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RESEARCH ARTICLE

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Sculpting infant soothability: the role of prenatal SSRI antidepressant exposure and neonatal *SLC6A4* methylation status

Maria A. Gartstein[1]* | Kaia V. Hookenson[2] | Ursula Brain[2] | Angela M. Devlin[2] | Ruth E. Grunau[2] | Tim F. Oberlander[2]

[1] Department of Psychology, Washington State University, Pullman, Washington

[2] Department of Pediatrics, University of British Columbia, Child and Family Research Institute, Vancouver, British Columbia, Canada

***Correspondence**
Maria A. Gartstein, Department of Psychology, Washington State University, P.O. Box 644820, Pullman, WA.
Email: gartstma@wsu.edu

Abstract

The role of prenatal Selective Serotonin Reuptake Inhibitor (SSRI) exposure and *SLC6A4* promoter methylation status in shaping soothability at 3 and 6 months of age, for infants exposed to antidepressant medication prenatally ($n = 46$) and those not exposed ($n = 69$) was investigated. SSRI exposure status and duration of exposure (number of days) were examined along with neonatal methylation status at mean CpG 9,10 and via factor analysis across 10 CpG sites yielding PC1 (CpGs sites: 3,4,5,7) and PC2 (CpG 1,8). Analyses revealed interactions for methylation markers and SSRI exposure variables. A significant interaction between SSRI exposure and mean *SLC6A4* methylation at CpG 9,10 and separately for PC1 emerged, controlling for multiple birth/medical and background covariates (e.g., Apgar scores, maternal education). Increased neonatal methylation status was associated with increased soothability changes from 3 to 6 months among infants prenatally exposed to SSRIs.

KEYWORDS
infant temperament, maternal depression, prenatal SSRI exposure, *SLC6A4* methylation

1 | INTRODUCTION

Elucidating early origins of soothability in infancy, defined as the reduction of fussing, crying, or distress in the presence of caregiver soothing (Rothbart, 1981) is critical to understanding pathways to mental and physical health across the life span (Rothbart, 2011). Capacity for self-regulation emerges in the first year of life. The ability to calm or reduce arousal with parental intervention is critical to emotional/behavioral control, and is related to key aspects of subsequent child behavior. Soothability has been linked with attachment security: low child soothability differentially predicted insecure ambivalent attachment quality (Mills-Koonce, Propper, & Barnett, 2012), as well as language development, wherein higher soothability was associated with more advanced skills (Dixon & Smith, 2000). In terms of biological foundations, the action of oxytocin and serotonin (5HT) has been associated with attention shifting, a key component underlying soothability in infancy (Clark et al., 2013; Leppanen et al., 2011). Behavioral genetics studies of temperament have found that patterns of genetic influence vary from trait to trait, with soothability explained primarily by shared environmental effects (e.g., Goldsmith, Lemery, Buss, & Campos, 1999).

Connections between infant soothability and maternal mental health have also been reported. Lower levels of soothability have been associated with higher levels of concurrent parenting stress in a clinical sample among mothers with substance abuse and psychiatric symptoms (Siqveland, Olafsen, & Moe, 2013). In addition, infant soothability moderated the impact of negative infant temperament on maternal sensitivity, whereby negative emotionality was positively related to maternal sensitivity if mothers perceived their infants as highly soothable, and negatively related when infant soothability was low (Ghera, Hane, Malesa, & Fox, 2006). Lower levels of soothability were also linked with early manifestations of callous-unemotional traits (Willoughby, Waschbusch, Moore, & Propper, 2011). In our work, we have found associations between infant soothability, and sleep, as well as early behavior problems (Gartstein, Potapova, & Hsu, 2014; Gartstein, Putnam, & Rothbart, 2012). For example, infants who were more easily soothed displayed fewer internalizing behavior problems from 3 to 5 years of age (Gartstein et al., 2012). In early childhood, soothability consistently clusters with other fine-grained dimensions measuring aspects of self-regulation (e.g., Gartstein, Knyazev, & Slobodskaya, 2005; Gartstein & Rothbart, 2003). Moreover, relative stability of soothability throughout the first year of life was recently demonstrated, with age, gender, preterm birth status, and birth-order exerting only a minimal influence on the stability of this attribute (Bornstein et al., 2015). Importantly, soothability is affected by environmental factors that shape parenting, family circumstances, and daily routine, such as the influence of culture (e.g., Sung, Beijers, Gartstein, de Weerth, & Putnam, 2015).

Increased use of Selective Serotonin Reuptake Inhibitors (SSRIs) antidepressants to manage maternal mood disturbances during pregnancy (Oberlander, 2012) has raised critical and unanswered questions about how changes in central 5HT (5-hydroxytryptamine or serotonin receptors) functioning related to these medications may also shape early child self-regulation. It is estimated that 7.6% of all pregnant women in the United States take an antidepressant, with use during pregnancy increasing from 1.5% in 1996 to 6.2% of all pregnant women in 2005 (Andrade et al., 2008).

Decisions regarding the use of antidepressants during pregnancy are often complicated, as symptoms of prenatal depression are known to have a negative impact on child functioning (e.g., O'Connor, Monk, & Fitelson, 2014). At the same time, emerging evidence suggests that exposure to SSRIs during gestation may affect offspring 5HT signaling, increasing sensitivity to negative social contexts for some individuals, and possibly conferring sensitivity to positive life circumstances in others. Such fetal programming effects may be best thought of as "plasticity factors," which could offer critical insights into explaining individual differences in developmental risk and resilience in the context of early changes in 5HT signaling (Oberlander, 2012).

