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Preeclampsia-induced alterations in brain and liver gene expression and DNA methylation patterns in fetal mice

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Brief Report

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Abstract

Exposure to pregnancy complications, including preeclampsia (PE), has lifelong influences on offspring's health. We have previously reported that experimental PE, induced in mice by administration of adenoviral sFlt1 at gestational day 8.5 combined with LPS at day 10.5, results in symmetrical growth restriction in female and asymmetrical growth restriction in male offspring. Here, we characterize the molecular phenotype of the fetal brain and liver with respect to gene transcription and DNA methylation at the end of gestation.

In fetal brain and liver, expression and DNA methylation of several key regulatory genes is altered by PE exposure, mostly independent of fetal sex. These alterations point toward a decreased gluconeogenesis in the liver and stimulated neurogenesis in the brain, potentially affecting long-term brain and liver function. The observed sex-specific growth restriction pattern is not reflected in the molecular data, showing that PE, rather than tissue growth, drives the molecular phenotype of PE-exposed offspring.

Introduction

Preeclampsia (PE), a severe complication of pregnancy affecting 2–8% of all pregnancies, is characterized by maternal hypertension and proteinuria arising in the second half of gestation. The etiology of PE is not completely understood, but likely involves poor trophoblast invasion, leading to placental dysfunction and the release of proinflammatory and antiangiogenic factors into the maternal circulation.[1] This poses a direct threat to maternal and fetal health. Offspring exposed to PE *in utero* have an elevated risk of cardiovascular and metabolic disorders later in life, a phenomenon known as fetal programming.[2]

We have recently described a novel double-hit mouse model of PE which closely resembles the human disease.[3] This double-hit mouse model of PE is based on two pronounced pathophysiological mechanisms: 1) a disrupted angiogenic balance and 2) an increased systemic inflammatory response. Here, we aim to characterize the molecular phenotype of the fetal brain and the liver in this mouse model of PE, to potentially get more insight into the mechanisms of fetal programming by PE.

Experimental PE was induced by a combination of the antiangiogenic soluble fms-like tyrosine kinase (sFlt-1; via a recombinant adenovirus) and proinflammatory lipopolysaccharide (LPS) at gestational day (GD) 8.5 and 10.5, respectively.[3] This double-hit approach leads to a PE-like phenotype with maternal hypertension and proteinuria. At GD 18.5, both male and female fetuses were growth restricted (PE-exposed males weigh 90.5% of controls, females 85%), with only the males showing an asymmetrically large brain, known as brain sparing (brain/liver ratio in PE-exposed males is 1.41 vs 1.21 in controls; in females 1.36 vs 1.24). Furthermore, dams, male fetuses and female fetuses show distinct profiles in plasma metabolome. Phenotypic data on dam and offspring are published in Stojanovska et al., 2019.[3] Based on these sex-specific growth patterns and brain sparing, we hypothesize sex-specific PE-induced molecular changes especially in fetal brain and liver. Therefore, these fetal tissues were selected for further analysis.

The liver is a major metabolic regulator prone to adverse fetal programming *in utero*. In adverse *in utero* circumstances, evident changes have been found in the liver. Persistence of these adaptations into postnatal life has the potential to promote obesity, diabetes and other facets of the metabolic syndrome.[4] Therefore, we were specifically interested in putative sex-specific molecular changes in our PE model. Based on relations between fetal liver gene expression and function and later life health, described by others, a number of genes were selected and analyzed. These genes belong to pathways regulating inflammation and oxidative stress

(*Dusp1, Hmox1, Id1, Maff, Nfkb1, Socs3*), maturation (*Afp, Cebpa, Tat, Tnfa*), and glucose, glucocorticoid, lipid, and cholesterol metabolism (*Fasn, Srebf2, Pgc1a, Ppara*).

