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RESEARCH ARTICLE

Fetal glucocorticoid exposure leads to sex-specific changes in drug-transporter function at the blood-brain barrier in juvenile guinea pigs

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Abstract

Antenatal synthetic glucocorticoids (sGCs) are a life-saving treatment in managing pre-term birth. However, off-target effects of sGCs can impact blood-brain barrier (BBB) drug transporters essential for fetal brain protection, including P-glycoprotein (P-gp/*Abcb1*) and breast cancer resistance protein (BCRP/*Abcg2*). We hypothesized that maternal antenatal sGC treatment modifies BBB function in juvenile offspring in a sex-dependent manner. Thus, the objective of this study was to determine the long-term impact of a single or multiple courses of betamethasone on P-gp/*Abcb1* and BCRP/*Abcg2* expression and function at the BBB. Pregnant guinea pigs ($N = 42$) received 3 courses (gestation days (GDs) 40, 50, and 60) or a single course (GD50) of betamethasone (1 mg/kg) or vehicle (saline). Cerebral microvessels and brain endothelial cells (BEC) were collected from the post-natal day (PND) 14 offspring to measure protein, gene expression, and function of the drug transporters P-gp/*Abcb1* and BCRP/*Abcg2*. P-gp protein expression was decreased ($p < .05$) in microvessels from male offspring that had been exposed to multiple courses and a single course of sGC, in utero. Multiple courses of sGC resulted in a significant decrease in P-gp function in BECs from males ($p < .05$), but not females. There was a very strong trend for increased P-gp function in males compared to females ($p = .055$). Reduced P-gp expression and function at the BBB of young male offspring following multiple prenatal sGC exposures, is clinically relevant as many drugs administered postnatally are P-gp

Abbreviations: BBB, blood-brain barrier; BCRP, breast cancer resistance protein; BEC, brain endothelial cell; BETA, betamethasone; C-AM, calcein-AM; Ce6, chlorin e6; DMEM, Dulbecco's modified eagle medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GD, gestation day; HBSS, Hank's balanced salt solution; MDR, multidrug resistance; P-gp, P-glycoprotein; PND, postnatal day; POLR2A, RNA polymerase II subunit A; sGC, synthetic glucocorticoid; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta.

substrates. These novel sex differences in drug transporter function may underlie potential sexual dimorphism in drug sensitivity and toxicity in the newborn and juvenile brain.

KEYWORDS

blood-brain barrier, breast cancer resistance protein (BCRP/Abcg2), drug transporters, fetal development, glucocorticoids (betamethasone), P-glycoprotein (P-gp/Abcb1)

1 | INTRODUCTION

Antenatal synthetic glucocorticoids (sGCs) are administered to women at risk of delivering pre-term, to mature the fetal lung and increase neonatal survival.[1] sGC administration is an essential, life-saving therapy, but can also lead to long-term programming of endocrine, metabolic, and neurologic function.[2,3] One system that may be vulnerable to the long-term programming effects of sGCs is the blood-brain barrier (BBB).[4] The BBB is essential for brain protection and homeostasis. It separates the brain from the systemic circulation, protecting it from potential neurotoxins. The BBB is established early in development and protects the developing fetal brain from environmental and endogenous toxins.[5] One way in which the BBB achieves this level of selectivity, is through the expression of nutrient and drug efflux transporters in brain endothelial cells (BECs) of the cerebral capillaries.[6] Several hormones (e.g., aldosterone, cortisol) and xenobiotics (e.g., antiepileptic, antiretrovirals, anti-cancer drugs, and sGCs) are substrates for P-glycoprotein (P-gp; encoded by *Abcb1*) and breast cancer resistance protein (BCRP/*Abcg2*). P-gp and BCRP are abundantly expressed at the mammalian BBB and are also known as multidrug resistance (MDR) transporters.[7–9] P-gp is an early marker of BBB formation in the fetus and is detectable as early as 8 weeks gestation in humans[10]; BCRP is also expressed at the BBB in early development, however, less is known about its role in fetal brain protection.[11] BBB permeability changes in response to microenvironmental cues such as xenobiotic exposure, stress, and inflammation, in part via modulation of drug transporters like P-gp and BCRP.[7,8]

In guinea pigs, antenatal sGC treatment has been shown to increase expression of *Abcb1* in the paraventricular nucleus and hippocampus of F1 prepubertal female offspring.[12] The guinea pig is an important model for studying programming effects of diverse insults[13] and provides several advantages over other rodent models including a longer length of gestation (70 days), a similar neurodevelopmental profile to humans,[14] and the same form of hemochorial placentation.[15,16] Further, both endogenous (cortisol), and exogenous glucocorticoids (e.g., dexamethasone) are potent modulators of P-gp expression and function at the developing BBB.[4,17–21] In BECs derived from guinea pigs at post-natal day (PND) 14, in vitro treatment (24 h) with cortisol, aldosterone, and the sGC dexamethasone increased P-gp activity.[4] In another study, maternal dexamethasone administration increased *Abcb1* mRNA and P-gp protein in BECs from fetuses derived 24 h after treatment.[19] However, nothing is known about the long-term effects of in utero GC exposure on P-gp and BCRP expression and function in the BBB after birth.