Prenatal SSRI exposure has been linked with multiple neonatal and infant behavioral disturbances, including birth/medical complications (Grigoriadis et al., 2014; Roca et al., 2011), developmental shifts (de Vries, van der Veere, Reijneveld, & Bos, 2013; Harrington, Lee, Crum, Zimmerman, & Hertz-Picciotto, 2014; Weikum, Oberlander, Hensch, & Werker, 2012), behavioral disturbances and physiological alterations in stress regulation (Hanley, Brain, & Oberlander, 2013; Oberlander et al., 2008; Zeskind & Stephens, 2004), yet direct measures of infant temperament following prenatal SSRI exposure remain limited, with existing studies failing to control for maternal mood. To date, temperament in SSRI exposed infants has been examined in two studies with considerable methodological limitations. The earlier of these, Nulman et al. (1997), did not identify significant differences in temperament for children ages between 16 and 86 months of 55 mothers using SSRI medication during pregnancy; however, temperament was inconsistently assessed, only directly reported among children under 24 months of age, with behavior problems examined for older children instead. Nulman et al. (2002) again failed to identify differences in a very small sample ($n = 18$) of SSRI-exposed children using limited measures of temperament.

SSRI-exposed infants' displayed decreased physiological and behavioral responses to a typical neonatal painful event (Davis, Glynn, Waffarn, & Sandman, 2011; Oberlander et al., 2002). As well, altered neonatal cortisol levels (Pawluski, Brain, Underhill, Hammond, & Oberlander, 2012) have been reported, even when controlling for maternal symptoms. Specifically, in SSRI-exposed infants, higher levels of neonatal corticosteroid-binding globulin (CBG) predicted a smaller diurnal change in infant salivary cortisol, independent of maternal depression (Pawluski et al., 2012). That is, there was a decrease in daily variability of infant cortisol concentrations for infants with higher CBG levels and a history of SSRI *in utero* exposure. At the same time, maternal depression/anxiety have been shown to affect expression of placental *SLC6A4* mRNA (Ponder et al., 2011) and neonatal cord blood *SLC6A4* methylation, which was not altered by maternal SSRIs (Devlin, Brain, Austin, & Oberlander, 2010). *SLC6A4* encodes the serotonin transporter (5HTT), and transmembrane 5HTT is a key regulator of 5HT concentrations that governs the intrasynaptic reuptake of 5HT into the presynaptic neuron (Lesch & Gutknecht, 2005). Thus, prenatal SSRI exposure may result in fetal programming effects that in turn influence brain development via altered levels of the key brain neurotransmitter 5HT during developmentally sensitive periods, and will be considered with respect to infant soothability in the present study.

Considerable attention is focusing on variants in 5HT-related genes associated with risk for child behavior and mental health problems (e.g., Wang et al., 2012), that could also reflect how developmental changes in 5HT levels influence brain function and subsequent behavior. Earlier efforts indicated that the short allele of a tandem repeat variant in the upstream region of *SLC6A4* (5HTTLPR) is associated with a risk for depression, particularly following early adverse life events (Caspi et al., 2003). However, findings have been inconsistent (Karg, Burmeister, Shedden, & Sen, 2011; Risch et al., 2009), and increasingly focus has shifted to examining associations between peripheral epigenetic marks that reflect how *SLC6A4* expression might link early life adversity and mental health. In particular, an emerging literature has begun to illustrate that peripheral DNA methylation changes could reflect how environment affects the serotonergic system and 5HT dependent neuronal processes via epigenetic modifications altering gene expression (Booij, Wang, Levesque, Tremblay, & Szyf, 2013; Wang et al., 2012). Epigenetic mechanisms, such as covalent modification of DNA by methylation of cytosine residues, are thought to be involved in fetal effects associated with prenatal exposure to SSRIs (Gurnot et al., 2015) and maternal mood/anxiety symptoms (Devlin et al., 2010). Although a number of epigenetic mechanisms have been identified, DNA methylation appears particularly critical to developmental processes (Lister et al., 2013; Szyf & Bick, 2013).

Recent attention has shifted from weighing genetic and environmental influences to understanding how methylation of the *SLC6A4* promoter may reflect a mechanism that alters 5HT signaling, involved in self-regulatory behaviors and their deficiencies, evident in mood disorders (Booij et al., 2013). In particular, studies have reported associations between *SLC6A4* methylation in peripheral tissues, such as blood and saliva, and early life adversity and depression (Booij et al., 2015; Chau et al., 2014). Rhesus monkeys reared in a nursery displayed higher *SLC6A4* methylation in peripheral blood mononuclear cells and increased separation anxiety compared with mother-reared infants (Kinnally et al., 2010). Using lymphoblast cell lines, Philibert et al. (2008) found that increased *SLC6A4* methylation was linked with a life history of depression and higher methylation was associated with lower mRNA expression. Childhood adversities also appear to be related to subsequently increased *SLC6A4* methylation in the saliva of children (Chau et al., 2014), and peripheral blood of adults (Beach, Brody, Todoroov, Gunter, & Philibert, 2011; Kang et al., 2013). Moreover, higher *SLC6A4* methylation in T cells and monocytes have been associated with lower *in vivo* brain 5HT synthesis (Wang et al., 2012), and greater *SLC6A4* methylation in whole blood was associated with lower hippocampal volumes (Booij et al., 2015). Even before birth, maternal depressive mood during the second trimester has been associated with lower methylation of the *SLC6A4* promoter in newborn cord blood leukocytes (Devlin et al., 2010), suggesting fetal programming of the serotonergic system via an epigenetic process. Together, these findings suggest that peripheral *SLC6A4* methylation may be a sensitive marker of the impact of early life experience, reflecting the effects on key brain regions and functions implicated in psychiatric disturbances such as depression. In this way, peripheral *SLC6A4* methylation status may offer a developmental "window" onto how early life stressors shape subsequent behavior. That is, if increased *SLC6A4* methylation suppresses transcription, less efficient 5HT transport/re-uptake would result, leading to increased levels of intrasynaptic serotonin, in a sense mimicking the effects of *in utero* SSRI exposure.