The second organ which we previously found to be affected in a sex-specific way was the brain: in our opinion, the brain sparing shown in male offspring warrants further investigation. Furthermore, exposure to PE has been shown to lead to alterations in neurodevelopment.[5] Dysregulation of genes involved in different neurodevelopmental pathways has been reported in intrauterine inflammation-exposed offspring and in fetal growth-restricted (FGR) fetuses. The genes influencing neuron differentiation and maturation, myelination, and axon guidance.[6–10] Therefore, a number of neurodevelopment-associated genes (*Auts2, Axin, Bdnf, Mag, Mbp, Mecp2, Nxrn1, Pou4f1, Pparg, Reln, Vldlr)*, genes belonging to pathways regulating inflammation and oxidative stress (*Dusp1, Hmox1, Id1, Maff, Nfkb1, Socs3*), and cholesterol metabolism (*Srebf2*) were analyzed. As fetal programming often involves long-lasting changes in epigenetics, global and gene-specific DNA methylation was assessed in the fetal brain.

Method

Animals and experimental procedures

Fetal tissue as described in our previously published double-hit pre-eclampsia model was used.[3] In short, C57Bl/6j mice (9 per experimental group; Charles River, France) between 9 and 12 weeks received either recombinant adenovirus encoding mouse sFlt-1 (Ad-sFlt1) or empty control adenovirus (Ad-null) at gestational day 8.5 and 25 ug/kg LPS (Ad-sFlt1 group) or PBS (Ad-null group), respectively, at gestational day 10.5. Fetal brain and liver were collected and snap frozen at gestational day 18.5. One male and one female fetus per dam, 18 dams in total, were randomly selected for further analysis.

DNA and RNA isolation

Both fetal brain and liver were homogenized before DNA and RNA isolation, to avoid region-specific effects. RNA from fetal brain was isolated using TRIzol Reagent (Invitrogen) following manufacturer's protocol. To optimize DNA isolation in brain tissue, TRIzol complemented by Back Extraction Buffer protocol was used. Fetal hepatic DNA and RNA were isolated using the NucleoSpin TriPrep kit and protocol (Macherey-Nagel). DNA and RNA quantities were assessed with Nanodrop 2000c (Thermo Scientific).

Gene expression

One microgram of RNA was converted into cDNA and used for gene expression analysis by quantitative polymerase chain reaction (qPCR). Gene expression was assessed using SYBR Green PowerUp in brain and SYBR Green PowerUp and Taqman Fast Advanced Master Mix in liver. qPCR runs took place using the Quantstudio 3 (for SYBR mastermix) and StepOnePlus (for Taqman mastermix) hardware and accompanying software (Applied Biosystems), following the protocol of the mastermix manufacturer. Primer and probe sequences are listed in Supplementary Table S1. The $2^{-\Delta Ct}$ method was used for relative quantification with stable expressed housekeeping genes (*Gapdh* and *36b4* for brain and *36b4* and *bactin* for liver) as reference.

Pyrosequencing

DNA methylation was analyzed in a select number of targets (brain: repetitive element *LINE1*, *Srebf2, Bdnf, Auts2*; Liver: *LINE1*, *Srebf2*) using pyrosequencing. Bisulfite conversion of 500 ng genomic DNA was performed with EZ DNA methylation gold kit (Zymo Research). Bisulfite-specific primers were using PyroMark Assay Design software (Qiagen) and are listed in Supplementary Table S2. For amplification of 50 ng bisulfite-treated DNA, HotStarTaq Master Mix (Qiagen) was used. The PCR product was analyzed for changes in methylation for selected CpG positions by pyrosequencing with the use of PyroMark Q24 (Qiagen) or PyroMark Q48 Autoprep (Qiagen).

Statistical analysis

Data are presented as mean ±SD with the use of Prism 7 (GraphPad). Statistical analysis was performed with IBM SPSS statistics 23 (IBM Corp.). Shapiro–Wilk test was applied to test for normal distribution of data. When not normally distributed, data were transformed before analysis. A two-way ANOVA test with Šídák multiple comparisons test was performed to examine the effect of gender and treatment on relative gene expression. The effect of gender and treatment on DNA methylation was tested with repeated measurements two-way ANOVA, with Huynh–Feldt correction. For all statistical test, $p < 0.05$ was considered significant.