While multiple courses of sGC were routinely administered to pregnant women at risk of preterm birth in the 90/00s, single course therapy is currently the recommended treatment.[22] However, it has been shown that the benefits of prenatal sGCs decline over time (7 days post-treatment), and, therefore, rescue courses are still being administered.[3,23] Currently, the Society of Obstetricians and Gynecologists of Canada recommends a single course of betamethasone (two doses, 24 h apart) for women at risk of delivering pre-term, between 24 and 34 weeks of pregnancy.[24] It is also important to note that ~35% of women who receive sGC treatment deliver at term.[22] The impact of multiple or single courses of antenatal sGCs on BBB function has not been investigated.

A large portion of neonatal adverse drug reactions are attributed to agents that target or access the nervous system, and some are also P-gp/BCRP substrates, including anti-epileptics (e.g., phenobarbital), anesthetics (e.g., midazolam), analgesics (e.g., morphine, fentanyl), and antiretrovirals (e.g., Zidovudine).[25] Transport of many of these drugs into the brain is regulated by drug transporters at the BBB.[26–30] Thus, prenatal exposure to sGCs that leads to long-term changes in BBB function may have consequences for post-natal drug disposition. There is also evidence for sex-specific expression and function of drug transporters in the brain and other tissues,[31–34] however, virtually nothing is known about potential sex differences at the developing BBB. In the present study, we hypothesized that prenatal exposure to multiple and single courses of sGCs would lead to long-term changes in P-gp/*Abcb1* and BCRP/*Abcg2* expression and function

at BBB in juvenile offspring and that these effects are sex-specific.

2 | METHODS

2.1 | Animals

All animal protocols were approved by the Animal Care Committee at the University of Toronto, in accordance with the Canadian Council on Animal Care. 12-week-old, nulliparous Female Dunkin-Hartley guinea pigs were purchased from a supplier (Charles River, Quebec) and singly housed in the Division of Comparative Medicine at U of T. They were kept on a 12-h light-dark cycle and given food and water ad libitum. Females (total $N = 42$) were time-mated with males as previously described.[35] Once pregnant, dams were randomly assigned to the control or treatment group. Pregnant dams were subcutaneously injected with either multiple courses or a single course of betamethasone (BETA; Betaject phosphate-acetate mix, Sabex, Boucherville, QC, Canada) or saline (veh). One course of prenatal BETA consisted of two injections of BETA (1 mg/kg) 24 h apart. This dose is four times that clinically administered to pregnant women (~0.25 mg/kg), as the guinea pig GR has a fourfold lower affinity for sGC.[36,37] Dams receiving multiple courses of BETA (1 mg/kg/day; $N = 15$) or saline ($N = 15$) were treated on gestation days (GD) 40, 41, 50, 51, 60, and 61, whereas dams in the single course group received BETA ($N = 6$) or veh ($N = 6$) treatment on GD50 and 51, as previously described.[14] Pregnant guinea pigs were allowed to deliver at term, and male and female offspring were housed with their mothers until tissue collection at PND 14. This age was chosen to represent an early life, pre-pubertal time-point. For all analyses, one individual conceptus from each litter was used, per sex (selected randomly by random number generator), thus the number of individuals, per sex, represents the number of dams. Microvessel and BEC samples were collected for each individual conceptus included in the study groups, thus each BEC culture was derived from a different individual. The average litter size in this study was 3.33 and the range of litter sizes was 1–5 with any singleton pregnancies being excluded from further analyses. No biological samples were pooled, and no data from same-sex littermates were pooled. In the main experiment, analysis of mRNA, protein, and transport function in females and males were designed and run in separate batches, due to experimental and analytic limitations, such that direct comparison of the long-term effects of betamethasone on transporter expression and function between females and males was not possible.

2.2 | Microvessel extraction

Brain microvessels were isolated from one cerebral cortex as previously described, with minor modifications.[4] Briefly, PND 14 (juvenile) offspring were euthanized under isofluorane anesthetic by cardiac puncture and exsanguination followed by decapitation. Brains were removed and one cerebral cortex was isolated for primary BEC culture, the other stabilized in RNA*later*™ (Invitrogen, MA, USA) for mRNA expression analysis. The cerebral cortex was dissected into small pieces using surgical scissors in a 50 ml conical tube (Falcon) with 10 ml of RNALater™ Stabilization Solution (Invitrogen, MA, USA) on ice, and stored overnight at 4°C, for qPCR analysis or homogenized (Potter-Evehljem Tissue Grinder; Sigma, MA, USA), resuspended in dextran (Millipore Sigma, MA, USA) solution [17.5% (wt/vol) in Hank's Balanced Salt Solution (HBSS, Wisent BioProducts, Quebec, Canada)] and centrifuged (3000 *g*, 30 min, 4°C) for microvessel isolation. After centrifugation, the brain parenchyma and dextran were aspirated, leaving the microvessel pellet. The microvessel pellet was resuspended into 3 ml of HBSS, aliquoted into three 2 ml Eppendorf tubes, and spun on a benchtop centrifuge (10 000 rpm, 10 min, 4°C). The HBSS was removed, and microvessel pellets were stored at −80°C for downstream applications.

