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

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Abcb2/BCRP1 mediated protection increases in the fetal brain with advancing gestation and Abcg2 mRNA is significantly regulated by synthetic glucocorticoid in a dose-, sex- and age-dependent manner.

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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 not known. Synthetic glucocorticoids (i.e. 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 that: 1) Abcg2 mRNA and BCRP1 protein expression increases with development (E15.5 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 Embryonic Day (E) 15.5 or E18.5 and fetal brains were collected and analyzed for [3H]mitoxantrone (BCRP1 specific substrate) accumulation and Abcg2/BCRP1 expression. In another six groups (n=4-5/gp), pregnant mice were treated with DEX (0.1m/kg or 1mg/kg) or vehicle (saline) from either E9.5-E15.5 (mid-gestation) or E12.5-E18.5 (late-gestation) and then injected with [3H]mitoxantrone. In conclusion, Abcg2 mRNA expression significantly decreases, while BCRP1-mediated neuroprotection increases with advancing gestation. Further, 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.
INTRODUCTION

Breast cancer-resistance protein (BCRP1; encoded by the Abcg2 gene) was first identified in MCF-7 cells, a human breast carcinoma sub-line, 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 H2-receptor antagonist), and glyburide (anti-diabetic) ([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; 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 plays 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, fetal brain-to-body ratio of genistein (phytoestrogen; BCRP1 substrate) increased 1.4 fold in Abcg2/BCRP1 knockout mice compared to wild-type at two weeks gestation [4]; suggesting that BCRP1 in the fetal brain plays 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 the brain development is not linear [12, 13]. In general, the first and second half of gestation and the 1st 7 days after birth in the mouse brain are considered to be equivalent to the first, second and third trimester respectively in the human fetal brain [14-16]. BCRP1 expression has been reported as early as 22 wks of gestation (human) and E13 (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 regards to regulation of Abcg2/BCRP1 in normal fetal tissues. In the breast cancer cell line, MCF-7/MX cells, dexamethasone (DEX) treatment decreased Abcg2 mRNA and BCRP1 protein expression in a dose- and time-dependent manner [20, 21]. Further, 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 (~10% of all pregnancies) to mature the fetal lungs and other organs [23, 24]. However, the consequences of synthetic glucocorticoid exposure on Abcg2/BCRP1 expression and function in the fetal brain are not known.

In the present study, we hypothesized that 1) Abcg2 mRNA and BCRP1 protein expression increases in the fetal brain with advancing gestation (E15.5 to E18.5) and that this corresponds to decreased accumulation of BCRP1 substrate into 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 after presence of vaginal plug and designated as embryonic (E) 0.5 (average gestation period ~ 19.5 days). All experiments were carried out in our facility at 0900h. These studies were performed using protocols approved by the Animal Care Committee at the University of Toronto and in accordance 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\text{H}\text{]mitoxantrone}\) (prototypical substrate for assessing BCRP1 function; 5mg/kg; Sigma Chemical Co. St. Louis, Missouri, USA and 1uCi/animal; Moravek Biochemicals, Brea, California, USA) 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, as they correspond to our previous study examining BCRP1 expression in the placenta [19], 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\mu l) were dissolved in SOLVABLE (1ml; PerkinElmer Inc., Boston, Massachusetts, USA) and hydrogen peroxide (30%; 100\mu l) was added to decolorize samples and optimize counting efficiency. Following addition of scintillation fluid (Ultima-Gold, PerkinElmer Inc., Boston, Massachusetts, USA), radioactivity (DPM) in the fetal brain and body was determined on a Tri-Carb Beta-Counter (PerkinElmer Inc., Boston, Massachusetts, USA). Levels of \[^3\text{H}\text{]mitoxantrone}\) were standardized as a drug equivalent per weight of tissue. Drug distribution was expressed as the ratio of fetal brain tissue concentration to fetal body tissue concentration; designated ‘drug ratio’. \[^3\text{H}\text{]Mitoxantrone}\) accumulation in the fetal body was used as an index of substrate availability for the fetal brain, as fetal blood collection is not possible in the mouse. Fetal body substrate accumulation may vary with fetal growth. However, accumulation of \[^3\text{H}\text{]mitoxantrone}\) in the fetal body was standardized to weight, thus correcting for differences in fetal growth. Fetal tails were collected and stored at -20ºC 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 (0900h) with either DEX (pharmacological doses of 0.1mg/kg; n=4 dams/gp or 1mg/kg; n=5 dams/gp) or vehicle (saline; n=5 dams/gp) from embryonic day (E) 9.5 to E15.5 (mid-gestation). In the second group, pregnant dams were injected (s.c.) daily (0900h) with DEX (0.1mg/kg; n=4 dams/gp or 1mg/kg; n=5 dams/gp or vehicle (saline; n= 4 dams/gp) from E12.5 to E18.5 (late-gestation). The dose of dexamethasone utilized in clinical management of threatened preterm labor is 0.2mg/kg [26]. Glucocorticoid treatment during the last week of gestation (E12.5-E18.5) has been used in murine models to mimic antenatal glucocorticoid treatment [27, 28]. We chose an equivalent treatment regimen (E9.5-E15.5) earlier in gestation based on the ontogenic profile and since fetal brain sensitivity to glucocorticoids is lower at this time [29]. On the last day (E15.5 or E18.5),
2h post final treatment of DEX or vehicle injection, $[^3]$Hmitoxantrone (5mg/kg and 1uCi/animal) was injected (i.v.) and dams were then euthanized 30 min later with isoflurane (AErrane, USP; Baxter Corportation, Mississauga, ON, Cananda). Maternal blood and fetal tissues were collected and processed as described above. Fetuses were separated by sex and data was averaged per litter per treatment group for analysis. An average 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) $[^3]$Hmitoxantrone accumulation in the fetal body (body excluding head) did not significantly differ with treatment.

