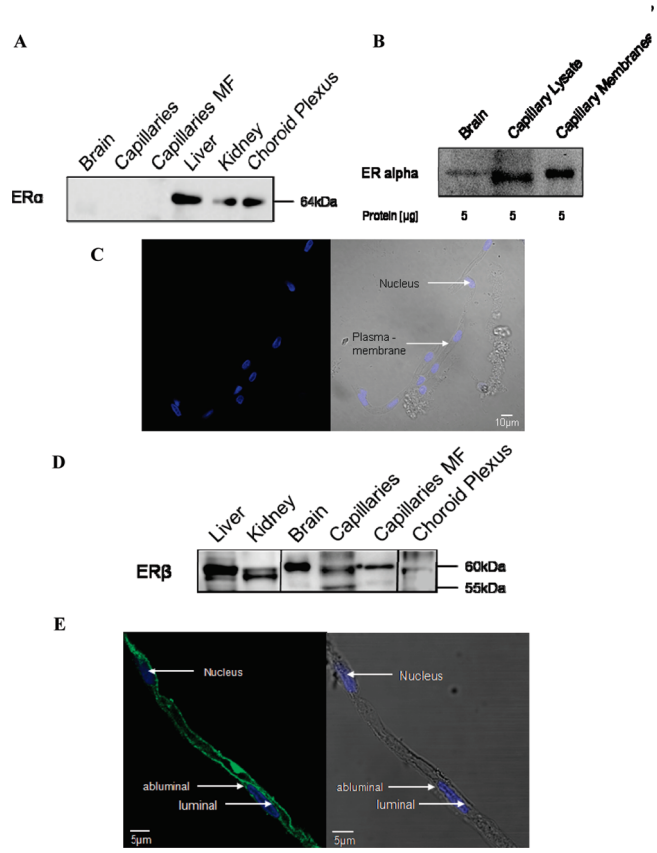

Figure 3. (A) Representative Western blot for ERα. ERα expression is detected in the liver, kidney, and choroid plexus at a molecular weight of 64 kDa. (B) Separate detection of ERα in the brain, capillaries, and the capillary membrane fraction reveals moderate signals in each tissue sample (protein was analyzed from 2−6 pooled male rat tissue samples). (C) Immunostaining for ERα in male rat brain capillaries. No fluorescent signal was detected (left image). Nuclei were stained with DAPI (blue). Transmitted light image of the capillary (right image). (D) Western blot for ERβ. ERβ is highly expressed in the kidney and brain and to a smaller extent in the liver, brain capillaries, and the capillary membrane fraction. A faint band is visible in the choroid plexus (protein was analyzed from 2−6 pooled male rat tissue samples). Bands at a height of 55 kDa present deglycosylated ERβ. (E) Localization of ERβ in endothelial cells of rat brain capillaries (green, left image). Nuclei were stained with DAPI (blue). Transmitted light image (right image).

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In contrast, the Western blot in Figure 3D demonstrates intense expression of ERβ in the rat liver and brain tissue at a molecular weight of 60 kDa. Moderate protein levels were detected in the kidney and only minor amounts in choroid plexus tissue. Considering ERβ expression at the blood−brain barrier, the receptor subtype is present in brain capillaries and the capillary membrane fraction, even though to a lower extent than in brain tissue. These findings indicate that ERβ is principally expressed in neurons, astrocytes, and glia. Besides that, the Western blot depicts an additional band in the brain, capillaries, and the capillary membrane fraction, which presumably results from deglycosylated ERβ. Similar data were obtained by Nealen et al. (2001) who reduced the molecular size of ERβ in human platelets by incubation with N-glycosidase F. In comparison to ERβ, ERα is known to be predominantly expressed in classical hormone-responsive tissues such as the uterus, ovary, testis, or pituitary glands.[47] In contrast, ERβ is preferentially found in nonreproductive tissues such as the lung, bladder, or brain and plays only a minor role in typical estrogen target tissues.[48−50]

The detection of ER by Western Blot was confirmed by an immunostaining of brain capillary endothelial cells (Figure 3E). In summary, these findings exhibit the presence of both ER subtypes in rat brain capillaries while ERβ appears to be higher expressed at the blood−brain barrier than ERα.

