Received: 2 December 2016 | Revised: 22 March 2017 | Accepted: 30 March 2017

DOI: 10.1111/jne.12475

ORIGINAL ARTICLE

WILEY Journal of Neuroendocrinology

Exposure to extrinsic stressors, social defeat or bisphenol A, eliminates sex differences in DNA methyltransferase expression in the amygdala

E. C. Wright[1] | S. A. Johnson[2,3,4] | R. Hao[1] | A. S. Kowalczyk[1] | G. D. Greenberg[5] | E. Ordoñes Sanchez[1] | A. Laman-Maharg[5] | B. C. Trainor[1,5] | C. S. Rosenfeld[2,3,6]

[1]Department of Psychology, University of California, Davis, CA, USA

[2]Bond Life Science Center, University of Missouri, Columbia, MO, USA

[3]Department of Biomedical Sciences, University of Missouri, Columbia, MO, USA

[4]Department of Animal Science, University of Missouri, Columbia, MO, USA

[5]Neuroscience Graduate Group, University of California, Davis, CA, USA

[6]Genetics Area Program and Thompson Center for Autism and Neurobehavioral Disorders, University of Missouri, Columbia, MO, USA

Correspondence
Cheryl S. Rosenfeld, Bond Life Science Center, University of Missouri, Columbia, MO, USA.
Email: rosenfeldc@missouri.edu
and
Brian C. Trainor, Department of Psychology, University of California, Davis, CA, USA.
Email: bctrainor@ucdavis.edu

Funding information
Foundation for the National Institutes of Health, Grant/Award Number: 5R21ES023150 and MH103322

Chemical and psychological stressors can exert long lasting changes in brain function and behaviour. Changes in DNA methylation have been shown to be an important mechanism mediating long lasting changes in neural function and behaviour, especially for anxiety-like or stress responses. In the present study, we examined the effects of either a social or chemical stressor on DNA methyltransferase (DNMT) gene expression in the amygdala, an important brain region modulating stress responses and anxiety. In adult California mice (*Peromyscus californicus*) that were naïve to social defeat, females had higher levels of *Dnmt1* expression in punch samples of the central amygdala (CeA) than males. In addition, mice that underwent social defeat stress showed reduced *Dnmt1* and *Dnmt3a* expression in the CeA of females but not males. A second study using more anatomically specific punch samples replicated these effects for *Dnmt1*. Perinatal exposure (spanning from periconception through lactation) to bisphenol A or ethinyl oestradiol (oestrogens in birth control pills) also abolished sex differences in *Dnmt1* expression in the CeA but not the basolateral amygdala. These findings identify a robust sex difference in *Dnmt1* expression in the CeA that is sensitive to both psychological and chemical stressors. Future studies should aim to examine the impact of psychological and chemical stressors on DNA methylation in the CeA and also investigate whether *Dnmt1* may have an underappreciated role in plasticity in behaviour.

KEYWORDS
California mouse, DNMT1, DNMT3A, endocrine disruptors, oestrogen receptor

1 | INTRODUCTION

Neurobehavioural programming is the product of both the intrinsic and extrinsic influences experienced across a lifespan. Experiences, such as social interactions or dietary alterations, can induce neuroadaptations that lead to long lasting changes in behaviour. The methylation of CpG sites can be an important mechanism for mediating these experience-driven effects.[1-3] One of the ways this process is controlled is by DNA methyltransferases (DNMT), with *Dnmt1* primarily targeting hemimethylated CpG and *Dnmt3a*, targeting both unmethylated and hemimethylated CpG sites.[4] The impact of DNMTs early in life is well documented,[5-7] and there is growing evidence to indicate that DNMTs can also mediate the effects of experience on brain function and behaviour in adults. For example, the infusion of a DNMT inhibitor in hippocampus blocked the formation of long-term fear memories.[8] The behavioral effects of social defeat stress, which normally induces anxiety-like and depression-like behaviours in male rodents, were reduced with infusions of a DNMT inhibitor into the nucleus accumbens.[9] It was also shown that defeat stress increased *Dnmt3a* expression, suggesting that experience can modulate DNMT activity, which in turn affects behavioural responses to stress.[6,7,10] Another form of experience that can alter DNMT expression within

the brain is through exposure to endocrine disrupting chemicals (EDC) in the environment. Bisphenol A (BPA) is an EDC that binds weakly to oestrogen receptors (ESR1 and ESR2, also referred to as ERα and ERβ respectively).[11] In utero exposure to BPA was shown to increase the expression of *Dnmt3a*, as well as hypermethylation of *Esr1*, in the male mouse prefrontal cortex, whereas the same dose induced down-regulation of *Dnmt3a* and hypomethylation of *Esr1* in the female mouse prefrontal cortex. In the same study, *Dnmt1* expression was decreased by BPA exposure in the female mouse hypothalamus. This suggests that BPA exposure may disrupt sexually dimorphic methylation patterns.[12] Prenatal BPA exposure also leads to increased expression of *Dnmt1* in the male mice hippocampus.[13] There is growing evidence that exposure to EDCs such as BPA early in life can interact with stressors experienced later in life. This idea has been conceptualised as the "two hit" hypothesis, which poses that a combination of external hits can produce a phenotype that is greater than the sum of their individual effects.[14,15] As a first step towards testing this hypothesis, we examined the individual effects of developmental BPA exposure (or ethinyl oestradiol: EE, positive oestrogen control for BPA studies) or social defeat experienced at adulthood on DNMT gene expression.

We chose to study the California mouse (*Peromyscus californicus*), a monogamous, biparental rodent species in which both males and females are vulnerable to social defeat.[10,16] This approach allowed us to study the effect of defeat in both males and females, an important aspect to consider because sex differences in the effects of psychosocial stress on DNMT function have been observed.[17] California mice are also sensitive to perinatal BPA exposure.[18-21] We examined the effects of these stressors in the amygdala, which is sensitive to environmental stressors,[10,22,23] and regulates behavioural responses to stress.[24] In female rats, perinatal BPA exposure reduced *Dnmt1* expression in the basolateral amygdala (BLA) and increased anxiety-like behaviour.[25] Sex differences in *Dnmt3a* expression have been found in the developing amygdala[26] but, to our knowledge, adults have not been studied. In the present study, we examined *Dnmt1* and *Dnmt3a* expression in the medial amygdala (MeA), central nucleus of the amygdala (CeA) and BLA. Two experiments were performed to examine the effects of social defeat stress on *Dnmt* expression in adult males and females, while a third experiment focused on developmental BPA exposure. Although specific subnuclei of the amygdala have not been examined, previous studies have generally reported stronger effects of chemical[12] or psychosocial[17] stressors on *Dnmt* expression in females versus males. Based on these results, we predicted that social defeat and BPA would have stronger effects on DNMT gene expression in females than males.