Results

In the fetal liver, a downregulation of gene expression was found for genes involved in inflammation and oxidative stress (*Dusp1, Hmox1* and *Socs3;* Fig. 1) for the PE-exposed offspring, while there was no difference for *Id1, Maff,* and *Nfkb1* (Supplementary Table S3). Analysis of the liver maturation markers only showed a significant decrease in mRNA expression of the proinflammatory and maturation-suppressing *Tnfa* (Fig. 1). Gene expression of the glycogenesis marker *Pepck* was significantly reduced, and a trend of reduced expression was found for *G6pc* and *Sirt1* in the PE-exposed offspring. A significant downregulation of *Srebf2* gene expression, encoding for an important marker of lipid and cholesterol metabolism, was found for PE-exposed offspring. DNA methylation analysis of the promotor site of *Srebf2* showed a significant decrease in methylation (Fig. 1 and Supplementary Fig. S1). Overall, gene expression differences found in the liver were not sex specific.

In fetal whole brain, a significant increase in expression was found for the genes involved in inflammation and oxidative stress *Nfkb1* and *Socs3*; and a trend was found for *Hmox1* in PE-exposed offspring (Fig. 2). For the neurodevelopment-associated genes, an increased expression was found for *Auts2, Axin2, Bdnf,* and *Mag* in PE-exposed offspring (Fig. 2). Other neurodevelopmental associated genes showed no difference in gene expression between males and females and between control or PE-exposed offspring (Supplementary Table S4). A significantly increased gene expression for the cholesterol biosynthesis marker *Srebf2* was found for PE-exposed offspring (Fig. 2). No sex-specific differences were found in the gene expression patterns of the fetal brain. DNA methylation analysis of LINE1 showed no difference in global methylation between groups (Supplementary Fig. S2). A significant decrease in DNA methylation was found in the promotor region of *Bdnf* exon IV for PE-exposed offspring (Fig. 2 and Supplementary Fig. S2). Multiple CpG positions in the promoter site of *Auts2* were analyzed. The first 12 and last three CpG positions were