Effect of ER Agonists. First, the contribution of each receptor subtype to the 17β-estradiol-mediated, genomic down-regulation of BCRP transport and protein expression at the blood−brain barrier was investigated by the use of selective ERα (PPT, $EC_{50} = 0.2$ nM) and ERβ (DPN, $EC_{50} = 0.85$ nM) agonists.[51,52] This information might be useful to reliably predict drug interactions of BCRP substrates with estrogens or antiestrogens according to their affinity to the ER subtypes at the blood−brain barrier.

Incubating male rat brain capillaries with 1 and 10 nM of the ERβ-selective agonist DPN for 6 h led to a significant down-regulation of BCRP transport activity (Figure 4A). Similarly, BCRP protein expression was also decreased by DPN with regard to control levels (Figure 4B). Since these results mirror the 17β-estradiol-mediated down-regulation of BCRP efflux activity and protein expression in rat brain capillaries, it may be anticipated that ERβ is responsible for the 17β-estradiol-induced genomic signaling of BCRP at the blood−brain barrier.

Rat brain capillaries from female rats were incubated with nanomolar concentrations of DPN as well, and a decline of both luminal BODIPY-Prazosin accumulation and thus BCRP activity (Figure 4C) as well as protein expression (Figure 4D) were determined. Consequently, it can be concluded that BCRP is decreased by ERβ in both male and female rats.

To further clarify the ER subtype that is involved in genomic signal transduction of BCRP function and expression at the blood−brain barrier, male rat brain capillaries were treated with the ERα-selective agonist PPT. Figure 4E and F illustrates that neither 0.5 nor 1.0 nM PPT provoked a significant decline in BCRP efflux activity or monomer expression as was observed with 17β-estradiol and the ERβ agonist DPN. Thus, the participation of ERα in the 17β-estradiol-mediated regulation of BCRP at the blood−brain barrier can be excluded after the use of ER agonists.

Effect of ER Antagonists. The participation of ERβ but not ERα in genomic estrogen signaling of BCRP was specified by the application of selective ERα and ERβ antagonists. In Figure 5A, male rat brain capillaries were exposed to the selective ERα inhibitor MPP for 15 min, which was followed by the addition of 10 nM 17β-estradiol for 6 h. MPP is a highly selective ERα antagonist that shows 200-fold selectivity over ERβ, and its K_i value amounts to 2.7 nM. In terms of BCRP-specific transport activity, 17β-estradiol significantly reduced BODIPY-Prazosin efflux, which was not reversed by the inhibition of ERα with MPP. Noticeably, the blockade of ERα did not impede the down-regulation of BCRP protein expression by 10 nM 17β-estradiol either, suggesting that ERα is not involved in the hormone-dependent regulation of BCRP at the blood−brain barrier (Figure 5B). Neither the stimulation of ERα nor the inhibition of the receptor subtype with MPP biased the effect of 17β-estradiol on BCRP expression and function.

Since no selective ERβ antagonist exists so far, the unspecific ER antagonist ICI182.780 was taken to define the role of ERβ in genomic BCRP signaling. In Figure 5C, male rat brain capillaries were preincubated with the ER antagonist ICI182.780 and subsequently exposed to the endogenous ER agonist 17β-estradiol for 6 h. Indeed, analysis of the BCRP-mediated BODIPY-Prazosin efflux showed that the estrogen-induced down-regulation was restored up to control levels by the unspecific ER antagonist ICI182.780. Since fulvestrant

