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Breast Cancer-Resistance Protein (BCRP1) in the Fetal Mouse Brain: Development and Glucocorticoid Regulation[1]

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ABSTRACT

Breast cancer-resistance protein (BCRP1), encoded by *Abcg2* mRNA, limits the penetration of a spectrum of compounds into the brain. The fetal brain is a primary target for many BCRP1 substrates; however, the developmental expression, function, and regulation of *Abcg2*/BCRP1 in the mouse fetal brain are unknown. Synthetic glucocorticoids (e.g., dexamethasone [DEX]) increase *Abcg2*/BCRP1 expression and function in vitro in endothelial cells derived from brain microvessels. A regulatory role of glucocorticoids on *Abcg2*/BCRP1 in the fetal brain is of importance given that approximately 10% of pregnant women are treated with synthetic glucocorticoid for threatened preterm labor. We hypothesized the following: 1) *Abcg2* mRNA and BCRP1 protein expression increases with development (from Embryonic Day [E] 15.5 to E18.5), corresponding to decreased accumulation of BCRP1 substrate in the fetal brain. 2) Maternal treatment with DEX will up-regulate *Abcg2* mRNA and BCRP1 protein expression in the fetal brain, resulting in decreased BCRP1 substrate accumulation. Pregnant FVB dams were euthanized on E15.5 or E18.5, and fetal brains were collected and analyzed for [^{3}H]mitoxantrone (BCRP1-specific substrate) accumulation and *Abcg2*/BCRP1 expression. In another six groups (n = 4–5/group), pregnant mice were treated with DEX (0.1 or 1 mg/kg) or vehicle (saline) from either E9.5 to E15.5 (midgestation) or E12.5 to E18.5 (late gestation) and then injected with [^{3}H]mitoxantrone. In conclusion, *Abcg2* mRNA expression significantly decreases with advancing gestation, while BCRP1-mediated neuroprotection increases. Furthermore, there is a dose-, sex-, and age-dependent effect of DEX on *Abcg2* mRNA in the fetal brain in vivo, indicating a complex regulatory role of glucocorticoid during development.

Abcg2, *BCRP1, blood-brain barrier, breast cancer-resistance protein, developmental biology, dexamethasone, fetal blood-brain barrier, gene regulation, [^{3}H]mitoxantrone, multidrug resistance, neuroprotection, pregnancy*

INTRODUCTION

Breast cancer-resistance protein (BCRP1), encoded by the *Abcg2* gene, was first identified in MCF-7 cells (a human breast carcinoma subline), where it resulted in chemoresistance [1]. In normal tissues, BCRP1 limits absorption and facilitates the excretion of a wide range of substrates. Substrates of BCRP1 include hormones such as 17-β estradiol or therapeutic agents such as nitrofurantoin (antibiotic), mitoxantrone (antineoplastic), cimetidine (histamine H_2-receptor antagonist), and glyburide (antidiabetic) [2–8]. As a result of maternal use of therapeutic drugs, the developing fetus may be inadvertently exposed to teratogenic factors present in maternal circulation, with the fetal brain being particularly susceptible to many of these BCRP1 substrates.

The blood-brain barrier (BBB), composed of capillary endothelial cells, is a dynamic structure that regulates the entry of endogenous and exogenous substrates into the brain. In the adult brain, BCRP1 is localized on the luminal surface of capillary endothelial cells [9, 10]. Here, BCRP1 has an important role in neuroprotection by limiting entry of BCRP1 substrates into the brain [11]. For example, in the presence of a BCRP1 inhibitor, uptake of prazosin and mitoxantrone (prototypical substrates of *Abcg2*/BCRP1) increased in both wild-type and CF1 mice (*Abcb1a*/P-gp knockout mutant strain) [11], demonstrating that *Abcg2*/BCRP1 restricts brain uptake of these substrates.

Abcg2/BCRP1 in the fetal brain has not been extensively investigated. In a recent study [4], the fetal brain:body ratio of genistein (phytoestrogen BCRP1 substrate) increased 1.4-fold in *Abcg2*/BCRP1 knockout mice compared with wild type at 2 wk of gestation, suggesting that BCRP1 in the fetal brain has an important role in limiting the entry of substrates into the fetal brain. Mapping neurodevelopment of the mouse brain onto that of the human is complex given that brain development is not linear [12, 13]. In general, the first half and second half of gestation and the first 7 days after birth in the mouse brain are considered to be equivalent to the first, second, and third trimesters, respectively, in the human fetal brain [14–16]. BCRP1 expression has been reported as early as 22 wk of gestation (in the human) and Embryonic Day (E) 13 (in the rat) in fetal brain capillary endothelial cells [17, 18]. We have previously shown that BCRP1 is localized to capillary endothelial cells in the fetal mouse brain on E18.5 [19]. However, the developmental expression and function of *Abcg2*/BCRP1 in the fetal brain remain to be determined.