2.3 | BEC culture

The methods for extracting BECs from the left hemisphere for culture are similar to microvessel extraction described above, and as described previously.[4] The cerebral cortex was dissected in sterile Medium199 (Gibco) and homogenized in a sterilized Potter-Evehljem Tissue Grinder (Millipore-Sigma, MA, USA). The homogenate was resuspended in dextran (D8821; Millipore-Sigma, MA, USA) solution [17.5% (wt/vol) in HBSS (Wisent BioProducts, Quebec, Canada)] and centrifuged (3000 *g*, 30 min, 4°C). Collagenase (10 ml; Millipore-Sigma) at 1 mg/ml in DMEM without phenol red (Wisent) was added to the microvessel pellet and incubated in a water bath at 37°C (30 min). After digestion, the cells were centrifuged (2000 *g*, 10 min, 4°C) and collagenase was removed. The BEC pellet was resuspended in warm BEC media made up of 20% FBS (Wisent) in DMEM w/ phenol red (Wisent) supplemented with heparin (Millipore-Sigma), endothelial cell growth supplement from bovine neural tissue (ECGS; Millipore-Sigma), insulin-transferrin-selenium × (ITSX; Gibco), and antibiotic-antimycotic (Anti-anti; Gibo). Cells were plated on attachment factor (0.1% gelatin; Gibco) coated 75 cm^2 tissue culture flasks and grown in 5% CO_2 at 37°C in a humidified incubator. BEC cultures have been characterized

previously.[4] After expansion, cells were frozen in liquid nitrogen for use in drug transporter functional assays.

2.4 | qPCR

RNA was extracted from microvessels using the Allprep® DNA/RNA/miRNA Universal Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, and RNA concentration and purity were determined. Samples with 260/280 values above 1.8 were kept for cDNA conversion. RNA integrity was assessed by RNA gel electrophoresis and confirmed by the identification of clear 28s and 18s rRNA bands. All RNA samples (1 μg/sample) were reverse transcribed to cDNA using the SensiFAST™ cDNA Synthesis Kit (Bioline, London, England) in one cycle. Three samples were randomly selected as no reverse transcriptase controls. The cDNA was diluted to 5 ng/μl and 10 ng of cDNA was loaded per sample for qPCR using the SensiFAST™ Sybr HI-Rox Mix (Bioline, London, England) at 20 μl/reaction, and run using the CFX96 Touch™ Real-Time PCR Detection System (https://www.bio-rad.com/en-us/product/cfx96-touch-real-time-pcr-detection-system?ID=LJB1YU15, RRID:SCR_018064). A list of gene targets, primer sequences, and Ensembl ID is provided in Table 1. Primers were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/, RRID:SCR_003095), and synthesized by Integrated DNA Technologies (IDT, Iowa, USA). The geometric mean of three reference genes, RNA Polymerase II Subunit A (*Polr2a*), Glyceraldehyde-3-Phosphate Dehydrogenase (*Gapdh*), and Tyrosine 3-Monooxygenase/Tryptophan 5-monooxygenase Activation Protein Zeta (*Ywhaz*), was used for normalization of gene targets. The primer sequences are presented in Table 1. The relative mRNA expression levels were calculated using the Livak method.[38]

2.5 | Western blot

Protein was extracted from microvessels as previously described.[4] Briefly microvessel pellets were homogenized with a bead in a Qiagen TissueLyser (Qiagen, #85300, Hilden, Germany) in 1× radioimmunoprecipitation assay (RIPA) lysis buffer with cOmplete™ Mini Protease Inhibitor Cocktail (Roche, #11836153001). Homogenates were incubated on ice (30 min), centrifuged (10 000 rpm, 20 min, 4°C), and the supernatant (containing total microvessel protein) stored at −80°C. Protein was quantified using a Pierce BCA protein assay. For detection of P-gp and BCRP, 30 and 15 μg of total proteins, respectively, were separated via SDS-PAGE using hand cast 7.5% Acrylamide TGX Stain-Free™ FastCast™ gels. StainFree™ gels (Bio-Rad) were imaged on the ChemiDoc™ MP Imaging system (Bio-Rad) and used as a total protein loading control. Separated proteins were then transferred to nitrocellulose membranes (Bio-Rad) using the Trans-Blot® Turbo™ Transfer System (Bio-Rad; 10 min). Membranes for P-gp were blocked with skim milk (5%; BioShop) in Tris-buffered saline with 0.1% Tween (TBS-T; 1 h). Blots were incubated overnight at 4°C on a shaker with the following primary antibodies: rabbit anti-P-gp (1:1000, abcam, #ab170904, RRID:AB_2687930); rabbit anti-BCRP (1:1000, abcam, #ab108312, RRID:AB_10861951). Blots were incubated with horseradish-peroxidase-conjugated anti-rabbit secondary antibody (1:10 000, Santa cruz, #sc2004, RRID:AB_631746), and subsequently with chemiluminescence substrate (Bio-rad) prior to imaging (ChemiDoc™ MP Imaging system; Bio-rad). Band densitometry was quantified using Image Lab™ (http://www.bio-rad.com/en-us/sku/1709690-image-lab-software, RRID:SCR_014210). Total P-gp and BCRP protein was normalized to a specific band on a StainFree™ gel.

