Epigenetic programming of the juvenile social brain: risk and resilience conferred by biological sex

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Se non sta andando bene, chiama la nonna .

This thesis is dedicated to my grandmothers.

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ABSTRACT:

The data presented in the following chapters of this dissertation are meant to address open questions about how the early environmental milieu shapes an individual's epigenetic landscape—and, in turn, may manifest as risk or resilience to certain mental health disorders. We hypothesized that experiential and hormonal influences on biological sex during development may produce differences in the epigenome, and that these differences play an important role in gating risk or resilience to a number of neurological and psychiatric disorders (**Chapter I**). One intriguing hypothesis is that the framework belying sex differences in the brain, consisting of differences in methylation and demethylation patterns, confer risk and resilience to mental health disorders. In our attempt to elucidate these mechanisms, we focused primarily on the amygdala given its seminal role in the formation of socioemotional and anxiety behaviors; we were furthermore interested in this region as disturbances in its activity are implicated in the etiology of a wide variety of psychiatric disorders. We present evidence suggesting that mRNA expression within the amygdala of the DNA demethylation factor, Gadd45b, is involved in the patterning of social behaviors (**Chapter II**). Furthermore, we describe amygdalar sex differences in Gadd45b expression during a critical period of neonatal brain development, and suggest this pathway may be a useful target in investigating the etiology of social deficits associated with some mental heath disorders (**Chapter III**).

Importantly, we provide the first evidence, to our knowledge, of the function and existence of 6-methyladenine (6mA) as a novel epigenetic regulator in the mammalian brain (**Chapter IV**). Specifically, we demonstrate that 6mA is present in the gene promoter of serotonin receptor type 2a (*Htr2a*), and that its presence predicts levels of gene expression. We furthermore show that

this epigenetic modification exhibits stable, developmental sex differences and stress responsivity in a novel, variable predator odor exposure (POE) paradigm of early life stress. Additionally, we characterize anxiety-like behavior in our neonatally predator odor exposed animals and find long-term changes; as Htr2a and the serotonergic system have a well-known role in the development of anxiety disorders, we suggest that 6mA may represent a valuable biomarker and pharmacological target in the their treatment. Given the unchallenged assumption of 5-methylcytosine (5mC) as the primary epigenetic regulator in the mammalian genome, we hope this research opens up new and exciting avenues into the investigation of stress-induced neuropathologies.

CHAPTER I: INTRODUCTION

EPIGENETIC MECHANISMS MAY UNDERLIE THE AETIOLOGY OF SEX DIFFERENCES IN MENTAL HEALTH RISK AND RESILIENCE

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INTRODUCTION

Biological sex is a significant risk factor for numerous mental health disorders, as there are differences in the diagnostic rates between genders of such seemingly disparate conditions as autism spectrum disorders (Baron-Cohen et al., 2005), early-onset schizophrenia (Abel et al., 2010), major depressive disorders (Dao et al., 2010), stress/anxiety-related disorders (Palanza, 2001), and anorexia nervosa (Bulik et al., 2006) (**Table 1**). Given this discrepancy, the obvious questions to arise are: what belies the etiology of sex differences in the brain, and how does this manifest as differential susceptibility to pathology? While alterations in the genetic code are implicated in many of these disorders, epigenetic modifications to the genome may also be a contributing factor. This introduction will attempt to address what is known about the organization of sex differences in the brain during development and to discuss important epigenetic factors associated with psychiatric disorders. It should be noted that we define epigenetics as a dynamic or maintained change to chromatin (i.e. DNA-histone complexes), which alters or has the potential to alter gene transcription without necessarily modifying the underlying DNA code. This definition differs slightly from that of Adrian Bird in its emphasis on the transient nature of these epigenetic marks, discussed further in **Chapter III** (Bird, 2007).

While neuronal sex differences are present at birth due to both genetic and hormonal organization (McCarthy and Arnold, 2011), chemical and morphological changes continue to occur postnatally and into adulthood. We will discuss briefly how these variances emerge and their translation to sex differences in the epigenome. A model will be presented in which sex-specific epigenetic programming of the amygdala is critical for typical juvenile social interactions. Additionally, we will discuss data associating variations in epigenetic factors with

mental health risk, making them a plausible culprit in conferring risk or resilience to mental health disorders.

I. HORMONAL ACTIVATION OF NEURAL CIRCUITS IN THE DEVELOPING BRAIN

The developing brain is exquisitely sensitive to the effects of circulating steroid hormones during so-called sensitive periods of perinatal development which are critical in defining a particular developmental trajectory (Roth and David Sweatt, 2010; Heim and Binder, 2012). While the timing of hormone sensitivity varies by species, the sex-determining mechanism is conserved between placental and marsupial mammals, dating it at some 148 million years (Wallis et al., 2008). Briefly, Sry on the Y chromosome is a transcription factor that serves to differentiate the bipotential gonad into testes. Leydig cells within the testes begin producing testosterone *in utero* and in substantially greater amounts than the female ovaries (Reyes et al., 1973). Testosterone can then enter the brain, where it is aromatized or reduced into estradiol or dihydrotestosterone, respectively. These metabolites subsequently bind to estrogen receptors (ERs) or androgen receptors (ARs) and result in masculinization and defeminization of the brain, especially those systems pertinent to reproduction ((Whalen and Edwards, 1967), for review, see (Baum, 1979)).

Generally speaking, hormone binding to its cognate nuclear receptor results in a conformational change that releases the receptor from heat shock proteins. Nuclear receptors can then dimerize and translocate to the nucleus where they bind to response elements in gene promoter regions and recruit coregulatory proteins. Coactivator proteins, such as cAMP-response element binding protein-binding protein (CBP) and steroid receptor coactivator 1 (SRC1), contain intrinsic histone acetylation (HAT) activity that relaxes the tight coiling of negatively charged DNA to

positively charged histones, allowing for more efficient access of the transcriptional machinery (for review, see (Tetel et al., 2009)) (**Figure 1**). Interestingly, both CBP and SRC-1 are expressed more highly in males during the early neonatal period in rat brain (Misiti et al., 1998; Auger et al., 2002) while corepressors are expressed more highly in females (see below for discussion), likely resulting in sex-specific epigenomic variation.

Perinatal hormonal surges occur mostly during discrete windows of time during development, but can have relatively stable organizational effects on an organism's behavior and physiology—thus rendering sexual differentiation a useful model by which we can begin to study lasting epigenetic processes in the brain. While hormones and experience significantly alter sexually dimorphic brain regions (e.g. hypothalamic regions related to stress, preoptic area (POA) control of reproduction), sex differences in the epigenetic reprogramming of gene function are also known to occur within the amygdala (Auger et al., 2011a), a region critical for socioemotional processes (LeDoux, 2007).

II. CRITICAL INVOLVEMENT OF THE AMYGDALA IN SOCIAL BEHAVIOR

The amygdala—so named from Greek due to its almond-like shape—is a small, complex structure in the medial temporal lobe known to be of central importance in emotion processing, conditioned fear learning, and modulation of social behaviors (for anatomical review, see (Phelps and LeDoux, 2005; LeDoux, 2007)). A primitive amygdalar structure can be found in amphibians, dating its evolutionary appearance sometime before the amphibian-amniote split, or some 315 million years (Laberge et al., 2006). Lesion studies have provided ample evidence of an amygdalar role in social/emotional integration in both human and non-human primates; they

furthermore implicate that the time of lesioning significantly impacts the severity of deficits, with childhood or congenital lesions having the most detrimental effects on later functioning (Shaw et al., 2004; LeDoux, 2007). Importantly, disruption of normal amygdala development has been noted in a plethora of neurodevelopmental disorders, e.g. autism, schizophrenia, anxiety disorders, childhood bipolar disorder (Schumann et al., 2011a; Hanson et al., 2015); reviewed in (Amaral et al., 2003; Tottenham, 2009)). The timing of amygdala development in relationship to other sexually differentiated brain regions is less clear. In rodents, this system remains plastic in response to hormone manipulation into adulthood (Johansen et al., 2004); in humans, the amygdala appears to develop approximately 5 months post-fertilization and remains relatively plastic until late adolescence, unlike other primate species (Phelps and LeDoux, 2005; LeDoux, 2007; Schumann et al., 2011b). It is well known that the amygdala is extremely steroid responsive, as high expression levels of ERs and ARs can be found in this structure early in development (Meaney and Stewart, 1981a; Meaney et al., 1983; Meaney and McEwen, 1986; Pellis et al., 1994; Goy and Deputte, 1996; Yokosuka et al., 1997; Olesen et al., 2005; Auger and Olesen, 2009), making it a region of considerable interest in understanding sexual differentiation of social behaviors and associated pathologies.

III. Organization of Juvenile Social Play Behavior by Steroid Hormones

One well-studied example of sexually dimorphic behavior is juvenile social play. Juvenile play behavior has emerged as an important developmental event that can both facilitate future social interactions and confer normal cognitive abilities later in life (for review, see (Auger and Olesen, 2009)). Sex differences in frequency of initiation of so-called “rough and tumble play” are observed in myriad species, including humans (Whiting and Edwards, 1973; DiPietro, 1981; Humphreys and Smith, 1987), non-human primates (Goy and Deputte, 1996), dogs (Ward et al.,

2008), cats (Caro, 1981), and rodents (Olioff and Stewart, 1978). Organization of play behavior occurs, in part, within the amygdala due to the presence of sex hormones, though the relative contributions of androgens and estrogens are unclear and may be species-specific (Meaney and Stewart, 1981a; Meaney et al., 1983; Meaney and McEwen, 1986; Pellis et al., 1994; Goy and Deputte, 1996; Olesen et al., 2005; Auger and Olesen, 2009). While there are many theories as to why animals play, abnormal play behavior may be a good experimental model for social anhedonia, a common component of multiple neurodevelopmental disorders (for reviews, see (Panksepp et al., 1984; Panksepp and Lahvis, 2007; Berridge and Kringelbach, 2008; Auger and Olesen, 2009)).

A. Studies in rodents

In rodents, males are more likely to initiate play and engage in more bouts overall as scored by tracking pouncing, boxing, pinning, biting, and chasing (Pellis et al., 1994). Implanting testosterone directly into the amygdalae of newborn female rat pups effectively masculinized play (Meaney and McEwen, 1986). Conversely, castrating males on the day of birth reduces bouts of play to female-typical levels (Meaney and Stewart, 1981a; Pellis et al., 1994). Furthermore, analysis of male play behavior in the testicular feminized mutation (*tfm)* rat, wherein a point mutation to the steroid-binding domain of AR renders it unable to bind androgens, shows reduced levels of play (Meaney et al., 1983). However, male *tfm* rats, while exhibiting less play behavior than control males, tended to play more frequently than females and certain components of play remained at male-typical levels, indicating androgen receptors alone are insufficient to completely masculinize this behavior (Meaney et al., 1983; Field et al., 2006).

Our lab has shown that systemic treatment of neonatal female rats with a dose of estradiol benzoate (EB) shown to result in male-like estradiol levels in the hypothalamus (Amateau et al., 2004) is sufficient to masculinize play behavior, and this effect could be prevented by pretreatment with the selective estrogen receptor modulator (SERM), tamoxifen (Olesen et al., 2005). A unifying explanation for this apparent discrepancy in hormonal organization may be that estrogen masculinizes some but not all components of play and otherwise "primes" the brain for androgen exposure, as estradiol can selectively induce expression of AR mRNA (McAbee and Doncarlos, 1999). Additional evidence for the masculinization of play behavior by hormone exposure comes from intrauterine position studies. In litter-bearing mammals, females located between two male fetuses have an increased anogenital distance and exhibit more masculine play behavior than females that are located between two other females (Ryan and Vandenbergh, 2002).

B. STUDIES IN HUMANS

In humans, rough-and-tumble play is quantified by measuring playful, non-harmful physical assault, physical assault on an object, and wrestling. These play gestures are found across a wide spectrum of cultures (Whiting and Edwards, 1973) and can furthermore be observed in non-human primates (Fedigan, 1972)—speaking to the evolutionary importance of such behaviors during development. Examples from human studies support sex differences in rough-and-tumble play behavior and implicate a role for early steroid hormone exposure.

1. Congenital adrenal hyperplasia (CAH)

In individuals diagnosed with congenital adrenal hyperplasia (CAH), normal cortisol production does not occur during the second month of gestation, ultimately leading to excess androgen accumulation in the body. Females with CAH frequently have ambiguous genitalia at birth, demonstrating the powerful effects *in utero* exposure to fetal testosterone can have on normal anatomical development (reviewed in (Hines and Kaufman, 1994)). Interestingly, behavioral studies on these children have revealed that females with CAH engage in more aggressive play during childhood, and tend to prefer male playmates when compared to sisters without this congenital abnormality, who themselves tend to prefer female playmates (Berenbaum et al., 2000).

2. Nonclinical population studies

Efforts have been made to correlate fetal testosterone exposure to gender role behavior and/or rough and tumble play behavior, with conflicting results depending on the method of analysis of fetal testosterone (Hines et al., 2002; Knickmeyer et al., 2005). The most recent data seem to suggest that higher levels of fetal testosterone exposure do in fact correlate positively to increased rough and tumble play in human children (Auyeung et al., 2009).

C. EFFECTS OF MATERNAL CARE ON PLAY BEHAVIOR

It has previously been shown in rats that there are natural variations in maternal care between dams, and that these variations drive epigenetic changes in factors responsible for stress reactivity, maternal behavior, and cognitive functioning (Francis et al., 1999; Liu et al., 2000; Weaver et al., 2004; Champagne et al., 2006). Pertinent here, male pups of dams exhibiting high levels of licking and grooming and arched back nursing (LG-ABN) play significantly less than

pups from low LG-ABN dams (Parent and Meaney, 2008). This effect was specific to males, as play fighting was not significantly different between females from high or low LG-ABN mothers. Our lab has focused on isolating the tactile stimulation component of maternal behavior to eliminate the possibility that some factor in the nursing milieu is responsible for outcomes due to variations in maternal care in a paradigm we call simulated maternal grooming (SMG). Our results recapitulate previous findings that male pups with higher levels of tactile stimulation play less overall and that this effect is sex-specific (Edelmann et al., 2013). It furthermore provides mechanistic data for reduced play behavior, namely that serotonin receptor type IIa (Htr2a) mRNA expression increases to female-typical levels. Since Htr2a expression seems to inversely correlate with aggression in both rats (Homberg et al., 2007) and non-human primates (Higley and Linnoila, 1997; Fairbanks et al., 2001), it would appear that maternal care impinges on serotonergic pathways in males only and thereby reduces aggression. One enticing explanation for the male-specific effect of maternal care on rough-and-tumble play is that hormonal cues induce dams to engage in behavior that will minimize overt sex differences. Specifically, rat dams tend to preferentially lick and groom their male offspring more than females (Moore and Morelli, 1979). Injecting neonatal females with estradiol, dihydrotestosterone, or testosterone all induce the mother to lick and groom them at male-typical levels, perhaps in part to temper the effects of hormones on play behavior (Meaney and Stewart, 1981b; Moore, 1982; Meaney et al., 1983).

While maternal care reduces sex differences in juvenile social play, it appears to masculinize some gene expression patterns in females, and variations in maternal care can epigenetically reprogram promoter regions of important transcription factors influencing behavior within the

developing hippocampus and preoptic area (POA) of neonatal rats (Weaver et al., 2004; Champagne et al., 2006). Our lab has shown that treatment of neonatal females with SMG increases amygdalar ERα promoter methylation, decreases ERα mRNA, and therefore phenocopies a male-typical ERα status (Edelmann and Auger, 2011). While it is unclear whether these maternal cues are used in other species and to what extent, the maternal rearing environment is known to be a critical component of normal psychological development. For example, the importance of touch in human development has been well documented (reviewed in (Field, 2010))—one study quantifying the amount of touch contact French children vs. American children received revealed that French infants, who receive more physical contact from their mothers than American children, are less aggressive (Field, 1999).

Conversely, the effects of neglect or abuse have also been shown; for example, Harry Harlow's controversial studies involving social isolation of rhesus monkeys clearly demonstrated the essential role of maternal care during infancy (Harlow, 1958; Harlow et al., 1965)—unsurprisingly, maternal deprivation results in lasting changes in the epigenome (Provencal et al., 2012). Examination of postmortem tissue from the brains of suicide victims found that childhood abuse altered glucocorticoid receptor (GR) expression (by increasing methylation of the promoter and decreasing mRNA expression) (McGowan et al., 2009); as GR expression aids in dampening the body's response to stress, it is postulated that early life adversity permanently inhibited these patients' ability to process stress appropriately. While much remains to be parsed out about the relative contributions of maternal care (or neglect) and the ensuing outcome on sex-typical behavior, major inroads have been made in terms of understanding altered epigenetic

processes due to maternal care in rodent models. It will be interesting to see how and if maternal care confers a protective effect against development of psychiatric symptoms.

IV. Epigenetic mechanisms governing repression and demethylation/ involvement in conferring risk or resilience to mental health disorders

While some co-activators are known to be involved in the etiology of genetic disorders, e.g. CREB-binding protein (CBP) deletion in Rubinstein-Taybi syndrome (Petrij et al., 1995) and E6-AP activity loss in Angelman Syndrome (Nawaz et al., 1999) (reviewed in (Tetel et al., 2009)), disruptions in a number of repressors/co-repressors have been identified in sex-skewed neurodevelopmental disease ontogeny (discussed below), making them a particularly interesting target for investigation. Below we will review various factors involved in gene repression and consider how sex differences in these proteins may contribute to sex differences in risk and resilience.

A. DNA methylation by DNMTs

Gene repression begins by enzymatic transfer of a methyl group to the 5' carbon of a cytosine base (5mC), usually in the context of CpG "islands", or regions of DNA rich in cytosine and guanine base pairs. Though methylation can occur at CpA, CpT, or CpC sites, it is less common (i.e. CpG methylation makes up approximately 40% of all CpN methylation) and does not occur as efficiently (Grafstrom et al., 1985; Ramsahoye et al., 2000). Methylation is accomplished by DNA methyltransferases, or DNMTs. These include DNMT1, which binds hemi-methylated CpGs and is thus primarily thought of as a maintenance enzyme (Pradhan et al., 1999); DNMT3a, which results in *de novo* 5mC synthesis and is expressed more robustly around birth;

DNMT3b, also capable of *de novo* 5mC formation but with high expression after conception that declines over time; and finally DNMT3L, the function of which is unclear, though it has been shown to lack enzymatic activity (Li et al., 1998; Okano et al., 1999; Feng et al., 2005).

1. Sex differences in DNMT expression—implications for psychiatric disorders?

Previous work from our lab has shown that at PN1, female rats express more DNMT3a than males in the amygdala, but not the hypothalamus or POA. Female expression levels were reduced towards male-typical levels by prior injection of estradiol benzoate or dihydrotestosterone, and sex differences were no longer observed by PN10. Furthermore, no sex difference at either time point was observed for DNMT1 (Kolodkin and Auger, 2011). Additional evidence substantiated our finding that there is no sex difference in DNMT1 expression, but found that enzymatic activity was altered—i.e., females had greater activity than males (Nugent et al., 2015). As DNMT overexpression has been noted in psychiatric disorders (Zhubi et al., 2009; Grayson and Guidotti, 2012) and there are sex differences in expression and activity, this may be one potential mechanism by which males and females exhibit sex differences in risk and resilience to mental illness.

B. Other forms of DNA methylation

Until recently, other, non-CpG forms of DNA methylation were thought to be the exclusive purview of bacteria and some unicellular organisms, and had even been considered as pharmacological targets in the development of new antibiotics (Wion and Casadesús, 2006). Now, in a trio of papers published earlier this year, there is firm evidence that algae, worms, and flies produce and use N^6-methyladenine (6mA) as an epigenetic mark *in vivo* (Fu et al., 2015;

Greer et al., 2015; Zhang et al., 2015). These exciting discoveries challenge the 5mC status quo, though very little is known about 6mA at present. Data presented in **Chapter IV** of this dissertation present evidence for the first time to our knowledge that the mammalian brain also uses 6mA as an epigenetic modification for gene transcription. However, much work remains—including identification of the enzymes responsible for organization of 6mA into the epigenome of higher eukaryotic species (Heyn and Esteller, 2015).

C. Recruitment of methyl binding domain (MBD) proteins and corepressors to ensure gene suppression

Upon methylation, gene repression is accomplished at several levels: first, methylation can physically block binding of transcription factors, thus preventing assembly of co-activators and recruitment of RNA polymerase II. Furthermore, MBD proteins (e.g. methyl-CpG-binding protein 2 (Mecp2), Mbd1, Mbd2, Mbd3, Mbd4, and Kaiso) recognize 5mC and can recruit corepressors like NCoR and SMRT (reviewed in (Auger and Jessen, 2009)), ultimately resulting in acquirement of histone deacetylase (HDAC) activity. HDACs remove the acetyl group masking positively charged lysine residues in histones and allow for tight packing of DNA and histones into chromatin, providing another mechanism by which access to a transcriptional start site is blocked (for review, see (Auger and Auger, 2011)). It is important to note that MBD protein binding may not always predict gene repression—for example, Mecp2 can bind CREB and increase gene expression (Chahrour et al., 2008), complicating the straightforward mechanism discussed above. The conditions under which this occurs are relatively unknown but appear to be an exception, not the rule.

1. Mecp2 and pervasive developmental disorders (Rett syndrome and autism)

Mecp2 is an X-linked gene that was identified in a mutational screen for Rett syndrome (Amir et al., 1999), a neurodevelopmental disease primarily affecting females as it is usually embryonic lethal for males. Rett syndrome, like autism, is considered a pervasive developmental disorder marked by severely impaired speech and social interactions, repetitive motor movements, and seizures (Amir et al., 1999; Ramocki et al., 2009). Interestingly, Janine LaSalle and colleagues found that aberrant *MECP2* promoter hypermethylation was a common feature of male autistic brain in post-mortem patient tissue samples, demonstrating that proper dosing of Mecp2 may be important in a variety of autism-spectrum disorders (ASD) (Nagarajan et al., 2006). We have observed a sex difference in neonatal rat Mecp2 mRNA on PN1, where females express more in the amygdala and the hypothalamus (but not the POA) and that is eliminated by PN10 (Kurian et al., 2007).

A lower level of Mecp2 expression at birth may put males at increased risk of developing symptoms inherent to Mecp2-related disorders. To test this concept, siRNA targeted to Mecp2 was directly infused into the amygdala of neonatal male and female rats for three days starting on the day of birth, and animals were subsequently raised, undisturbed, to the juvenile period (Kurian et al., 2008). Mecp2 siRNA-infused males engaged in less juvenile social play behavior though no alterations in female social play behavior were observed. While the mechanism by which this occurs remains to be fully established, neonatal Mecp2 siRNA treatment reduced male-typical expression of arginine vasopression (AVP)—a peptide hormone critical for proper social recognition in males that is organized by steroid hormone exposure during developmentally sensitive windows of time (Han and de Vries, 2003; Forbes-Lorman et al.,

2012). These data support the idea that male juvenile social interactions can be disrupted by perturbations in Mecp2 levels during a critical time point of amygdala development, and that disruptions in AVP expression may be involved. It is not known why females appear more resilient to perturbations in Mecp2 expression within the amygdala, but this may be due to their normally higher levels of Mecp2 expression or to the presence of a compensatory Mecp2 allele on the X chromosome.

2. NCoR—evidence for involvement in anxiolytic aspects of mental health disorders

Nuclear receptor co-repressor 1 (NCoR) was one of the first corepressors to be discovered (Hörlein et al., 1995), is ubiquitously expressed in the brain (Van Der Laan et al., 2005), and exhibits a sex difference within the amygdala and medial basal hypothalamus in rats which can be reversed by treatment at birth with steroid hormones (Jessen et al., 2010). Despite NCoR's known binding affinity to important epigenetic regulators like Mecp2 and Kaiso (Kokura et al., 2001; Yoon et al., 2003) and its known disregulation and abnormal localization in Huntington's disease (Boutell et al., 1999), very little work has been directed at understanding what involvement it may have in the development of psychiatric symptoms. A relatively recent study from our lab demonstrated that by infusing the neonatal rat amygdala with siRNA targeting NCoR, juvenile play behavior was 'hypermasculinized' in males specifically, and anxiety was elevated in both sexes (Jessen et al., 2010). It will be interesting to determine if there are links between heightened anxiety and increased rough-and-tumble play behavior in males. These data illustrate that reductions in an epigenetic factor early in development can increase juvenile social interactions in males. This further supports the idea that the epigenome is sexually differentiated

and perturbations to epigenetic regulatory factors may yield different outcomes depending on biological sex.

D. Histone modifications

Both co-activators and co-repressors recruit histone-modifying enzymes or possess intrinsic modifying activity—importantly, there are sex differences in the acetylation and methylation of mouse histone H3 (Tsai et al., 2009) and in the expression of murine histone demethylases (Xu et al., 2008a; 2008b). Histone modifications play an important role in regulation of gene transcription and repression and thus may help guide sexual differentiation of the brain. For example, males have a larger volume and more cells within the principal nucleus of the bed nucleus of the stria terminalis compared to females, and treatment with the HDAC inhibitor valproic acid disrupts this sex difference (Murray et al., 2009). Sex differences in histone acetylation patterns are important not only for brain morphology, but also for regulating adult behavior. Specifically, treating newborn males with trichostatin A (TSA), an HDAC inhibitor, or infusing them with antisense oligodeoxynucleotides targeting HDAC2 and HDAC4, partially disrupts some adult male sexual behavior (Matsuda et al., 2011). Additionally, TSA can facilitate pair-bonding in adult female prairie voles (Wang et al., 2013), Together, these data indicate that histone modifications occurring early in postnatal development are important in shaping lasting sex differences in brain and behavior, and that chromatin modifications remain plastic throughout the lifespan.

