The epigenetic organization of sex differences in vasopressin expression within the rat amygdala

Robin Forbes-Lorman

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The dissertation is approved by the following members of the Final Oral Committee:
Anthony Auger, Professor, Psychology, University of Wisconsin-Madison.
Catherine Auger, Assistant Professor, Psychology, University of Wisconsin-Madison.
Wilson Chung, Assistant Professor, Biological Sciences, Kent State University
Catherine Marler, Professor, Psychology, University of Wisconsin-Madison.
Lauren Riters, Professor, Zoology, University of Wisconsin-Madison

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Abstract

Sex differences in the brain may underlie risk for neurological disorders. Arginine vasopressin (AVP) is implicated in several behavioral disorders and acts in the brain to influence social behaviors in rats. Male rats express more AVP than females within several brain regions, and this is largely organized by testosterone and its metabolites during early postnatal development. However, the mechanism by which these hormones establish male-typical AVP remains unclear. **I hypothesized that hormones organize sex differences in AVP through epigenetic modifications.**

First, I examined whether methyl-CpG-binding protein 2 (MeCP2) plays a role in the organization of AVP expression within the amygdala by infusing MeCP2 siRNA into the amygdala during the first three days of postnatal life. I found that a transient reduction in MeCP2 decreases AVP, galanin, and AR expression within the male amygdala. Only the effect on AVP lasts into adulthood, highlighting a unique function of MeCP2 in sexual differentiation.

I then examined whether there are sex differences in DNA methylation within the *Avp* promoter region that correspond to the sex difference in AVP expression, and whether these sex differences are organized by hormones during development. I found that females had higher levels of methylation than males at two CpG sites within the *Avp* promoter region, and estradiol decreased methylation at one of these sites.

While the importance of AVP regulation by testosterone is well known, data also suggest that progesterone acts upon progestin receptors (PRs) to suppress AVP and AVP-dependent social recognition. I examined the role of PRs in the organization of AVP-dependent behaviors and AVP expression during early postnatal life. Blocking PRs improved social recognition in juvenile animals and increased juvenile social play in females to male-typical levels. AVP mRNA was increased within the female, but not the male amygdala. Furthermore, females had higher methylation at two CpG sites within the *Avp* promoter region than males, and blocking PRs decreased methylation in females.

These data suggest that testosterone organizes AVP in part through epigenetic modifications. Furthermore, PRs also play a role in its organization. This research contributes to our understanding of the epigenetic regulation of sex differences.

Abbreviations

ACHe, acetylcholinesterase
AR, androgen receptor
AVP, arginine vasopressin; *Avp,* AVP gene
BST, bed nucleus of the stria terminalis
BSTMP, medial posterior division of the bed nucleus of the stria terminalis
CBP, CREB-binding protein
CMA, centromedial amygdala
CpG, cytosine-guanine
CRE, cAMP response element
CREB, cAMP response element-binding protein 1
DHT, dihydrotestosterone
DNMT3a, DNA methyltransferase 3a
EIA, enzyme immunoassay
EPM, elevated plus maze
ER, estrogen receptor
ERE, estrogen response elements
Foxg1, forkhead box protein G1
GAPDH, glyceraldehyde-3- phosphate dehydrogenase
GFAP, glial fibrillary acidic protein; *GFAP*, GFAP gene
GRE, glucocorticoid response element
HDAC, histone deaceytlase
HPRT, hypoxanthine-guanine phosphoribosyltransferase
ICC, immunocytochemistry
ICV, intracerebroventricular
-ir, immunoreactive
LS, lateral septum
LSV, ventral lateral septum
M, Marie
MeA, medial amygdala
MeCP2, methyl-CpG-binding protein 2; *MECP2,* MeCP2 human gene; *Mecp2,* MeCP2 rat gene
MSREs, methylation-sensitive restriction enzymes
NCOR, nuclear receptor corepressor
Pir, piriform cortex
PN, postnatal day
POA, preoptic area
PR, progestin receptor
PRE, progestin response element
PRKO, PR knockout
PVN, paraventricular nucleus of the whypothalamus
RID, ratio of discrimination
RTT, Rett syndrome
SCN, suprachiasmatic nucleus
SDN, sexually dimorphic nucleus of the preoptic area
siRNA, small interfering RNA
SON, supraoptic nucleus
TBS, tris-buffered saline
VMHdm, dorsomedial ventromedial hypothalamus
Ywhaz, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

Chapter 1

Introduction

Not surprisingly, there are differences between male and female brains in most species. Often, these differences relate to functional sex differences in behavior. For example, males have a larger sexually dimorphic nucleus of the preoptic area (SDN-POA) than females and this appears to be important for male sexual behavior (De Jonge *et al.*, 1989). There are sex differences in numerous neurological disorders, such as Rett syndrome (RTT), autism, schizophrenia, attention deficient disorder, and affective disorders (Hagberg, 1985; Tang *et al.*, 1996; Aleman *et al.*, 2003; American Psychiatric Association, 2013), and understanding sex differences in the brain may provide insight into risk and resilience for these neurological disorders. Arginine vasopressin (AVP) is a small peptide that is implicated in several sexually dimorphic behavioral disorders in humans, including autism, schizophrenia, and affective disorders (Frank and Landgraf, 2008; Surget and Belzung, 2008; Lukas and Neumann, 2013). Interestingly, there are also sex differences in AVP expression in several brain regions. In rats, males express higher levels of AVP than females within the medial amygdala (MeA) and bed nucleus of the stria terminals (BST), as well as in the terminal fields of these AVP cells (Van Leeuwen *et al.*, 1985; De Vries and al-Shamma, 1990). However, the molecular mechanisms by which hormones organize this sex difference remain unclear. My dissertation research focused on elucidating some of the epigenetic factors involved in the hormonal regulation of AVP.

AVP is an endocrine hormone and neuropeptide.

AVP was first identified as an endocrine hormone, when extracts of the pituitary gland were found to regulate blood pressure (Oliver and Schafer, 1895). With the discovery that this hormone was synthesized in the hypothalamus (Bargmann, 1951) and the isolation and characterization of the peptide (Turner *et al.*, 1951), researchers began to elucidate the role of AVP in the hypothalamic-neurohypophyseal system. AVP is synthesized primarily in large magnocellular neurons within two hypothalamic regions: the paraventricular nucleus of the hypothalamus (PVN) and the supraoptic nucleus (SON). These neurons project into the

posterior pituitary and release AVP from their axon terminals and into the bloodstream, where it regulates peripheral functions such as water absorption in the kidneys, constriction of smooth muscles, and glucose metabolism (Heller, 1965; Altura and Altura, 1977; Brownstein *et al.*, 1980; Gainer and Wray, 1994). There are also magnocellular AVP neurons within the suprachiasmatic nucleus (SCN), which are important for the regulation of circadian rhythms (Vandesande *et al.*, 1975).

The ability of AVP to work within the central nervous system was first proposed with the finding that it played a role in conditioned place preference (De Wied, 1965; De Wied, 1971). This led to the "neuropeptide concept", which suggested that neuropeptides have non-hormonal functions within the central nervous system (De Wied, 1984). Many of these functions are regulated by smaller, parvocellular AVP neurons located outside of the hypothalamus, within the MeA and the BST (Caffe and Van Leeuwen, 1983; van Leeuwen and Caffe, 1983; Sofroniew, 1985). AVP cells within these two brain regions are sexually dimorphic, and have similar morphology, projection sites, and responsiveness to steroids. However, differences between the AVP cells within these brain regions are discussed in the General Discussion. These neurons project to the lateral septum (LS), ventral septum, olfactory tubercle, lateral habenular nucleus, midbrain central grey, dorsal raphe nucleus, pontine peripeduncular nucleus, locus coeruleus, and ventral hippocampus (De Vries and Miller, 1998). The AVP within these regions is involved in a diverse number of functions, including social recognition, aggression, male sex behavior, and thermoregulation (De Vries and Miller, 1998), some of which will be discussed below.

AVP binds to three main types of receptors: V1a, V1b, and V2; as well as V3, which appears to be restricted to the anterior pituitary (De Keyzer *et al.*, 1994). While all of these types are found throughout various tissues in the periphery (Jard, 1981), V1a and V1b receptors are prevalent in many brain regions (Jard *et al.*, 1987; Tribollet *et al.*, 1988; Phillips *et al.*, 1988), while V2 receptors are less common (Foletta *et al.*, 2002). V1a binding does not occur within the PVN or SON (Phillips *et al.*, 1988), but is prevalent throughout the MeA, BST, and their

projection sites (Johnson *et al.*, 1993; Veinante and Freund-Mercier, 1997). Unlike AVP, V1a receptor mRNA is not sexually dimorphic (Szot *et al.*, 1994). V1b receptors have been studied less, partially due to methodological difficulties localizing V1b receptors, but more recent data suggests that these receptors are also important in the brain (Stevenson and Caldwell, 2012). Both V1a and V1b receptors are G-protein coupled receptors that activate phospholipase C and mobilize calcium; and the binding of AVP to these receptors can have inhibitory, excitatory, and modulatory activity, depending on the brain region and context (Jard *et al.*, 1987; Landgraf and Neumann, 2004; Raggenbass, 2008).

AVP within the rat amygdala is socially relevant.

AVP is an important nonapeptide hormone in most mammals. The homolog in non-mammalian vertebrates is vasotocin, and vasopressin and oxytocin, which only differ by one amino acid, arose following gene duplication. Interestingly, the functions of the various vasotocin-related nonapeptides within the PVN and SON as a neurohypophyseal hormone are largely conserved across species, but their role within the MeA and BST in influencing behaviors are more complex and diverse (Goodson, 2008). Although AVP within the rodent brain is the focus of this research, it is interesting to note that these peptides play diverse, yet important, roles in social behaviors across species. The AVP neurons located within the MeA, BST, and their projection sites are involved in a diverse number of social behaviors. In rats, AVP within these regions influences social recognition, juvenile social play behavior, anxiety-like behavior, and sex behavior.

AVP within the male LS is necessary for social recognition, which is the ability to retain the memory of a previously encountered conspecific individual, primarily through acting on V1a receptors (Dantzer *et al.*, 1988; Everts and Koolhaas, 1999; Bielsky *et al.*, 2004; Bielsky *et al.*, 2005a). V1b receptors play a similar, but smaller role in males as well (Wersinger *et al.*, 2002). However, although AVP facilitates social recognition in females, it is not necessary (Bluthe and

Dantzer, 1990; Engelmann *et al.*, 1998; Bielsky *et al.*, 2005b). AVP within the MeA and BST may also influence juvenile social play behavior in rats. Several studies have found a correlation between decreased play and decreased AVP expression within the MeA and BST (Forbes-Lorman *et al.*, 2012; Taylor *et al.*, 2012), and another study found that intracerebroventricular injection of a V1a receptor antagonist decreased play in males and increased it in females, while an injection into the LS increased play in males and decreased it in females (Veenema *et al.*, 2013).

AVP within the LS facilitates anxiety-like behavior in male rats, as blocking AVP receptors decreases anxiety-like behavior (Landgraf *et al.*, 1995; Liebsch *et al.*, 1996; Everts and Koolhaas, 1999), and appears to do so through binding V1a receptors (Bielsky *et al.*, 2004). AVP does not appear to modulate anxiety-like behavior in females (Bielsky *et al.*, 2005b). Lastly, lesion studies have demonstrated that the BST and MeA are important for both male and female sex behavior (Nance *et al.*, 1974; Harris and Sachs, 1975; Valcourt and Sachs, 1979), and AVP has been implicated in both. For example, AVP administration can reverse the decline in male sex behavior following castration (Bohus, 1977) and V1a receptor antagonists administered centrally facilitate female sex behavior (Sodersten et al., 1985).

The amygdala is sexually dimorphic and socially relevant.

The amygdala is an almond-shaped structure located in the medial temporal lobe. The amygdala is diverse and complex, and is broadly involved in emotional processing across species (Phelps and LeDoux, 2005). More specifically, the amygdala is involved in aggression, fear, anxiety, and social behaviors (Sah *et al.*, 2003). Importantly, the amygdala plays a role in the organization of juvenile social play behavior. Male rats normally play more than female rats and lesions to the amygdala disrupted social play behavior in males but not females (Meaney *et al.*, 1981).

The amygdala is comprised of many subnuclei, which are typically grouped into the basolateral, cortical-like, and centromedial groups (LeDoux, 2007). AVP is located within the medial nucleus (MeA), which along with the central nucleus, makes up the centromedial amygdala (CMA). The projections from the CMA are primarily GABAergic, in contrast to the majority of the amygdala, which contains glutamatergic projections (Swanson and Petrovich, 1998).

The MeA is a particularly sexually dimorphic and hormonally responsive region within the rat brain (Sar and Stumpf, 1973; Sheridan, 1979; Simerly *et al.*, 1990; Shughrue *et al.*, 1997). In addition to having more AVP-expressing neurons, males also have more CCK-expressing cells (Micevych *et al.*, 1988), substance P fibers (Malsbury and McKay, 1989), and a larger total volume in posterodorsal region (Mizukami *et al.*, 1983; Hines *et al.*, 1992). Interestingly this latter sex difference is due to a difference in excitatory synapses, not neuron number or soma size (Cooke and Woolley, 2005). There are also sex differences in several epigenetic proteins within the developing amygdala, with females expressing higher levels of Methyl-CpG-binding protein 2 (MeCP2) (Kurian *et al.*, 2007), DNA methyltransferase 3a (DNMT3a) (Kolodkin and Auger, 2011), and nuclear receptor corepressor (NCoR) (Jessen *et al.*, 2010) than males.

Sex differences depend on both organizational and activational effects of hormones.

Early research clearly demonstrated that circulating hormone levels in adult animals are required for normal sex behavior. For example, castration in adulthood dramatically decreases male sex behavior and it can be restored by testosterone administration in male rats (Beach and Holz, 1946) and guinea pigs (Grunt and Young, 1952). Likewise, female sex behavior is dependent on circulating estrogen and progesterone levels (Frank and Fraps, 1945; Ward, 1946). It had also been demonstrated that testicular secretions are necessary for the development of the male reproductive system (Jost, 1947; Jost, 1948; Jost, 1957), and that

testosterone specifically during early postnatal life has the ability to disrupt normal female reproduction (Wilson *et al.*, 1940; Bradbury, 1941; Wilson *et al.*, 1941; Wolfe *et al.*, 1944).

In 1959, Phoenix et al. showed that females exposed perinatally to doses of testosterone below that which altered morphology of the genitalia displayed decreased female sex behavior in adulthood, as well as increased male sex behavior at higher doses (Phoenix *et al.*, 1959). This suggested that, similar to sexual differentiation of the gonads, sexual differentiation of the brain requires the appropriate hormones during a critical time in development. They proposed that steroid hormones act directly on neural tissue both during development to permanently *organize* sex differences and in adulthood to reversibly *activate sex differences*.

Although evidence suggests that steroid hormones also organize sex differences during puberty (Schulz *et al.*, 2004; Schulz and Sisk, 2006; Ahmed *et al.*, 2008), many sex differences are organized due to steroid hormone action during early postnatal development (Cooke *et al.*, 1998; Simerly, 2002a). Male rats experience a surge in serum testosterone levels during late embryonic and early postnatal life (Weisz and Ward, 1980; Rhoda *et al.*, 1984), and testosterone is metabolized into two principle metabolites: estradiol, which binds to estrogen receptors (ERs) and the androgen, dihydrotestosterone (DHT), which binds to androgen receptors (ARs) (Naftolin *et al.*, 1971). The binding of the steroid hormone to its receptor induces a conformational change, which allows the complex to bind to a response element on the DNA and act as a transcription factor (Jensen *et al.*, 1968; Walters, 1985; Carson-Jurica *et al.*, 1990). Testosterone and its metabolites are critical for masculinizing and defeminizing the brain (Tonjes *et al.*, 1987; Casto *et al.*, 2003; Macleod *et al.*, 2010). Masculinization refers to the increase of male-typical traits, and is primarily dependent on ERα and AR; while defeminization is the decrease of female-typical traits, and is primarily dependent on ERβ (Kudwa *et al.*, 2006; Zuloaga *et al.*, 2008). Females are resistant to the masculinization and defeminization effects of testosterone because they do not have testes that release testosterone, perinatal ovarian

production of estrogen is minimal, and they express an estrogen-sequestering protein during development (Germain *et al.*, 1978).

Testosterone organizes sex differences in AVP via phenotypic differentiation.

