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Sex differences in Gadd45b expression and methylation in the developing rodent amygdala

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Abstract

Precise spatiotemporal epigenetic regulation of the genome facilitates species-typical development; sexual differentiation of the brain by gonadal hormones and sex chromosomes causes extensive epigenetic reprogramming of many cells in the body, including the brain, and may indirectly predispose males and females to different psychiatric conditions. We and others have demonstrated sex differences in DNA methylation, as well as in the enzymes that form, or 'write', this epigenetic modification. However, while a growing body of evidence suggests that DNA methylation undergoes rapid turnover and is dynamically regulated *in vivo*, to our knowledge no studies have been done investigating whether sex differences exist in the epigenetic 'erasers' during postnatal development. Here we report sex differences in the expression of growth arrest and DNA damage inducible factor β (Gadd45b), but not family members α (a) or γ (g), in the neonatal and juvenile rodent amygdala.

Keywords

Epigenetics; Gadd45b; sex differences; amygdala

1. Introduction:

The developing mammalian brain is uniquely sensitive to environmental and hormonal cues; during so-called 'sensitive periods', extensive epigenetic programming and reprogramming is occurring (A. P. Auger et al., 2011; Roth and David Sweatt, 2010; Weaver et al., 2004). Abnormalities in the epigenome acquired during development can cause long-lasting and

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adverse effects on such far-reaching behavioral and physiological traits as memory (Korosi et al., 2012), parental behavior (Champagne et al., 2006), sexual differentiation (A. P. Auger and Jessen, 2009; Matsuda et al., 2011; Nugent et al., 2015), drug-seeking behavior (Massart et al., 2015), and are furthermore linked to psychiatric dysfunction (Bagot et al., 2014; Labonté et al., 2013; McGowan et al., 2009). In fact, drugs directed at epigenetic factors are a proposed target in the treatment of psychiatric disorders (Szyf, 2015). Interestingly, in addition to altered programming of the neural epigenome, biological sex is a highly salient risk factor in the development of psychiatric disorders ((Chase et al., 2015), reviewed in (Kigar and A. P. Auger, 2013)). This makes it a priority to understand the basic biological signaling mechanisms at play in controlling the epigenome during sexual differentiation.

We and others have previously demonstrated sex differences in the abundance of epigenetic modifications such as 5-methylcytosine (5mC) (Kurian et al., 2010; Nugent et al., 2015) and histone acetylation or methylation (Tsai et al., 2009). We have also shown that there are sex differences in the expression of epigenetic factors that increase or decrease gene transcription; specifically, males have higher levels of co-activators such as steroid receptor coactivator-1 (Src-1) (A. P. Auger et al., 2000) and cyclic-AMP response element-binding protein (CBP) (A. P. Auger et al., 2002), whereas females have higher levels of co-repressors such as methyl cytosine binding protein 2 (MeCP2) (Kurian et al., 2007) and nuclear receptor corepressor (NCoR)(Jessen et al., 2010). Finally, sex differences have been observed in the expression or activity of enzymes that catalyze formation of epigenetic modifications, including DNA methyltransferase 3a (Dnmt3a) (Kolodkin and A. P. Auger, 2011), Dnmt1 (Nugent et al., 2015), and several histone deacetylases (Xu et al., 2008a; 2008b).

While DNA methylation was previously thought to be a static and/or permanent epigenetic modification (Wu and Zhang, 2010), a growing body of evidence suggests it undergoes rapid turnover and is dynamically regulated *in vivo* (C. J. Auger et al., 2011; Feng et al., 2010; Ma et al., 2009). Briefly, ten-eleven translocation (Tet) enzymes are able to catalyze conversion of 5mC into 5-hydroxymethylcytosine (5hmC); 5hmC will eventually be converted to a uracil analog, resulting in a DNA base pair mismatch that will be excised. Importantly, the growth arrest and DNA damage inducible factor (Gadd45) β (b), α (a) and γ (g) family of proteins appear to participate in DNA demethylation through an as-yet unidentified mechanism (Niehrs and Schäfer, 2012); in particular, Gadd45b is required for stimulus-induced DNA demethylation (Ma et al., 2009).