E. Active DNA demethylation

Gene repression vis-à-vis cytosine methylation was previously thought to be a permanent modification. In cases where DNA demethylation was observed, the process was assumed to be passive in the sense that methylation marks were lost through successive rounds of cell division by, for example, inhibition of DNMT1 (for discussion, see (Wu and Zhang, 2010)). As most neurons are held in a quiescent state it became clear that this could not fully account for DNA demethylation in the brain, and an older body of literature demonstrating the widespread existence of 5-hydroxymethylcytosine (5hmC) (Penn et al., 1972) was subsequently unearthed. We now know 5hmC to be both a transitional state towards active DNA demethylation and the product of *ten eleven translocation* (TET) protein family oxidation. TETs were originally described as tumor suppressors, because chromosomal translocation mutations resulted in acute myeloid leukemia (Delhommeau et al., 2009). It was later realized that TET family proteins shared homology with trypanosomal 2-oxoglutarate/Fe(II)-dependent oxygenases, leading to their discovery as the enzymes responsible for formation of 5hmC (Tahiliani et al., 2009).

Importantly, 5hmC represents ~40% of all modified cytosines in the brain and is enriched relative to 5mC in some cell types—including Purkinje cells, where it represents 0.6% of all nucleotides (Kriaucionis and Heintz, 2009; Szulwach et al., 2011), suggesting a significant role for this moiety in neuroepigenetic signaling. As seen in Figure 2, in lieu of further Tet1 oxidation, 5hmC can be modified by the activation-induced deaminase/apolipoprotein B mRNA editing cytokine (AID/APOBEC3) family, which converts the amine functional group of cytosine to a carbonyl, effectively generating uracil—specifically 5-hydroxymethyluracil (5hmU) (Conticello, 2008). This molecule can be recognized by enzymes responsible for base excision repair (BER) via generation of a T:G mismatch, and appears to require Mbd4 and

thymine DNA glycosylase (TDG) (Guo et al., 2011). Gadd45b (growth arrest and DNA-damage-inducible, beta) is required for neuronal activity-induced DNA demethylation, and is thought to act by recruiting AID/APOBEC3 and/or TDG to specific promoters (namely, BDNF and FGF) (Ma et al., 2009). Alternatively, 5hmC can be further oxidized by TET1 to form 5-formylcytosine and 5-carboxycytosine **(Figure 2).** While little is currently known about these molecules and what they interact with, it is feasible that they serve to assemble signaling complexes. Future studies directed at understanding whether these oxidative intermediates interact with transcription factors would be illuminating.

1. Involvement in psychosis

It has recently been found that both Gadd45b and Tet1 are overexpressed in postmortem tissue from patients diagnosed with major psychosis (encompassing both schizophrenia and bipolar disorder) (Dong et al., 2012; Gavin et al., 2012). Furthermore, Apobec3 was downregulated and 5hmC was seen to accumulate. To assess the effects of these alterations in gene expression, BDNF and Gad67 were examined—BDNF is an important neurotrophin implicated in depression (as part of the so called "neurotrophin hypothesis of depression", reviewed in (Martinowich et al., 2007)), whereas Gad67 is the enzyme responsible for making GABA, an important neurotransmitter in the brain previously shown to be disrupted in patients with schizophrenia (Lewis et al., 2005). In the case of both BDNF and Gad67, promoters were found to be hyper-hydroxymethylated while mRNA levels declined, indicating that the presence of 5hmC alone does not predict increased gene expression.

Interestingly, there appears to be a dynamic interplay between Tet1 hydroxylation and Mecp2 binding, suggesting that Mecp2 may protect certain promoter regions from being targeted for active DNA demethylation. In a Mecp2 knockout mouse model, 5hmC increases by approximately 20% whereas Mecp2 overexpression causes a 25% reduction in 5hmC (Szulwach et al., 2011). As Mecp2 expression has been clearly implicated in pervasive developmental disorders like autism and Rett syndrome (Amir et al., 1999; Nagarajan et al., 2006; Ramocki et al., 2009), the ensuing misregulation of Tet1 hydroxylation may be an important avenue for future exploration. Further adding to this complexity, we present data in **Chapter II** that in neonatal rats, a transient reduction in Gadd45b expression (via targeted siRNA delivery into the neonatal amygdala) decreases Mecp2 expression ~30% (Kigar et al., 2015), suggesting there are several pathways by which aberrant Mecp2 expression can be accomplished. Future work should be directed at understanding whether Gadd45b and/or Tet1 expression is altered in ASD or Rett syndrome.

F. Maintenance of DNA methylation by steroid hormones

Emerging evidence suggests that—at least in the brain—DNA methylation must be maintained to ensure normal function (Dong et al., 2010; Auger et al., 2011b). It has been shown that cycling between a methylated or demethylated state can happen rapidly—on the order of minutes *in vitro* (Métivier et al., 2008). While it remains to be determined how fast this switching occurs *in vivo*, it was recently demonstrated that circulating testosterone levels are required for maintenance of AVP methylation patterns and expression within the bed nucleus of the stria terminalis in adult male rat brain. Cessation of testosterone signaling following castration resulted in less promoter methylation of AVP and increased AVP mRNA, while the opposite pattern was seen in ERα

(Auger et al., 2011b). These data indicate that the presence of steroid hormones maintains neuronal methylation status. It will be important to elucidate which methyltransferases or demethylases are responsible for this active switching in the brain.

As hormones appear to maintain methylated or demethylated states in rodents, it will be important to understand if the same holds true in humans. Aging is a natural phenomenon that results in a decrease or cessation of steroid hormone levels, as in menopause where there is a gradual discontinuation of estrogen production from the ovaries. Notably, the symptoms of psychiatric disorders like schizophrenia or neurodegenerative disorders like Alzheimer's disease increase significantly after menopause, giving rise to the idea that estrogen is 'neuroprotective' for women ((Tang et al., 1996) reviewed in (Seeman, 1997)). It is possible that a sustained decline in hormone levels results in changes to the epigenome that increase risk for some disorders. Future work examining the underlying molecular mechanisms will be of significant interest in treating these illnesses.

V. Mental health disorders as epigenetic diseases

Here we have outlined some of what is known about one of the first non-mother directed social behaviors (namely, juvenile social play), and addressed the involvement of a variety of epigenetic factors in its organization. We have also presented a parsimonious model by which gene repression by 5mC can trigger recruitment of corepressors or factors responsible for active DNA demethylation; however it should be noted that 5mC does not *de facto* indicate repression, e.g. methylation occurring in the promoter of a co-repressor would be predicted to increase gene expression, for example.

Despite the inherent complexities, establishing a more causative role for epigenetic molecules in human disease will be vital going forward as many mental health disorders are now being recognized as neurodevelopmental in origin, including schizophrenia (Pedersen and Mortensen, 2001; Lewis and Levitt, 2002; Abel et al., 2010), autism (Baron-Cohen et al., 2005; Knickmeyer et al., 2006; Surén et al., 2013; Volk et al., 2013), anorexia nervosa (Cnattingius et al., 1999; Bulik et al., 2006; Kaye et al., 2013) , and depression/anxiety (Zahn-Waxler et al., 2008; Hopkins et al., 2013). In each of these cases, perinatal exposure to a chemical (e.g. testosterone, folic acid) or an environmental cue (e.g. nurturance, maternal depression) can create a lasting change in mental health risk or resilience. This is by nature epigenetic, further supported by twin studies showing discordance in heritability of these disorders (Sullivan et al., 2003; Bulik et al., 2006; Arseneault et al., 2008; Rosenberg et al., 2009). Much remains to be determined about the advantages and disadvantages conferred by biological sex, and others have stressed the need for both investigating basic sex differences in the brain as well as for including females in studies (McCarthy et al., 2012). There is, however, growing support for the culpability of hormones and the rearing environment as organizers of an epigenetic framework determining risk and resilience to disease between the sexes.

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Disorder[a]	Sex Ratio (M:F)
Attention Deficit Hyperactivity Disorder	80:20
Autism	80:20
Schizophrenia[b]	58:42
Alzheimer's disease[c]	30:70
Anorexia nervosa	7:93
Anxiety disorder	33:67
Bulimia	10:90
Depression	33:67

Table 1: Data compiled from the *Diagnostic and statistical manual of mental disorders IV tr* (APA, 2000) except where noted (b. Aleman et al., 2003; c. Tang et al., 1996). Importantly, while the determined sex difference in schizophrenia is small, age of onset is earlier in males and is accompanied by more severe negative symptoms.

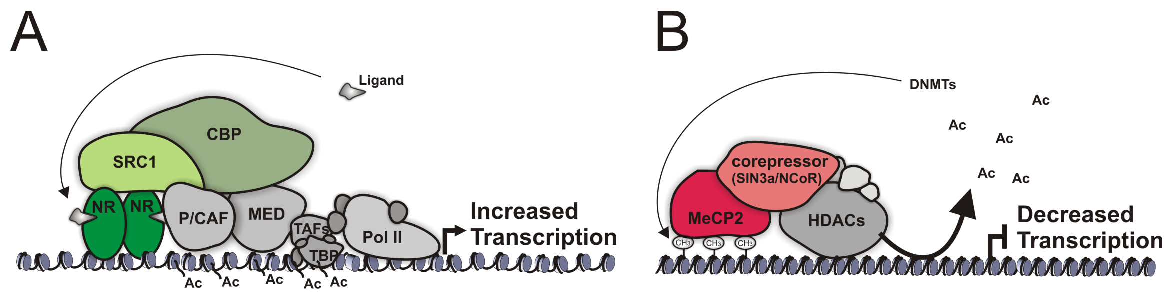

Figure 1: (A) Ligand binding to nuclear receptors (NR) induces dimerization and translocation to the nucleus, where assembly of co-activators (SRC-1, CBP) acetylates and unwinds DNA from histones and recruits transcription factors (including RNA Polymerase II) to allow for gene transcription. (B) Cytosine methylation by DNMTs recruits methyl-binding domain proteins (MBDs) like Mecp2 and co-repressors (SIN3a/NCoR), which deacetylate histones and tightly wind DNA, thus preventing gene transcription.

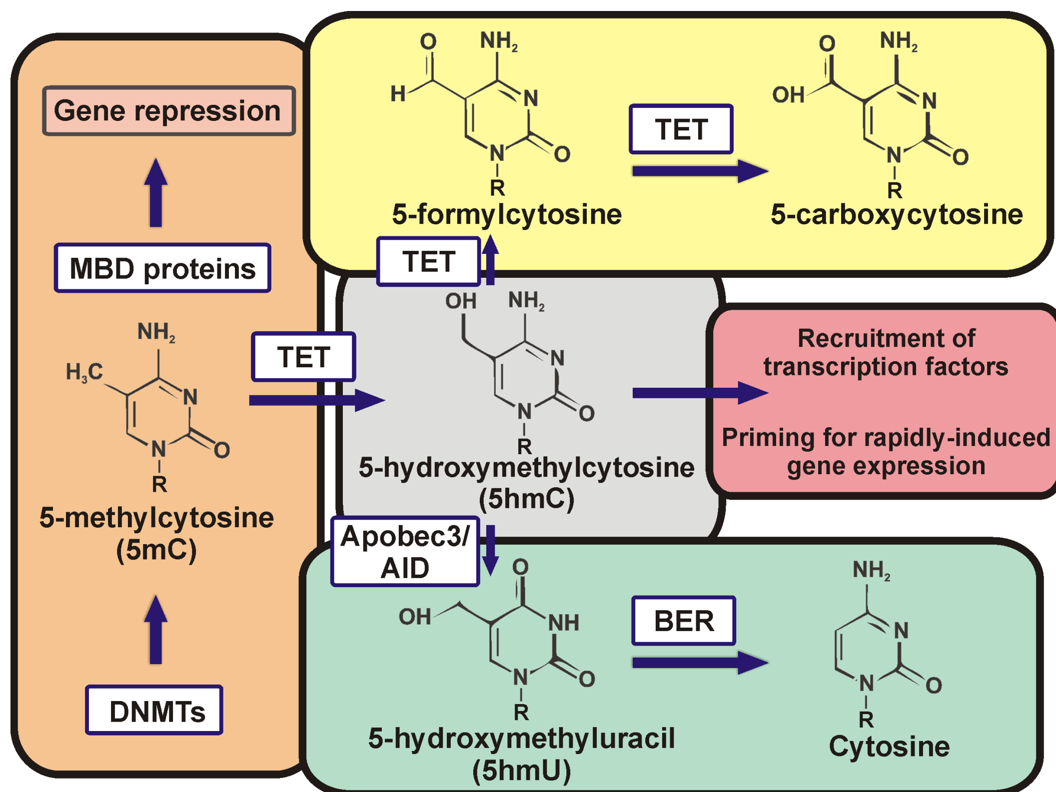

Figure 2: DNA methyltransferases (DNMTs) add a methyl group to the 5' carbon of cytosine. Methyl binding domain (MBD) proteins recognize this modification and recruit transcription factors to deacetylate histones and otherwise repress gene transcription. Alternatively, Tet oxidation of 5-methylcytosine (5mC) leads to the formation of 5-hydroxymethylcytosine (5hmC), which can be further oxidized to 5-formylcytosine and 5-carboxycytosine (presumably these molecules can act as signaling scaffolds in their own rite, though this remains to be seen). 5hmC can be deaminated by Apobec3 or AID, leading to base excision repair in an active DNA demethylation pathway. Alternatively, 5hmC can serve as an intermediate signaling molecule, recruiting unique transcriptional complexes or otherwise "priming" for gene expression.

CHAPTER II: GADD45B IS AN EPIGENETIC REGULATOR OF JUVENILE SOCIAL BEHAVIOR AND ALTERS LOCAL PRO-INFLAMMATORY CYTOKINE PRODUCTION IN THE RODENT AMYGDALA

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ABSTRACT

Precise regulation of the epigenome during perinatal development is critical to the formation of species-typical behavior later in life. Recent data suggests that Gadd45b facilitates active DNA demethylation by recruiting proteins involved in base excision repair (BER), which will catalyze substitution of 5-methyl- cytosine (5mC) for an unmodified cytosine. While a role for Gadd45b has been implicated in both hippocampal and amygdalar learning tasks, to the best of our knowledge, no study has been done investigating the involvement of Gadd45b in neurodevelopmental programming of social behavior. To address this, we used a targeted siRNA delivery approach to transiently knock down Gadd45b expression in the neonatal rat amygdala. We chose to examine social behavior in the juvenile period, as social deficits associated with neurodevelopmental disorders tend to emerge in humans at an equivalent age. We find that neonatal Gadd45b knock-down results in altered juvenile social behavior and reduced expression of several genes implicated in psychiatric disorders, including methyl-CpG-binding protein 2 (MeCP2), Reelin, and brain derived neurotrophic factor (BDNF). We furthermore report a novel role for Gadd45b in the programmed expression of a2-adrenoceptor (Adra2a). Consistent with Gadd45b's role in the periphery, we also observed changes in the expression of pro-inflammatory cytokines interleukin-6 (Il-6) and interleukin-1beta (Il-1beta) in the amygdala, which could potentially mediate or exacerbate effects of Gadd45b knockdown on the organization of social behavior. These data suggest a prominent role for Gadd45b in the epigenetic programming of complex juvenile social interactions, and may provide insight into the etiology of juvenile behavioral disorders such as ADHD, autism, and/or schizophrenia.

INTRODUCTION

While there is a growing body of literature investigating mechanisms involved in DNA methylation in the brain, less is known about the molecules involved in DNA demethylation. The growth arrest and DNA damage inducible factor 45 (Gadd45) family appears to be involved in active demethylation of the epigenome (Niehrs and Schäfer, 2012), and, in particular, Gadd45b appears necessary for rapid DNA demethylation in response to neuronal signaling (Ma et al., 2009). Interestingly, in patients with major psychosis, Gadd45b is overexpressed in regions of the cerebral cortex receiving projections from the amygdala (Gavin et al., 2011). Furthermore, aberrant epigenetic marks are found in the post-mortem neural tissue of adult psychiatric patients (Grayson et al., 2005; Nagarajan et al., 2006; McGowan et al., 2009; Gavin et al., 2011; Dong et al., 2012). Given the Gadd45 family's involvement in demethylation (Niehrs and Schäfer, 2012) and localization to acetylated histones (Carrier et al., 1994), there is strong support for a role in the precise temporal and spatial regulation of DNA- and histone-modifying enzyme activity, which may in turn be critical for neurotypical development.

Gadd45b transcription occurs in response to environmental stressors (Takekawa and Saito, 1998), immune signaling (Liu et al., 2013), neuronal activity (Ma et al., 2009), and both hippocampal and amygdalar learning tasks (Keeley et al., 2006). While the relative importance of Gadd45b expression in the developmental programming of behavior remains unclear, two recent studies using adult Gadd45b knock-out mice demonstrated a role for Gadd45b in both hippocampal and striatal memory and learning tasks (Leach et al., 2012; Sultan et al., 2012). However, to our knowledge no studies have addressed a role for Gadd45b in the formation of social behaviors. Given the induction of Gadd45b in response to amygdala-related learning tasks

(Keeley et al., 2006), we chose to investigate this brain region during development. We were specifically interested in the effect of Gadd45b on juvenile social behavior, a critical component of which is juvenile social play, that is thought to be both highly rewarding and a salient indicator of typical social development (Panksepp et al., 1984).

We have previously reported the sensitivity of this behavior to neonatal perturbations in epigenetic programming; specifically, a transient disruption to Mecp2 expression in the developing amygdala decreases juvenile social play behavior (Kurian et al., 2008). Though the role of Mecp2 in regulating gene expression is complex (Yasui et al., 2007; Chahrour et al., 2008), it is generally associated with gene repression. This raises the intriguing possibility that some genes must be repressed (e.g., those targeted by MeCP2) to activate play behavior; conversely, some genes may need to undergo transcriptional activation (e.g., through Gadd45b-assisted DNA demethylation) to inhibit play in order to achieve a species-typical level of the behavior. This led us to hypothesize that Gadd45b expression may be required to prevent an over-active play drive. We thus examined the consequence of siRNA-mediated, amygdalar Gadd45b knockdown on juvenile social development by assessing its impact on juvenile social play, sociability, and anxiety-like behavior.

We furthermore were interested in local expression of cytokines, as peripheral levels of Gadd45b are reported to regulate their availability (Lu et al., 2004). In particular, Gadd45b knockout mice produced less Il-6, a cytokine whose role in the central nervous system (CNS) may be to promote and stabilize excitatory synapse formation (Wei et al., 2012). To the best of our knowledge, Gadd45b has not been studied in the brain with respect to its potential effects on

proinflammatory cytokine expression, despite a known role for these molecules in suppressing social behavior (Weil et al., 2006; Hennessy et al., 2014). We also examined genes known to influence play behavior, e.g., MeCP2, Adra2a (Kurian et al., 2008; Vanderschuren et al., 2008), and investigated a variety of genes previously associated with Gadd45b's activity as an epigenetic regulator. This included BDNF, Reelin, and glutamate decarboxylase-67 (Gad67) (Ma et al., 2009; Matrisciano et al., 2011).

MATERIALS AND METHODS

Animals and weaning environment: Untimed-pregnant Sprague Dawley female rats (~15d pregnant) were purchased from Charles River Laboratories (Wilmington, MA) and allowed to deliver normally. Cages were checked regularly to determine the day of birth (P0). On P0, litters were culled to 10 and were composed of 4 untreated females, 3 control siRNA males, and 3 Gadd45b siRNA males to maintain typical litter size and sex composition. Stimulus animals of the same strain that were uninfused, used for the sociability task, were also weaned into mixed sex litters. Animals raised to the juvenile period for behavioral testing were left with dams undisturbed following siRNA treatment until weaning. Juveniles were weaned at P21 into cages of 5 with mixed gender and treatment groups. Animals were housed under standard laboratory conditions (light/dark cycle of 12/12 h, food and water ad libitum). Standard 1200 cm^2 cages were used throughout the duration of the experiment. All procedures were approved by the University of Wisconsin– Madison Animal Care and Use Committee.

Gadd45b siRNA treatment: Gadd45b siRNA (Santa Cruz Biotechnology; catalog #sc-270211) and non-targeting control siRNA (Santa Cruz Biotechnology; catalog #sc-37007) were

resuspended to 100 µM in ribonuclease-free water and Oligofectamine reagent (Invitrogen; catalog #12252-011) at a ratio of 2:1. Infusions directly into the neonatal rat amygdala were performed using a modified stereotaxic device (David Kopf Instruments; model #003445R), and coordinates from center and bregma suture lines were 1 mm lateral, 2 mm caudal, and 5.5 mm ventral as done previously in our lab (McCarthy et al., 2000; Kurian et al., 2008; Jessen et al., 2010; Forbes-Lorman et al., 2012; 2014). After cold anesthetization, rats were bilaterally infused with 1 µl (100 nmol) of either Gadd45b or control siRNA and allowed to recover under a warm lamp for approximately 20 min before being returned to the dam. Using this technique, we have reported that infusion of a dopamine D1 receptor agonist induces c-Fos expression localized to the injection site in the neonatal amygdala (Forbes-Lorman et al., 2012). In the current study, a separate set of 30 animals spread across P0–P2 was used for bilateral ink infusions to ensure targeting accuracy of the technique; the right and left amygdalae were successfully hit in all animals, as confirmed with rapid decapitation and microdissection.

To confirm knockdown in the first experiment, 12 animals (6 Gadd45b siRNA, 6 control siRNA) received two infusions 24 h apart from P0–P1 and were sacrificed 24 h later on P2. For behavioral analysis in the second experiment, 20 animals (9 Gadd45b siRNA, 11 control siRNA) received three infusions 24 h apart from P0–P2 and were then left to develop undisturbed with the dam until weaning. Animals were sacrificed at P33 after behavior testing.

Behavior Testing: All behavioral tests were performed under dim red light and began 1–2 h after the dark phase of the light cycle began. Each animal was tested in all paradigms but was not repeat-tested in any task. Each behavior was video recorded with the exception of the open field

test and the light/dark chamber, which were scored in real time. Videos were analyzed and scored using The Observer software (Noldus Information Technologies) by a trained observer who was blind to all treatments.

Juvenile social play: The social play behavior paradigm was adapted from previously reported methods (Meaney and McEwen, 1986; Olesen et al., 2005). Animals were weaned on P21 and housed with littermates in groups of five, containing animals from each treatment condition (male control siRNA, male Gadd45b siRNA, and uninfused females to maintain normal sex ratios per litter). Animals were tail- and back-marked with a Sharpie and video recorded in their home cages twice per day (1 and 3 h after lights off) for 5 min trials over 5 d (P25–P29) for a total observation time of 50 min per animal. Play behavior was scored using the following criteria: (1) biting: one rat bites another; (2) chasing: one rat chases another; (3) pouncing: one rat pounces or lunges at another; (4) pinning: one rat stands over another, with its forepaws on the ventral surface of the opposing rat; and (5) boxing: both rats stand on hind legs and engage each other with forepaws.

Juvenile sociability: The sociability task was adapted from previous methods (Yang et al., 2001) using a three-chambered apparatus with removable dividers. On P30/31, test animals were placed in the chamber and allowed to freely explore for a 5 min acclimation period. The animal was then confined to the middle chamber and social and nonsocial stimuli were placed in opposing outside chambers. The dividers were removed and the test animal was allowed to explore the entire apparatus for 10 min. The social stimulus was an age- and sex-matched novel juvenile rat of the same strain that had not received infusions, held in a perforated plastic container (7 x 7 x

16 cm) that permitted sight, sound, scent, and minimal tactile contact. The nonsocial stimulus was an empty, identically perforated plastic container. Location in any chamber was scored as having all 4 paws inside the chamber.

Juvenile anxiety-like behavior: Under red light, 2 h after the dark phase of the light cycle began, animals were tested on three separate 5 min tasks – the open field arena, the elevated plus maze, and the light/dark box. Each animal (aged 31–32 d) was tested in this order, with 5 min inter-test intervals. Lux meter analysis revealed that there were approximately 15 (dim), 125 (low/moderate), and 7000 (bright/aversive) lumens for the EPM, open field, and light dark box, respectively.

Open field test: Rats were placed into a corner of an 80 cm square arena with opaque walls 40 cm high and allowed to move freely for 5 min. The arena floor had markings that divided it into 25 equal square (16 x 16 cm) divisions. A light was shone onto the middle of the test while the perimeter remained dark. The rat's movement in the arena was scored as the number of crossings between squares during the 5 min testing period and perimeter crosses, middle crosses, groomings, defecations, and stretching from one square to another were scored.

Elevated plus maze: The elevated plus maze is a Plexiglas structure standing 50 cm off the floor and consisting of two opposing 100 cm runways that cross at the center. One runway is open, whereas the other is closed with 39-cm-high Plexiglas walls with an opening in the center to allow crosses. Rats were placed in the center of the maze facing an open arm and maze exploration was recorded for 5 min. Parameters quantified were entries into as well as time spent

in the open and closed arms and the center of the maze arms. An entry was counted when all four paws crossed into a certain portion of the maze.

Light/dark box: The light/dark chamber is a large, Plexiglas chamber split by an opaque Plexiglas insert into two compartments: a light side where a white incandescent lamp was shining (35 x 38 x 39 cm), and smaller opaque, dark side (25 x 38 x 39 cm). An opening in the lower corner of the Plexiglas insert (6 x 10 cm) allows the animal to move freely between the light and dark chambers. The animal was placed in the corner of the light side of the box facing the opening and was observed for 5 min. Latency to enter the dark chamber and total time in light were recorded.