2.6 | Drug transporter functional assays

P-gp and BCRP functional assays were performed as described previously by our research group with modifications.[4,18,19,39–41] BECs were seeded at 10 000 cells/cm^2 on 24-well plates (Falcon) in BEC Media supplemented with 10% FBS. One day before the assay, BEC Media was withdrawn and replaced with DMEM without phenol red, supplemented with 10% charcoal-stripped FBS (CS-FBS). On the day of assay, media was withdrawn, and cells were washed twice with warm Tyrode salts

TABLE 1 RT-qPCR primer sequences

Target	Forward	Reverse	Ensembl ID
Abcb1	GCC ATG TTT CGC TAT TC	CAC TGA CTT CAC TTT TAT CTA ATG	ENSCPOT00000012540
Abcg2	TGG AAA CTC AC TAG AGT GGC AG	CCC TTC AGT ACG GTT CCT CAG	ENSCPOT00000014737
Gapdh	TGT ACT GGA GGT CAA TGA AGG	GTC GGA GTG AAC GGA TTT G	ENSCPOT00000043852
Ywhaz	TGG CCC ATC ATG ACA TTG GG	GCA CAT GGC CAC CAA ATA GG	ENSCPOT00000035714
Polr2a	GAT GAT TGT CAC GGT GCT GC	CGT CCT CCG CGA TAA CAT GA	ENSCPOT00000014957

solution (Sigma, #T2145) supplemented with sodium bicarbonate (1 g/L, Sigma, #S6014). For the P-gp functional assay, cells were incubated with calcein-AM (C-AM; sigma #17783) at 10 μM for 1 h at 37°C and 5% CO_2. For the BCRP assay, cells were incubated with 5 μM chlorin e6 (Ce6, Santa Cruz Biotechnology, #SC-263067) for 1 h at 37°C and 5% CO_2. After incubation, plates were placed on ice, and cells were washed twice with ice-cold Tyrode salts solution. Cells were lysed with cold 1% Triton X-100 (Sigma #X100) in HBSS. Accumulation of the fluorescent substrates C-AM and Ce6 was measured using Gen5 (https://www.biotek.com/products/software-robotics-software/gen5-micro plate-reader-and-imager-software/, RRID:SCR_017317) on a Synergy Mx Microplate Reader (BioTek, Vermont, USA) at excitation/emission wavelengths of 485/510 nm (C-AM/P-gp) and 407/667 nm (Ce6/BCRP). Cell lysate was collected, and protein was quantified using a Pierce microBCA assay. Relative fluorescence units (RFUs) obtained from the microplate reader were then normalized to protein content. Three technical replicates were included per *N*, and $N = 3–8$ in each group. Each *N* represents BECs derived from one litter that received prenatal BETA or vehicle. Data are presented as a percent change from the average of the vehicle group (prenatal treatment with saline). To directly analyze potential sex differences in P-gp and BCRP function, BEC functional analysis was undertaken in control female and male offspring from the multiple course experiment. Data for sex differences are displayed as fluorescent substrate accumulation normalized to total protein. A decrease in substrate accumulation is indicative of an increase in P-gp and BCRP function. Inhibitors of P-gp and BCRP, verapamil (10^{-5}M; sigma, #V4629), and Ko143 (10 μM; sigma, #K2144) were included for assay validation. Verapamil and Ko143 increased P-gp and BCRP substrate accumulation in BECs, respectively (Figure S1).

2.7 | Statistical analyses

Statistical analyses were performed using Prism 9 (http://www.graphpad.com, RRID:SCR_002798). For drug transporter activity assays, statistical analyses were performed on RFU/protein normalized data. The data were first assessed for normal distribution using a Shapiro-Wilk test. Any outliers were detected and excluded using a ROUT test. Gene, protein, and functional assay data were analyzed using a Student's *t*-test. $N = 3–11$ per group, specified in each figure. Data are presented as mean ± standard error of the mean (SEM) and differences were considered significant if $p < .05$.

3 | RESULTS

3.1 | Effect of prenatal betamethasone on P-gp/*Abcb1* and BCRP/*Abcg2* expression

Exposure to multiple and single courses of prenatal BETA led to a significant decrease in P-gp protein levels in brain microvessels of male ($p < .05$) guinea pigs, but not females (Figure 1). There was no effect of multiple courses or a single course of BETA on BCRP protein levels in microvessels. Unlike protein, there was no effect of multiple courses or a single course of prenatal BETA on *Abcb1* (encoding P-gp) or *Abcg2* (BCRP) mRNA in brain microvessels (Figure 2).

3.2 | P-gp and BCRP activity in BECs

Prenatal exposure to multiple courses of BETA led to a significant decrease in P-gp function in BECs derived from juvenile male offspring (*$p < .05$, Figure 3B), but not females (Figure 3A). There was no change in BCRP function after prenatal exposure to multiple courses of BETA. Single-course sGC exposure did not alter P-gp and BCRP function in BECs derived from male or female offspring (Figure 3).

3.3 | Sex differences in P-gp and BCRP function

In the vehicle animals from the multiple course cohort, (Figure 4A), there was a very strong trend for reduced calcein-AM accumulation in males ($p = .0548$), compared to females. This would suggest a higher P-gp function in male offspring. Baseline BCRP function was not different between males and females (Figure 4B).

4 | DISCUSSION

In the present study, we have identified, for the first time, long-term effects of prenatal sGC exposure on specific drug transporters at the juvenile BBB. Multiple courses of prenatal sGC administered in late gestation resulted in decreased P-gp protein expression and function in the male BBB; without changing male BCRP expression and activity. There were no effects of multiple sGC exposure on drug transporters in the female juvenile BBB. In addition, while a single course of sGC exposure (GD50) led to decreased P-gp protein expression, this did not translate to altered transporter function. Again, there were no effects observed in females, after a single course of BETA.