2 | MATERIALS AND METHODS

2.1 | Animals

For Experiments 1 and 2, California mice were obtained from a pathogen-free breeding colony at the University of California (UC), Davis. They were group housed (two or three same-sex animals per cage) and maintained in a temperature-controlled room under a 16 : 8 hour light/cycle with water and food available ad libitum (Harlan Teklad 2016; Harlan Teklad, Madison, WI, USA). Cages comprised polycarbonate plastic with Sani-Chip bedding (Harlan Laboratories, Indianapolis, IN, USA), Nestlets (Ancare, Bellmore, NY, USA) and Enviro-Dri (Eco-bedding, Fibercore, Cleveland, OH, USA).[27] All procedures were approved by the UC Davis Internal Animal Care and Use Committee and conformed to National Institutes of Health (NIH) guidelines.

For Experiment 3, outbred adult (60-90 days of age) founder California mouse females and males, free of common rodent pathogens, were purchased from the *Peromyscus* Genetic Stock Center (PGSC) at the University of South Carolina (Columbia, SC, USA). They were placed in quarantine at the University of Missouri for a minimum of 8 weeks to ensure that they did not carry any transmittable and zoonotic diseases. From the time that the animals had been captured between 1979 and 1987, *P. californicus* captive stocks have been bred by the PGSC to maintain their outbred status. Mice were housed in polypropylene cages (Allentown Inc., Allentown, NJ, USA) with aspen shaving bedding (Bourn Feed, Columbia, MO, USA) and nestlets. All experiments were approved by the University of Missouri Animal Care and Use Committee and confirmed to NIH guidelines.

2.2 | Experiment 1: Effects of social defeat on gene expression in the CeA and MeA

Male and female California mice were randomly assigned to either social defeat stress or to remain naïve to defeat. Each mouse assigned to social defeat was placed in the cage of an aggressive same-sex mouse resident mouse.[28] Each episode lasted 7 minutes or until the resident attacked the focal mouse seven times (whichever occurred first), and this was repeated on three consecutive days with three different residents. Naïve mice were introduced into a clean cage for 7 minutes, again for three consecutive days. Immediately after defeat/control conditions, mice were returned to their home cage. To focus on long-term behavioural changes, behaviour was assessed via a social interaction test 2 weeks after the last episode of defeat. Behavioural and neurobiological effects of defeat persist for at least 10 weeks.[10,29] The social interaction test consisted of three phases, 3 minutes each.[30] In the open field phase, animals were introduced into a large open field (89×63×60 cm). Durations within a centre zone located 14 cm from the sides were recorded using the Any-Maze video tracking system (Stoelting, Wood Dale, IL, USA). During the acclimation phase, a small wire cage was introduced against one side of the arena, the amount of time the mouse spent within 8 cm of the empty cage was recorded. During the social interaction phase, an unfamiliar, same-sex virgin stimulus mouse was placed into the wire cage. We recorded the amount of time the focal mouse spent interacting with the wire cage and the duration spent in the two corners opposite the wire cage. Behavioural data for these mice have been published.[30] We did not include separate groups for different stages of the oestrous cycle because previous studies observed that oestrous cycle[10] and gonadectomy[28] had no effect on behaviour after defeat.

Immediately after social interaction testing, each mouse was lightly anaesthetised with isoflurane and rapidly decapitated. Although it is possible that isoflurane exposure could affect gene expression, exposure was minimised (90 seconds or less). Furthermore, a recent study reported that 5 minutes of isoflurane exposure did not affect expression of four different genes in hippocampus.[31] Although it is possible that the specific genes we quantified were affected by isoflurane, all experimental groups were euthanised in a consistent manner. Each brain was rapidly removed and placed into a brain matrix chilled on ice to collect 2-mm slices.[32] Punch samples of the MeA and CeA were collected with a 1-mm diameter punch tool (Figure 1). The punches of the CeA may have contained a small amount of adjacent BLA. Samples were frozen on dry ice and stored at −40°C.

2.3 | Experiment 2: Effects of social defeat on gene expression in the CeA and BLA

Male and female California mice were randomly assigned to defeat stress or control and then tested in the social interaction test as described in Experiment 1. However, instead of euthanising mice immediately after the social interaction test mice were euthanised by isoflurane anesthesia and decapitation 12-14 hours later. Brains were rapidly removed and a chilled brain matrix was used to collect 1-mm slices. Slices containing the amygdala were stored in RNAlater (Ambion, Austin, TX, USA) overnight at 4°C and bilateral punch samples were collected the next day. Storing brain slices in this manner stabilises RNA and results in a more rigid tissue that facilitates dissection. In the present study, we used a 0.36-mm diameter (20 gauge) punch tool to obtain non-overlapping samples of the CeA and BLA (Figure 1). Samples were frozen on dry ice and stored at −40°C.

2.4 | Experiment 3: Effects of BPA exposure on gene expression in the CeA and BLA

Two weeks prior to breeding, virgin females (8-12 weeks of age) were randomly assigned to receive one of three diets: (i) a low phytoestrogen AIN 93G diet (Research Diets, New Brunswick, NJ, USA) supplemented with 7% by weight corn oil to minimise potential phytoestrogenic contamination that would otherwise be present with inclusion of soybean oil in the diet; (ii) a diet supplemented with 50 mg BPA kg^{-1} feed weight that we have documented to lead to internal serum concentrations close to those measured in pregnant women unknowingly exposed to this chemical;[33] and (iii) a control diet supplemented with 0.1 ppb feed weight that served as an oestrogen positive control, as requested by the Food and Drug Administration for any BPA study considered to guide policy decisions. Females were maintained on these diets throughout gestation and lactation, as described previously.[21,33,34] Diets were assigned at the time that males were paired with females and maintained until the offspring were weaned. Thus, this exposure regimen to BPA or EE is unlikely to affect the caudal epididymal spermatozoa, which developed and matured prior to being placed on the diets. However, it is possible that direct exposure to EDCs might have affected paternal care, as discussed below. At weaning (30 days of age), F1 male and female offspring were placed on the AIN control diet, and they were maintained on this diet throughout the remainder of the study. At adulthood, which ranged from 60 to 194 days of age, mice were killed by cervical dislocation. Each brain was rapidly removed and then frozen on dry ice and stored −80°C.