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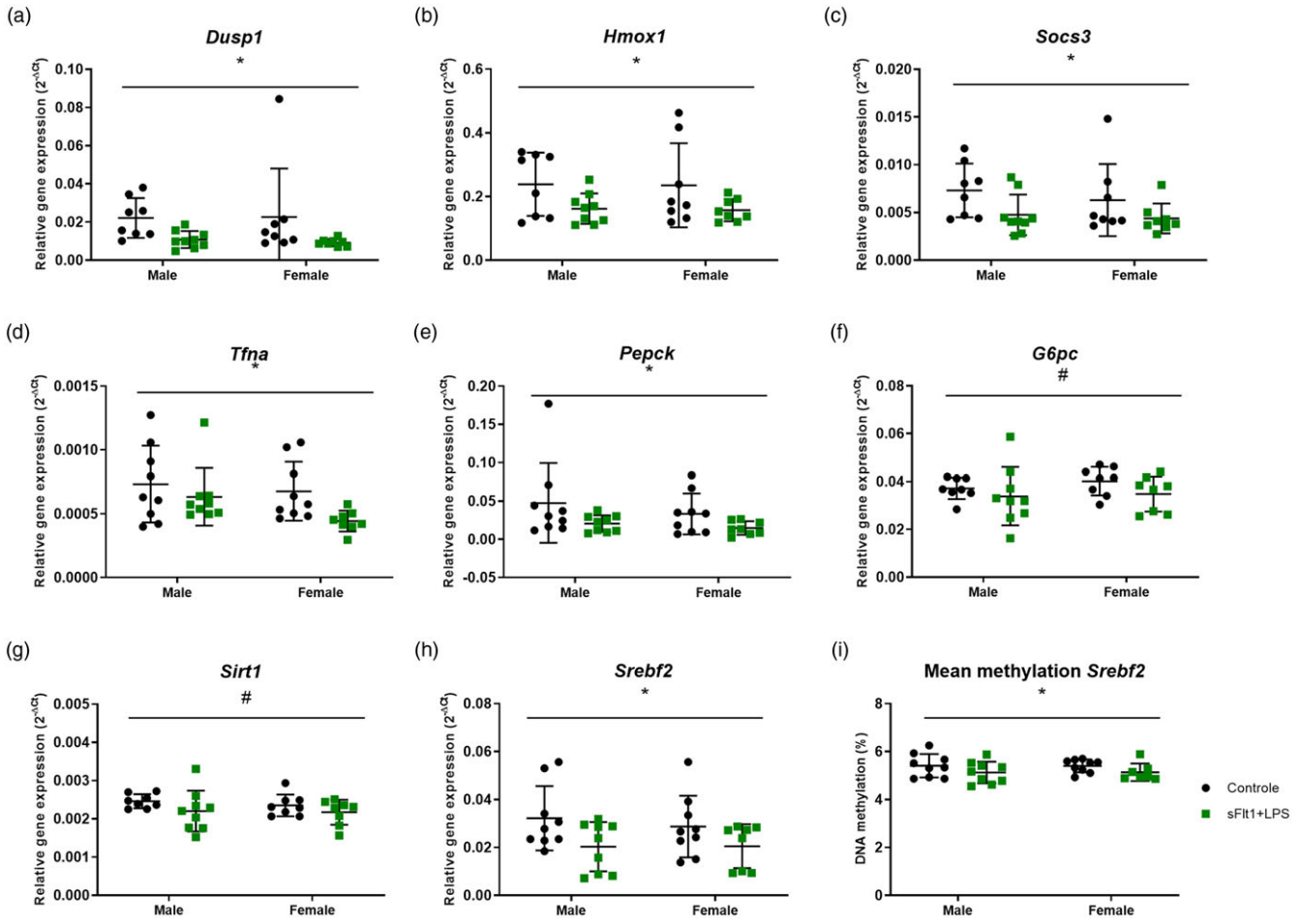

Fig. 1. Gene expression of inflammation, oxidative stress and metabolic markers and DNA methylation of *Srebf2* in fetal liver of preeclampsia-exposed offspring. Shown are the relative gene expressions of inflammatory and oxidative stress genes *Dusp1* (a), *Hmox1* (b), S*ocs3* (c), maturation marker *Tnfa* (d), gluconeogenesis markers *Pepck* (e), *G6pc* (f), *Sirt1* (g), cholesterol biosynthesis marker *Srebf2* (h), and the mean DNA methylation of the *Srebf2* promotor site (i). Additional hepatic gene expression data are shown in Supplementary Table S3 and the DNA methylation per CpG position of the *Srebf2* promotor site is shown in Supplementary figure S1. Control groups and sFlt1+LPS male $n = 9$, sFtl1+ LPS female $n = 8$. Relative gene expression was calculated using the $2^{-\Delta Ct}$ method, normalized against housekeeping genes *36b4* and *bactin,* and analyzed using two-way ANOVA. DNA methylation was analyzed with repeated measure two-way ANOVA. Data are presented as mean ±SD. * $p < 0.05$ for treatment, # $p = 0.076$ for treatment.

significantly decreased for male PE-exposed offspring (Supplementary Fig. S2). No difference was found in the female offspring (Fig. 2).

Discussion

We have previously described a reduction of fetal liver weight in our PE double-hit model. This phenotype raised the question whether the livers are well developed but small, or lagging behind in maturation. Analysis of maturation markers *Tat, Cebpa,* and *Afp* showed no signs of abnormal maturation.[11,12] A significant decrease in expression of the proinflammatory and maturation-suppressing *Tnfa* was found, which is in accordance with the decreased expression of other proinflammatory genes, shown later. Therefore, we conclude that, although the livers of PE-exposed fetuses are small, they are at a similar stage of maturation.

