RESEARCH PAPER

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Prenatal antidepressant exposure associated with *CYP2E1* DNA methylation change in neonates

Cécile Gurnot[1,2], Ignacio Martin-Subero[3], Sarah M Mah[4], Whitney Weikum[5], Sarah J Goodman[4], Ursula Brain[5], Janet F Werker[6,7], Michael S Kobor[4,7,8], Manel Esteller[9,10,11], Tim F Oberlander[5,8,*], and Takao K Hensch[1,2,7,*]

[1]Center for Brain Science; Department of Molecular Cellular Biology; Harvard University; Cambridge, MA USA; [2]FM Kirby Neurobiology Center; Department of Neurology; Children's Hospital Boston; Harvard Medical School; Boston, MA USA; [3]Department of Anatomic Pathology, Pharmacology and Microbiology; University of Barcelona; Barcelona, Spain; [4]Centre for Molecular Medicine and Therapeutics; Child and Family Research Institute; Department of Medical Genetics; University of British Columbia; Vancouver, BC Canada; [5]Developmental Neuroscience and Child Health; Child & Family Research Institute; University of British Columbia; Vancouver, BC Canada; [6]Department of Psychology; University of British Columbia; Vancouver, BC Canada; [7]Child Brain Development Program; Canadian Institute for Advanced Research; Toronto, ON Canada; [8]Human Early Learning Partnership; University of British Columbia; [9]Cancer Epigenetics and Biology Program (PEBC); Bellvitge Biomedical Research Institute (IDIBELL); L'Hospitalet, Barcelona, Catalonia, Spain; [10]Department of Physiological Sciences II; School of Medicine; University of Barcelona; Barcelona, Catalonia, Spain; [11]Institucio Catalana de Recerca i Estudis Avan, cats (ICREA); Barcelona, Catalonia, Spain

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Abbreviations: CNV, copy number variation; HR, heart rate; SRI, serotonin reuptake inhibitor; HAMD, Hamilton Rating Scale for Depression.

Some but not all neonates are affected by prenatal exposure to serotonin reuptake inhibitor antidepressants (SRI) and maternal mood disturbances. Distinguishing the impact of these 2 exposures is challenging and raises critical questions about whether pharmacological, genetic, or epigenetic factors can explain the spectrum of reported outcomes. Using unbiased DNA methylation array measurements followed by a detailed candidate gene approach, we examined whether prenatal SRI exposure was associated with neonatal DNA methylation changes and whether such changes were associated with differences in birth outcomes. Prenatal SRI exposure was first associated with increased DNA methylation status primarily at *CYP2E1*($\beta_{Non\text{-}exposed} = 0.06$, $\beta_{SRI\text{-}exposed} = 0.30$, FDR = 0); however, this finding could not be distinguished from the potential impact of prenatal maternal depressed mood. Then, using pyrosequencing of *CYP2E1* regulatory regions in an expanded cohort, higher DNA methylation status—both the mean across 16 CpG sites ($P < 0.01$) and at each specific CpG site ($P < 0.05$)—was associated with exposure to lower 3rd trimester maternal depressed mood symptoms only in the SRI-exposed neonates, indicating a maternal mood x SRI exposure interaction. In addition, higher DNA methylation levels at CpG2 ($P = 0.04$), CpG9 ($P = 0.04$) and CpG10 ($P = 0.02$), in the interrogated *CYP2E1* region, were associated with increased birth weight independently of prenatal maternal mood, SRI drug exposure, or gestational age at birth. Prenatal SRI antidepressant exposure and maternal depressed mood were associated with altered neonatal *CYP2E1* DNA methylation status, which, in turn, appeared to be associated with birth weight.

Introduction

Prenatal exposure to maternal mood disturbances alter fetal development and may set pathways that have long-term developmental consequences.[1] Prenatal exposure to maternal depression is also associated with lower birth weight, short gestational length, and neurobehavioral alterations during childhood.[2–8] To treat these prenatal mood disturbances, serotonin reuptake inhibitor (SRI) antidepressants can be prescribed, but such prenatal SRI exposure was associated in some studies with preterm delivery, lower birth weight, changes in perceptual learning, congenital malformations, and neurobehavioral disturbances extending from fetal periods to early childhood. [9–20] Therefore, in both cases, adverse outcomes may occur, explaining why there are not yet clear guidelines on how to treat depression during pregnancy.

Studies on epigenetic mechanisms are increasingly contributing to the improvement of our current understanding on the association between prenatal exposure to maternal mood disorders and/or antidepressants and the spectrum of neonatal outcomes. Recent evidence now points to both exposures as factors modulating the epigenetic regulation of gene expression.[22] For example, in mice, maternal stress increases transcription of the DNA methyltransferases DNMT1 and DNMT3a in a tissue-specific manner, as well as the DNA methylation level of the placental enzyme 11β-hydroxysteroid dehydrogenase type 2 (*11β-HSD2*) gene.[23] Likewise, adult exposure to fluoxetine has been

*Correspondence to: Tim F Oberlander; Email: toberlander@cw.bc.ca; Takao K Hensch; Email: takao.hensch@childrens.harvard.edu
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shown to increase global expression of histone deacetylase HDAC5,[28] HDAC2 in the caudate putamen,[29] and miR-16 levels in serotonergic raphe neurons in rodents.[30] Other studies report that chronic exposure to escitalopram leads to a reduction in DNA methyltransferase (DNMT1 and 3a) and hypomethylation of the *P11* promoter leading to an increase of P11 protein.[31] Conversely, direct administration of inhibitors of histone deacetylase like sodium butyrate[32] or MS-275,[33] or of DNMT, such as RG108[34] or zebularine,[35,36] or overexpression of histone methyltransferases, such as G9A[33,37] and SETDB1,[38] exhibit antidepressant-like effects in several behavioral assays.

Additionally, some studies have focused on the effect of prenatal maternal mood disorders on neonatal DNA methylation, suggesting an association with *NR3C1*,[24,39] *SLC6A4*,[27,39] *NFKB2*, *FKBP5,* and *CRHR1*[39]; however, none of these genes have been identified in studies taking an epigenome-wide approach.[39,40] Similarly, prenatal SRI exposure has been found to be associated with changes in neonatal DNA methylation in *NR3C1*,[24,25,39] *NFKB2*, *SLC6A4*, *FKBP5*, and *DNMT3a,*[39] but never by epigenome-wide approach.[39,40] Instead, epigenome-wide studies, have identified 10[39] novel candidate genes associated with prenatal maternal mood disorders and 2 (*TNFRSF21* and *CHRNA2)*[40] with prenatal SRI exposure. However, in all of these cases, the biological significance of these findings was challenged by the small mean DNA methylation difference between groups. Therefore, additional studies may deepen our understanding on the potential epigenetic link between prenatal mood, antidepressant exposure, and DNA methylation.