We have recently demonstrated a role for Gadd45b in dampening juvenile male rats' drive to engage in 'rough and tumble play' (Kigar et al., 2015), suggesting it may be an important risk factor in the development of abnormal social behaviors. We were specifically looking at the function of Gadd45b within the developing amygdala—a region of the brain critically important in the formation of socioemotional behaviors (LeDoux, 2007; Phelps and LeDoux, 2005; Shaw et al., 2004). While sex differences in Gadd45b mRNA expression have been observed in the adult prefrontal cortex (Blaze and Roth, 2013), it is unknown whether sex differences exist in its expression in steroid hormone-responsive brain regions, e.g. the amygdala and hypothalamus. Here we address this knowledge gap by examining the

expression of the Gadd45 protein family at two important developmental time points—twenty-four hours after birth and the beginning of the juvenile social play period.

2. Results

mRNA sex differences in demethylase factors at postnatal day 1 (P1)

Sex differences in the expression of Gadd45b, but not related family members Gadd45a and Gadd45g, were found in the PI amygdala, where females had higher levels of Gadd45b mRNA than males: [Gadd45b (female: 0.6337 ± 0.05103, N=9; male: 0.4061 ± 0.02500, N=8. p=0.0016); Gadd45a (female: 0.5947 ± 0.03712, N=8; male: 0.5194 ± 0.04059, N=9. p=0.1947); Gadd45g (female: 0.5548 ± 0.1090, N=10; male: 0.5148 ± 0.1017, N=8. p=0.7958)] (Fig. 1A). We observed no sex differences in Gadd45 family members in the PI hypothalamus: [Gadd45b (female: 0.6698 ± 0.05339, N=8; male: 0.5863 ± 0.03181, N=9. p=0.1877); Gadd45a (female: 0.5775 ± 0.07341, N=8; male: 0.4809 ± 0.04452, N=10. p=0.2566); Gadd45g (female: 0.6181 ± 0.06382, N=8; male: 0.5893 ± 0.04411, N=9. p=0.7108)] (Fig. 1B).

Effect of hormone treatment on Gadd45 expression in females

Neonatal females were injected subcutaneously with hormone or vehicle on P0 and P1 before sacrifice on P2. There was a significant overall effect of hormone treatment on the expression of Gadd45b mRNA in females, where dihydrotestosterone (DHT)-treated females showed less expression than oil-treated females (one-way ANOVA; $F_{(3,27)} = 4.130$, $p = 0.0170$. Tukey's *post hoc*; $q = 4.873$) (Fig. 2A). We observed no effects of hormones on remaining family members Gadd45a or Gadd45g: [Gadd45a (oil: 0.4269 ± 0.04353, N=8; testosterone: 0.4621 ± 0.05316, N=7; estrogen: 0.4041 ± 0.06577, N=6. dihydrotestosterone: 0.3387 ± 0.02716, N=7. p=0.3371); Gadd45g (oil: 0.4424 ± 0.09015, N=6; testosterone: 0.4833 ± 0.08918, N=7; estrogen: 0.4458 ± 0.1031, N=6. dihydrotestosterone: 0.4555 ± 0.1221, N=7. p=0.9917)] (Fig. 2B–C).

mRNA sex differences in Gadd45 family members at postnatal day 25 (P25)

Sex differences in the expression of Gadd45b, but not related family members Gadd45a and Gadd45g, were also found in the P25 amygdala, where females had higher levels of Gadd45b mRNA than males: [Gadd45b (female: 0.6490 ± 0.04799, N=10; male: 0.4676 ± 0.04679, N=8. p=0.0170); Gadd45a (female: 0.5579 ± 0.04441, N=10; male: 0.6194 ± 0.06860, N=9. p=0.4535); Gadd45g (female: 0.5682 ± 0.08165, N=10; male: 0.6458 ± 0.02790, N=8. p=0.4268)] (Fig. 3A). We observed no sex differences in Gadd45 family members in the P25 hypothalamus: [Gadd45b (female: 0.6683 ± 0.03507, N=11; male: 0.6340 ± 0.03228, N=8. p=0.4976); Gadd45a (female: 0.6206 ± 0.03230, N=11; male: 0.5424 ± 0.01264, N=7. p=0.0820); Gadd45g (female: 0.5959 ± 0.02852, N=10; male: 0.6353 ± 0.03555, N=9. p=0.3961)] (Fig. 3B).

Methylation changes in the Gadd45b promoter

We used a methylation sensitive restriction enzyme (MSRE) assay to examine relative amounts of DNA methylation at an estrogen receptor α (ERα) response element (ERE) in the P25 amygdala Gadd45b promoter. Doing so, we found sex differences in methylation at

an AciI (CCGC) cut site, where males had more than females: Gadd45b (female: 0.3972 ± 0.04209, N=10; male: 0.6356 ± 0.1068, N=9. p=0.0453) (Fig. 4).