The present study was undertaken to investigate biological influences on the development of self-regulation (soothability) in the first 6 months of life, via epigenetic mechanisms (neonatal methylation status of *SLC6A4*) thought capable of altering developmental levels in 5HT. We sought to study the impact of prenatal SSRI exposure and neonatal *SLC6A4* methylation status on soothability assessed in early and mid-infancy (at 3 and 6 months of age), as well as change in soothability from 3 to 6 months. This strategy enabled us to examine contributions of two important serotonergic influences to the development of soothability during a critical period known for rapid growth across a number of areas (e.g., motor capabilities, attentional skills; e.g., Shonkoff & Phillips, 2000). In particular, we sought to investigate the impact of the following: (i) prenatal SSRI exposure and (ii) duration of *in utero* SSRI exposure on the development of self-regulation-related behaviors. We focused on the interactions between SSRI exposure status and *SLC6A4* methylation markers, testing hypotheses that these dual 5HT-signaling factors would jointly influence emerging soothability. Specifically, we hypothesized that prenatal SSRI exposure would moderate the impact of *SLC6A4* methylation at key CpG regions on soothability, after accounting for the effects of maternal mood/anxiety and infant sex (Else-Quest, Hyde, Goldsmith, & Van Hulle, 2006). We predicted that higher methylation levels in the context of SSRI exposure (i.e., increased fetal 5HT signaling) would be associated with increased soothability, irrespective of maternal mood.

2 | METHOD

2.1 | Participants

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 ($N = 191$) of pregnant women was recruited in their early second trimester as part of a study of the impact of prenatal SSRI exposure on neonatal health. Of the original cohort, 185 mothers (i.e., six mothers withdrew for personal reasons prior to delivery) and their neonates ($n = 140$) were available for study at delivery (45 samples were not available because of inadequate DNA yield or infant cord blood sample was not obtained at birth). The final sample ($N = 115$) included participants providing data for all of the variables examined in this study: prescribed an SSRI antidepressant medication prenatally ($n = 46$) and those not prescribed SSRIs ($n = 69$) during pregnancy. Mothers were only included in the study if they took no other serotoninergic medications or other psychotropic medications during their pregnancy. All mothers were taking folic acid during their pregnancies, either as a component of a prenatal vitamin supplement or on its own.

2.2 | Measures

2.2.1 | Infant behavior questionnaire (IBQ; Rothbart, 1981)

This widely used caregiver-report questionnaire comprised 94 items referring to specific/observable infant behaviors. Items are rated on a 7-point Likert scale, indicating the frequency of occurrence for various manifestations of temperament characteristics in the past week, or 2 weeks for less frequent events. These items form six scales assessing temperament dimensions, with soothability utilized as the dependent variable in this study. Parents are asked if they had tried specific soothing techniques, and how often the various methods soothed the baby. For example: "When patting or gently rubbing some part of the baby's body, how often did s/he soothe immediately?"; "When showing the baby something to look at, how often did s/he soothe immediately?" Mean item-scale correlations for the IBQ have ranged from .41 to .77, with Chronbach's alphas ranging from .67 to .84 (median = .79). Moderate correlations (between .45 and .69) were also reported for pairs of adults ($N = 24$) that shared caregiving responsibilities for a particular infant (Rothbart, 1981). Convergence between laboratory observation ratings of temperament and the IBQ scales has been consistently reported (Bridges, Palmer, Morales, Hurtado, & Tsai, 1993; Crockenberg & Acredolo, 1983; Goldsmith & Rothbart, 1991), along with relative stability over the first year of life (Bornstein et al., 2015; Rothbart, 1986).

2.2.2 | Maternal internalizing symptoms

The Edinburgh Postnatal Depression Scale (EPDS; Cox, Holden, & Sagovsky, 1987) is a commonly employed measure of perinatal depressive symptoms, was used at 33–36 weeks gestation. This questionnaire contains 10 items, rated on a 4-point Likert scale (reflecting the frequency of various symptoms), summed to produce a scale score, with higher scores indicative of more significant symptomatology. Reliability and validity have been established for this 10-item self-report instrument in the prenatal period. Maternal mood and anxiety were assessed at 33–36 weeks gestation by trained research assistants using the Hamilton Rating Scale for Depression (HAMD; Hamilton, 1960) and the Hamilton Rating Scale for Anxiety, (HAMA; Hamilton, 1959). The 21-item HAMD and the 14-item HAMA represent clinician rated measures that reflect symptom severity of depression and anxiety. Individual items, rated on a 5-point Likert scale, are summed, with higher scores indicting greater severity of symptoms. A sum of the EPDS, HAMD, and HAMA scale scores was subsequently computed, creating a multi-method Internalizing Symptom Composite, as previously described (Gartstein et al., 2012; Oddi et al., 2013; Potapova, Gartstein, & Bridgett, 2014). This approach was deemed optimal in light of data reduction considerations, as these variables represent covariates in the design of this study, and because of substantial intercorrelations among the anxiety and depression scores in the present sample (range .75–.89; mean = .82). As these measures all address similar dimensions of emotional symptoms, we used this approach to quantify the magnitude of distress without making distinctions between the nature of the symptoms (anxiety/depression) or the source of information. Two indicators of depression were included, because these were not redundant, and derived via different methodological approaches (self vs. clinician report), enabled us to develop a multi-method internalizing construct.

2.2.3 | Methylation status: bisulfite pyrosequencing DNA methylation analysis

Cord blood was collected at delivery and genomic DNA was extracted from newborn whole blood using the Flexigene DNA Blood Kit (Qiagen, Valencia, CA). The methylation status of 10 CpG sites in the *SLC6A4* promoter was quantified by bisulfite pyrosequencing (Dupont, Tost, Jammes, & Gut, 2004). Genomic DNA samples (1 µg) were bisulfite-treated using EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA) following the manufacturer's protocol, and stored at −20°C until further analysis. A 130 bp fragment of the *SLC6A4* promoter was amplified by PCR from bisulfite-treated DNA using HotStar Taq DNA Polymerase (Qiagen) and the following primers: PMHSERTF, 5′-GTATTGTTAGG TTTTAGGAAGAAAGAGAGA-3′ and PMHSERTR, 5′-AAAAATCCTAACTTTCCTACTCT TTAACTT-3′ (IDT Inc, Coralville, IA), with the reverse primer containing a biotin at the 5′ end. Cycling conditions were 94°C for 15 min followed by 50 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min with a final extension of 10 min at 72°C. PCR products were purified and sequenced using a PyroMark MD System (Biotage, Foxboro, MA) following the manufacturer's protocol and the following sequencing primer: PMHSERTS, 5′-AA ACTACACAAAAAAACAAAT-3′ (IDT). The percent methylation at each CpG site was quantified using the Pyro Q-CpG software, version 1.0.9 (Biotage), and used as independent variables in this study.