**Sex Determination**
DNA was extracted from fetal tails utilizing Sigma REDExtract-N-AMP Tissue PCR Kit (XNAT), and PCR was performed to determine fetal sex using Sry forward: 5' TCATGAGACTGCAACCACAG 3'; Sry reverse: 5' CATGACCACCACCACCAAA 3' primers [30] according to manufacturer’s guidelines (Sigma Chemical Co. St Louis, MO). 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 as per manufacturer’s instructions (Invitrogen Canada Inc, Burlington, Ontario, Canada). Total RNA was subjected to reverse-transcription using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, California), as we have described previously [31].

Real-time PCR was performed using the FAM-labeled TaqMan gene expression assays for Abcg2 mRNA and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) mRNA (Mm00496364_m1 and 4352932E; Applied Biosystems, Foster City, CA) and the TaqMan Universal PCR Mix (Applied Biosystems) [31]. Abcg2 mRNA expression was quantified on a Chromo4 real-time PCR detector (Bio-Rad Laboratories) with the following cycling conditions: activation at 50ºC for 2 min, initial denaturation at 95ºC for 10 min. 35 cycles were then performed of denaturation at 95ºC for 15s, annealing and extension at 60ºC for 60s, plate read. All samples were run in triplicate. Data were analyzed using Opticon software (Bio-Rad Laboratories) and relative quantification was calculated $\Delta \Delta CT$ 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 CT values were obtained for Gapdh with treatment (vehicle versus dexamethasone; data not shown). The stability of Gapdh expression validated the use of this gene for normalization. Further, similar amplification efficiencies were obtained for both the target gene (Abcg2) and our house-keeping gene (data not shown).

**Western Blot Analysis**
BCRP1 protein expression was assessed by Western blotting, as described previously [19]. Briefly, frozen brain (one male and one female were arbitrarily selected per litter per treatment group; 4-5 litters/ treatment) was prepared for electrophoresis. Samples (50µg protein total) were subjected to SDS-PAGE electrophoresis (8% resolving gel) and transferred to a nitrocellulose membrane. Nitrocellulose membranes were blocked overnight (4ºC) in skim milk (5% wt/vol of PBS with Tween 20; PBS-T). Membranes were washed with PBS-T and cut at the
50kDa mark. The upper half was then incubated with BXP-21 mouse monoclonal antibody (1:250 dilution, 1h, 23°C; Calbiochem, Cat# OP191; La Jolla, California) and the lower half incubated with β-actin (β-actin; 1:5000 dilution, rabbit polyclonal A2066; Sigma Chemical Co. St Louis, MO). The membranes were then washed and incubated with anti-mouse or anti-rabbit IgG respectively (1:5000 dilution, 1h, 23°C; NEN, Boston, USA) followed by Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer). Bands were visualized by exposure to Kodak Blue X-OMAT film (Perkin Elmer). The relative optical density (ROD) 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, UK). Western analysis was performed in duplicates for each fetal brain. The specificity of the antibody which detects the 72 kDa band has previously been confirmed in our laboratory [19].