Frozen brains were shipped to UC Davis where they were sliced on a cryostat at a thickness of 500 μm at −10°C and immediately immersed into RNAlater for approximately 24 hours at 4°C.[35] A 0.36-mm diameter punch tool was used to collect bilateral samples of BLA and CeA. Samples were then kept frozen at −80°C until RNA extraction.

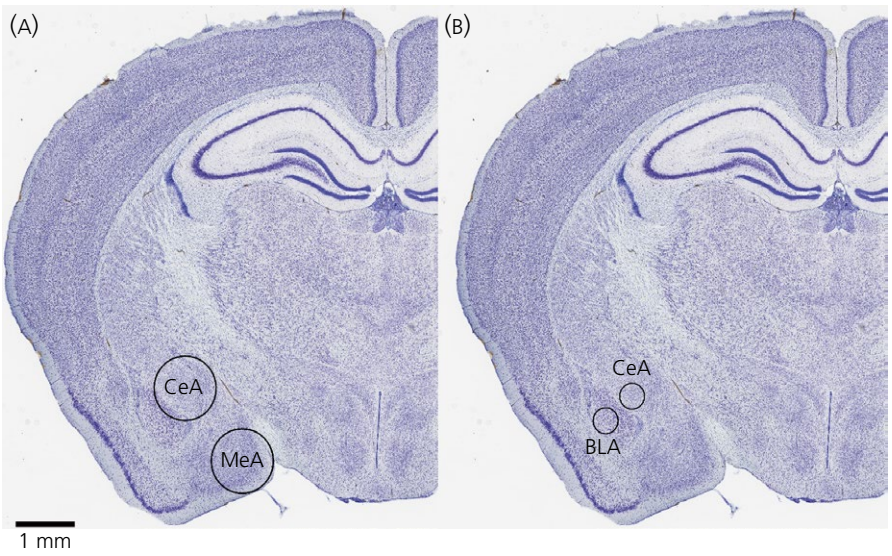

FIGURE 1 (A) Diagram of 1-mm punch sample taken from the central amygdala (CeA) and medial amygdala (MeA) in Experiment 1. (B) Diagram of 0.36-mm punch sample taken from the CeA and basolateral amygdala (BLA) in Experiment 2. Nissl images from the *California Mouse Brain Atlas* at brainmaps.org

2.5 | RNA extraction and real-time PCR

RNA was extracted from punch samples by using RNAqueous-Micro Total RNA Isolation Kit (AM1931; Ambion) and underwent a DNase I treatment before reverse transcription. For each sample, the RNA concentration and quality was assessed using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). Samples were reverse transcribed by using the iScript cDNA Synthesis Kit (#1708891; Bio-Rad, Hercules, CA, USA).

For real-time PCR analysis, SYBR green chemistry was used to detect genes of interest. For Experiment 1, specific primers for *Dnmt1a* and *Dnmt3a* were used for both CeA and MeA samples. In addition, *Bdnf* was also quantified for CeA. We also examined *Esr1* (oestrogen receptor 1) and *Esr2* (oestrogen receptor 2) in MeA as a result of the high abundance of these transcripts in MeA. In Experiments 2 and 3, *Dnmt1*and *Bdnf* were quantified for both BLA and CeA samples. Because of technical difficulties, *Dnmt3a* was not examined in the samples for Experiment 2. The small amount of RNA recovered from the smaller punch size prevented us from re-running this analysis. All gene expression measurements were normalised to *B2m1* expression and normalised to control males as described previously.[29] Primer sequences are referenced in Table 1.

2.6 | Statistical analysis

Gene expression data were analysed by two-way ANOVA for Experiments 1 and 2 followed by planned comparisons testing[36] for the effects of sex or stress using SPSS (SPSS Inc., Chicago, IL, USA). Planned comparisons were limited to comparing control males and females (baseline sex difference) and the effects of stress within each sex (three comparisons total per variable). In Experiment 3, we tested for effects of diet on gene expression data (within sex) using Kruskal-Wallis nonparametric analyses because of heterogeneous variability between the experimental groups (as indicated by Levene's test). Mann-Whitney tests were used for pairwise comparisons in all three experiments. For Experiment 1, we also used Spearman nonparametric correlations to correlate gene expression data with previously published behaviour data. The behavioural variables considered included total distance in an open field test, time spent in the centre of the open field, time spent within 8 cm of an empty cage, and time spent within 8 cm of a cage containing an unfamiliar same-sex mouse.

3 | RESULTS

3.1 | Experiment 1

When 1-mm punches were used to collect CeA samples, main effects of sex were detected for *Dnmt1* ($F_{1,25}$=4.8, P<.05) (Figure 2A) and *Dnmt3a* ($F_{1,25}$=6.5, P<.05) (Figure 2B). Although there was no significant sex by stress interaction for either transcript, planned comparisons revealed that control females had higher *Dnmt1a* (P<.05) and *Dnmt3a* (P<.05) expression than control males, whereas these sex differences were not significant in stressed mice. There were no differences in *Bdnf* expression. In MeA samples, there were no differences in *Dnmt1*, *Dnmt3a* or *Esr1* expression (all P>.2) (Figure 3). However, there was a significant main effect of sex for *Esr2* in the MeA ($F_{1,25}$=11.5, P <.01) (Figure 3D). Again, although the interaction between sex and (A, B, C) stress was not significant, planned comparisons showed that control males had higher *Esr2* expression than control females (P<.001), whereas this difference was not significant in stressed mice. In correlational analyses, *Esr2* in the MeA was negatively correlated with total distance in the open field for females (ρ=−.61, P=.01) but not males (ρ=−.17, P=.58). Gene expression data for *Dnmt1*, *Dnmt3a* or *Bdnf* were not significantly correlated with behaviour in the social interaction test.