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^{TM} Reverse Transcription System (Promega; catalog #A3800). Real-time quantitative polymerase chain reaction (RT-PCR) was conducted using a Stratagene Mx3000PTM 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 was performed to ensure the purity of PCR products. cDNA levels were normalized to a housekeeping gene, Ywhaz, using the ΔC_t method (**Table 1).**

Quantification of DNA methylation: DNA methylation was assessed using an adapted version of the methylation sensitive restriction enzyme (MSRE) assay (Hashimoto et al., 2007; Auger et al., 2011). Briefly, 240 ng of DNA from each rat was divided equally into two tubes and digested with either HpaII (New England Biolabs; catalog #R0171) or HhaI (New England Biolabs; catalog #R0139) 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 either HhaI or HpaII cut sites were contained within the region of interest, but not both. To assess relative amounts of DNA methylation, RT-qPCR was performed as described above.

Statistical analysis: Behavioral data and PCR data were analyzed using either a two-tailed Student's t test or a one-way ANOVA with repeated measures as necessary in the sociability task and EPM (chamber duration as a within-subjects variable) using Prism 5 (GraphPad Software, Inc.). Potential outliers were screened for using the Grubbs test for outliers (http://graphpad.com/quickcalcs/ Grubbs1.cfm). All reported measures are listed as mean ± SEM. Significance was defined as a p value of <0.05.

RESULTS:

Confirmation of Gadd45b siRNA targeting and knockdown

To confirm specificity of Gadd45b siRNA in the amygdala, animals receiving two rounds of infusions 24hrs apart and sacrificed at PN2 were examined for Gadd45b expression, as well as for the expression of Gadd45 family members Gadd45a and Gadd45g. Gadd45b siRNA-infused males showed an approximate 20% reduction in Gadd45b mRNA levels (Fig. 1). In an adjacent

brain region, the hypothalamus, no knockdown was observed, suggesting the amygdala was successfully targeted and diffusion of siRNA to surrounding areas did not occur. Additionally, no significant knockdown of either Gadd45a or Gadd45g mRNA levels was observed, suggesting specificity of Gadd45b siRNA in the amygdala.

Neonatal Gadd45b siRNA increases frequency of juvenile social play behavior

Normal levels of neonatal Gadd45b mRNA in the amygdala appear to be important for the organization of juvenile social play, as a transient disruption in its expression altered this behavior later in development. Animals given siRNA treatment from PN0-PN2 and allowed to develop to the juvenile period were recorded in the home cage with mixed group littermates two times daily from PN25-PN29. Measures are reported as the average frequency across all observations. One control male outlier was removed from the analysis. T-test analysis revealed that Gadd45b siRNA treatment more than doubled (~2.5x) levels of juvenile social play (Fig. 2A). Individual components of play including boxing, chasing, and pouncing were also increased in Gadd45b siRNA treated animals (Fig. 2B-D), though frequency of pinning was not significantly different between treatment groups (Fig. 2E). Biting was infrequently observed and no statistically significant differences were found (data not shown).

Gadd45b siRNA treatment alters juvenile sociability

As seen in Figure 3, control males spent significantly more time in the social chamber of a 3-chambered sociability apparatus during a 10min test when compared to Gadd45b siRNA males (control: 62.31 ± 5.85%, N=9 vs. Gadd45b: 39.25 ± 9.01%, N=7; p=0.0423). No significant

differences in the percent time spent in the non-social (control: 26.42 ± 4.41%, N=9 vs. Gadd45b: 36.64 ± 9.45%; p=0.308) or middle (control: 11.26 ± 2.10%, N=9 vs. Gadd45b: 11.45 ± 2.03%, N=7; p=0.952) chambers were observed. There were no differences in the number of exits and entries into the social chamber (control: 25.8 ± 1.33, N=8 vs. Gadd45b: 23.9 ± 1.64, N=6; *p*=0.380) indicating that overall locomotion wasn't affected in this task.

Gadd45b siRNA treatment does not alter juvenile anxiety-like behavior

Gadd45b siRNA infusion had no significant effects on anxiety, as tested by the light/dark chamber, open field maze, and the elevated plus maze (EPM) (Fig. 4). Student's t-tests for percent time spent in the closed arm (control: 72.2 ± 6.09%, N=8; Gadd45b: 67.5 ± 7.94%, N=7. p=0.640), open arm (control: 12.1 ± 3.39%, N=8; Gadd45b: 13.9 ± 4.26%, N=7. p=0.742), and the middle (control: 15.7 ± 4.30%, N=8; Gadd45b: 18.6 ± 4.12%, N=7. p=0.636) reveal no significant differences.

siRNA knockdown of Gadd45b expression is transient

Juvenile amygdala samples were analyzed for Gadd45b mRNA levels to examine whether siRNA treatment was transient or lasting (Fig. 5). As expected, Gadd45b mRNA levels show no significant differences between control and Gadd45b siRNA treated groups in the amygdala on P33. Gadd45a mRNA levels were also unchanged, but intriguingly Gadd45g expression was significantly reduced by 33% at this later time point. Gadd45b mRNA expression was not altered in the juvenile hypothalamus.

Analysis of gene expression in neonatal and juvenile samples

At P2, Reelin mRNA levels showed a marked, 75% decrease compared to controls (control: 0.422 ± 0.162, N=6; Gadd45b: 0.106 ± 0.0219, N=6. p=0.041), while there was no change in Gad67 (control: 0.304 ± 0.00529, N=4; Gadd45b: 0.290 ± 0.00524, N=5. p=0.107). MeCP2 mRNA levels were reduced ~30% (control: 0.432 ± 0.0366, N=6; Gadd45b: 0.307 ± 0.0373, N=6. p=0.0384). BDNF and Adra2a mRNA levels were unchanged (control: 0.350 ± 0.0161, N=5; Gadd45b: 0.388 ± 0.0422, N=5. p=0.414; control: 0.212 ± 0.0184, N=6; Gadd45b: 0.250 ± 0.0474, N=6. p=0.472, respectively). Bcl2 mRNA was unchanged (control: 0.407 ± 0.0295, N=5; Gadd45b: 0.385 ± 0.0601, N=6. p=0.769), and GFAP levels were not significantly different (control: 0.152 ± 0.0658, N=6; Gadd45b: 0.279 ± 0.102, N=5. p=0.306) (Fig. 6A). In the juvenile period, we found that the Reelin effect was transient (control: 0.232 ± 0.0298, N=8; Gadd45b: 0.238 ± 0.0375, N=7. p=0.901) and that again there was no change in Gad67 levels (control: 0.486 ± 0.0475, N=8; Gadd45b: 0.529 ± 0.0380, N=7. p=0.504). The MeCP2 effect was also transient (control: 0.381 ± 0.0879, N=9; Gadd45b: 0.328 ± 0.0856, N=7. p=0.683) Both BDNF and Adra2a (BDNF (control: 0.584 ± 0.0476, N=9; Gadd45b: 0.437 ± 0.0227, N=7. p=0.0239); Adra2a (control: 0.469 ± 0.0460, N=8; Gadd45b: 0.350 ± 0.0227, N=7. p=0.0450)) exhibited a long-term change in gene expression, showing an approximate 25% reduction. There was no change in Bcl2 (control: 0.190 ± 0.0507, N=9; Gadd45b: 0.151 ± 0.0409, N=6. p=0.599) or GFAP (control: 0.215 ± 0.0606, N=9; Gadd45b: 0.180 ± 0.0713, N=7. p=0.706) (Fig. 6B).

Gadd45 family members were originally described in white blood cells of the periphery; we thus examined the expression of several cytokines in the amygdala and observed a transient decrease in pro-inflammatory cytokines Il-6 and Il-1beta but not in transforming growth factor beta (Tgf-beta) at postnatal day 2 (Fig. 7A). See figure for stats.

Gadd45b siRNA epigenetically alters methylation of the Adra2a promoter in the amygdala

We chose to further examine the promoter region of Adra2a, shown in Table 2, using a methylation-sensitive restriction enzyme method. Site 1 primers were designed around a cAMP responsive element-binding protein (CREB) transcription-factor binding site distal to the transcriptional start site (TSS), and Site 2 primers were designed around an early growth response protein 1 (Egr-1) site more proximal to the TSS. In the neonatal period, the Gadd45b siRNA infused animals showed a significant 52% increase in Adra2a promoter methylation at Site 1 (Fig. 8A), with no effect on methylation at Site 2 (Fig. 8B). We found a significant, 51% increase in methylation of the Adra2a promoter in the juvenile amygdala at Site 1 (Fig. 8C), but again no significant change at Site 2 (Fig. 8D).

DISCUSSION

Our findings indicate that a transient knockdown of Gadd45b expression in the neonatal amygdala can cause lasting changes in juvenile social behaviors. Specifically, we report that Gadd45b siRNA treatment results in a dramatic increase in the overall frequency of juvenile social play behavior, recorded twice a day for 5min over the course of five days for a total of 50min of play per animal and reported as an average frequency (Fig. 2A). 'Play' involves a complex suite of behaviors, including pouncing, chasing, boxing and pinning; all of these components were increased in the Gadd45b siRNA-treated animals relative to controls, with the exception of pinning (Fig. 2B-E). This may be indicative of a generally increased drive to engage in play. We have previously reported that MeCP2 knockdown within the developing amygdala results in decreased juvenile social play (Kurian et al., 2008), suggesting that epigenetic processes associated with gene repression are important for programming, and possibly

activating, typical levels of juvenile social play. In the current study, disruption to a molecule involved in DNA demethylation increased play, which may indicate Gadd45b is involved in the formation of inhibitory pathways governing this behavior. Together, these data indicate that the developmental programming of juvenile social play behavior is perhaps under repressive control by epigenetic modifications within the newborn amygdala.

To further examine Gadd45b's role in juvenile social interactions, we used the three-chambered test for sociability to investigate anti-social behavior in rodents (Yang et al., 2001; Moretti et al., 2005). We find that neonatal Gadd45b disruption results in lower levels of juvenile sociability; specifically, Gadd45b siRNA-treated animals spent 37% less time in the social chamber than control siRNA-treated animals (Fig. 3). While these behavioral results may appear contradictory when compared to the play behavior data, an increased drive to play could impede performance in the sociability task. Specifically, by depriving Gadd45b-siRNA treated rats of the ability to engage in play with the stimulus rat, higher impulsivity behavior and thus greater exploration of the non-social chamber may occur. Alternatively, the molecular pathways regulating play and sociability could be regulated differently and therefore be dissociable. The seemingly disparate behavioral phenotype observed with Gadd45b siRNA treatment has been previously observed in spontaneously hypertensive rats (SHRs), which are a commonly used animal model of ADHD. Specifically, SHRs show increased play behavior towards conspecifics (Ferguson and Cada, 2004) and decreased social interaction (Calzavara et al., 2011) when compared to the parental Wistar-Kyoto strain.

The similar behavioral phenotype of Gadd45b-siRNA treated rats and SHRs with regard to play and sociality also appears to be supported by parallel disruptions in Adra2a. That is, SHRs show reduced levels of the norepinephrine-binding autoreceptor Adra2a in the brain (Olmos et al., 1991), and Gadd45b siRNA treatment also results in significantly decreased expression of Adra2a in the juvenile amygdala (Fig. 6B). The reduced levels of Adra2a may be a result of reduced Gadd45b expression allowing for increased methylation near a CREB-binding site in the promoter (Fig. 8A,C). The effect of Gadd45b siRNA on Adra2a promoter methylation at Site 1 was observed during the neonatal period and lasts into the juvenile period; however, the change in mRNA did not occur until P33. This suggests that the organization of Adra2a expression occurs early in development and that epigenetic programming of CpG sites near the CREB-binding site may be important for driving Adra2a expression later in life. That is, while reducing Gadd45b resulted in increased methylation of the Adra2a promoter, it did not result in a functional consequence on Adra2a mRNA levels until the juvenile period when social play emerges. Importantly, previous data have implicated Adra2a as an important molecule in regulating juvenile social play behavior. Pharmacological Adra2a antagonism increases juvenile social play in rats (Siviy and Baliko, 2000). Furthermore, pretreatment with an Adra2a antagonist can significantly reverse deficits in play behavior incurred by the administration of either amphetamine or methylphenidate (Vanderschuren et al., 2008). These data suggest that Adra2a acts to suppress juvenile social play. The directionality of Gadd45b regulated Adra2a expression is consistent with its suppressive role on juvenile social play behavior; thus the Gadd45b-mediated programming of the Adra2a promoter observed in this study may provide some mechanistic insight into a potent regulator of complex social behavior.

We furthermore report a 30% decrease in Mecp2 mRNA expression in the neonatal amygdala that was no longer detected at P33 (Fig. 6). A behavioral study using Mecp2$^{308/Y}$ mice, in which the Mecp2 protein is truncated, revealed significantly decreased social interactions with an unfamiliar stimulus animal in the three-chambered sociability task, whereas social behavior towards familiar animals did not differ from wild type controls (Moretti et al., 2005), which appears to be consistent with our current behavioral data set. However, a previous study from our lab in which we targeted the amygdala with Mecp2 siRNA resulted in decreased juvenile social play behavior (Kurian et al., 2008). It is possible that the decrease in Adra2a expression due to Gadd45b siRNA treatment outcompetes the deficits in social play behavior induced by Mecp2 knockdown, as Adra2a antagonists profoundly affect the frequency of juvenile social play (Vanderschuren et al., 2008). It is also possible that genetic manipulation of the Mecp2 protein and the effect of Gadd45b siRNA alter as yet unidentified binding partners of Mecp2 that would change its effect on gene transcription (Yasui et al., 2007; Chahrour et al., 2008). Nevertheless, Mecp2 deficiency is implicated in both Rett syndrome and autism spectrum disorders (ASDs), both of which are characterized by abnormal social interactions. Future work is necessary to determine if pharmacological α_2-adrenoceptor manipulation can overcome some of the social deficits observed in Mecp2 deficient rodent models.

It is important to note that there were no alterations in anxiety-like behavior (Fig. 4), suggesting that the behavioral disruptions were not universal, but may be limited to juvenile social interactions. Furthermore, genes involved in cell death or cellular differentiation were unaltered by Gadd45b siRNA treatment within the amygdala. Specifically, expression of either the anti-apoptotic protein Bcl2 (Garcia et al., 1992) or GFAP— an intermediate filament found in glia,

astrocytes and neural stem cells (Imura et al., 2003), which is typically altered in response to damage (Bignami and Dahl, 1974)—was not different between treatment groups at either time point (Fig. 6), suggesting that we have not globally altered cell death or response to damage within the amygdala. Finally, lesioning the amygdala decreases social play behavior (Meaney et al., 1981), whereas Gadd45b siRNA resulted in the opposite effect. Combined, these data indicate that it is unlikely that Gadd45b siRNA infusions resulted in global amygdala disruption.

To further characterize the molecular effects of Gadd45b knockdown, we examined the mRNA levels of several candidate genes linked to Gadd45b expression. A recent study using Gadd45b ChIP revealed binding to the promoter regions of Reelin, Gad67, and BDNF in the hippocampus (Matrisciano et al., 2011)—genes previously implicated in the etiology of psychiatric disorders. Interestingly, expression of these genes was differentially affected by Gadd45b siRNA between the neonatal and juvenile amygdala data sets. While Gad67 failed to reach significance at either time point, there was a dramatic 75% neonatal reduction in Reelin expression that was no longer present at P33. Conversely, the expression of BDNF was not different from controls at P2, but was significantly decreased during the juvenile period (Fig. 6). The temporally specific effect of Gadd45b manipulation warrants future study, and may depend on the availability of as yet unidentified co-factors to localize it to each promoter region.

Reduced expression of BDNF was suggested as a mechanism to explain GABAergic interneurons' vulnerability to environmental stressors in the amygdala (Guilloux et al., 2011), though the ontology of this reduction in BDNF remains elusive. Our data suggests that Gadd45b-mediated expression of Reelin early in development may belie this change; Reelin, a large

glycoprotein preferentially excreted from GABAergic neurons (Pesold et al., 1998), colocalizes with integrins (Rodriguez et al., 2000) and is thought to stabilize dendritic spines and connections with interneurons (Guidotti et al., 2000). The large reduction in the expression of Reelin seen in our experiment could result in abnormal neurite outgrowth and synapse stabilization of GABAergic interneurons from or within the amygdala. Furthermore, while little is known about Gadd45g, it was previously demonstrated that BDNF expression in the ventral tegmental area (VTA) is necessary to maintain typical Gadd45g levels in the nucleus accumbens (Koo et al., 2012). Thus, a cascade of gene expression changes beginning with early, perinatal programming of synapse-stabilizing genes like Reelin by Gadd45b may have lasting consequences on the integrity of GABAergic interneurons, which could in turn, through changes in BDNF expression, influence the expression profile of their postsynaptic targets, as with the reduced levels of Gadd45g seen in this study (Figure 5). Consequently, Gadd45b manipulation may disproportionally affect the formation of GABAergic signaling pathways in and from the amygdala to other brain regions, which may underlie the behavioral disinhibition of social play observed here.

Finally, we investigated the expression of several pro-inflammatory cytokines, as Gadd45b has been studied extensively with respect to innate immunity and cytokine production in the periphery (for excellent reviews see (Hoffman and Liebermann, 2007; Schmitz, 2013)). Importantly, a functional role for Gadd45b in the production of Il-6 was demonstrated in activated white blood cells of a Gadd45b knockout mouse (Lu et al., 2004). To our knowledge, no study has examined whether Gadd45b alters cytokine production in the brain, despite both a neuroprotective role for neuronally-expressed Il-6 (Gadient and Otten, 1997) and emerging data

to suggest proinflammatory cytokines—including Il-6 and Il-1beta—can affect social behavior (Weil et al., 2006; Hennessy et al., 2014). We observed a 40% reduction in the expression of Il-6 in the neonatal amygdala of our Gadd45b siRNA animals (Fig. 7A). We also observed a 45% reduction in the expression of a potent inducer of Il-6 expression, Il-1beta (Ringheim et al., 1995). Tgf-beta, previously shown to induce expression of Gadd45b in retinal ganglion cells (Liu et al., 2013), is linked to Gadd45b signaling in the dentate gyrus (Haditsch et al., 2013), and may be upstream of Gadd45b signaling whereas Il-6 and Il-1beta may be downstream effectors. Consistent with this idea, we observed no change in Tgf-beta expression at either time point (Fig. 7).

At present, we are unable to discern whether reduced expression of Il-6 and Il-1beta is a consequence of less Gadd45b activity, as seen previously in white blood cells, or if the significantly reduced availability of Reelin causes an indirect change through altered synapse formation. It is of interest that Il-6 has been shown to specifically promote the formation and stability of tyrosine hydroxylase (TH)-positive neurons during postnatal rat brain development (Kushima et al., 1992)—TH being the rate-limiting step in production of catecholamines, including norepinephrine. Although a social defeat paradigm did establish a role for β-adrenergic receptor signaling in Il-1beta-dependent anxiety behavior (Wohleb et al., 2011), it remains to be determined whether there are links between the availability of Il-6 and expression of Adra2a, which could help integrate the signaling pathways altered in our current data set.

While the functional consequences of cytokine regulation in programming juvenile social interactions is unclear, reduced levels of cytokines, including Il-6 and Il-1beta, are associated

with aggressive behavior in adult humans (Provençal et al., 2013) and mice (Alleva et al., 1998). Juvenile rough and tumble play behavior as described herein is not thought to be an aggressive behavior—rather, it is reported to be a joyful and rewarding activity (Burgdorf et al., 2008). Generally speaking, the aggressive behaviors linked to cytokine levels may be ascribed to alterations in sociability, consistent with previous observations (Weil et al., 2006; Hennessy et al., 2014); mechanisms to understand these links are of considerable interest, as altered levels of cytokines have been linked to a variety of environmental stressors and neuropsychiatric disorders (for review, see (Bilbo and Schwarz, 2012))

Herein we have demonstrated a novel role for Gadd45b-mediated programming of social behaviors, altered epigenetic programming of Adra2a, and raised the intriguing idea of reduced cytokine expression as a mediating pathway for altered juvenile social development. These effects are rather complex in that they increase juvenile social play but disrupt sociability; however, the behavioral and molecular changes seen here appear to be consistent with social alterations found in several animal models of human neurodevelopmental disorders. Interestingly, Gadd45b knockdown resulted in both changes specific to social behaviors and to an epigenetically programmed decrease in the expression of Adra2a. It will be important for future work to determine if pharmacologically targeting this receptor would be therapeutic in the treatment of neurodevelopmental disorders presenting with social deficits. A parsimonious explanation of the data presented here is that increased gene expression in the amygdala is required to inhibit certain aspects of juvenile social play behavior; reducing the cell's ability to demethylate genes, such as Adra2a, may disinhibit the system and cause atypical levels of play due to abnormal epigenetic programming.

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Primer	Accession	Forward	Reverse
Ywhaz	NM_013011	TTGAGCAGAAGACGGAAGGT	GAAGCATTGGGGATCAAGAA
Gadd45b	NM_001008321	GCTGGCCATAGACGAAGAAG	GCCTGATACCCTGACGATGT
Gadd45a	NM_024127	GCTACTGGAGAACGACAAGAG	CCATTGTGATGAATGTGGGTTC
Gadd45g	NM_001077640	CTGAATGTGGACCCTGACAAT	AACGCCTGGATCAACGTAAA
Reelin	NM_080394	AGAGGACAATGCACTCGACATGGT	AAGCTGACTTCAGCACCACGGATA
Gad67	M76177.1	CTGCCATCCTGGTCAAGGAA	GAATCGCCTTGTCCCCTGTA
MeCP2	NM_022673	ACAGACTCACCAGTTCCTGCTTTG	ATGGAATCCTGTTGGAGCTGGTCT
BDNF	AY176065.1	GCCCATGAAAGAAGCAAACGTCGA	TTTCTTCGTTGGGCCGAACCTTCT
Adra2a	NM_012739	CTCGCTGAACCCTGTTGTCTA	TCACACGATGCGCTTTCT
Bcl2	L14680	GTGGATGACTGAGTACCTGAAC	GAGACAGCCAGGAGAAATCAA
Gfap	NM_017009.1	GGTGTGGAGTGCCTTCGTAT	TACGATGTCCTGGGAAAAGG
Il-6	NM_012589	ATATGTTCTCAGGGAGATCTTGGAA	GTGCATCATCGCTGTTCATACA
Il-1beta	NM_031512	CCCTGCAGCTGGAGAGTGTGG	TGTGCTCTGCTTGAGAGGTGCT
Tgf-beta	NM_021578	ACCAACTACTGCTTCAGCTCCACA	TGTACTGTGTGTCCAGGCTCCAAA

Table 1: mRNA Primer sequences and Pubmed accession numbers for RT-qPCR

Rat Adrenoceptor α_{2A}

accession number: U21241

186gttcccgcttaccccacccccgagctcagtccaaaactgcccccaagacaggaaaaaaaaaaaaaggggaggagagg
gttccagaggcacctggagcccttaagtcgct**ttccagccgtgacgaccagcg**gccagcaggtgtcagtg**CG[1]**ctctcgttctg
tcacagctcagggcctgcccc**366…1686**accagctggccccactcgatcctgccgcttagaaataaaacttgactgtgtttgt
ggagctgggagagag**aaagcacccaccCG[2]gag**catcggaaagacgaac**CG[2]**gtgccagttcgcgggctcc**1809**

Methylation Site	Forward primer 5' to 3'	Reverse primer 5' to 3'	MSRE	Cut Site
Site 1	CCCGAGCTCAGTCCAAAACT	AGCTGTGACAGAACGAGAGC	HhaI	GCG^C
Site 2	CGATCCTGCCGCTTAGAAAT	GGTTCGTCTTTCCGATGCT	HpaII	C^CGG

Table 2: Methodological details for methylation sensitive restriction enzyme (MSRE) analysis of CpG sites within the rat Adra2a promoter. Primer annealing sequences used for examining methylation at sites 1 and 2 are underlined. Each methylation site examined is bolded and numbered. MatInspector predicted transcription factor binding sites are indicated (CREB site shown orange, Egr-1 is shown in blue). Primers used to investigate the methylation status at two distinct promoter sites are indicated. Cut sites for each MSRE are indicated by an arrow. Methylation at these CpG sites protects against enzyme digestion and allows for PCR amplification.

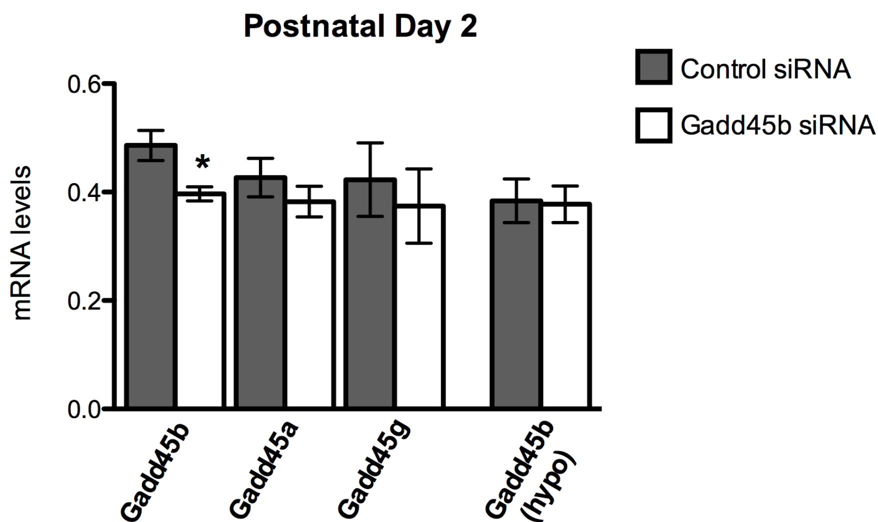

Figure 1*:* Gadd45b siRNA specificity in the amygdala. Two days of Gadd45b siRNA gives approximately 20% knockdown of mRNA (control: 0.4861 ± 0.0276, N=5; Gadd45b: 0.3967 ± 0.0130, N=5); p=0.0191. Gadd45b siRNA does not significantly decrease mRNA levels of closely related family members Gadd45a (control: 0.4267 ± 0.03536, N=6; Gadd45b: 0.3825 ± 0.02830, N=6; p=0.3525) or Gadd45g (control: 0.423 ± 0.06764, N=6, Gadd45b: 0.374 ± 0.06849, N=6; p=0.6229). Gadd45b siRNA does not spread to the adjacent hypothalamus (control: 0.3838 ± 0.04025, N=4; Gadd45b: 0.3775 ± 0.03378, N=6; p=0.9079).