AVP expression within the BST and MeA is regulated by gonadal steroid hormones in most mammals. In rats, males express higher levels of AVP than females within the MeA and BST, as well as in the terminal fields of these AVP cells (Van Leeuwen *et al.*, 1985; De Vries and al-Shamma, 1990). AVP neurons in the MeA and BST, but not the PVN and SON, are colocalized with ERs (Axelson and Leeuwen, 1990), ARs (Zhou *et al.*, 1994), and progestin receptors (PRs) (Auger and De Vries, 2002), and AVP expression within these neurons is regulated by the steroid hormones that bind all three of these receptors.

The sex difference in AVP is largely regulated by the activational effects of circulating testosterone levels, with dramatically decreased AVP in castrated adult males, and restoration of normal AVP expression following testosterone administration (De Vries *et al.*, 1984; DeVries *et al.*, 1985; Van Leeuwen *et al.*, 1985). The ability of testosterone to regulate AVP levels appears to be through its metabolism into both estradiol and DHT. Although estradiol alone had a larger effect in restoring normal levels than DHT, only administration of both fully restored AVP (De Vries *et al.*, 1986; De Vries *et al.*, 1994; Wang and De Vries, 1995).

However, females still have less AVP even when gonadectomized and treated with the same hormone regimen as males in adulthood (De Vries and al-Shamma, 1990; De Vries *et al.*, 1994; Wang and De Vries, 1995), suggesting that testosterone during early postnatal life also plays a role in the organization of AVP expression patterns. Like many other sex differences, castration of neonatal males results in female-typical levels of AVP and testosterone administration to neonatal females results in male-typical levels (De Vries *et al.*, 1983; Wang *et al.*, 1993; Han and De Vries, 2003). The sensitive period for the organization of AVP extends through the first week of postnatal life (De Vries *et al.*, 1983; Wang *et al.*, 1993). The

organizational effect of testosterone on AVP expression depends primarily on its metabolism into estradiol; however, the sex difference in AVP is also influenced by DHT. That is, administering estradiol, but not DHT, to neonatal females increases AVP to male levels, but DHT still increases expression compared to control females (Han and De Vries, 2003). Interestingly, although DHT acts on ARs, DHT appears to stimulate AVP expression through the action of its metabolite 5α-androstane-3β,17β-diol on ERβ (Pak *et al.*, 2007; Pak *et al.*, 2009), as 5α-androstane-3β,17β-diol has a higher affinity for ERβ than it does AR (Weihua *et al.*, 2002).

Within the MeA, there is a sex difference in the number of AVP cells, but not in the amount of AVP mRNA per cell (Wang and De Vries, 1995), suggesting that individual cells within the male and female MeA have the same ability to express AVP in response to circulating hormones, but the number of cells that have this ability differs between the sexes. However, the mechanism by which testosterone and its metabolites act during development to organize this sex difference remains unclear. Testosterone can organize sex differences in the brain though apoptosis (Chung *et al.*, 2000), neuronal migration, neurogenesis, and neurotransmitter plasticity (Simerly, 2002b). However, the sex difference within AVP is not likely due to differential neurogenesis or neuronal migration, as the cells are born on embryonic day 12-13, before the surge in testosterone induces sexual differentiation (al-Shamma and De Vries, 1996; Han and De Vries, 1999). Furthermore, a more recent study demonstrated that the sex difference is not due to differential apoptosis (De Vries *et al.*, 2008). AVP in the amygdala and BST is co-expressed with galanin (Miller *et al.*, 1993), and it is thought that AVP expression is induced in a subset of these neurons in a sexually dimorphic manner (Planas *et al.*, 1995; Han and De Vries, 1999). This suggests that males and females have the same number of cells that have the potential to express AVP and that testosterone induces AVP expression in these cells. The mechanism by which hormones establish the phenotypic differences in this cell population is unknown.

Progesterone regulates AVP expression.

While testosterone and its metabolites clearly play an important role in both the organization and regulation of AVP expression, it has been demonstrated that progesterone acts upon PRs to regulate expression levels in the adult brain. For example, systemic administration of progesterone to adult male rats decreases AVP expression within the CMA and BST, but not the paraventricular thalamic nucleus, which does not contain sexually dimorphic AVP (Auger and Vanzo, 2006). PRs also regulate AVP-dependent social behavior in adult males. Progesterone administration impairs social recognition in males (Bychowski and Auger, 2012) and infusion of AVP into the lateral septum rescues this behavior (Bychowski and Auger, 2011).

However, the role of PRs during development is less clear. Several lines of evidence suggest that PRs are important for the development of the male brain. Males express PR as early as embryonic day 20 in many brain areas (Quadros *et al.*, 2007) and express more PR than females within the medial preoptic area (mPOA) and anteroventral periventricular nucleus until PN10 (Wagner *et al.*, 1998; Quadros *et al.*, 2002). This transient sex difference in neonatal PR expression suggests that PRs are important for the organization of the male-typical brain during this critical period of development. However, data suggests that PR does not play a role in organizing sex differences in AVP, because although knocking out PRs increases AVP expression, it does so in both males and females and the former still express higher levels (Rood *et al.*, 2008). This suggests that PRs regulate AVP expression in the amygdala and BST; however, as PR knockout (PRKO) animals lack PRs both during and after development, its role in organizing male and female levels of AVP remains unclear.

The regulation of AVP is largely influenced by a promoter region

The *Avp* gene contains three exons, which encode a putative signal peptide, AVP, neurophysin 2, and copeptin (Schmale *et al.*, 1983). Known regulatory elements include a

promoter region that begins 1 kilobases upstream of the transcriptional start site (Mohr and Richter, 1990) and a downstream enhancer region (Gainer *et al.*, 2001). The genes encoding oxytocin and AVP are closely linked in rodents and humans, in tail-to-tail orientation, and the enhancer region is within an intergenic region.

Within the promoter region, several response elements have been identified, including two estrogen response elements (EREs), a glucocorticoid/progestin response element (GRE/PRE), two cAMP response elements (CREs), and several activator protein (AP) 1 and 2 binding sites (Mohr and Richter, 1990; Iwasaki *et al.*, 1997; Shapiro *et al.*, 2000). Estrogen acting on both ERα and ERβ facilitates *Avp* gene transcription through one of the EREs (Shapiro *et al.*, 2000). Interestingly, the presence of the GRE/PRE provides a mechanism by which progestins action on PRs may regulate AVP (Auger and Vanzo, 2006; Rood *et al.*, 2008). There are four predicted CpG islands, where many CpG sites are located in close proximity, within the *Avp* gene. One island is located within the promoter region, one spans the second and third exons, and two are within the downstream enhancer region (Weber *et al.*, 2007).

DNA methylation can regulate gene transcription.

Epigenetics refers to marks on the chromatin that can alter gene transcription without altering the genetic code, and typically includes histone modification and DNA methylation. While the majority of CpG sites in the mammalian genome, including gene bodies, intergenic regions, and transposons, are highly methylated (Rabinowicz *et al.*, 2003; Rakyan *et al.*, 2004; Eckhardt *et al.*, 2006), CpG sites within gene regulatory regions tend to be unmethylated, and changes in methylation of these regions corresponds to changes in gene expression (Bird *et al.*, 1985; Bird, 1986). Methylation of CpG sites typically results in the silencing of gene transcription. For example, DNA methylation contributes to X-inactivation (Mohandas *et al.*, 1981) and genomic imprinting (Li *et al.*, 1993).

While the methyl marks that contribute to genomic imprinting and X-inactivation are thought to be stable throughout the lifespan, not all methyl marks are permanent. Active demethylation is a complicated process that removes methyl groups through enzymatic reactions (Wu and Zhang, 2010; Bhutani *et al.*, 2011; Teperek-Tkacz *et al.*, 2011), and plays a critical role in both developing (Wossidlo *et al.*, 2011; Gu *et al.*, 2011) and mature organisms (Martinowich *et al.*, 2003). For example, cell differentiation involves the regulation of gene expression through changes in both methylation and demethylation at key developmental time points (Ehrlich, 2003).

DNA methylation can repress gene transcription through two different mechanisms. The first is by directly interfering with the binding of a transcription factor (Comb and Goodman, 1990). The second is through recruitment of methyl-binding proteins, which then change the conformational state of the chromatin. Methyl-binding proteins typically recruit corepressor complexes, resulting in the compaction of the chromatin and transcriptional repression. DNA is negatively charged, while lysine residues on the tails of histones are positively charged. Without any modifications to the histones, the positive and negative charges attract, causing chromatin compaction and thereby transcriptional repression. The presence of acetyl groups on the histone tails neutralizes the positive charge, which repels the DNA and opens up the chromatin for gene transcription. While coactivators hyperacetylate the histone tails, thus loosening the chromatin and increasing transcription, corepressors typically contain histone deaceytlase (HDACs) (McKenna *et al.*, 1999). HDACs remove acetyl groups from the histone tails, thus reintroducing the positive charge, resulting in the compaction of the chromatin and transcriptional repression.

MeCP2 is a methyl-binding protein implicated in neurodevelopmental disorders.

A well-known methyl binding protein is MeCP2, which binds to methylated DNA and typically recruits the corepressor complex, Sin3a, to repress gene transcription (Meehan *et al.*,

1992; Nan *et al.*, 1997; Nan *et al.*, 1998). However, recent evidence suggests that MeCP2 can also activate gene transcription through interactions with the coactivator cAMP response element-binding protein 1 (CREB) (Chahrour *et al.*, 2008). In fact, the majority of genes altered in both the hypothalamus and cerebellum of MeCP2-deficient mice are downregulated and it was confirmed that MeCP2 binds directly to several of these genes, suggesting that MeCP2 can activate gene expression (Chahrour *et al.*, 2008; Ben-Shachar *et al.*, 2009). Furthermore, MeCP2 can regulate specific genes, such as BDNF (Chen *et al.*, 2003; Martinowich *et al.*, 2003), as well as global gene expression (Nan *et al.*, 1997; Skene *et al.*, 2010), and may also play a role in RNA splicing (Young *et al.*, 2005).

MeCP2 is encoded by an X-linked gene, *MECP2,* which is critical for normal brain function, as disruptions in typical MeCP2 expression are associated with several neurological disorders (Gonzales and LaSalle, 2010). For example, mutations in *MECP2* cause Rett syndrome, an X-linked disorder that is diagnosed more often in females; and reductions in MeCP2 expression or function can also occur in autism, which is more prevalent in males. Interestingly, there are sex differences in MeCP2 expression in the developing brain, with females having higher levels of MeCP2 mRNA and protein than males within the amygdala and POA on PN1, but not PN10 (Kurian *et al.*, 2007).

Sex differences are regulated by epigenetic phenomena.

Several studies suggest that hormones regulate gene expression through altering methylation patterns. For example, males express higher levels of AR mRNA than females, and testosterone administration decreases AR mRNA and increases methylation of the AR promoter (Kumar and Thakur, 2004). Additionally, castration of adult males reduces AVP expression in the BST and increases methylation of the *Avp* promoter region; while testosterone replacement reverses these patterns (Auger *et al.*, 2011). These data suggest that sex differences in the adult brain can be maintained by differential methylation.

It appears that the surge in testosterone during early development organizes some sex differences in the brain through differential methylation. For example, female rats express less ERα in the POA than males (Rainbow *et al.*, 1982; DonCarlos and Handa, 1994; Yokosuka *et al.*, 1997), and have lower levels of methylation within the ERα exon1b promoter region in the POA than both males on PN10 (Kurian *et al.*, 2010). Additionally, administering testosterone to neonatal females results in male-typical mRNA and methylation patterns.

Epigenetic proteins also appear to be involved in organizing sex differences. For example, a transient reduction in MeCP2 within the developing amygdala during early postnatal life reduces the levels of juvenile social play in males to female-typical levels (Kurian *et al.*, 2008). Juvenile male rats play at higher frequencies than females, and this is organized by testosterone action within the developing male amygdala (Meaney and Stewart, 1981; Smith *et al.*, 1997). Combined, these data suggest that some sex differences are organized by epigenetic phenomena, specifically differential methylation and methyl-binding proteins.

Thesis outline and aims

Although much is known about the hormones involved in the organization and regulation of AVP expression, the mechanisms by which these hormones exert their effects remain unclear. It appears that the sex difference in AVP is due to phenotypic differences (al-Shamma and De Vries, 1996; Han and De Vries, 1999; De Vries *et al.*, 2008), and is possibly mediated by epigenetic phenomena. The proposed research will determine whether 1) MeCP2 within the developing amygdala plays a role in organizing AVP expression, 2) the sex difference in AVP expression within the amygdala is organized by differential DNA methylation, and 3) PRs within the developing amygdala organize AVP and AVP-dependent behaviors.

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Chapter 2

Neonatal MeCP2 is important for the organization of sex differences in vasopressin expression.

RM Forbes-Lorman, JJ Rautio, JR Kurian, AP Auger, CJ Auger (2012) Epigenetics 7(3):230-8

Abstract

Several neurodevelopmental disorders are marked by atypical methyl-CpG-binding protein 2 (MeCP2) expression or function; however, the role of MeCP2 is complex and not entirely clear. Interestingly, there are sex differences in some of these disorders, and it appears that MeCP2 has sex-specific roles during development. Specifically, recent data indicate that a transient reduction in MeCP2 within developing amygdala reduces juvenile social play behavior in males to female-typical levels. These data suggest that MeCP2 within the amygdala is involved in programming lasting sex differences in social behavior. In the present study, we infused MeCP2 or control siRNA into the amygdala of male and female rats during the first three days of postnatal life in order to assess the impact of a transient reduction in MeCP2 on arginine vasopressin (AVP), a neural marker that is expressed differentially between males and females and is linked to a number of social behaviors. The expression of AVP, as well as several other genes, was measured in two-week old and adult animals. Two-week old males expressed more AVP and galanin mRNA in the amygdala than females, and a transient reduction in MeCP2 eliminated this sex difference by reducing the expression of both gene products in males. A transient reduction in MeCP2 also decreased androgen receptor (AR) mRNA in two-week old males. In adulthood, control males had more AVP-immunoreactive cells than females in the medial amygdala, bed nucleus of the stria terminalis, and in the fibers that project from these cells to the lateral septum. A transient reduction in MeCP2 eliminated this sex difference. Interestingly, there were no lasting differences in galanin or AR levels in adulthood. Reducing MeCP2 levels during development did not alter estrogen receptorα, neurofilament, or Foxg1. We conclude that a transient reduction in MeCP2 expression in the developing male amygdala has a transient impact on galanin and AR expression but a lasting impact on AVP expression, highlighting the importance of MeCP2 in organizing sex differences in the amygdala.

Introduction

Methyl-CpG-binding protein 2 (MeCP2) is encoded by an X-linked gene, *MECP2,* that is critical for normal brain function, as disruptions in MeCP2 are associated with several neurological disorders (Gonzales and LaSalle, 2010). Typically, MeCP2 plays an important role in gene repression by binding to methylated DNA (Meehan *et al.*, 1992; Nan *et al.*, 1997) and recruiting corepressor proteins to reduce gene transcription (Nan *et al.*, 1997; Martinowich *et al.*, 2003; Skene *et al.*, 2010). However, recent evidence suggests that MeCP2 can also have a number of other functions, such as activating gene transcription (Chahrour *et al.*, 2008), modifying RNA splicing (Young *et al.*, 2005), and affecting neural differentiation/maturation and synaptic plasticity (Jung *et al.*, 2003; Tsujimura *et al.*, 2009; Na and Monteggia, 2010). Mutations of *MECP2* cause Rett syndrome (RTT), an X-linked disorder that is diagnosed more often in females. Reductions in MeCP2 expression or function can also occur in autism, which is more prevalent in males. Therefore, in order to understand sex differences in some neurodevelopmental disorders, it becomes important to elucidate the function of MeCP2 in the context of sexual differentiation of the brain.

