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Developmental administration of valproic acid alters DNA methylation and maternal behavior

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Abstract

Exposure to adversity in early development has powerful and potentially lasting consequences on behavior. Previous work in our laboratory using female Long-Evans rats has demonstrated that exposure to early-life maltreatment manifests into alterations in dam behavior, including a perpetuation of the maltreatment phenotype. These observed behavioral changes coincide with changes in epigenetic activity in the prefrontal cortex (PFC). Further, treating dams with a chromatin modifying agent (Zebularine) normalizes methylation and maltreatment phenotypes, suggesting a link between epigenetic programming and phenotypic outcomes. Here, we sought to investigate if administration of a chromatin modifying agent concurrent with the experience of maltreatment normalizes epigenetic activity associated with maltreatment and alters behavioral trajectories. Administration of valproic acid (VPA) transiently lowered levels of global DNA methylation in the PFC, regardless of exposure to nurturing care or maltreatment. When VPA-exposed animals reached adulthood, they engaged in more adverse behaviors toward their offspring. These data provide further evidence linking epigenetic changes in the developing brain with effects on behavior.

Keywords

early-life adversity, maltreatment, prefrontal cortex, valproic acid

1 | INTRODUCTION

Early-life adversity, in the form of caregiver maltreatment, has been demonstrated to impart numerous consequences to health across the lifespan. This includes immune challenges (Carpenter et al., 2010; Coelho et al., 2013; Lehto et al., 2012), poor physical health, (Danese et al., 2007; Kaufman et al., 2007; L. Li et al., 2015; Sun et al., 2018), increased psychopathology (Doherty et al., 2017; Fonzo et al., 2015; Humphreys et al., 2020; Jaffee, 2017; Weaver et al., 2004), or combinations of all of the above (Danese et al., 2009), making maltreatment a significant public health problem. In an effort to elucidate a biological underpinning to help understand root causes of outcomes and targets for intervention and prevention, work continues to explore epigenetic activity.

One epigenetic mark that continues to be explored is DNA methylation, or the addition of methyl groups to cytosines, often near a promoter sequence and at CG dinucleotides (Moore et al., 2012), which typically (albeit not exclusively) leads to less gene expression (Razin & Cedar, 1991). Work from our laboratory (Blaze & Roth, 2017; Doherty et al., 2019; S. M. Keller et al., 2019; Roth et al., 2009) and others (Hoye et al., 2019; McGowan et al., 2009, 2011; Murgatroyd et al., 2009; Weaver et al., 2004) has demonstrated aberrant DNA methylation in response to early-life adversity, which has the propensity to perpetuate into further generations (Anway et al., 2005; Dias & Ressler, 2013; Franklin et al., 2010; Heijmans et al., 2008; Mulligan et al., 2012; Pilkay et al., 2020; Roth et al., 2009), often perpetuating poor cognitive and mental health. Indeed, because our experiences impact both our health and the health of future generations, exploring how perturbation of the

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epigenome may propagate or mitigate health problems is an important and timely research focus.

While the epigenome displays the remarkable ability to respond to environmental input throughout the lifespan (reviewed in Collins et al., 2020), evidence suggests the presence of sensitive periods where the epigenome is perhaps most attuned to these inputs (Curley & Champagne, 2016; Faulk & Dolinoy, 2011), including early infancy (Dunn et al., 2019). This period is critical for proper brain growth and development (Gilmore et al., 2018), with neural network connectivity already beginning to take shape (Haartsen et al., 2016). Early intervention strategies associated with maltreatment have proven efficacious in ameliorating epigenetic aging (Brody et al., 2015), promoting differential methylation in gene pathways associated with neuronal differentiation and development (Hoye et al., 2019), and improving developmental outcomes (Bernard et al., 2017; Brody et al., 2015; Dozier & Bernard, 2017; Jankowski et al., 2016; Miller, 2015; Nelson et al., 2007). Perturbing epigenetic markers known to be associated with early-life maltreatment is critical to further understanding how health outcomes can be improved.

While typically used in cancer research, the utility of histone deacetylase inhibitors (HDACi) to mitigate epigenetic activity associated with disease continues to be appreciated. Indeed, pharmacological compounds including but not limited to trichostatin A (TSA), sodium butyrate (NaB), or valproic acid (VPA) have proven to be neuroprotective and neurorestorative in several preclinical animal models (Covington et al., 2009, 2015; Kilgore et al., 2009; Revenga et al., 2018; Rumbaugh et al., 2015; Schmauss, 2015). HDACis have demonstrated the capacity to lower DNA methylation (Doherty et al., 2019; Kao et al., 2012; Sarkar et al., 2011; Tremolizzo et al., 2005; Weaver et al., 2004) and have been proposed as potential therapeutics for epigenetic therapy associated with disease states (Szyf, 2009).

Using the scarcity adversity paradigm of limited nesting and bedding, our laboratory has demonstrated that early-life maltreatment causes increased methylation of *brain-derived neurotrophic factor (Bdnf)* DNA in offspring (Doherty et al., 2019; Roth et al., 2009), which persists into adulthood, and is associated with maltreatment behavior in offspring (S. M. Keller et al., 2019; Roth et al., 2009). Indeed, aberrant methylation of *Bdnf* has been associated with psychopathology (D'Addario et al., 2012; Fuchikami et al., 2011; Kang et al., 2013; S. Keller et al., 2010), which work from our laboratory (e.g., Blaze & Roth, 2017; Doherty et al., 2019; S. M. Keller et al., 2019; Roth et al., 2009), and others (Kundakovic et al., 2015) has demonstrated to be significantly impacted by the early caregiving environment. Considering we know that maltreatment in early development induces changes to *Bdnf* methylation and behavior, the epigenome is a sound target to explore how perturbations of methylation impact phenotypic outcomes.

Prior work in our laboratory has demonstrated that administering epigenetic-modifying drugs ameliorates epigenetic marks from animals with a history of caregiver adversity (S. M. Keller et al., 2019; Roth et al., 2009), and normalizes caregiving behaviors of dams exposed to maltreatment in early life (S. M. Keller et al., 2019). However, this approach was exclusively employed in adult animals. If environmental factors in early development can trigger epigenetic alterations and alter behavior, then agents administered to animals concurrent with adverse experiences that can block or diminish methylation could potentially change behavioral trajectories. Utilizing the HDACi NaB, we recently determined its ability to lower maltreatment-induced DNA methylation, though at the doses explored this was only effective in males (Doherty et al., 2019). Work from other laboratories has shown that VPA, when administered to infant rats at the time of daily maternal separation, prevents separation-induced decreases in cued light-potentiated startle in adult rats (Kao et al., 2012).