Nothing is known with regard to regulation of *Abcg2*/BCRP1 in normal fetal tissues. In the breast cancer cell line MCF-7/MX, dexamethasone (DEX) treatment decreased *Abcg2* mRNA and BCRP1 protein expression in a dose- and time-dependent manner [20, 21]. Furthermore, in isolated adult rat brain endothelial cells, DEX treatment increased BCRP1 activity and expression [22]. Clinically, synthetic glucocorticoids are prescribed to women at risk of premature delivery (approximately 10% of all pregnancies) to mature the fetal lungs and other organs [23, 24]. However, the consequences of

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synthetic glucocorticoid exposure on *Abcg2*/BCRP1 expression and function in the fetal brain are unknown.

In the present study, we hypothesized the following: 1) *Abcg2* mRNA and BCRP1 protein expression increases in the fetal brain with advancing gestation (from E15.5 to E18.5), and this corresponds to decreased accumulation of BCRP1 substrate in the fetal brain. 2) Maternal treatment with synthetic glucocorticoid (DEX) will up-regulate *Abcg2* mRNA and BCRP1 protein expression and function in the fetal brain.

MATERIALS AND METHODS

Female FVB mice (Charles River, Germantown, NY) were bred in our colony. Pregnancy was defined by the presence of a vaginal plug and was designated as E0.5 (average gestation period, approximately 19.5 days). All experiments were carried out in our facility at 0900 h. These studies were performed using protocols approved by the University of Toronto Animal Care Committee in accord with the Canadian Council for Animal Care.

In Vivo Drug Distribution Studies

Functional changes of fetal BBB Abcg2*/BCRP1 across gestation.* On either E15.5 (n = 4 dams) or E18.5 (n = 5 dams), pregnant FVB dams were injected (i.v.) with [^{3}H]mitoxantrone (prototypical substrate for assessing BCRP1 function; prepared with 5 mg/kg of unlabeled mitoxantrone [Sigma Chemical Co., St. Louis, MO] and 1 μCi/animal of [^{3}H]mitoxantrone [Moravek Biochemicals, Brea, CA]) and euthanized 30 min following the mitoxantrone injection using isoflurane (AErrane, USP; Baxter Corporation, Mississauga, ON, Canada) [2, 11, 25]. E15.5 and E18.5 were chosen to investigate *Abcg2*/BCRP1 expression and function in the fetal brain because they correspond to findings in our previous study [19] examining BCRP1 expression in the placenta, the primary barrier between maternal circulation and the fetus. Fetal brains and bodies (excluding the fetal head) were collected and stored at −80°C until further use. Homogenized whole fetal brain and body (200 μl) were dissolved in SOLVABLE (1 ml; PerkinElmer Inc., Boston, MA), and hydrogen peroxide (30%, 100 μl) was added to decolorize samples and optimize counting efficiency. Following addition of scintillation fluid (Ultima-Gold; PerkinElmer Inc.), radioactivity (disintegrations per minute [DPM]) in the fetal brain and body was determined on a Tri-Carb Beta-Counter (PerkinElmer Inc.). Levels of [^{3}H]mitoxantrone were standardized as a drug equivalent per weight of tissue. Drug distribution was expressed as the fetal brain tissue concentration:fetal body tissue concentration ratio, designated "drug ratio." [^{3}H]Mitoxantrone accumulation in the fetal body was used as an index of substrate availability for the fetal brain, as fetal blood collection is impossible in the mouse. Fetal body substrate accumulation may vary with fetal growth. However, accumulation of [^{3}H]mitoxantrone in the fetal body was standardized to weight, thus correcting for differences in fetal growth. Fetal tails were collected and stored at −20°C for sex determination.

Glucocorticoid regulation of Abcg2 *and BCRP1 expression and function.* Glucocorticoid regulation was examined in two groups of pregnant FVB mice. In the first group, pregnant dams were injected (s.c.) daily (at 0900 h) with either DEX (pharmacological doses of 0.1 mg/kg [n = 4 dams/group] or 1 mg/kg [n = 5 dams/group]) or vehicle (saline [n = 5 dams/group]) from E9.5 to E15.5 (midgestation). In the second group, pregnant dams were injected (s.c.) daily (at 0900 h) with DEX (0.1 mg/kg [n = 4 dams/group] or 1 mg/kg [n = 5 dams/group]) or vehicle (saline [n = 4 dams/group]) from E12.5 to E18.5 (late gestation). The dose of DEX utilized in clinical management of threatened preterm labor is 0.2 mg/kg [26]. Glucocorticoid treatment during the last week of gestation (from E12.5 to E18.5) has been used in murine models to mimic antenatal glucocorticoid treatment [27, 28]. We chose an equivalent treatment regimen (from E9.5 to E15.5) earlier in gestation based on the ontogenic profile and because fetal brain sensitivity to glucocorticoids is lower at this time [29]. On the last day (E15.5 or E18.5) at 2 h after final treatment of DEX or vehicle injection, [^{3}H]mitoxantrone (1 μCi/animal and 5 mg/kg of unlabeled mitoxantrone) was injected (i.v.), and dams were then euthanized 30 min later with isoflurane (AErrane, USP; Baxter Corporation). Maternal blood and fetal tissues were collected and processed as already described. Fetuses were separated by sex, and data were averaged per litter per treatment group for analysis. Averages of 3.0 and 4.4 male and female fetuses, respectively, were used per dam per treatment group for analysis of mitoxantrone transfer. Absolute (DPM/g) [^{3}H]mitoxantrone accumulation in the fetal body (excluding the head) did not significantly differ with treatment.