In the present study, we investigated whether *in utero* SRI exposure was associated with variations in the neonatal DNA methylation status of > 27,000 CpG sites using umbilical cord blood white blood cells (WBC). Guided by our microarray findings, we undertook pyrosequencing of one putative candidate gene, *CYP2E1*, using an expanded cohort to further determine the impact of prenatal SRI exposure and to examine whether variations in DNA methylation were influenced by antidepressant exposure, antenatal maternal mood, or a combination of both. Finally, we examined whether these outcomes were associated with birth outcomes.

Results

The study cohort consisted of 44 women, including 19 depressed and SRI-treated and 25 not SRI-treated, prospectively recruited during pregnancy (see Methods below). All mothers were assessed at approximately 26 weeks and again at 36 weeks of their pregnancy.We used a mean score of the 3rd trimester measures of maternal mood (**Table 1A**). Because maternal mood varied both within and between groups, it was used as a continous variable and treated as a covariable, where appropriate. With the exception of higher depressed mood scores (mean prenatal) and psychotropic medication use, maternal characteristics did not vary significantly between groups ($P > 0.05$). All mothers took a prenatal vitamin containing the typical prenatal folic acid dose (0.8–1 mg).

Infant characteristics are presented in **Table 1A**. All infants were full term, although gestational age at birth was significantly reduced in SRI-exposed neonates ($GA_{NO\_SRI} = 40.3$ wks, $GA_{SRI} = 37.0$ wks, $P < 0.01$), as was head circumference (HeadCircumf.$_{NO\_SRI} = 35.3$ cm, HeadCircumf.$_{SRI} = 32.5$ cm, $P = 0.01$) and apgar at 1 min (Apgar1$_{NO_SRI} = 8.8$, Apgar1$_{SRI} = 7.2$, $P < 0.01$). SRI maternal and cord drug levels varied between medications. However, fetal (cord)-maternal drug ratio remained stable between drugs (mean: 0.73, sd: 0.33) (**Table 1B***)*, reflecting the extent of transplacental transfer, fetal drug exposure, and maternal and fetal capacity to metabolize the drug.

Microarray findings

To examine whether *in utero* exposure to SRIs alters neonatal DNA methylation patterns at an epigenome level, we analyzed the methylation profiles of > 27,000 CpG sites in genomic DNA from cord blood in a subset of the whole cohort, which included neonates with (n =12) and without (n = 11) prenatal SRI antidepressant exposure. After correcting for multiple testing, increased methylation levels at 3 CpG sites were associated with prenatal antidepressant exposure (FDR = 0): 1) *CYP2E1 cg13315147* ($\beta_{Non\text{-}exposed} = 0.06$, $\beta_{SRI\text{-}exposed} = 0.30$) (**Fig. 1**); 2) *EVA1 cg18399703* ($\beta_{Non\text{-}exposed} = 0.04$, $\beta_{SRI\text{-}exposed} = 0.06$) and; 3) *SLMAP cg11743795* ($\beta_{Non\text{-}exposed} = 0.02$, $\beta_{SRI\text{-}exposed} = 0.03$).

Among these candidates, we performed further analysis for the CpG site with the highest methylation difference between non-SRI and SRI-exposed neonates, *CYP2E1,* as it had the highest likelihood of functional relevance. For this CpG site, a high degree of correlation was found between maternal mood scores and *CYP2E1* DNA methylation values in the SRI-exposed group (r^2: −0.81, $P < 0.01$, n = 11), but not in the non-SRI group (r^2: 0.17, $P = 0.58$, n = 12), suggesting a mood x drug interaction. To further test this hypothesis, we examined associations between mean maternal 3rd trimester depression symptoms, *in utero* SRI exposure status, and methylation status at the *CYP2E1* CpG site in the whole cohort. This analysis revealed a main effect for SRI exposure status ($F(1,23) = 63.975$, $P < 0.01$, $\acute{\eta}^2 = 0.771$), as well as a significant interaction between maternal mood and exposure status ($F(1,23) = 29.229$, $P < 0.01$, $\acute{\eta}^2 = 0.606$), but not for maternal mood alone ($F(1,23) = 3.93$, $P = 0.06$, $\acute{\eta}^2 = 0.741$) (DNA methylation differences 0.087 vs. 0.36, non-SRI vs. exposed; **Fig. 2; Table S1**).

CYP2E1 has been associated twice previously with psychogenic stressors. In C57BL/6 mouse liver, chronic psychoemotional stress reduces CYP2E1 protein levels 2-fold.[42] Lam et al. (2012)[43] analyzed *CYP2E1* DNA methylation in adult human peripheral blood mononuclear cells (PBMC) (n = 55), early social economic status (SES), and depression values and revealed a main effect for early SES ($F(1,92) = 4.41$, $P = 0.039$, $\acute{\eta}^2 = 0.048$), but not for depression alone ($F(1,92) = 0.545$, $P = 0.462$, $\acute{\eta}^2 = 0.006$) or for the interaction between early SES and depression ($F(1,92) = 1.57$, $P = 0.213$, $\acute{\eta}^2 = 0.18$) (**Table S1**). The first study implicates that stress factors play a role in CYP2E1 levels, and the latter suggests that early stress exposure may induce long-term epigenetic changes on *CYP2E1*.