3. Discussion:

Herein we present data describing neurodevelopmental sex differences in the mRNA expression of Gadd45b, a protein involved in DNA demethylation (Ma et al., 2009). Of the three Gadd45 family members, Gadd45b alone exhibited hormone responsivity in the amygdala, but not the hypothalamus (Fig. 1, 3). Specifically, females expressed greater levels of Gadd45b mRNA neonatally (Fig. 1A) and this effect was also present during the juvenile period (Fig. 3A). The reduced mRNA expression observed in males furthermore corresponds with increased methylation at an ERE site within the Gadd45b promoter (Fig. 4), suggesting that the demethylation factor Gadd45b is itself under epigenetic control. Moreover, steroid hormone treatment at PO and PI caused a repressive effect on Gadd45b mRNA expression in the neonatal female amygdala (Fig. 2A), suggesting that Gadd45b levels are sensitive to changes in peripheral gonadal hormones. Interestingly, only treatment with dihydrotestosterone, and not estradiol or testosterone, resulted in decreased Gadd45b levels in the amygdala (Fig. 2A). As dihydrotestosterone binds with high affinity to androgen receptors (Wilson and French, 1976), this suggests androgen receptors may be responsible for the sex difference found in Gadd45b levels within the developing amygdala and supports previous literature reporting that androgens are important for this region's masculinization (Cooke et al., 2003; Meaney and Stewart, 1981). As hormones reduce the expression of Gadd45b, it is possible that Gadd45b facilitates some of the changes induced by steroid-mediated sexual differentiation of the brain. Indeed, lowering Gadd45b levels using siRNA results in increased juvenile social play behavior, which is typically higher in males (Kigar et al., 2015). As reducing Gadd45b levels increases male-typical behavior later in life, it is not surprising that females exhibit higher levels of Gadd45b during brain sexual differentiation.

The Gadd45 family is a highly conserved group of small, nuclearly-localized proteins whose peripheral expression can be rapidly induced by a variety of environmental stimuli, including gamma irradiation (Engelmann et al., 2007), ultraviolet treatment (Gupta et al., 2006), and histone deacetylase inhibition (Chen et al., 2002). Interestingly, environmentally responsive changes to neuronal Gadd45b expression have also been reported. For example, its expression is upregulated by photons of light in the superchiasmatic nucleus (Porterfield et al., 2007), in response to electroconvulsive therapy in the hippocampus (Ma et al., 2009), and increases rapidly following hippocampal, amygdalar, and striatal learning tasks (Keeley et al., 2006; Sultan et al., 2012). Our current data indicates that Gadd45b is also sensitive to hormones and biological sex during brain development.

The swift induction of Gadd45b expression in response to environmental cues appears to be an important component of the DNA demethylation pathway. Specifically, a study of neuronal-activity induced changes to DNA methylation in the hippocampus of Gadd45b knock-out mice showed that Gadd45b was required for rapid demethylation of the brain derived neurotrophic factor (BDNF) promoter at exon IV (Ma et al., 2009). Furthermore, recent data from our lab demonstrated that early life perturbations to Gadd45b expression in

the amygdala resulted in both immediate and long-term increases in promoter methylation of the norepinephrine-binding autoreceptor, α_2-adrenoceptor (Adra2a)—consistent with Gadd45b's role as a DNA demethylase (Kigar et al., 2015). While the mechanism by which this feat is accomplished has yet to be determined as Gadd45b itself is non-enzymatic, it may involve recruitment of deaminases or other factors involved in base excision repair [e.g. activation-induced deaminase/apolipoprotein B mRNA editing cytokine, or AID/APOBEC); for review, see (Grayson and Guidotti, 2012; Kigar and A. P. Auger, 2013)].

Clinically, Gadd45b overexpression was observed in the postmortem tissue of patients whom, while living, were diagnosed with either schizophrenia (SZ) or bipolar disorder (BPD) (Gavin et al., 2012); the authors concluded that this overexpression may be compensatory, as it has been extensively studied that in postmortem SZ tissue, chromatin is in a restricted state (Abdolmaleky et al., 2006; Chase et al., 2015; Gavin et al., 2009; Grayson et al., 2005; Huang and Akbarian, 2007; Huang et al., 2007; Ruzicka et al., 2007; Veldic et al., 2004; 2005; 2007). Presently it is unclear what factors may lead to the overexpression seen in postmortem SZ and BPD tissue; however, in an animal model of early life stress, Gadd45b mRNA levels were repressed in the adult prefrontal cortex (PFC) (Blaze and Roth, 2013). This may suggest that perturbations during the sensitive period of neonatal development may somehow influence its expression later in life, though this hypothesis remains to be tested.