Sex Determination

DNA was extracted from fetal tails utilizing a REDExtract-N-AMP Tissue PCR kit (Sigma Chemical Co.), and PCR was performed to determine fetal sex using *Sry* forward (5′ TCATGAGACTGCCAACCACAG 3′) and *Sry* reverse (5′ CATGACCACCACCACCACCAA 3′) primers [30] according to the manufacturer's guidelines. Amplification product was detected by 1% gel electrophoresis.

Real-Time PCR

Total RNA was extracted from frozen brains (one male and one female brain were arbitrarily selected per litter per treatment group) using TRIzol (Invitrogen Canada Inc., Burlington, ON, Canada) per the manufacturer's instructions. Total RNA was subjected to reverse transcription using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) as we have described previously [31].

Real-time PCR was performed using FAM-labeled TaqMan gene expression assays for *Abcg2* mRNA and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) mRNA (Mm00496364_m1 and 4352932E; Applied Biosystems) and TaqMan Universal PCR mix (Applied Biosystems) [31]. *Abcg2* mRNA expression was quantified on a Chromo4 real-time PCR detector (Bio-Rad Laboratories, Hercules, CA) with activation at 50°C for 2 min and initial denaturation at 95°C for 10 min. Thirty-five cycles were then performed of denaturation at 95°C for 15 sec, annealing and extension at 60°C for 60 sec, and plate reading. All samples were run in triplicate. Data were analyzed using Opticon software (Bio-Rad Laboratories), and relative quantification was calculated using the ΔΔCT method with *Gapdh* as the endogenous control [32]. Selection of an appropriate endogenous control for mouse studies was performed by analyzing the expression of placental *Gapdh* and with treatment. Similar amplification efficiencies and threshold cycle (CT) values were obtained for *Gapdh* with treatment (vehicle versus DEX [data not shown]). The stability of *Gapdh* expression validated the use of this gene for normalization. Furthermore, similar amplification efficiencies were obtained for both the target gene (*Abcg2*) and our housekeeping gene (data not shown).

Western Blot Analysis

BCRP1 protein expression was assessed by Western blot analysis as described previously [19]. Briefly, frozen brain (one male and one female were arbitrarily selected per litter per treatment group [n = 4–5 litters/treatment]) was prepared for electrophoresis. Samples (50 μg of protein total) were subjected to SDS-PAGE electrophoresis (8% resolving gel) and transferred to a nitrocellulose membrane. Nitrocellulose membranes were blocked overnight at 4°C in skim milk (5% wt/vol of PBS with Tween 20/PBS-T). Membranes were washed with PBS-T and cut at the 50-kDa mark. The upper half was then incubated with BXP-21 mouse monoclonal antibody (1:250 dilution for 1 h at 23°C, catalog No. OP191; Calbiochem, La Jolla, CA), and the lower half was incubated with β-actin (1:5000 dilution, rabbit polyclonal A2066; Sigma Chemical Co.). The membranes were then washed and incubated with anti-mouse and anti-rabbit IgG, respectively (1:5000 dilution for 1 h at 23°C; NEN Life Science Products, Boston, MA), followed by Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Inc.). Bands were visualized by exposure to Kodak Blue X-OMAT film (PerkinElmer Inc.). The relative optical density of the bands was measured using computerized image analysis and was standardized against the β-actin signal (MCID TM Core 7.0; Imaging Research Inc., Interfocus Imaging Ltd., Cambridge, England). Western blot analysis was performed in duplicate for each fetal brain. The specificity of the antibody that detects the 72-kDa band has previously been confirmed in our laboratory [19].

Statistical Analysis

Group data are presented as the mean ± SEM and are analyzed using Prism (GraphPad Software Inc., San Diego, CA). *Abcg2* mRNA expression was normalized to the endogenous control *Gapdh* mRNA. Analysis was undertaken on data obtained from sex-separated fetal brains averaged per dam. For comparison of treatment with vehicle, data were expressed as percentage vehicle and then $\log_{10}$ transformed. One-sample *t*-test against a hypothetical value of 2 ($\log_{100} = 2$ represents vehicle value) was performed, followed by Holm modification of Bonferroni correction [33]. For comparison of treatment with vehicle drug ratios, treatment was normalized to corresponding control as the percentage vehicle mean. Data were $\log_{10}$ transformed and analyzed with one-sample *t*-test for differences between treatment and vehicle. Unpaired Student *t*-test for all column combinations using Holm modification of Bonferroni correction to control for multiplicity was then performed. Two-way ANOVA was utilized to determine differences among treatment doses, sex, and gestational age, and where significance was demonstrated, post hoc analysis was undertaken using Student *t*-test for all column combinations using Holm modification of Bonferroni correction. Significance was set at $P < 0.05$.