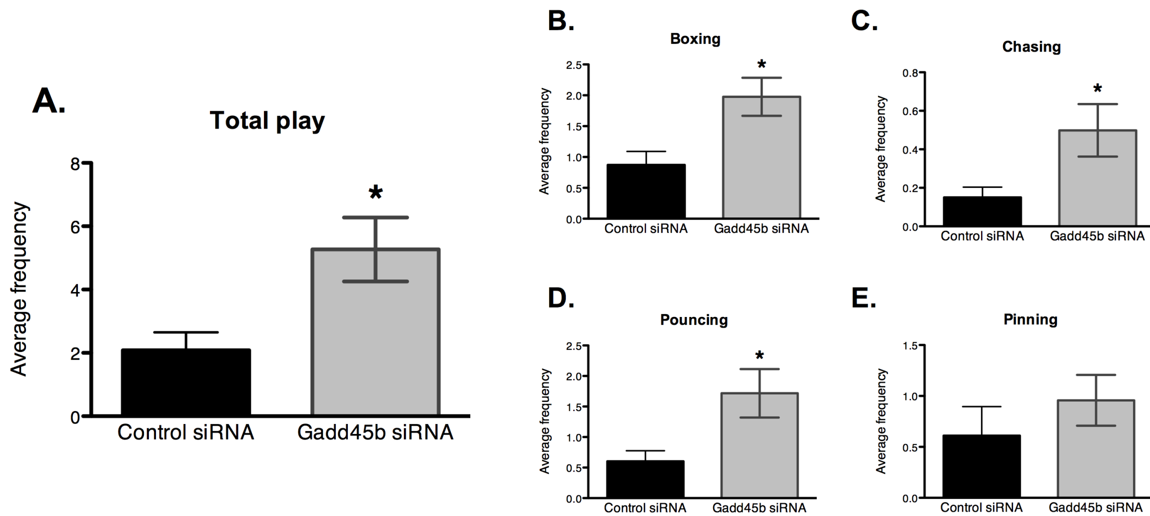

Figure 2*:* Neonatal Gadd45b siRNA infusion increases the initiation of play fighting. ***A***. Transient neonatal disruption of Gadd45b expression in the amygdala more than doubles the frequency of juvenile social play (control siRNA males: 2.064 ± 0.5007, N=9. Gadd45b siRNA males: 4.664 ± 1.060, N=8. *p*=0.0361.) ***B***. Average frequency of boxing increased 2.27-fold in Gadd45b siRNA treated males (1.976 ± 0.3082, N=7) compared to control siRNA treated males (0.8708 ± 0.2199, N=8); *p*=0.0107. ***C.*** Chasing increased 3.32-fold in Gadd45b siRNA treated males (0.4984 ± 0.1364, N=7) compared to controls (0.1500 ± 0.05345, N=8); *p*=0.0265. ***D.*** Pouncing increased 2.85-fold in Gadd45b siRNA males (1.717 ± 0.3971, N=7) relative to control males (0.6028 ± 0.1723, N=8); *p*=0.0183. ***E.*** Pinning was not statistically different between Gadd45b siRNA males (0.9571 ± 0.2494, N=7) and control males (0.6111 ± 0.2840, N=9); *p*=0.3909.

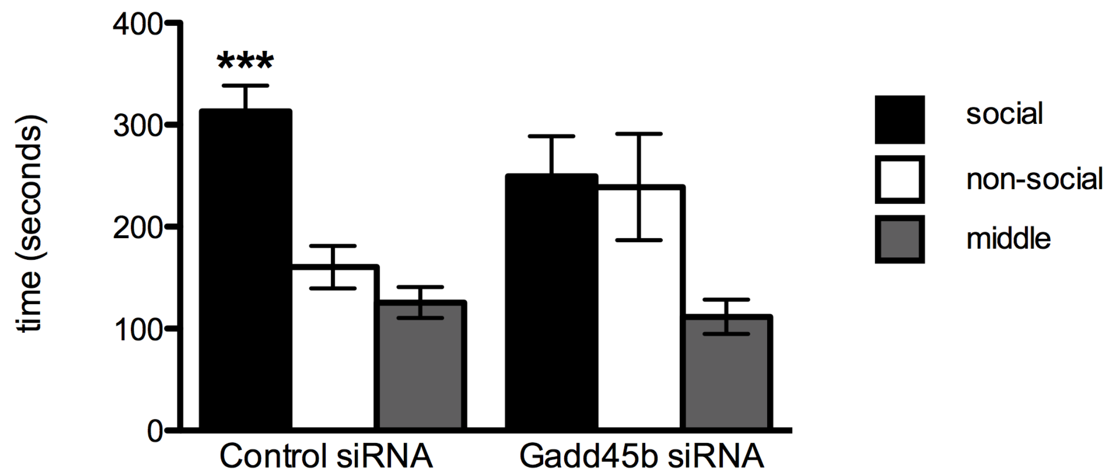

Figure 3*:* Gadd45b siRNA infused males exhibit abnormal social behavior, with no preference for the social chamber. Repeated measures ANOVA analysis reveals a strong preference for the social chamber in control siRNA males (F=15.23, $p < 0.0005$), which Gadd45b siRNA males appear to lack (F=2.599, p=0.1233).

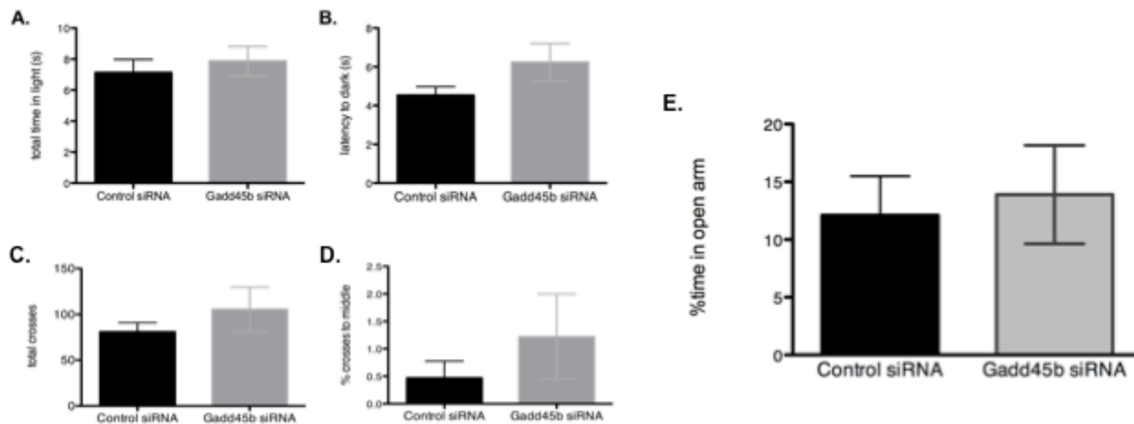

Figure 4*:* No significant changes were seen in anxiety behavior. Light/dark box: ***A.*** total time in light (control: 7.13s ± 0.856, N=8 vs. Gadd45b: 7.87s ± 0.950, N=6; p=0.575), ***B.*** latency to enter the dark chamber (control: 4.53 ± 0.446, N=9 vs. Gadd45b: 6.23 ± 0.972, N=7; p=0.109). Open field task: ***C.*** total crosses (control: 81.1 ± 9.99, N=9 vs. Gadd45b: 105.4 ± 24.2, N=7; p=0.329) or ***D.*** percent crosses from periphery into the middle (control: 0.467 ± 0.306, N=8 vs. Gadd45b: 1.22 ± 0.778, N=6; p=0.339). Elevated plus maze: ***E.*** Percent time spent in the open arm of the elevated plus maze (see text for statistics).

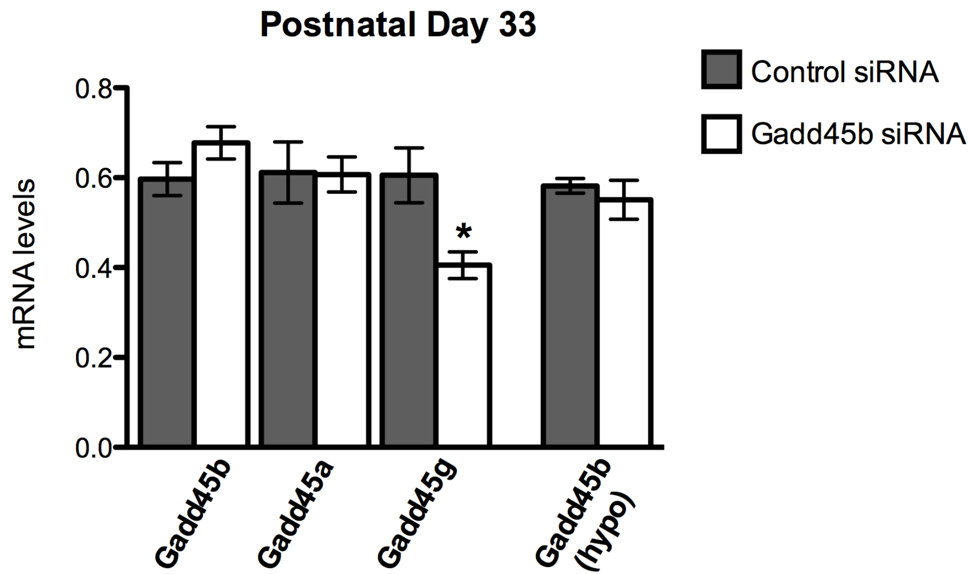

Figure 5: The decrease in Gadd45b expression is transient (control: 0.5970 ± 0.03689, N=8 vs. Gadd45b: 0.6779 ± 0.03609, N=7; *p*=0.1433). Gadd45a expression remains unchanged at the juvenile time point (control: 0.612 ± 0.0677, N=9; Gadd45b: 0.607 ± 0.0390, N=7; *p*=0.958). Gadd45g expression was decreased by 33% (control: 0.605 ± 0.0610, N=9; Gadd45b: 0.406 ± 0.0296, N=6; *p*=0.0255). No change in Gadd45b expression is seen in the hypothalamus at P33 (control: 0.5818 ± 0.01635, N=7; Gadd45b: 0.5507 ± 0.04331, N=7; *p*=0.5151).

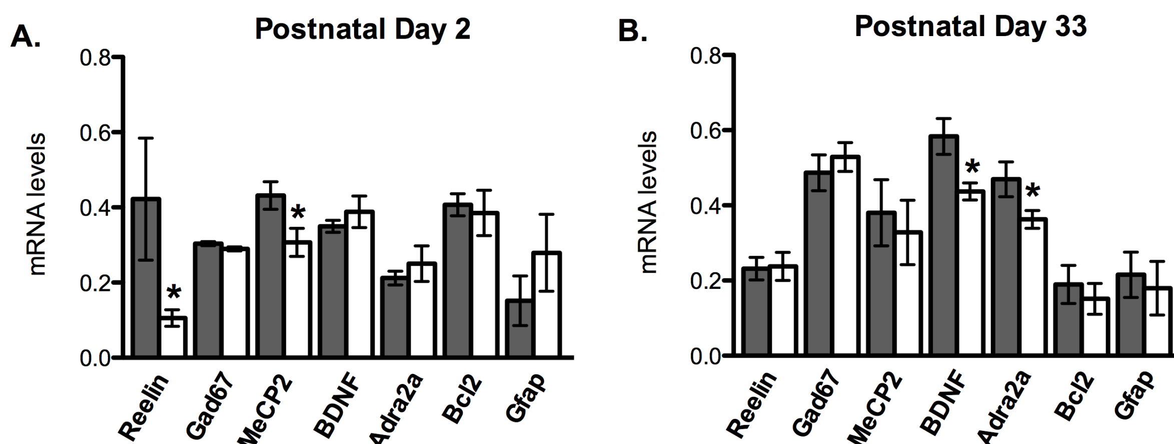

Figure 6*:* Gadd45b siRNA alters the expression of several genes implicated in psychiatric disorders differentially at ***A.*** P2 and ***B.*** P33. Reelin exhibited a transient but dramatic 75% reduction in expression of mRNA at P2. We saw no change in expression of Gad67 at either time point. Levels of BDNF were unchanged at P2; however, MeCP2 was decreased by approximately 30% neonatally. This effect was transient, as levels of MeCP2 had returned to control levels in the juvenile samples. Though no difference was seen at P2, BDNF mRNA was decreased at P33 by 25%. Additionally, Adra2a showed a 25% decrease in expression at the juvenile time point, but not at the neonatal time point, suggesting a lasting organizational effect of Gadd45b siRNA on gene expression. B-cell CLL/Lymphoma 2 (Bcl2) was not significantly different at either time point. Furthermore, expression of glial fibrillary acidic protein (GFAP) was highly variable at the neonatal time point but not significantly different in either sample set. Control siRNA shown in dark gray; Gadd45b siRNA shown in white.

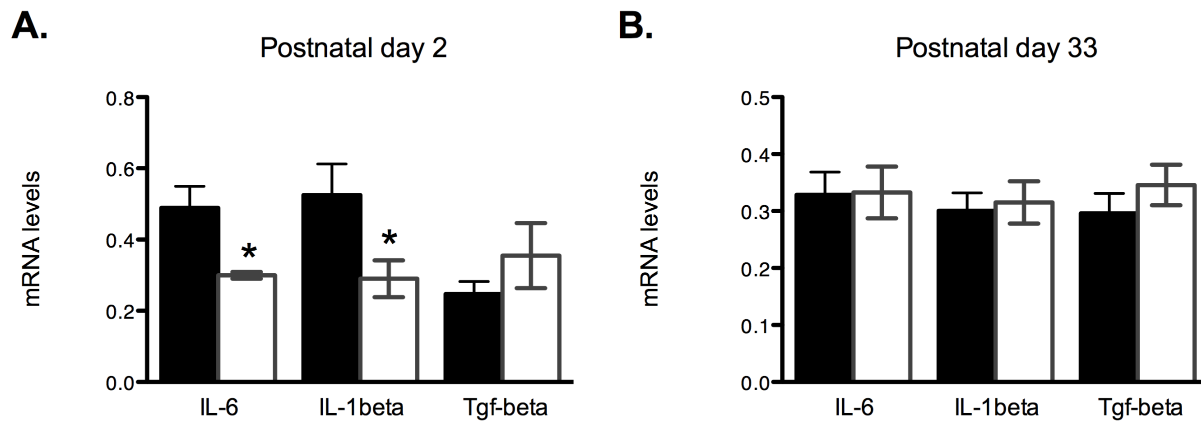

Figure 7: ***A.*** Pro-inflammatory cytokines Il-6 (control: 0.489 ± 0.135, N=5; Gadd45b: 0.300 ± 0.0184, N=4. *p*=0.029) and Il-1beta (control: 0.525 ± 0.213, N=6; Gadd45b: 0.290 ± 0.0127, N=6. *p*=0.043), but not Tgf-beta (control: 0.247 ± 0.0353, N=6; Gadd45b: 0.355 ± 0.0914, N=6. *p*=0.294) show less expression at PN2. ***B.*** This effect was transient; no changes in these cytokines was observed in the juvenile period (Il-6: control: 0.329 ± 0.0398, N=8; Gadd45b: 0.333 ± 0.0455, N=7. *p*=0.947. Il-1beta: control: 0.300 ± 0.0314, N=9; Gadd45b: 0.315 ± 0.0370, N=6. *p*=0.766. Tgf-beta: control: 0.296 ± 0.0348, N=8; Gadd45b: 0.346 ± 0.0355, N=7. *p*=0.337).

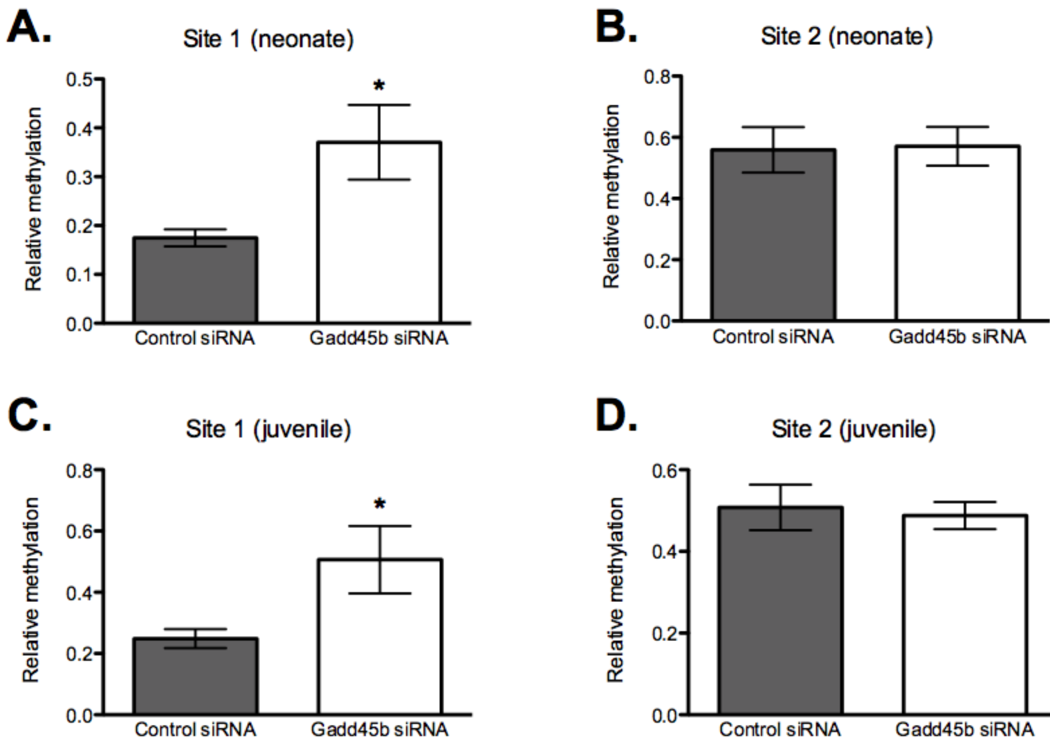

Figure 8*:* Neonatal Gadd45b siRNA treatment epigenetically alters Adra2a promoter DNA methylation near a CREB binding site at P2 (control: 0.175 ± 0.0175, N=5 vs. Gadd45b: 0.371 ± 0.0764, N=6; p=0.0489) and P33 (control: 0.249 ± 0.0310, N=8 vs. Gadd45b: 0.506 ± 0.110, N=7; p=0.0328) (***A,C***) but a downstream Egr-1 site shows no methylation difference at either P2 (control: 0.558 ± 0.0744, N=6; Gadd45b: 0.571 ± 0.0634, N=6; p=0.902) or P33 (control: 0.508 ± 0.0557, N=9; Gadd45b: 0.488 ± 0.0333, N=7; p=0.781) (***B,D***).

CHAPTER III: SEX DIFFERENCES IN THE EXPRESSION OF GADD45B ARE EPIGENETICALLY PROGRAMMED BY STEROID HORMONES IN THE RODENT AMYGDALA

Manuscript submitted to *Brain Research*.

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.

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 (Weaver et al., 2004; Roth and David Sweatt, 2010). Abnormalities in the epigenome acquired during development can cause long-lasting and adverse effects on such far-reaching behavioral and physiological traits as memory (Korosi et al., 2012), parental behavior (Champagne et al., 2006), sexual differentiation (Matsuda et al., 2011; Nugent et al., 2015), drug-seeking behavior (Massart et al., 2015), and are furthermore linked to psychiatric dysfunction (McGowan et al., 2009; Labonté et al., 2013; Bagot et al., 2014). 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 **Chapter I, Table 1**). 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). Our lab has also shown that there are sex differences in the expression of proteins which initiate or amplify an epigenetic signal, e.g. methyl cytosine binding protein 2 (MeCP2) (Kurian et al., 2007) or 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 Auger, 2011), several histone deacetylases (Xu et

al., 2008a; 2008b), and Dnmt1 (Nugent et al., 2015). However, whereas epigenetic modifications were previously thought to be static and/or permanent (for review, see (Wu and Zhang, 2010)), a growing body of evidence suggests that DNA methylation undergoes rapid turnover and is dynamically regulated *in vivo* (Ma et al., 2009; Feng et al., 2010; Auger et al., 2011).

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. Base excision repair enzymes remove the modified cytosine base completely, replacing it with an unmodified cytosine (see **Chapter I**, **Figure 2**). Growth arrest and DNA damage inducible factor (Gadd45) family members are thought to participate in base excision repair through an as yet unknown mechanism (Barreto et al., 2007; Jin et al., 2008) that may involve recruitment of deaminases (e.g. activation-induced deaminase/apolipoprotein B mRNA editing cytokine, or AID/APOBEC) and DNA glycosylases (e.g. thymine DNA glycosylase (TDG) or methyl-binding domain 4 (MBD4); for review, see (Grayson and Guidotti, 2012; Kigar and Auger, 2013)).

Though sex differences exist in the prevalence of psychiatric disorders, to the best of our knowledge there have been no studies examining sex differences in the expression or regulation of epigenetic 'erasers', or proteins involved in the active process of DNA demethylation. Here we attempt to address this knowledge gap by examining the expression of a variety of proteins recognized for their role in the DNA demethylation process.

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 = 10 males, n = 10 females). Brains were collected, and the amygdala and hypothalamus were microdissected with razorblades.

Hormone-treated data set: As published previously (Kolodkin and 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^{TM} 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 was performed to ensure the purity of PCR products. cDNA levels were normalized to a housekeeping gene, Ywhaz, using the $\Delta\Delta C_t$ method. 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 (Hashimoto et al., 2007; Auger et al., 2011). 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 AciI but not ClaI 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 either a two-tailed Student's t test or a one-way ANOVA for the hormone-treated data set using Prism 5 (GraphPad Software, Inc.). Potential outliers were screened for using the Grubbs test for outliers (http://graphpad.com/quickcalcs/ Grubbs1.cfm). All reported measures are listed as mean ± SEM. Significance was defined as a p value of <0.05.

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 P1 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 P1 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**).

We observed no sex differences in any other demethylation factor examined in either the P1 amygdala: [Apobec3 (female: 0.5633 ± 0.08219, N=8; male: 0.4338 ± 0.05019, N=9. p=0.1877); Mbd4 (female: 0.4484 ± 0.05580, N=9; male: 0.5191 ± 0.05724, N=9. p=0.3899); Tdg (female: 0.5510 ± 0.07345, N=9; male: 0.4452 ± 0.04830, N=10. p=0.4558); Tet1 (female: 0.5086 ± 0.02352, N=9; male: 0.5218 ± 0.03909, N=9. p=0.7752)] (**Fig. 2A**) or P1 hypothalamus: [Apobec3 (female: 0.6378 ± 0.02593, N=9; male: 0.6406 ± 0.04489, N=11. p=0.9594); Mbd4 (female: 0.5928 ± 0.03719, N=10; male: 0.5489 ± 0.02149, N=10. p=0.3204); Tdg (female: 0.5999 ± 0.02455, N=9; male: 0.6090 ± 0.0292, N=11. p=0.8204); Tet1 (female: 0.6195 ± 0.03842, N=9; male: 0.6023 ± 0.04404, N=11. p=0.7778)] (**Fig. 2B**).

Effect of hormone treatment on Gadd45 expression in females

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. 3A**). 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. 3B-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. 4A**).

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. 4B**).

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. 5**).

DISCUSSION:

Herein we present data describing neurodevelopmental sex differences in the mRNA expression of proteins involved in DNA demethylation. Of the proteins we investigated in this study, only Gadd45b exhibited hormone responsivity, and only in the amygdala. Specifically, females expressed greater levels of Gadd45b mRNA neonatally (**Fig. 1A**) and this effect was also present during the juvenile period (**Fig. 4A**).). Interestingly, the reduced expression observed in males corresponded to increased methylation at an ERE site within the Gadd45b promoter Furthermore, the reduced expression observed in males corresponded to increased methylation at an ERE site within the male Gadd45b promoter (**Fig. 5**), Furthermore, steroid hormone treatment at P0 and P1 caused a repressive effect on Gadd45b mRNA expression in the neonatal female amygdala (**Fig. 3A**), suggesting that Gadd45b levels are sensitive to changes in peripheral gonadal hormones. Interestingly, only treatment with dihydrotestosterone (DHT), and not estradiol or testosterone, resulted in decreased Gadd45b levels in the amygdala (**Fig. 3A**). Because DHT 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 (Meaney and Stewart, 1981; Cooke et al., 2003). The hormonal reduction of Gadd45b expression suggests a mediating role in 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 (**Chapter** II, 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

Because of these initial results, we became especially interested in the Gadd45 family—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, changes in neuronal Gadd45b expression have previously been reported; 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 the environment 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 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 (Veldic et al., 2004; Grayson et al., 2005; Veldic et al., 2005; Abdolmaleky et al., 2006; Huang and Akbarian, 2007; Huang et al., 2007; Ruzicka et al., 2007; Veldic et al., 2007; Gavin et al., 2009; Chase et al., 2015). 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 (Kigar and Auger, 2013; Chase et al., 2015), 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 (Kurian et al., 2008; Jessen et al., 2010; Kolodkin and Auger, 2011) 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 hormonal or non-hormonal factors involved in brain sexual

differentiation (Auger and Jessen, 2009; Kigar and Auger, 2013). Nonetheless, these data further support the idea that some brain sex differences can be observed at the level of the epigenome.