Recent data indicate that MeCP2 is important for typical functioning of mature neurons. For example, re-expression of MeCP2 in juvenile or adult mice restores many of the deficits induced by MeCP2 deletion (Guy *et al.*, 2007; Giacometti *et al.*, 2007). Likewise, using an inducible MeCP2 knockout model, reductions in MeCP2 later in life can recapitulate many of the deficits seen in the MeCP2 knockout (McGraw *et al.*, 2011). Together, these data suggest that some of the neurological outcomes induced by MeCP2 disruption may not be programmed during development, but rather are a consequence of loss of adult MeCP2 function. However, the importance of MeCP2 during development in organizing lasting changes in behavior has also been recently established. Specifically, a transient reduction in MeCP2 within the developing amygdala during the first three days of postnatal life reduces the levels of juvenile social play in males to female-typical levels (Kurian *et al.*, 2008). Given the intriguing finding that

typical male juvenile social behavior is disrupted by decreased MeCP2 expression in the developing amygdala, we wanted to examine if MeCP2 disrupts sex differences in gene expression within the amygdala. Juvenile male rats play at higher frequencies than females, and this sex difference is organized by testosterone action within the developing male amygdala (Meaney and Stewart, 1981; Smith *et al.*, 1997). Testosterone is metabolized into two principle ligands: estradiol, which binds to estrogen receptors (ERs) and the androgen, dihydrotestosterone, which binds to androgen receptors (ARs) (Naftolin *et al.*, 1971) and these receptors are critical for organizing many sex differences in the brain and behavior. It is unclear, however, what genes are altered by reduced MeCP2 expression within the developing amygdala to effect this change in social behavior in juvenile male rats. AVP expression within the bed nucleus of the stria terminalis (BST) and the medial amygdala (MeA) is known to be involved in a number of social behaviors (Winslow *et al.*, 1993) (Bielsky and Young, 2004; Bielsky *et al.*, 2005), including social play behavior (Veenema and Neumann, 2009). Additionally, it has been recently suggested that MeCP2 can regulate vasopressin (AVP) expression within the hypothalamus (Murgatroyd *et al.*, 2009). These data suggest AVP as a potential candidate when examining genes that are regulated by MeCP2 and influence social behavior.

AVP expression within the BST and MeA is highly sexually dimorphic, as adult males express 2-3 times more AVP in these areas (De Vries and al-Shamma, 1990). Moreover, this sex difference is organized during early postnatal life by steroid hormone actions on ER and AR (Wang *et al.*, 1993; De Vries *et al.*, 1994; Han and De Vries, 2003a). Therefore, we hypothesized that transiently reducing MeCP2 during this time period would have a lasting impact on AVP expression levels within the amygdala, possibly through its action on ER or AR (Wilson *et al.*, 2008; Westberry *et al.*, 2010). Also, as AVP is co-expressed in a subset of galanin expressing neurons (Miller *et al.*, 1993a) we also examined galanin expression.

Methods

General:

Subjects. Sprague-Dawley rats supplied by Charles River Labs (Wilmington, MA) were bred in our animal facility. Dams were checked daily to determine the day of birth, and were allowed to deliver normally. The rats were housed under a 12:12 light/dark cycle with food and water available *ad libitum.* This research was approved by the University of Wisconsin Institutional Animal Care and Use Committee.

Infusions into the amygdala. Infusions were done with a stereotaxic device modified for neonatal rats, and coordinates from center and bregma suture lines were empirically determined for direct infusion to the amygdala (1mm lateral, 2mm caudal, and 5.5mm ventral from the pial surface at our nose cone angle). Under cold anesthesia, the amygdala was bilaterally infused with either MeCP2 or control siRNA (experiments 1 and 2) or SKF 38393 (experiment 3). Further details are described below. After infusions, pups were allowed to recover under a warm lamp before being returned to the dam.

Statistical analysis. All statistical comparisons were carried out using SigmaStat statistical software version 3.5 (Systat Software, Inc.). Statistical comparisons for experiments 1 and 2 were carried out using a two-way ANOVA and post hoc tests were conducted using a Fisher LSD when a significant interaction was found. Statistical comparisons for experiment 3 were carried out using a student's t-test and linear regression. Outliers were determined using GraphPad Software, Inc. (http://www.graphpad.com). Effects with a p value of < 0.05 were considered statistically significant.

Experiment 1: Impact of neonatal infusions of MeCP2 siRNA on mRNA expression on PN14

Subjects and general procedures: Twenty-four pups were collected from 4 dams on postnatal day (PN) 0 (day of birth). Neonatal pups were pooled and assigned to a treatment group (5 male control siRNA, 7 male MeCP2 siRNA, 7 female control siRNA, and 5 female

MeCP2 siRNA). Neonatal pups were then sorted to form litters consisting of mixed sex and treatment groups and then placed back with a dam. Pups were infused with MeCP2 or control siRNA on PN0-2 and then remained with the dam until sacrificed two weeks later on PN14.

MeCP2 siRNA infusions. MeCP2 (sc-35893, Santa Cruz Biotechnology) and control siRNA (sc-37007, Santa Cruz Biotechnology) were resuspended to 100μM in the supplied siRNA diluent with Lipofectamine LTX reagent (15338100, Invitrogen). The amygdala was bilaterally infused with 1μL (100pmol) of either MeCP2 or control siRNA. Each animal received three infusions 24 h apart (i.e., on PN0, PN1, and PN2). We have previously shown that this amount of MeCP2 siRNA induced a transient and localized reduction in MeCP2 expression within the amygdala (Kurian *et al.*, 2008).

Tissue processing. Animals were sacrificed by rapid decapitation on PN14. Brains were extracted and the amygdala and hypothalamus were dissected and snap frozen in isopentane on dry ice. Total RNA was isolated using the AllPrep DNA/RNA Mini Kit (80204, Qiagen). RNA concentrations were determined using a Qubit Fluorometer (Invitrogen) and cDNA was generated using ImProm-II™ Reverse Transcription System (A3800, Promega) in an Eppendorf MasterCycler Personal PCR machine. The samples were stored at -80˚C.

Real-time polymerase chain reaction (RT-PCR). Amplifications were done using a Stratagene Mx3000P™ real-time PCR system using GoTaq® Colorless Master Mix (M7132, Promega), SYBR green, and ROX as a reference dye. Primers designed to target AVP, galanin, AR, ERα, NF-L, Foxg1 MeCP2, Ywhaz, and HPRT1 were run in duplicate (Table 1). HPRT1 and Ywhaz were used as normalizing genes to control for subtle variations in sample concentrations. The amplification protocol was as follows: an initial denaturing step at 95˚C for two minutes, followed by 40 cycles of a 95˚C melting step for 30 seconds, 60˚C annealing step for 30 seconds, and a 72˚C elongation step for 30 seconds. Following amplification, a dissociation melt curve analysis was performed to ensure the purity of PCR products. Data were

analyzed with the following program term settings based on Invitrogen recommendations: (1) amplified based threshold, (2) adaptive baseline set v1.00 to v3.00 algorithm and (3) smoothing moving average with amplification averaging three points. Relative cDNA levels were calculated using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001).

Experiment 2: Impact of neonatal infusions of MeCP2 siRNA on immunocytochemistry in adulthood.

Subjects and general procedures: To examine the stability of the alterations in AVP, galanin, and AR expression observed on PN14, we replicated and extended the findings of experiment 1. AVP, galanin, and AR immunoreactivity (ir) was examined in brains collected from adult rats that were used in a previous study examining the impact of neonatal infusions of MeCP2 siRNA on juvenile social play behavior and juvenile sociability (Kurian *et al.*, 2008). The infusion paradigm was the same as in experiment 1 above. Briefly, 40 pups from 5 dams were collected on the day of birth and assigned to a treatment group (10 male control siRNA, 10 male MeCP2 siRNA, 10 female control siRNA, and 10 female MeCP2 siRNA). The amygdala was bilaterally infused with 1µL (100pmol) of either MeCP2 or control siRNA on PN0, PN1, and PN2. All animals were gonadectomized and then implanted with Silastic® tubing (2.5cm long, 1.5mm inner diameter, 2.4mm outer diameter, Dow Corning Corp.) filled with testosterone (Sigma-Aldrich, Inc.) at 6 months of age to standardize testosterone levels, as AVP expression is highly regulated by circulating levels of testosterone.

Tissue processing. At 7.5 months of age, rats were deeply anaesthetized with isoflurane and rapidly decapitated. At least 1ml of trunk blood was collected from each animal, immediately placed on ice, and centrifuged for 10 min at 10,000g's. The serum was removed and stored in a clean tube at -20°C until used in a testosterone assay. The brains were fixed by immersion in 5% acrolein in 0.1M Tris-buffered saline (TBS) for 24 h followed by 72 h in 30% sucrose dissolved in TBS. Following fixation, the brains were rapidly frozen in methylbutane and stored

at -80°C until tissue processing. Brains were cut using a cryostat into 4 series of 40μm sections each and stored at -20°C in a cryprotectant solution (60% sucrose, 30% ethylene glycol, 2% polyvinylpyrrolidone) until use in immunocytochemistry.

Immunocytochemistry for AVP, galanin, and AR. All steps in were carried out at room temperature unless otherwise noted. All of the tissue targeted by each antibody was processed at the same time. All washes were done three times for 5 min each in either TBS or 0.3% TBS-Triton X-100 (TBS-T). For each antibody, one of every fourth section of the brain was washed and incubated for 15 min in 0.1% sodium borohydride in TBS. After washes, the tissue was placed in a blocking solution containing 20% normal goat serum and 3% hydrogen peroxide in TBS for 60 min. The tissue was incubated for 18 h with rabbit anti-vasopressin (1:10,000; 20069, Immunostar, Inc), rabbit anti-galanin (1:7000; T-4333, Bachem Americas), or rabbit anti-androgen receptor, PG-21 (1:2000; 06-680, Millipore) in a solution of 2% goat serum and 0.5% gelatin in TBS-T. After washes, the tissue was incubated for 90 min in biotinylated goat anti-rabbit IgG (1:500; BA-1000, Vector Labs) in TBS-T with 0.5% gelatin. The tissue was then washed and incubated in Vectastain Elite ABC peroxidase (PK-6101; Vector Laboratories) using half the concentration recommended by the manufacturer for 60 min. After washes, the tissue was incubated for 10 min using a Vector SG kit (SK-4700, Vector Laboratories) diluted in TBS-T with 0.5% gelatin using the concentration recommended by the manufacturer. After three final washes, the sections were mounted, air dried, and cover-slipped with Permount (SP15-100, Fisher Scientific).

Immunocytochemical Analysis. The following areas were chosen for analysis: the bed nucleus of the stria terminalis (BST) and the medial amygdala (MeA), which express steroid-responsive AVP cells; the lateral septum (LS), which receives projections from these cells; and the paraventricular thalamic nucleus (PVT), which receives AVP projections from the suprachiasmatic nucleus, a hypothalamic region that contains non-steroid sensitive AVP cells

(De Vries *et al.*, 1984; DeVries *et al.*, 1985). The Atlas of Paxinos and Watson (Paxinos and Watson, 1986) was used to match a bilateral plate for each animal containing the LS, corresponding to plate 18; BST, corresponding to plate 21; amygdala, corresponding to plate 30; and PVT, corresponding to plate 33. These areas were inspected under bright-field illumination using an Olympus BX-61 microscope fitted with a 10x (for cell counts) or 20x (for fiber analysis) objective and an Olympus FV II digital camera. The number of AVP or galanin-immunoreactive (ir) cells in the amygdala and BST was counted at 10x by an experimenter blind to treatment groups. AVP-ir fiber area in the LS and PVT and AR-ir cell number in the CMA were determined using Olympus MicroSuite (Soft Imaging Corp.). For each of these brain regions, the same sized area was analyzed for each animal. The threshold to detect foreground was set at 15% (for AVP-ir fibers) or 5% (for AR-ir cells) above the background grey value mean that was determined individually for each animal. Microscope light intensity was kept constant throughout the imaging session for each brain region analyzed to ensure consistent measurements.

Quantification of serum testosterone levels. An enzyme immunoassay (EIA) for testosterone (582701, Cayman Chemical Company) was run on the serum collected from the animals. This EIA is based on the competition between testosterone and a testosterone-acetylcholinesterase conjugate for a limited number of testosterone-specific binding sites. Testosterone standards, serum samples diluted 1:8, and the necessary controls were loaded onto a 96-well plate coated with mouse anti-rabbit IgG. Testosterone-specific acetylcholinesterase (ACHe) tracer and rabbit anti-testosterone antiserum were added to all wells on the plate, except several of the control wells. The plate was incubated for 2 h at room temperature on an orbital shaker to allow for competitive binding. After this incubation, the plate was washed 5 times with a wash buffer. The concentration of testosterone was determined by measuring the enzymatic activity of ACHe with Ellman's Reagent, which contains the substrate

for ACHe. The product of this enzymatic reaction absorbs at 412nm. The plate developed in the dark for 1 h and absorbance was read on a plate reader equipped with a filter that reads wavelengths between 405 and 420 nm. The assay specificity is 100% for testosterone, and the intra-assay coefficient was 9.0%. The detection limit of this assay was 6pg/ml. Results were calculated using a computer spreadsheet program provided by the assay manufacturer (http://www.caymanchem.com/eiatools/promo/kit).

Experiment 3: Immunocytochemical assessment of infusion area.

Subjects and procedures: To determine possible spread of infusions into the amygdala, we infused varying doses of SKF 38393 into the amygdala and examined c-Fos-ir cells in the amygdala and BST. SKF 38393 is a D1-receptor agonist that induces c-Fos protein in several brain areas(Meredith *et al.*, 1997; Shearman *et al.*, 1997), including the amygdala and BST (Olesen and Auger, 2008), within an hour following a systemic infusion, suggesting that the number of c-Fos-ir cells in the BST would increase if the SKF 38393 infusions spread to the BST. On PN1, female rats were bilaterally infused into the amgydala with 0, 300, 600, or 1200 ng of SKF in 1µL of sterile saline. Pups recovered under a warm lamp and were sacrificed 1 h after the single infusion. Brains were fixed by immersion in 5% acrolein (Sigma-Aldrich, Inc.) in 0.1M TBS for 24 h followed by 24 h in 20% sucrose dissolved in TBS. Following fixation, the brains were then rapidly frozen in methylbutane on dry ice and stored at -80°C until tissue processing. Brains were cut into 50µm sections using a cryostat and stored in cryprotectant at -20°C. Immunocytochemistry was carried out following the procedure described in experiment 1 using a rabbit anti-c-Fos primary antibody (1:2000; sc-52, Santa Cruz Biotechnology) in a solution of 2% goat serum and 0.5% gelatin in TBS-T. The number of c-Fos-ir cells was measured with a 10x objective in the amygdala and BST (plates 30 and 21 in the atlas of Paxinos and Watson, respectively) using Olympus MicroSuite (Soft Imaging Corp.), as described above.

Results

Experiment 1: A transient, reduction in MeCP2 reduced AVP, galanin, and androgen receptor (AR) mRNA at PN14.

AVP mRNA in the amygdala. As AVP expression is sexually dimorphic and is relevant to a number of social behaviors, we examined the impact of neonatal MeCP2 siRNA on AVP mRNA expression in the amygdala two weeks after infusion. We found an interaction between sex and treatment on relative AVP mRNA expression ($F(1,15)=6.82$, $p=0.02$, Fig 1A). Post hoc comparisons indicate that control males expressed higher levels of AVP mRNA in the amygdala than both control females, ($p=0.011$), and MeCP2 siRNA-treated males ($p=0.007$).

Galanin mRNA in the amygdala. As AVP is co-expressed in a subset of galanin cells, we examined the effect of neonatal MeCP2 siRNA on galanin mRNA expression in the amygdala two weeks after infusion. Interestingly, there was a main effect of sex ($F(1,18)=4.98$, $p=0.039$) and an interaction between sex and treatment ($F(1,18)=5.41$, $p=0.032$) on relative galanin mRNA expression (Fig 1B). Post hoc comparisons indicate that control males expressed higher galanin mRNA levels in the amygdala than both control females ($p=0.005$) and MeCP2 siRNA-treated males ($p=0.008$).

AR and ERα mRNA in the amygdala. As AVP expression is organized during development by steroid action on ER and AR, we examined the effect of neonatal MeCP2 siRNA on AR and ERα mRNA expression in the amygdala two weeks after infusion. There was a main effect of treatment ($F(1,19)=8.1$, $p=0.01$) and an interaction between sex and treatment ($F(1,19)=4.5$, $p<0.05$) on relative AR mRNA (Fig 1C). Post hoc comparisons indicate that control males expressed higher levels of AR mRNA in the amygdala than MeCP2 siRNA-treated males ($p=0.002$). In contrast, there was no effect of sex ($F(1,19)=2.17$, $p=0.16$), treatment ($F(1,19)=0.03$, $p=0.86$), or an interaction ($F(1,19)=0.59$, $p=0.45$) on relative ERα mRNA in the amygdala (Fig 1D).