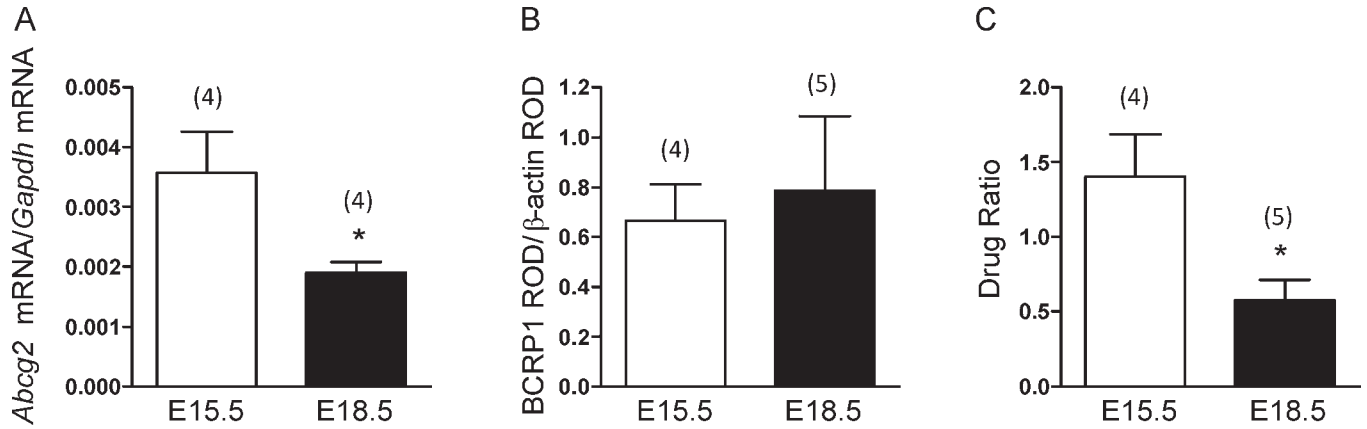

FIG. 1. Developmental expression of fetal brain (**A**) *Abcg2* mRNA on E15.5 and E18.5. The error bar represents the mean ± SEM expressed as relative units of mRNA standardized against *Gapdh*. **B**) Relative levels of BCRP1 protein expressed on E15.5 and E18.5. The error bar represents the mean ± SEM BCRP1:β-actin ratio. **C**) Function of fetal brain BCRP1 on E15.5 and E18.5 determined by accumulation of transporter-specific substrate and accumulation of [^{3}H]mitoxantrone (5 mg/kg) in whole fetal brain on E15.5 and E18.5. The error bar represents the mean ± SEM drug ratio (DPM/g fetal brain:DPM/g fetal body). The number in parentheses represents the number of dams in each group. *$P < 0.05$ versus E15.5. ROD indicates relative optical density.

RESULTS

Development of Abcg2/*BCRP1 Expression and Function in the Fetal Brain*

There were no sex differences in the expression of *Abcg2* mRNA or BCRP1 protein or in function with advancing gestation. As such, data were combined from male and female fetal brains for the developmental assessment of expression and function. *Abcg2* mRNA significantly decreased on E18.5 compared with E15.5 ($P < 0.05$) (Fig. 1A). However, there was no corresponding decrease in BCRP1 protein (Fig. 1B). [^{3}H]Mitoxantrone accumulation in the fetal brain significantly decreased with advancing gestation ($P < 0.05$) (Fig. 1C), indicating increased BCRP1 function with advancing gestation in the fetal brain.

Glucocorticoid Regulation

Two-way ANOVA revealed a significant interaction between treatment and gestational age in the expression of *Abcg2* mRNA in the male brain ($P = 0.0005$). Subsequent post hoc analysis revealed a significant dose-dependent effect of DEX on *Abcg2* mRNA expression on E15.5 and E18.5 ($P < 0.05$). Furthermore, a significant age-dependent effect was observed for high-dose DEX (1 mg/kg) in the male brain ($P < 0.01$) between E15.5 and E18.5. In the female brain, two-way ANOVA also revealed a significant interaction between treatment and gestational age ($P = 0.0018$). Subsequent post hoc analysis demonstrated a significant effect of treatment on E18.5 ($P < 0.001$). Furthermore, there was a significant effect of gestational age on *Abcg2* mRNA expression with low-dose DEX (0.1 mg/kg) ($P < 0.05$).

In the male fetal brain, low-dose DEX (0.1 mg/kg) treatment significantly increased *Abcg2* mRNA expression on both E15.5 and E18.5 ($P < 0.001$ and $P < 0.01$, respectively) (Fig. 2). At E15.5, high-dose DEX increased *Abcg2* mRNA ($P < 0.001$), whereas at E18.5, the same dose of DEX had no effect on *Abcg2* expression (Fig. 2). In the female fetal brain, low-dose DEX (0.1 mg/kg) treatment during midgestation (between E9.5 and E15.5) had no significant effect on *Abcg2* mRNA at E15.5 (Fig. 2A) but on E18.5 resulted in a significant increase in *Abcg2* mRNA ($P < 0.001$) (Fig. 2B). In contrast, high-dose DEX (1 mg/kg) treatment during midgestation (between E9.5 and E15.5) and late gestation significantly decreased *Abcg2* mRNA on E15.5 and E18.5 ($P < 0.05$) in the female fetal brain (Fig. 2).

Glucocorticoid treatment during midgestation and late gestation had no significant effect on BCRP1 protein expression in the fetal brain in either sex (Fig. 3). Furthermore, there were no significant effects of glucocorticoid treatment on BCRP1 function in the fetal brain. Accumulation of [^{3}H]mitoxantrone in the brains of fetuses exposed to DEX did not differ significantly from those exposed to vehicle at either age (Fig. 4).