The genes *Socs3, Hmox1, Dusp1, Maff, Id1,* and *Nfkb1* are known to be markers of inflammation and oxidative stress. *In utero* exposure to a high-fat diet leads to expression of these genes, which indicates an elevated risk of later life metabolic syndrome.[13] Cellular stress and inflammation are proposed links between adverse circumstances *in utero* and later life risk of metabolic syndrome. An induction of these genes, due to a pre-eclamptic and thereby proinflammatory state in the dams, was expected. However, slight but significant downregulation of *Socs3, Hmox1, Dusp1* was found, showing that there is no lasting proinflammatory state in fetal liver after the original induction of PE by sFlt-1 and LPS at GD8.5 and 10.5, respectively. This indicates that either the dam is not in a proinflammatory state or the fetuses (or fetal liver) are protected against this. Even though there are no signs of inflammation anymore, it is still very likely that the fetuses were exposed to short-lasting inflammation. Based on other studies, a peak inflammation should have taken place in the first 24 hours after injection at GD 10.5, in the dam and the fetus.[14] The major long-term consequence of short-term exposure to LPS-induced inflammation at GD10.5 is increased food intake and weight gain when exposed to a western-style diet later in life, likely caused by disturbed hypothalamic pathways, in female offspring only.[15]

Metabolic markers related to gluconeogenesis, lipid, and cholesterol biosynthesis were analyzed in the fetal liver. Unlimited gluconeogenesis is a risk factor in growth-restricted fetuses, as this increases plasma glucose levels which can result in insulin resistance.[13] In our study, mRNA levels of the gluconeogenesis-promoting genes *Pepck*, *G6pc,* and *Sirt1* were all significantly or

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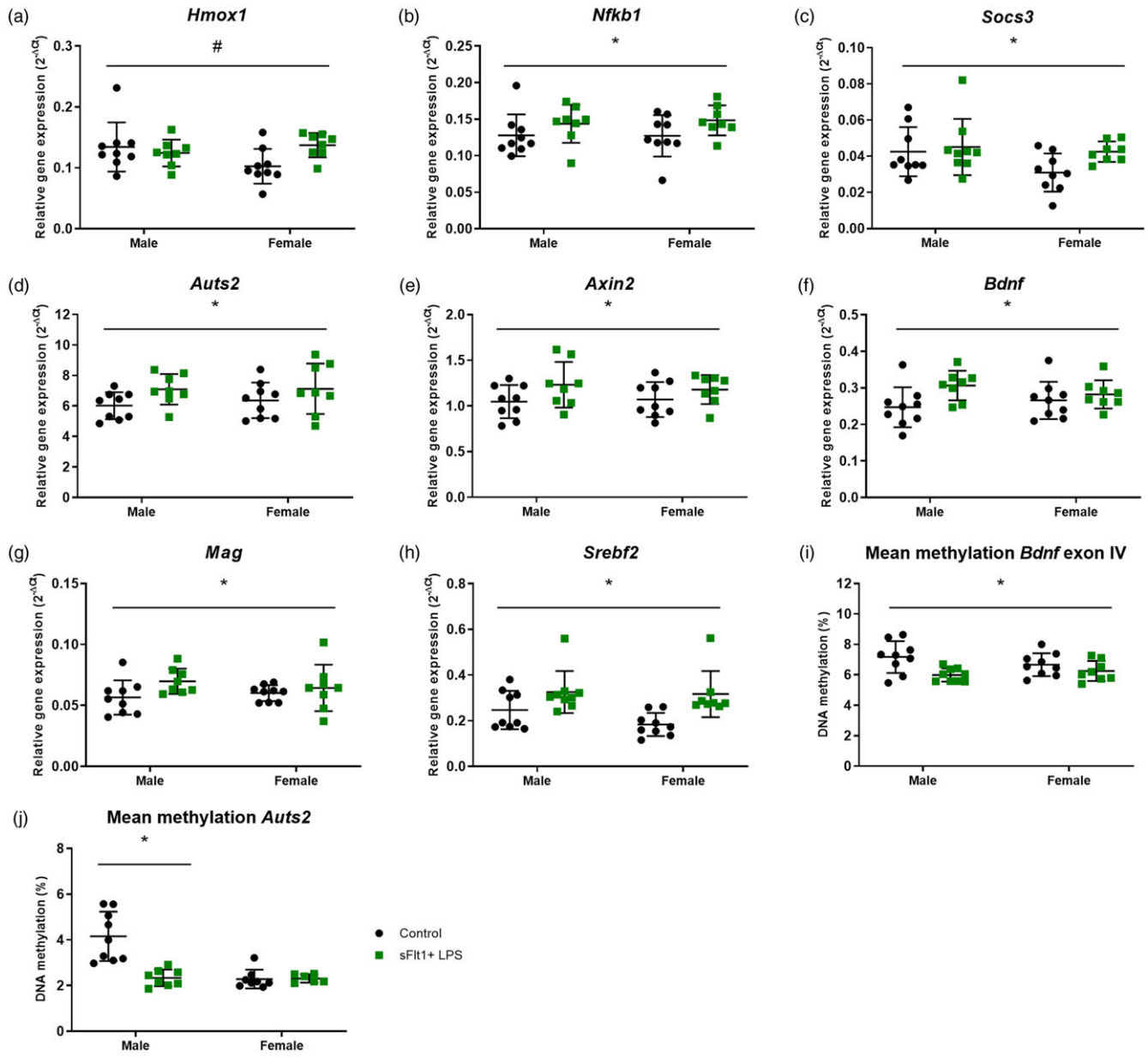