3.2 | Experiment 2

When a smaller punch was used to collect the CeA, a main effect of sex on *Dnmt1* was detected in the CeA region ($F_{1,31}$=4.21, P<.05) (Figure 4A). As in Experiment 1, although the sex by treatment interaction was not significant, planned comparisons detected increased *Dnmt1* expression in control females compared

TABLE 1 Primer sequences from all quantitative PCR ran in Experiments 1-3

	Genbank accession	Forward primer	Reverse primer
Bdnf	JX977026	CCA TAA GGA CGC GGA CTT GTA T	GCA GAG GAG GCT CCA AAG G
Dnmt1	XM_006987030.2	AGC CGG AGA GCA GAA ATG GC	ACT GTC CGA CTT GCT TCT CC
Dnmt3a	XM_006981412.2	TCT TGA GTC CAA CCC CGT GAT G	CCT CAC TTT GCT GAA CTT GGC T
Esr1	XM_015996803.1	TGC ACC AGA TCC AAG GGA AC	TCG GGG TAG TTG AAC ACA GC
Esr2	XM_006973152.2	CAC GCT TCG AGG GTA CAA GT	AGG CAG CCA TAA GAT GAC GC
B2m	XM_3006995122.1	TCT AGT GGG AGG TCC TGT GG	TGC GTT AGA CCA GCA GAA GG

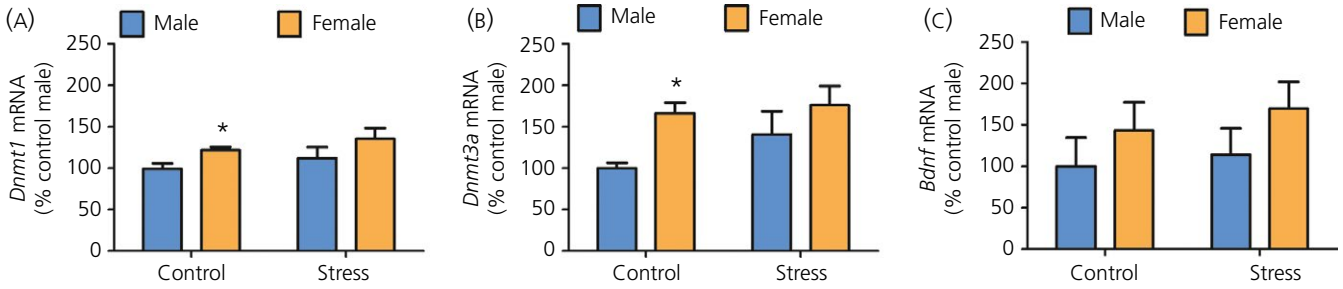

FIGURE 2 Effects of sex and social defeat on gene expression in 1 mm diameter punch samples of the central amygdala. Control females (n=7) had higher *Dnmt1* (A) and *Dnmt3a* (B) expression than control males (n=7). No sex differences were observed between stressed males (n=7) and stressed females (n=8). There were no differences in *Bdnf* expression (C). **P*<.05 vs control male. *Dnmt1*, DNA methyltransferase 1; *Dnmt3a*, DNA methyltransferase 3a; *Bdnf*, brain-derived neurotrophic growth factor

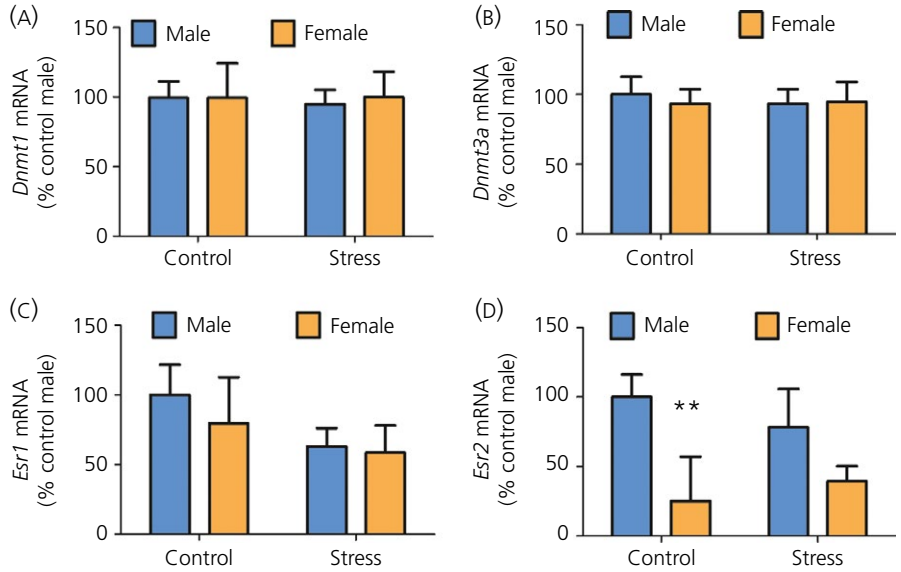

FIGURE 3 Effects of sex and social defeat on gene expression in the medial amygdala. There were no differences in *Dnmt1* (A), *Dnmt3a* (B) or *Esr1* (C) expression. Control males (n=8) had significantly more *Esr2* expression (D) than control females (n=8), although this difference was not detected between stressed males (n=7) and stressed females (n=8). ***P*<.01 vs control male. *Dnmt1*, DNA methyltransferase 1; *Dnmt3a*, DNA methyltransferase 3a; *Esr1*, oestrogen receptor 1; *Esr2*, oestrogen receptor 2

to control males, whereas no sex difference was detected in stressed mice. In addition, stress reduced *Dnmt1* expression in females (*P*<.05) (Figure 4A) but not males. There were no differences in *Bdnf* expression in the CeA (all *P*>.07) (Figure 4B). Analyses of punch samples from the BLA showed no differences in *Dnmt1a* or *Bdnf* (all *P*>.14).

3.3 | Experiment 3

Developmental exposure to one of the two EDCs led to sex and anatomically specific effects on gene expression. First, Kruskal-Wallis analyses showed significant effects of diet on *Dnmt1a* expression in the CeA in males (Kruskal-Wallis, *H*=6.1, *P*<.05) (Figure 5A) but not females (Kruskal-Wallis, H = 1.1, *P* = .56) (Figure 5A). Pairwise comparisons showed that males raised by EE exposed dams had significantly higher *Dnmt1* expression than males derived from dams on the control diet (*P*<.05). No effect of diet was observed in *Dnmt3a* or *Bdnf* expression in the CeA (all *P*>.23) (Figure 5A). The BLA Kruskal-Wallis analyses showed significant effects of treatment on *Bdnf* expression in males (Kruskal-Wallis, *H*=6.1, *P*<.05) (Figure 5F) but not females (Kruskal-Wallis, *H*=6.1, *P*<.05) (Figure 5A). Pairwise comparisons showed that males raised by EE exposed dams had significantly lower *Bdnf* expression than males raised by dams on the control diet (*P*<.05).

For mice raised by dams on the control diet, females had significantly higher *Dnmt1* expression than males in the CeA (Mann-Whitney, *U*=80, *P*<.05) (Figure 5A) but not BLA (Figure 5D). In mice raised by dams on the EE diet, females had higher *Dnmt1* (Mann-Whitney *U*=39, *P*<.01) (Figure 5D) and *Bdnf* (Mann-Whitney, *U*=37, *P*<.05) (Figure 5F) expression in the BLA. For these same mice, no sex differences were observed in the CeA (all *P*>.1). Intriguingly, no sex differences were observed in either the CeA or BLA for mice raised by dams on the BPA diet. Thus, developmental exposure to BPA reduced the sex differences otherwise observed in the CeA region for *Dnmt1*.