DISCUSSION

The present study demonstrates a significant developmental reduction in fetal mouse brain *Abcg2* mRNA expression, with no corresponding reduction in BCRP1 protein expression. Furthermore, there was a substantial decrease in BCRP1 substrate accumulation in the fetal brain, suggesting an increase in that BCRP1 efflux capacity in late gestation. Glucocorticoids appear to regulate *Abcg2* mRNA in the fetal brain in a dose-, age-, and sex-dependent manner. However, there were no changes in BCRP1 protein or activity. Together, these data suggest that there is a substantial disconnect between *Abcg2* gene regulation and BCRP1 function during development.

In the present study, BCRP1 protein levels remained unchanged in late gestation, despite the decrease in *Abcg2* mRNA. Cygalova and colleagues [3] reported a similar decrease in fetal rat brain *Abcg2* mRNA on E21 compared with E18; this group did not measure BCRP1 protein. To our knowledge, no other studies have quantified BCRP1 protein expression in the fetal brain. However, several studies [19, 34–36] have demonstrated that levels of *Abcg2* mRNA and BCRP1 protein do not correlate in BeWo cells and placenta. The disparity in expression may be potentially attributed to altered posttranscriptional processing, mRNA stability, or delayed protein synthesis. A limited relationship between total protein and active protein has been previously reported [37]. In most studies, including the present study, protein is measured from total tissue extract. However, functional BCRP1 protein is localized to the membrane, and as such, protein measurements from the membrane fraction would provide a more accurate index of active protein. Further studies are required to

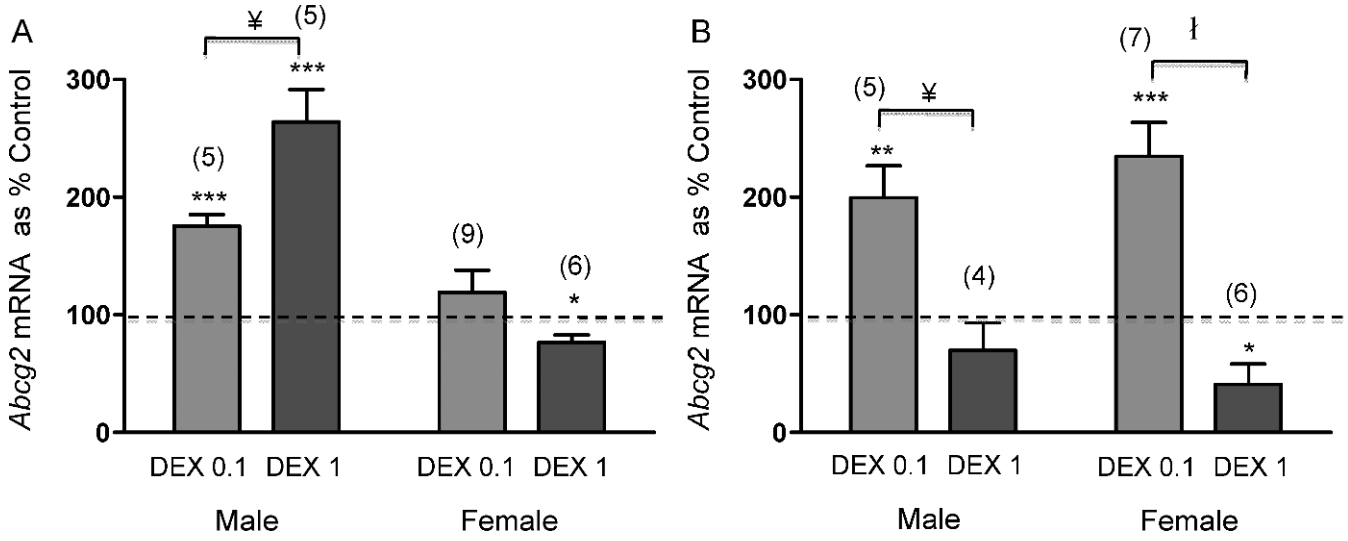

FIG. 2. *Abcg2* mRNA on E15.5 (**A**) and E18.5 (**B**) from male and female brains following DEX treatment (0.1 and 1 mg/kg). The error bar represents the mean ± SEM expressed as relative units of mRNA standardized against *Gapdh*. Treatment data were then normalized to respective vehicle and presented as percentage vehicle. The dotted line represents the control. The number in parentheses represents the number of dams per treatment group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control, two-way ANOVA, followed by post hoc analysis comparing dose. $¥P < 0.05$, $łP < 0.001$.

determine the relationship between total cellular BCRP1 and that present in the membrane fraction.