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Primer	Accession	Forward	Reverse
Ywhaz	NM_013011	TTGAGCAGAAGACGGAAGGT	GAAGCATTGGGGATCAAGAA
Gadd45b	NM_001008321	GCTGGCCATAGACGAAGAAG	GCCTGATACCCTGACGATGT
Gadd45a	NM_024127	GCTACTGGAGAACGACAAGAG	CCATTGTGATGAATGTGGGTTC
Gadd45g	NM_001077640	CTGAATGTGGACCCTGACAAT	AACGCCTGGATCAACGTAAA
Apobec3	NM_001033703	GCCTTGGATGAGACTGAGAATAA	CCGGTGGCTGTTGTTAATTG
Mbd4	XM_002726448	CTGTGGATGGGAAAGAGTTGT	TTGATCCTTGTGGGCTGATAAA
Tdg	NM_053729	ACATGGATGACCACACCTTAC	CCTCCTTCCCGGAATTCTTTAC
Tet1	NM_001107643	ATTGCTGGAGACTGTCGACTTGGT	TGTCACGGCCATCTTCTCGAATCA

Table 1: mRNA Primer sequences and Pubmed accession numbers for RT-qPCR

Primer	Cut Site	Forward	Reverse
G45b ERE	AciI (CCGC)	CTCGATTCGCTGGCAGTC	GATTGGCTGGAGGTAGGAAAG

Table 2: Methylation primers used to examine the Gadd45b promoter

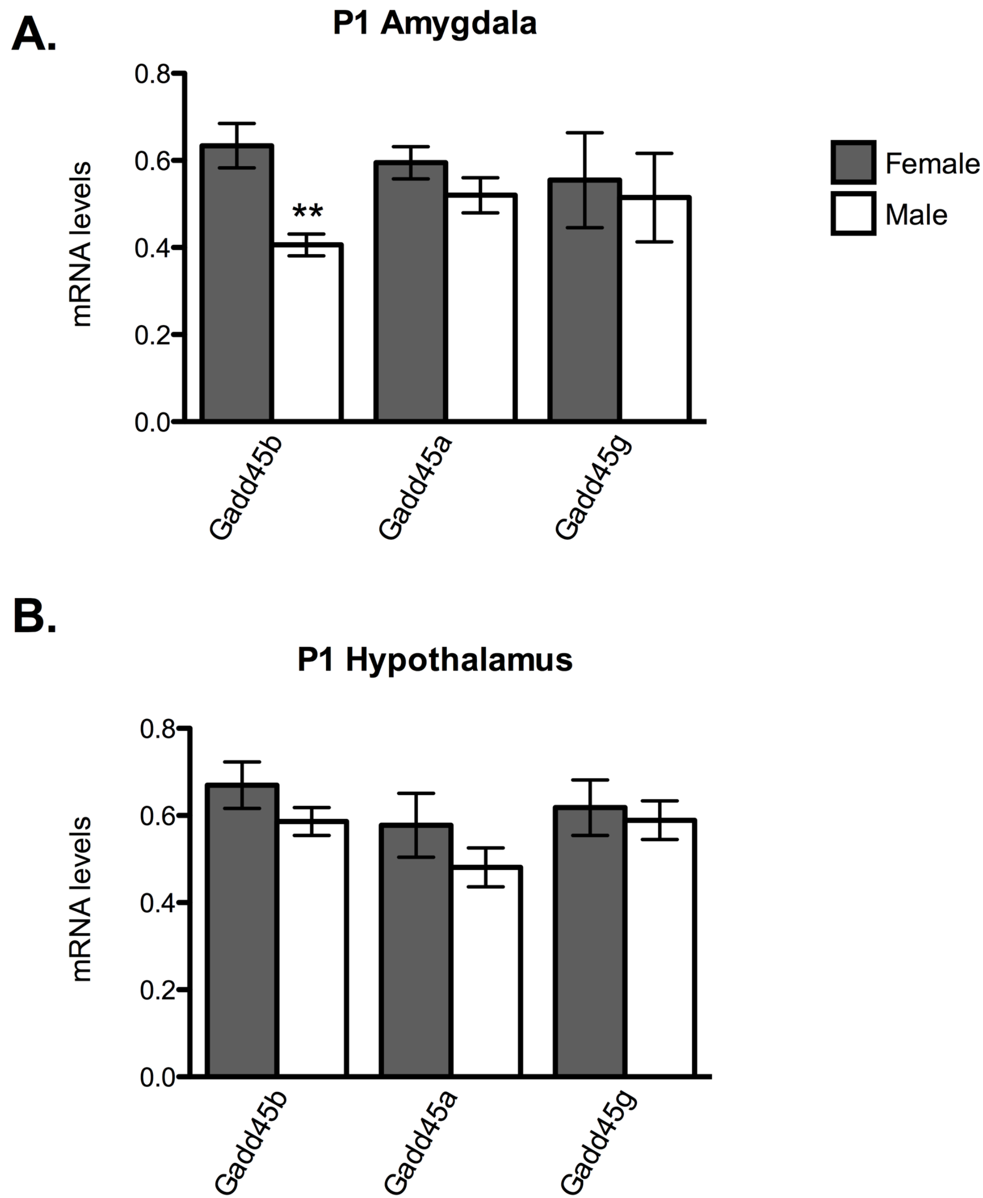

Figure 1: Sex differences in the mRNA expression of Gadd45 family members Gadd45b, Gadd45a, and Gadd45g at postnatal day 1 (P1) in two brain regions ***A.*** amygdala and ***B.*** hypothalamus known to be steroid hormone-responsive during early neonatal development. $^{**}p<0.01$.

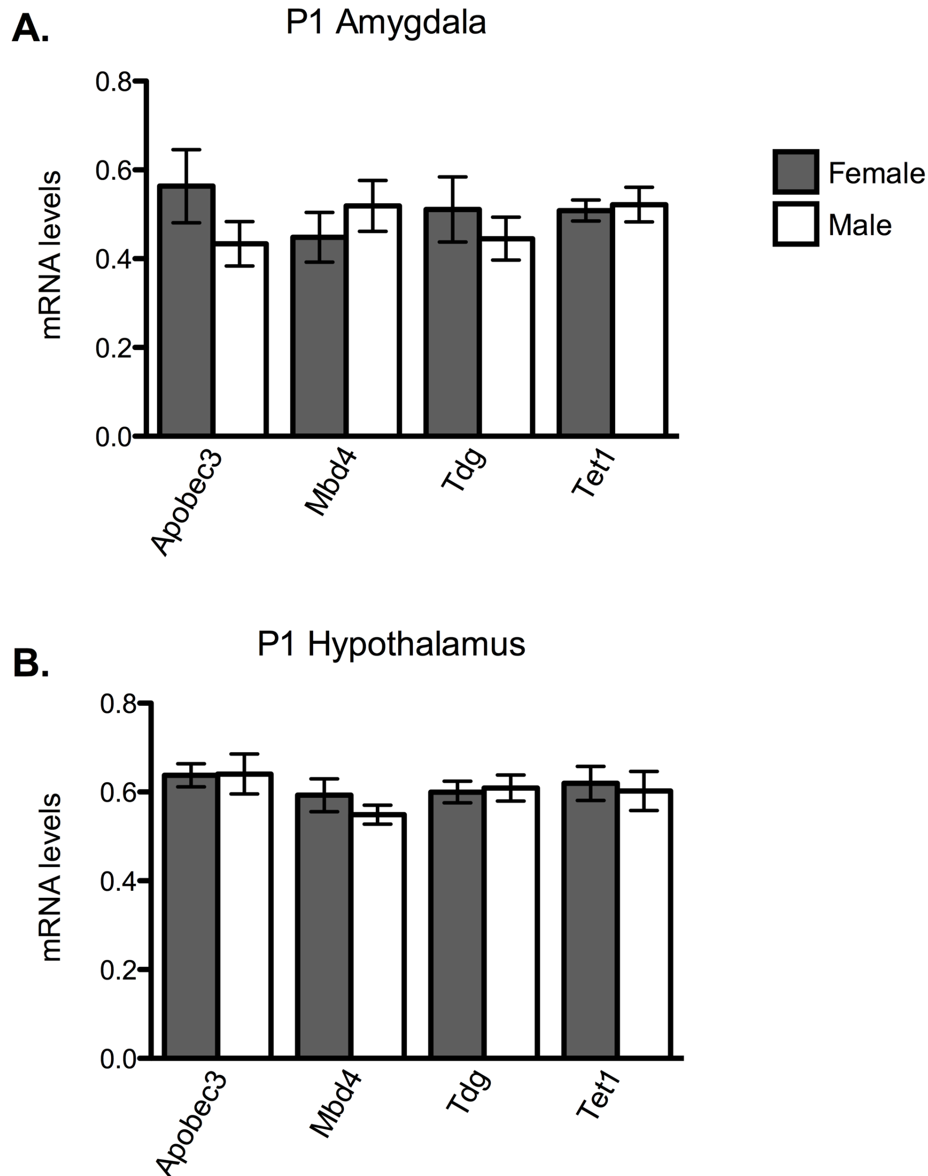

Figure 2: No amygdalar sex differences were observed in other enzymes known to facilitate active DNA demethylation at postnatal day 1 (P1) in either ***A.*** the amygdala or ***B.*** the hypothalamus. Abbreviations: apolipoprotein B mRNA editing cytokine 3 (Apobec3), methyl-binding domain 4 (Mbd4), thymine DNA glycosylase (Tdg), ten-eleven translocation 1 (Tet1).

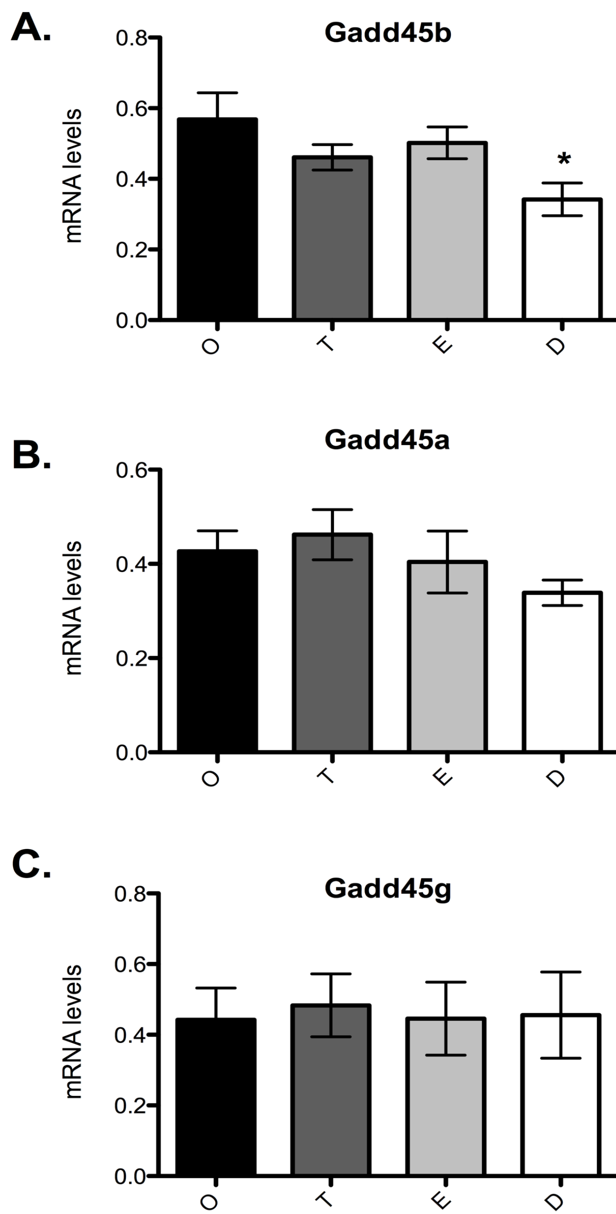

Figure 3: Gadd45 family members' expression in the amygdala of hormone-treated females: ***A.*** Gadd45b, ***B.*** Gadd45a, and ***C.*** Gadd45g. Females were treated with hormone or vehicle twice and then sacrificed at P2. *p<0.05

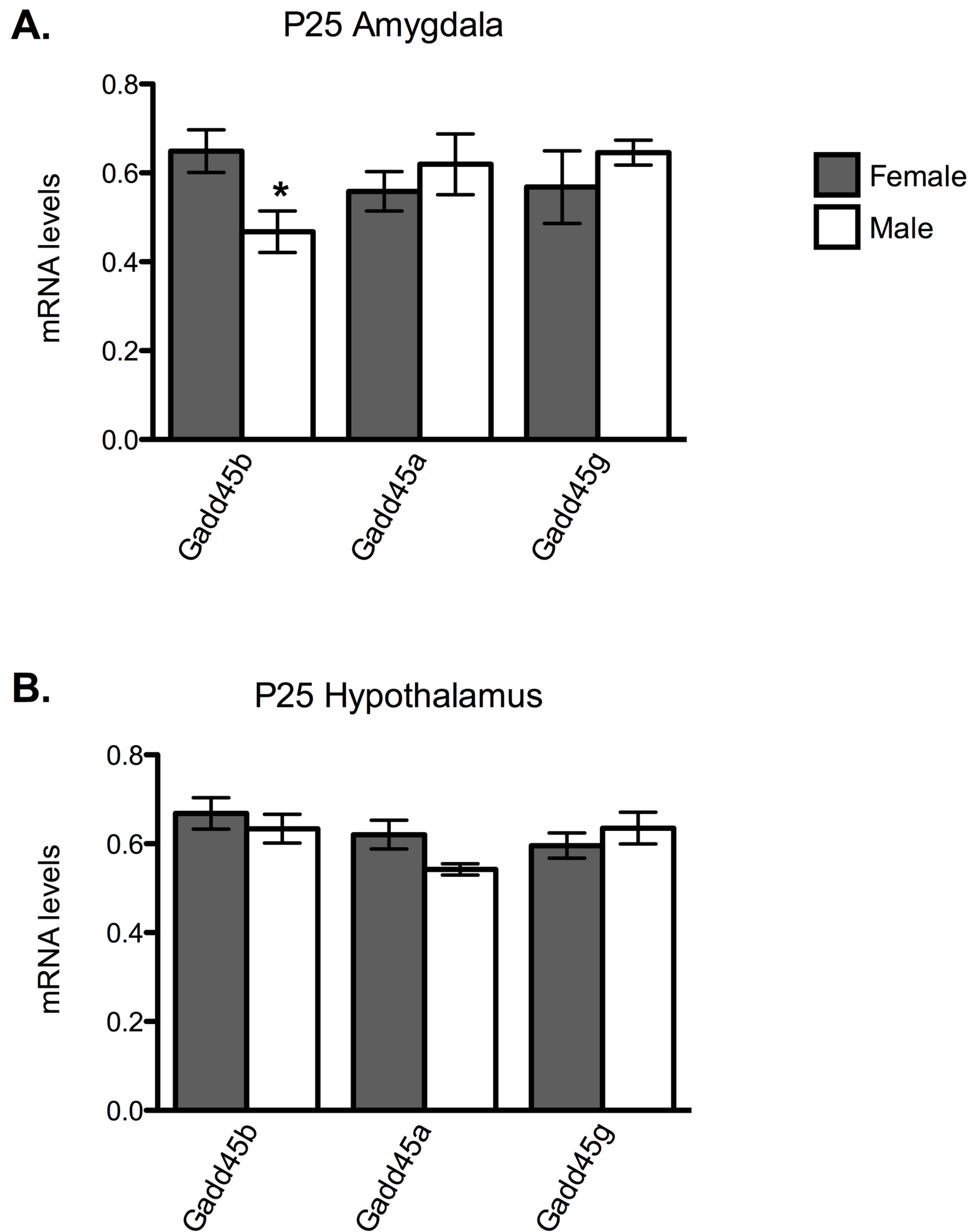

Figure 4: Sex differences in Gadd45 family members in the postnatal day 25 (P25) ***A.*** amygdala and ***B.*** hypothalamus. *$p<0.05$

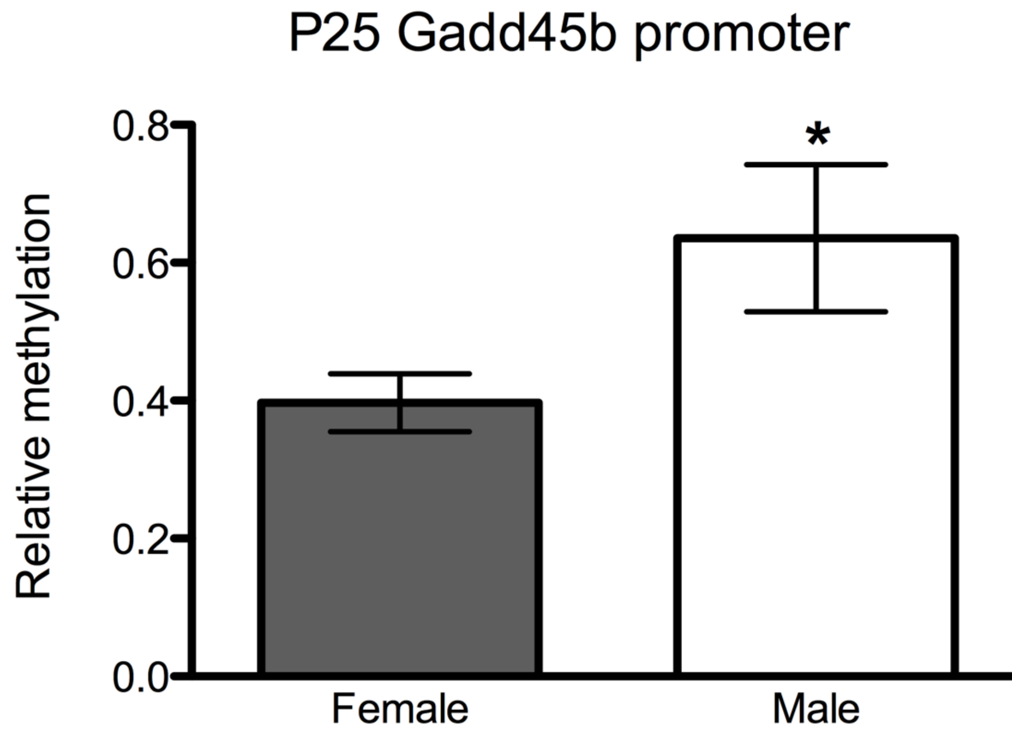

Figure 5: Sex differences in Gadd45b promoter methylation at an estrogen receptor α (ERα) response element (ERE) in the juvenile amygdala (postnatal day 25 (P25)). *$p<0.05$

CHAPTER IV: N^6-METHYLADENINE IS AN EPIGENETIC MARKER OF EARLY LIFE STRESS

Manuscript under review at Proceedings of the National Academy of Sciences.

ABSTRACT:

Very recent evidence described the existence of 6-methyladenine (6mA) as a novel epigenetic regulator in a variety of multicellular species and tissues, including the adult *Drosophilia* brain. However, the question of whether 6mA was used as an epigenetic mark in mammals remained unanswered. As early life stress is a potent long-term regulator of the epigenome, we developed the variable predator odor exposure (POE) paradigm described herein to examine lasting effects of an ecologically relevant stressor on the developing rodent amygdala. Our results provide the first evidence to our knowledge that 6mA is not only present in the gene promoter of a well-known mediator of anxiety-like behavior, serotonin receptor *Htr2a*, but that N^6-methyladenine levels are sensitive to early life stress and are associated with gene repression. Predator odor exposure altered 6mA levels in females, but not males—an intriguing, sex-specific effect of early life stress. Furthermore, we report sex differences in baseline 6mA abundance within the *Htr2a* promoter that are present neonatally and last into the juvenile period. This lasting sex difference in 6mA abundance observed in the juvenile amygdala suggests that 6mA may participate in normal mammalian sexual differentiation of the brain and may underlie sex specific risk or resilience to early life perturbations. Together, these data indicate that, complementarily to 5mC, 6mA in mammalian DNA is a useful biomarker for investigating the development of stress-induced neuropathology.

INTRODUCTION:

There is considerable evidence suggesting that precise regulation of 5-methylcytosine (5mC) during brain maturation is necessary to achieve species-typical behaviors later in life, and that early life experiences can alter epigenetic programming (Weaver et al., 2004; McGowan et al., 2009; Roth et al., 2009). Of particular clinical interest, early life stress and adverse childhood experiences can negatively affect one's wellbeing into adulthood, potently regulating the epigenome (for review, see (Bagot et al., 2014; Provençal and Binder, 2014; Jawahar et al., 2015)). Affective disorders such as anxiety and post-traumatic stress disorder (PTSD) increase markedly in individuals who have experienced early life stress (Sanchez et al., 2001; Edwards et al., 2003; Chapman et al., 2004; Heim et al., 2008), and the rate of diagnosis varies greatly by biological sex (Kessler et al., 1994). Understanding how biological sex confers risk or resilience during organismal development is critically important for improved treatment strategies, as has been stressed previously (McCarthy et al., 2012).

We and others have previously described sex differences in the neuronal epigenome with respect to 5-methylcytosine (5mC) (Kurian et al., 2010; Nugent et al., 2015). However, recent studies suggest that a variety of multi-cellular organisms —including *Chlamydomonas* (Fu et al., 2015), *C. elegans* (Greer et al., 2015), and *Drosophila* (Zhang et al., 2015)—possess the ability to produce and use 6-methyladenine (6mA) as an epigenetic modification *in vivo*. These discoveries herald a major paradigm shift; 6mA was previously thought to exist exclusively in bacteria and some unicellular organisms, so much so that the enzymes responsible for methylation were once considered for antibiotic drug development (Wion and Casadesús, 2006). Given the environmentally-sensitive nature of this modification in bacteria (Hale et al., 1994), and data

suggesting that 6mA is enriched in adult brain tissue of *Drosophila* (Zhang et al., 2015), we sought to determine whether 6mA could be found in the mammalian brain and whether it was responsive to the neonatal environment.

To do so, we adapted to neonates the well-established paradigm of predator stress for adult Sprague Dawley rats implemented by the Bakshi lab (Baisley et al., 2011; Bakshi et al., 2012; Rajbhandari et al., 2015) in order to model the lasting effects of early life adversity in the adolescent brain (Fig. 1a). We included both males and females in our study, first examining whether "fear of harm" induced by neonatal predator odor exposure (POE) would have an effect on anxiety-like behavior, as seen previously with other rodent models of ELS (Eiland and McEwen, 2010) We focused specifically on the amygdala—a region of the brain important for its modulatory role in fear acquisition and anxiety (LeDoux, 2007)—and 5-hydroxytryptamine (5-HT, or serotonin) receptors Htr2a and Htr1a, which are well-known for mediating anxiety-like behavior (Benekareddy et al., 2011; Cryan, 2014). Finally, we examined *Htr2a* and *Htr1a* promoter methylation and chromatin status to assess whether ELS or biological sex epigenetically impacted expression of these genes.

MATERIALS AND METHODS:

Animals: Untimed-pregnant Sprague Dawley female rats (~15 d pregnant) were purchased from Charles River Laboratories (Wilmington, MA) and allowed to deliver normally. Cages were checked regularly to determine the day of birth (P0). On P0, approximately 6 hours after birth, litters were culled to 10 (5 males and 5 females). Animals raised to the juvenile period for behavioral testing were left with dams undisturbed following predator odor exposure until

weaning. Juveniles were weaned at P21 into cages of 5 with littermates into mixed-sex groups. Two separate cohorts of animals that were unhandled, weaned at P21, raised to P35, and sacrificed to examine sex differences were used for methylated DNA and chromatin immunoprecipitation (MeDIP and ChIP). Animals were housed under standard laboratory conditions (light/dark cycle of 12h/12h, food and water *ad libitum*). All procedures were approved by the University of Wisconsin-Madison Animal Care and Use Committee.

Predator odor exposure: 12 randomly assigned litters were used in this study; 3 neonatal stress litters, 3 neonatal control litters, 3 juvenile stress litters, and 3 juvenile control litters. Pups in the experimental stress group were removed from the dam and taken to a separate room, where they were placed in a novel (non-conditioned) odorant environment. Stress group animals were exposed to cat odor, novel adult male rat odor, and ferret odor on P1-3, respectively, for 5 minutes at a time (Fig. 1a). Animals in the control group were handled in the same manner but were not exposed to any predator odor. All animals were returned to the dam in <10 minutes. A subset of 6 litters was sacrificed 30 minutes after the last odor exposure on P3. The remaining 6 litters were reared normally until behavioral testing before sacrifice on P33.

Elevated Plus Maze: The elevated plus maze (EPM) is a Plexiglas structure standing 50 cm off the floor and consisting of two opposing 100 cm runways that cross at the center. One runway is open whereas the other is closed, with an opening in the center to allow crosses. Rats were placed in the center of the maze facing an open arm and maze exploration was recorded for 5 min. Parameters quantified were entries into as well as time spent in the open and closed arms and the center of the maze arms. An entry was counted when all four paws crossed into any arm

of the maze. The experiment was performed under dim red light beginning 2 hours after the dark phase of the light cycle began. Animals were not repeat-tested in the task. Each animal was video recorded and behavior was analyzed and scored using The Observer software (Noldus Information Technologies) by a trained observer blind to all treatments.