The present study sought to explore the efficacy of VPA, prior to daily exposure to caregiver maltreatment, to prevent maltreatment-induced DNA methylation increases and associated deleterious consequences for maternal behavior. We sought to first establish an effective dose of the HDACi VPA in altering methylation levels at either a *Bdnf* locus known to be affected by maltreatment, or genome wide global-5mC content in the female whole prefrontal cortex (PFC; Experiment 1). To assess this, a dose of 200 mg/kg, 400 mg/kg, or 600 mg/kg was used in exploring the extent to which this HDACi perturbed methylation levels in animals exposed to aversive or nurturing caregiving conditions. Once an effective dose was established (400 mg/kg), a separate cohort of female animals underwent the paradigm, were grown to adulthood, and incidences of altered behavior were assessed along with *Bdnf* Exon IX methylation and genome-wide 5-mC content in the whole PFC (Experiment 2).

2 | METHODS

2.1 | Experiment 1

2.1.1 | Subjects

All animal procedures were conducted following approval by the University of Delaware Institutional Animal Care and Use committee (IACUC) following NIH established guidelines. All animals were maintained on a 12 h light cycle (7 a.m.–7 p.m.) and were provided food and water ad libitum. Male and female breeding pairs (acquired from Charles River Laboratories) were bred in-house. The pregnant female was then single housed and given ample wood shavings. The day of parturition was classified as postnatal day (PN) 0, and on PN 1, litters were culled to maintain 4–6 males and 4–6 females per litter. One hundred and seventeen female pups (8–12 per group) were used for all behavioral and biochemistry assays. No first-time mothers were used as caregivers or stimulus dams.

2.1.2 | Caregiving manipulations

As previously described by our laboratory elsewhere, (Blaze & Roth, 2017; Doherty et al., 2017; Doherty et al., 2019; S. M. Keller et al., 2019; Roth et al., 2009), rat neonates were exposed to a variation of the scarcity adversity paradigm of limited bedding and nesting. Using a within-litter design for 30 min per day on PN 1–7, male and female

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rat pups were exposed to either a nurturing care condition (with either their home-cage dam or cross-foster dam), or a maltreatment condition. Immediately prior to the paradigm on PN 1–7, rats were injected (intraperitoneally) each day with either 200 mg/kg, 400 mg/kg, or 600 mg/of VPA or saline control. The nurturing condition had adequate nesting materials (~2–3 cm of wood shavings) in either their home cage with their biological dam, or in a plexi chamber with a dam matched for postpartum age and diet and given ample time (1 h) to habituate to the chamber. In the maltreatment condition, pups were exposed to a dam likewise matched for postpartum age and diet but given limited nesting material (~100 ml of wood shavings) and inadequate time (5 min) to habituate to the plexi chamber. All pups were weighed and marked daily, and returned to their home-cage dam at the end of the paradigm and left undisturbed. There were no significant differences between incidences of nurturing care for the normal care group and cross-foster care group, which replicates previous findings in our laboratory, (e.g., Doherty et al., 2019; Roth et al., 2009). Only the normal care condition was used, which represents the nurturing care condition depicted in all analyses. Whenever it was possible, litters were evenly split among the different experimental conditions (VPA or saline, and nurturing or maltreatment).

Caregiving behaviors were recorded, and a subset of caregiving videos from all litters on PN 1, PN 4, and PN 7 were scored by two trained observers via random shuffle, who were blind to the experimental condition in order to replicate the previously established findings from this model (i.e., Blaze et al., 2013; Roth et al., 2009). Videos were coded for adverse (rough handling, stepping, dropping, dragging, stepping, or actively avoiding pups), and nurturing (anogenital licking, pup-licking, nursing, or hovering) behaviors, recorded in 5-min time bins and measured discretely for later statistical analyses. Incidences of individual behaviors were analyzed in addition to the proportion of aversive behaviors to nurturing behaviors calculated as a percentage.

Concurrent with caregiving manipulations on PN 1–7 for 30 min, ultrasonic vocalizations (USVs) were recorded using a Batbox III D detector tuned to 40 kHz. A subset of USVs from PN 1, PN 4, and PN 7 of all F0 litters were later scored by two trained observers, measured discretely in one minute time bins for the 30 min recording, and later used for statistical analyses. The proportion of USVs was calculated and averaged across PN 1, PN 4, and PN 7 to get the average percent 40 kHz USVs emitted in each condition. The interrater reliability of scorers was calculated using Pearson's correlation, and was $r > 0.80$ for all behavioral measurements in Experiment 1.

2.1.3 | Drug injections

98% VPA sodium salt in powder form was obtained from Sigma-Aldrich, and stored according to the manufacturer's suggested storage conditions. Prior to the behavioral paradigm, separate aliquots were dissolved with saline, which were made according to the solubility of VPA (50 mg VPA/1 ml saline). Fresh aliquots were made each day, and rat pups were weighed and injected in the intraperitoneal cavity (IP) with doses of either 200 mg/kg, 400 mg/kg, or 600 mg/kg before being placed in the behavioral paradigm. Saline was administered as a control, with left and right sides of the IP cavity alternated daily to reduce soreness.

2.1.4 | Locus-specific DNA methylation

Work in our laboratory has focused on the *Bdnf* gene. *Bdnf* is a growth factor that is critical for proper dendritic synapse formation and subsequent stabilization (Cohen-Cory et al., 2010; Kowiański et al., 2018; Lu et al., 2014; Reichardt, 2006; Vicario-Abejón et al., 2002), learning and memory (Bekinschtein et al., 2008; Jeon & Ha, 2017), and neuronal survival (Ghosh et al., 1994). Using the scarcity adversity paradigm of limited nesting and bedding, our laboratory has demonstrated epigenetic modifications of *Bdnf* associated with early maltreatment (e.g., Blaze et al., 2013; Doherty et al., 2019; Duffy & Roth, 2020; Roth et al., 2009), with associated alterations in maternal behavior (Roth et al., 2009) that can be ameliorated with adult administration of the DNA methylation inhibitor Zebularine (S. M. Keller et al., 2019). Taken together, *Bdnf* is of interest as stable epigenetic activity at this locus has been associated with early-life maltreatment, with related behavioral modifications observed in adulthood.