Table 1. A. Maternal and Fetal details

		PYROSEQUENCED COHORT			MICROARRAY COHORT		
		Non-SRI-exposed	SRI-exposed	Sig.	Non-SRI-exposed	SRI-exposed	Sig.
		Mean ± SD (n)	Mean ± SD (n)		Mean ± SD (n)	Mean ± SD (n)	
MATERNAL DETAILS	Education (yrs)	17.46 ± 2.98 (24)	16.65 ± 4.57 (19)	0.93	16.59 ± 2.81 (12)	17.55 ± 3.83 (11)	0.5
	HAM-D† Score (Mean 26-36 wks Gest.)	7.06 ± 5.10 (25)	10.51 ± 5.73 (18)	0.02*	7.25 ± 5.75 (12)	11.64 ± 6.84 (11)	0.11
	Smoking (yes/no)	0 (25)	0.05 ± 0.23 (18)	0.33	0 (12)	0.09 ± 0.30 (11)	0.34
	Alcohol (no. of drinks for entire pregnancy)	3.96 ± 6.55 (25)	4.03 ± 5.92 (18)	0.88	4.79 ± 8.28 (12)	5.27 ± 7.36 (11)	0.87
	Maternal Age at birth (years)	34.3 ± 5.2 (25)	31.3 ± 7.9 (19)	0.28	34.0 ± 5.9 (12)	31.9 ± 4.9 (11)	0.36
	Delivery type (vaginal/c/s)	20/5 (25)	16/3 (19)		11/1 (12)	11/0 (11)	
	Length Prenatal SRI exposure (days)	0 (25)	272.1 ± 9.6 (19)		0 (12)	270.8 ± 10.2 (11)	
	Prenatal SRI Medications (median mg/d)						
	Paroxetine	—	25 ± 7.1 (2)		—	25 ± 7.1 (2)	
	Fluoxetine	—	46.7 ± 23.1 (3)		—	60 ± 0 (2)	
	Sertraline	—	175 ± 35.4 (2)		—	175 ± 35.4 (2)	
	Citalopram	—	40 ± 0 (2)		—	40 (1)	
	Venlafaxine	—	159.5 ± 53.9 (10)		—	145.3 ± 69.2 (4)	
FETAL DETAILS	Gender (m/f)	(15/10) (25)	(8/11)	0.25	6/6)	(5/6)	0.84
	Birth Gest. Age (wks)	40.3 ± 1.1 (25)	37.0 ± 8.8 (19)	5.10^{-4}*	39.9 ± 1.0 (12)	38.4 ± 1.4 (11)	0.01*
	Birth Weight (g)	3621 ± 408 (25)	3245 ± 768 (19)	0.22	3614 ± 312 (12)	3352 ±356 (11)	0.08
	Head Circumference (cm)	35.34 ± 0.99 (25)	32.53 ± 7.76 (19)	0.01*	35.29 ± 1.13 (12)	33.70 ±1.28 (11)	5.10^{-3}*
	Apgar at 1 min.	8.76 ± 0.52 (25)	7.22 ± 2.15 (19)	1.10^{-3}*	8.58 ± 0.67 (12)	7.91 ± 1.51 (11)	0.19
	Apgar at 5 min.	9.04 ± 0.35 (25)	8.55 ± 1.98 (19)	0.57	9.0 ± 0.43 (12)	9.0 ± 0 (11)	1

B. SRI levels in the pyrosequensed cohort (ng/ml): Maternal (delivery), Neonatal (cord blood) and Fetal/Maternal ratio (delivery)

	Maternal Drug Level (Delivery)	Neonatal Drug Level (Cord)	Fetal/Maternal ratio Drug Level (Delivery)
	Mean ± SD (n)	Mean ± SD (n)	Mean ± SD (n)
Fluoxetine	278.1 ± 249.0 (3)	177.4 ± 151 (3)	0.70 ± 0.18 (3)
Norfluoxetine	228.3 ± 154.3 (3)	153.2 ± 101.0 (3)	0.73 ± 0.26 (3)
Paroxetine	7.9 ± 1.7 (2)	5.8 ± 5.5 (2)	0.69 ± 0.6 (2)
Sertraline	54.6 ± 27.1 (2)	20.9 ± 6.4 (2)	0.47 ± 0.35 (2)
Citalopram	27.8 ± 10.8 (2)	22.3 ± 9.5 (2)	0.80 ± 0.03 (2)
Venlafaxine	21.5 ± 15.6 (6)	17.0 ± 9.9 (7)	0.86 ± 0.34 (6)
MEAN			0.74 ± 0.33 (19)

Pyrosequencing findings

To confirm *CYP2E1* DNA methylation values obtained by microarray, we pyrosequenced the region including and surrounding the relevant *CYP2E1* CpG site (ch10:135,341,528, NCBI36/hg19) interrogated on the microarray. We found that DNA methylation values obtained by pyrosequencing and for the CpG site on the microarray were correlated ($r^2 = 0.65$, $P < 0.01$, n = 21). The pyrosequenced region contained 15 additional CpG loci in close proximity of the array CpG. All were localized between +626 and +742 from the ATG of the *CYP2E1-001 ENST00000463117* transcript (**Fig. 3**), and their DNA methylation values were correlated with each other ($0.81 < r^2 < 0.98$) (**Fig. 4**), suggesting use of the DNA methylation mean as a reliable marker for this region. Moreover, DNA methylation status (either by microarray or pyrosequencing) did not vary with cord-maternal drug ratios (all $P > 0.05$).

These results encouraged us to further investigate the *CYP2E1* DNA methylation difference observed between SRI and non-SRI neonates in an expanded cohort that would enable us to examine the possible concurrent influence of maternal mood disturbances. Similar to the array findings, we found that increased 3rd trimester maternal mood was associated with lower *CYP2E1* methylation only in the exposed (r^2: −0.63, $P < 0.01$, n = 17) and not for the non-SRI (r^2: 0.11, $P = 0.57$, n = 25) infants across the whole cohort (**Fig. 5; Table S1**).

When run on the whole pyrosequenced cohort, regression analysis confirmed the main SRI exposure effect and the interaction between maternal mood and SRI status on *CYP2E1* mean

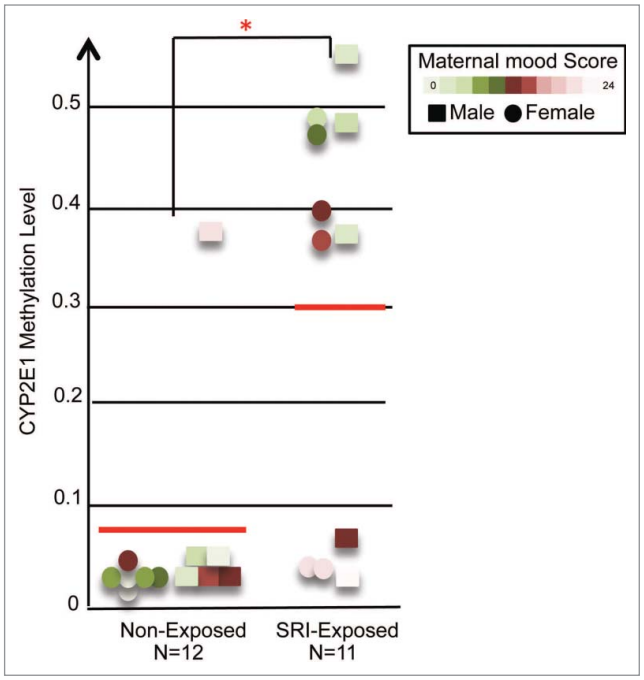

Figure 1. Methylation level for *CYP2E1* CpG in neonates exposed or not to antidepressants. Range from 0 to 1. Differences between groups was 0.24. Colors represent mean HAMD maternal mood. Note exposed neonates with high *CYP2E1* DNA methylation level had mothers with low HAMD maternal mood score, while exposed neonates with low DNA methylation level had mothers with high HAMD scores.