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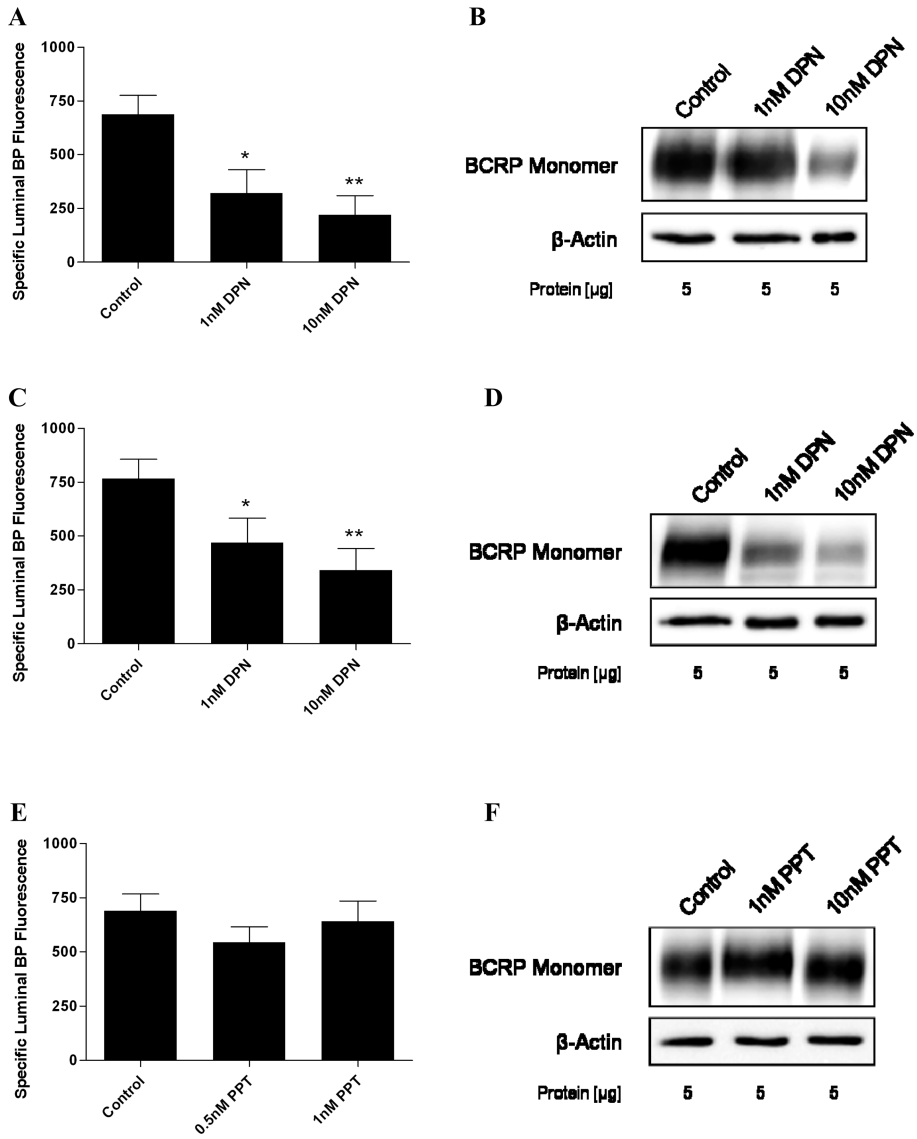

Figure 4. (A) Influence of the selective ERβ agonist DPN on BCRP transport and protein expression after 6 h in male rat brain capillaries. Luminal BODIPY-Prazosin accumulation is significantly decreased in comparison to BCRP control efflux. This is paralleled by a decline of BCRP protein expression (B). (C) Influence of the selective ERβ agonist DPN on BCRP transport and protein expression after 6 h in female rat brain capillaries. BCRP efflux activity is decreased by incubation with 1 and 10 nM DPN (C), which could also be confirmed on the protein level (D). (E) Influence of the selective ERα agonist PPT on BCRP transport and protein expression after 6 h in male rat brain capillaries. No effect was observed on BCRP efflux. BCRP protein expression remains unchanged as well (F) (each experiment, mean $\pm$ SEM, $n = 7-10$ capillaries from 6 pooled SD rats, $p^* < 0.05$, $p^{**} < 0.01$).

blocks both ER subtypes, but the involvement of ERα in the long term regulation of BCRP was excluded in the previous section (Figure 4E and F, and Figure 5A and B), it can be concluded that inhibition of ERβ must cause the recovery of 17β-estradiol-induced down-regulation of BCRP transport function. Also on the translational level, the BCRP protein was decreased after 6 h of exposure toward 10 nM 17β-estradiol, and inhibition of both ER subtypes restored BCRP expression up to control levels (Figure 5D). This Western blot confirms that ERβ mediates the decline of BCRP function and expression after 6 h of incubation with 17β-estradiol at the blood−brain barrier.