Within 24 h of the conclusion of caregiving manipulations (PN 8), rats were sacrificed and whole brains were collected. DNA and RNA were extracted from PFC tissue utilizing manufacturer protocols from the Qiagen Allprep DNA/RNA Mini Kit, and stored at −80°C. To assess nucleic acid quality and concentration, spectrophotometry was performed (NanoDrop 2000). The DNA was then bisulfite converted (Y. Li & Tollefsbol, 2011), using the Qiagen Epitect Bisulfite Kit, and stored at −20°C. *Bdnf* IX methylation levels were determined using methylation specific real-time PCR (MSP; Bio-Rad CFX96) as described elsewhere (Hattori & Ushijima, 2011; e.g., Roth et al., 2009). Tubulin was used as a reference gene, and methylation was quantified using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

2.1.5 | Global DNA methylation

DNA extracted from the same PFC tissue for locus-specific DNA methylation was used to quantify global methylation. MethylFlash Global DNA Methylation ELISA Easy Colorimetric Kits were used according to the manufacturer's instructions (Epigentek, Farmingdale, NY, USA) to quantify genome-wide methylation (5-mC) by measuring light absorbance. DNA samples, along with standards (diluted to a concentration of 0.1, 0.2, 0.5, 1.0, 2.0, or 5.0) were assayed simultaneously and run in vertical duplicates. The methylated DNA was detected using capture and detect antibodies on both the samples and standards. DNA methylation was then colorimetrically quantified by measuring the optical density (OD) using the Infinite F50 microplate reader (Tecan, Männedorf, Switzerland). A standard regression curve was formulated using the OD readings from the standards, with a cutoff R^2 of 0.90. The amount of global 5-mC in each sample was proportional to the intensity of the optical density, which was compared to the stan-

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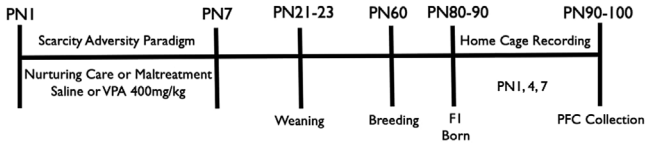

FIGURE 1 Schematic of the timeline of Experiment 2. Female rat pups were exposed to the scarcity adversity paradigm for the first 7 days of life, and were randomly assigned to either the nurturing or maltreatment care condition, and to the saline or valproic acid (VPA) 400 mg/kg condition. Pups were then left undisturbed in their home cage until weaning, which occurred starting on postnatal day (PN) 21. Rat pups were pair housed, matching for infant and drug condition, and left undisturbed except for weekly weighing until PN 60. Starting on PN 60, rats were outbred with males housed in our colony generating an F1 generation. On PN 1, 4, and 7 of the F1 generation, home-cage recordings were taken for 30 min per session from our colony room. At the end of the behavioral paradigm, dams were sacrificed, whole brains were collected, and prefrontal cortex (PFC) was isolated.

dard curve. DNA concentrations were strictly 100 ng/well with total volume added being between 2–5 μl of DNA. Global methylation was quantified using the fold-change method relative to normal care saline animals.

2.2 | Experiment 2

2.2.1 | Subjects

After an effective dose of VPA was established, Experiment 2 used 45 Long-Evans female rats that were bred in-house, exposed to the scarcity adversity model, raised to adulthood (PN 90; 8–12 per group), and assessed in their home cage within the animal colony for behavior exhibited toward their offspring. Animals were injected with either saline or VPA 400 mg/kg as described previously during PN1-7 concurrent with the paradigm (Figure 1).

2.2.2 | Home-cage caregiving behaviors

After the paradigm on PN 1–7, pups were left in their home cage until PN 21, at which point they were weaned with littermates and matched by group. Animals were weighed on PN 1, PN 7, PN 14, PN 21, PN 35, PN 45, and PN 60 to assess normal weight gain. Outside of weight checks, females were left undisturbed until PN 60, at which point they were single housed and bred with breeder males. Dams were then left undisturbed until the new F1 generation was PN 1, at which point F1 litters were culled to 4–6 males and 4–6 females. Home-cage recordings took place on PN 1, PN 4, and PN 7 for 30 min, and maternal caregiving behaviors were scored as described previously (adverse or nurturing). Since there were numerous adverse behaviors occurring in any particular time bin, these variables were scored continuously. In addition, self-grooming data from the dam was recorded both in terms of average duration (in seconds) and a count of the number of times the animal self-groomed, as our laboratory has previously reported an increase in self-grooming as a consequence of early-life maltreatment (Roth et al., 2009).

USVs were recorded as previously described in Experiment 1, from the F1 home cage. The interrater reliability of scores for all behavior measurements in Experiment 2 was calculated using Pearson's correlation, and was $r > 0.80$.

2.2.3 | Locus-specific DNA methylation

Within 24 h of the conclusion of home-cage recordings, the dams (PN 90–100) were sacrificed and whole brains were collected. The PFC was dissected at the time of brain removal and homogenized in 600 μl (PN 90–100) of lysis buffer as described previously for later processing. DNA/RNA extraction, bisulfite modifications, and methylation specific real-time PCR were performed as described above.

2.2.4 | Global DNA methylation

The same PFC tissue used for locus-specific methylation analyses was used to assess global methylation. Global methylation was colorimetrically quantified as described above.

2.2.5 | Statistical analyses

Behavioral data were analyzed using *t*-tests, or one and two-way analysis of variances (ANOVAs) (infant condition by caregiving behavior) where appropriate. Locus-specific DNA methylation was analyzed using two-way ANOVA (infant condition by drug condition). Global methylation was analyzed using the fold-change method, using one-sample students *t*-tests (two tailed) relative to saline controls. Body weight data were analyzed using a two-way mixed model ANOVA (postnatal day by drug condition), and a Greenhouse–Geisser correction was applied (Experiment 2) where the assumption of sphericity was not assumed. Tukey and Bonferonni corrected alpha were performed for post hoc analyses where appropriate. Nonparametric Mann–Whitney tests were used if the assumption of homogeneity of variance was violated for *t*-tests. Statistical significance was set at a threshold of $p < .05$.