Statistical Analysis
Group data are presented as means ± standard error of the mean (SEM) and analyzed using Prism (GraphPad Software Inc., San Diego, California, USA). Abcg2 mRNA expression was normalized to endogenous control, Gapdh mRNA. Analysis was undertaken on data obtained from sex separated fetal brains averaged per dam. For comparison of treatment to vehicle, data was expressed as percent vehicle and then Log_{10} transformed. One sample t-tests against a hypothetical value of 2 (log (100)= 2, represents vehicle value) was performed followed by the Holm modification of the Bonferroni correction [33]. For comparison of treatment to vehicle ‘drug ratios’, treatment was normalized to corresponding control as percent vehicle mean. Data was Log_{10} transformed and analyzed with one sample t-test for differences between treatment and vehicle. Unpaired Student’s t-test for all column combinations, using the Holm modification of the Bonferroni correction to control for multiplicity was then performed. Two-way ANOVA was utilized to determine differences between treatment doses, sex and gestational age and where significance was demonstrated, post-hoc analysis was undertaken using Student’s t-test for all column combinations, using the Holm modification of the Bonferroni correction. Significance was set at P<0.05.

RESULTS
Development of Abcg2/BCRP1 Expression and Function in the Fetal Brain
There were no sex differences in the expression of Abcg2 mRNA, BCRP1 protein or 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 when compared to E15.5 (Fig. 1A; P<0.05). However, there was no corresponding decrease in BCRP1 protein (Fig. 1B). [\textsuperscript{3}H]Mitoxantrone accumulation in the fetal brain significantly decreased with advancing gestation (Fig. 1C; P<0.05), 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). Further, a significant age-dependent effect was observed for high-dose DEX (1mg/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). Further, there was a significant effect of gestational-age on Abcg2 mRNA expression with low-dose DEX (0.1mg/kg) (P<0.05).

In the male brain, low-dose DEX (0.1mg/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 (0.1mg/kg) treatment during mid-gestation (E9.5-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 (1mg/kg) treatment during mid-gestation (E9.5-E15.5) and late gestation significantly decreased Abcg2 mRNA on E15.5 and E18.5 (P<0.05; Fig. 2) in the female fetal brain.

Glucocorticoid treatment during mid-gestation and late gestation had no significant effect on BCRP1 protein expression in the fetal brain in either sex (Fig. 3). Further, there were no significant effects of glucocorticoid treatment on BCRP1 function in the fetal brain. Accumulation of [3H]mitoxantrone in the brains of fetuses exposed to DEX did not differ significantly from 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 reported a similar decrease in fetal rat brain Abcg2 mRNA on E21 when compared to E18; this group did not measure BCRP1 protein [3]. To our knowledge, no other studies have quantified BCRP1 protein expression in the fetal brain. However, a number of studies have demonstrated that levels of Abcg2 mRNA and protein do not correlate in BeWo cells and placenta [19, 34-36]. The disparity in expression may be potentially attributed to either altered post-transcriptional processing, mRNA stability or delayed protein synthesis. A limited relationship between protein and active protein has been previously reported [37]. In the majority of 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 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., demonstrated reduced brain accumulation of mitoxantrone and prazosin when BCRP1 was inhibited with GF120918 [11].
However, utilizing a similar protocol, another study suggested that the functional role of BCRP1 is minimal at the BBB [38]. Knowledge pertaining to the functional role of BCRP1 in the fetal brain is even more limited. Fetal brain-to-body ratios of genistein concentrations (BCRP1 substrate) increased 1.4 fold in BCRP1 knockout fetus compared to wild-type [4]. To date, one other study 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 to E18 (preterm), while accumulation in the fetal body was unchanged, suggesting an increase in BCRP1 mediated BBB function with advancing gestation [3]. We now report a similar finding in the fetal mouse brain between E15.5 (mid-gestation) and E18.5 (late-gestation). Interestingly, 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), comprised of capillary epithelial cells of the choroid plexus, limits entry of substrates from the CSF 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 4th 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 microvessel is not possible. 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 attribute 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. Clearly, further studies are required to investigate the relative role of BCRP1 in both the BBB and the BCSFB in both 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-dependant changes in Abcg2 mRNA. Glucocorticoids are known to exhibit biphasic effects [45]. Further,
dose-dependent regulation of ABC transporters with DEX has been previously demonstrated in the placenta, fetal brain, liver and small intestine [46-49]. Studies have shown that DEX activates an intracellular network of nuclear transporters including, the glucocorticoid receptor (GR) and pregnane xenobiotic transporter (PXR) [22, 50-54]. 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 (100nM), 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 amongst 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-dependant. 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. 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, until recently pregnant women 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 labour.