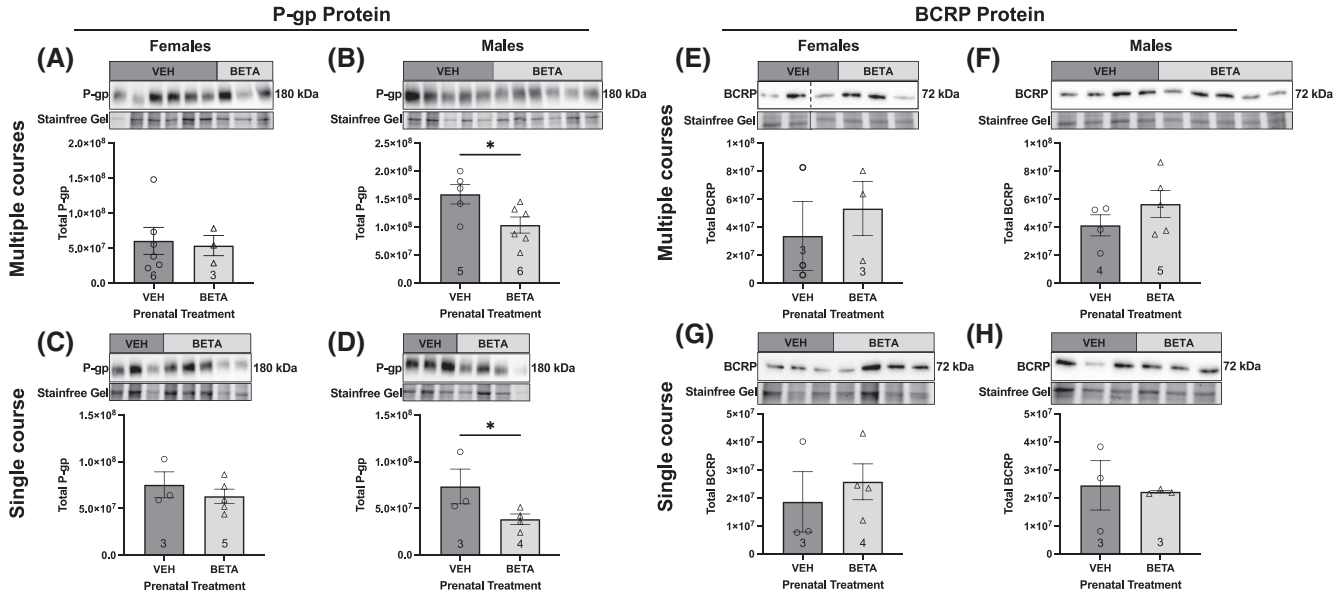

FIGURE 1 Multiple courses and a single course of prenatal betamethasone result in sex-specific decreases in P-gp protein in brain microvessels. P-gp (A–D) and BCRP (E–H) protein expression by western blot in brain microvessels derived from female and male post-natal day (PND) 14 guinea pigs prenatally exposed to betamethasone (BETA; 1 mg/kg) or saline (VEH). PND14 offspring were prenatally exposed to either multiple courses of BETA or VEH on gestation days (GD) 40, 50, and 60 (A,B,E,F), or a single course of BETA or VEH on GD40 (C,D,G,H). Images of each blot for P-gp, BCRP, and Stainfree Gel (loading control) are shown above each bar graph. Each data point on the graph represents a single lane on the gel, which includes microvessel protein from one individual conceptus per litter, per sex. Dashed line indicates where a sample was removed due to a loading error. *N* values are displayed within bars. Data are presented as mean ± SEM. Statistical analysis: unpaired Student's *t*-test. *$p < .05$, multiple courses two-tailed, single-course one-tailed based on findings from multiple courses cohort

sGC are commonly administered to pregnant women threatened with preterm labor.[24] In the current study, we aimed to identify whether prenatal sGC exposure would induce long-term changes in BBB function, by altering levels of key efflux transporters, P-gp and BCRP. We and others have previously shown that P-gp/*Abcb1* expression and function are modified acutely the following exposure to exogenous and endogenous glucocorticoids at the developing BBB.[4] In previous studies, we observed that P-gp expression increases with advancing gestation, coincident with the late gestation fetal cortisol surge.[42,43] Subsequently, we demonstrated that the sGC dexamethasone acutely affected the expression and function of P-gp in BEC derived at different developmental time points in the guinea pig, but that the nature of the effect differed with age. P-gp function in BECs derived at GD40 was not affected by acute GC exposure, but P-gp function was responsive to GC in BECs derived in later gestation (GD 50, 65) and postnatal life (PND14).[4] To our knowledge, the present study is the first to identify the long-term effects of fetal sGC exposure on drug transporter function at the BBB.