3 | RESULTS

3.1 | Experiment 1

3.1.1 | Caregiving behavior

Adverse and nurturing behaviors were averaged across PN 1, 4, and 7 during the first 7 days of life for either the nurturing care or maltreatment condition (Figure 2a). Specific behaviors (stepping, dropping, dragging, actively avoiding, rough handling, nursing, or licking and grooming) are depicted in pie charts (Figure 2b) for each infant condition. A two-way between subjects ANOVA examining the inci-

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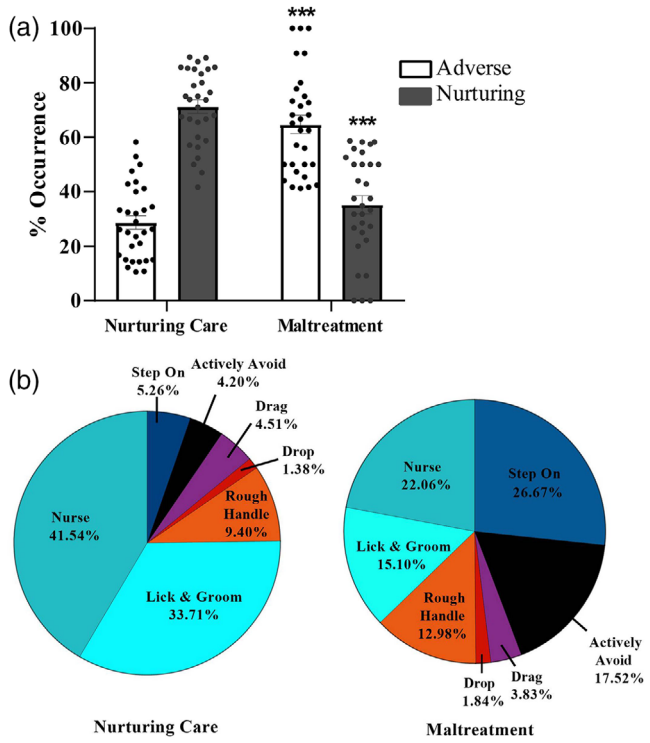

FIGURE 2 Incidences of adverse or nurturing behaviors across either the nurturing care or maltreatment infant condition, depicted as overall percent occurrence (a) or individual behaviors (b). Data are collapsed across all litters for postnatal days (PN) 1, 4, and 7 for each infant condition. $n = 30$ litters/condition. $^{***}p < .001$ versus nurturing care controls. Error bars represent SEM.

dence of adverse or nurturing behaviors between infant conditions revealed a main effect of behavior type, $F(1,116) = 4.84$, $p = .0298$, $\eta^2 = 0.01807$, and a significant behavior type by infant condition interaction, $F(1,116) = 146.8$, $p < .0001$, $\eta^2 = 0.55$. Post hoc comparisons utilizing Tukey's correction criterion revealed that the maltreatment group experienced a significantly larger proportion of adverse behaviors ($M = 64.75$, SD = 18.61) compared to nurturing behaviors ($M = 35.25$, SD = 18.61), $p < .0001$, and the nurturing care group experienced a significantly larger proportion of nurturing behaviors ($M = 71.29$, SD = 13.57) compared to adverse behaviors ($M = 28.72$, SD = 13.57), $p < .0001$. Furthermore, the nurturing care group experienced a significantly lower proportion of adverse behaviors compared to the maltreatment group ($p < .0001$), and a significantly larger proportion of nurturing behaviors compared to the maltreatment group ($p < .0001$).

An *F*-test revealed that the step on ($p = .0092$), drag ($p = .0349$), actively avoiding ($p = .0003$), and rough handling ($p = .0108$) behaviors violated the homogeneity of variance assumption, so these data were analyzed using the nonparametric Mann–Whitney *U* test. The Mann–Whitney *U* test indicated that the maltreatment group experienced a greater amount of stepping (Mdn = 2.09) compared to the nurturing care group (Mdn = 0.33), $U = 74$, $p < .0001$, and a greater amount of actively avoiding (Mdn = 1.50) compared to the nurturing care group (Mdn = 0.17), $U = 141.50$, $p < .0001$. There were no significant differences in incidences of dragging in the maltreatment group (Mdn = 0.09) compared to the nurturing care group (Mdn = 0.17), $U = 425$, $p = .6987$, or incidences of rough handling (Mdn = 0.75) compared to the nurturing care group (Mdn = 0.67), $U = 431$, $p = .7817$. Independent samples *t*-tests revealed that there were no significant differences in incidences of dropping between the maltreatment group ($M = 0.14$, SD = 0.27) and the nurturing care group ($M = 0.12$, SD = 0.20), $t(58) = 0.35$, $p = .7242$. However, the nurturing care group experienced significantly more incidences of pup licking ($M = 2.99$, SD = 1.06) and nursing ($M = 3.68$, SD = 1.62) compared to the maltreatment group licking ($M = 1.18$, SD = 0.85), $t(58) = 7.29$, $p < .0001$, and nursing ($M = 1.73$, SD = 1.32), $t(58) = 5.13$, $p < .0001$.

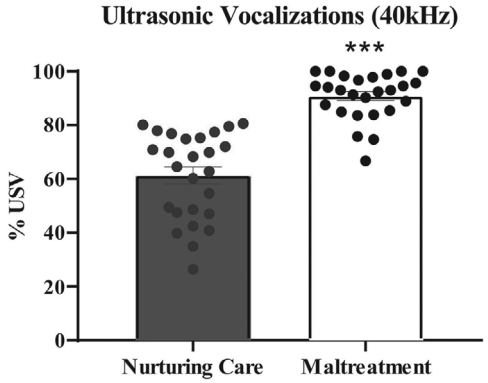

FIGURE 3 Ultrasonic vocalizations at a frequency of 40 kHz were obtained concurrent with caregiving manipulations across the first 7 days of life. $n = 26$ litters/condition. $^{***}p < .001$ versus nurturing care controls. Error bars represent SEM.

3.1.2 | Ultrasonic vocalizations

USVs were averaged across PN 1, 4, and 7 during the first 7 days of life for both the nurturing care and the maltreatment condition (Figure 3). An *F*-test to compare variances indicated that the homogeneity of variance assumption was violated ($p = .0026$), so the nonparametric Mann–Whitney *U* test was implemented in the analysis. Results indicated that the maltreatment group emitted significantly more 40 kHz USVs (Mdn = 93.01) compared to the nurturing care group (Mdn = 66.40), $U = 27$, $p < .0001$.

Taken together, these data along with the findings from the caregiving manipulations replicate previous findings from our laboratory using this model (e.g., Blaze & Roth, 2017; Doherty et al., 2019; S. M. Keller et al., 2019; Roth et al., 2009), such that pups in the maltreatment condition are being exposed to more adverse experiences as a function of experimental conditions created.