Tissue collection: Both neonatal and juvenile rats were sacrificed via rapid decapitation. The amygdala was dissected as previously described (Edelmann et al., 2013) and immediately snap frozen in isopentane on dry ice. Tissue samples were first randomized and then homogenized; total RNA and DNA were collected using an AllPrep DNA/RNA Mini Kit (Qiagen; catalog #80204). The P3 amygdala set contained 6 control females, 6 stress females, 6 control males, and 6 stress males; 3 distinct litters were represented in each group to minimize any effects of variation in maternal care. The P33 amygdala set contained an n=10 animals per group with at least three litters in each as before.

Quantitative PCR Analysis: RNA conversion to cDNA for RT-qPCR analysis was performed as previously described (Edelmann et al., 2013). Following amplification, a dissociation melt curve and DNA gel analysis was performed to ensure specificity of the primers. cDNA levels were normalized to three averaged housekeeping genes—Ywhaz, Hprt, and Rpl13a—using a modified version of the ΔΔCT method. mRNA primer sequences listed in **Table 1**. Line1 mRNA primers taken from the literature (Hunter et al., 2012).

Enzyme-based methylation analysis: 5mC DNA methylation was assessed using an adapted version of the methylation sensitive restriction enzyme (MSRE) assay (Hashimoto et al., 2007),

as previously described (Auger et al., 2011; Edelmann and Auger, 2011; Kigar et al., 2015). Primers were designed such that some combination of HpaII (New England Biolabs; catalog #R0171), HhaI (New England Biolabs; catalog #R0139), or Hpy188III (New England Biolabs; catalog #R0622) cut sites were inside or outside of the primer sequence. 6mA DNA methylation was determined by methylation dependent restriction enzyme (MDRE) analysis. Briefly, 240ng of DNA from each rat was divided equally into two tubes and digested with either DpnI (Invitrogen; catalog #5242-019) or BstUI (Thermo; catalog #ER0922) in the same buffer conditions at 37°C for 3 hours. Enzymes were subsequently inactivated by heating to 70°C for 15min. A no-DNA control was added to ensure purity of the restriction enzyme reaction. For MDRE, primers were designed such that DpnI cut sites, but not BstUI cut sites, were contained within the region of interest. After cutting, RT-qPCR was performed in as described above to quantify relative amounts of DNA methylation. Genomic DNA primer sequences are listed in **Table 2**.

Methylated DNA immunoprecipitation (MeDIP): Genomic DNA, isolated from non-handled P35 males and females (n=6 per group), was fragmented using probe-based sonication. Shearing was confirmed via DNA gel analysis to be between 300-1000bp. 800ng of sonicated DNA was incubated at 4°C overnight with either 6mA antibody (Millipore; catalog #ABE572) or 5mC antibody (Millipore; catalog #MABE146). An IgG negative control reaction was included to test for non-specific DNA-binding. The antibody-DNA sample was then incubated with a Dynabead immunoprecipitation kit (Invitrogen; catalog #10007D) for 2 hours at 4°C and the manufacturer's protocol followed. RT-qPCR analysis was performed to quantify relative

amounts of methylated DNA enrichment; immunoprecipitated (IP) samples were normalized to input.

Chromatin immunoprecipitation (ChIP): ChIP was performed on bilateral amygdalae sections collected as described above from non-handled P35 males and females (n=6 per group). Snap-frozen tissue (~37mg) was fixed in 1% formaldehyde for 15 min at room temperature with gentle shaking. The reaction was quenched with 160uL of 1.25M glycine for 5 min at room temp with gentle shaking. Samples were washed 2x with PBS and cOmplete protease inhibitor (CPI) tablets (Roche; catalog #04693116001) then resuspended in 400µL RIPA lysis buffer + CPI tablets. Samples were homogenized with a pestle then lightly sonicated via probe-based sonication to fully lyse cells and the nuclear envelope. Chromatin shearing was obtained using 1000 gel units of Micrococcal Nuclease (MNase) (New England Biolabs; catalog #M0247S); samples were incubated with Proteinase K to ensure efficient shearing of DNA (between 300bp-900bp). MNase reaction was stopped with 1.25µmol EGTA. Debris was removed by centrifugation at 15,000 g for 10 m at 4°C; 100µL of each lysate was diluted in 200µL IP buffer (PBS + 0.05% Triton X-100) and incubated overnight with 1µg of antibody. Antibodies used were H3K9Ac (Upstate; catalog #06-942) and Sp1 (Santa Cruz; catalog #sc-59x). After overnight antibody incubation, 25µL of magnetic beads (Life Technologies; catalog #10004D) was added and mixed for 2 h at 4°C with gentle rocking. Samples were washed sequentially with 500µL Low Salt, 500µL High Salt, and 500µL TE Buffers, then resuspended in Digestion Buffer (50mM Tris, 10mM EDTA, 0.5% SDS, pH 8.0) + Proteinase K for 2h at 62°C. DNA was isolated via column clean-up. PCR was then performed on samples as described above.

Statistical analysis: Statistics were run using SigmaPlot 11. Behavioral data, mRNA PCR data, and MDRE/MSRE data were analyzed using two-way ANOVA and Tukey post-hoc; MeDIP and ChIP data were analyzed using t-tests. Potential outliers were screened for using the Grubbs test for outliers (http://graphpad.com/quickcalcs/Grubbs1.cfm). All reported measures are listed as mean ± SEM. Significance was defined as a p value of <0.05.

RESULTS:

POE-induced ELS increases anxiety-like behavior

Examination of percent time spent in the open arm of the elevated plus maze (EPM) revealed a significant effect of stress, indicating both males and females have increased anxiety-like behavior with POE-treatment (**Fig. 1B**). There was a main effect of stress (two-way ANOVA; $F_{(1,37)} = 10.621$, $p = 0.003$), though neither an interaction was observed (stress by sex; two-way ANOVA; $F_{(1,37)} = 0.337$, $p = 0.565$), nor was there a main effect of sex (two-way ANOVA; $F_{(1,37)} = 1.870$, $p = 0.181$).

POE results in temporal- and sex-specific changes to serotonin receptor mRNA expression

At P3, there was a main effect of sex on Htr2a expression, where females expressed higher levels than males (two-way ANOVA; $F_{(1,23)} = 7.773$, $p = 0.011$). Furthermore, a main effect of stress revealed increased Htr2a expression (treatment; two-way ANOVA; $F_{(1,23)} = 10.124$, $p = 0.005$) in both males and females [(Females: Tukey's *post hoc*; $q = 2.984$, $p < 0.048$); (Males: Tukey's *post hoc*; $q = 3.379$, $p < 0.027$)]. There was no interaction (stress by sex; two-way ANOVA; $F_{(1,23)} = 9.116$, $p = 0.846$). Htr1a mRNA expression was unaltered by sex or stress (stress by sex; two-way ANOVA; $F_{(1,23)} = 0.263$, $p = 0.613$). There was no main effect of sex (two-way

ANOVA; $F_{(1,23)} = 0.261$, $p = 0.615$) or stress (two-way ANOVA; $F_{(1,23)} = 0.00193$, $p = 0.965$) (**Fig. 1C**).

As at P3, there was a main effect of sex at P33 on the expression of Htr2a (two-way ANOVA; $F_{(1,38)} = 18.896$, $p < 0.001$). An interaction was also observed (stress by sex; two-way ANOVA; $F_{(1,38)} = 9.116$, $p = 0.005$). Post hoc analysis revealed a sex difference in control males and females and a stress effect in females where stressed females had less mRNA [(Tukey's *post hoc*; $q = 7.269$, $p < 0.001$); treatment effect in females (Tukey's *post hoc*; $q = 4.642$, $p = 0.002$)]. When we examined Htr1a mRNA expression at P33, an interaction was observed (group by sex; two-way ANOVA; $F_{(1,37)} = 4.207$, $p = 0.048$). In this case, post hoc analysis revealed a sex difference in control males and females where males had more, and stressed females had male-like levels of mRNA [(Tukey's *post hoc*; $q = 3.954$, $p = 0.009$) and a stress effect in females (Tukey's *post hoc*; $q = 3.412$, $p = 0.022$)] (**Fig. 1D**).

Impact of stress on adenine methylation in the Htr2a promoter

We first exploited the use of methylation-dependent restriction enzymes (MDREs) to examine the two GmATC DpnI cut sites, arbitrarily referred to as A and B, found in the neuron-specific region of the *Htr2a* promoter (Toth, 1996). At P3, sex differences in relative 6mA levels were observed, where males had higher levels than females, at Site A (two-way ANOVA; $F_{(1,21)} = 7.288$, $p = 0.015$). No interaction (stress by sex; two-way ANOVA; $F_{(1,21)} = 0.469$, $p = 0.502$) or effect of treatment was observed (two-way ANOVA; $F_{(1,21)} = 0.0227$, $p = 0.882$). At Site B, we also observed a main effect of sex, where males had greater levels of 6mA (two-way ANOVA;

$F_{(1,23)} = 6.094$, $p = 0.023$), though there was no main effect of stress (two-way ANOVA; $F_{(1,23)} = 1.218$, $p = 0.283$) or an interaction (two-way ANOVA; $F_{(1,23)} = 0.499$, $p = 0.488$) (**Fig. 2B**).

At P33, both stressed and control males showed higher levels of 6mA than control females, and stressed females had more male-typical levels (**Fig. 2C**). At Site A, an interaction was observed (stress by sex; two-way ANOVA; $F_{(1,39)} = 5.206$, $p = 0.029$). Post hoc analysis revealed a sex difference between control males and females, where males had higher levels of methylation (Tukey's *post hoc*; $q = 3.786$, $p = 0.011$), and also a stress effect in females, where stressed females had higher levels of methylation (Tukey's *post hoc*; $q = 3.631$, $p = 0.015$). There was no main effect of sex (two-way ANOVA; $F_{(1,39)} = 2.262$, $p = 0.141$) or stress (two-way ANOVA; $F_{(1,39)} = 1.821$, $p = 0.186$). At Site B, there was no main effect of sex (two-way ANOVA; $F_{(1,37)} = 2.303$, $p = 0.138$) or treatment (two-way ANOVA; $F_{(1,37)} = 0.001$, $p = 0.975$), nor was there an interaction (two-way ANOVA; $F_{(1,37)} = 2.20 \times 10^{-5}$, $p = 0.996$).

Impact of stress on cytosine methylation in the Htr2a promoter

We next used a variety of methylation-sensitive restriction enzymes (MSREs) to investigate CpG methylation sites. No interactions or main effects were observed for 5mC levels in the *Htr2a* promoter at either P3 or P33. At P3 (**Fig. 2D**): [Site #1 (Hpy188III), stress by sex; two-way ANOVA, $F_{(1,22)} = 3.419$, $p = 0.08$; there was no main effect of sex (two-way ANOVA; $F_{(1,22)} = 0.978$, $p = 0.335$) or stress (two-way ANOVA; $F_{(1,22)} = 0.0487$, $p = 0.828$); Site #2 (HpaII), stress by sex; two-way ANOVA; $F_{(1,21)} = 1.751$, $p = 0.202$; there was no main effect of sex (two-way ANOVA; $F_{(1,21)} = 0.000113$, $p = 0.992$) or stress (two-way ANOVA; $F_{(1,21)} = 0.241$, $p = 0.629$); Site #3 (HhaI) (*no methylation observed)*; Site #4 (HpaII), stress by sex; two-way ANOVA;

$F_{(1,23)} = 0.201$, $p = 0.659$; there was no main effect of sex (two-way ANOVA; $F_{(1,23)} = 0.241$, $p = 0.629$) or stress (two-way ANOVA; $F_{(1,23)} = 0.00243$, $p = 0.961$); Site #5 (HpaII), stress by sex; two-way ANOVA; $F_{(1,23)} = 0.848$, $p = 0.368$; there was no main effect of sex (two-way ANOVA; $F_{(1,23)} = 0.736$, $p = 0.401$) or stress (two-way ANOVA; $F_{(1,23)} = 1.096$, $p = 0.308$)]. At P33 (**Fig. 2E**): [Site #1, stress by sex; two-way ANOVA; $F_{(1,35)} = 0.0422$, $p = 0.839$; there was no main effect of sex (two-way ANOVA; $F_{(1,35)} = 4.083$, $p = 0.052$) or stress (two-way ANOVA; $F_{(1,35)} = 0.173$, $p = 0.68$); Site #2, stress by sex; two-way ANOVA; $F_{(1,35)} = 0.170$, $p = 0.683$; there were no main effects of sex (two-way ANOVA; $F_{(1,35)} = 0.0475$, $p = 0.829$) or stress (two-way ANOVA; $F_{(1,35)} = 1.007$, $p = 0.323$); Site #3, stress by sex; two-way ANOVA; $F_{(1,35)} = 0.0777$, $p = 0.782$; there were no main effects of sex (two-way ANOVA; $F_{(1,35)} = 0.0082$, $p = 0.929$) or stress (two-way ANOVA; $F_{(1,35)} = 1.349$, $p = 0.254$); Site #4, stress by sex; two-way ANOVA; $F_{(1,35)} = 0.31$, $p = 0.582$; there was no main effect of sex (two-way ANOVA; $F_{(1,35)} = 01.425$, $p = 0.241$) or stress (two-way ANOVA; $F_{(1,35)} = 0.465$, $p = 0.500$); Site #5, stress by sex; two-way ANOVA; $F_{(1,36)} = 0.46$, $p = 0.502$; there was no main effect of sex (two-way ANOVA; $F_{(1,36)} = 0.400$, $p = 0.532$) or stress (two-way ANOVA; $F_{(1,36)} = 0.229$, $p = 0.636$)].

Confirmation of Htr2a methylation findings via MeDIP analysis

We isolated and prepared genomic DNA samples from a separate group of non-handled males and females aged P35 for MeDIP analysis. With a 6mA antibody that was previously used for this technique (Zhang et al., 2015), we found sex differences in 6mA abundance that matched those found with the MDRE assay described above [Site A: (*t* test; $t(9) = 2.438$, $p = 0.0375$); Site B: (*t* test; $t(7) = 1.321$, $p = 0.2280$)] (**Fig. 3A**). Figure 3C is a DNA gel showing that our assay worked as expected, with single bands appearing in the input and IP lane, and no non-specific

DNA pull-down with an IgG antibody. There is one CpG site contained within the Site A primer set; however, no sex difference was observed at this location using a 5mC antibody (*t* test; $t(9) = 0.8075$, $p = 0.4402$). There were also no sex differences in 5mC levels using primers for Site B (*t* test; $t(8) = 0.8704$, $p = 0.4094$) (**Fig. 3B**).

Comparison of 6mA presence in Htr2a to retrotransposons

The recent identification of 6mA in the vertebrate genome found vast enrichment in *Drosophila* retrotransposon elements. We thus examined the presence of 6mA in an abundant retrotransposon of the mammalian genome, long interspersed nuclear element 1 (Line1). Although 6mA was highly enriched in an untranslated region of the Line1 promoter (**Fig. 3D**), we saw no significant difference in the expression of Line1 mRNA [stress by sex (two-way ANOVA; $F_{(1,37)} = 0.0288$, $p = 0.866$; sex (two-way ANOVA; $F_{(1,37)} = 0.272$, $p = 0.605$); and stress (two-way ANOVA; $F_{(1,35)} = 1.011$, $p = 0.322$)] (**Fig. 3E**).

Methylation analysis of the Htr1a promoter

We observed no long-term methylation changes in either 6mA [Site A, stress by sex; two-way ANOVA; $F_{(1,37)} = 0.165$, $p = 0.687$; there was no main effect of sex (two-way ANOVA; $F_{(1,37)} = 0.769$, $p = 0.387$) or stress (two-way ANOVA; $F_{(1,37)} = 1.347$, $p = 0.254$); Site C, stress by sex; two-way ANOVA; $F_{(1,38)} = 0.418$, $p = 0.522$; there was no main effect of sex (two-way ANOVA; $F_{(1,38)} = 0.856$, $p = 0.361$) or stress (two-way ANOVA; $F_{(1,38)} = 0.773$, $p = 0.385$)] (**Fig. 4B**) or 5mC [Site #1 (Hpy188III), stress by sex; two-way ANOVA; $F_{(1,36)} = 0.581$, $p = 0.451$; there was no main effect of sex (two-way ANOVA; $F_{(1,36)} = 1.182$, $p = 0.285$) or stress (two-way ANOVA; $F_{(1,36)} = 2.39\text{x}10^{-4}$, $p = 0.988$); Site #2 (HpaII), stress by sex; two-way ANOVA; $F_{(1,36)} = 0.234$, *p*

= 0.632; there was no main effect of sex (two-way ANOVA; $F_{(1,36)} = 0.0929$, $p = 0.762$) or stress (two-way ANOVA; $F_{(1,36)} = 3.03 \times 10^{-3}$, $p = 0.956$)] (**Fig. 4C**) using enzyme-based methods.

For confirmation, we used a MeDIP assay as before. Again, no sex differences were observed in either 6mA [Site A: (*t* test; t = 1.267, df = 9, $p = 0.2369$); Site C: (*t* test; t = 1.469 df = 7, $p = 0.1854$)] (**Fig. 4D**) or 5mC [Site A: (*t* test; t = 0.5224, df = 9, $p = 0.6140$); Site C: (*t* test; t = 0.03275, df = 8, $p = 0.9747$)] (**Fig. 4E**).

Effects on chromatin and Sp1 transcription factor binding

To examine whether N^6-methyladenine was associated with a less-active chromatin state, we used a second cohort of non-handled P35 males and females for ChIP and probed for histone acetylation on core histone protein H3 at lysine 9 (H3K9), as this is a well-known marker of active gene expression (Karmodiya et al., 2012) (**Fig. 5A**). We observed a sex difference in H3K9 acetylation, where females had more, at Site A in the *Htr2a* promoter (*t* test; $t(8) = 2.436$, $p = 0.0408$). There was no sex difference at Site B (*t* test; $t(9) = 0.8783$, $p = 0.4026$).

Sp1 has previously been characterized as a transcription factor important for Htr2a mRNA transcription (Falkenberg et al., 2011). We observed a sex difference in Sp1 pull-down at Site A, but not Site B, with females having more Sp1 pulldown [Site A: (*t* test; $t(9) = 3.263$, $p = 0.0098$); Site B: (*t* test; $t(8) = 1.899$, $p = 0.0941$)] (**Fig. 5B**).

DISCUSSION:

Our findings suggest that 6mA is a novel epigenetic regulator in the mammalian brain. After first confirming that an ecologically relevant form of early life stress, neonatal predator odor exposure (POE), had a profound and lasting impact on later juvenile anxiety-like behavior—notably decreasing time spent in the open arm for both males and females (**Fig. 1B**)—we observed sex-specific changes in gene expression in the amygdala. The amygdala plays a well-characterized role in socioemotional processing, and fear learning in particular (Fanselow and LeDoux, 1999); furthermore, other popular models of early life stress such as maternal separation (MS) have been shown to increase anxiety-like behavior later in life (Eiland and McEwen, 2010). While an immediate upregulation of Htr2a expression occurred around the time of POE in both the males and female amygdala (**Fig. 1C**), POE resulted in a lasting decrease in Htr2a gene expression in juvenile females only (**Fig. 1D**). Conversely, while no changes were observed in the expression of Htr1a neonatally, stressed females showed increased mRNA levels at P33 (**Fig. 1C-D**). These data indicate that POE can elicit lasting changes in Htr2a expression within the juvenile amygdala of females, but not males. This is consistent with previously published data from our lab in which we observed that Htr2a expression is influenced by both biological sex and the neonatal environment in juveniles (Edelmann et al., 2013).

To determine whether these sex-specific changes in mRNA levels were due to epigenetic programming, we examined a region in the *Htr2a* gene promoter that is reportedly used exclusively by neurons (Toth, 1996) (**Fig. 2A**). We first adapted a methylation-dependent restriction enzyme (MDRE) assay in tandem with qPCR amplification (Auger et al., 2011) to answer this question, which allowed us to examine the occurrence of N^6-adenine methylation at

specific GATC sites (GmATC sequences are recognized and cut by the DpnI restriction enzyme). It is of note that a major obstacle in studying vertebrate 6mA results from its extremely low abundance in the overall genome (Luo et al., 2015). We reasoned that selective examination of specific sites using this highly-sensitive, RT-qPCR-based method of detection may reveal ELS-induced changes to N^6-adenine methylation in the amygdala. At a well-conserved area of the promoter (Zhu et al., 1995), we observed both sex differences and a juvenile female-specific stress effect on 6mA methylation (**Fig. B-C**). An overall sex difference in 6mA was present at both GATC sites in the *Htr2a* promoter neonatally, but was not seen proximal to the transcriptional start site (TSS) in juveniles (**Fig. 2C**). At both time points, the sex-difference in 6mA methylation is consistent with a repressive effect on mRNA expression; additionally, the ELS-induced increase in 6mA levels is consistent with a repressive effect on Ht2a mRNA levels in the female juvenile brain (**Fig. 1D**). These data suggest that N^6-adenine methylation levels within the developing amygdala are sensitive to both biological sex and ELS. We observed no significant differences in 5mC at either time point (**Fig. 2D-E**), suggesting that adenine methylation may exert more control over Htr2a gene expression than cytosine methylation within the developing amygdala. No effects of 6mA or 5mC methylation were observed in the *Htr1a* promoter (**Fig. 4B-C**), suggesting a level of specificity for adenine methylation within gene promoters.

The inversely proportional relationship of adenine methylation and mRNA expression in this area of the promoter was surprising, as evidence in *C. Elegans* and *Drosophila* indicated 6mA may facilitate gene expression (Greer et al., 2015; Zhang et al., 2015). To further confirm our findings, we employed a methylated DNA immunoprecipitation (MeDIP) assay in a separate

cohort of non-handled male and female rat amygdalae collected at P35. We replicated the sex difference in which increased enrichment of 6mA within the *Htr2a* promoter was observed in males (**Fig. 3A**). Also consistent with the enzyme-based methods presented in this study, we found no significant differences in *Htr2a* 5mC methylation (**Fig. 3B**) or *Htr1a* methylation (**Fig 4D-E**). Finally, given the robust presence of 6mA in *Drosophila* retrotransposons (Zhang et al., 2015), we examined enrichment in a common mammalian retrotransposon, Line1. While no changes in the mRNA levels of Line1 were observed across treatment groups (**Fig. 3E**), 6mA was highly enriched in our MeDIP assay, suggesting some commonality in 6mA regulation across speciation (**Fig. 3D**).

Finally, we performed a ChIP assay to examine chromatin states, looking at H3K9 acetylation—a well known marker of active gene transcription (Karmodiya et al., 2012). We saw that males had significantly less H3K9 acetylation at Site A (**Fig. 5A**), consistent with the notion that 6mA may act as a marker of gene repression in the *Htr2a* promoter as males also had more 6mA at this location. We also looked at the transcription factor Sp1, as it is a known regulator of Htr2a expression in this region (Falkenberg et al., 2011); we found that females had significantly more Sp1 bound at this site (**Fig. 5B**). This supports a scenario in which the presence of 6mA causes increased condensation of chromatin, reduced binding of Sp1, and ultimately reduced expression of Htr2a, summarized in **Figure 6**. At present, it is unclear what regulates Htr1a mRNA expression; this will be an important avenue for future study.

To the best of our knowledge, this study reports the first evidence that 6mA is both present and regulated by environmental perturbations such as ELS in the mammalian brain. Though the

enzymes responsible for organization of 6mA into the epigenome remain to be determined, candidates based on sequence homology to bacterial proteins have been proposed (Heyn and Esteller, 2015). Conventional wisdom has, to this point, held that 5-methylcytosine (5mC) and its oxidated derivatives were the sole mediators of epigenetic regulation in mammalian DNA. Building on recent evidence suggesting some multi-cellular organisms produce and use N^6-methyladenine (6mA) as an epigenetic modification, this work challenges that dogma as we now report that 6mA is both present and regulated by early life stress (ELS) and biological sex in the developing juvenile rat brain. Due to the dynamic nature of 6mA in mammalian DNA, these data raise the intriguing possibility that 6mA may be an important biomarker for investigating the stress-induced development of neuropathology.

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Primer	Accession	Forward	Reverse
Ywhaz	NM_013011	TTGAGCAGAAGACGGAAGGT	GAAGCATTGGGGATCAAGAA
Hprt	NM_012583	GCAGACTTTGCTTTCCTTGG	CCGCTGTCTTTTAGGCTTTG
Rpl13a	NM_173340	AGCAGCTCTTGAGGCTAAGG	GGGTTCACACCAAGAGTCCA
Htr2a	NM_017254	AACGGTCCATCCACAGAG	AACAGGAAGAACACGATGC
Htr1a	NM_012585	CCGCACGCTTCCGAATCC	TGTCCGTTCAGGCTCTTCTTG

Table 1: mRNA Primer sequences and Pubmed accession numbers for RT-qPCR.

Promoter	Location:	Forward	Reverse
Htr2a	Site #1 – Hpy188III	AAAGTAAGCTAGTTGCGAGATGTA	GTCAGGAAGTCTGTGGCAATAA
Htr2a	Site A - DpnI	CCTGACTCCTCTGAACGTGT	GTCCTGGGATCTAGGGGCAT
Htr2a	Site #2 - HpaII	CCTAGGCTGTCTCCCCATTC	TGCCTCCTTCCTCAGACCTC
Htr2a	Site #3 - HhaI	TGCGGCTCTTTTGTGTGACT	AGCAGCCCAGGAACTTACAT
Htr2a	Site #4 - HpaII	TGCGGCTCTTTTGTGTGACT	AGCAGCCCAGGAACTTACAT
Htr2a	Site B - DpnI	CTCCCTCCTCGTTTGGATCT	GCTGTAAGTTCTCACGGAAGC
Htr2a	Site #5 - HpaII	CTCCCTCCTCGTTTGGATCT	GCTGTAAGTTCTCACGGAAGC
Htr1a	Site A - DpnI	AGGAGGCGGGGTTTAATCTG	CCCCCACCACCATCTAACAC
Htr1a	Site #1 – Hpy188III	AGGAGGCGGGGTTTAATCTG	CCCCCACCACCATCTAACAC
Htr1a	Site #2 - HpaII	GAGAGAAGCAACCAGGAGATG	GGATTCTCCCGCCTAACAAA
Htr1a	Site B - DpnI	TGAGTGCTCTTCTCAGATGCC	TTTTCTGGGGAGTTTCAGAGGG
Htr1a	Site C - DpnI	TCCCTCTGAAACTCCCCAGAA	AGGTCACGTCGGAGATGCTA
Line1	----	GAAAGCACCAAATGCCACTGG	GTAGTCTGCTATCGGGCGTT

Table 2: Genomic DNA promoter primer sequences for RT-qPCR. *Htr2a* accession number: L31546. *Htr1a* accession number: AF217200.