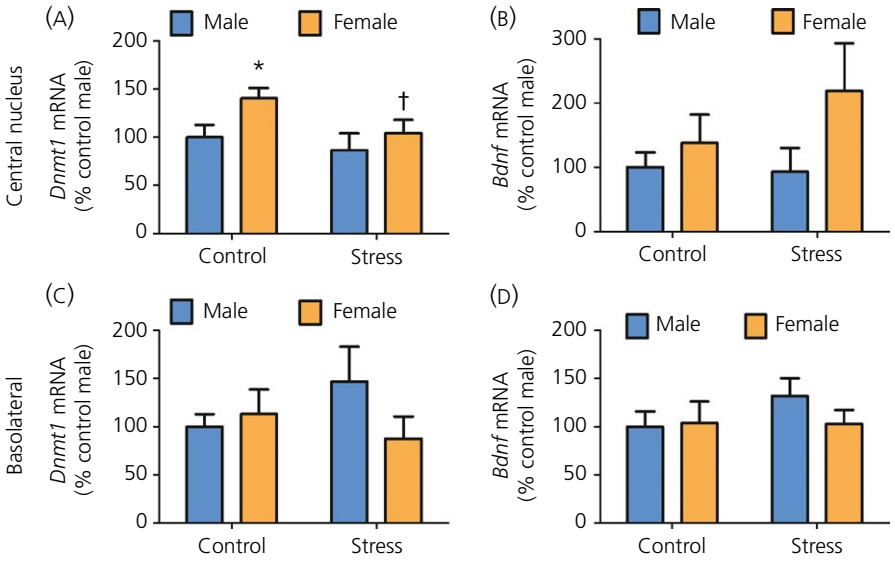

FIGURE 4 Effects of stress and social defeat in 0.36-mm diameter punch samples of the central amygdala (CeA) and basolateral amygdala (BLA). Control females (n=9) had significantly more *Dnmt1* expression in the CeA compared to control males (n=12) (A). *Dnmt1* expression was reduced in stressed females (n=7) compared to control females. There was no difference in *Dnmt1* expression between stressed males (n=7) and control males. No differences in *Dnmt1* were observed in BLA (C). No differences in *Bdnf* were observed in CeA (B) or BLA (D). **P*<.05 vs control male. †*P*<.05 vs control female. *Dnmt1*, DNA methyltransferase 1; *Bdnf*, brain-derived neurotrophic growth factor

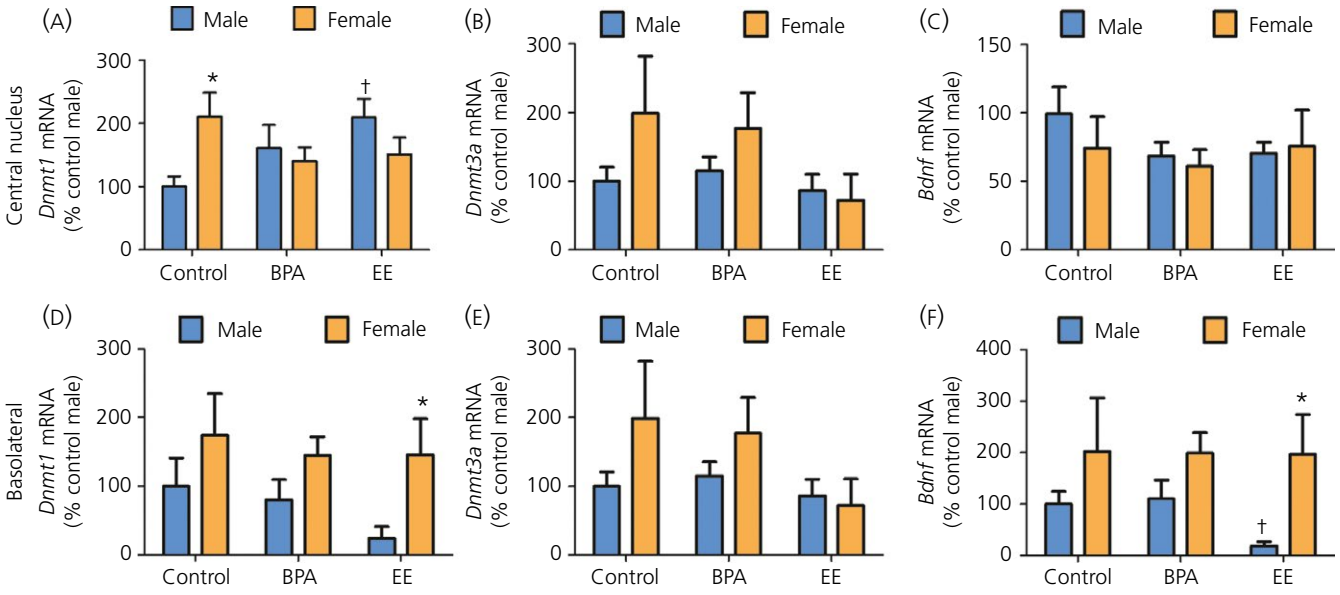

FIGURE 5 Effects of sex, bisphenol A (BPA) and ethinyl oestradiol (EE) on gene expression in the central amygdala (CeA) and basolateral amygdala (BLA). Control females (n=11) had higher *Dnmt1* expression in CeA than control males (n=11) (A), although this differences was absent in BPA (females, n=10, males, n=9) and EE treated mice. EE diet increased *Dnmt1* expression in males. In the BLA, females treated with EE (n=9) had higher *Dnmt1* expression than EE treated males (n=7) (D). No differences in *Dnmt3a* were observed in CeA (B) or BLA (E). The EE diet reduced male *Bdnf* expression in the BLA (F) but not CeA (C). **P*<.05 effect of sex within diet, †*P*<.05 effect of diet within sex. *Dnmt1*, DNA methyltransferase 1; Bdnf, brain-derived neurotrophic growth factor

4 | DISCUSSION

Across the three experiments, we observed that *Dnmt1* expression in the CeA was higher in adult females compared to adult males. Furthermore, this sex difference was reduced in mice assigned to social defeat stress after sexual maturity or BPA exposure during development. This difference was anatomically specific because no baseline sex differences or changes in *Dnmt1* expression were observed in MeA or the adjacent BLA. The subregions of the amygdala have different anatomical connections and cell types,[37] which may contribute to the anatomically specific patterns

in *Dnmt* expression. This may explain why a previous study, which examined samples of the entire amygdala, reported no sex difference in *Dnmt1* expression.[26] An intriguing finding of the present study was that differences in *Dnmt1* expression were long lasting, detected weeks after the termination of BPA exposure or social defeat.