The functional role of *Abcg2*/BCRP1 in the adult BBB remains somewhat controversial [4, 11, 38, 39]. Using *Abcb1a* knockout mice, Cisternino et al. [11] demonstrated reduced brain accumulation of mitoxantrone and prazosin when BCRP1 was inhibited with GF120918. However, utilizing a similar protocol, another study [38] suggested that the functional role

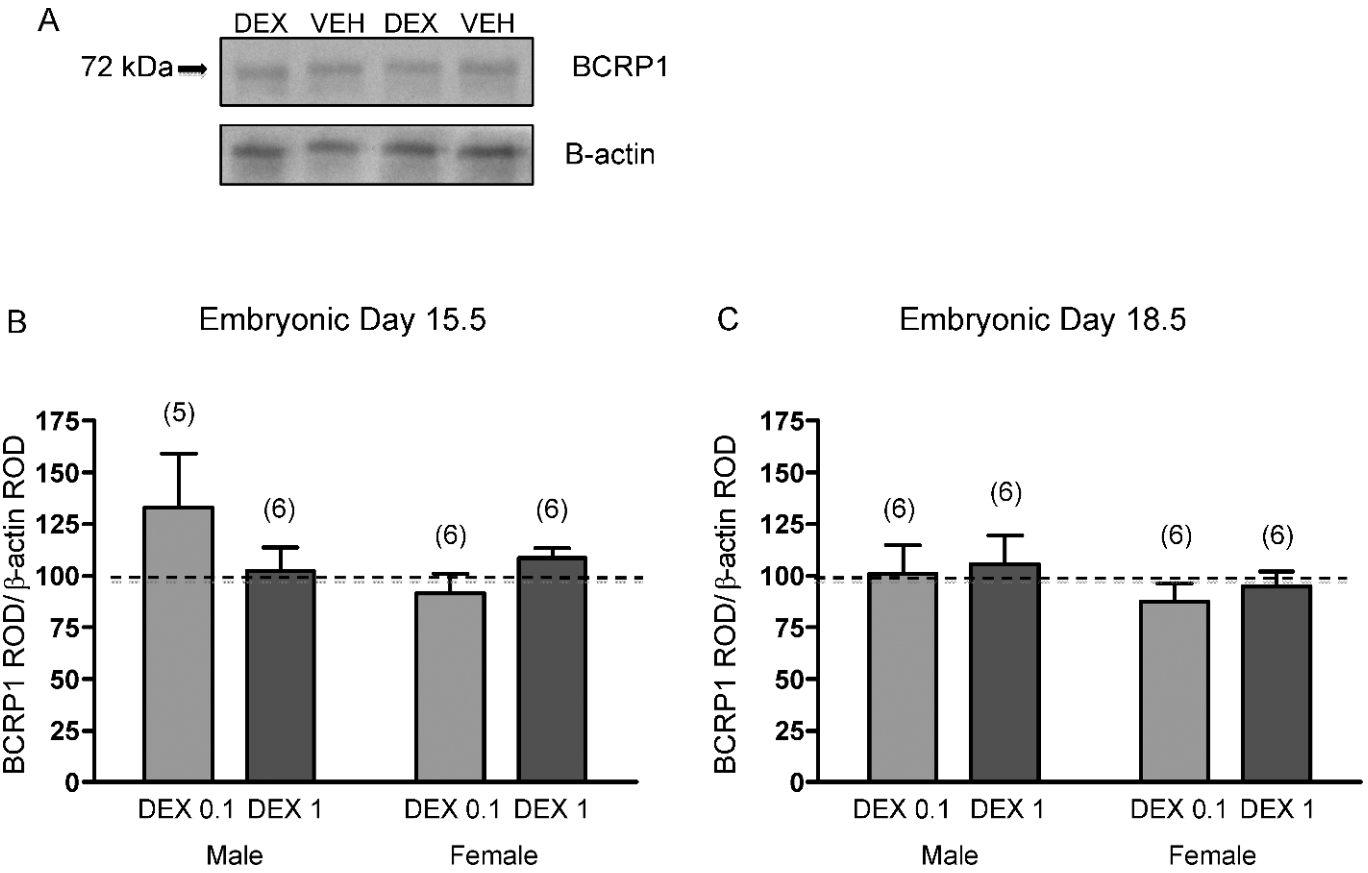

FIG. 3. **A**) Representative immunoblot of fetal brain BCRP1 protein following treatment with DEX (0.1 mg/kg) or vehicle (VEH). BCRP1 protein on E15.5 (**B**) and E18.5 (**C**) from male and female brains following DEX treatment (0.1 mg/kg [DEX 0.1] or 1 mg/kg [DEX 1]). The error bar represents the mean ± SEM expressed as relative optical density (ROD) of BCRP1 standardized against β-actin ROD. Treatment data were then normalized to respective vehicle. The dotted line represents the control. The number in parentheses represents the number of dams per treatment group.