Neurofilament, Foxg1, and MeCP2, mRNA in the amygdala. In order assess the possibility that the changes observed in gene expression were not simply the impact of overall transcriptional dysregulation, we examined the effect of neonatal MeCP2 siRNA on neurofilament, light polypeptide (NF-L), MeCP2, and Foxg1 mRNA expression in the amygdala. NF-L is a neuron-specific cytoskeletal gene (Lee and Cleveland, 1994), and Foxg1 is a gene that encodes for a transcription factor that is important for brain development and has been recently identified to be deleted or inactivated in RTT (Jacob *et al.*, 2009; Takahashi *et al.*, 2011). MeCP2 mRNA was also assessed in order to ensure that expression levels returned to normal two weeks following the infusions on PN0-2, as previously shown (Kurian *et al.*, 2008). There was no effect of neonatal MeCP2 siRNA on the expression of any of these genes. There was no effect of sex ($F(1,18)=0.07$, $p=0.79$) or treatment ($F(1,18)=0.71$, $p=0.41$) on relative NF-L mRNA in the amygdala. There was also no effect of sex ($F(1,19)=0.57$, $p=0.46$) or treatment ($F(1,19)=0.03$, $p=0.86$) on relative MeCP2 mRNA in the amygdala. Finally, there was no effect of sex ($F(1,18)=0.09$, $p=0.77$) or treatment ($F(1,18)=0.32$, $p=0.58$) on relative Foxg1 mRNA in the amygdala.

AVP mRNA in the hypothalamus. In order to assess the possibility that the infusion of MeCP2 siRNA may have spread to the hypothalamus and altered AVP gene expression in that brain region, we examined the effect of neonatal infusion of MeCP2 siRNA into the amygdala on AVP mRNA expression in the hypothalamus. There was no effect of sex ($F(1,18)=0.001$, $p=0.97$) or treatment ($F(1,18)=0.33$, $p=0.57$) on relative AVP mRNA in the hypothalamus.

Experiment 2: Neonatal infusions of MeCP2 siRNA reduced AVP, but not galanin or AR-ir, in adulthood.

AVP-ir cell number and fiber density in the amygdala, BST and lateral septum. In order to assess if the impact of neonatal MeCP2 siRNA on AVP expression in males was maintained into adulthood; we examined AVP-ir cell number in adulthood. Our data replicate previous

findings of a sex difference in AVP-ir cell number, with males have higher AVP-ir cell number than females. More importantly, we find a lasting effect of transient reduction in MeCP2 on AVP-ir cells. That is, MeCP2 siRNA-treated males had lower AVP-ir cell number than control males. An interaction between sex and siRNA treatment was found in number of AVP-ir cells within the amygdala ($F(1,29)=8.34$, $p=0.007$, Fig 2A&4A), BST ($F(1,30)=4.58$, $p=0.04$, Fig 2B), and one of the projection sites of these cells, the lateral septum (LS) ($F(1,31)=6.55$, $p=0.016$, Fig 2C). Post hoc comparisons indicate that control males expressed more AVP-ir cells than control females in adulthood within the amygdala ($p<0.001$) and BST ($p<0.001$), as well as more AVP-ir fibers in the LS ($p<0.001$). Additionally, control males had more AVP-ir cells compared to MeCP2 siRNA-treated males within the amygdala ($p<0.001$) and BST ($p<0.001$), as well more AVP-ir fibers in the LS ($p=0.003$). As in experiment 1, there were no differences between MeCP2 siRNA-treated females and control females in any brain region examined. As expected, the number of AVP-ir cells within the paraventricular thalamic nucleus (PVT), a control region as this area is innervated by AVP cells that are not sexually dimorphic, did not differ by sex ($F(1,29)=0.006$, $p=0.97$) or treatment ($F(1,29)=0.002$, $p=0.94$, Fig 2D).

Galanin-ir cell number in the amygdala and BST. In order to assess if the impact of neonatal MeCP2 siRNA on galanin expression in males was maintained into adulthood; we examined galanin-ir cell number in adulthood. Interestingly, there was no lasting effect of a transient reduction in MeCP2 on galanin-ir cells in adulthood. There was no effect of sex ($F(1,31)=1.23$, $p=0.81$) or treatment ($F(1,31)=0.06$, $p=0.28$) on the number of galanin-ir cells in the amygdala (Fig 3A). Likewise, there was no effect of sex ($F(1,32)=0.13$, $p=0.78$) or treatment ($F(1,32)=0.08$, $p=0.73$) on the number of galanin-ir cells in the BST.

AR-ir cell number in the amygdala. In order to assess if the impact of neonatal MeCP2 siRNA on AR expression in males was maintained into adulthood, we examined AR-ir cell number in adulthood. Interestingly, there was no lasting effect of a transient reduction in MeCP2

on AR in adults. There was no effect of sex ($F(1,30)=2.4$, $p=0.13$) or treatment ($F(1,30)=0.15$, $p=0.70$) on the number of AR-ir cells in the amygdala (Fig 3B).

Testosterone EIA. We measured testosterone concentrations in adult animals that had been gonadectomized and implanted with testosterone-filled implants in order to ensure that levels did not differ between sex or treatment groups and were in the physiological range for adult males. As expected, there was no effect of sex ($F(1,29)=0.002$, $p=0.96$) or treatment ($F(1,29)=0.423$, $p=0.52$) on serum testosterone in castrated and testosterone-implanted adults (data not shown). Average testosterone levels were 1.31±0.31 ng/mL in control females, 1.29±0.35 ng/mL in control males, 1.08±0.27 ng/mL in MeCP2 siRNA-treated females, and 1.49±0.25 ng/mL in MeCP2 siRNA-treated males. These levels fall within the range of typical adult male levels of circulating testosterone (Lee *et al.*, 1975).

Experiment 3: An infusion of SKF into the amygdala did not activate c-Fos in the BST.

As we observed a decrease in AVP protein levels in the BST, we wanted to determine if our infusion spread from the amygdala to the BST. To test this, we infused SKF 38393, a dopamine D1-like receptor agonist, into the developing amygdala and examined c-Fos-ir cell number. As SKF 38393 increases c-Fos protein levels, it provides a functional marker of altered cell activity and should help us determine the spread of the infusion. Within the amygdala, animals treated with any dose SKF had more c-Fos-ir cells than saline animals ($t(9)=2.99$, $p=0.02$) and this effect was dose-dependent ($r^2=0.43$, $F(1,9)=6.88$, $p=0.03$). However, there was no effect of SKF infusion into the amygdala on c-Fos-ir cells in the BST ($t(7)=5.17$, $p=0.62$). While c-Fos-ir cells were detected around the site of infusion, there was no apparent spread of SKF 38393 medial to the optic tract, such as in the hypothalamus. These data, as well as a lack of alterations in AVP expression within the hypothalamus, suggest that there was little spread outside the amygdala.

Discussion

These data indicate that MeCP2 participates in the developmental organization of the AVP system in a sex-specific manner. In two-week old and adult male rats, a transient reduction in MeCP2 during neonatal development reduced AVP levels within the amygdala, a region critical for social and emotional behavior. We found that two-week old control males expressed more AVP mRNA within the amygdala compared to females, and a transient reduction in MeCP2 within the amygdala during the first three days of postnatal life decreased AVP mRNA expression to female levels. A reduction in MeCP2 during development had no effect on female AVP expression. In addition, these effects are lasting into adulthood, suggesting that MeCP2 is involved in the organization of male-typical AVP expression. That is, adult males had more AVP-ir cells within the amygdala, BST and fibers within the lateral septum compared to females; a transient reduction in MeCP2 within the developing male amygdala eliminates this sex difference. The functional significance of reduced AVP, including its possible influence on juvenile social play behavior, is not clear; however, these data indicate that MeCP2 is important for the organization of AVP within the amygdala in a sex-specific manner.

As AVP-ir cells in the amygdala project to the LS**,** we expect a decrease in AVP-ir fibers in this area. However, it is less clear how the infusion of MeCP2 siRNA into the amygdala decreased AVP-ir cell number in the BST. It is possible that the infusion could have spread to the BST and directly altered AVP-ir cell number; however, we have previously confirmed that the MeCP2 siRNA infusion into the amygdala does not reduce MeCP2 levels in the hypothalamus (Kurian *et al.*, 2008). Furthermore, we found that an injection of SKF 38393 into the amygdala increases c-Fos-ir labeling in the amygdala but not the BST, even though systemic SKF 38393 administration can increase c-Fos-ir label in both the amygdala and BST (Olesen and Auger, 2008). These data provide an indirect yet functional assay of spread to the BST from infusion site in the amygdala. More importantly, previous data have shown that the BST and amygdala are interconnected, and lesions of the BST disrupt AVP expression in the

amygdala (De Vries and Buijs, 1983). Based on this information, it is not surprising that a disruption in the developing amygdala would result in a similar disruption in the BST. We also show that there was no influence of reduced MeCP2 on AVP mRNA within the hypothalamus in two week-old animals. As this area is rich in AVP, but not interconnected with the amygdala, this suggests that the disruption within the BST may not have been due to the spread of siRNA.

AVP in the amygdala and BST is co-expressed with galanin (Miller *et al.*, 1993a), and it is thought that AVP expression is induced in a subset of these neurons in a sexually dimorphic manner (Planas *et al.*, 1995; Han and De Vries, 1999). While some studies have reported no sex difference in galanin (Planas *et al.*, 1994), others have reported a relatively small sex difference in galanin in both rats (Han and De Vries, 1999) and mice (Rajendren *et al.*, 2000). In the present study, two-week old males expressed more galanin mRNA than females in the amygdala; however, there was no sex difference detected in the number of galanin-ir cells in either the amygdala or the BST in adulthood. Galanin is regulated by circulating testosterone levels (Miller *et al.*, 1993b), and the adult animals in this study received testosterone-filled implants; whereas, the two-week old animals did not. As there is a sex difference in endogenous testosterone levels around this time-point (Dohler and Wuttke, 1975; Banu *et al.*, 2002), this could contribute to a sex difference in galanin mRNA expression in the two-week old animals. Interestingly, it was recently reported that young adult males express higher levels of galanin than females, and this difference declines within age as levels of galanin decrease in males (Garcia-Falgueras *et al.*, 2011). Therefore, it is possible that the sex difference in galanin levels within rodents similarly declines with age. It is also possible that sex differences in galanin expression are easier to detect with quantitative PCR. Importantly, a transient reduction in MeCP2 within the developing amygdala decreased galanin mRNA expression within the amygdala in two-week old males, but not females, and this effect was not lasting into adulthood. These data suggest that MeCP2 plays an important role in programming AVP expression into adulthood; however, the influence of MeCP2 on galanin appears transient.

The mechanism by which a transient reduction in MeCP2 disrupts the expression of these peptides is not clear. Research has shown that MeCP2 binds to the promoter region of the *Avp* gene within the paraventricular nucleus of the hypothalamus (PVN) to repress gene transcription (Murgatroyd *et al.*, 2009). Although this is not in agreement with the present findings that a reduction in MeCP2 decreases AVP expression in the BST and amygdala; this could point toward distinct functions of MeCP2 in different brain regions or at different time points, depending on the activity of other coregulatory proteins. Although MeCP2 is typically thought to mediate a decrease in gene transcription, it can also increase gene transcription through its interaction with cAMP response element-binding protein 1 (CREB1) (Chahrour *et al.*, 2008). Importantly, sex differences in cAMP pathways are thought to be critical for the organization of some sex differences. For example, CREB-binding protein (CBP) and phosphorylated CREB are both higher in males in several brain areas (Auger *et al.*, 2001; Auger *et al.*, 2002) and CBP is necessary for the organization of some sex differences in behaviors (Auger *et al.*, 2002). These data suggest that higher levels of CREB activity in males within sexually dimorphic brain regions may facilitate MeCP2-induced gene transcription and organize sex differences. Indeed, studies have shown that CREB binds to the *Avp* promoter region to activate gene transcription *in vitro* (Iwasaki *et al.*, 1997). In contrast to its role in the repression of AVP within the PVN, where the AVP is relatively steroid insensitive, MeCP2 may interact with CREB within the developing amygdala to increase steroid-dependent *Avp* gene transcription. Therefore, a reduction in MeCP2 during development could disrupt the organization of steroid hormone-dependent AVP expression within the amygdala.

Although a correlation between AVP expression and juvenile social play behavior has previously been observed (Veenema and Neumann, 2009), it is unclear whether a reduction in AVP expression could explain the reduction in juvenile social play behavior recently seen in the males by Kurian *et al.* (Kurian *et al.*, 2008). As a reduction in MeCP2 in the developing male amygdala results in lasting disruptions in both juvenile social play behavior and AVP expression,

MeCP2 may be critical for the organization of the male-typical amygdala and its related behaviors. Like many sexually dimorphic systems, juvenile social play behavior and AVP expression are organized largely by testosterone exposure in early life (Meaney and Stewart, 1981; De Vries *et al.*, 1994; Smith *et al.*, 1997; Han and De Vries, 2003b). A reduction in MeCP2 disrupts sex differences in both social play and AVP expression, suggesting that MeCP2 may be important for steroid hormone activity. Therefore, reducing neonatal MeCP2 may have a lasting impact through altering the function or expression of steroid receptors. Our data suggest that this is the case for AR, as a reduction in MeCP2 during the first three days of postnatal life decreases its expression in two week-old males. The reduction in AR expression may play a role in the decrease in AVP expression in MeCP2-siRNA treated males. This is in agreement with data indicating that neonatal treatment with dihydrotestosterone, which binds to AR, contributes to male-like AVP mRNA expression in adult rat brain (Han and De Vries, 2003a). As AR is critical in the development of social play behavior (Meaney *et al.*, 1983), a reduction in AR expression during development could also contribute to the lasting decrease in juvenile social play behavior in males.

As MeCP2 has been shown to be involved promoting neuronal differentiation of neural precursor cells (Tsujimura *et al.*, 2009), it is possible that a reduction in MeCP2 altered cell differentiation or cell death; however, previous findings from other labs indicate that sex differences in the levels of AVP are not due to cell death, but rather most likely due to phenotypical differentiation (De Vries *et al.*, 2008). Furthermore, our data indicate several other genes are unaffected by siRNA treatment, including neurofilament, Foxg1, ERα, and MeCP2. As all AVP cells are co-expressed with galanin and AR, and these co-expressed genes were not altered in adults, these data suggest that AVP differences are unlikely due to cell death, and consistent with the idea MeCP2 plays a role in the organization of sexually-dimorphic AVP cells.

While numerous studies have confirmed that MeCP2 disruptions occur in RTT, as well as several other neurodevelopmental disorders, less is known about what genes MeCP2 may

regulate within socially-relevant brain regions. As a transient reduction in MeCP2 levels has lasting consequences on AVP, but not galanin or AR expression, MeCP2 may be important for organizing certain neuronal phenotypes. Our data suggest that reductions in MeCP2 may impact male juvenile social behavior by disrupting the organization of AVP within the developing amygdala. The consequences of a lasting decrease in AVP expression in males on other social and cognitive behaviors remain to be fully explored. Interestingly, the programming of AVP expression was not altered in females, suggesting a greater resistance to neonatal disruptions of MeCP2 expression than males. It is important to note that while many of the consequences of MeCP2 loss can be reversed by postnatal restoration of MeCP2 expression (Guy *et al.*, 2007; McGraw *et al.*, 2011), the present study demonstrates that a reduction in MeCP2 during a critical time point in brain development can have lasting consequences.