As sex differences in risk and resilience to psychiatric disorders have been noted, and sex-specific expression of epigenetic factors appear to underlie this phenomenon (Chase et al., 2015; Kigar and A. P. Auger, 2013), it will be an important future direction to establish whether Gadd45b dictates a repressive or permissive chromatin environment, and whether this is also sex-specific. One intriguing possibility is that Gadd45b acts not to compensate for a restrictive environment with respect to chromatin, but instead mobilizes the expression of repressive factors in some brain regions, including the amygdala, and may thus exacerbate transcriptional repression. For example, we have recently demonstrated that Gadd45b knockdown decreased the expression of MeCP2 in the neonatal amygdala of male rats (Kigar et al., 2015), and have previously reported that there are sex differences in the expression of MeCP2 (Kurian et al., 2007). If Gadd45b is directly responsible for MeCP2 expression, the directionality would be consistent with the Gadd45b sex difference reported here (Fig. 1A).

In conclusion, we find that Gadd45b levels are different between males and females at a critical time point in amygdalar development. These data suggest that both increased and decreased gene expression is a necessary component for typical sexual differentiation of the brain, as females appear to express both more repressive epigenetic factors (Jessen et al., 2010; Kolodkin and A. P. Auger, 2011; Kurian et al., 2008) and higher levels of a putative DNA demethylation factor, Gadd45b. It remains to be determined if increased Gadd45b underlies the increased expression of repressive epigenetic factors observed in females. It is possible that the increased expression of repressive machinery in females may function to protect the developing female brain from the masculinizing effects of either hormonal or non-hormonal factors involved in brain sexual differentiation (A. P. Auger and Jessen, 2009;

Kigar and A. P. Auger, 2013). Nonetheless, these data bolster and support preexisting evidence from the literature that sex differences exist at the level of the neural epigenome.

4. Materials and Methods

Animals and weaning environment

Sprague Dawley female rats, purchased from Charles River Laboratories (Wilmington, MA), were bred in our colony and allowed to deliver normally. Cages were checked regularly to determine the day of birth (P0). Juveniles were weaned at P21 into cages of 5 with mixed sex littermates. Animals were housed under standard laboratory conditions (light/dark cycle of 12/12 h, food and water ad libitum). All procedures were approved by the University of Wisconsin-Madison Animal Care and Use Committee.

Sex difference data sets

Males and females from multiple litters were sacrificed via rapid decapitation at 24 h (P1; n = 10 males, n = 10 females) or first anesthetized with isoflourane and then rapidly decapitated 25 days after birth (P25; n = 9 males, n = 11 females). Brains were collected, and the amygdala and hypothalamus were microdissected with razorblades.

Hormone-treated data set

As published previously (Kolodkin and A. P. Auger, 2011), newborn female rats were given subcutaneous injections of 100μg testosterone propionate (T; n = 7), 100μg of estradiol benzoate (E; n = 6), 250μg of dihydrotestosterone (D; n = 7), or sesame oil (O; n = 8). Selected drug dosages were based on literature demonstrating these concentrations of hormone are required to attain male-typical levels in the hypothalamus during development (Amateau et al., 2004). Animals were injected with hormone or oil both immediately and 24 h after birth. Female brains were collected 48 h (P2) after birth.

Quantification of mRNA

RNA concentrations were determined using the Qubit Quantification Platform (Invitrogen; catalog #Q32857). RNA conversion to cDNA was performed in an Eppendorf MasterCycler Personal PCR machine via the ImPromII™ Reverse Transcription System (Promega; catalog #A3800). Real-time quantitative polymerase chain reaction (RT-PCR) was conducted using a Stratagene Mx3000P™ real time PCR system, and cDNA was amplified with GoTaq Colorless Master Mix (Promega; catalog #M7132), SYBR green (Invitrogen; catalog #S33102), and ROX as a reference dye (Invitrogen; 12223-012). Following amplification, a dissociation melt curve and DNA gel analysis were performed to ensure the purity of PCR products. cDNA levels were normalized to a housekeeping gene, Ywhaz, using the $\Delta\Delta C_t$ method. Ywhaz was chosen based on previous literature citing its remarkable stability in rat brain gene expression when compared to other commonly used housekeeping genes (Bonefeld et al., 2008; Gubern et al., 2009). Primers used in this experiment are found in Table 1.