Fig. 2. Gene expression of inflammation, oxidative stress, and neurodevelopment-associated genes and DNA methylation of *Bdnf* and *Auts2* in fetal whole brain of preeclampsia-exposed offspring. Shown are the relative gene expressions of inflammatory and oxidative stress genes *Hmox1* (a), *Nfkb1* (b), *Socs3* (c), neurodevelopment-associated genes *Auts2* (d), *Axin2* (e), *Bdnf* (f), *Mag* (g), cholesterol biosynthesis marker *Srebf2* (h), the mean DNA methylation for *Bdnf* exon IV (i), and *Auts2* (j). Additional brain gene expression data are shown in Supplementary Table S4. DNA methylation per CpG position for *Bdnf* exon IV and *Auts2* promotor site is shown in Supplementary figure S2. Control groups and sFlt1+LPS male $n = 9$, sFlt1+ LPS female $n = 8$. Relative gene expression was calculated using the $2^{-\Delta Ct}$ method, normalized against housekeeping genes *Gapdh* and *36b4* and analyzed using two-way ANOVA. DNA methylation was analyzed with repeated measure two-way ANOVA. Data are presented as mean ±SD. * $p < 0.05$ for treatment, # $p = 0.065$ for treatment.

trending to be decreased, suggesting this experimental PE model is not promoting high glucose-induced insulin resistance. On the other hand, decreased gluconeogenesis could contribute to the observed fetal growth restriction. In adult mice, increased levels of *Pepck* in muscle are associated with increased physical activity. Both pre- and postnatal growth restrictions are associated with a reduction in physical activity.[16,17] Therefore, alternatively or concomitantly and when also shown in muscle, reduced levels of *Pepck* could be a molecular mechanism to reduce physical activity in a nutrient-deprived environment. It would be of interest to assess this further in a long-term study.

Gene expression of *Srebf2*, a master regulator of cholesterol biosynthesis, was found decreased in the current study and in a rat model of diabetic pregnancy as well.[18] Fetal cholesterol production is essential for growth and development, and lower *Srebf2* expression, although not a direct read-out of cholesterol levels, could therefore be related to the decreased fetal weight.[19] Lasting abnormal cholesterol homeostasis can be both cause and consequence of adverse metabolic programming in the fetus.[19] The *Srebf2* gene was hypomethylated in the experimental PE-exposed fetuses across several CpG positions, indicating long-lasting changes in *Srebf2* gene expression.