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Tables

Gene	Accession #	Forward sequence	Reverse sequence	Efficiency	Product size
AVP	NM_016992.2	TGCCTGCTACTTCCAGAACTGC	AGGGGAGACACTGTCTCAGCTC	93-94%	55 bp
Galanin	M18102	GCGCTGGCTACCTTCTGG	GCTCTCAGGCAGGGGTAC	96-104%	125 bp
MeCP2	NM_022673.2	CGTCCCCTTGCCTGAAGGTTGG	CTTTCCAGCAGAGCGACCAG	92-98%	42 bp
AR	NM_012502.1	GGCAAAGGCACTGAAGAGAC	CCCAGAGCTACCTGCTTCAC	96-101%	114 bp
ERα	NM_012689.1	TCCGGCACATGAGTAACAAA	TGAAGACGATGAGCATCCAG	100-103%	109 bp
NF-L	NM_031783	ACCAGCGTGGGTAGCATAAC	GAAGAGCAGTCAGAGGTGG	96-104%	157 bp
Foxg1	NM_012560.2	GTTCAGCTACAACGCGCTCATCAT	TCACGAAGCACTTGTTGAGGGACA	93-101%	173 bp
Ywhaz	NM_013011.3	TTGAGCAGAAGACGGAAGGT	GAAGCATTGGGGATCAAGAA	95-100%	136 bp
HPRT	NM_012583	GCAGACTTTGCTTTCCTTGG	CAAGCCTAAAAGACAGCGG	100-105%	239 bp

Table 1. Primers used for real-time PCR

Figures

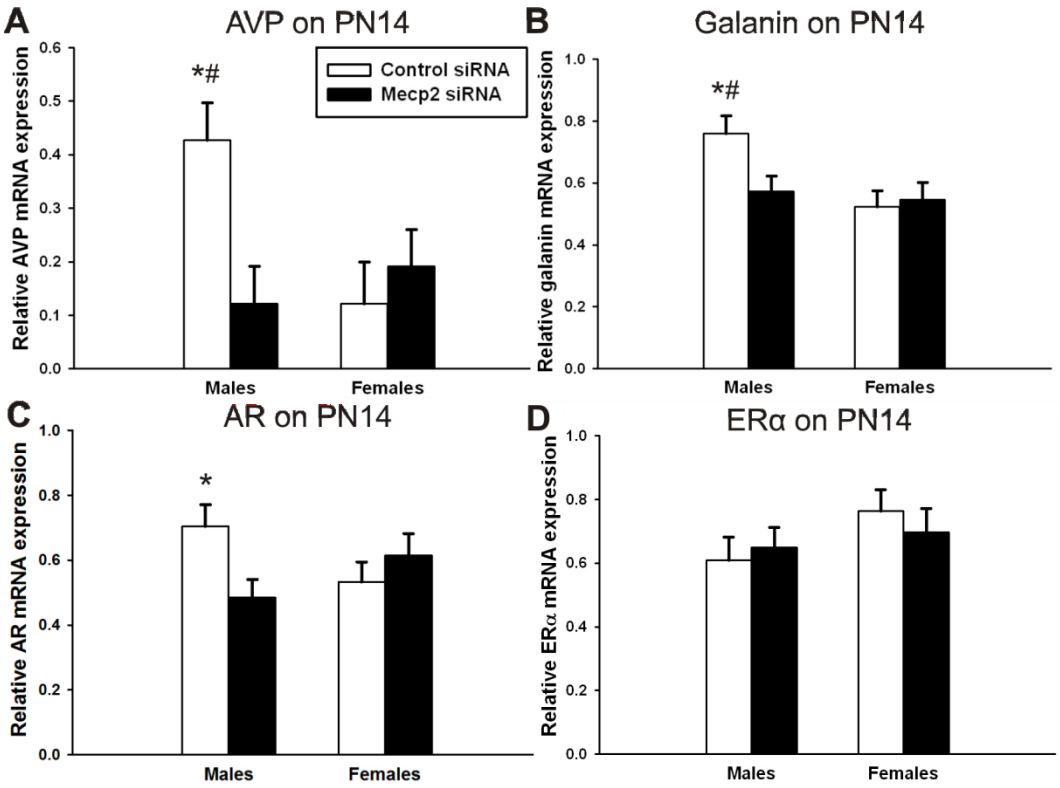

Figure 1. Relative mRNA expression in the amygdala of PN14 animals infused with MeCP2 or control siRNA on PN0-2. A) Post hoc tests indicate that control males expressed more relative AVP mRNA than control females (**p*=0.011) and MeCP2 siRNA-treated males (#*p*=0.007). B) Post hoc tests indicate that control males expressed more relative galanin mRNA than control females (**p*=0.005) and MeCP2 siRNA-treated males (#*p*=0.008). C) Post hoc tests indicate that control males expressed more relative AR mRNA than MeCP2 siRNA-treated males (**p*=0.002). D) There was no effect on relative ERα mRNA.

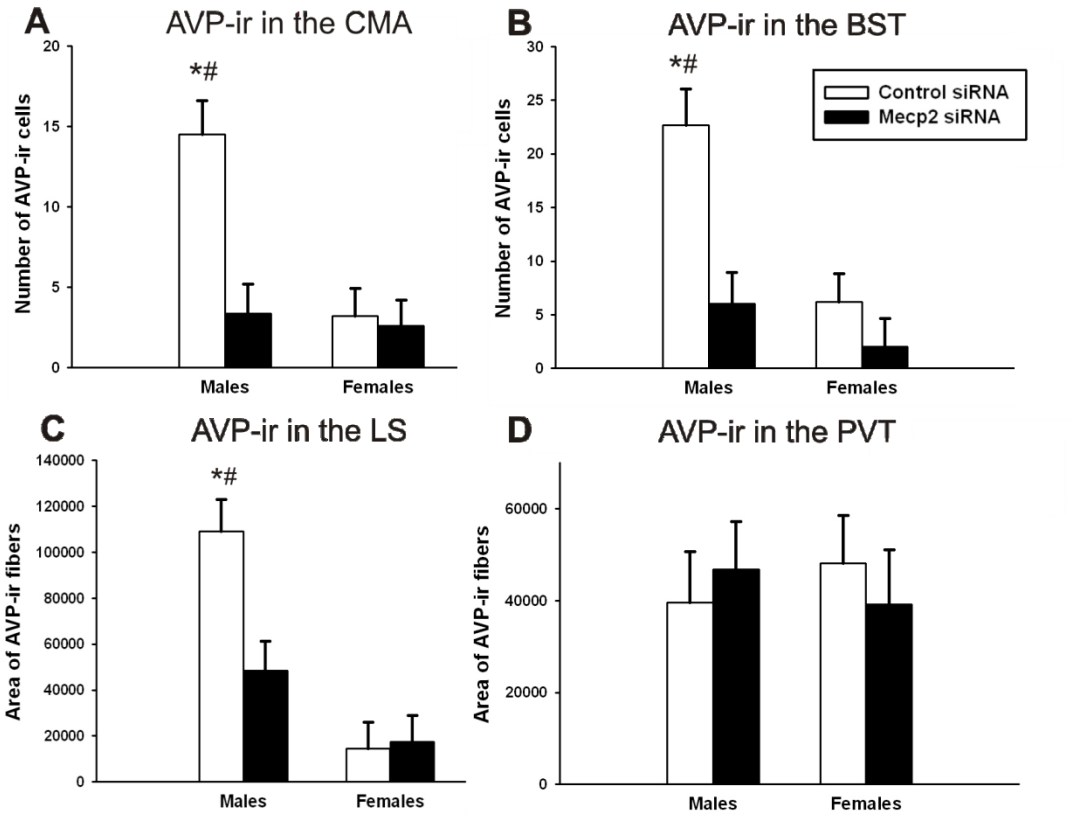

Figure 2. AVP-ir in adult animals infused with MeCP2 or control siRNA on PN0-2. A) Post hoc comparisons indicate that control males expressed more AVP-ir cells than control females (*p<0.001) and MeCP2 siRNA-treated males (#p<0.001) in the amygdala. B) Post hoc comparisons indicate that control males expressed more AVP-ir cells than control females (*p<0.001) and MeCP2 siRNA-treated males (#p<0.001) in the BST. C) Post hoc comparisons indicate that control males expressed a larger area of AVP-ir fibers than control females (*p<0.001) and MeCP2 siRNA-treated males (#p=0.003) in the LS. D) There was no effect of MeCP2 siRNA treatment on AVP-ir fiber area in the paraventricular nucleus of the thalamus (PVT).

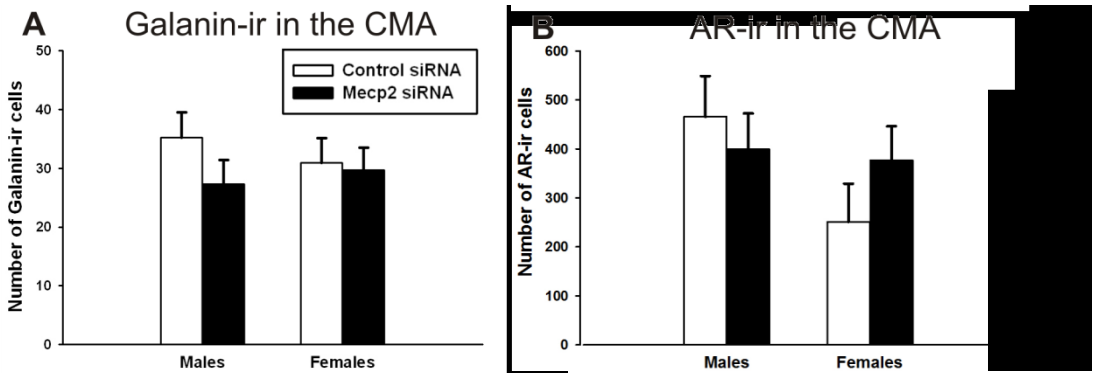

Figure 3. Immunoreactive cells in the amygdala of adults infused with MeCP2 or control siRNA on PN0-2. A) There was no effect of sex (p=0.81) or treatment (p=0.28) on the number of galanin-ir cells. B) There was no effect of sex (p=0.78) or treatment (p=0.73) on the number of AR-ir cells.

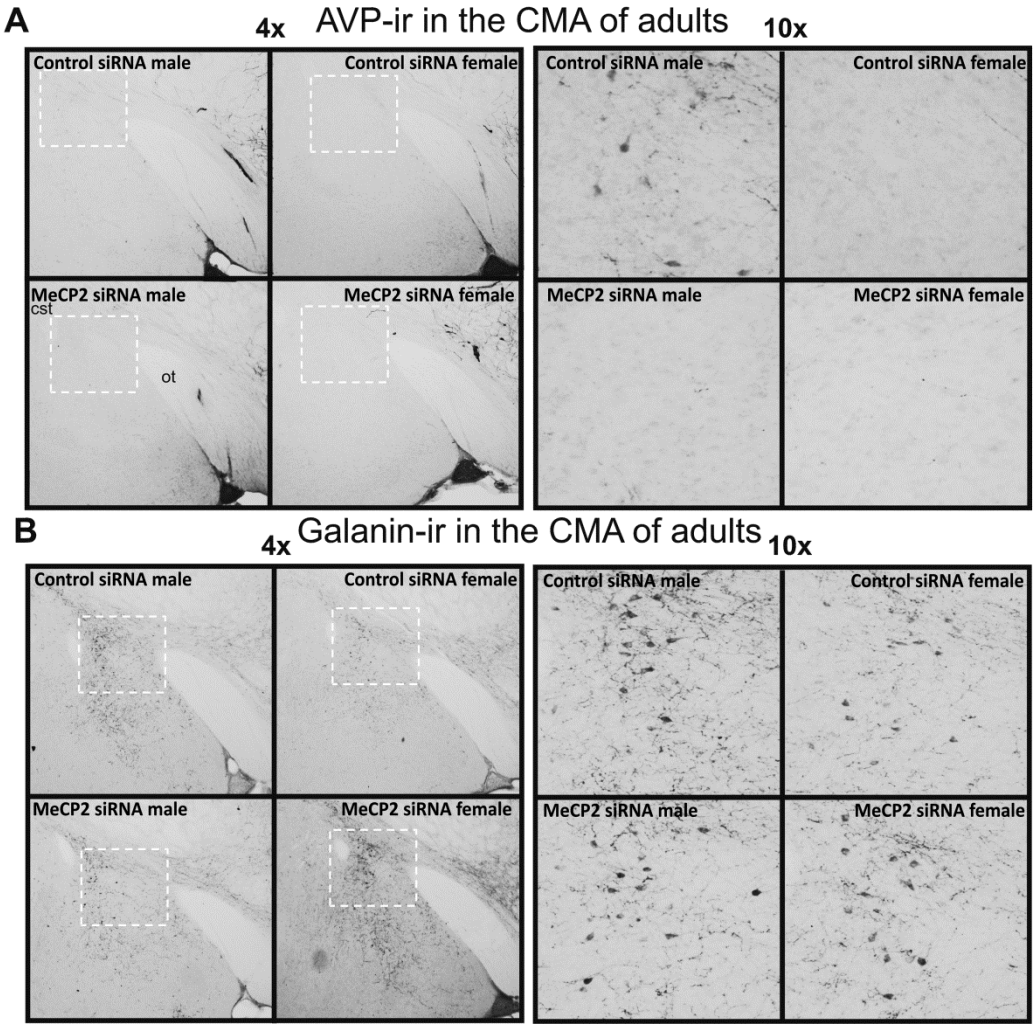

Figure 4. Photomicrographs of adults infused with MeCP2 or control siRNA on PN0-2.
A) AVP-ir cells in the amygdala at 4x (left) and 10x (right). B) galanin-ir cells in the amygdala at 4x (left) and 10x (right). White dashed box represents the area analyzed.

Chapter 3

Epigenetic marks on the vasopressin promoter within the amygdala: Implications for the organization of sex differences in expression

RM Forbes-Lorman, J Chi, AP Auger, CJ Auger

Abstract

Arginine vasopressin (AVP) plays an important role in social behavior and is implicated in several behavioral disorders in humans. In rats, males express higher levels of AVP than females within the medial amygdala and bed nucleus of the stria terminalis. Previous research demonstrates that these differences are not likely due to differential cell birth, death, or migration, suggesting that another mechanism establishes these phenotypic differences. One mechanism that is implicated in the control of gene expression is DNA methylation, which typically results in gene repression. Previous data suggest that testosterone maintains AVP expression in adult male rats by altering the methylation profile of the *Avp* promoter region. Therefore, we hypothesized that sex differences in AVP expression within the amygdala are also a product of differential methylation.

First, we examined AVP mRNA levels in the amygdala of postnatal day (PN) 1 and PN10 male and female rats using real-time PCR as well as methylation of individual CpG sites within the *Avp* promoter region using methylation sensitive restriction enzymes. While no sex differences in AVP mRNA or methylation were found on PN1, males expressed higher levels of AVP mRNA than females on PN10, consistent with previous data. Additionally, corresponding to the higher levels of AVP expression, males had lower levels of methylation than females at two CpG sites within the *Avp* promoter region on PN10. Because estradiol, a metabolite of testosterone, plays a large role in the organization of the sex difference in AVP expression, we administered estradiol or oil vehicle to female rats on PN0, PN2, and PN4 and measured AVP mRNA and CpG methylation. Estradiol treatment increased AVP mRNA and decreased methylation at one CpG site on PN10. Although the functional significance of the methylation marks remains unknown, our data suggest that differential methylation may be involved in the regulation of sex differences in AVP expression. Testosterone may organize sex difference in AVP within the amygdala in part through decreasing CpG methylation of the *Avp* promoter region.

Introduction

Sex differences are largely the result of steroid hormone action in the developing brain (Cooke *et al.*, 1998; Simerly, 2002). Male rats have higher levels of serum testosterone than females during late embryonic and early postnatal life (Weisz and Ward, 1980; Rhoda *et al.*, 1984), which is metabolized into two principle ligands: estradiol, which binds to estrogen receptors (ERs) and dihydrotestosterone (DHT), which binds to androgen receptors (ARs) (Naftolin *et al.*, 1971). These complexes are critical for organizing many sex differences in the brain and behavior. For example, male rats express more arginine vasopressin (AVP) than females within the medial amygdala (MeA), bed nucleus of the stria terminalis (BST), and the terminal fields of these AVP cells (Van Leeuwen *et al.*, 1985; De Vries and al-Shamma, 1990). This is largely organized during the first week of postnatal development, when a surge in testosterone in males induces AVP expression, primarily through its metabolism into estradiol (Wang *et al.*, 1993; De Vries *et al.*, 1994; Wang and De Vries, 1995; Han and De Vries, 2003). Although the sex difference is highly dependent on circulating testosterone levels, females still express less AVP even when gonadectomized and treated with the same hormone regimen as males (De Vries and al-Shamma, 1990; De Vries *et al.*, 1994; Wang and De Vries, 1995). However, the mechanism by which testosterone and its metabolites induce male-typical AVP expression patterns remains unclear. Previous research has demonstrated that the sex difference in AVP is not due to differential cell birth, death, or migration, suggesting that another mechanism establishes these phenotypic differences (al-Shamma and De Vries, 1996; Han and De Vries, 1999; De Vries *et al.*, 2008).

Recent data indicate that a number of sex differences in the brain are mediated by epigenetic phenomena, such as DNA methylation. Methylation of CpG sites within the promoter region of a gene is a common epigenetic mechanism that typically reduces gene transcription. DNA methylation of a gene promoter region can repress gene transcription through two different mechanisms: by directly interfering with the binding of a transcription factor (Comb and

Goodman, 1990) and through recruitment of methyl-binding proteins, which recruit corepressor complexes that cause histone deacetylation and transcriptional repression (Nan *et al.*, 1998; Jones *et al.*, 1998). Differential methylation between males and females may be one way in which testosterone organizes sex differences in the brain. For example, female rats express more ERα within the preoptic area than males (Rainbow *et al.*, 1982; DonCarlos and Handa, 1994; Yokosuka *et al.*, 1997) and have lower levels of methylation of the ERα exon1b promoter region on postnatal day (PN) 10. Furthermore, treatment with estradiol in females decreases ERα mRNA and increases methylation to male-typical levels (Kurian *et al.*, 2010). Additionally, testosterone maintains AVP expression in the BST of adult male rats by regulating the methylation profile of the *Avp* gene. Specifically, castration of adult male rats increased methylation of two CpG sites within the promoter region of *Avp*, corresponding to a decrease in AVP expression. Moreover, testosterone replacement reduced methylation at these same CpG sites and increased AVP expression to normal levels (Auger *et al.*, 2011). These data indicate that sex differences in the brain are organized by steroid modulation of epigenetic factors and that epigenetic factors are important in maintaining expression levels of AVP. Therefore, we hypothesize that sex differences in AVP expression are organized through differential methylation in males and females.