A consistent finding was that females had higher *Dnmt1* expression in the CeA than males. This sex difference in *Dnmt1* expression was observed in California mice raised in colonies at different universities, with varying early life experiences, and contrasting experimental procedures. This is despite the inevitable differences in husbandry practices that are present between different animal facilities. The difference in expression is a robust sex difference and does not appear to be dependent on factors such as husbandry.[38] If these differences in mRNA expression influence DNMT activity, it would suggest that there could be important sex differences in DNA methylation patterns in the CeA. This in turn could affect the transcription of other genes. Social defeat reduced Dnmt1 expression in females, however we found no significant correlations between *Dnmt1* expression and behaviour in the social interaction test. One possible explanation for the lack of correlations is that DNMT1 may act immediately after episodes of defeat to facilitate long-term changes in behaviour. If this hypothesis is correct, then DNMT inhibitors administered at the time of defeat would have stronger effects on behaviour than inhibitors administered after defeat. This would follow the same pattern as DNMT inhibition in the hippocampus, where the behavioural effects of fear conditioning are blocked when a DNMT inhibitor (targeting both DNMT1 and DNMT3a) is given immediately after a conditioning episode but not when administered 6 hours later.[8]

Traditionally, *Dnmt1* has been considered to play a more defining role during the early developmental stages because of its role in maintaining DNA methylation patterns.[39] However, *Dnmt1* has important functions in the adult brain. When a conditional knockout mouse was used to inactivate *Dnmt1* in the forebrain (including amygdala) 2-3 weeks after birth, synaptic plasticity in the hippocampus and spatial memory were impaired.[40] Male conditional *Dnmt1* knockout mice also had reduced anxiety-like behaviour in the open field and increased social interaction behaviour.[41] The loss of *Dnmt1* may have broad effects on transcription that could contribute to increases in anxiety-like behaviour. The results of the present study suggest that *Dnmt1* inhibition may have even more robust effects in females. Further studies are needed to determine the impact of decreases in DNMT1 activity in the adult brain on transcription. It is likely that the effects of DNMT1 on methylation and transcription will be dependent on social/environmental stress exposure.

BPA exposure elicited the same changes to *Dnmt1* expression as social defeat. Developmental exposure to BPA or EE reduced sex differences in *Dnmt1* expression in the CeA. Developmental exposure to BPA also reduced sex differences in behaviour by decreasing exploratory behaviour in females and reducing territorial marking behaviour in males.[21] These results support the hypothesis the BPA exposure reduces sexually dimorphic characteristics. The effects of developmental EE exposures were stronger than BPA, driven primarily by increased *Dnmt1* expression in males exposed to EE. It is unclear how EE can have such sex-dependent effects on *Dnmt1* expression in the CeA, a region of the amygdala with few nuclear oestrogen receptors (ERs).[42,43] One possibility is that EE acts in other nuclei with inputs into the CeA. For example, the bed nucleus of the stria terminalis has abundant ERα and ERβ expression in California mice[44] and sends dense projections to the CeA.[45]

When interpreting the effects of developmental exposure to BPA or EE in Experiment 3, it is important to consider the potential impact that these diets may have had on parental behaviour. Although developmental exposure to BPA and EE can affect the parental behaviour of adult male and female California mice,[19] it remains to be determined whether adult exposure to these diets induces similar disruptions. Cross-fostering approaches could be useful in teasing apart the direct effects of developmental BPA/EE exposure on gene expression vs indirect effects of BPA/EE on parental care. For example, one study showed that mice exposed to BPA during gestation and reared by a foster dam demonstrated increases in anxiety-like behaviour compared to controls.[46] Yet even cross-fostered offspring might also bear a permanent stamp as a result of prenatal exposure that may be sensed by the foster parents. This could in turn affect parental investment. Thus, there is currently no ideal way of dissecting the neurobehavioural effects attributed to direct exposure to EDCs vs those potentially arising from altered parental care.

Data from the other amygdala subregions differed from findings in the CeA. In the MeA, we observed that males had a higher expression of *Esr2* than females and this difference was weakened by defeat stress. Currently, it is unclear whether sex differences in *Esr2* in the MeA are evolutionarily conserved. One study in rats reported increased *Esr2* mRNA expression in males vs females in the MeA,[47] although but this difference was not replicated.[48] Quantitative analysis of ERβ protein has been hampered by cross-reactivity of ERβ antibodies in mice.[49] The development of *Esr2* reporter mice is a creative solution to this problem, but no sex differences in ERβ-enhanced green fluorescent protein were observed in the MeA between postnatal day 0 and 21.[50] Work with Syrian hamsters shows strong evidence that *Esr1* acting in the MeA has important effects on social behaviours,[51] although almost nothing is known about the role of *Esr2* in the MeA. In the CeA and BLA, we did not observe differences in *Bdnf* expression. Previous work in Syrian hamsters showed that *Bdnf* expression in BLA was increased 2 hours after experiencing social defeat.[52] In Experiment 2, brains were collected 2 weeks after defeat, suggesting that elevated *Bdnf* transcription after defeat may be transitory. Another possibility is that BDNF protein levels could be elevated in the absence of *Bdnf* mRNA.[30,53]

5 | CONCLUSIONS

A consistent finding across all three experiments is that females had a higher expression of *Dnmt1* in the CeA than males. This difference was robust across different experiments and procedures. Although

DNA methylation is considered to be an important mechanism for inducing behavioural and neurobiological plasticity, it is usually assumed that, in the adult brain, *Dnmt3a* plays a larger role in this process than *Dnmt1*. The results of the present study suggest that further investigation of *Dnmt1* function in the amygdala of adults may be warranted. Interestingly, both BPA and defeat stress weakened sex differences in *Dnmt1* expression in the CeA, which supports the idea that, in some respects, BPA acts as a chemical stressor. Even though these stressors occurred during different developmental time points, they both manifested with a similar molecular change in adults. Currently, it is unclear whether developmental BPA exposure would alter the impact of social defeat on brain function and behaviour. An environmental stressor experienced during development might render an individual more susceptible to a second extrinsic stressor experienced later in life, as predicted by the two-hit hypothesis.[15,54-56] Our results indicate that future research investigating epigenetic changes in the amygdala following stress will need to consider the possibility of sex differences in DNMT expression leading to sex-specific transcriptional responses to extrinsic stressors.