2.3 | Procedure

2.3.1 | Medication status/days on SSRI; questionnaires and ratings

In a prospective cohort design, pregnant women provided information regarding demographic characteristics, status with respect to SSRI medication, as well as other pharmaceutical and substance use and the duration of SSRI use over the course of the pregnancy; infant and medical indicators were collected related to birth (Table 1). At 33–36 weeks of pregnancy the women responded to questions concerning symptoms of depression and anxiety, and provided self-report of depression. Mother-report of infant temperament was obtained at 3 and 6 months of age.

2.3.2 | Analytic strategy

Two approaches were used to identify key regions in methylation status in the 10 CpG sites in the *SLC6A4* promoter. First, principal component analysis (PCA) was used as a data driven approach for methylation data reduction. Two principal components had an eigenvalue > 1, and together they accounted for 76% of the total variance in *SLC6A4* methylation. Principal Component 1 (PC1) (eigenvalue = 5.9) explained 59% of variance (CpG sites: 3,5,7,9), while PC2 (eigenvalue = 1.7) explained 17% of variance (CpG 1,8). As both PC1 and PC2 were the predominant components, they were both used to represent the most significant aspect of methylation status in *SLC6A4* CpG 1–10. Second, a mean of CpGs 9 and 10 methylation was used as a measure of candidate site-specific CpG methylation status previously shown to have central neural correlates (Wang et al., 2012; Frodl et al., 2015). The methylation detection limit for a single CpG by bisulfite pyrosequencing is estimated to be ~5% (Mikeska, Felsberg, Hewitt, & Dobrovic, 2011), thus only values above this threshold were considered.

Descriptive statistics (Table 1) were computed for background and infant birth/medical indicators, and study measures, comparing infants with and without *in utero* SSRI exposure, using independent groups *t*-tests (χ^2 for categorical data).

Using hierarchical multiple regression models, associations were examined between SSRI exposure, *SLC6A4* methylation status, and temperament, first controlling for relevant covariates; second, entering individual predictors (e.g., SSRI medication status, *SLC6A4* methylation) and third, entering the interaction terms (e.g., SSRI medication status**SLC6A4* methylation). In a similar model, duration of *in utero* SSRI exposure and its interaction with *SLC6A4* methylation was examined. These interaction terms were of primary importance, as our hypotheses involved joint action of SSRI exposure and *SLC6A4* methylation status, with moderation effects anticipated for the exposure related variables (Figure 1).

To interpret significant interactions between SSRI medication status, duration of *in utero* SSRI exposure, and *SLC6A4* methylation, we followed a twofold strategy. For exposure status, a significant interaction effect was followed-up by examining the contribution of CpG 9,10 percentage methylation separately for the two exposure conditions. We planned to follow-up significant interaction effects by

TABLE 1 Descriptive statistics: SSRI exposure, no SSRI exposure, and total sample

Groups	SSRI (*n* = 46)	No SSRI (*n* = 69)	Total sample (*N* = 115)
Variables	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**
Gestational age at birth	39.05 (1.60)**	39.76 (1.75)	39.47 (1.72)
Apgar (1 min)	7.48 (1.63)**	8.24 (1.39)	7.93 (1.54)
Apgar (5 min)	8.80 (.74)[#]	8.99 (.57)	8.91 (.65)
Weight at birth (g)	3,323.12 (517.72)*	3,492.05 (545.32)	3,423.57 (539.28)
Maternal age at birth	32.70 (5.43)	33.65 (5.04)	33.26 (5.21)
Maternal years of education	16.36 (3.21)**	17.69 (3.32)	17.15 (3.30)
Maternal alcohol use (number of drinks during pregnancy)	4.05 (9.38)	3.42 (6.40)	3.68 (7.73)
Mothers' internalizing symptoms[a]	33.92 (18.22)**	19.65 (17.49)	25.37 (19.07)
SLC6A4 CpG PC1 methylation status (principal component score)	−.07(1.0)	.04 (.97)	.00 (1.0)
SLC6A4 CpG PC2 methylation (principal component score)	.13 (1.6)	−.09 (.32)	.00 (1.00)
SLC6A4 CpG 9,10 methylation (mean methylation)	8.58 (2.66)	8.58 (1.80)	8.58 (2.18)
Age at 6-month assessment	.52 (.04)	.51 (.03)	.51 (.03)
Days on SSRIs	235.28 (67.87)	.00 (.00)	–
	Frequency	**Frequency**	**Frequency**
Sex	26F/20M	36F/33M	62F/53M
Vaginal delivery	33	50	83
C-section delivery	13	19	32
Benzodiaepine use	11*	2*	13
Anti-depressant (non-SSRI) use	5	0	5

[a]Sum of scores derived from the Edinburgh Postnatal Depression Scale (EPDS; Cox, Holden, & Sagovsky, 1987), the Hamilton Rating Scale for Depression (HAMD; Hamilton, 1960) and the Hamilton Rating Scale for Anxiety, respectively (HAMA; Hamilton, 1959).
*$p < .05$; **$p < .01$; [#]$p < .10$.

predicting soothability with *SLC6A4* CpG 9,10 methylation for prenatally SSRI-exposed and non-exposed infants, as these "natural" groups are meaningful in interpreting the interaction effect, and exposure has been conceptualized as a moderator (Figure 1).

In both analyses, we initially examined three separate hierarchical multiple regression models, wherein SSRI and *SLC6A4* methylation status variables first predicted soothability at 3 months, then at 6 months of age, and were subsequently examined as predictors of change in soothability (Δ soothability) between 3 and 6 months. The latter was accomplished by considering the 3-month indicator of soothability prior to our predictors of interest in regression equations, controlling for that portion of the 6-month scores that corresponds to the stability of this attribute. Thus, the remaining portion of the 6-month soothability score represents developmental change (Δ soothability), subsequently predicted by our independent variables of interest (e.g., SSRI status, *SLC6A4* methylation). The latter is of particular importance, as examining this change in an early appearing aspect of self-regulation provides an opportunity to consider potential serotonergic contributions to its development between early and mid-infancy.