The present study examines AVP mRNA expression and the methylation of individual CpG sites within the *Avp* gene promoter region in the amygdala of male and female rats on PN1 and PN10. We predict that male rats will have lower levels of methylation of specific CpG sites than females within the amygdala and that this sex difference will not emerge until PN10, as testosterone does not appear to fully differentiate AVP expression until PN7 (Wang *et al.*, 1993). Because the sex difference in AVP expression is primarily organized by estradiol during early postnatal life (Han and De Vries, 2003), we examined whether administration of estradiol to female rats during early postnatal life would result in male-typical methylation patterns.

Methods

Subjects and treatment. Sprague-Dawley rats supplied by Charles River Labs were bred in our animal facility. Dams were checked daily to determine the day of birth and were allowed to deliver normally. For Experiment 1, pups were collected from multiple dams on PN1 (12 males, 12 females), which is during the postnatal surge in testosterone in males, and on PN10 (9 males, 10 females), which is after the sensitive period for sexual differentiation of AVP (Weisz and Ward, 1980; Rhoda *et al.*, 1984; Wang *et al.*,1993). For Experiment 2, 23 female pups were collected from multiple dams and given subcutaneous injections of 100µg estradiol benzoate in 0.1mL sesame oil vehicle (11 animals) or 0.1mL sesame oil vehicle (12 animals) on PN0, PN2, and PN4. This dose has previously been shown to increase AVP expression within the BST (Han and De Vries, 2003). The rats were housed under a 12:12 light/dark cycle with food and water available *ad libitum.* This research was approved by the University of Wisconsin Institutional Animal Care and Use Committee.

Tissue collection and processing. Animals were sacrificed by rapid decapitation on PN1 or PN10. Brains were extracted and the amygdala was dissected and snap frozen in isopentane on dry ice. To dissect the amygdala, the whole brain was placed ventral side up on a cold surface. Using a razor blade, two coronal cuts were made, one caudal to the optic chiasm and one caudal to the hypothalamus. This section of tissue was placed rostral side up and a cut was made along the optic tract followed by another cut at approximately 60 degrees to form an approximate triangle. Both sides of the amygdala were collected, pooled, and frozen. Total RNA and DNA were isolated using the AllPrep DNA/RNA Mini Kit (80204, Qiagen) and concentrations of each were determined using a Qubit Fluorometer (Invitrogen). The samples were stored at -80˚C.

Quantification of mRNA. cDNA was generated from the RNA using ImProm-II™ Reverse Transcription System (A3800, Promega) in an Eppendorf MasterCycler Personal PCR machine. Real-time PCR was done using a Stratagene Mx3000P™ real-time PCR system using GoTaq®

Colorless Master Mix (M7132, Promega), SYBR green, and ROX as a reference dye. Primers designed to target AVP and HPRT1 were run in duplicate (Table 1) and each primer set was used in three separate runs to replicate the results. A standard curve was included to ensure primers had efficiencies between 90 and 110%; all primers had between 93 and 105% efficiency. HPRT1 was used as normalizing genes to control for subtle variations in sample concentrations. The amplification protocol was as follows: an initial denaturing step at 95˚C for two minutes, followed by 40 cycles of a 95˚C melting step for 30 seconds, 60˚C annealing step for 30 seconds, and a 72˚C elongation step for 30 seconds. Following amplification, a dissociation melt curve analysis was performed to ensure the purity of PCR products. Data were analyzed with the following program term settings based on Invitrogen recommendations: (1) amplified based threshold, (2) adaptive baseline and (3) smoothing moving average with amplification averaging three points. Relative cDNA levels were calculated using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001).

Quantification of DNA Methylation. To determine relative methylation of specific CpG sites, we followed an adaptation of a previously published method (Hashimoto *et al.*, 2007; Auger *et al.*, 2011; Edelmann and Auger, 2011). This method uses a restriction enzyme that will bind a specific CpG site and cut the DNA unless it has been methylated. Therefore, designing primers to surround the targeted CpG site will produce a PCR product only if that site has been methylated. This is a powerful technique because it uses quantitative PCR to assess the relative methylation of all DNA isolated from the tissue sample. DNA was digested using one of three methylation-sensitive restriction enzymes; HhaI, HpaII, or Hpy188II (New England Biolabs). To examine the methylation status of CpG sites sensitive to cleavage by each enzyme, each sample was divided equally into three tubes that were incubated with one of the enzymes for 1 h at 37°C, followed by 20 min at 65°C to inactivate the enzyme. After the enzyme digest, the amount of amplification of each DNA sample was examined using real-time PCR. Real-time PCR was done using a Stratagene Mx3000P™ real-time PCR system using GoTaq® Colorless

Master Mix (M7132, Promega), SYBR green, and ROX as a reference dye. Primers designed to encompass one of the five different CpG sites within the *Avp* promoter region were run in duplicate (Table 1, Fig 1) and each primer set was used in three separate runs to replicate the results. The amplification protocol was as follows: an initial denaturing step at 95˚C for two minutes, followed by 35 cycles of a 95˚C melting step for 30 seconds, 60˚C annealing step for 30 seconds, and a 72˚C elongation step for 30 seconds. Following amplification, a dissociation melt curve analysis was performed to ensure the purity of PCR products. Data were analyzed with the following program term settings based on Invitrogen recommendations: (1) background-based threshold and (2) adaptive baseline. Relative methylation levels were calculated using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). In this case, the ΔC_T for each sample was determined by calculating the difference between the average C_T of the reference sample (sample digested with an enzyme that does not cleave within the amplicon) and the average C_T of the sample of interest (sample digested with an enzyme that does cleave within the amplicon).

Statistical analysis. Statistical comparisons were carried out using a two-tailed t-test for Experiment 1 and a one-tailed t-test for Experiment 2, as we predicted that estradiol treatment would increase AVP mRNA and decrease methylation. Effects with a p value of < 0.05 were considered statistically significant. Outliers were determined using GraphPad Software, Inc. (http://www.graphpad.com).

Results

Experiment 1. On PN1, there was no sex difference in AVP mRNA levels within the amygdala ($t(19)=0.45$, $p=0.66$, Fig 1A) and no sex difference in methylation at CpG site 1 ($t(20)=0.38$, $p=0.71$, Fig 1B), CpG site 2 ($t(22)=0.04$, $p=0.97$, Fig 1C), CpG site 3 ($t(20)=0.05$, $p=0.96$, Fig 1D), CpG site 4 ($t(22)=0.43$, $p=0.67$, Fig 1E) or CpG site 5 ($t(20)=0.53$, $p=0.60$, Fig 1F). On PN10, there was a sex difference in AVP mRNA levels within the amygdala (Fig 2A).

Males expressed higher levels of AVP mRNA than females ($t(16)=2.37$, $p=0.03$). There was no sex difference in methylation at CpG site 1 ($t(16)=0.39$, $p=0.71$, Fig 2B), CpG site 2 ($t(17)=0.33$, $p=0.74$, Fig 2C), CpG site 3 ($t(17)=0.73$, $p=0.48$, Fig 2D); however, males had lower levels of methylation at sites 4 ($t(16)=2.47$, $p=0.03$, Fig 2E) and 5 ($t(17)=2.90$, $p=0.01$, Fig 2F).

Experiment 2. As expected, estradiol increased AVP mRNA within the female amygdala ($t(17)=2.39$, $p=0.01$, Fig 3A) and decreased methylation at CpG site 4 ($t(17)=1.9$, $p=0.04$, Fig 3E). There was no effect at CpG site 1 ($t(18)=1.22$, $p=0.12$, Fig 3B), CpG site 2 ($t(18)=0.76$, $p=0.23$, Fig 3C), CpG site 3 ($t(18)=0.04$, $p=0.49$, Fig 3D), or CpG site 5 ($t(18)=0.52$, $p=0.31$, Fig 3F).

Discussion

Epigenetic mechanisms are an exciting area of investigation as they provide for a possible explanation for how early life experiences can have lasting outcomes on gene expression and behavior. The present study offers a mechanism by which testosterone and its metabolites may organize the sex difference in AVP expression within the amygdala. There was no sex difference in AVP mRNA levels or methylation at any of the five CpG sites examined within the *Avp* gene promoter on PN1. This is consistent with previous data suggesting that the sex difference in AVP expression is not fully organized until PN7 (Wang *et al.*, 1993). It appears that the AVP system is bipotential in males and females through the first week of life, and it is hormonal exposure during this time that drives the system to male-like expression profiles (Wang *et al.*, 1993; Han and De Vries, 2003). In contrast, there was a sex difference in AVP mRNA within the amygdala on PN10, with males having higher levels of mRNA than females. In a separate group of animals, neonatal estradiol treatment increased AVP mRNA in the female amygdala. Importantly, males had lower levels of methylation than females at CpG sites 4 and 5, and estradiol treatment decreased methylation at CpG site 4 in females. It appears that

hormones organize the sex difference in AVP expression in part through altering DNA methylation at this site.

If DNA methylation is in part responsible for the repression of the *Avp* gene prior to the increase in expression in males during the first week of postnatal life, then CpG site 4 may be demethylated in response to hormonal exposure in order to facilitate male-like expression levels. Hormone activity has been shown to induce active demethylation via MBD4 (Kouzmenko *et al.*, 2010), and estradiol could regulate a similar process. Alternatively, if mechanisms other than DNA methylation repress *Avp* prior to the first week of postnatal life, then the differential methylation could be due to increased methylation in females, and estradiol could prevent this from occurring. Some factor that is present at higher levels within the female brain may induce methylation, such as DNA-methyltransfersase 3, which induces *de novo* methylation (Kolodkin and Auger, 2011) and/or some factor may protect males against methylation. Although males express lower levels of MeCP2 (Kurian *et al.*, 2007), MeCP2 can bind to unmethylated DNA (Hansen *et al.*, 2010) and may be critical for protecting against methylation in males. Interestingly, CpG site 4 is also near an AT rich region, which makes it a potential MeCP2 binding site (Klose *et al.*, 2005). This could also explain why disrupting MeCP2 in the neonatal amygdala decreases AVP expression in males (Chapter 2). In this case, we would expect MeCP2 siRNA to increase methylation at CpG site 4 in males, which remains unknown.

The lack of an effect of estradiol treatment on methylation at CpG site 5 suggests that some other factor establishes this methyl mark. First, there may be an activational effect of testosterone on DNA methylation. Males have higher levels of circulating testosterone at PN10, and this could result in demethylation or the inhibition of methylation at this site within the male amygdala. Alternatively, as sex chromosomes play a role in organizing sex differences in the brain (De Vries *et al.*, 2002), the sex difference in methylation at this site may have a genetic influence.

Importantly, CpG sites 4 and 5 are near transcription factor response elements that are important for regulating *Avp*. CpG site 4 is near the glucocorticoid/progestin response element (GRE/PRE) and CpG site 5 is near the cAMP response element (CRE) (Mohr and Richter, 1990; Shapiro *et al.*, 2000). The sex difference in methylation at these sites may be particularly important because of their proximity to these response elements. For example, higher levels of methylation near these hormonal responsive regions in females may serve as protection from steroid-induced sexual differentiation of the AVP system, similar to the protective effects of alpha-fetoprotein in females against the masculinizing effects of hormonal exposure early in life (McEwen *et al.*, 1975; Bakker *et al.*, 2006). In this way, the potential for male-like programming of the AVP system by hormonal exposure may be reduced in females through the presence of higher levels of methylation marks on the *Avp* promoter around PN10. Our data suggest that differential methylation near these transcription factor response elements may be one mechanism for the regulation of sex differences in AVP expression. Higher methylation of the *Avp* promoter region in females may also repress gene expression through the recruitment of methyl-binding proteins resulting in histone deacetylation and chromatin compaction. Additional studies are needed to determine the functional significance of the methylation at this site.

Prior studies demonstrated that the sex difference in AVP expression is not due to cell death, birth, or migration, but is likely due to phenotypic differentiation (al-Shamma and De Vries, 1996; Han and De Vries, 1999; De Vries *et al.*, 2008). This supports the results of the present study, which suggests that the sex difference is in part due to differential methylation of the *Avp* promoter region. Furthermore, as estradiol administration decreases methylation of a CpG site near a GRE/PRE in females, the surge in testosterone in males may organize AVP expression through demethylation. Whether differential methylation of the other CpG site is influence by sex chromosomes and/or circulating hormones remains unclear.

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Tables

Gene	Accession #	Forward sequence	Reverse sequence	Product size	Enzyme
AVP	NM_016992	TGCCTGCTACTTCCAGAACTGC	AGGGGAGACACTGTCTCAGCTC	55 bp	NA
HPRT	NM_012583	GCAGACTTTGCTTTCCTTGG	CCGCTGTCTTTTAGGCTTTG	239 bp	NA
CpG site 1	AF112362	GAACGGCCTGACTCTGTCTC	GCTTCTAACCGCTTAGGCCT	284 bp	HpaII
CpG site 2	AF112362	GTAGACCGCCACACCTGATT	GGTCACACAGCAATGTCTGG	236 bp	HpaII
CpG site 3	AF112362	CGCCTTCAAAGCTCTAGTGG	CACTTCCCTTGGGGTTCATA	203 bp	HpaII
CpG site 4	AF112362	ACCCTAGAAGGCCTGAAACTCACA	AACTTGGTGAGACCCTGTCTCGAT	124 bp	Hpy188III
CpG site 5	AF112362	GGCCTTTGGCTCTATGTTCA	GATTTCCAGGTGACCCTCA	188 bp	HhaI

Table 1. Primers used for real-time PCR.

Figures

AVP promoter targeted CpG site 1:
1388- GAACGGCCTGACTCTGTCTC**CCGG**GTGCTGGCATTGAAGATCTGTGCC
ACCATCATCAGGCTGGGTTTTAAAAGATTTAGTGGTTTTTATTTAATGTGTATGAGTGTTTTGTTT
GCATGTATATCTGTACATGACAGGTGTGCCTGGTGTTTACAGAGGCCAGAAGAACATACCAGAT
CCCCCTGGAACTGAAGTTACAGACAGTCGTGAGCCATCTCGGGGTTGCTGGGGACAGAATCC
GAGGGCTCTTCTTGAGTAGCAAGTGCTTCTAACCGCTTAGGCCT

AVP promoter targeted CpG site 2:
2131- GTAGACCGCCACACCTGATTTTTAAAAGCTCTCAGTGAAACTGAGCATGGTAGCACA
TGTTTGTAATCCCAGCAGACATGTGGGGAGACAAAGGAATGGACTCAGACTCAG**CCGG**AGAG
CAAGTTCACGGCTAGACTGGACCATTCCTACAATGAGGTAGGAATTGGGGTTAGCACATCAAGT
AAGTAACCCTGGAAACAAGTTTGACTTGTCCAAGGTCACACAGCAATGTCTGG

AVP promoter targeted CpG site 3:
3736- CGCCTTCAAAGCTCTAGTGGATGTGATTGCCCCAGACAAGTCTGCCCCAAAGCTCAT
CTTCGTCCATTAATAGAAAAAAAGGTTTCTTCTTGACCAAGGAAGCTGTTCTCTCTGGAAACAAT
CACTTAACAAGGACATTACTAACACGAAGCTGCTGT**CCGG**ATCACATCACCATGACGCAAGCAC
TTCCCTTGGGGTTCATA

AVP promoter targeted CpG site 4:
4751- ACCCTAGAAGGCCTGAAACTCACAGAAATTCTCCTGCCTCTGCTTTCCAATGGCTGG
GGTTAAAAGCATG*TGTCACAACTGTCCTT*TTTATTCTTTTAATA**TCGAGA**CAGGGTCTCACCAAG
TT

AVP promoter targeted CpG site 5:
4910- GGCCTTTGGCTCTATGTTCAGTCTTGACTCCATGACTGTGGCCGCTAGCCCATGAG
GCT**GCGC**GTGGGAATTTCCTTCTGAAAGCTCACCTGGTATCGATGCTTCCTCTTATCCTACACC
ACAACTAACAACCTCTAGTCTGTTGAGAGCTGCTGAAATGCTCAACTATGATTTCCAGGTGACC
CTCAGTCGGCTCACCTCCCTGATTGCACAGCACCAATCACTGTGGCGGTGG*CTCCCGTCA*

Figure 1. Primer sequences used to assess relative methylation of individual CpG sites. Forward and reverse primers are underlined, enzyme recognition sites containing the targeted CpG sites are bolded, and other regions of interest are italicized. Base numbering from GenBank accession no. AF112362. HpaII restriction enzyme was used to target the CCGG site encompassed by primers in CpG sites 1-3. Hpy188III restriction enzyme was used to target the TCGAGA site encompassed by primer in CpG site 4. This CpG site is near a GRE/PRE (*TGTCACAACTGTCCTT*). HhaI restriction enzyme was used to target the GCGC site encompassed by primer in CpG site 5. This CpG site is near a CRE (*CTCCCGTCA*).