3.1.3 | Locus-specific DNA methylation

A two-way between subjects ANOVA (Figure 4a) examining the effect of infant condition (nurturing care or maltreatment) and drug dose,

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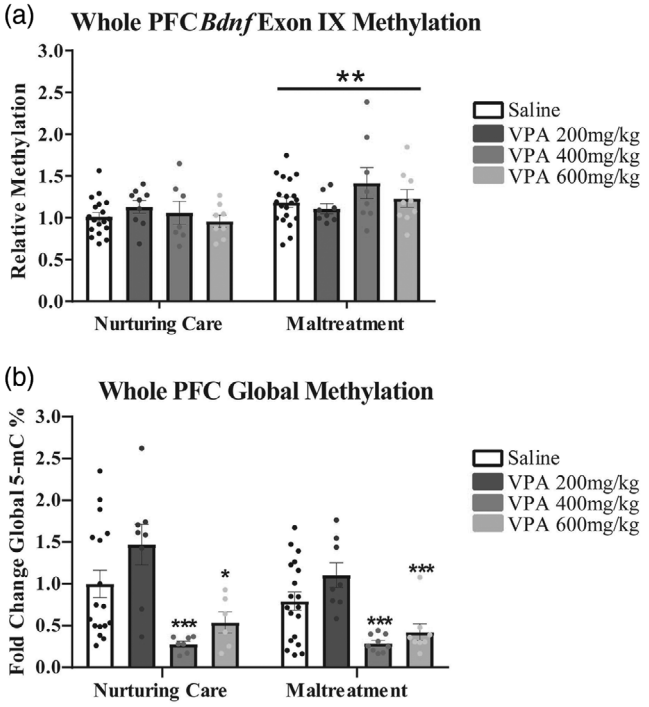

FIGURE 4 Locus-specific *Bdnf* Exon IX methylation (a) and fold-change global-5mC (b) compared to saline nurturing care controls were assessed in whole female prefrontal cortex (PFC) after exposure to the paradigm. $n = 6–19$/group. $^{**}p < .01$ versus nurturing care (a), $^{*}p < .05$ versus nurturing care saline, $^{***}p < .001$ versus nurturing care saline (b). Error bars represent SEM.

(VPA 200 mg/kg, 400 mg/kg, or 600 mg/kg) on *Bdnf* Exon IX methylation in the female PFC was conducted. A significant main effect of infant condition was observed, $F(1,79) = 8.56$, $p = .0045$, $\eta^2 = 0.0925$, such that females in the maltreatment group had significantly higher *Bdnf* Exon IX methylation in the PFC ($M = 1.22$, SD = .33) compared to the nurturing care group ($M = 1.04$, SD = .25). There was no significant main effect of drug dose, $F(3,79) = 0.91$, $p = .4379$, and no significant interaction, $F(3,79) = 1.28$, $p = .2867$.

Maltreated-animals possessing higher *Bdnf* Exon IX methylation compared to nurturing care animals replicates previous findings from our laboratory (e.g., Doherty et al., 2019; Roth et al., 2009). While VPA did not lower methylation at this time point, other HDACis have proven efficacious at doing so in males (Doherty et al., 2019), suggesting that VPA has differential specificity. To elucidate this further, and since VPA has been demonstrated to cause widespread epigenetic reprogramming of DNA methylation (Milutinovic et al., 2007), global methylation levels were quantified.

3.1.4 | Global DNA methylation

The same subset of PFC tissue used to assess locus-specific *Bdnf* Exon IX methylation was used to quantify fold-change global-5mC levels (Figure 4b). Using a two-tailed students *t*-test, VPA at a dose of 400 mg/kg in both the nurturing ($M = 0.28$, SD = .09), $t(6) = 20.38$, $p < .001$) and maltreatment ($M = 0.29$, SD = 0.10), $t(8) = 20.56$, $p < .001$ animals produced a significant fold-change decrease in global-5mC percent compared to nurturing saline animals. Likewise, VPA at a dose of 600 mg/kg in both the nurturing ($M = 0.54$, SD = 0.31), $t(5) = 3.67$, $p = .0145$, and maltreatment ($M = 0.42$, SD = 0.28), $t(7) = 5.84$, $p = .0006$ animals produced a fold-change decrease in global-5mC percent compared to nurturing saline animals.

Together, these results indicate that VPA at either a dose of 400 mg/kg or 600 mg/kg is efficacious in lowering global-5mC levels in the whole PFC, despite having no effect on locus-specific *Bdnf* Exon IX methylation in females. However, since VPA at a dose of 600 mg/kg significantly lowered PN 7 body weight compared to saline animals (Table 1), 400 mg/kg was chosen as the effective dose of VPA and used in Experiment 2.

3.2 | Experiment 2

3.2.1 | Body weight and litter health

A two-way mixed model ANOVA examining the effect of drug condition (saline or VPA 400 mg/kg) on body weight over time (PN 1–60) revealed a significant interaction, $F(6,192) = 17.43$, $p < .0001$, $\eta^2 = 0.0032$. Since the variability of body weight increases with age, sphericity was not assumed and a Greenhouse–Geisser correction was applied. Post hoc analyses using Bonferroni adjusted alpha criterion revealed significant differences of body weight between saline and 400 mg/kg VPA injected animals (Table 2), such that starting at PN 7 and continuing to PN 60, drug injected animals weighed significantly less at each time point compared to their saline counterparts. However, the body weights of drug injected animals were within normative developmental weights as indicated by growth charts provided by the vendor (Charles River Laboratories).

To examine the health effects of the F1 generation, body weight (Figure 5a), F:M ratio (Figure 5b), and litter size (Figure 5c) were recorded. A two-way mixed model ANOVA examining the effect of drug condition on body weight over time (PN 1–7) revealed no significant interaction $F(1,211) = .01$, $p = .9070$, but a significant main effect of time, $F(1,211) = 12,183$, $p < .0001$, $\eta^2 = 0.94$, such that all pups gained weight throughout development. We next examined litter size and F:M ratio, as stress is known to bias this ratio toward females (Trivers & Willard, 1973). Separate two-way ANOVAs examining the effect of infant and drug condition on litter composition were conducted. We found no significant difference in the F:M ratio, including no main effect of drug, $F(1,37) = 0.17$, $p = .6807$, no main effect of infant condition, $F(1,37) = 0.76$, $p = .3880$, and no interaction effect, $F(1,37) = .14$, $p = .706$. However, there was a significant interaction for litter size, $F(1,36) = 4.90$, $p = .0333$, $\eta^2 = 0.11$. Tukey's post hoc analyses indicated that the maltreatment animals injected with saline had a significantly reduced litter size ($M = 12.00$, SD = 2.68) compared to nurturing care animals injected with saline ($M = 14.75$, SD = 1.91), $p = .0433$.