ERKO Mice. As a third tool, ERKOα and β mice were used to investigate the effect of lacking ER subtypes on the 17β-estradiol-induced decline of BCRP on functional and translational levels. Brain capillaries from wild-type and ERKOα mice were isolated, and transport function and BCRP protein expression were compared to control values. As shown in Figure 6A, no difference was observed in basal BCRP transport and protein expression between wild-type and ERKOα mice brain capillaries. Comparable to rats, the exposure of wild-type mice brain capillaries toward 10 nM 17β-estradiol caused a drop in BCRP efflux activity and protein expression. Incubation of brain capillaries from

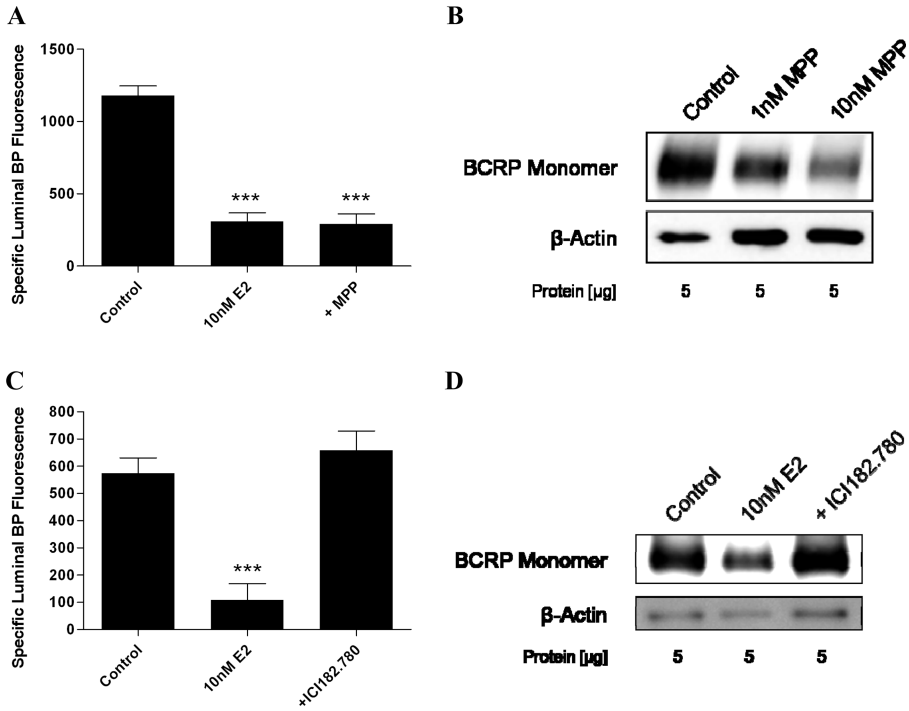

Figure 5. (A) Incubation of male rat brain capillaries with the ERα-selective antagonist MPP for 6 h. Brain capillaries were isolated and preincubated with MPP for 15 min, which was followed by a 6 h lasting exposure toward 10 nM 17β-estradiol. BCRP-specific BODIPY-Prazosin efflux is reduced by the hormone but cannot be reversed by the blockade of ERα (A). BCRP protein expression is down-regulated by 17β-estradiol but remains unchanged, although ERα is inhibited (B). (C) Incubation of male rat brain capillaries with the unspecific ER antagonist ICI182.780 for 6 h. The 17β-estradiol-mediated decline of BCRP efflux activity is reversed after incubation with ICI182.780 (C). On the translational level, BCRP expression, which is down-regulated by 10 nM 17β-estradiol, is restored up to control levels by the unspecific ER antagonist (D) (each experiment, mean ± SEM, $n = 7-10$ capillaries from 6 pooled SD rats, $p^{***} < 0.001$).

ERKOα mice with 10 nM 17β-estradiol, however, demonstrated that even though ERα was lacking, the steroid hormone was still able to down-regulate BCRP transport function and also protein expression (Figure 6A). These findings were equal to those of wild-type mice that possess both ER subtypes, meaning that ERα is not involved in the pathway of BCRP down-regulation by estrogens. Otherwise, a lack of ERα in ERKO mice would have impeded the 17β-estradiol-induced reduction of BCRP at the blood−brain barrier.

Continuing with male ERKOβ mice, no difference in basic BCRP transport or protein expression between wild-type and ERKOβ mice was detected, proposing that ER subtypes do not influence the control BCRP function and expression (Figure 6B). When brain capillaries from ERKOβ mice were treated with 10 nM 17β-estradiol for 6 h, the lack of ERβ prevented the estrogen-mediated decline in BCRP transport and expression, evidencing that ERβ and not α is responsible for the reduction of BCRP at the blood−brain barrier.