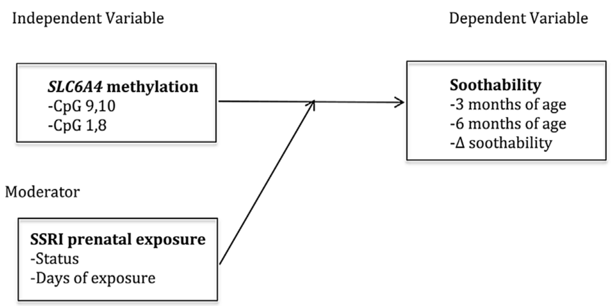

FIGURE 1 Schematic representation of the hypothesized moderation effects

3 | RESULTS

3.1 | Cohort description

Demographic and behavioral outcomes for the mothers and their infants are presented in Table 1. Significant group differences were observed, with the SSRI exposed group showing lower gestational age at birth ($p = .01$), lower weight at birth ($p = .04$), lower 1 min APGAR scores ($p = .00$), lower maternal education ($p = .01$), and higher maternal internalizing symptomatology (i.e., mood/anxiety) at 33–36 weeks ($p = .00$) compared to the non-exposed group. None of the infants were small for gestational age.

3.2 | *In utero* SSRI exposure status (exposed vs. non-exposed)

Comparing infants with SSRI exposure and those without exposure yielded a number of significant results (Table 1). Differences in gestational age at birth, Apgar (total scores at 1 and 5 min), weight at birth, and maternal years of education were detected, and these variables were all considered as covariates, along with prenatal maternal internalizing composite and infant sex. However, these maternal and neonatal characteristics did not account for significant amounts of variance in the hierarchical multiple regression models examining SSRI exposure and mean *SLC6A4* CpG 9,10 methylation effects, predicting temperament at 3 and 6 months, or change in temperament occurring between these assessments. Thus, only infant sex and prenatal maternal internalizing symptoms were controlled statistically in the final models (presented in Tables 2–5), as the remaining covariates were "trimmed" in order to increase the power to detect hypothesized effects (Cohen, Cohen, West, & Aiken, 2003). It should be noted, however, that the significant effects reported below were also evident with the covariates in the regression equation (results available upon request from the first author).

Interactions between SSRI exposure and *SLC6A4* PC1 and also for mean CpG 9,10 methylation, emerged as key predictors, accounting for significant amounts of variance in infant soothability at each of the two assessment times (at 3 and 6 months of age; Table 2). The interaction effect predicted greater change in infant soothability between 3 and 6 months of age (i.e., after controlling for 3-month soothability; Table 2), for mean *SLC6A4* CpG 9,10. The joint contribution of SSRI medication status and neonatal *SLC6A4* CpG 9,10 methylation accounted for developmental changes in soothability (Δ soothability) between early and mid infancy ($\beta = .28$; $p = .03$). At the same time, neither methylation nor SSRI exposure status made a significant independent contribution in predicting soothability, or change over time, and prenatal maternal internalizing symptoms made only a marginal contribution accounting for 3-month soothability.

Further models examined outcomes for SSRI-exposed and non-exposed infants separately. Among SSRI exposed infants, higher levels of neonatal *SLC6A4* PC1 and mean CpG 9,10 methylation predicted higher soothability scores at 6 months, and mean CpG 9,10 methylation was associated with increased Δ soothability between 3 and 6 months of age. Moreover, at 6 months higher soothability was associated with higher levels of *SLC6A4* PC2 methylation ($p = .04$), for SSRI-exposed infants only (Table 4).

In contrast, among non-exposed infants, only maternal internalizing symptoms made a statistically significant contribution to 3-month soothability, so that higher levels of prenatal symptomatology was associated with lower infant soothability. Not surprisingly, 3-month soothability also emerged as a significant predictor of 6-month soothability (Table 3).

3.3 | Duration of *in utero* SSRI exposure (days of exposure)

Duration (days) of SSRI medication exposure during pregnancy was considered as a predictor of infant soothability, along with *SLC6A4* PC2 (CpG 1,8) methylation. The impact of duration of exposure could only be quantified for the infants in our exposed group. An interaction between *SLC6A4* PC2 methylation status and days of prenatal SSRI use emerged that predicted both 3 and 6-month soothability (Table 4). It should be noted that the distribution of days of SSRI exposure variable deviated from normality, and was thus logarithmically transformed. This transformation of the days of exposure did not change the pattern of results observed in the context of hierarchical multiple regression analyses, and the initial non-transformed model results are provided in Tables 4 and 5.

Follow-up analyses were subsequently conducted for high and low number of exposure days, in groups based on the median split (Table 5). In the high exposure-days group only, greater extent of PC2 methylation was associated with higher soothability, accounting for 17% of the variance. Overall, results obtained with respect to the duration of exposure also suggest a protective effect of prenatal SSRIs, prescribed to treat symptoms of depression during pregnancy.

4 | DISCUSSION

This study investigated the impact of *in utero* SSRI exposure on infant temperament development. Specifically, we examined the influence of neonatal *SLC6A4* methylation on the development of soothability at 3 and 6 months of age in the context of SSRI exposure. We observed a significant interaction between SSRI exposure and *SLC6A4* methylation (PCI) and mean CpG 9,10, controlling for multiple birth/medical and background covariates (e.g., Apgar scores, maternal education). Namely, among SSRI exposed infants, higher levels of mean neonatal *SLC6A4* CpG 9,10 methylation predicted higher soothability scores at 6 months, and increased Δ soothability between 3 and 6 months of age. Among SSRI-exposed infants greater soothability was also associated with greater *SLC6A4* PC1 methylation, whereas for the non-exposed infants, methylation and soothability were not related. PC2 also predicted soothability at 6 months of age for infants whose mothers were treated with SSRIs during pregnancy. Significant interactions between PC2 methylation and days of prenatal SSRI exposure shaped this effect, as follow-up analyses revealed that a significant positive relationship was observed for those experiencing longer prenatal SSRI exposure only. Moreover, among non-exposed infants, maternal internalizing symptoms were associated with lower 3-month soothability, suggesting that prenatal treatment with an SSRI might confer a form of developmental benefit, buffering the infant from the physiological effects of the mother's mood. As temperament is relatively stable even in the first year of life, 3-month soothability also emerged as a significant predictor of 6-month soothability (Table 3). Overall, results of this study suggest that pattern of associations between *SLC6A4* CpG methylation and infant soothability vary dependent on presence and duration of SSRI exposure (Figure 1). Together this pattern of findings suggests that increased prenatal SSRI exposure may confer a developmental benefit which was not observed in non-exposed infants where mothers' depressed mood was associated with lower infant self-regulation.