Taken together, these results suggest that dams exposed to VPA 400 mg/kg were gaining weight at a slower rate compared to saline

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TABLE 1 Drug-dose response of valproic acid (VPA) on pup body weight (grams)

	Saline		VPA 200 mg		VPA 400 mg		VPA 600 mg					
Age	M	SD	M	SD	M	SD	M	SD		*t*	*df* (294)	*p*
PN 1	7.12	0.81	7.01	0.54	8.04	0.81	7.2	0.80				
									Saline vs. 200 mg	0.38		>.9999
									400 mg	2.14		=.1982
									600 mg	0.20		>.9999
PN 7	17.56	2.52	15.45***	1.55	16.39*	1.70	13.08***	1.62				
									Saline vs. 200 mg	7.17		<.0001
									400 mg	2.70		=.0436
									600 mg	11.13		<.0001

Note: Animals were weighed in all conditions at PN 1 and PN 7. A mixed-model ANOVA examining drug condition by postnatal weight changes over time revealed a significant interaction, $F(3,147) = 46.39$, $p < .0001$, $\eta^2 = 0.18$. All data in the table are representative of post hoc analyses utilizing Bonferroni's adjusted alpha criterion, and represent comparisons against saline controls.
Abbreviation: PN, postnatal day
*$p<.01$ for each respective drug dose compared to saline.
***$p < .001$ for each respective drug dose compared to saline. $n = 24$ litters.

TABLE 2 The effect of 400 mg/kg valproic acid (VPA) on developmental body weight (grams)

Age	Saline		400 mg/kg VPA		*t*	*df*	*p*
	M	SD	*M*	SD			
PN 1	6.92	0.62	6.74	0.55	.87	30.10	>.9999
PN 7	16.53	1.67	13.05***	1.75	5.82	27.27	<.0001
PN 14	33.98	3.57	27.91***	2.94	5.43	31.06	<.0001
PN 21	55.73	2.69	45.82***	3.45	9.01	23.49	<.0001
PN 35	135.43	6.96	115.89***	6.27	8.54	29.88	<.0001
PN 45	183.69	11.60	161.28***	9.67	6.12	30.92	<.0001
PN 60	238.56	17.20	212.57***	14.30	4.80	30.95	=.0003

Note: Animals were weighed in all conditions at PN 1, PN 7, PN 14, PN 21, PN 35, PN 45, and PN 60 to examine the effect of drug on body weight at these time points. Data in the table are representative of post hoc analyses, utilizing Bonferroni's adjusted alpha criterion.
Abbreviation: PN, postnatal day.
***$p < .001$ compared to saline control at each respective timepoint. $n = 14$–20 dams per drug condition.

controls, which became more apparent throughout development. The drug effect on body weight is ameliorated in the progeny in which there is also no drug effect on litter size or F:M ratio. This suggests that valproate does not interfere with dam sexual development or the capacity for normative parturition. However, these data suggest that exposure to early-life maltreatment, through the scarcity adversity paradigm of limited nesting and bedding, can impart implications in regard to future litter size.

3.2.2 | Caregiving behavior

Figure 6a displays the number of adverse incidences these mothers engaged in toward their F1 offspring, averaged across home-cage recordings from PN 1, 4, and 7. A two-way between subjects ANOVA indicated no significant interaction $F(1,37) = .02$, $p = .8856$, and no significant main effect of infant condition, $F(1,37) = .10$, $p = .7595$. However, there was a main effect of drug condition, $F(1,37) = 7.70$, $p = .0086$, $\eta^2 = 0.17$, such that moms who were exposed to VPA 400 mg/kg in infancy engaged in significantly more adverse behaviors ($M = 24.29$, SD = 1.00) toward their own offspring compared to the saline injected animals ($M = 15.62$, SD = 0.36) irrespective of previous infant condition.

Separate two-way between subjects ANOVAs were conducted for each adverse behavior to elucidate the type of aversive behavior the dams were engaging in. There was no significant main effect of drug condition observed for incidences of stepping, $F(1,35) = .01$, $p = .9178$, dropping, $F(1,37) = 0.82$, $p = .3700$, or actively avoiding, $F(1,37) = 3.29$, $p = .0780$. However, there was a main effect of drug condition observed for incidences of rough handling, $F(1,37) = 7.400$, $p = .0099$, $\eta^2 = 0.16$, with drug-exposed dams engaging in significantly more incidents of rough handling ($M = 12.72$, SD = 1.58) toward their F1 offspring, compared to saline-exposed animals ($M = 7.35$, SD = 0.75). Furthermore, there was a significant main effect of drug condition observed for inci-

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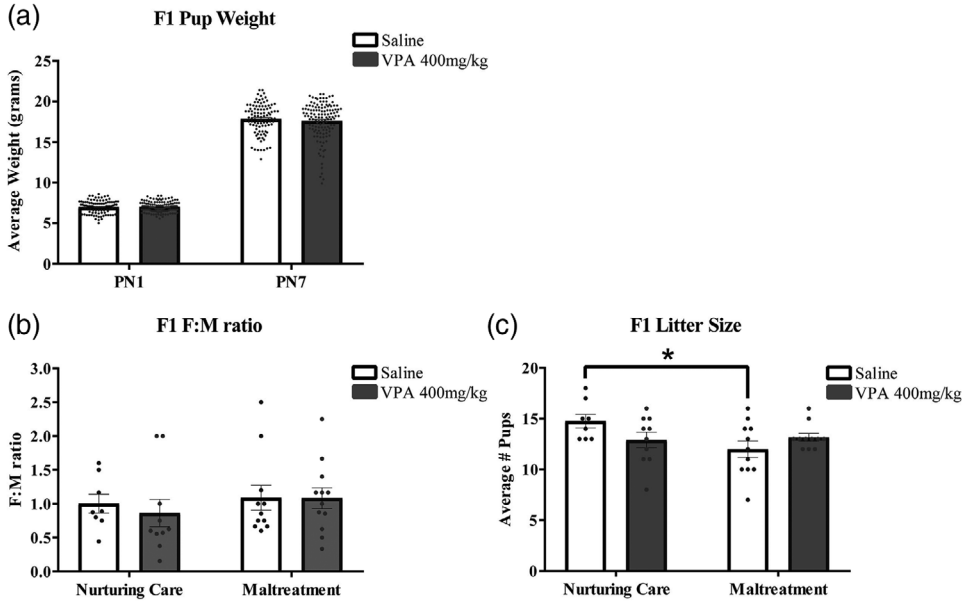

FIGURE 5 F1 pup weight (a), F1 F:M ratio (b), and F1 litter size (c) were recorded to examine the effects of valproic acid (VPA) 400 mg/kg on parturition and health of the progeny, which were averaged across each infant condition. $n = 8–12$ litters/group. $^{*}p < .05$, nurturing care saline versus maltreatment saline. Error bars represent SEM.