Inhibition of Proteasome-Dependent-Degradation. The down-regulation of BCRP function and protein expression could exclusively result from a transcriptional and also a post-translational process, such as a proteasome-dependent degradation pathway. To prove whether 17β-estradiol affects the reduction of BCRP activity and expression not only by a decreased transcription rate but also via degradation in the proteasome, male rat brain capillaries were pretreated with lactacystin, an inhibitor of the 20S proteasome subunit. Ten nanomolar 17β-estradiol was added for 6 h, and BCRP-specific BODIPY-Prazosin efflux was quantified. In Figure 7A, the blockade of the proteasome-mediated degradation pathway restored the 17β-estradiol-mediated decrease of the ABC transporter function up to control levels. Moreover, BCRP protein expression was reduced by the hormone after 6 h, whereas preincubation with lactacystin evoked the inhibition of the 17β-estradiol-induced degradation of BCRP in the proteasome (Figure 7B).

Discussion

BCRP is expressed in organs such as the liver, intestine, or placenta to control the absorption, metabolism, and secretion of drugs but also to protect from harmful substances.[53,54] At the blood−brain barrier, BCRP is discussed to play either only a minor role or to work in concert with P-glycoprotein compensating its loss in knockout

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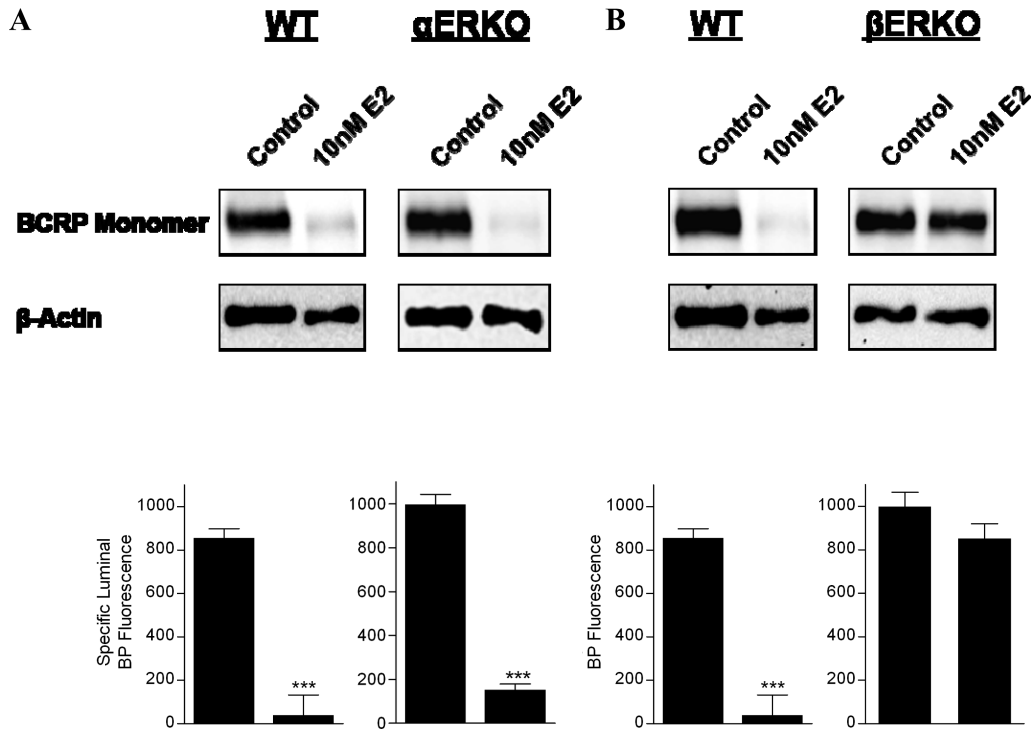

Figure 6. (A) Incubation of male wild-type mice and ERKOα (A) and ERKOβ mice (B) with 10 nM 17β-estradiol for 6 h. Transport was compared between ERKO and wild-type mice. No difference in BCRP transport or protein reduction was determined in ERKOα mice after incubation with 10 nM 17β-estradiol (A). Absence of ERβ, however, impeded the decrease of BCRP transport and protein expression supposing that ERβ is required for genomic down-regulation of BCRP expression and function by hormones (B) (mean ± SEM, $n = 7-10$ capillaries from 6 pooled mice, $p^{*} < 0.05$, $p^{***} < 0.001$).