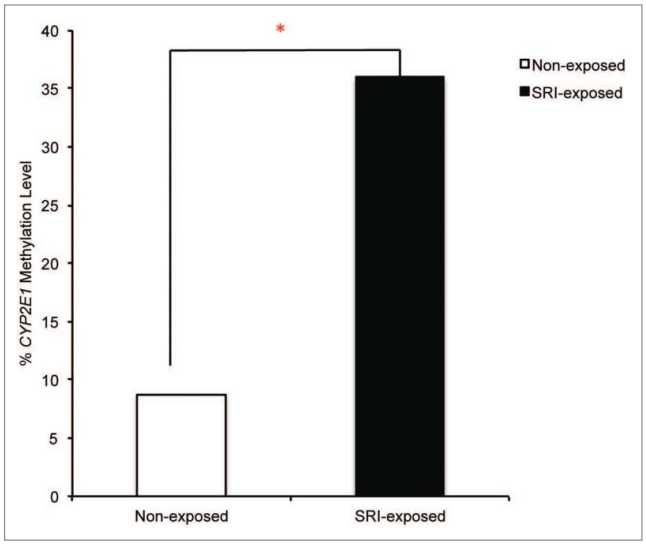

Figure 2. Estimated *CYP2E1* CpG methylation level using covariance analysis for neonates exposed or not to antidepressants. DNA methylation levels for SRI- and non-exposed were 36% and 8.7%, respectively ($P < 0.05$, controlling for mean HAMD maternal mood and interaction between mean HAMD maternal mood and SRI exposure).

CpG ($P < 0.01$ for both), as well as on each specific CpG site ($P < 0.05$ for both) (**Table S1**). Moreover, we found a significant maternal mood effect on *CYP2E1* mean CpG methylation and on 12 out of 16 pyrosequenced CpG sites.

Follow-up analysis to examine the direction of this interaction was done by regression analysis on both non-SRI and exposed groups with methylation status at CpG sites as the dependent variable, and mean 3rd trimester maternal mood as predictor. These were run separately for each group so there was no interaction term in these analyses—that is, SRI exposure was not a factor. We found that increased prenatal 3rd trimester depressed mood symptoms predicted lower levels of neonatal methylation—but only in the SRI-exposed group for the CpG mean ($\beta = -0.696$, $P = 0.001$) (**Fig. 6**) and for all specific CpG sites (**Table S1**). In the interaction between SRI exposure and maternal mood, SRI exposure moderated the effect of mothers' antenatal mood on DNA methylation, whereby maternal mood only mattered to DNA methylation status with SRI exposure.

Birth outcomes

To examine whether neonatal *CYP2E1* CpG methylation status predicted indices of neonatal health, key birth outcome measures were examined in separate regression models with SRI exposure status, mean maternal prenatal mood and an interaction term between these key covariates. With the exception of birth weight, no neonatal outcomes (gestational age, length, head circumferance) were associated with methylation status at any of the 16 CpG sites in *CYP2E1*. Decreased birth weight was predicted by decreased methylation status at CpG2 ($\beta = 0.359$; $P = 0.04$), CpG9 ($\beta = 0.352$; $P = 0.04$), and CpG10 ($\beta = 0.439$; $P = 0.02$), regardless of SRI exposure and controlling for gestational age at birth, mean antenatal depressed mood symptoms and the interaction between antenatal maternal mood and SRI exposure status (**Fig. 7**, **Table S1**). Neonatal pyrosequencing of *CYP2E1* DNA methylation status did not vary significantly with mode of delivery (cesarean section vs. vaginal delivery) ($P > 0.05$).

Discussion

CYP2E1 DNA methylation is associated with maternal mood in SRI-exposed neonates

Using measures of both mean methylation status across 16 CpG sites and at specific CpG sites, this study reports an association between higher DNA methylation status of *CYP2E1* and exposure to lower 3rd trimester maternal depressed mood symptoms in SRI-exposed neonates. Although it is not entirely clear why *CYP2E1* DNA methylation was associated only with maternal mood in SRI exposed neonates, one possibility is that CYP2E1 functions as a "buffer" against the adverse effects of SRI exposure and thus acts to "protect" the developing fetus. Consistent with this hypothesis, suppression of CYP2E1 expression in mothers and neonates during late pregnancy has been hypothesized to "protect" the fetus from oxidative stress in several studies.[44,45]

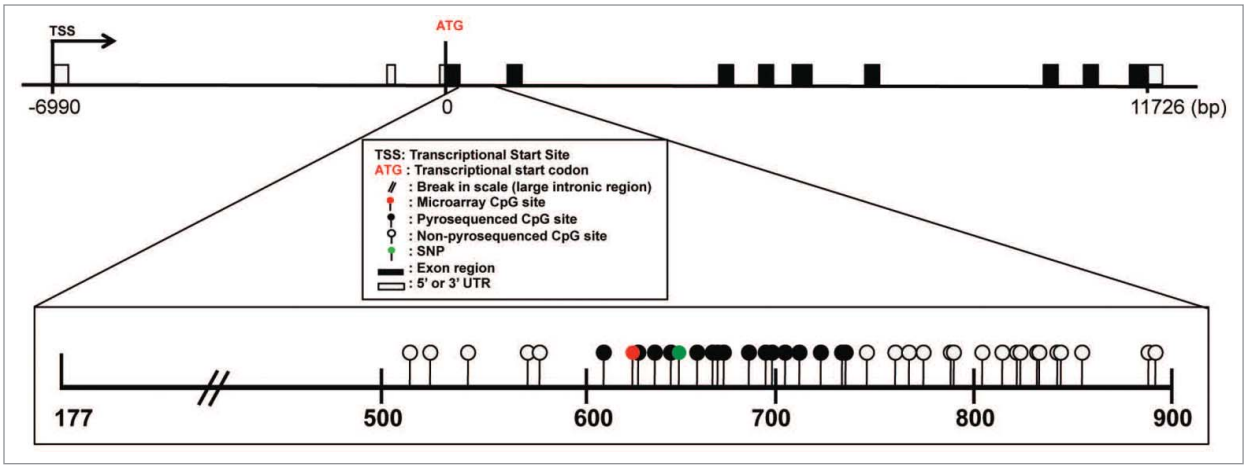

Figure 3. CYP2E1-001 (ENST00000463117) transcript structure. TSS, ATG, and CpG on the microarray were mapped to chromosome coordinates with respect to the human genome build ch10:135,333,910, ch10:135,340,900 and ch10:135,341,528, respectively. Pyrosequenced CpGs were localized between +626 and +742 from the ATG site.