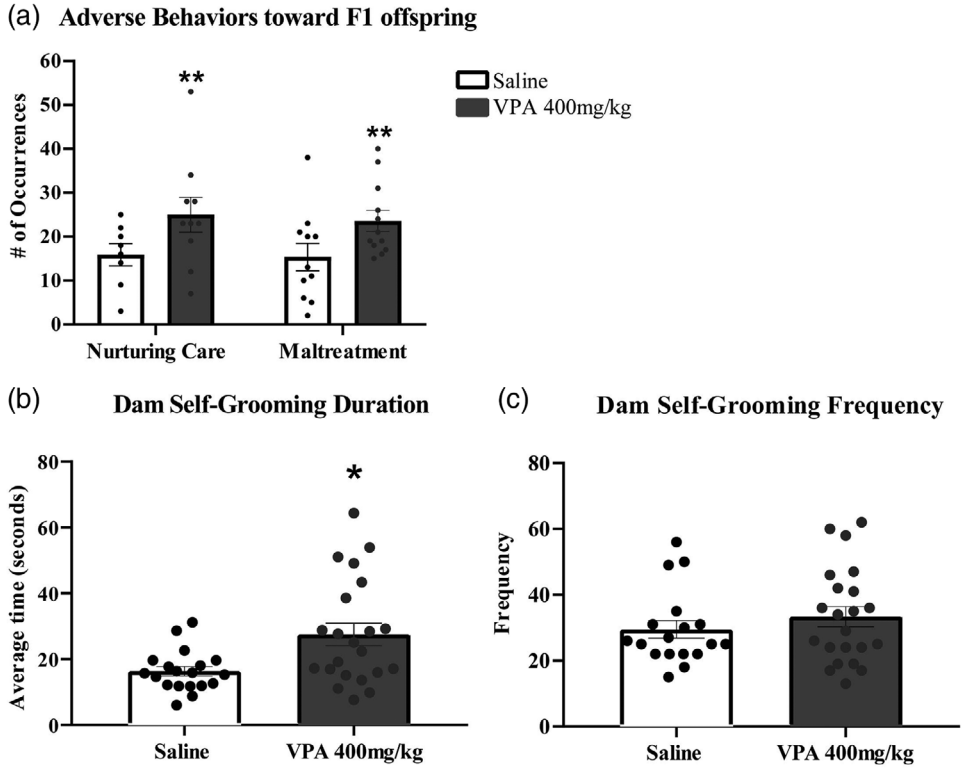

FIGURE 6 Home-cage recordings for each dam took place on postnatal days (PN) 1, 4, and 7, and the number of occurrences were averaged by infant and drug condition for these recordings (a). $n = 8–12$ dams/condition. $^{**}p < .01$, main effect of drug versus saline. Additionally, dam self-grooming duration (b) and frequency (c) were recorded throughout the home-cage recording, and averaged by drug condition. $n = 8–12$ dams/condition. $^{*}p < .05$ versus saline controls. All error bars represent SEM.

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dences of dragging, $F(1,35) = 7.92$, $p = .0080$, $\eta^2 = 0.18$, such that drug-exposed animals engaged in significantly more occurrences of dragging ($M = 3.20$, SD = 0.28) toward their F1 offspring compared to saline-exposed animals ($M = 0.54$, SD = 0.05).

Together, these results suggest that VPA at a dose of 400 mg/kg transiently injected in development increases later maternal maltreatment toward F1 offspring. This may be associated with the pharmacological efficacy of this drug in lowering global 5-mC methylation at PN 8.

3.2.3 | Grooming behavior

Figure 6b,c depicts the average duration and frequency of grooming for the dams exposed to either saline or VPA 400 mg/kg in infancy. The assumption of homogeneity of variance was violated for grooming duration ($p < .001$). A nonparametric Mann–Whitney test revealed that the drug-exposed dams self-groomed for longer durations (Mdn = 23.68) compared to saline-exposed dams (Mdn = 15.68), $U = 120$, $p = .0195$. However, an independent samples t-test on grooming frequency found no differences between the drug-exposed ($M = 33.36$, SD = 14.48) and saline-exposed ($M = 29.50$, SD = 11.34) dams.

3.2.4 | Locus-specific DNA methylation

A two-way between subjects ANOVA examining the effect of previous infant condition and drug condition on *Bdnf* Exon IX methylation in the PFC of PN 90 dams revealed no significant interaction, $F(1,40) = 1.32$, $p = .2583$, and no significant main effect of infant condition $F(1,40) = 1.78$, $p = .1895$ or drug condition $F(1,40) = .6055$, $p = .4411$ (data not shown).

3.2.5 | Global DNA methylation

Compared to saline nurturing care, there were no fold-change differences in global 5-mC detected for the drug-exposed nurturing care dams, $t(9) = 1.08$, $p = .3093$, saline-exposed maltreated dams, $t(10) = 0.82$, $p = .4292$, or drug-exposed maltreated dams, $t(11) = 1.30$, $p = .2192$ (data not shown).

Taken together, these data indicate that the differences seen in infancy in both locus-specific *Bdnf* Exon IX methylation in response to maltreatment, and global-5mC methylation in response to VPA, are transient effects.

4 | DISCUSSION

Here, we show that animals exposed to caregiver maltreatment displayed increased *Bdnf* Exon IX methylation in the PFC during infancy. While VPA was ineffective at lowering this locus-specific methylation, it proved efficacious in lowering global-5mC content in the PFC in animals with either a history of maltreatment or nurturing care. This decrease in global-5mC was transient, as no differences were detected in adulthood. Despite this transient decrease, administration of this compound produced behavioral modifications later in life that included an increased propensity to maltreat progeny and engage in longer bouts of self-grooming.

Rodent pups were exposed to the scarcity adversity model of limited nesting and bedding, with variations of this model employed widely (e.g., Davis et al., 2020; Gallo et al., 2019; Ivy et al., 2008) and with translational relevance (Kentner et al., 2018; Walker et al., 2017). As previously established by our lab, rat pups exposed to the scarcity adversity paradigm of limited nesting and bedding outside of the home cage emit a greater proportion of 40 kHz USVs, and are exposed to more adverse caregiving behaviors, including stepping, dropping, dragging, actively avoiding, or rough handling (e.g., Doherty et al., 2019; Roth et al., 2009). Note that 40 kHz USVs are emitted by pups immediately after birth (Allin & Banks, 1971) and are indicative of distress or anxiety (Portfors, 2007; Schwarting & Wöhr, 2012; Simola, 2015). Data from the present study are in alignment with previous findings.