These findings are consistent with emerging literature suggesting that peripheral *SLC6A4* methylation may also reflect neural correlates of central serotonergic signaling. First, a high correlation was reported between *SLC6A4* methylation status in nine of 69 CpG sites evaluated

TABLE 2 Predicting soothability with SSRI status and *SLC6A4* CpG 9,10 and Principal Component 1 (PC1) methylation

	3 months					6 months					Δ soothability
Variable	Model 1 β	Model 2 β	Model 3 β	Model 4 β	Model 5 β	Model 1 β	Model 2 β	Model 3 β	Model 4 β	Model 5 β	Model 6[a] β
Infant sex	−.05	−.06	−.06	−.07	−.10	.00	.00	−.01	−.02	−.06	−.01
3-month soothability											.53**
Mother internalizing		−.14	−.14	−.18#	−.20#		−.02	.00	−.03	−.06	.04
SLC6A4 9,10 methylation			.01	.00	-.24			.12	.12	.23	−.11
SSRI status				.09	.10				.08	.09	.06
SSRI status 9,10 *methylation					.30*					.45**	.28*
F Δ	.31	2.35	.01	.77	4.20*	.00	.05	1.73	.65	9.89**	4.84*
R^2 Δ	.00	.02	.00	.01	.04	.00	.02	.02	.01	.08	.03
	3 months					6 months					Δ soothability
Variable	Model 1 β	Model 2 β	Model 3 β	Model 4 β	Model 5 β	Model 1 β	Model 2 β	Model 3 β	Model 4 β	Model 5 β	Model 6[a] β
Infant sex	−.05	−.06	−.06	−.07	−.09	.00	.00	−.01	−.01	−.04	.00
3-month soothability											.55**
Mother internalizing		−.14	−.15	−.19#	−.20*		−.02	−.06	−.05	−.07	.04
SLC6A4 PC1 methylation			−.05	−.05	−.22#			.09	.09	−.16	−.06
SSRI Status				.09	.11				.05	.11	.07
SSRI Status PC1 * methylation					.27*					.32**	.16
df	1, 114	2, 113	3, 112	4, 111	5, 110	1, 114	2, 113	3, 112	4, 111	5, 110	6, 109
F Δ	.31	2.35	.25	.80	4.75*	.00	.00	.83	.27	7.25**	2.17
R^2 Δ	.00	.02	.02	.01	.04	.00	.05	.01	.00	.06	.01

[a]Results obtained for Model 6 represent contributions to Δ soothability (i.e., 3-month soothability controls for stability of this attribute), and only the final model (i.e., Model 6) estimates are presented.
$^*p < .05$; $^{**}p < .01$; $^{\#}p < .10$.

TABLE 3 Follow-up for predicting soothability with SSRI status, *SLC6A4* CpG 9,10 and Principal Component 1 (PC1) methylation

	SSRI							No SSRI						
	3 months			6 months			Δ soothability	3 months			6 months			Δ soothability
CpG 9,10 methylation	Model 1 β	Model 2 β	Model 3 β	Model 1 β	Model 2 β	Model 3 β	Model 4 β	Model 1 β	Model 2 β	Model 3 β	Model 1 β	Model 2 β	Model 3 β	Model 4[a] β
Infant sex	−.14	−.14	−.20	−.03	−.03	−.15	−.09	.01	−.02	−.04	.01	.00	−.02	.03
Mother internalizing		−.09	−.06		−.06	.02	.02		−.21[#]	−.27*		−.05	−.10	.05
SLC6A4 9,1 methylation			.23			.47**	.34*			−.22[#]			−.19	−.08
3-month soothability							.51**							.52**
F Δ	.89	.35	2.05	.03	.13	10.76**	7.22*	.01	3.07[#]	3.20[#]	.00	.16	2.43	.53
R^2 Δ	.02	.01	.05	.00	.00	.20	.10	.00	.04	.04	.00	.00	.04	.01
PC1 methylation														
Infant sex	−.14	−.14	−.19	−.03	−.03	−.09	−.04	.01	−.02	−.04	.01	.00	−.02	.03
Mother internalizing		.09	−.07		−.06	−.03	−.01		−.21[#]	−.26*		−.05	−.08	.07
SLC6A4 PC1 methylation			.21			.36*	.21			−.24[#]			−.16	−.05
3-month soothability							.58**							.53**
df	1, 45	2, 44	3, 45	1, 45	2, 44	3, 43	3, 44	1, 68	2, 67	3, 66	1, 68	2, 67	3, 66	4, 65
F Δ	.89	.35	1.85	.03	.13	6.07**	2.52	.01	3.07[#]	4.00[#]	.00	.16	1.66	.17
R^2 Δ	.02	.01	.04	.00	.00	.13	.04	.00	.04	.04	.00	.00	.02	.00

[a]Results obtained for Model 6 represent contributions to Δ soothability (i.e., 3-month soothability controls for stability of this attribute), and only the final model (i.e., Model 6) estimates are presented.
*$p < .05$; **$p < .01$; [#]$p < .10$.