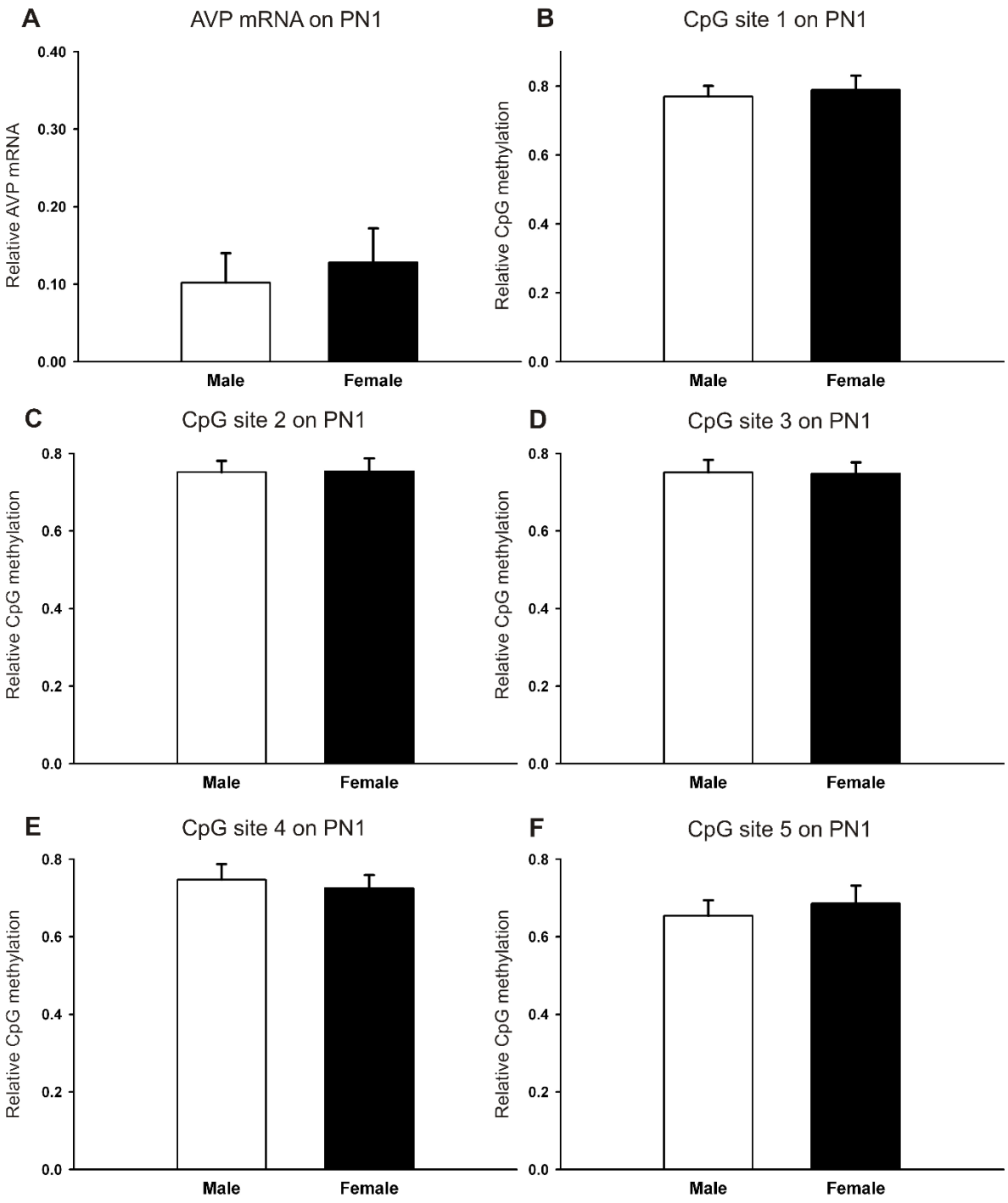

Figure 2. AVP mRNA and methylation of the *Avp* promoter region in the amygdala on PN1. There was no sex difference in AVP mRNA expression (A, p=0.66). There was no sex difference in methylation at CpG site 1 (B, p=0.71), CpG site 2 (C, p=0.97), CpG site 3 (D, p=0.96), CpG site 4 (E, p=0.67), or CpG site 5 (F, p=0.60).

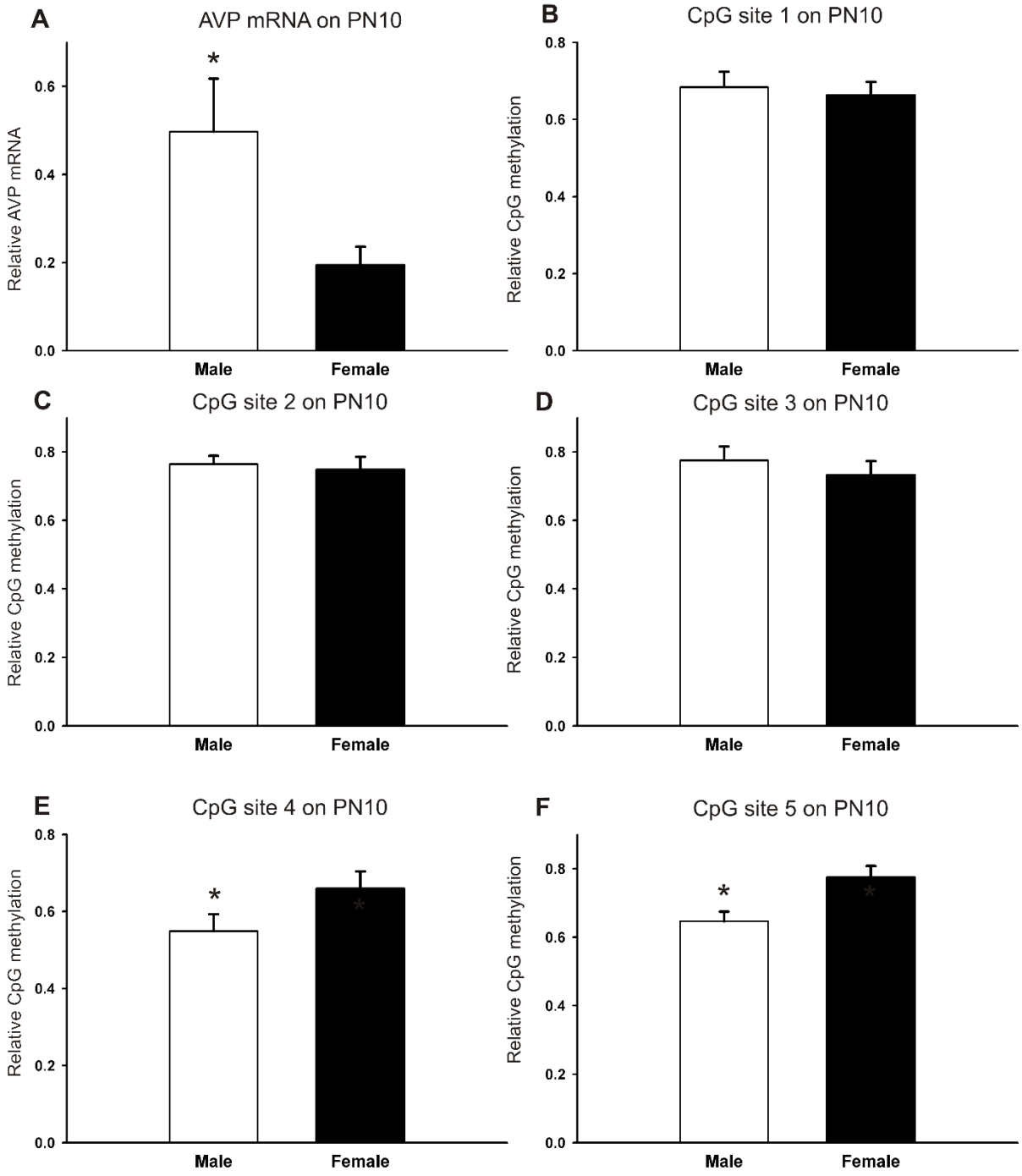

Figure 3. AVP mRNA and methylation of the *Avp* promoter region in the amygdala on PN10. Males expressed higher levels of relative AVP mRNA than females (A, p=0.03). There was no sex difference in methylation at CpG site 1 (B, p=0.71), CpG site 2 (C, p=0.74), or CpG site 3 (D, p=0.48); however, females had higher levels of methylation at CpG site 4 (E, p=0.03) and CpG site 5 (F, p=0.01).

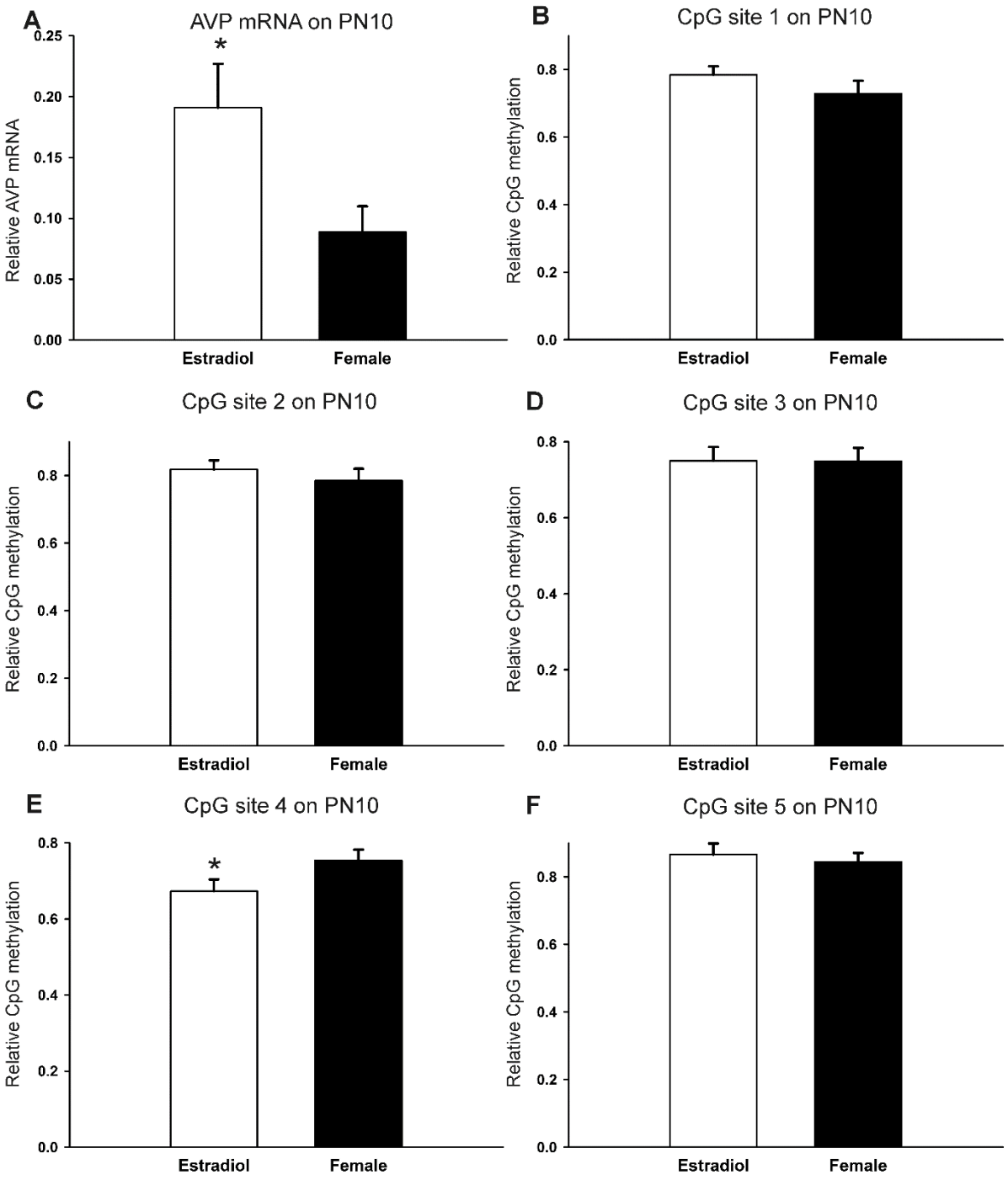

Figure 4. Effects of estradiol on AVP mRNA and methylation of the *Avp* promoter region in the female amygdala on PN10. Estradiol increased relative AVP mRNA compared to oil-treated females (A, p=0.01). There was no effect of estradiol on relative methylation at CpG site 1 (B, p=0.12), CpG site 2 (C, p=0.23), CpG site 3 (D, p=0.49), or CpG site 5 (F, p=0.31), but estradiol decreased relative methylation at CpG site 4 (E, p=0.04).

Chapter 4

Neonatal progestin receptor antagonism influences juvenile behavior and vasopressin expression in rats.

RM Forbes-Lorman, J Chi, AB Walker, AP Auger, CJ Auger

Abstract

Arginine vasopressin (AVP) is implicated in sexually dimorphic disorders, and there are sex differences in its expression in several brain areas in many species. While testosterone and its metabolites play an important role in both the organization and regulation of this sex difference, data have also demonstrated that progesterone acts upon progestin receptors (PRs) to regulate protein levels in the adult brain. For example, systemic administration of progesterone to adult male rats decreases AVP in the centromedial amygdala and bed nucleus of the stria terminalis. PRs also regulate AVP-dependent social recognition in adult males. In the present study, we examined the role of PRs in organizing AVP-dependent juvenile behaviors and AVP expression during the juvenile period. Social play behavior, anxiety-like behavior, and social recognition were examined in juvenile male and female rats that had been treated with CDB, a PR antagonist, during the first week of postnatal life. Neonatal PR antagonism improved social recognition, and this was significant in males, and increased play initiation in females. We then examined AVP mRNA levels and methylation of the *Avp* gene promoter within the amygdala on postnatal day 35. There was a trend of increased AVP mRNA within the female, but not the male, amygdala. Additionally, females had higher levels of methylation at two of the five CpG sites examined, and CDB treatment decreased methylation at these sites to male levels. These data suggest that PRs play an important role in organizing certain aspects of behavior and potentially in the organization of sex differences in the brain and behavior.

Introduction

Arginine vasopressin (AVP) is a small peptide that is implicated in several behavioral disorders in humans, including autism, schizophrenia, and affective disorders (Frank and Landgraf, 2008; Surget and Belzung, 2008). Interestingly, there are sex differences in these disorders as well as sex differences in AVP expression within the rodent brain. Males express higher levels of AVP within the medial amygdala (MeA), bed nucleus of the stria terminalis (BST), and their projection sites (Van Leeuwen *et al.*, 1985; De Vries and al-Shamma, 1990), such as the lateral septum (LS) (De Vries and Miller, 1998). The AVP neurons located within these brain regions are involved in social behaviors. For example, AVP within the male LS is necessary for social recognition, which is the ability to retain the memory of a previously encountered conspecific, primarily through acting on V1a receptors (Dantzer *et al.*, 1988; Everts and Koolhaas, 1999; Bielsky *et al.*, 2004; Bielsky *et al.*, 2005a). However, although AVP facilitates social recognition in females, it is not necessary (Bluthe and Dantzer, 1990; Engelmann *et al.*, 1998; Bielsky *et al.*, 2005b). AVP within the MeA and BST may also influence juvenile social play behavior in rats. Several studies have found a correlation between decreased play and decreased AVP expression within the MeA, BST, and LS (Chapter 2), (Kurian *et al.*, 2008; Taylor *et al.*, 2012). However, a study examining the causal relationship between the two revealed a complicated relationship, with sex-specific and brain region-specific effects of a V1a receptor antagonist (Veenema *et al.*, 2013a).