ACKNOWLEDGEMENTS

We thank Angela B. Javurek for technical assistance. This work is supported by NIH R01MH103322 to BCT and 5R21ES023150 to CSR.

REFERENCES

1. Van den Veyver IB. Genetic effects of methylation diets. *Annu Rev Nutr*. 2002;22:255-282.
2. Anier K, Malinovskaja K, Aonurm-Helm A, Zharkovsky A, Kalda A. DNA methylation regulates cocaine-induced behavioral sensitization in mice. *Neuropsychopharmacology*. 2010;35:2450-2461.
3. Edelmann MN, Auger AP. Epigenetic impact of simulated maternal grooming on estrogen receptor alpha within the developing amygdala. *Brain Behav Immun*. 2011;25:1299-1304.
4. Robertson KD. DNA methylation, methyltransferases, and cancer. *Oncogene*. 2001;20:3139-3155.
5. Roth TL, Lubin FD, Funk AJ, Sweatt JD. Lasting epigenetic influence of early-life adversity on the BDNF gene. *Biol Psychiatry*. 2009;65:760-769.
6. Jensen Peña C, Monk C, Champagne FA. Epigenetic effects of prenatal stress on 11b-hydroxysteroid dehydrogenase-2 in the placenta and fetal brain. *PLoS ONE*. 2012;7:e39791.
7. Matrisciano F, Tueting P, Dalal I, et al. Epigenetic modifications of GABAergic interneurons are associated with the schizophrenia-like phenotype induced by prenatal stress in mice. *Neuropharmacology*. 2013;68:184-194.
8. Miller CA, Sweatt JD. Covalent modification of DNA regulates memory formation. *Neuron*. 2007;53:857-869.
9. LaPlant Q, Vialou V, Covington HE, et al. Dnmt3a regulates emotional behavior and spine plasticity in the nucleus accumbens. *Nat Neurosci*. 2010;13:1137-1143.
10. Trainor BC, Pride MC, Villalon Landeros R, et al. Sex differences in social interaction behavior following social defeat stress in the monogamous California mouse (*Peromyscus californicus*). *PLoS ONE*. 2011;6:e17405.
11. Vandenberg LN, Maffini MV, Sonnenschein C, Rubin BS, Soto AM. Bisphenol-A and the great divide: a review of controversies in the field of endocrine disruption. *Endocr Rev*. 2009;30:75-95.
12. Kundakovic M, Gudsnuk K, Franks B, et al. Sex-specific epigenetic disruption and behavioral changes following low-dose in utero bisphenol A exposure. *Proc Natl Acad Sci USA*. 2013;110:9956-9961.
13. Kundakovic M, Gudsnuk K, Herbstman JB, Tang D, Perera FP, Champagne FA. DNA methylation of BDNF as a biomarker of early-life adversity. *Proc Natl Acad Sci USA*. 2015;112:6807-6813.
14. Crews D, Gore AC. Life imprints: living in a contaminated world. *Environ Health Perspect*. 2011;119:1208-1210.
15. Rosenfeld CS, Trainor BC. Environmental health factors and sexually dimorphic differences in behavioral disruptions. *Curr Environ Health Rep*. 2014;1:287-301.
16. Steinman MQ, Trainor BC. Sex differences in the effects of social defeat on brain and behavior in the California mouse: insights from a monogamous rodent. *Semin Cell Dev Biol*. 2017;61:92-98.
17. Hodes GE, Pfau ML, Purushothaman I, et al. Sex differences in nucleus accumbens transcriptome profiles associated with susceptibility versus resilience to subchronic variable stress. *J Neurosci*. 2015;35:16362-16376.
18. Javurek AB, Spollen WG, Johnson SA, et al. Effects of exposure to bisphenol A and ethinyl estradiol on the gut microbiota of parents and their offspring in a rodent model. *Gut Microbes*. 2016;7:471-485.
19. Johnson SA, Javurek AB, Painter MS, et al. Disruption of parenting behaviors in California mice, a monogamous rodent species, by endocrine disrupting chemicals. *PLoS One*. 2015;10:e0126284.
20. Johnson SA, Painter MS, Javurek AB, et al. Sex-dependent effects of developmental exposure to bisphenol A and ethinyl estradiol on metabolic parameters and voluntary physical activity. *J Dev Orig Health Dis*. 2015;6:539-552.
21. Williams SA, Jasarevic E, Vandas GM, et al. Effects of developmental bisphenol A exposure on reproductive-related behaviors in California mice (*Peromyscus californicus*): a monogamous animal model. *PLoS ONE*. 2013;8:e55698.
22. Zhou R, Bai Y, Yang R, et al. Abnormal synaptic plasticity in basolateral amygdala may account for hyperactivity and attention-deficit in male rat exposed perinatally to low-dose bisphenol-A. *Neuropharmacology*. 2011;60:789-798.
23. Yu CJ, Fang QQ, Tai FD. Pubertal BPA exposure changes central ERα levels in female mice. *Environ Toxicol Pharmacol*. 2015;40:606-614.
24. Cardinal RN, Parkinson JA, Hall J, Everitt BJ. Emotion and motivation: the role of the amygdala, ventral striatum, and prefrontal cortex. *Neurosci Biobehav Rev*. 2002;26:321-352.
25. Zhou R, Chen F, Chang F, Bai Y, Chen L. Persistent overexpression of DNA methyltransferase 1 attenuating GABAergic inhibition in basolateral amygdala accounts for anxiety in rat offspring exposed perinatally to low-dose bisphenol A. *J Psychiatr Res*. 2013;47:1535-1544.
26. Kolodkin MH, Auger AP. Sex difference in the expression of DNA methyltransferase 3a in the rat amygdala during development. *J Neuroendocrinol*. 2011;23:577-583.
27. Duque-Wilckens N, Steinman MQ, Laredo SA, et al. Anxiolytic effects of vasopressin V1a receptor in the medioventral bed nucleus of the stria terminalis: sex specific effects in social and nonsocial contexts. *Neuropharmacology*. 2016;110: 59-68.
28. Trainor BC, Takahashi EY, Campi KL, et al. Sex differences in stress-induced social withdrawal: independence from adult gonadal hormones and inhibition of female phenotype by corncob bedding. *Horm Behav*. 2013;63:543-550.
29. Steinman MQ, Duque-Wilckens N, Greenberg GD, et al. Sex-specific effects of stress on oxytocin neurons correspond with responses to intranasal oxytocin. *Biol Psychiatry*. 2016;80:406-414.
30. Greenberg GD, Laman-Maharg A, Campi KL, et al. Sex differences in stress-induced social withdrawal: role of brain derived neurotrophic factor in the bed nucleus of the stria terminalis. *Front Behav Neurosci*. 2014;7:223.