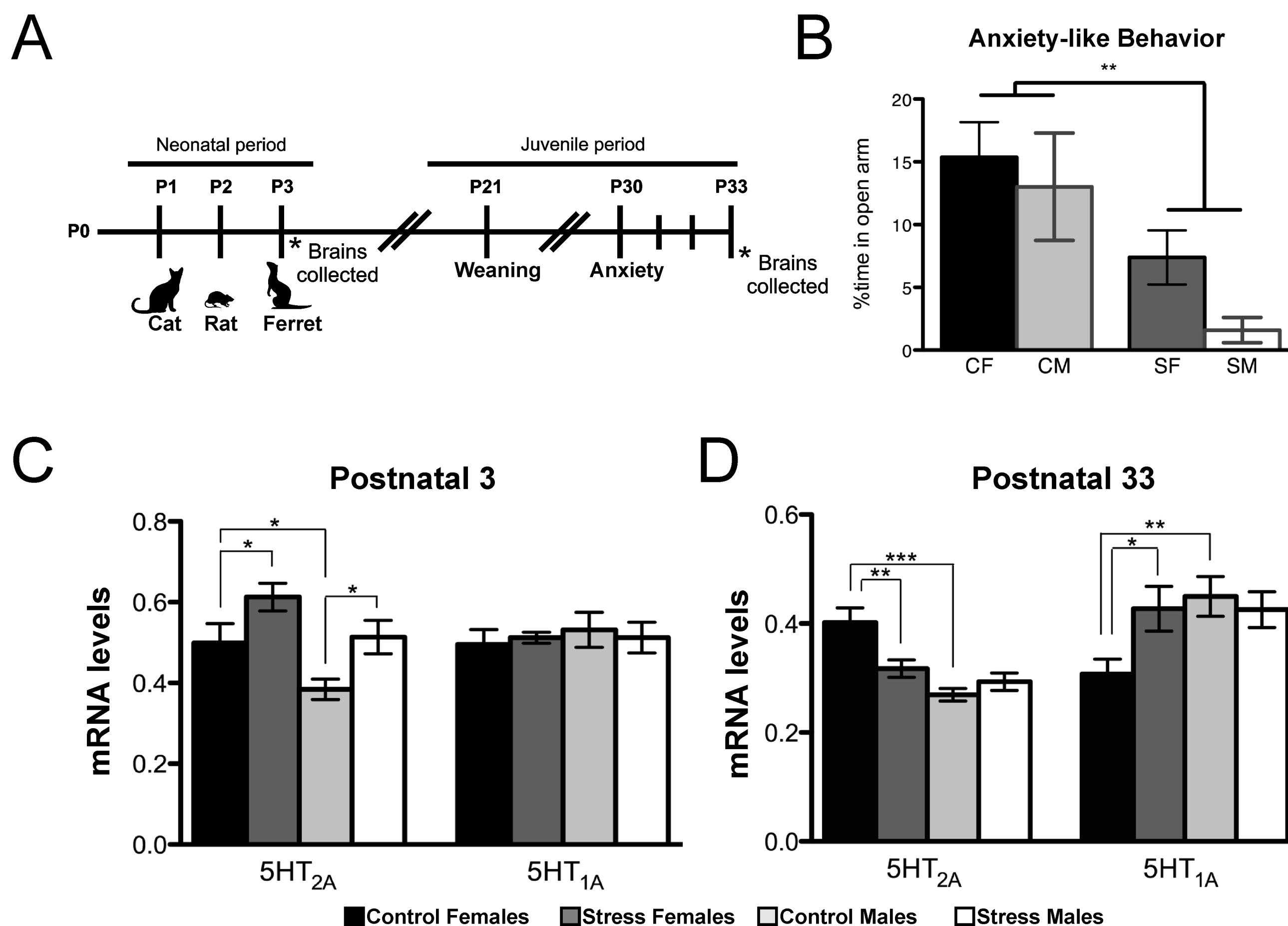

Figure 1: *(A)* Mixed-sex litters were exposed to variable predator odor. Half of the animals were sacrificed for gene expression analysis 30m post-exposure on P3; the remaining half was raised undisturbed before behavior testing and sacrifice at P33. *(B)* Anxiety-testing in the EPM revealed a significant main effect of stress. *(C)* RT-qPCR analysis of the P3 amygdala shows a main effect of sex and stress on Htr2a mRNA. *(D)* Changes in Htr2a and Htr1a mRNA are seen in the juvenile amygdala. Values shown as mean ± SEM. ***p<0.001, **p<0.01, *p<0.05.

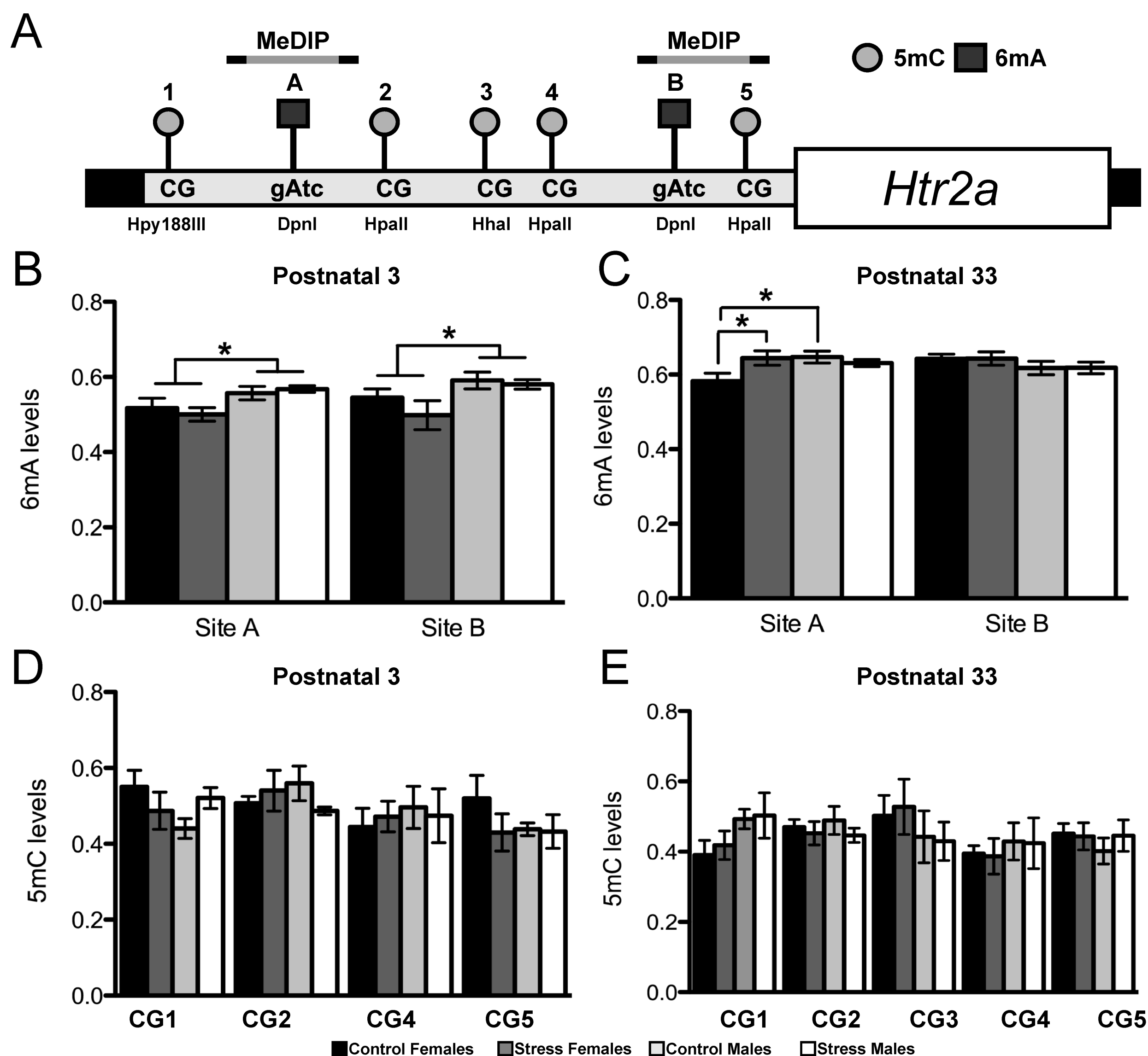

Figure 2: *(A)* Schematic of *Htr2a* gene promoter, showing individual methylation sites analyzed. Vertical bars indicate regions also examined in Figure 3. *(B)* MDRE analysis shows higher 6mA in males at two distinct sites in the promoter. *(C)* A sex difference persists in juveniles at Site A, but is lost at Site B. Stress females show increased 6mA compared to control females. *(D, E)* No changes in 5mC were observed at either time point. No methylation was detected at CG3 in P3 animals. Values shown as mean ± SEM, *p<0.05.

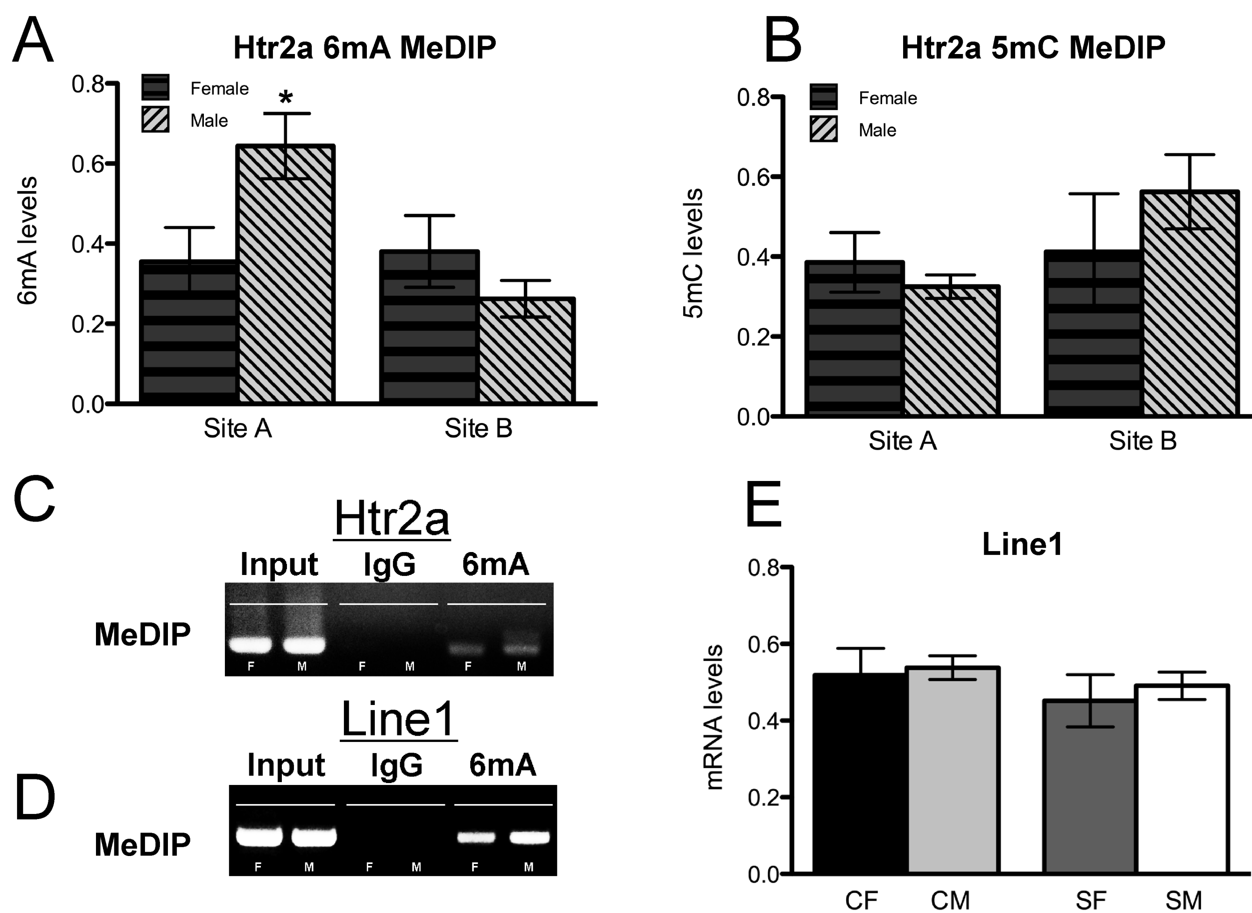

Figure 3: *(A)* Confirmation of a juvenile sex difference in 6mA abundance in the amygdala of unhandled P35 animals using MeDIP as indicated in Figure 2a. *(B)* No significant differences found using 5mC MeDIP, consistent with Figure 2E. *(C)* DNA gel for 6mA MeDIP of *Htr2a* promoter DNA at Site A showing modest enrichment; input and IgG controls show assay worked as expected. *(D)* DNA gel for 6mA MeDIP of Line1 showing robust enrichment, as well as input and IgG controls. *(E)* RT-qPCR data showing no group differences in expression of Line1 mRNA. Values shown as mean ± SEM, *p<0.05.

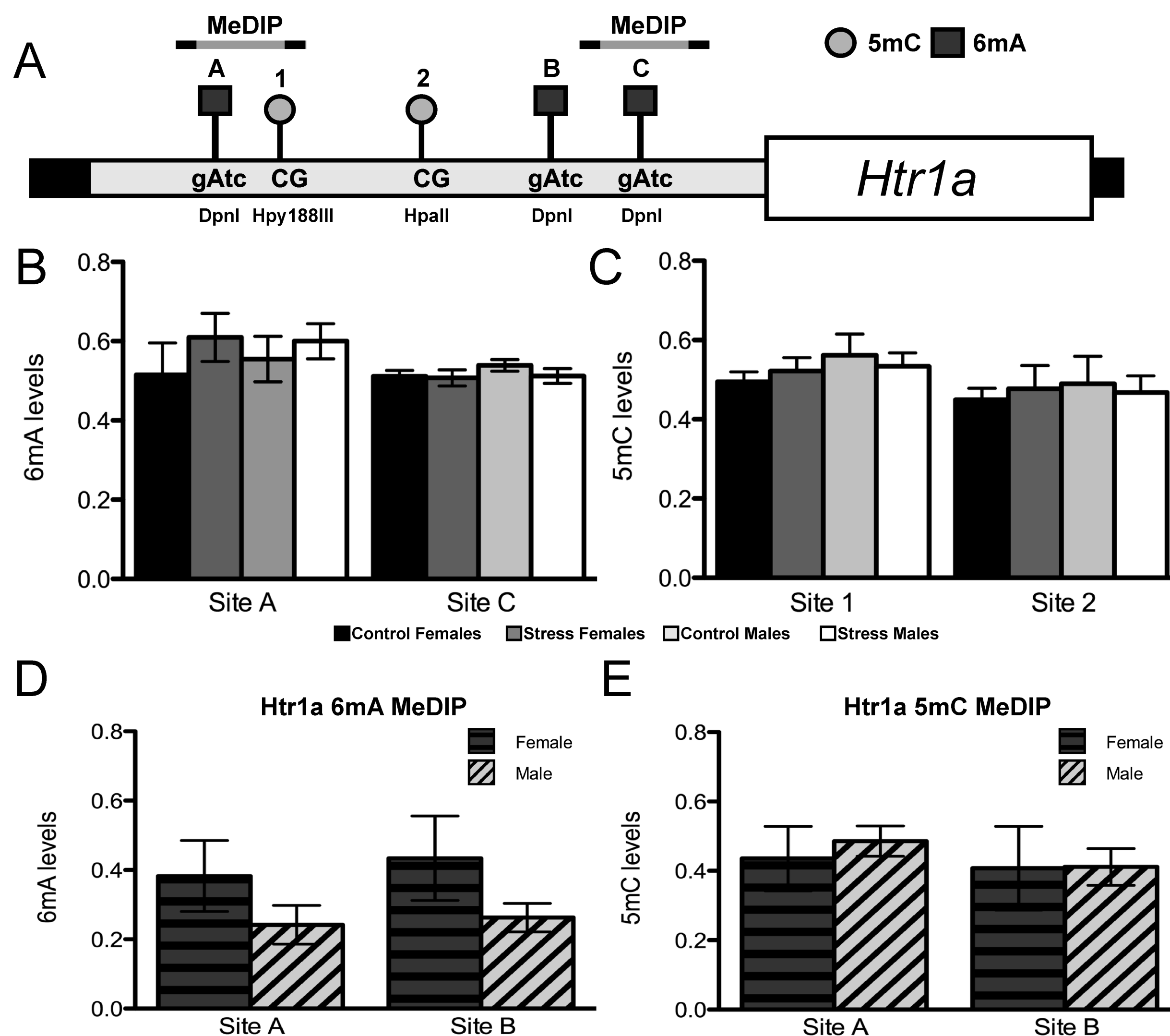

Figure 4: *(A)* Schematic of *Htr1a* gene promoter, showing individual methylation sites analyzed. Vertical bars indicate regions also examined using MeDIP. Note: no methylation was detected at GATC Site B. *(B)* MDRE analysis shows no differences in 6mA methylation at two distinct GATC sites in the *Htr1a* promoter. *(C)* MSRE analysis shows no differences in 5mC methylation at two distinct CG sites *(D)* 6mA MeDIP analysis shows no significant differences in 6mA abundance. *(E)* 5mC MeDIP analysis shows no significant differences in 5mC abundance.

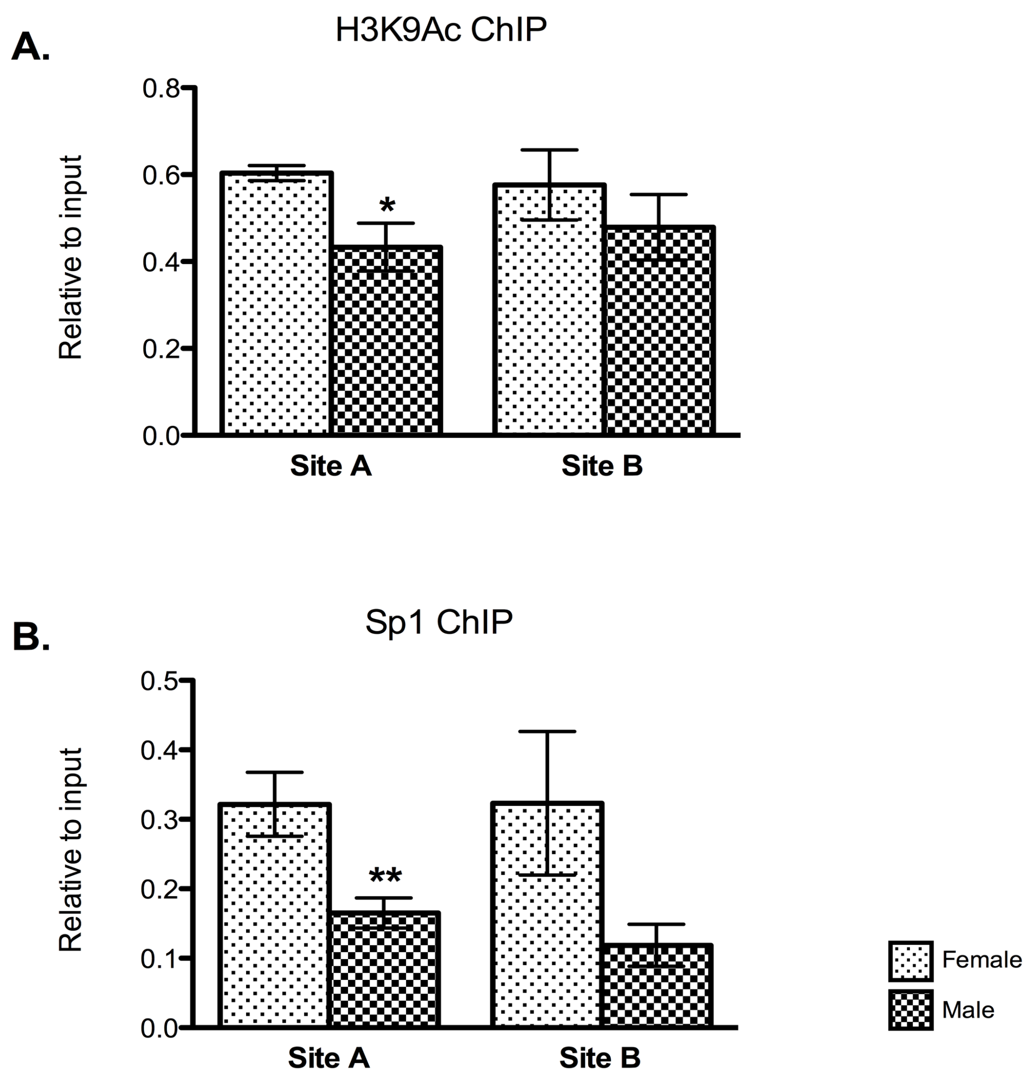

Figure 5: Chromatin immunoprecipitation (ChIP) data for two different antibodies in unhandled P35 male and female amygdalae, using primers to examine the *Htr2a* promoter at Sites A and B. *(A)* Females show significantly greater levels of H3K9 acetylation at Site A, indicating there is more transcriptional activity when compared to males. *(B)* Females also show increased Sp1 transcription factor binding at Site A. $^{**}p<0.01$, $^{*}p<0.05$.

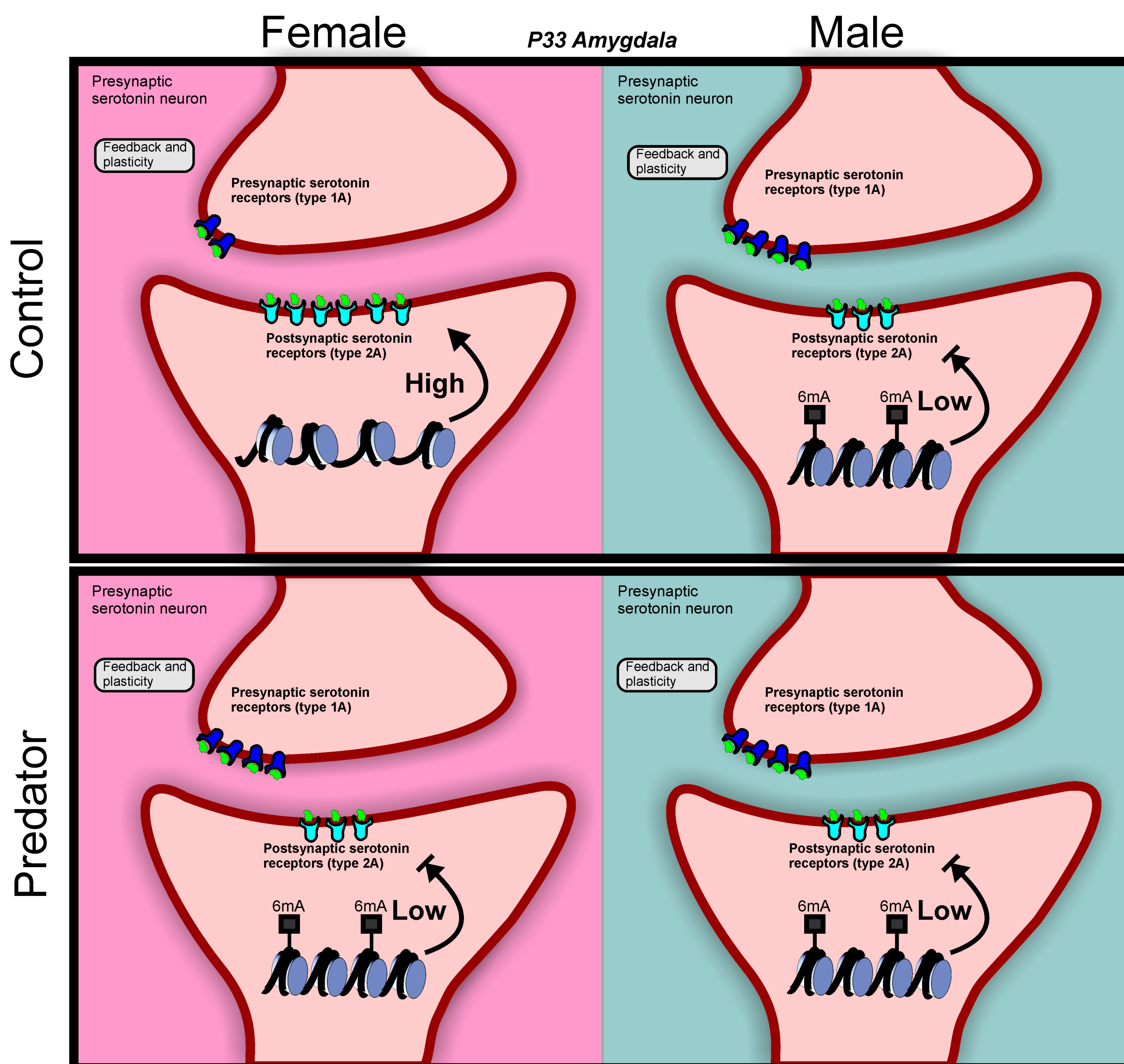

Figure 6: Model showing predicted effect of biological sex and stress on 5-HT signaling in the juvenile amygdala. Htr1a is depicted as a pre-synaptic autoreceptor, capable of inhibiting 5-HT release into the synaptic terminal. Htr2a is shown post-synaptically, capable of initiating signal transduction events in response to 5-HT signaling. Chromatin states for the *Htr2a* promoter are predicted to relaxed or condensed based on 6mA status. *Clockwise from top left*: Control females

have low levels of Htr1a and high levels of Htr2a, indicating heightened sensitivity to 5-HT signaling. Low levels of 6mA allow for a relaxed chromatin state in the *Htr2a* promoter and greater access by the transcriptional machinery. Control males have both higher levels of 6mA in the *Htr2a* promoter, leading to condensed chromatin and diminished transcription of Htr2a, and higher levels of Htr1a presynaptically. This would be predicted to cause blunted activation by 5-HT release. Stress males show no significant differences when compared to control males; stress females phenocopy control and stress males.