CYP2E1 is a member of the hepatic cytochrome P450 oxidase system found to be involved in the metabolism of numerous xenobiotics, such as carcinogenic nitrosamines, benzene, low-molecular-weight compounds and key enviromental exposures including acetaminophen, volatile anesthetics, ethanol, and tobacco, but not yet for antidepressants.[46] The cytochrome P450 (CYP) 2D6 enzyme has been shown to be inhibited by some SRIs, such as fluoxetine,[47] suggesting the possibility that another CYP family member like CYP2E1 might be indirectly inhibited through SRI. Importantly, other key influences, such as CYP450 genetic variations, also influence SRI pharmacology and, presumably, *in utero* drug exposure. While CYP450 genotyping was beyond the scope of this study, fetal drug exposure (as reflected by cord/maternal drug ratios) as a reflection of metabolic capacity was not associated with DNA methylation status, offering some, albeit indirect, evidence that our findings may reflect an epigenetic mechanism that could potentially link SRI exposure with birth outcomes.

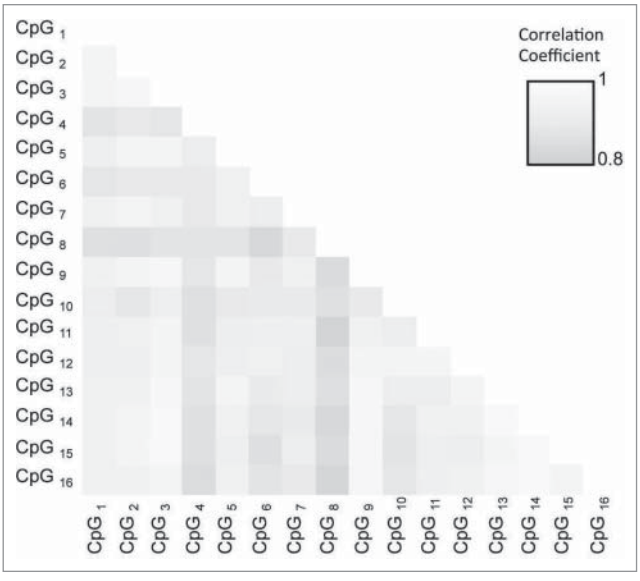

Figure 4. Pearson correlation matrix describing methylation status of the promoter region of *CYP2E1* across 16 CpGs of all neonates. Scores range from 0.8 to 1.

The mechanism underlying regulation of neonatal *CYP2E1* DNA methylation remains to be determined. A potential candidate for increased cord blood DNA methylation in SRI-exposed neonates could involve the upregulation of DNA methyltransferase levels. Previous studies have shown an increase in placental DNMT3a mRNA and DNMT1 in hypothalamus and cortex in response to maternal stress.[23,26] Furthermore, SRI antidepressants have been associated with changes in gene expression associated with chromatin remodeling,[48,49] histones,[50] and increased expression of methyl binding proteins MeCP2 and MBD1.[29] Together, these studies suggest that either SRI and/or maternal mood may have a direct or indirect effect on these molecules and may participate in *CYP2E1* DNA methylation levels.

Previous attempts using an epigenome-wide approach to identify changes associated with prenatal SRI exposure have yielded mixed results.[39,40] One study failed to identify DNA methylation changes, and maternal mood was not accounted for in their analytic models.[39] This could be explained by a lack of data on the severity of depression/anxiety (no HAM-D scores were used), and, therefore, an inability to account for it in their analytic models. Moreover, the specific *CYP2E1* site identified in our current study was not included in the 450 K Illumina platform used in that study. In another report,[40] methylation differences at 2 (*TNFRSF21*and *CHRNA2)* CpG sites were associated with prenatal SRI exposure. Importantly, the difference between exposure groups was small (1–3%) raising questions about biological significance. Their cohort also included mothers with a variety of mental health disorders (bipolar, schizophrenia) and

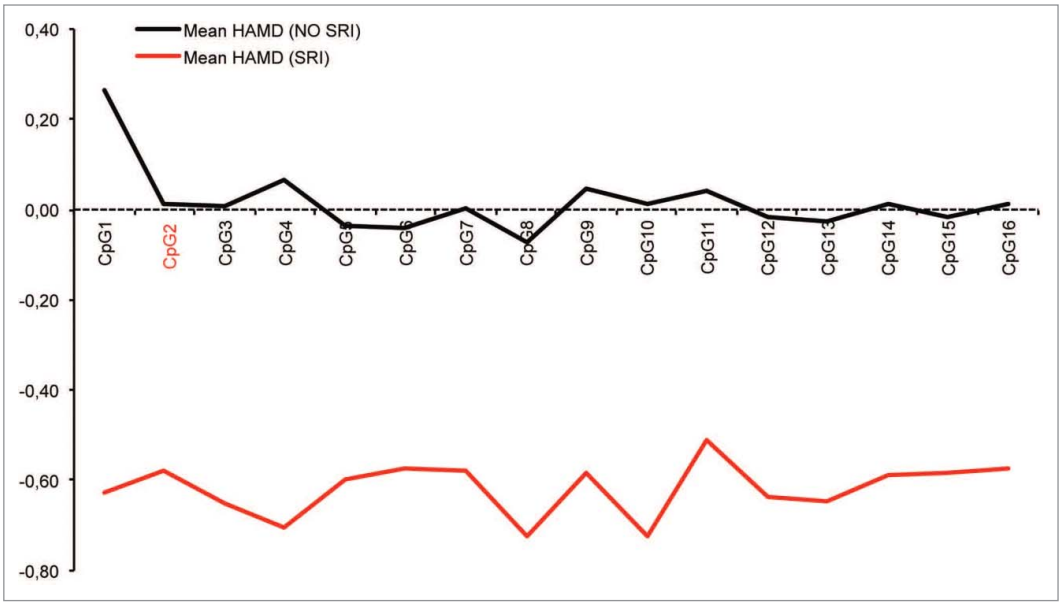

Figure 5. Pearson correlation between maternal mood and neonatal methylation status of 16 CpGs within the promoter region of CYP2E1. Scores were low for the non-exposed and high for SRI-exposed groups. The CpG2, highlighted in red, was included on the microarray (ch10:135,341,528).

exposure to additional psychotropic medications (e.g., hypnotics, benzodiazepines, antiemetics, bupropion, TCAs, atypical antipsychotics), which may have introduced a spectrum of illness severity and pharmacological factors beyond the characteristics of our cohort. Future studies are needed to examine epigenetic changes related to illness severity and non-serotonin specific psychotropic medications.