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 effect Abcg2 mRNA in a dose-, age- and sex-dependent manner; with no corresponding change in total cellular BCRP1 protein. Further, 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 labour. The present study also suggests that the natural rise in endogenous glucocorticoids that
occur in the fetal circulation in late gestation are 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 fetal brain health, particularly when therapeutic strategies are utilized in pregnancy.

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REFERENCES


FIGURE LEGENDS

Figure 1. Developmental expression of fetal brain (A) 

Abcg2 mRNA on embryonic day (E) 15.5 and E18.5. Bar represents mean ± SEM expressed as relative units of mRNA standardized against Gapdh. B) Relative levels of BCRP1 protein expressed on E15.5 and E18.5. Each bar represents mean ± SEM BCRP1/GNB ratio. Representative Western blot of BCRP1 and GNB protein is shown in inset. (C) Function of fetal brain BCRP1 on embryonic day (E) 15.5 and E18.5, determined by accumulation of transport specific substrate. Accumulation of [3H]mitoxantrone (5mg/kg) in whole fetal brain on E15.5 and E18.5. Values are expressed as male and female ‘drug ratios’ averaged per dam. Bar represents mean ± SEM drug ratio (DPM/g fetal brain: DPM/g fetal body). The number (n) in parentheses represents the number of dams in each group. *P<0.05 vs. E15.5.
**Figure 2.** *Abcg2* mRNA on (A) embryonic day (E) 15.5 and (B) E18.5 from male and female brains following dexamethasone (DEX) treatment (0.1mg/kg and 1mg/kg). Bar represents mean ± SEM expressed as relative units of mRNA standardized against *Gapdh*. Treatment data was then normalized to respective vehicle and presented as percent vehicle. Dotted line represents control. Number (n) in parentheses above bar represents number of dams per treatment group. *P<0.05, **P<0.01, ***P<0.001 vs. control; Two-way ANOVA followed by post-hoc analysis comparing dose.

**Figure 3.** (A) Representative immunoblot of fetal brain BCRP1 protein following treatment with either dexamethasone (DEX; 0.1mg/kg) or vehicle (VEH). BCRP1 protein on (B) embryonic day (E) 15.5 and (C) E18.5 from male and female placentas following DEX treatment (0.1mg/kg; DEX 0.1 or 1mg/kg; DEX 1). Bar represents mean ± SEM expressed as relative optical density (ROD) of BCRP1 standardized against β-actin ROD. Treatment data was then normalized to respective vehicle. Dotted line represents control. Number (n) in parentheses above bar represents number of dams per treatment group.

**Figure 4.** [3H]Mitoxantrone (5mg/kg) accumulation in the ‘fetal-unit’ on (A) embryonic day (E) 15.5 after daily injections of dexamethasone (0.1mg/kg; DEX 0.1 or 1mg/kg; DEX 1) or vehicle (VEH) from E9.5-E15.5. (B) E18.5 after daily injections with dexamethasone (0.1mg/kg or 1mg/kg) or vehicle from E12.5-E18.5. Bar represents group mean ± SEM drug ratio (DPM/g fetal brain: DPM/g fetal body). Since no sex differences were observed, male and female brain-to-body accumulation was combined per litter. Number (n) in parentheses above bar represents number of dams per treatment group.
**Figure 1**

A) Abcg2 mRNA/Gapdh mRNA

B) BCRP1 ROD/β-actin ROD

C) Drug Ratio

* denotes statistical significance.
Figure 2

Abcg2 mRNA as % Control

A

Male

DEX 0.1
DEX 1

Female

DEX 0.1
DEX 1

B

Male

DEX 0.1
DEX 1

Female

DEX 0.1
DEX 1

Note: The symbols ¥, ¥, ¥, *, **, *** indicate statistical significance.
Figure 3

A

72 kDa

BCRP1

B-actin

B

Embryonic Day 15.5

DEX 0.1  DEX 1

Male

Female

(C)

Embryonic Day 18.5

DEX 0.1  DEX 1

Male

Female

(#5) #6 #6

(6) (6) (6) (6) (6) (6)
Figure 4

Embryonic Day 15.5

A  Male  B  Female

Drug Ratio

VEH  DEX 0.1  DEX 1  VEH  DEX 0.1  DEX 1

(5)  (4)  (4)

Embryonic Day 18.5

C  Male  D  Female

Drug Ratio

VEH  DEX 0.1  DEX 1  VEH  DEX 0.1  DEX 1

(4)  (4)  (3)  (4)  (4)  (4)