Quantification of DNA methylation

DNA methylation was assessed using an adapted version of the methylation sensitive restriction enzyme (MSRE) assay (C. J. Auger et al., 2011; Edelmann and A. P. Auger, 2011; Hashimoto et al., 2007; Kigar et al., 2015). Briefly, 240 ng of DNA from each rat was divided equally into two tubes and digested with either AciI (New England Biolabs; catalog #R0551) or ClaI (New England Biolabs; catalog #R0197) in the same buffer conditions at 37°C for 90 min. Enzymes were subsequently inactivated by heating to 65°C for 20 min. A no-DNA control was added to ensure purity of the restriction enzyme reaction. Primers were designed such that Acil but not Clal cut sites were contained within the region of interest. To assess relative amounts of DNA methylation, RT-qPCR was performed as described above. Primers used in this experiment are found in Table 2.

Statistical analysis

PCR data were analyzed using a two-tailed Student's t test for sex difference data and one-way ANOVA with Tukey's posthoc for pairwise comparison was used for the hormone-treated data set (Prism 5; GraphPad Software, Inc.). To eliminate any technical error for the PCR data, outliers were screened for using the Grubbs test with a significance level of alpha = 0.05. All reported measures are listed as mean ± SEM. Significance was defined as a p value of <0.05.

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Highlights

- Females express higher levels of Gadd45b within the developing amygdala
- Juvenile males have more DNA methylation in the Gadd45b promoter
- Steroid hormones decrease Gadd45b levels in the female amygdala