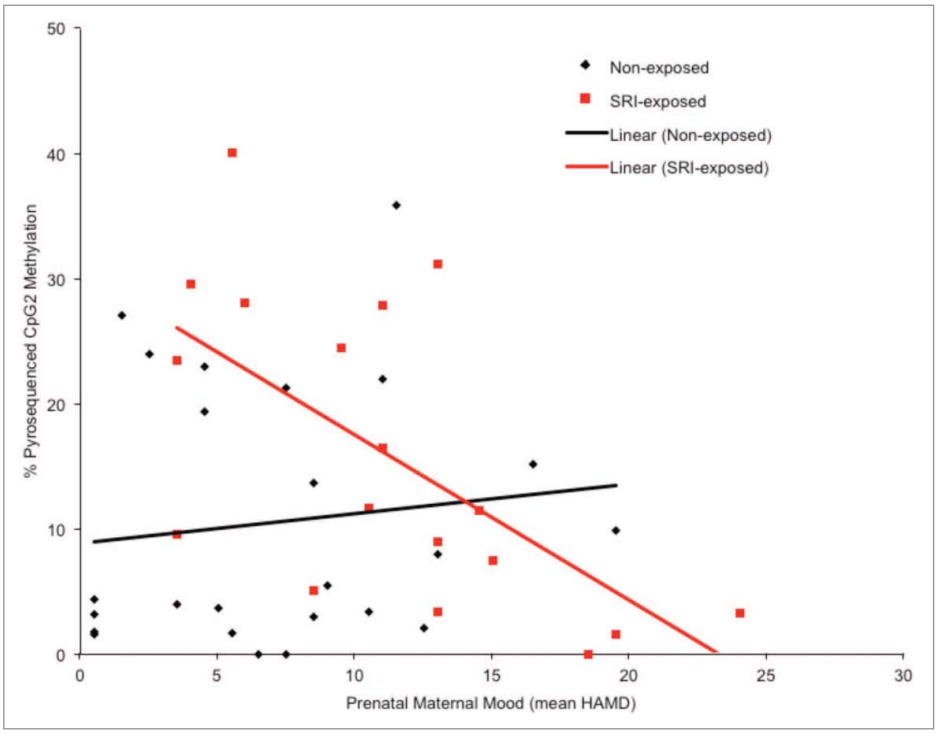

Figure 6. Methylation status of pyrosequenced CpG2 (ch10:135,341,528), maternal prenatal mood (mean 3rd trimester), and SRI interaction. Increased prenatal maternal mood (mean HAMD) predicted lower levels of neonatal DNA methylation in SRI-exposed, but not in the non-exposed group.

CYP2E1 DNA methylation level and birth weight

Previous studies have identified several epigenetically regulated genes associated with fetal growth trajectories. In placenta, high *WNT2* promoter methylation (*WNT2PrMe*), high methylation of the glucocorticoid receptor, and low miR-16 and miR-21, were found to be associated with low birth weight.[51–53] In cord blood, low methylation of the imprinted insulin-like growth factor 2 (*IGF2*) gene was associated with high birth weight.[54]

Our study links epigenetic variation to birth weight and further links higher *CYP2E1* DNA methylation for the CpG2, 9, and 10 at birth and increased birth weight that is independent of maternal mood, SRI drug exposure, and gestational age at birth. These assocations may suggest that high *CYP2E1* methylation levels could reflect an as yet unknown mechansim that could "protect" the fetus from adverse SRI effects, such as low birth weight. This is supported by the observation that neonates of mothers who received SRI treatment but remained symptomatic had lower DNA methylation levels and lower birth weights, while neonates of mothers who received SRI treatment but did not remain symptomatic had higher DNA methylation levels and higher birth weights.

Indeed, maternal allelic variations in *CYP2E1* have been associated with reduced birth weights and lengths, independent of maternal smoking status.[55] However, another study has not reported an association between the 3 *CYP2E1* genotypes tested and reduced birth- weight,[56] suggesting that other important factors are involved in birth weight outcomes. In fact, CYP2E1 may

have other developmental effects that were not considered in this study. It is a key cellular prooxidant and a source of ROS,[57] associated with increased risk for cellular injury. Studies report its implication in risks associated with obesity,[58] fasting,[59] diabetes,[60,61] liver disease,[62] Parkinson's disease,[63] and non-syndromic oral cleft.[64]

Limitations

Previous studies have shown an association between *CYP2E1* DNA methylation and *CYP2E1* gene expression in different human tissues, such as lung, liver, placenta, skin, and neuroma,[65–68] but not in adult human PBMC for this specific *CYP2E1* CpG.[43] Botto and colleagues, using a 3' probe corresponding to positions 749–1,623 of the cDNA, showed that hypomethylation of the 3' end of the coding region resulted in reduced expression of the gene.[69] It remains unclear whether the modest differences in *CYP2E1* DNA methylation levels between SRI- and non-exposed neonates, and between low- and high-birth weights, had an effect in terms of gene expression, fetal physiological function, or clinical relevance, especially in a cohort in which birth weight remained within the normal range (range: 2,679–4,805 grams).

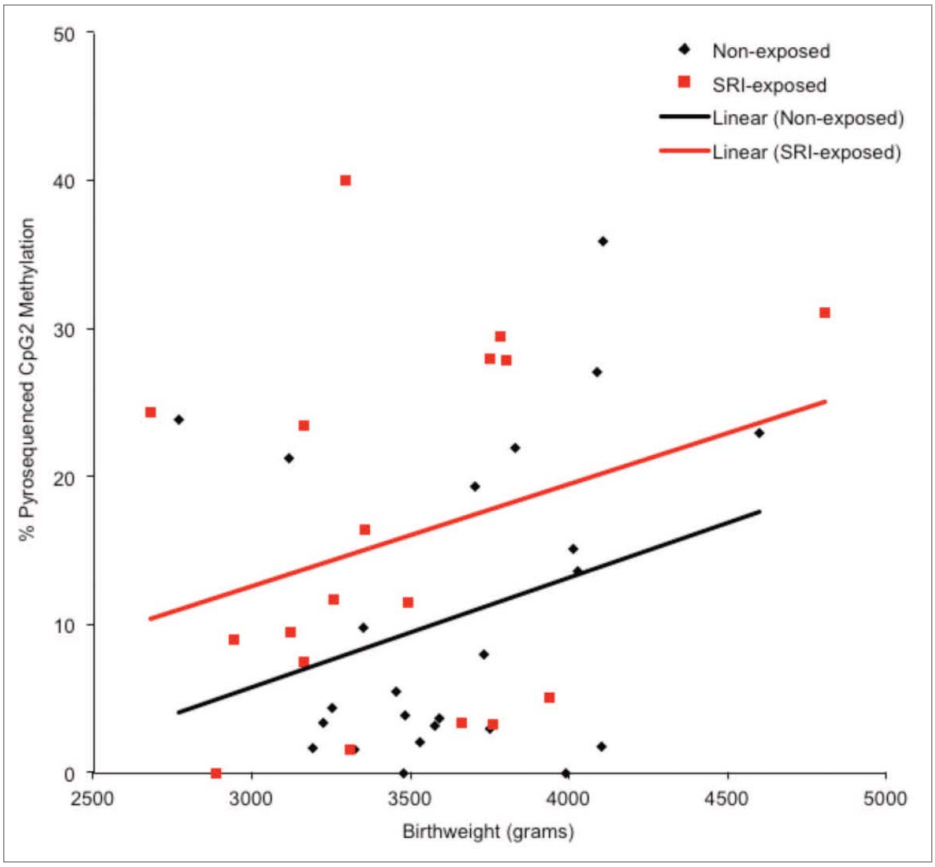

Figure 7. Methylation status of pyrosequenced CpG2 (ch10:135,341,528), birth weight, and prenatal SRI exposure. Increased birth weight predicted higher levels of neonatal DNA methylation status in both groups.