mice.[55,56] On the opposite site, the transporter has been demonstrated to extrude several cytostatics from the brain, limiting their therapeutic benefit in the treatment of brain tumors.[57−62] In the present study, the 17β-estradiol-dependent effect on BCRP efflux activity and mRNA and protein expression was investigated in brain capillaries from rats and mice. Here, physiologically relevant nanomolar concentrations of the endogenous hormone were used to determine the regulation of BCRP in an ex vivo model after 6 h.[63] It was found, that 17β-estradiol down-regulates BCRP-mediated efflux activity as well as transcripts and protein expression in male and female rat brain capillaries, proposing a genomic signaling mechanism. As was demonstrated by ER-selective agonists, antagonists, and ERKO mice, this effect was triggered by ER subtype β. Besides, the 17β-estradiol-induced decrease of BCRP was also abrogated by inhibition of proteasome-dependent degradation assuming

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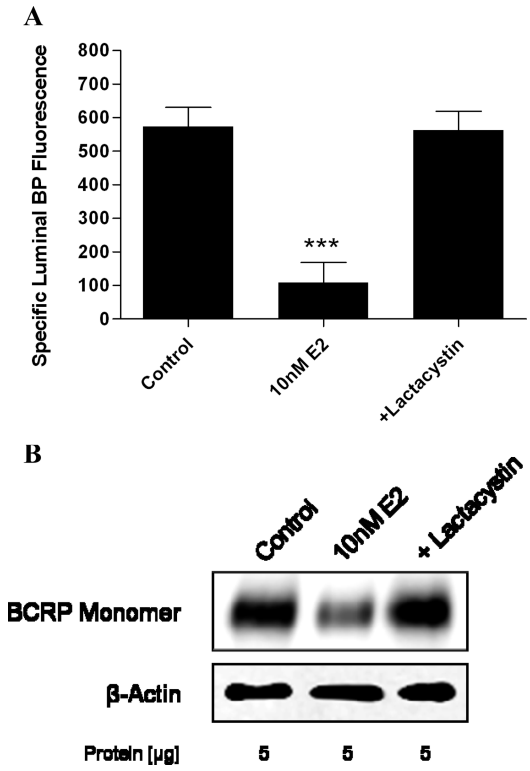

Figure 7. (A) Effect of proteasome inhibition on BCRP transport and expression after 6 h in the male rat brain capillaries. BCRP activity is reduced by 10 nM 17β-estradiol but restored upon inhibition of the proteasomal degradation pathway. The same issue is presented on the protein level (B) (mean $\pm$ SEM, n = 7−10 capillaries from 4 pooled SD rats, $p^{***} < 0.001$). Lactacystin was previously shown to exert no effect on BCRP transport nor protein expression.[42]

that the transcriptional regulation of the transporter is paralleled by a post-translational pathway. These data about the long-term regulation of BCRP efflux, gene, and protein expression by hormones at the blood−brain barrier can be perceived as a sequel to the nongenomic, rapid decrease of BCRP transport recently introduced.[42]

Contradictory data have been published about the influence of hormones on BCRP ranging from down-regulation via ERβ in human BeWo cells and brain microvessel cells to up-regulation via ERα in hormone-sensitive breast cancer cell lines.[32,64,65] However, it is important to note, that the differentiation between exogenous and endogenous transporter expression, the use of micromolar and physiologic nanomolar concentrations particularly in the placenta cell line BeWo have augmented the diversity of results.[64,66] More specifically, Ee et al. described a significant up-regulation of endogeneous BCRP transcripts after incubation with nanomolar concentrations of 17β-estradiol in T47D breast cancer cells, whereas Imai et al. ascertained post-translational down-regulation of BCRP protein in the same cell line. Against this background, the use of an ex vivo model of isolated brain capillaries is more likely to reflect in vivo situations and to provide more relevant data than immortalized cell lines. Moreover, the estradiol-dependent suppressive effect on BCRP in male rat liver as well as at the rat and mouse blood−brain barrier but the induction in primary rat placenta syncytiotrophoblasts points out to a tissue-specific regulation mechanism which could be associated with different expression patterns of both ER subtypes in the respective tissue.[31,67−69]