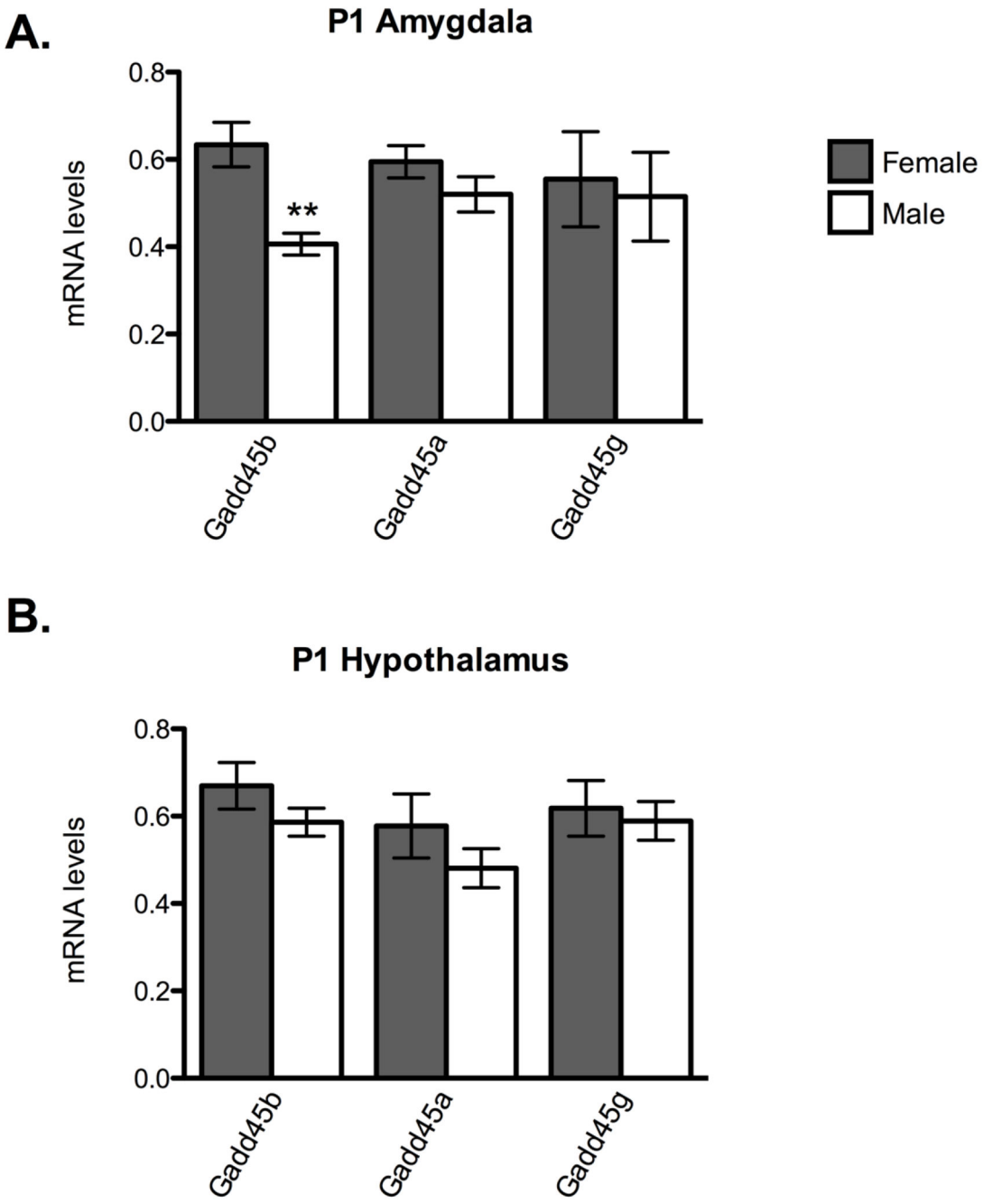

Figure 1:

Sex differences in the mRNA expression of Gadd45 family members Gadd45b, Gadd45a, and Gadd45g at postnatal day 1 (P1) in two brain regions ***A.*** amygdala and ***B.*** hypothalamus known to be steroid hormone-responsive during early neonatal development. ** $p<0.01$

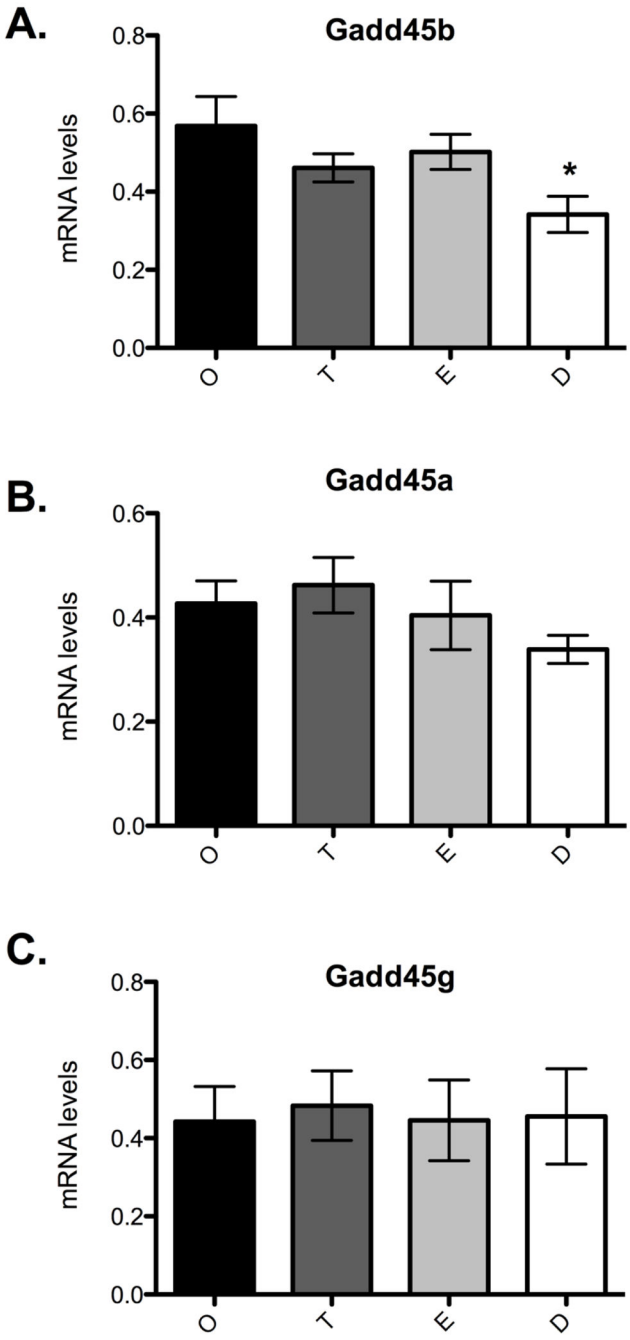

Figure 2:

Gadd45 family members' mRNA expression in the amygdala of hormone-treated females: ***A.*** Gadd45b, ***B.*** Gadd45a, and ***C.*** Gadd45g. Females were treated with either vehicle (O = oil) or hormone [T = testosterone, E = estrogen, D = dihydrotestosterone (DHT)] twice and then sacrificed at P2. Posthoc analysis of Gadd45b mRNA levels revealed a significant difference between oil-treated and DHT-treated females. *$p<0.05$