A number of studies support a negative correlation between DNA methylation and CYP2E1 expression within a single sample. For example, DNA methylation of specific 5' residues in the *CYP2E1* gene has been associated with lack of *CYP2E1* transcription in fetal liver.[67] Similarly, *CYP2E1* transcription has been associated with specific demethylation of CpG sites located in the first-exon-first-intron region, suggesting that the location of methylated CpG sites could play a role in the modulation of CYP2E1 transcription rate. However, as was previously published for a number of genes, *CYP2E1* DNA methylation patterns were not linked to expression levels when comparing multiple samples.[43]

In addition, *CYP2E1* is also within a known region of copy number variation with common duplication alleles among different populations.[70] Duplicated *CYP2E1* alleles have a probability of 0.029 (n = 206) in caucasians and have been found in two 5' promoter region repeat alleles, *1C (6 repeats) and *1D (8 repeats, frequency of 1% in caucasian) but never in *CYP2E1**2, *3, *4, *5, *6, or *7 variants.[71] Although the biological implication of these findings remain unclear, they could potentially alter the metabolism of drugs such as acetaminophen, isoniazid, tamoxifen, and procarcinogens by modifying CYP2E1 enzyme activity or by altering ethanol oxidation in the liver. To date, only alleles of *CYP2E1* (e.g., *1D, *5B, and *6) have been associated with alcoholism and cancer development in different ethnoracial groups.[72-76] Whether CNV has implications for fetal or neonatal development following *in utero* SRI exposure remains to be determined.

While we used fetal/maternal drug ratios as an index of fetal drug exposure that might reflect maternal and neoantal drug metabolic capacity, this is a distal measure that was not able to distinguish exact contributing mechanisms. Beyond *in utero* SRI exposure alone, factors such as maternal drug dose, differences in SRI potency, maternal and fetal drug metabolism, and the role of pregnancy itself may all influence the extent of fetal drug exposure, thereby potentially contributing to inter-individual variability of SRI responses in neonatal outcomes.[1,21] Genetic variations in CYP450 enzymes have been shown to alter SRI response in adults,[77] but limited evidence exists in neonates or infants.[78] It is conceivable that variability in outcomes following *in utero* SRI exposure could be due to genetic variations in key regulatory genes (e.g., 3A4, 2C19, 2D6).[77] In the current study, we included maternal and cord drug levels as well as fetal/maternal ratios as index of the exent of fetal drug exposure. Given

differences in drug pharmacology and our small-sized cohort, further study is needed to examine the impact of *CYP450* genetic variations on fetal drug exposure, DNA methylation status, and developmental outcome.

The relationship between *CYP2E1* DNA methylation and *CYP2E1* gene expression would then appear to be more complex than a simple association of high DNA methylation levels with low gene expression, in particular, when looking at its expression across a large number of subjects. Notably, several factors are involved in epigenetic variation, which may indirectly explain why gene expression and DNA methylation correlation is not always strong.[79] Another important concern regards the further interpretation of these DNA methylation differences in cell types beyond venous cord blood (in this case, the brain).[69] Notably, early SRI exposure has been linked to later emerging neurological symptoms, such as sensory map abnormalities,[80] increased risk for developmental delay,[81] or ADHD.[82] It will be important to determine whether early *CYP2E1* DNA methylation can contribute to these later phenotypes.

Conclusion

Prenatal exposure to maternal mood disturbances and SRI antidepressant treatment are increasingly associated with altered birth outcomes. Why some but not all infants are at-risk has led to studies of epigenetic factors. Higher DNA methylation status of *CYP2E1*, both across 16 CpG sites (mean) and at each CpG site, was associated with exposure to lower 3rd trimester maternal depressed mood symptoms, but only in SRI-exposed neonates. Higher methylation levels at CpG2, CpG9, and CpG10 were associated with increased birth weight, independently of maternal mood, SRI drug exposure, and gestational age at birth. Instead, neonates of mothers who received SRI treatment but remained symptomatic (i.e., partial or non-responder) had the opposite impact, namely lower DNA methylation levels and lower birth weights. Molecular mechanisms underlying the links between prenatal SRI/maternal mood exposure and neonatal epigenetic changes, which might influence birth outcomes, remain to be elucidated.

Methods

Subjects

With approval from the University of British Columbia Research Ethics Board, Children's and Women's Health Center of British Columbia Research Review Committee, and informed parent consent, a cohort (n = 91) of mothers was recruited in their early second trimester as part of a study of the impact of -prenatal psychotropic medication exposure on neonatal health. Of the original 91 mothers who completed a second trimester data collection, 79 participated in a fetal speech perception study at 36 weeks gestation.[20] From these, a convenience sample of cord blood obtained at delivery was chosen on the basis of SRI exposure status and heart rate (HR) response during the fetal language discrimination task.

An SRI-exposed group (n = 12) with greatest fetal HR response was compared to a non-exposed group (n = 12) in which the HR response was the weakest or non-existent on the fetal language task. One sample from the SRI-exposed group was excluded for a technical reason. These samples were then used to study epigenetic marks associated with speech perception using a genome-wide array approach to detect differences across >27,000 sites. However, as this approach did not detect whether any epigenetic differences were a result of SRI exposure, fetal capacity to perceive speech, or maternal mood, DNA methylation differences were examined in a larger cohort using pyrosequencing.

The follow-up pyrosequencing sample included 21 out of 23 samples from the microarray cohort and 21 further samples from the whole cohort. Two samples from the microarray cohort were not included in the pyrosequenced cohort due to lack of DNA. Nine neonates from the whole cohort were excluded either for unavailable blood samples, or for experimental errors (n = 3). In addition, we excluded subjects for either incomplete fetal perceptual HR records (n = 35) or fetal movement during the prenatal perceptual task (n = 23).

Thirty-five neonates from the whole cohort were excluded for either incomplete fetal perceptual HR records or fetal movement during the prenatal perceptual task (n = 23), unavailable blood samples (n = 9), or experimental errors (n = 3). Mothers were included if they took no other serotonergic medications or psychotropic medications during their pregnancy.

Demographic characteristics between infants in the array cohort and the 21 additional infants of the pyrosequenced cohort did not differ significantly with regard to maternal depressed mood symptoms, maternal age at delivery, gestational age at delivery, birth weight, length, or apgar scores, but differed significantly for head circumference (SRI_array: 35.38, SRI_additional samples: 35.19, $P = 0.02$) for the SRI-exposed groups.