CHAPTER V: SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

We hypothesized that sex differences in methylation and demethylation patterns would confer risk and resilience to altered development; specifically, that environmental stressors may synergize with a sex-typical epigenomic framework to produce sex-specific maladaptive behavior and/or physiology. The data presented here supports this hypothesis, and furthermore adds to the literature by identifying a novel epigenetic regulator in the mammalian brain, N^6-methyladenine (6mA).

SUMMARY

In **Chapter II**, we report that neonatal perturbation to the amygdalar expression of a putative player in DNA demethylation, growth arrest and DNA damage inducible factor beta (Gadd45b), results in long-lasting changes to gene expression and juvenile social behavior, but not anxiety-like behavior. Specifically, males given intra-amygdalar infusions of Gadd45b siRNA from P0-P2 exhibit a dramatically increased drive to engage in social play behavior, but no preference for a social stimulus in the three-chambered task for sociability. These results appear on the surface to be contradictory; the heightened frequency of 'rough and tumble' play would typically be classified as an increased drive to participate in social interactions, which are rewarding to the individual (Panksepp and Beatty, 1980; Panksepp et al., 1984; Panksepp and Lahvis, 2007). However, when this was not born out in the social interaction test, we came to the conclusion that the status of the social stimulus was an important factor, i.e., novel vs. familiar individuals ('play' is scored in the home cage with littermates; the social interaction test takes place in a novel environment with an unfamiliar, age- and sex- matched individual). We can only speculate as to the neural circuitry involved in mediating these behavioral responses, as samples

were micro-dissected and processed for qPCR, thus losing anatomical resolution and fiber distribution between brain regions.

One possibility may be disturbances to the arginine vasopressin (AVP) and/or oxytocin (the so-called "love hormone") systems, which facilitate social recognition (Dantzer et al., 1987; Bluthe et al., 1990), pair-bonding (Cho et al., 1999; Ross et al., 2009; Walum et al., 2012), and trust (Kosfeld et al., 2005). These peptide hormones also promote in-group vs. out-group affiliative behaviors (Declerck et al., 2010; De Dreu et al., 2011) and are thought to be involved in the social deficits associated with autism spectrum disorders (ASDs) and psychopathology (Guastella et al., 2010; Dadds et al., 2014). Given the Gadd45b siRNA-infused animals' atypical lack of preference to spend time in the social chamber in the sociability test—a test developed to study mouse models of autism (Yang et al., 2001; Crawley, 2005; Carter et al., 2011)—and their strong drive to engage in play with conspecifics, this peptide system would be an attractive candidate for future analysis into Gadd45b-mediated social impairments. Indeed, the transient decrease we observed at postnatal day 2 (P2) of MeCP2 expression raises the important possibility that Gadd45b may play a role in the etiology of ASDs.

More concretely, we show evidence in **Chapter II** that expression of the α_2-adrenoceptor (Adra2a) is significantly altered in the juvenile brain of Gadd45b siRNA treated animals. This finding is consistent with data obtained from a naturally arising animal model of attention deficit and hyperactivity disorder (ADHD), the spontaneous hypertensive rat (SHR). Specifically, SHRs engage in increased play behavior (Ferguson and Cada, 2004), show decreased social exploration (Calzavara et al., 2011), and have decreased expression of Adra2a in the brain (Olmos et al.,

1991). Importantly, when we examined DNA methylation in the Adra2a promoter of Gadd45b siRNA treated animals we found an immediate increase in 5-methylcytosine (5mC) methylation at P2 that lasted into the juvenile period. This change in methylation is consistent with the expected role of Gadd45b in mediating DNA demethylation.

In **Chapter III** we examined whether there were sex differences in Gadd45b expression given our finding that Gadd45b knockdown resulted in increased play behavior—a sexually dimorphic behavior observed across a wide variety of mammalian species (Whiting and Edwards, 1973; Olioff and Stewart, 1978; Caro, 1981; DiPietro, 1981; Humphreys and Smith, 1987; Goy and Deputte, 1996; Ward et al., 2008). Gadd45b is one of three proteins in its family, along with Gadd45a and Gadd45g. We examined its expression in two regions of the brain that are known to densely express steroid hormone receptors and exhibit sexually dimorphic gene expression: the amygdala and the hypothalamus. As discussed in **Chapter I**, the male testes begin synthesizing and secreting testosterone, which causes permanent reorganization of the neural epigenome and masculinization of the brain. In rats, there are two major surges of testosterone—one on embryonic day 18, and one again on P0, or the day of birth. We have shown previously in our lab that there are sex differences in a number of epigenetic regulators, such as MeCP2 (Kurian et al., 2007), DNMT3a (Kolodkin and Auger, 2011), and NCoR (Jessen et al., 2010) at P1. In **Chapter III** we provide evidence that of the three family members, only Gadd45b exhibits a sex difference shortly after birth, and—as far as we know— only in the amygdala. We went on to show that steroid hormones organize this difference in mRNA expression levels, as dihydrotestosterone (DHT)-treated females show reduced expression of Gadd45b when compared to oil-treated females. We found that at P25, an important developmental time point

when juvenile social play behavior begins to emerge, the sex difference in Gadd45b remained. Furthermore this could be explained at least in part by increased cytosine methylation at an estrogen receptor α (ERα) response element (ERE) in the male *Gadd45b* gene promoter.

Gadd45b is an immediate early gene; its expression is induced in response to a wide variety of environmental stressors (Liu et al., 2005; Gupta et al., 2006; Engelmann et al., 2007; Porterfield et al., 2007; Sultan et al., 2012).We were interested in Gadd45b induction in response to cytokines (Liu et al., 2013), as altered immune system functioning is becoming increasingly well-known as a risk factor in the etiology of psychiatric conditions and altered social behavior (Alleva et al., 1998; Weil et al., 2006; Bilbo and Schwarz, 2012; Cole et al., 2012; Taylor et al., 2012; Provençal et al., 2013; Hennessy et al., 2014; Hodes et al., 2014). Gadd45b itself acts as a potent inducer of cytokine expression in peripheral white blood cells (Lu et al., 2004), and we observed local changes in the amygdala to the expression of proinflammatory cytokines interleukin (IL)-6 and IL-1β (**Chapter II**, (Kigar et al., 2015)). In unpublished work supplemented here, we present data suggesting that an ecologically relevant form of early life stress (ELS), predator odor exposure (POE), is sufficient to increase expression of Gadd45b at P3 (**Fig. 1**); this increase in expression is eliminated peripubertally at P33 (**Fig. 2**). When we examined play behavior in POE animals, we found a strongly reducing effect of stress (**Fig. 3**). The directionality of gene expression (increased Gadd45b expression) and play behavior (decreased play) is opposite to, but consistent with, what we found in **Chapter II** (wherein reduced Gadd45b via siRNA infusion led to increased play behavior at the juvenile period). It is also consistent with baseline sex differences wherein males have less amygdalar Gadd45b during development and exhibit higher levels of play behavior in the juvenile period.

Pressingly, it is unclear why we do not see a sex difference in Gadd45b in the P3 early life stress study. We hypothesize that this may be due to differences in the neonatal environment; specifically, the early life stress study presented in **Chapter IV** was conducted at a different institution where there was environmental enrichment and different food, bedding, and caging requirements compared to the experiments presented in **Chapters II** and **III**. In addition, animals used in sex difference experiments were unhandled, and it has previously been suggested that neonatal handling, as was necessary in the stress study, can disrupt sexual differentiation by altering circulating levels of testosterone (Grota, 1971; Moore et al., 1991). We cannot say at present whether there was some residual stress effect of handling the controls animals that may be obscuring a sex difference.

Perhaps unsurprisingly, we observe that neonatal predator odor exposure (POE) causes a lasting increase in anxiety-like behavior. Our Gadd45b manipulation was local to the amygdala and did not result in changes to anxiety, whereas stress is holistic in the sense that it affects every tissue of the body vis-à-vis the hypothalamic pituitary adrenal (HPA) axis. Briefly, a stress-inducing event will initiate production of corticotrophin releasing hormone (CRH) and AVP in the periventricular nucleus (PVN) of the hypothalamus; these peptide hormones are released into the median eminence, where they gain access to the anterior pituitary. The anterior pituitary will then produce and release adrenocorticotropic hormone (ACTH), ultimately stimulating release of corticosteroids (cortisol in humans, corticosterone in rodents) from the adrenal gland. These corticosteroids permeate all tissues of the body, mobilizing energy stores in preparation for the sympathetic nervous system’s fight or flight response.

To better understand the 'output' underlying POE-induced increases in anxiety, we turned to the serotonergic system, as it is well-characterized for its role in anxiety and depression (i.e., the most commonly prescribed drugs for these conditions are selective serotonin reuptake inhibitors, or SSRIs). Previously published work from our lab showed that changes in the neonatal environment impinge on the expression of serotonin receptor type 2a (Htr2a) and furthermore that there are sex differences in its expression within the juvenile amygdala (Edelmann et al., 2013). In **Chapter IV**, we extend these data with the observation that there are sex differences in Htr2a expression as early as P3, and confirm our previous finding that this sex difference persists into the juvenile period. In humans, females are more typically diagnosed with anxiety disorders (see **Chapter I**), but our data and others' (Goel and Bale, 2010) consistently show that, in rodents, juvenile females have higher levels of Htr2a mRNA—suggesting resiliency. Interestingly, sex differences in the anxiety-like behavior of adult rats show that females explore the open arm of the elevated plus maze more frequently (Johnston and File, 1991; Zimmerberg and Farley, 1993), and additional analyses indicate that puberty is a critical factor in development of anxious temperament (Imhof et al., 1993). We did not observe a sex difference in percent time spent investigating, but our Htr2a data may be an indication that post-pubertally, females would show resilience in the task.

Importantly, we describe for the first time that a 'new' mammalian epigenetic mark, N^6-methyladenine (6mA), is spatially and temporally regulated by early life stress (ELS) in the *Htr2a* promoter, and that this phenomenon is sex-specific. While 6mA has been well characterized in prokaryotes and a handful of unicellular species (Hale et al., 1994; Wion and

Casadesús, 2006), until recently it was thought to be nonexistent in most metazoan species. In a series of pioneering papers using an advanced form of high performance liquid chromatography (HPLC) published by Chuan He's group in the journal *Cell*, total 6mA levels were characterized in green algae (Fu et al., 2015), nematodes (Greer et al., 2015), and fruit flies (Zhang et al., 2015). Groundbreaking though it undoubtedly was, open questions as to whether substantive levels of 6mA existed in mammalian cells remained. We show for the first time that 6mA acts as a repressive epigenetic marker in the mammalian genome, negatively regulating Htr2a expression, preventing Sp1 transcription factor binding at a well-conserved promoter site (Zhu et al., 1995; Falkenberg et al., 2011), and associating with a less active chromatin state. Given the canonical status of 5mC in mammalian epigenomic research, and researchers' myopic focus on it, we feel this discovery opens up new and exciting future directions to understand gene transcription. The presence of a sex difference in 6mA abundance also offers the tantalizing possibility of its acting in concert with 5mC and the histone code to facilitate sexual differentiation of the brain.

CONCLUSIONS AND FUTURE DIRECTIONS

As it is now well established that there are periods of heightened brain 'sensitivity', or plasticity, which are critical in defining a particular developmental trajectory, it becomes imperative that we understand what factors may engender a negative outcome. This dissertation set out to address whether sex differences in the epigenome determine risk or resilience to what may loosely be defined as maladaptive behavioral and physiological changes. We have provided evidence that an epigenetic 'eraser' of 5mC, Gadd45b, plays an important role in the species-typical formation of social behaviors, that this protein is environmentally sensitive to a

psychosocial stressor, and that there are sex differences in its expression that may partly underlie a well-studied example of sexually dimorphic social behavior (i.e., juvenile social play). One major conclusion we draw from this data is that Gadd45b may be 'putting the brakes' on juvenile social play behavior; reducing its levels via intraamygdalar siRNA infusions caused an increase in play behavior, whereas early life stress—which increased neonatal expression of Gadd45b—resulted in a dramatically decreased level of play.

We have furthermore shown that, though this particular epigenetic signaling pathway may be a mitigating factor in one behavioral aspect of our POE rodent model of early life stress, it by no means acts exclusively. Our unexpected discovery that 6mA levels are higher in males and increased specifically in the *Htr2a* promoter of POE-stressed females, and that this may predispose to increased anxiety-like behavior, provides important new avenues to explore and raises many urgent and important questions, discussed below.

What proteins are involved in the manipulation of 6mA levels?

Presently, there is a gap in our knowledge about the proteins responsible for regulating 6mA. Of note, we have attempted to examine methylation and expression of the *Htr2a* gene in our Gadd45b siRNA-treated samples and found no evidence to support a role for Gadd45b (data not shown). However, this does not eliminate the possibility that Gadd45b may play an indirect role in the development of 6mA-linked anxiety-like behaviors. Specifically, we saw increased neonatal Gadd45b expression and decreased juvenile social play in our POE-stressed animals (**Fig. 1, 3**). Juvenile social play is a critical and rewarding component of social development (see **Chapter I** for review), and lack of play during the juvenile period profoundly alters

neurobiology in the rat (Vanderschuren et al., 1995; Robbins et al., 1996; Hall, 1998; Hol et al., 1999; Sciolino et al., 2010). It is possible that the lack of play we see in our animals may exacerbate an anxious phenotype, and indirectly affects 6mA in the brain, via a reduction in juvenile social interactions. Conversely, this interplay between behavioral phenotypes may work in reverse, such that animals are socially anxious and thus not playing; however, given our P3 data, where there is a stress effect on Gadd45b expression but not on 6mA levels, this seems unlikely.

In terms of the enzymatic manipulation of 6mA levels in mammals, it has been suggested that either DNA methyltransferase 1 (Dnmt1) or methyltransferase 4 (Mettl4) may do the job, based on sequence homology to the bacterial protein deoxyadenosine methylase (Dam) (Heyn and Esteller, 2015; Luo et al., 2015), though there is no biochemical evidence at this time to say definitively. In terms of demethylation, the *Drosophila* equivalent of mammalian ten eleven translocation (Tet), DNA 6mA demethylase (Dmad) is clearly implicated in the removal of 6mA during embryogenesis (Zhang et al., 2015). The authors provide evidence that, while Dmad is capable of acting enzymatically on both 5mC and 6mA as substrates *in vitro*, *in vivo* it shows strong preference for 6mA; however, they do not speculate on why this might be.

We have previously knocked down Tet1 with siRNA in the neonatal rat amygdala, using methods equivalent to those seen in **Chapter II**; our initial attempts to characterize whether levels of 6mA were altered gave positive, though inconclusive, results. Specifically, at P2, there appears to be an increase in Htr2a mRNA and a corresponding decrease in 6mA; however, this is not consistent with the known role of Tet1 as a demethylase and requires further examination

(data not shown). We speculate that the affinity of Tet1 for 5mC vs. 6mA may depend on the availability of transcriptional cofactors, and that this may vary over the course of development. In any event, this will be an important future area for study given that 6mA may predict the development of anxiety (**Chapter IV**), and is of very low abundance in the epigenome of mammals (Heyn and Esteller, 2015), thus representing an attractive future pharmacological target.

Why have both 5mC and 6mA to perform the same function?

Our evidence suggests that 6mA acts as an epigenetic marker of repression; recall that increased levels were associated with decreased transcription factor binding and decreased acetylation of histone H3, in addition to the decrease in gene transcription. In essence this would then appear to be a redundant epigenetic modification, given that 5mC is a well-established repressive factor in gene transcription. One attractive explanation that has previously been posited is that, when compared to 5mC, 6mA is a more stable epigenetic modification. That is, C → T transitions are the single most common mutation in the genome, perhaps owing to thymidine's relatively late appearance over the course of evolution (Poole et al., 2001). This appears to be due to the spontaneous ability of cytosine to deaminate in an aqueous environment, which results in a uracil-like analog. In fact, mouse models in which the base excision repair (BER) enzyme methyl binding domain 4 (Mbd4) is knocked out accumulate C → T transitions and also develop tumors (Millar et al., 2002). Adenine does not appear to be subject to these same mutational susceptibilities, and thus may be used preferentially by stem cells or during development. In fact, whereas 5mC was previously believed to be a very stable modification, emerging evidence suggests it undergoes rapid turnover (Wu and Zhang, 2010; Auger et al., 2011; Auger and Auger,

2013). 6mA is perhaps a more stable DNA modification than 5mC and therefore preferred in a context where absolute integrity of the epigenome is paramount, e.g. in stem cells, during embryogenesis, etc.

In conclusion, we have shown here that subtle changes to the epigenome can have profound and lasting consequences on behavior. We also present exciting evidence suggesting that 5mC may not be the only player in epigenetic development, and propose that 6mA may be equally important in laying the groundwork for risk or resilience to mental health disorders over the course of development.

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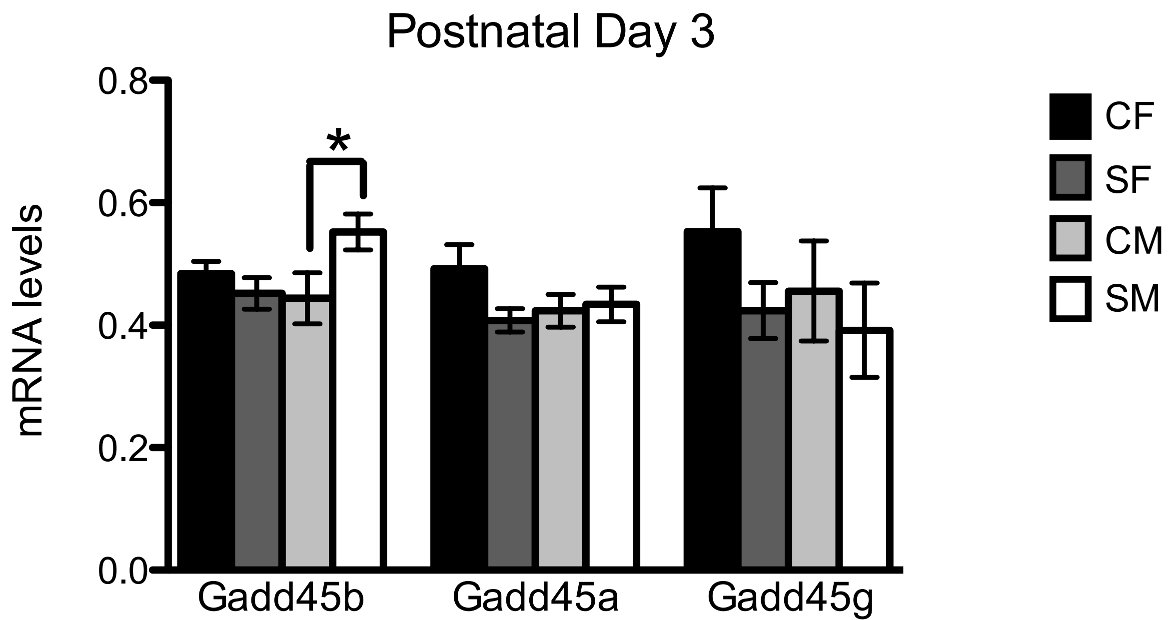

Figure 1: mRNA expression of Gadd45 family members in the postnatal day 3 (P3) amygdala of control females (CF), stressed females (SF), control males (CM), and stressed males (SM). There was an overall effect of stress, and post hoc analysis revealed this effect was in males specifically. *p<0.05

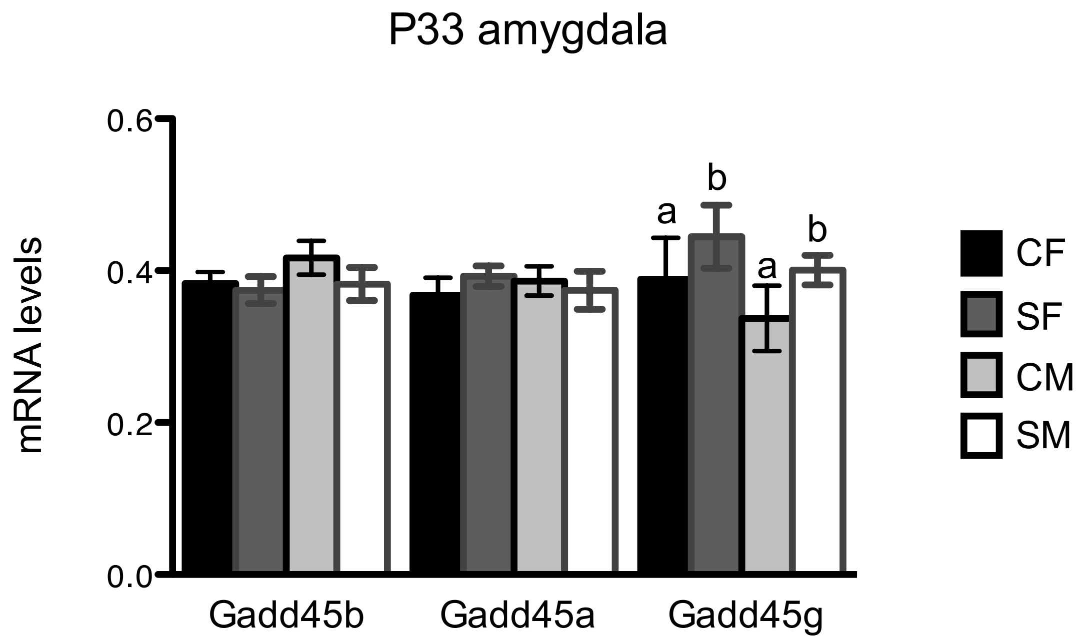

Figure 2: mRNA expression of Gadd45 family members in the postnatal day 33 (P3) amygdala of control females (CF), stressed females (SF), control males (CM), and stressed males (SM). There was an overall effect of stress on the expression of Gadd45g in both males and females, indicated by ‘b’.

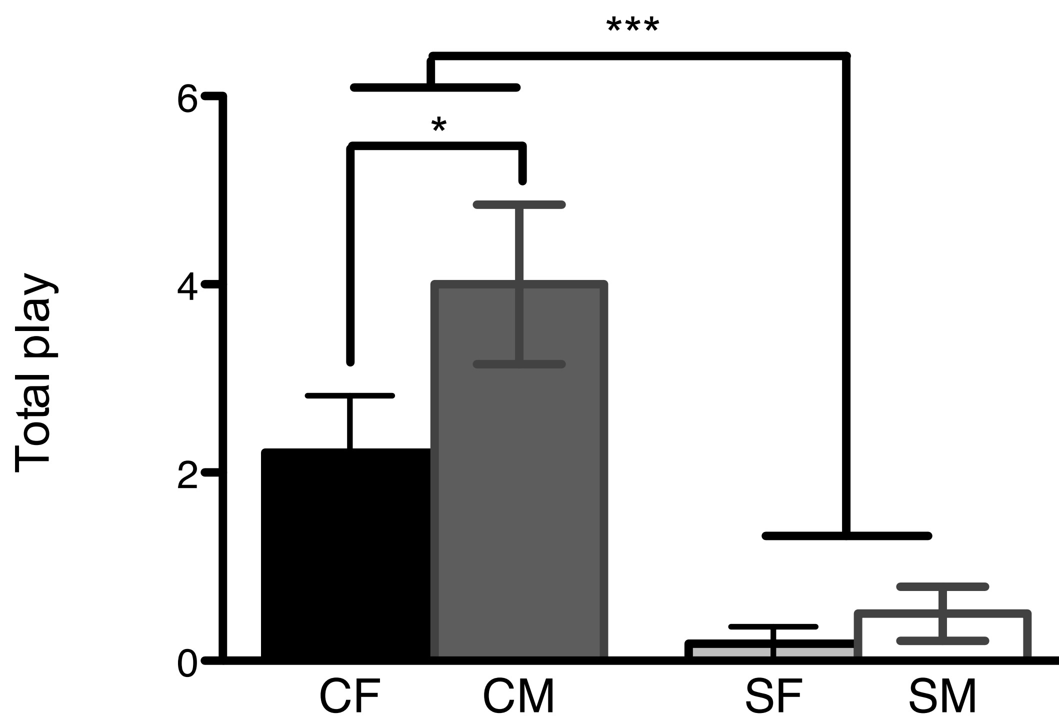

Figure 3: Overall incidence of juvenile social play behaviors (including pinning, pouncing, chasing, and boxing) on postnatal day 25 (P25)—typically considered the beginning of the play behavior period. Control females (CF), stressed females (SF), control males (CM), and stressed males (SM). There was an overall interaction, and an overall effect of stress. This effect is consistent with previously published work (Takahashi et al., 1992). Post hoc analysis revealed an expected sex difference in the controls. ***p<0.001, *p<0.05