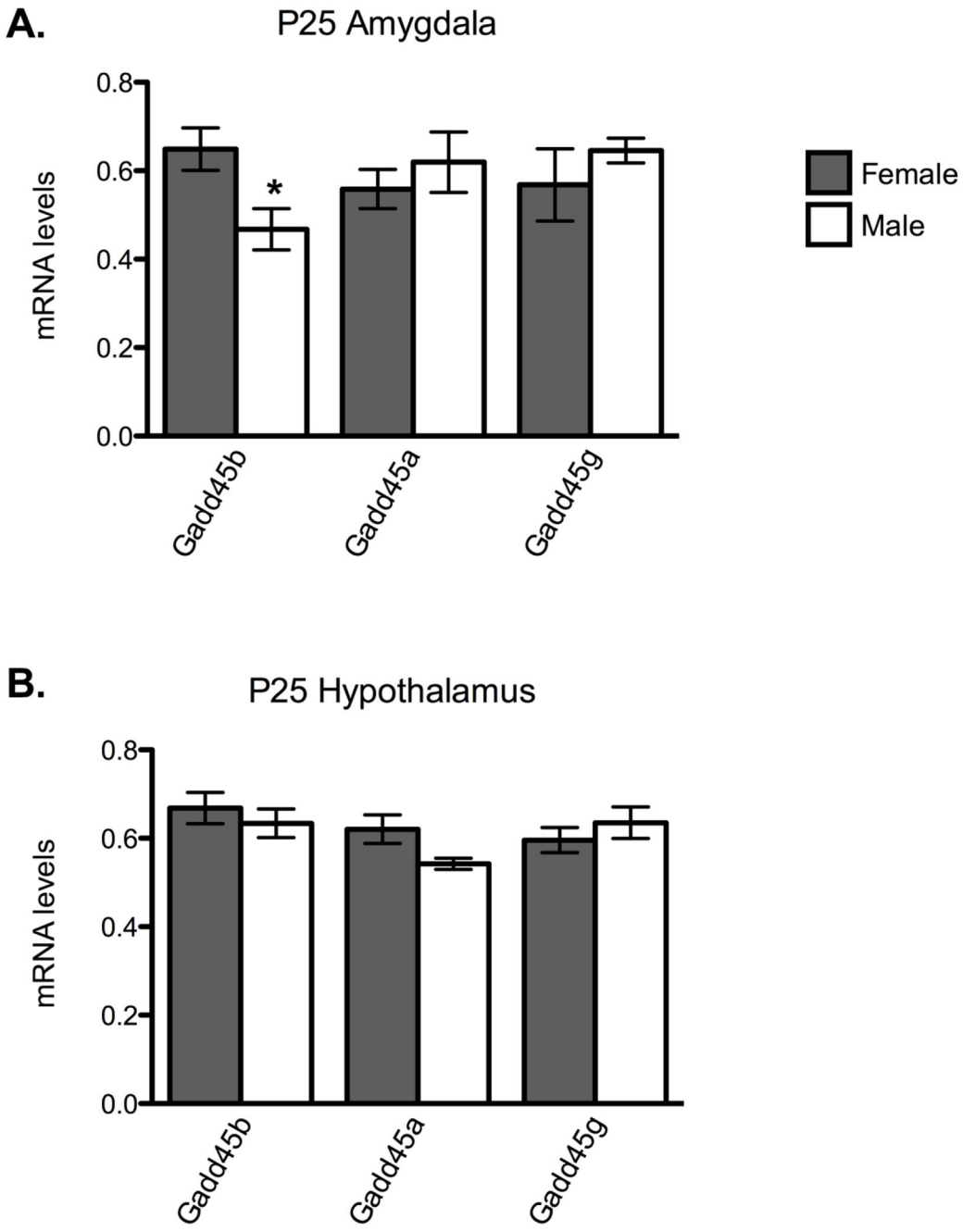

Figure 3:
Sex differences in mRNA expression of Gadd45 family members in the postnatal day 25 (P25) ***A.*** amygdala and ***B.*** hypothalamus. *p<0.05