As a next step, we showed that ERβ and, albeit to a lower extent, ERα are expressed in the rat brain, brain capillary endothelial cells, and even in the plasma membrane of brain capillaries. This expression profile reveals that both ER subtypes reside in the cytoplasm where they usually dimerize and move to the nucleus upon ligand binding to cause long-lasting, genomic signaling pathways. In contrast to their classical function as transcription factors, ERs are also able to mediate rapid, nongenomic actions that are supposed to be initiated by membrane-associated ERs and often comprise phosphorylation steps or quick changes in intracellular Ca^{2+} concentrations.[26,70,71] With the use of ER agonists and ERKO mice, the rapid down-regulation of BCRP transport activity at the blood−brain barrier was recently demonstrated to be communicated by both ER subtypes α and β.[42] Here, we document the expression of membrane-associated ERs in brain capillaries that are supposed to be responsible for the induction of nongenomic BCRP signaling within minutes. However, since ERα and ERβ are also expressed in the homogenate of brain capillaries, both receptors have to be taken into consideration to trigger the genomic regulation of BCRP at the blood−brain barrier. Notwithstanding, ERβ was more expressed in brain and capillaries than

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ERα measured by the expression level in the kidney, liver, or choroid plexus, which is consistent with data from Kuiper et al.[47]

The 17β-estradiol-dependent down-regulation of BCRP in the present study is derived from a transcriptional and from a post-translational mechanism. Alternatively, a 17β-Estradiol-mediated physiologic response can also be controlled by the ligand-dependent degradation of the receptor itself. Such a decrease of ERβ mRNA and a rapid turnover of ERs due to proteasome-dependent degradation upon ligand binding were described.[64,72,73] Consequently, our findings at the blood−brain barrier could also be a result of decreasing ER levels. This assumption gives reason to the supposition that ER-mediated efflux activity and BCRP expression are sustained under low estrogen concentrations but that any further addition of steroids interrupts agonistic ER signaling due to hormone-induced ER degradation. Alternatively, ER target genes have already been demonstrated to be negatively regulated by corepressors such as IL-6.[74] However, the stability of ER subtypes after incubation with 17β-estradiol remains to be determined.

In the experiments of the present investigation, BCRP protein expression in the plasma membrane but not in the cytoplasm was determined. The trafficking of the transporter to the membrane is reported to be impeded by improper protein folding and not by lacking glycosylation.[75,76] Henriksen et al. demonstrated that the formation of an intra- but not intercellular disulfide bond is essential for proper membrane targeting and function.[75,77] Likewise, Wakayabashi et al. determined an increased proteasome degradation of misfolded ABCG2 proteins lacking the intramolecular disulfide bond between C-592 and C-608.[78] Similar to that reported by Imai et al., who supposed that the estrogen-mediated repression of quiescin Q6, an enzyme that catalyzes disulfide bond formations in the endoplasmatic reticulum, is the reason for the post-translational reduction of BCRP expression in breast cancer cells, misfolded BCRP could also lead to a reduced insertion into the plasma membrane of the blood−brain barrier. [79] Thus, the profound effect of 17β-estradiol on BCRP mRNA expression is possibly superimposed by the proteasome-dependent degradation of misfolded BCRP. A time course would provide further information about the onset of each process assuming that the transcriptional regulation might begin later. A further mechanism that has been described only recently to mediate 17β-estradiol-dependent down-regulation of BCRP at the blood−brain barrier includes ERβ signaling via the PTEN/PI3K/Akt/GSK3 pathway. [65] These findings suggest that the decline of BCRP in rat brain endothelial cells could be run by several parallel and interdigitating mechanisms.

The present data provide ER subtype β as a new target receptor to reduce BCRP function and expression at the blood−brain barrier upon 17β-estradiol-mediated stimulation. This genomic signaling cascade offers a new opportunity to selectively bypass BCRP-mediated multidrug resistance at the blood−brain barrier and thus to enhance the bioavailability of CNS drugs in the brain. Nonetheless, the clinical benefit of estrogens in the treatment of CNS diseases, which optimize drug delivery across the blood−brain barrier, remains to be determined by in vivo studies about the onset and the dwindling of BCRP reduction, for example. Moreover, as a consequence of a hormone-dependent regulation, varying BCRP expression levels independent from pregnancy, obesity, or age could be deduced, which would make an individualized CNS medication necessary.

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