31. Bekhbat M, Merrill L, Kelly SD, Lee VK, Neigh GN. Brief anesthesia by isoflurane alters plasma corticosterone levels distinctly in male and female rats: implications for tissue collection methods. *Behav Brain Res*. 2016;305:122-125.
32. Trainor BC, Bird IM, Alday NA, Schlinger BA, Marler CA. Variation in aromatase activity in the medial preoptic area and plasma progesterone is associated with the onset of paternal behavior. *Neuroendocrinology*. 2003;78:36-44.
33. Jasarevic E, Sieli PT, Twellman EE, et al. Disruption of adult expression of sexually selected traits by developmental exposure to bisphenol A. *Proc Natl Acad Sci USA*. 2011;108:11715-11720.
34. Jasarevic E, Williams SA, Vandas GM, et al. Sex and dose-dependent effects of developmental exposure to bisphenol A on anxiety and spatial learning in deer mice (*Peromyscus maniculatus bairdii*) offspring. *Horm Behav*. 2013;63:180-189.
35. Perea-Rodriguez JP, Takahashi EY, Amador TM, Hao R, Saltzman W, Trainor BC. Effects of reproductive status on central expression of progesterone, oxytocin, and vasopressin V1a receptors in male California mice (*Peromyscus californicus*). *J Neuroendocrinol*. 2015;27:245-252.
36. Letner MB, Bishop T. *Experimental Design and Analysis*. Blacksburg, VA: Valley Book Company; 1993.
37. Swanson LW, Petrovich GD. What is the amygdala? *Trends Neurosci*. 1998;21:323-331.
38. Villalon Landeros R, Yoo HJ, Morisseau C, Fu S, Hammock BD, Trainor BC. Corncob bedding reverses the effects of estrogens on aggressive behavior and reduces estrogen receptor alpha expression in the brain. *Endocrinology*. 2012;153:949-953.
39. Fagiolini M, Jensen CL, Champagne FA. Epigenetic influences on brain development and plasticity. *Curr Opin Neurobiol*. 2009;19:207-212.
40. Feng J, Campbell SL, Le T, et al. Dnmt1 and Dnmt3a maintain DNA methylation and regulate synaptic function in adult forebrain neurons. *Nat Neurosci*. 2010;13:423-430.
41. Morris MJ, Na ES, Autry AE, Monteggia LM. Impact of DNMT1 and DNMT3a forebrain knockout on depressive- and anxiety like behavior in mice. *Neurobiol Learn Mem*. 2016;135:139-145.
42. Shughrue P, Merchenthaler I. Distribution of estrogen receptor beta immunoreactivity in the rat central nervous system. *J Comp Neurol*. 2001;436:64-81.
43. Mitra SW, Hoskin E, Yudkovitz J, et al. Immunolocalization of estrogen receptor beta in the mouse brain: comparison with estrogen receptor alpha. *Endocrinology*. 2003;144:2055-2067.
44. Trainor BC, Finy MS, Nelson RJ. Paternal aggression in a biparental mouse: parallels with maternal aggression. *Horm Behav*. 2008;53:200-207.
45. Dong HW, Petrovich GD, Watts AG, Swanson LW. Basic organization of projections from the oval and fusiform nuclei of the bed nuclei of the stria terminalis in adult rat brain. *J Comp Neurol*. 2001;436:430-455.
46. Cox KH, Gatewood JD, Howeth C, Rissman EF. Gestational exposure to bisphenol A and cross-fostering affect behaviors in juvenile mice. *Horm Behav*. 2010;58:754-761.
47. Cao J, Patisaul HB. Sex specific expression of estrogen receptors α and β and Kiss1 in the postnatal rat amygdala. *J Comp Neurol*. 2013;521:465-478.
48. Cao J, Joyner L, Mickens JA, Leyrer SM, Patisaul HB. Sex-specific Esr2 mRNA expression in the rat hypothalamus and amygdala is altered by neonatal bisphenol A exposure. *Reproduction (Cambridge, England)*. 2014;147:537-554.
49. Snyder MA, Smejkalova T, Forlano PM, Woolley CS. Multiple ERbeta antisera label in ERbeta knockout and null mouse tissues. *J Neurosci Methods*. 2010;188:226-234.
50. Zuloaga DG, Zuloaga KL, Hinds LR, Carbone DL, Handa RJ. Estrogen receptor beta expression in the mouse forebrain: age and sex differences. *J Comp Neurol*. 2014;522:358-371.
51. Ervin KS, Lymer JM, Matta R, Clipperton-Allen AE, Kavaliers M, Choleris E. Estrogen involvement in social behavior in rodents: rapid and long-term actions. *Horm Behav*. 2015;74:53-76.
52. Taylor SL, Stanek LM, Ressler KJ, Huhman KL. Differential BDNF expression in limbic brain regions following social defeat or territorial aggression. *Behav Neurosci*. 2011;125:911-920.
53. Tropea D, Capsoni S, Tongiorgi E, Giannotta S, Cattaneo A, Domenici L. Mismatch between BDNF mRNA and protein expression in the developing visual cortex: the role of visual experience. *Eur J Neurosci*. 2001;13:709-721.
54. Lesse A, Rether K, Gröger N, Braun K, & Bock J. Chronic postnatal stress induces depressive-like behavior in male mice and programs second-hit stress-induced gene expression patterns of OxtR and AvpR1a in adulthood. *Mol Neurobiol*. 2016;1-7. https://doi.org/10.1007/s12035-016-0043-8.
55. Groger N, Bock J, Goehler D, et al. Stress in utero alters neonatal stress-induced regulation of the synaptic plasticity proteins Arc and Egr1 in a sex-specific manner. *Brain Struct Funct*. 2016;221:679-685.
56. Novak G, Fan T, O'Dowd BF, George SR. Postnatal maternal deprivation and pubertal stress have additive effects on dopamine D2 receptor and CaMKII beta expression in the striatum. *Int J Dev Neurosci*. 2013;31:189-195.

How to cite this article: Wright EC, Johnson SA, Hao R., et al Exposure to extrinsic stressors, social defeat or bisphenol A, eliminates sex differences in DNA methyltransferase expression in the amygdala. *J Neuroendocrinol*. 2017;29:1–9. https://doi.org/10.1111/jne.12475