TABLE 4 Predicting soothability with days on SSRIs and *SLC6A4* PC2 (CpG 1,8) methylation

Variable	3 months					6 months					Δ soothability
	Model 1 β	Model 2 β	Model 3 β	Model 4 β	Model 5 β	Model 1 β	Model 2 β	Model 3 β	Model 4 β	Model 5 β	Model 6[a] β
Infant sex	−.14	−.14	−.13	−.15	−.25	−.03	−.03	.00	−.02	−.12	−.04
3-month soothability											.51**
Mother internalizing		−.09	−.07	−.08	−.08		−.06	.00	−.08	−.01	.01
SSRI days			.09	.03	.03			.27#	.23	.24	.21
SLC6A4 methylation				−.15	.74				−.12	.85**	.53
SSRI days methylation**					.97*					.94**	.64#
df	1, 45	2, 44	3, 43	4, 42	5, 41	1, 45	2, 44	3, 43	4, 42	5, 41	6, 40
F Δ	.89	.35	.30	.91	5.01**	.03	.13	3.30#	.60	6.77**	2.78#
R^2 Δ	.02	.01	.01	.02	.11	.00	.00	.08	.01	.13	.04

[a]Results obtained for Model 6 represent contributions to Δ soothability (i.e., 3-month soothability controls for stability of this attribute), and only the final model (i.e., Model 6) estimates are presented.
*$p < .05$; **$p < .01$; #$p < .10$.

in peripheral blood leukocytes and amygdala tissue, including CpG 10 examined in this study (Riese et al., 2014). Second, using positron emission technology, higher mean methylation in monocytes across CpG sites 5 and 6 (corresponding to CpG 10,9 in this study) was associated with lower *in vivo* 5HT synthesis in the lateral right and left orbitofrontal cortex (Wang et al., 2012). Third, lower hippocampal volume was associated with higher mean *SLC6A4* methylation in whole blood across 10 CpG sites in the promoter region, as well as CpG 5 and 6 (CpG 10,9 in this study; Booij et al., 2015). Fourth, mean *SLC6A4* methylation in whole blood across 10 CpG sites as well as the mean of CpGs 5 and 6 (CpG 10,9 in this study) were significantly associated with fMRI BOLD responses in the insula/inferior frontal operculum, in the context of an emotional attention-shifting task (Frodl et al., 2015). Lastly, a large region of the *SLC6A4* promoter containing 81 CpG sites was quantified in a lymphoblast cell line, and the methylation levels at four CpG sites were associated with *SLC6A4* mRNA levels (Philibert et al., 2007), with CpG 8 in our study being one of them. Thus, in combination with emerging findings supporting the biological significance of these CpG methylation sites, our results suggest a plausible link between infant soothability, 5HT-related neural correlates, and peripheral epigenetic marks, which should be confirmed by future research.

Importantly, suppression of *SLC6A4* transcriptional activity has also been achieved via *in vitro* methylation of the promoter in luciferase reporter constructs, demonstrating a functional role of methylation on *SLC6A4* gene expression (Wang et al., 2012). Combined with the results of the emerging literature concerning critical *SLC6A4* CpG sites, these findings indicate that higher levels of methylation may downregulate *SLC6A4* gene expression, resulting in less efficient 5HT transport. In the present study, infants with prolonged *in utero* SSRI exposure showed better soothability with greater percent neonatal *SLC6A4* CpG 1,8 (PC2) methylation; a pattern that was not present for infants exposed for fewer days. It is most conceivable that greater methylation reflected decreased gene expression and less 5HT transport, leading to an increased "effective" dose of 5HT contributing to increased soothability. This explanation is consistent with the mechanism described for the impact of prenatal maternal depression, wherein decreased *SLC6A4* methylation in exposed neonates (Devlin et al., 2010) was interpreted as leading to increased *SLC6A4* expression and availability of 5-HTT, in turn resulting in increased 5HT reuptake and lower intrasynaptic 5HT (Waters, Hay, Simmonds, & van Goozen, 2014).

Prenatal SSRI exposure was shown to alter central fetal 5HT concentrations (Laine, Heikkinen, Ekblad, & Kero, 2003), as SSRIs can extensively transfer across the placenta and blood-brain barrier (Kim et al., 2006; Rampono, Simmer, & Ilett, 2009). This crossover to the fetus can be expected to result in more intrasynaptic 5HT for infants whose mothers were using SSRIs during pregnancy, and our results suggest a compounded effect with *SLC6A4* methylation levels. That is, joint influences of SSRI exposure and *SLC6A4* CpG methylation likely increased intrasynaptic 5HT, as highest levels of soothability and greater increases in soothability from 3 to 6 months were observed among SSRI-exposed infants with higher percent methylation at CpG 9,10, and CpG 1,8 (PC2) methylation exhibiting a parallel functional

TABLE 5 Follow-up for predicting soothability with days on SSRI and *SLC6A4* PC2 (CpG 1,8) methylation

	>Median # of days[a]							<Median # of days						
	3 months			6 months			Δ soothability	3 months			6 months			Δ soothability
Variable	Model 1 β	Model 2 β	Model 3 β	Model 1 β	Model 2 β	Model 3 β	Model 4 β	Model 1 β	Model 2 β	Model 3 β	Model 1 β	Model 2 β	Model 3 β	Model 4[b] β
Infant sex	-.01	-.01	-.11	.06	.06	-.04	-.13	-.27	-.26	-.33	-.11	-.08	-.17	.04
Mother internalizing		-.01	.06		.16	.20	.23		-.18	-.18		-.29	-.28	-.20
SLC6A4 methylation			.42[#]			.43**	.34			-.34			-.40[#]	-.18
3-month soothability							.39[#]							.61**
df	1, 22	2, 21	3, 20	1, 22	2, 21	3, 20	4, 19	1, 22	2, 21	3, 20	1, 22	2, 21	3, 20	4, 19
F Δ	.00	.00	3.91[#]	.07	.59	4.80**	2.51	1.46	.64	2.47	.24	1.68	3.48[#]	.10
R^2 Δ	.00	.00	.16	.00	.03	.17	.09	.07	.03	.11	.01	.08	.15	.03

[a]Median = 266 days.
[b]Results obtained for Model 6 represent contributions to Δ soothability (i.e., 3-month soothability controls for stability of this attribute), and only the final model (i.e., Model 6) estimates are presented.
*$p < .05$; **$p < .01$; #$p < .10$.

relationship with our days-of-exposure variable. The resulting compounded exposure during developmentally sensitive periods could change the nature of subsequent 5HT signaling, down-regulating the serotonergic system (Popa, Lena, Alexandre, & Adrien, 2008), in turn leading to infants presenting as more soothable. In this context, early changes in 5HT signaling can be regarded as a plasticity factor that affects development in a manner that can lead to "better and worse" outcomes (Belsky, 1997), a possibility which should be explored in future research.