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In utero exposure to PE is associated with multiple adverse neurodevelopmental outcomes[5] and dysregulation of neurodevelopmental-associated genes influencing neuron differentiation and maturation, myelination, and axon guidance.[6–10] After induction of PE by sFlt-1 and LPS at GD8.5 and 10.5, a slight but significant upregulation of *Socs3, Hmox1, Nfkb1* was observed in the fetal brain at GD18.5. This is opposite of what is found in liver and indicates a lasting proinflammatory and oxidative stress response in fetal brain during maternal PE.

In PE-exposed fetal brain, increased gene expression was found for the neurodevelopmental-associated genes *Auts2, Axin2, Bdnf,* and *Mag*. *Auts2* is highly expressed in the central nervous system during development and is needed for neuronal migration and regulates synapse homeostasis. Multiple neurological disorders are associated with dysregulated *Auts2*.[20] *Axin2* is essential for myelination,[9] and *Mag* is a crucial myelin-related protein needed to maintain myelination of the axon.[7] Furthermore, the neurothrophin encoding gene *Bdnf* is essential for neuronal survival, axonal growth, and synaptic function. Increased transcription of *Bdnf* was observed in stress-resilient animals and could have long-term consequences as it increases neurogenesis in the hippocampus.[21] Chronical overexpression of *Bdnf* leads to learning and memory impairment, increased anxiety-like traits and seizure susceptibility.[22] The expression of cholesterol biosynthesis *Srebf2*, in contrast to the liver, is enhanced in the brain. Cholesterol metabolism plays an important role in the production of myelin and is crucial for synaptic structure and function in the brain.[23] The selected genes analyzed in the brain are directly linked to neurodevelopment. The alterations indicate an increase in neurogenesis in PE-exposed offspring in a non-sex-specific manner, possibly associated with brain sparing. However, not all neurodevelopmental-associated genes were differentially expressed in the PE-exposed offspring.

We need to keep in mind that the gene expression was measured for the whole brain, while some of the altered gene expressions in the literature were measured in selected brain regions or specific cells.[6,7,9] The gene expression of the specific regions of the brain could differ from the expression found for the whole brain.

Epigenetic changes in offspring exposed to PE *in utero* were observed, which can result in long-term adverse health effects. DNA methylation is a stable but reversible epigenetic marker regulating gene transcription.[2] Stable global DNA methylation between the groups, analyzed in repetitive element LINE1, shows that the overall DNA methylation machinery works properly. The transcription of *Bdnf* is highly regulated by DNA methylation of exon IV promotor sites.[21,24] The decreased DNA methylation found in the promotor site, including the two-transcription factor binding motifs, corresponds with the enhanced *Bdnf* expression in the fetal brain. Sex-specific differences in *Auts2* methylation were observed. Control males have a relatively high level of DNA methylation, while PE-exposed males show levels similar to both groups of female offspring. This might point to a neurodevelopmental difference between control and PE-exposed male offspring. However, as there is no correlation with the observed *Auts2* gene expression, the consequences of this DNA methylation difference remain uncertain.

Conclusion

In our PE mouse model, we have previously reported symmetrical growth restriction in female offspring, while male offspring showed brain sparing.[3] Brain sparing, a relatively small body but conserved brain size, is known as a protective event in growth restriction. Remarkably, those sex-specific growth restriction patterns are not reflected in the molecular data shown here. Our data in PE-exposed offspring point to decreased gluconeogenesis and *Srebf2* expression in the liver and potentially stimulated neurogenesis in the brain, independent of fetal sex. Only *Auts2* methylation was affected in a sex-specific manner. Although the growth restriction seemed worse in the PE-exposed female offspring and only males showed brain sparing, the molecular changes are generally similar between both sexes. This indicates PE, rather than the extend of growth restriction and brain sparing, is the driver of the molecular phenotype in the offspring.

Supplementary materials. For supplementary material for this article, please visit https://doi.org/10.1017/S2040174422000344

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Conflicts of interest. None.

Ethical standards. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Groningen (DEC licence #6803).

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