Glucocorticoid response elements have been identified in the promoter region of *Abcb1* of the hamster, with homologous regions identified in the human *ABCB1* promoter[44] suggesting direct transcriptional regulation of expression by GCs. However, in the current study, we did not find differences in drug transporter mRNA expression only P-gp protein and function. As such, the effects of sGC may not be mediated by long-term induction of *Abcb1* transcription. Other studies have shown rapid and reversible inhibition of P-gp after endothelin-1 treatment, an effect that was independent of any changes in gene transcription, and which the authors postulated was due to rapid trafficking of P-gp away from the plasma membrane.[45] P-gp trafficking occurs via direct transport to the plasma membrane in vesicles, or an intracellular storage pool via the endosomal pathway. It is possible that antenatal sGC exposure induces changes in protein trafficking machinery of the cell affecting levels of P-gp available for transport at the BBB. It has been shown that the endosomal recycling pathway is mediated by Rab GTAses. In this context, GCs stabilize the GDI-Rab4 complex, increasing the recycling of proteins between the early endosome and plasma membrane, via glucocorticoid-serum inducible kinase (SGK-1).[46] While some studies have identified a direct role for Rab4 in the regulation of P-gp trafficking, they were performed in cancer cell lines. Additional research is required to understand this relationship in

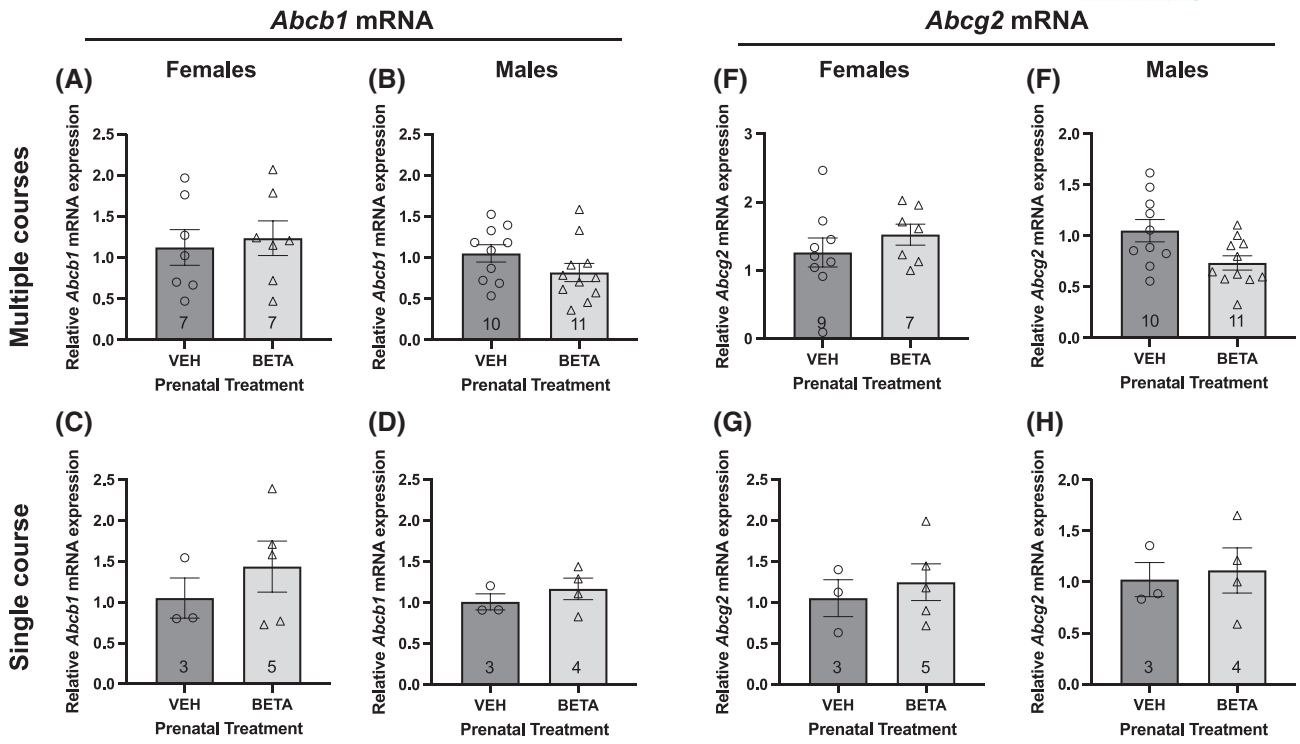

FIGURE 2 Drug transporter gene expression in brain microvessesls is not altered by prenatal betamethasone. *Abcb1* (A–D) and *Abcg2* (E–H) mRNA expression by RT-qPCR in brain microvessels derived from female and male post-natal day (PND) 14 guinea pigs prenatally exposed to betamethasone (BETA; 1 mg/kg) or saline (VEH). PND14 offspring were prenatally exposed to either multiple courses of BETA or VEH on gestation days (GD) 40, 50, and 60 (A,B,E,F), or a single course of BETA or VEH on GD40 (C,D,G,H). *N* values are displayed within bars. mRNA expression was quantified by RT-qPCR and normalized to the geometric mean of three reference genes, *Gapdh*, *Ywhaz*, and *Polr2a*, and displayed as relative quantity compared to VEH. Data are presented as mean ± SEM. Statistical analysis: unpaired Student's *t*-test

normal tissues.[47] In the present study, P-gp expression and function in microvessels and BECs, respectively, were reduced at PND14, 25 days after the last exposure to sGC in the multiple courses group. It is possible that long-term changes in protein trafficking machinery could lead to altered levels of P-gp at the plasma membrane without any observed changes in mRNA, a hypothesis that requires further investigation.