We replicate previous findings in our laboratory such that maltreated-pups have increased methylation of *Bdnf* Exon IX DNA in the PFC. Valproic acid at all doses tested in the present study was ineffective at altering *Bdnf* methylation levels in these animals or nurturing care controls. Prior work in our laboratory using NaB, an alternate HDACi, had demonstrated its efficacy in lowering methylation associated with maltreatment in male pups, while having no effect on either female or control animals (Doherty et al., 2019). Further, we have demonstrated the capacity of other pharmacological agents, including the DNA methyltransferase inhibitor Zebularine, to normalize *Bdnf* methylation and adult behavior (maternal behavior) disrupted by maltreatment (S. M. Keller et al., 2019; Roth et al., 2009), while disrupting gene levels and behavior in animals without a history of maltreatment (S. M. Keller et al., 2019).

In the present study, while VPA was ineffective at lowering *Bdnf* IX methylation in either nurtured- or maltreated-animals, it significantly lowered global methylation at either 400 mg/kg or 600 mg/kg doses in both care conditions. Taken together, data here and from our prior work demonstrate the ability to alter the epigenome with pharmacological approaches, but the efficacy of these compounds is dependent on contextual factors, including sex and early-life experience. Indeed, there are numerous classes of HDACis (I, IIa, IIb, III, IV) which have different protein structures and use different signaling pathways (Morris & Monteggia, 2013; Seto & Yoshida, 2014), and thus can impart different epigenetic and behavioral consequences, especially when interacting with contextual factors like disease states. For example, administration of TSA, a Class 1 and II HDACi, has been used to delay neurodegeneration (Kim et al., 2019), reverse memory impairment and anxiety associated with binge drinking, (Montagud-Romero et al., 2019; Sakharkar et al., 2014), has been shown to alter locus specific methylation associated with low licking and grooming (Weaver et al., 2004), and has been reported to decrease global methylation content in human cancer cell lines (Ou et al., 2007). Administration of VPA has been

shown to prevent epigenetic marks and behavioral changes associated with schizophrenia etiology in mice (Tremolizzo et al., 2005), while also impairing contextual memory (Sintoni et al., 2013) and spatial working memory (Umka et al., 2010). One study found impairments of neurogenesis in the hippocampus upon administration of VPA (Umka et al., 2010), while others found in the context of Alzheimer's disease (Zeng et al., 2019) or ischemic stroke (Liu et al, 2012), there is an improvement in neurogenesis.

Developmentally, the animals injected with 400 mg/kg gained weight more slowly, but all animals were within normal developmental growth as suggested by the animal vendor (Charles River Laboratories). While we report no bias toward female ratio as a function of stress (Trivers & Willard, 1973), our data indicated that exposure to early-life stress impacted litter size, with reductions observed in maltreated-exposed animals compared to nurturing care-exposed animals. Previous work in our laboratory using this paradigm found no significant differences in litter size, or female to male ratio (S. M. Keller et al., 2019). One potential reason for this discrepancy is daily injection and handling early in development in the present study, which has been demonstrated in animal models to enhance glucocorticoid signaling and stress (Deutsch-Feldman et al., 2015; Drude et al., 2011), which was not present in our previous study. While some work has suggested that restraint stress in mice reduces litter size (Wiebold et al., 1986), other work has suggested that the reduction in litter size may improve maternal care toward rat offspring (Enes-Marques & Giusti-Paiva, 2018). While it is not clear how early-life maltreatment in the scarcity adversity paradigm may impart deleterious consequences on parturition health in Long-Evans dams later in life, more work is needed to examine how litter size or health is impacted by this paradigm, and the consequences for behavioral outcomes.

In the present study, we report that IP exposure to VPA during the first week of life imparts consequences for later behavior, leading to higher occurrences of aversive caregiving behaviors regardless of infant care condition. In prior studies, we have observed that maltreated-animals grow up and mistreat their own pups (S. M. Keller et al., 2019; Roth et al., 2009), a finding that was not observed here. Likewise, we failed to observe the finding of increased methylation at *Bdnf* Exon IX in adulthood for maltreated (saline controls) animals. Methodologically speaking, there are a number of differences between this study and our prior, including decreased litter size with possible consequences on maternal behavior (Enes-Marques & Giusti-Paiva, 2018), and the daily handling and injecting of animals (with their own possible physiological consequences) that precludes direct comparison. Finally, we report that rodent pups exposed to VPA grow up with an increased propensity to maltreat progeny and self-groom for a longer duration. Rodent self-grooming is a repetitive, stereotyped behavior consisting of both a facial stroke phase and a body licking phase (Berridge et al., 1987). Increased incidences of self-grooming, including duration or frequency of engagement, are associated with numerous translationally relevant psychiatric disorders (for review, see Kalueff et al., 2016), and are notably increased during periods of stress (Reis-Silva et al., 2019).

We note several limitations in the present study. First, since an alteration in global DNA methylation was a transient effect, our data are correlative and it is possible that changes in maternal behavior were induced by an additional or alternative mechanism of VPA. Indeed, VPA is a commonly prescribed mood stabilizer, acting in the brain by enhancing GABA signaling (Chateauvieux et al., 2010) blocking voltage-gated ion channels (Ghodke-Puranik et al., 2013), and increasing levels 5-HT in the hippocampus (Biggs et al., 1992). It is possible that transient or stable alterations in these pathways, and not per-say lowered global methylation, lead to behavioral changes in adulthood.

Second, while VPA is commonly prescribed to treat epilepsy (Romoli et al., 2019) and has reported neuroprotective effects (Zhang et al., 2014), including mitigating cognitive impairments associated with Alzheimer's disease (Yao et al., 2014; Xuan et al., 2015) and alleviating depressive symptoms (Smith et al., 2010), VPA administration has consequences for the progeny if given during pregnancy (Ornoy, 2009), including inducing autism-spectrum disorder (Christensen et al., 2013; Zhao et al., 2019). Likewise, administering VPA to young children increases the propensity for liver damage (Mindikoglu et al., 2011; Star et al., 2014). Thus, the developmental timing of administration can have deleterious or beneficial consequences for behavior and disease etiology, which should be a consideration and avenue for future work in the utility of VPA as an effective HDAC inhibitor.

Third, we were unable to establish effective drug parameters to improve behavioral outcomes. Additional work is warranted to assess this possibility with other epigenetic agents at varying doses. Nonetheless, data from this study demonstrate the responsivity of the epigenome to adversity, and its malleability via pharmacological intervention with consequences for behavior.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Tiffany S. Doherty initiated this project. Nicholas J. Collins, Tiffany S. Doherty, and Tania L. Roth designed the study. Natalia L. H. Phillips provided training on experimental procedures, and Nicholas J. Collins, Catherine W. Zimmerman, SF, and Tiffany S. Doherty collected the data. Nicholas J. Collins and Tania L. Roth interpreted the results. Nicholas J. Collins wrote the first draft of the manuscript, and all authors edited the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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