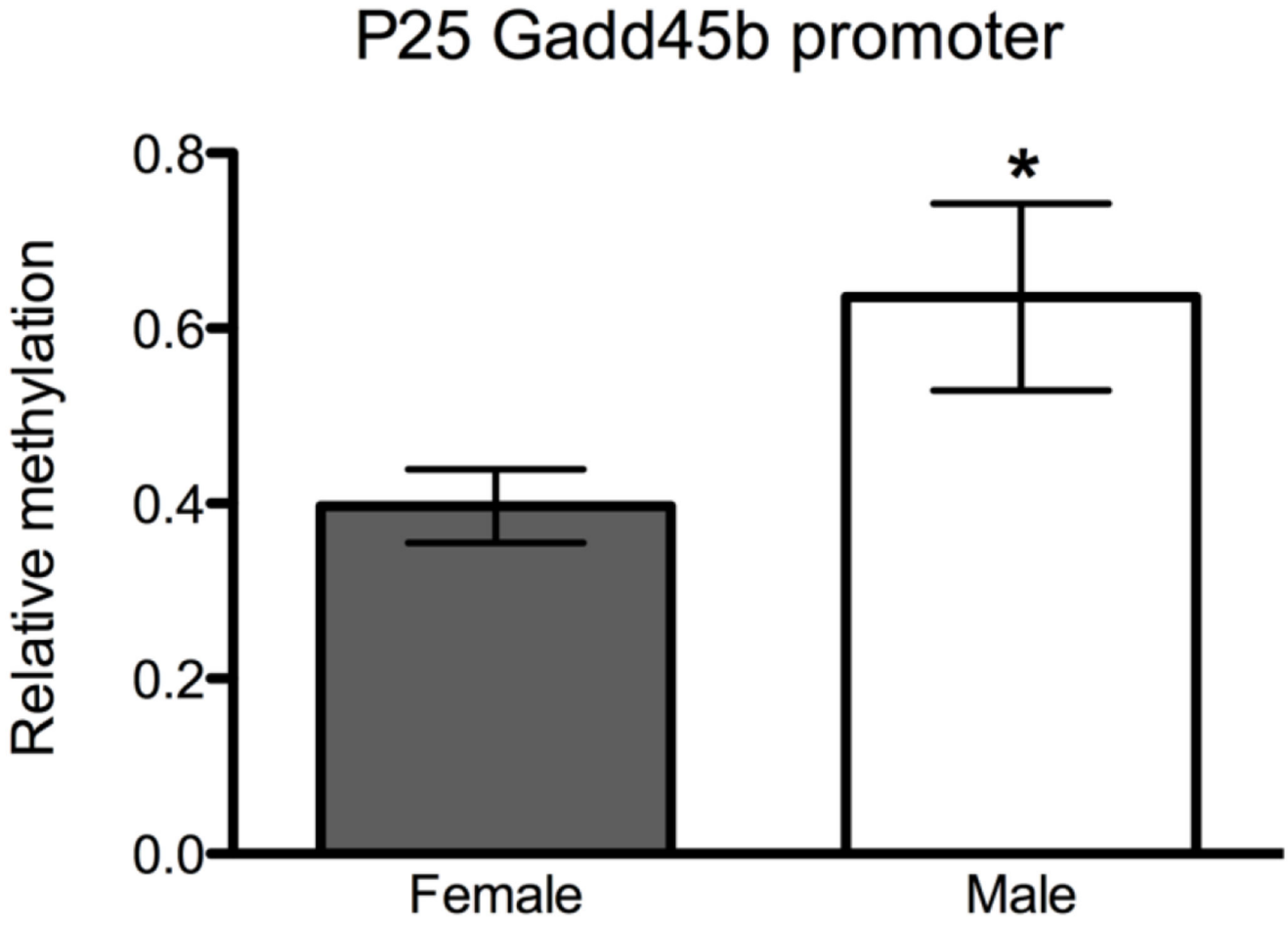

Figure 4:
Sex differences in Gadd45b promoter methylation at an estrogen receptor α (ERα) response element (ERE) in the juvenile amygdala (postnatal day 25 (P25)). *$p<0.05$

Table 1:

mRNA Primer sequences and Pubmed accession numbers for RT-qPCR

Ywhaz	NM_013011	TTGAGCAGAAGACGGAAGGT	GAAGCATTGGGGATCAAGAA
Gadd45b	NM_001008321	GCTGGCCATAGACGAAGAAG	GCCTGATACCCTGACGATGT
Gadd45a	NM_024127	GCTACTGGAGAACGACAAGAG	CCATTGTGATGAATGTGGGTTC
Gadd45g	NM_001077640	CTGAATGTGGACCCTGACAAT	AACGCCTGGATCAACGTAAA

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Table 2:

Methylation primers used to examine the Gadd45b promoter

G45b ERE	AciI (CCGC)	CTCGATTCGCTGGCAGTC	GATTGGCTGGAGGTAGGAAAG