Another potential mechanism for long-term changes in P-gp protein/function is GC-mediated changes in miRNAs. There is evidence that GC exposure leads to long-term changes in miRNA expression profiles. Late-gestation maternal stress in rats leads to altered miRNA signatures in the brains of offspring, including down-regulation of miRNA-145,[48] which in another study was found to block *Abcb1* translation in rat intestinal epithelial cells via binding of the 3′ UTR. miRNAs also mediate xenobiotic/drug-induced changes in P-gp expression. miR-466b-1-3p down-regulates P-gp in rat cerebrovascular endothelial cells and decreased expression was associated with phenobarbital induced over-expression of P-gp.[49] This is important, as phenobarbital shares a common xenobiotic signaling pathway (the nuclear family receptor CAR) with sGCs in activating P-gp.[50,51] Knowledge of differential regulation of miRNAs by antenatal sGCs could provide valuable insight into the mechanisms underlying long-term changes in drug transporter protein expression at the BBB and should be further investigated.

In BECs derived from adult rats, acute dexamethasone exposure increased activity and expression of both P-gp and BCRP. Little is known about glucocorticoid regulation of BCRP at the developing BBB, however, sGCs acutely modify BCRP/*Abcg2* and P-gp/*Abcb1* expression and function in a timing and sex-dependent manner in the mouse brain.[17,52] Therefore, while BCRP may be regulated acutely by sGCs, we have shown in the current study that these effects do not persist long-term. Finally, BCRP does not show the same increase as was observed with P-gp at the BBB in late gestation, coincident with the cortisol surge (unpublished observation). This likely indicates that glucocorticoid exposure plays a less important role in the regulation of BCRP at the developing BBB.

One goal of our study was to examine the effects of prenatal sGCs on long-term BBB function in both males and females. We identified both an effect of sGCs in males only and a sex difference in baseline P-gp activity. While little

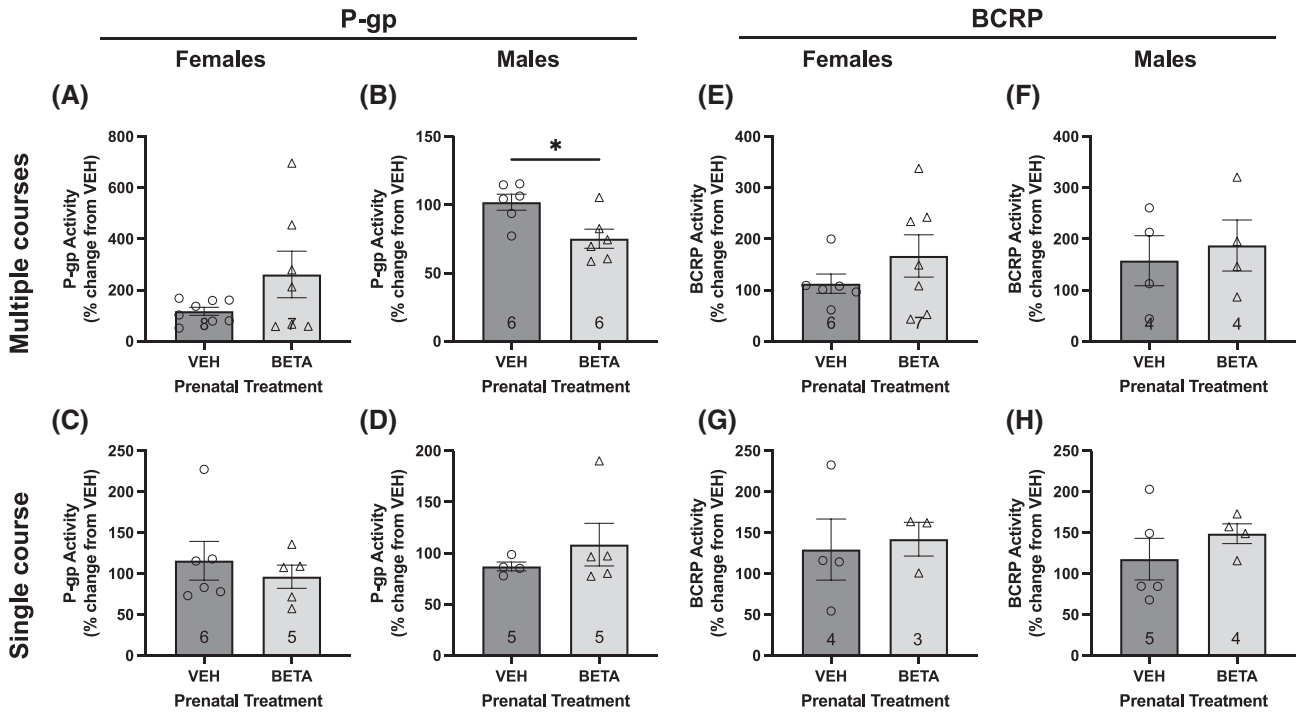

FIGURE 3 Multiple courses of prenatal betamethasone result in a sex-specific decrease in P-gp activity in brain endothelial cells from males. P-gp (A–D) and BCRP (E–H) activity by in vitro calcein-AM and chlorin e6 functional assay, respectively, in brain endothelial cells (BEC) derived from female and male post-natal day (PND) 14 guinea pigs prenatally exposed to betamethasone (BETA; 1 mg/kg) or saline (VEH). PND14 offspring were prenatally exposed to either multiple courses of BETA or VEH on gestation days (GD) 40, 50, and 60 (A,B,E,F), or a single course of BETA or VEH on GD40 (C,D,G,H). P-gp and BCRP activities are displayed as percent change from the VEH group. *N* values are displayed within bars. Data are presented as mean ± SEM. Statistical analysis: unpaired Student's *t*-test. $^*p < .05$