Regulatory skills emerging in infancy, that in this study appear to be in part a function of 5HT signaling, have been linked with a variety of important developmental processes, serving a protective role with respect to behavior problem/symptoms, social competence, and school readiness (Belsky, Friedman, & Hsieh, 2001; Eisenberg et al., 2004; Gartstein et al., 2012). The present study does not permit us to ascertain definitively if higher levels of soothability observed in exposed infants with greater percent methylation are adaptive, and speak to possible protective effects of SSRI medication treatment during pregnancy, as this apparent soothability could also represent an early manifestation of "under-arousal," noted in children with a history of prenatal SSRI exposure at 6 years of age (Weikum, Mayes, Grunau, Brain, & Oberlander, 2013). Soothability is generally related to self-regulation (Gartstein & Rothbart, 2003; Putnam, Gartstein, & Rothbart, 2008), clustering with the regulatory capacity/orienting factor in infancy and the effortful control factor for toddlers, and future research will need to determine if similar patterns of results, suggesting a protective function of greater soothability, emerge for infants prenatally exposed to SSRIs.

In addition, this study contributes to the conversation concerning decisions in the care of pregnant women requiring medication treatment for depression and/or anxiety. This study provides preliminary information about how prenatal maternal mood and antidepressant treatment may shape the development of one dimension of temperament, namely soothability. The emergence of these early self-regulatory skills may be especially important to consider in the context of how maternal symptoms of depression/anxiety and related treatment issues shape subsequent emotional/behavioral regulation during early childhood (e.g., Babineau et al., 2015; O'Connor et al., 2014).

Our findings with respect to maternal symptoms are somewhat nuanced and deserve additional mention. First, it is notable that mothers treated with SSRI medication during pregnancy exhibited considerably higher levels of symptomatology, relative to women who were not prescribed SSRI medication during pregnancy (Table 1). These ongoing residual symptoms of depression and anxiety observed are consistent with the existing literature suggesting that SSRIs may not always be sufficient for symptom management in all mothers. In addition, one significant effect of prenatal mood/anxiety symptoms emerged in our multiple regression analyses. Namely, greater maternal report of depression/anxiety symptoms predicted lower levels of 3-month soothability for non-exposed infants. As mood symptoms tend to be relatively stable (e.g., Green, 1998; Heron et al., 2004), it is likely these mothers continued to experience higher symptomatology at 3 months, which either interfered with their ability to facilitate soothability, or influenced their perceptions of infants' ability to regulate their arousal with maternal intervention. Future research should examine these possibilities further, utilizing observations of infant emerging regulation, along with parent-report.

A number of limitations of this work should be noted. First, while *SLC6A4* PC1 and mean CpG 9,10 methylation emerged as critical in the context of SSRI exposure status analyses, CpG 1,8 methylation also made a contribution to soothability in addressing links with days of prenatal SSRI antidepressant medication exposure, it remains unclear how to optimally quantity links between exposure and epigenetic marks. Although there has been some variability in the specific sites, or groups of sites, emerging as contributing important methylation markers related to emotion regulation (Booij et al., 2015; Frodl et al., 2015), existing studies support the overall importance of *SLC6A4* CpG methylation in this context (Booij et al., 2013). Caution is needed when interpreting results as methylation in the brain may differ from a peripheral tissue such as whole blood, which consists of different cell types that carry different epigenetic marks (Talens et al., 2010). However, studies finding similarities between *SLC6A4* methylation levels in peripheral and central tissues (Riese et al., 2014), and associations between *SLC6A4* methylation in whole blood and central 5HT synthesis (Wang et al., 2012) provide evidence that peripheral *SLC6A4* methylation status may have potential as a biomarker of central function. Furthermore, given that low percent methylation was observed in the *SLC6A4* promoter and *SLC6A4* mRNA levels were not quantified, the biological significance of our findings needs to be further clarified. Finally, our finding remain confounded by the inherent difficulty to distinguish the impact of maternal mood from the impact prenatal SSRI exposure and address the possibility that mothers prescribed/using medication may be inherently different from those not treated by medication (i.e., confounding by indication) (Marroun, White, Verhulst, & Tiemeier, 2014).

Analyses conducted to further examine relationships between CpG 1,8 methylation status (PC2) and days of prenatal exposure in SSRI exposed infants only were limited by a small sample size. As we utilized maternal report of infant soothability exclusively, future studies need to include a structured behavioral observation, and possibly physiological indicators, to more fully index self-regulation. Finally, we recognize that a very limited number of methylation of *SLC6A4* promoter CpG sites were the focus of our study and future studies should consider contributions of additional genetic and epigenetic targets. For example, Clark et al. (2013) recently linked levels of cerebral spinal fluid oxytocin in the neonates with subsequent soothability, thus methylation of genes associated with this neuro-transmitter, such as the oxytocin receptor (*OXTR*) gene, could be examined in the future. Alternatively, a genome-wide approach successfully utilized to examine methylation in animal models (e.g., Skinner & Guerrero-Bosagna, 2014), and more recently pursued with human infants (Cardenas et al., 2015; Gurnot et al., 2015; Non, Binder, Kubzansky, & Michels, 2014), could be carried out in the context of the variables examined in this study. Gurnot et al. (2015) for example, examined 27,000 CpG sites in genomic DNA, identifying CYP2E1 as the site providing the greatest discrimination between SSRI-exposed and non SSRI-exposed neonates with respect to methylation levels. The CYP2E1 has been linked with psychogenic stress, and the epigenetic changes taking place at this site could be important for infant regulation as well.

In summary, the present study suggests that infant soothability may be shaped by *in utero* exposure to SSRI medication and neonatal *SLC6A4* CpG methylation status. Such findings add to the emerging literature illustrating how fetal programming and biological processes contribute to temperament development.

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