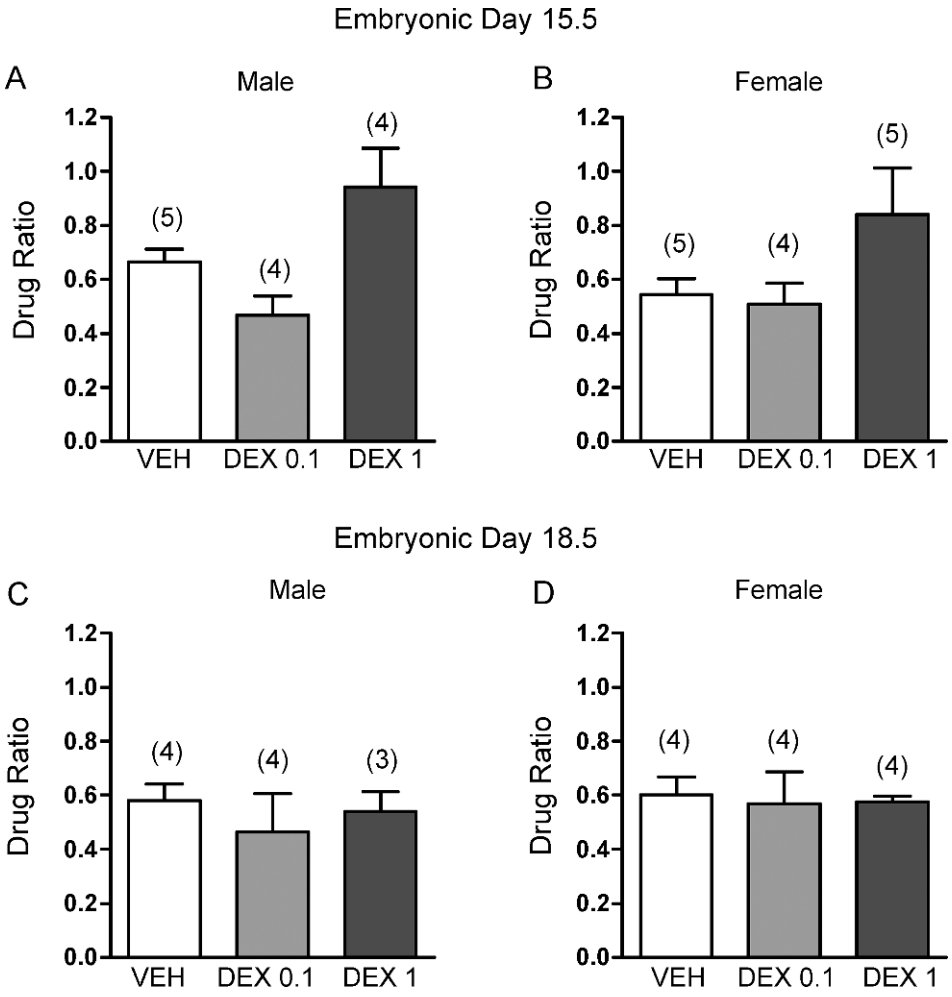

FIG. 4. [^{3}H]Mitoxantrone (5 mg/kg) accumulation in the fetal brain in males (**A**, **C**) and females (**B**, **D**). **A**, **B**) Embryonic day (E) 15.5 after daily injections of dexamethasone (0.1 mg/kg; DEX 0.1 or 1 mg/kg; DEX 1) or vehicle (VEH) from E9.5-E15.5. **C**, **D**) E18.5 after daily injections with DEX (0.1 or 1 mg/kg) or vehicle from E12.5 to E18.5. The error bar represents the group mean ± SEM drug ratio (DPM/g fetal brain:DPM/g fetal body). The number in parentheses represents number of dams per treatment group.

of BCRP1 is minimal at the BBB. Knowledge pertaining to the functional role of BCRP1 in the fetal brain is even more limited. Fetal brain:body ratios of genistein concentrations (BCRP1 substrate) increased 1.4-fold in BCRP1 knockout fetus compared with wild type [4]. To date, one other study [3] has investigated BCRP1 function in the fetal rat brain during development. [^{3}H]Cimetidine (BCRP1 substrate) accumulation was shown to significantly decrease (2.3-fold) in the fetal brain on E21 (term) compared with E18 (preterm), while accumulation in the fetal body was unchanged, suggesting an increase in BCRP1-mediated BBB function with advancing gestation. We now report a similar finding in the fetal mouse brain between E15.5 (midgestation) and E18.5 (late gestation). In both studies, *Abcg2* mRNA decreased with development, demonstrating a disparity between expression and function. Given that different substrates were utilized to assess BCRP1 function, it is unlikely that this discrepancy between expression and function is due to substrate pharmacokinetics or the involvement of other transporters. Moreover, a functional BBB has been confirmed as early as E11 in the fetal mouse by the presence of tight junctions and high electrical resistance [40–42].

In addition to the BBB, the blood-cerebral-spinal fluid barrier (BCSFB), composed of capillary epithelial cells of the choroid plexus, limits entry of substrates from the cerebrospinal fluid into the brain [43]. In the choroid plexus, BCRP1 is localized to the basolateral membranes, suggesting a role in neuroprotection [18]. Recently, it has been shown in the fetal rat brain that *Abcg2* mRNA expression in the lateral and fourth choroid plexus peaks on E15.5 (earliest time in gestation measured) but then decreases to term [18]. The functional significance of *Abcg2*/BCRP1 in the BCSFB is not understood. In the fetal mouse, determination of *Abcg2*/BCRP1 expression and function specifically in the BBB is difficult, as isolation of brain microvessels is impossible. As such, our studies were conducted on whole fetal brain, and measurements of *Abcg2*/BCRP1 expression and function relate to both the BBB and BCSFB; nonetheless, both barriers are suggested to contribute to neuroprotection. We propose that the disconnect between expression and function may relate to an interplay between BCRP1 function at the BBB and the BCSFB. Relative changes in either expression or function within these two barriers with advancing gestation could contribute to a net decrease in fetal brain exposure of xenobiotics. Studies examining the functional significance of *Abcg2*/BCRP1 during development in the BCSFB have not been undertaken to date. Clearly, further studies are required to investigate the relative role of BCRP1 in both the BBB and the BCSFB in adult and fetal brains.