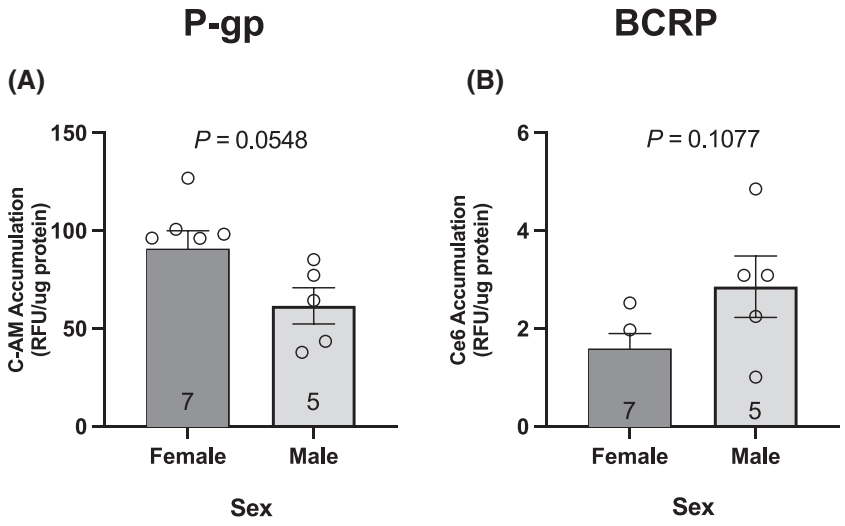

FIGURE 4 P-gp activity in brain endothelial cells is higher in males than females. Brain endothelial cell (BEC) P-gp (A) and BCRP (B) activity data were collected from control male and female guinea pig offspring at postnatal day (PND) 14 that received 3 courses of saline. Control offspring from the multiple courses cohort were analyzed to identify baseline sex differences in P-gp and BCRP function. Data are displayed as fluorescent substrate accumulation normalized to total protein; calcein-AM (C-AM) for P-gp (A) and chlorin e6 (Ce6) for BCRP (B). A decrease in substrate accumulation is indicative of an increase in P-gp/BCRP activity. *N* values are displayed within bars. Data are represented as mean ± SEM. Statistical analysis: unpaired Student's *t*-test

is known about sex differences in drug transporters at the BBB, some studies have shown sex differences to be highly species- and tissue-dependent. In the human adult BBB, P-gp activity was found to be higher in young men than women,[34] in line with the findings from the current study in guinea pigs. However, in mice and rats, P-gp activity at

the BBB is reported to be higher in females than males, despite the fact that P-gp protein expression was higher in males than females.[53,54] These differences could be attributed to the presence of two transcript variants *Abcb1a* and *Abcb1b* present in rats and mice, but not in humans or guinea pigs.[55] *Abcb1a* is reported to be the dominant transcript variant at the BBB of mice and rats.[56] A limitation of our study is that, due to experimental and analytical limitations, we were unable to undertake simultaneous analysis of males and females, to assess our primary outcome; the effects of prenatal glucocorticoid exposure on drug transporter expression and function. This precluded us from assessing the potential interaction of sex in our overall analyses.

Few studies have investigated the role of sex hormones in the direct regulation of P-gp/*Abcb1*, and often with conflicting findings. In human and mouse renal cell culture, estradiol increased digoxin clearance, and P-gp/*Abcb1* expression, while testosterone had no effect.[57] However, at the blood-spinal cord barrier of rats, testosterone supplementation in vivo led to increased P-gp protein expression.[58] In male guinea pigs, testosterone levels during the post-natal androgen surge peak at around PND3[59]; it is possible that high levels of testosterone have a positive regulatory effect on P-gp, leading to sexual dimorphism in transporter expression and function. There is a wide range of evidence supporting sex differences in drug metabolism in adults,[60] however, literature in the neonate is scarce. Ultimately, more research is required to elucidate the mechanisms underlying these sex differences in P-gp expression and function at the BBB.

In conclusion, our study has shown that prenatal sGCs exposure leads to long-term changes in BBB P-gp. While BBB P-gp protein expression was reduced in males after multiple courses and a single course of BETA, this only had functional consequences (decreased P-gp activity) after multiple courses of prenatal sGCs. We have also identified sex-specific effects of prenatal sGC on post-natal BBB function. Alterations in P-gp function can change uptake of drugs into the CNS; long-term changes in P-gp activity could, therefore, have implications for neonatal/post-natal drug disposition. Infants and neonates represent a vulnerable population exposed to a wide spectrum of drugs when they require perinatal care. Indeed, many of the most commonly used drugs are P-gp and BCRP substrates, and a number have been associated with toxicity in newborns (e.g., morphine). Thus, prenatal exposure to sGCs that leads to long-term changes in BBB function may have consequences for postnatal brain drug disposition. Future studies investigating the pharmacokinetics of clinically relevant P-gp substrates in offspring exposed antenatally to sGCs are warranted to determine whether in utero exposure to sGCs has a long-term effect on offspring drug pharmacokinetics.

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DISCLOSURES

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Margaret E. Eng: designed research, performed research, analyzed data, visualization, writing—original draft, Enrrico Bloise: analyzed data, writing—review and editing, and supervision Stephen G. Matthews: designed research, analyzed data, writing—review and editing, supervision, project administration, and funding acquisition.

DATA AVAILABILITY STATEMENT

Data used to support the findings from this study are available from the corresponding author upon request.

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