Maternal mood

Prenatal maternal mood was assessed by averaged clinician-rated measures (blinded to medication group status) derived from assessements at the time of study enrollment (approximately 26 weeks) and at 36 weeks gestation. The *Hamilton Rating Scale for Depression (HAM-D)*,[83] a 21-item clinician administered scale designed to assess the severity of depression symptoms was used. Scores on this scale have a possible range of 0–63, with higher scores being associated with higher levels of depression in the patient (**Table 1**).

Drug levels

Blood was drawn from SRI-treated mothers and venous cord at delivery to assess medication plasma concentrations. Samples were stored at −80°C before being sent to CANTEST Ltd., where plasma concentrations of paroxetine, fluoxetine, norfluoxetine (the major active metabolite of fluoxetine), sertraline, venlafaxine, and citalopram were analyzed by high performance liquid chromatography tandem mass spectrometry using atmospheric

pressure electrospray ionization in positive mode. The limit of quantification was 0.1 ng/L for all compounds analyzed.

DNA Extraction and Illumina Infinium HumanMethylation27 array

Genomic DNA from neonate whole cord blood was extracted using Qiagen DNA/RNA kit as per standard conditions and bisulfite modified with the EZ DNA Methylation Kit (Zymo Research) as per manufacturer's instructions. This step converted unmethylated cytosine into uracile but left methylated cytosine (5-methylcytosine and 5-hydroxymethylcytosine) or other potential methylated nucleotides, such as methyladenine, unaffected. Then, bisulfite-converted DNA was amplified, fragmented, and hybridized to Illumina Infinium HumanMethylation27 BeadArray chips (Illumina, Inc.) using Illumina supplied reagents and conditions. This array enabled the simultaneous quantitative measurements of 27,578 CpG sites of 14,475 well-annotated human genes for 24 neonates whole cord blood.

DNA methylation array quality controls and data normalization

Arrays were scanned on the Illumina iScan system and imported into GenomeStudio for further analysis (2,010.2) using BeadStudio (versions 3.1.3.0 Illumina, Inc.). Potential unreliable CpGs or potential unreliable samples were removed, as well as sexual chromosomes due to mixed sex population. A CpG was considered unreliable if >10% of the samples had a detection *P*-value > 0.1, and a sample was considered unreliable if >10% of its CpGs had a detection *P*-value > 0.1. No CpG site or sample was found to be unreliable. However, 1,092 CpG sites localized on X or Y chromosomes were removed from the original dataset. Then, we performed quantile normalization in R 2.11.0 using the limma package.[84]

Statistical tests for DNA methylation differences

The non-parametric Wilcoxon test was used to identify methylation differences between SRI- and non-exposed newborns using the built-in function of the SAMR package in R. Significance Analysis of Microarrays (SAM) is a statistical technique that has been developed to find significant gene expression differences in a set of microarray experiments and that corrects for multiple testing.[85] It uses repeated permutations of the data to estimate false discovery rate. The cutoff for significance is determined by a tuning parameter delta, chosen by the user. The FDR value is given by the q-value. Here, we used a 0.05 threshold.

Data reduction and statistical analyses

To examine relationships between antenatal SRI exposure and neonatal DNA methylation status across 26,486 sites, we first explored DNA methylation status using microarray data. Relationships between *CYP2E1* neonatal DNA methylation, antenatal SRI exposure, and maternal mood were studied with the univariate model using *CYP2E1* DNA methylation level as dependent variable, prenatal SRI exposure as independent variable, and maternal mood as a covariate. Then, to confirm array findings, SRI exposure-related methylation differences were examined at specific CpG sites using pyrosequencing.

Relationships between *CYP2E1* neonatal (cord) DNA methylation, antenatal SRI exposure and maternal mood were studied with linear regression models. As maternal mood (delivery) measures were continuous and given that maternal mood was highly correlated with SRI use, they were used as predictors in all regression models. This also allowed us to account for the wide range of depressive symptoms observed among both SRI-treated and untreated groups across time, as some mothers in our untreated group became depressed and some crossed over to the SRI-treated group.

Relationships between newborn DNA methylation status and birth outcomes were studied with linear regression models for each neonatal outcome (i.e., gestational age, birth weight) using prenatal maternal mood as a covariate. Given the exploratory nature of these approaches, Bonferroni corrections were not applied due to the heightened risk of associated Type II errors; instead, effect sizes for the regressions (squared semi partial correlations, $\acute{\eta}^2$) were computed to allow examination of the magnitude of the predictive relationships.[41]

Validation and pyrosequencing

The BLAST program from NCBI was used to determine whether candidate probe sequences mapped to a single unique location in the genome or multiple sites. The repeat masker from UCSC was used to determine whether candidate probes were located in repetive elements. The UCSC site was also used to determine whether the candidate probe sequences and their surrounding regions (100 upstream and downstream) contained any SNP.

Bisulfite pyrosequencing was used to validate the array study for *CYP2E1* and was performed in an expanded cohort. Pyrosequencing is a technique based on DNA sequencing in which sequential incorporation of complementary nucleotides leads to the production of a visible light that is proportional to the number of nucleotides incorporated. Preparation for pyrosequencing was carried out using the PyroMark Q96 Vacuum Prep Workstation (Qiagen) and the PyroMark Q96 ID pyrosequencer (Qiagen) according to the manufacturer's protocol.

In brief, for the samples that were not included on the array in the expanded cohort or if additional bisulfite-converted DNA used on the array was not available, genomic DNA (500 ng) samples were bisulphite-treated and purified using Zymogen EZ DNA Methylation Gold Kit (Zymo Research). Both these samples and those that were run on the array for which we still had bisulphite-converted DNA were amplified by PCR. The PCR product was then bound to streptavidin-sepharose high performance (GE healthcare), and the beads were agitated for at least 5 min.

After a series of denaturing and wash steps resulting in the isolation of single-stranded DNA, primer annealing was conducted at 80°C for 2 min. Pyrosequencing was then performed in a PyroMark Q96 ID pyrosequencer (Qiagen) according to the manufacturer's instructions. The DNA methylation status of each locus was analyzed individually using the Pyromark CpG 1.0.11

software (Qiagen). Pearson correlation was then performed between pyrosequencing and array DNA methylation values.

Pyrosequencing primers

CYP2E1 pyrosequencing primers were designed by EpigenDx (http://www.epigendx.com/) and able to test up to 18 different CpGs, but only 16 gave us pyrosequencing values. To assess DNA methylation quality, we used samples with different global methylated levels (0, 10, 25, 50, 75, 100%). The equation of the curve obtained with the pyrosequenced values for all CpGs was $y = 1.22x - 3.45$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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