While BCRP1 has been shown to limit the penetration of therapeutic agents into the fetal brain, there is no information concerning potential involvement of BCRP1 in normal physiological function. Dehydroepiandrosterone sulfate (DHEAS), an important precursor for estrogen synthesis, and estrone-3-sulfate (E3S), a metabolite of estrogens, are both substrates of *Abcg2*/BCRP1 [44], suggesting that *Abcg2*/BCRP1 may be involved in the elimination of these sulfated conjugates from the fetal brain. Given that *Abcg2*/BCRP1 may alter the dynamics of DHEAS and E3S in other tissues

(placenta) [44], it is logical to assume that *Abcg2*/BCRP1 may regulate estrogen dynamics in the fetal brain. The physiological significance of *Abcg2*/BCRP1 during fetal brain development remains to be elucidated.

In the present study, fetal glucocorticoid exposure resulted in dose-, age-, and sex-dependent changes in *Abcg2* mRNA. Glucocorticoids are known to exhibit biphasic effects [45]. Furthermore, dose-dependent regulation of ATP-binding cassette (ABC) transporters with DEX has been previously demonstrated in the placenta, fetal brain, liver, and small intestine [46–49]. Studies [22, 50–54] have shown that DEX activates an intracellular network of nuclear transporters, including the glucocorticoid receptor (GR) and pregnane xenobiotic transporter (PXR). The GR and PXR are expressed in endothelial cells of brain microvessels [50, 55], providing evidence for the potential involvement of the GR and PXR pathways in the regulation of *Abcg2*/BCRP1 in endothelial cells. Recently, both GR and PXR have been implicated in the DEX-mediated regulation of brain capillary *Abcg2*/BCRP1 expression using isolated endothelial cells from adult rat brain [22]. Synthetic glucocorticoids have been shown to stimulate *CYP3A4* gene expression in vitro through two different pathways depending on the dose utilized. At lower doses of DEX (100 nM), *CYP3A4* gene expression was modulated by GR-mediated up-regulation of PXR, whereas at higher doses (50 μM), DEX directly activated PXR [52]. Regulation of *Abcg2*/BCRP1 by glucocorticoids likely involves cross talk between GR and PXR.

While there were no sex differences in the expression of *Abcg2*/BCRP1 in the fetal brain during development, effects of fetal glucocorticoid exposure were highly sex dependent. Overall, glucocorticoid treatment significantly increased *Abcg2* mRNA expression in male brains and significantly decreased expression in female brains. To our knowledge, this is the first study to show a sexually dimorphic effect of glucocorticoid on *Abcg2* mRNA expression. A similar phenomenon has been previously demonstrated for the liver drug-metabolizing enzyme CYP3A4 [56, 57], where glucocorticoid treatment increased *CYP3A4* expression in males but decreased expression in females [56, 57]. Further studies are required to investigate the physiological significance of this sexual dimorphic regulation of *Abcg2* mRNA in late gestation.

The effects of glucocorticoids on BCRP1 function in the brain have not been previously investigated to our knowledge. The present study demonstrates little effect of DEX on BCRP1 function in the fetal brain. Approximately 10% of pregnant women are treated with synthetic glucocorticoid for threatened preterm labor. Preterm labor is difficult to diagnose; as such, pregnant women until recently often received repeat treatments with synthetic glucocorticoid [58]. Given the potential protective function of BCRP1 in the fetal brain, the lack of effect of DEX may be reassuring with respect to the clinical use of glucocorticoids in the management of preterm labor.

In late gestation, there is an exponential increase in endogenous glucocorticoid (cortisol in the human and corticosterone in the rodent) in maternal and fetal circulation [59–62]. Given the lack of effect of DEX on BCRP1-mediated protection in the fetal brain, our studies also suggest that the late gestational rise in maternal and fetal glucocorticoid is unlikely to represent a major factor in mediating the profound increase in BCRP1 activity that occurs in the fetal brain in late gestation.

In conclusion, BCRP1 function increases with advancing gestation in the fetal brain, likely in preparation for life ex utero. The present study also demonstrates that synthetic glucocorticoid treatment can affect *Abcg2* mRNA in a dose-, age-, and sex-dependent manner, with no corresponding change in total cellular BCRP1 protein. Furthermore, treatment with glucocorticoids does not alter BCRP1-mediated exclusion of xenobiotics from the fetal brain. The latter is reassuring given the extensive use of synthetic glucocorticoid for the management of preterm labor. The present study also suggests that the natural rise in endogenous glucocorticoids that occurs in the fetal circulation in late gestation is unlikely responsible for the dramatic rise in fetal brain BCRP1 activity observed in late gestation. The mechanisms of *Abcg2*/BCRP1 regulation in the fetal brain are complex and require further investigation. Understanding the regulation of *Abcg2*/BCRP1 expression and function in the fetal brain during normal development and under pathological conditions is critical for maintaining fetal brain health, particularly when therapeutic strategies are utilized in pregnancy.

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