While it is clear that testosterone and its metabolites play an important role in both the organization and regulation of AVP expression through their actions on estrogen receptors (ERs) and androgen receptors (ARs) (Wang *et al.*, 1993; De Vries *et al.*, 1994; Wang and De Vries, 1995; Han and De Vries, 2003), data have also demonstrated that progesterone acts upon progestin receptors (PRs) to regulate expression levels in the adult brain. For example, systemic administration of progesterone to adult male rats decreases AVP in the centromedial amgydala and BST (Auger and Vanzo, 2006). Likewise, knocking out PRs increases AVP

expression in both male and female mice (Rood *et al.*, 2008). Moreover, PRs, through decreasing AVP, also regulates some AVP-dependent social behavior in adult males. For example, progesterone administration impairs social recognition in adult males and an infusion of AVP into the LS rescues this behavior (Bychowski and Auger, 2012; Bychowski *et al.*, 2013).

Several lines of evidence suggest that PRs are important for the development of the male brain. Males express PRs as early as embryonic day 20 in many brain areas (Quadros *et al.*, 2007) and express more PRs than females within the medial preoptic area (mPOA) and anteroventral periventricular nucleus during on postnatal day (PN) 1 but not PN10 (Wagner *et al.*, 1998; Quadros *et al.*, 2002). This transient sex difference in PR expression suggests that PRs are important for the organization of the male-typical brain. Also, our own data indicate that blocking PRs during development influences the expression of ARs in the adult male brain (Appendix B). Data from PRs knockout (PRKO) mice suggests that PRs do not play a role in organizing sex differences in AVP. However, as PRKO mice lack PRs both during and after development, its role in organizing male and female levels of AVP remains unclear.

In the present study, we examined the role of PRs in organizing juvenile behaviors that are influenced by AVP. Social play behavior (Chapter 2), (Kurian *et al.*, 2008; Taylor *et al.*, 2012; Veenema *et al.*, 2013b), anxiety-like behavior (Liebsch *et al.*, 1996; Appenrodt *et al.*, 1998; Bielsky *et al.*, 2004), and social recognition (Dantzer *et al.*, 1988; Bielsky *et al.*, 2004; Bielsky *et al.*, 2005a) are influenced by AVP, therefore these behaviors were examined in juvenile male and female rats that had been treated with a subcutaneous injection of CDB, a PR antagonist, during the first week of postnatal life. In order to examine whether PRs play a role in organizing expression patterns in AVP, we then examined AVP mRNA levels within the amygdala on PN35 using real-time PCR. Lastly, in order to determine whether PRs regulated AVP mRNA through epigenetic modifications, we measured methylation of the *Avp* gene promoter using methylation-sensitive restriction enzymes (MSREs).

Methods

Subjects and treatment. Sprague-Dawley rats supplied by Charles River Labs were bred in our animal facility. Dams were checked daily to determine the day of birth and were allowed to deliver normally. Twenty-seven male and 25 female pups were pooled from 5 different litters and assigned to a treatment group. Following treatment, pups were foot-marked with India ink and placed back with the dams so that each new litter contained approximately equal numbers from each sex and treatment group. Pups were treated subcutaneously with either 75μg/0.01cc/g body weight of the PR antagonist CDB-4124 in a 0.2% benzyl alcohol, 0.6% benzyl benzoate, and sesame oil vehicle or 0.01cc/g body weight of vehicle on PN0 (day of birth), PN2 and PN4. The weight of the pups ranged from 5-12 grams over the 3 days of treatment. All pups remained with dams until weaning at PN21, at which time they were separated into 7 cages of 6 animals and 2 cages of 5 animals, each with at least one animal per treatment group and sex per cage. Final group numbers were 13 CDB-treated females, 12 oil-treated females, 13 CDB-treated males, and 14 oil-treated males. The rats were housed under a 12:12 light/dark cycle with food and water available *ad libitum.* This research was approved by the University of Wisconsin Institutional Animal Care and Use Committee.

Behavioral testing. All behavioral tests were performed and analyzed as previously reported (Kurian *et al.*, 2008; Jessen *et al.*, 2010). Briefly, behavioral tests were performed under dim red light approximately 1-3 h after the beginning of the dark phase of the light cycle. Each behavior was recorded and then analyzed by a trained technician blind to all treatments using The Observer® (Noldus Information Technologies) or Stopwatch+ (Center for Behavioral Neuroscience, Atlanta, GA) with the exception of the light/dark chamber, which was scored in real time.

Play Behavior. The play behavior paradigm and scoring criteria were adapted from Meaney and McEwen, 1986, Casto *et al,* 2003, and Olesen *et al.*, 2005. On PN25-29, play behavior was digitally recorded in two 4-minute sessions per day in the home cage covered with a clear

plastic lid. There were 5-6 animals in each cage, randomly numbered and coded by tail marks. An observer blind to the treatment groups scored the recordings for the following behaviors: pin, pounce, bite, and chase. The frequency of play behavior was calculated by summing each component of an animal's play behavior over the entire observation time. The animals from one cage were removed from the analysis because this cage contained animals of two different ages. We thought it was best to only use data from age-matched animals, as we have seen decreases in play over the five days of observation (unpublished observations). Indeed, social play decreases in both quantity and complexity with age (Thor and Holloway, Jr., 1984; Pellis and Pellis, 1990).

Elevated plus maze. On PN30 or 31, rats were tested for anxiety-like behavior within the elevated plus maze (EPM). The EPM consists of two opposing runways, one closed (with walls) and one open (no walls), each measuring 100 cm in length and constructed of black Plexiglas. Each arm of the closed runway is fitted with 39-cm-high Plexiglas walls on either side of the arms. The maze stands 50 cm off the floor. Rats were placed into the center of the maze facing an open arm, and their behavior was video recorded for 5 min as they explored the maze. Time spent in open and closed arms was later scored by dividing the total time spent in the open arms by the total time spent in the closed arms. An entry was scored when all four paws crossed into the new portion of the maze.

Light/dark chamber. After testing in the elevated plus maze, rats were tested immediately in the light/dark chamber task. The light/dark chamber is one large Plexiglas chamber that is divided into two chambers by an opaque Plexiglas insert: one large lit side (35 × 38 × 39 cm) constructed of clear Plexiglas and one smaller dark side (25 × 38 × 39 cm) constructed entirely of opaque material with a lid. An opening in the lower corner on the insert (6 × 10 cm) allows the animal to move freely between the two sides. A white incandescent light is situated above the light side. The animals were placed into the middle of the light side of the box and observed for

5 min. Total time in the light was recorded. Entry into the light or dark side was scored when all four paws were on the new side.

Social recognition. On PN32 or 33, rats were tested for social recognition using a social discrimination paradigm adapted from Engelmann et al. 1995 . Experimental animals were isolated for 4 hours prior to testing. Although it is typical to isolate adult animals for 1 to 10 days prior to testing (Bielsky *et al.*, 2004; Bielsky *et al.*, 2005a; Bychowski and Auger, 2012; Bychowski *et al.*, 2013), we chose to isolate the juvenile animals for only 4 hours, as social isolation is considered a severe stressor for juvenile animals (Dunlap *et al.*, 1978; Jones *et al.*, 1992; Lukkes *et al.*, 2009; Lukkes *et al.*, 2012; Inta *et al.*, 2013). In trial 1, an age and sex-matched juvenile rat was placed in the home cage of the experimental animal and the experimental animal was allowed to freely investigate for 5 minutes. After 5 minutes, the juvenile was removed and the experimental animal was alone in its cage for 30 minutes. After this 30-minute intertrial interval, the juvenile from trial 1 and a novel juvenile were placed in the experimental animal's cage, and the experimental animal was again free to investigate for 5 minutes. The juvenile rats were distinguishable to the researcher scoring the video by unique tail marks drawn with permanent marker. Investigation of the juvenile(s) was scored to include direct contact between the nose of the experimental animal and the body of the juvenile and close following behavior. Cage directed behavior was also scored as a measure of basic locomotor and investigatory activity. Social recognition was scored by calculating the ratio of discrimination (RID) (Engelmann *et al.*, 1995). The RID score was calculated by dividing the time spent investigating the familiar animal over the time spent investigating the novel animal. Smaller RID scores indicate recognition.

Tissue collection and processing. Animals were sacrificed by rapid decapitation before puberty on PN35. Beginning on PN30, females were checked daily to monitor vaginal opening, and none began opening before PN33. Brains were extracted and the amygdala was dissected and snap frozen in isopentane on dry ice. To dissect the amygdala, the whole brain was placed

ventral side up on a cold surface. Using a razor blade, two coronal cuts were made, one caudal to the optic chiasm and one caudal to the hypothalamus. This section of tissue was placed rostral side up and a cut was made along the optic tract followed by another cut at approximately 60 degrees to form an approximate triangle. Both sides of the amygdala were collected, pooled, and frozen. Total RNA and DNA were isolated using the AllPrep DNA/RNA Mini Kit (80204, Qiagen) and concentrations of each were determined using a Qubit Fluorometer (Invitrogen). The samples were stored at -80˚C. Because of the large number of samples, 10 samples were removed from the total, equally from each group. Final group numbers were 8 CDB-treated females, 10 oil-treated females, 10 CDB-treated males, and 11 oil-treated males.

Quantification of mRNA. cDNA was generated from the RNA using ImProm-II™ Reverse Transcription System (A3800, Promega) in an Eppendorf MasterCycler Personal PCR machine. Real-time PCR was done using a Stratagene Mx3000P™ real-time PCR system using GoTaq® Colorless Master Mix (M7132, Promega), SYBR green, and ROX as a reference dye. Primers designed to target AVP and HPRT1 were run in duplicate. A standard curve was included to ensure primers had efficiencies between 90 and 110%; all primers had between 93 and 105% efficiency. HPRT1 was used as normalizing genes to control for subtle variations in sample concentrations. The amplification protocol was as follows: an initial denaturing step at 95˚C for two minutes, followed by 40 cycles of a 95˚C melting step for 30 seconds, 60˚C annealing step for 30 seconds, and a 72˚C elongation step for 30 seconds. Following amplification, a dissociation melt curve analysis was performed to ensure the purity of PCR products. Data were analyzed with the following program term settings based on Invitrogen recommendations: (1) amplified based threshold, (2) adaptive baseline and (3) smoothing moving average with amplification averaging three points. Relative cDNA levels were calculated using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). All samples were run on the same plate.

Quantification of DNA Methylation. To determine relative methylation of specific CpG sites, we followed an adaptation of a previously published method (Hashimoto *et al.*, 2007; Auger *et al.*, 2011; Edelmann and Auger, 2011). This method uses a restriction enzyme that will bind a specific CpG site and cut the DNA unless it has been methylated. Therefore, designing primers to surround the targeted CpG site will produce a PCR product only if that site has been methylated. This is a powerful technique because it uses quantitative PCR to assess the relative methylation of all DNA isolated from the tissue sample. DNA was digested using one of three MSREs; HhaI, HpaII, or Hpy188II (New England Biolabs). To examine the methylation status of CpG sites sensitive to cleavage by each enzyme, each sample was divided equally into three tubes that were incubated with one of the enzymes for 1 h at 37°C, followed by 20 min at 65°C to inactivate the enzyme. After the enzyme digest, the amount of amplification of each DNA sample was examined using real-time PCR. Real-time PCR was done using a Stratagene Mx3000P™ real-time PCR system using GoTaq® Colorless Master Mix (M7132, Promega), SYBR green, and ROX as a reference dye. Primers designed to encompass one of the five different CpG sites within the *Avp* promoter region (Chapter 3) were run in duplicate and each primer set was used in three separate runs to replicate the results. The amplification protocol was as follows: an initial denaturing step at 95˚C for two minutes, followed by 35 cycles of a 95˚C melting step for 30 seconds, 60˚C annealing step for 30 seconds, and a 72˚C elongation step for 30 seconds. Following amplification, a dissociation melt curve analysis was performed to ensure the purity of PCR products. Data were analyzed with the following program term settings based on Invitrogen recommendations: (1) background-based threshold and (2) adaptive baseline. Relative methylation levels were calculated using the $\Delta\Delta C_T$ method(Livak and Schmittgen, 2001). In this case, the ΔC_T for each sample was determined by calculating the difference between the average C_T of the reference sample (sample digested with an enzyme that does not cleave within the amplicon) and the average C_T of the sample of interest (sample digested with an enzyme that does cleave within the amplicon).

Statistical analyses. All statistical comparisons were carried out using SigmaStat statistical software version 3.5 (Systat Software, Inc.). Statistical comparisons were carried out using a two-way ANOVA and Tukey *post hoc* tests were conducted when a significant or trend toward an effect was found. Outliers were determined using GraphPad Software, Inc. (http://www.graphpad.com). Effects with a p value of < 0.05 were considered statistically significant and effects with a p value of < 0.1 were considered trending.

Results

Play behavior. There was a trend in main effect of sex ($F(1,43)=3.1$; $p=0.09$) a trend in an interaction of sex x treatment ($F(1,43)=3.9$, $p=0.05$) on the initiation of play behavior (Fig 1). Post hoc comparisons indicate that control males initiated play more than control females ($p=0.01$) and there was a trend of increased play initiation in CDB-treated females compared to control females ($p=0.07$).

Anxiety-like behavior. There was no effect of sex or treatment on anxiety-like behavior as measured by the EPM ($F(1,45)=1$; $p=0.3$, $F(1,45)=0.1$; $p=0.7$, respectively) or the light/dark chamber ($F(1,45)=0.1$; $p=0.7$, $F(1,45)=0.5$; $p=0.5$, respectively), (data not shown).

Social recognition. There was no sex difference ($F(1,42)=2.2$; $p=0.1$) but there was a main effect of treatment on social recognition ($F(1,42)=7.2$; $p=0.01$), with CDB-treated animals showing better recognition than oil-treated animals (Fig 2). Post hoc comparisons indicate that the improvement in social recognition was significant in males ($p=0.03$), but not in females ($p=0.1$).

AVP mRNA. There was an interaction between sex and treatment on relative AVP mRNA ($F(1,33)=4.9$; $p=0.03$, Fig 3A). Post hoc comparisons indicate a trend of lower mRNA in oil-treated females compared to CDB-treated females ($p=0.08$), while there was no effect in males ($p=0.2$).

Methylation of the Avp *promoter.* There was a trend of an interaction between sex and treatment on methylation at CpG site 1 ($F(1,35)=3.8$; $p=0.06$, Fig 3B). Post hoc comparisons indicate that oil-treated females had higher methylation than both oil-treated males ($p=0.03$) and CDB-treated females ($p=0.04$). There was no effect of sex or treatment on methylation at CpG sites 2, 3, or 4 (CpG site 2: $F(1,35)=1.2$; $p=0.3$, $F(1,35)=0.07$; $p=0.8$, Fig 3C), CpG site 3: ($F(1,34)=0.42$; $p=0.5$, $F(1,34)=2.0$; $p=0.2$, Fig 3D), or CpG site 4: ($F(1,35)=0.63$; $p=0.4$, $F(1,35)=3.8$; $p=0.06$, Fig 3E)). There was a trend of an interaction between sex and treatment on methylation at CpG site 5 ($F(1,34)=2.8$; $p=0.1$, Fig 3F). Post hoc comparisons indicate a trend of higher methylation in oil-treated females compared to both oil-treated males ($p=0.09$) and CDB-treated females ($p=0.09$).

Discussion

The present data suggest that PRs play a role in organizing some AVP-dependent behaviors and AVP expression. Neonatal antagonism of PRs improved juvenile social recognition in both males and females, and increased play behavior in females. This was coincident with an increase in AVP mRNA and a decrease in methylation at two CpG sites within the *Avp* gene promoter region within the female amygdala on PN35. Although previous data have demonstrated that PRs regulate AVP expression and AVP-dependent behavior in adults (Auger and Vanzo, 2006; Rood *et al.*, 2008; Bychowski and Auger, 2012; Bychowski *et al.*, 2013), the present study is the first to show effects on these systems following a transient neonatal manipulation of PRs.

Play Behavior.

The increase in play in females may be in part due to the increase in AVP mRNA. This is supported by studies showing correlations between play behavior and AVP expression in the MeA, BST, and LS in males (Chapter 2), (Kurian *et al.*, 2008; Taylor *et al.*, 2012). Specifically, male rats had reductions in both social play and AVP expression, suggesting that AVP may

facilitate play behavior. This is also consistent with the fact that males have higher levels of AVP within these brain regions and exhibit higher levels of juvenile social play (Meaney and Stewart, 1981; De Vries *et al.*, 1981; Beatty *et al.*, 1981; Meaney *et al.*, 1981; Van Leeuwen *et al.*, 1985). Furthermore, another study showed that intracerebroventricular (ICV) injection of a V1a receptor antagonist decreased play in males, although the same injection into the LS increased play in these animals (Veenema *et al.*, 2013b). In the same study, the ICV injection of a V1a receptor antagonist increased play in females and the injection in the LS decreased it. These authors suggest that AVP acting throughout the brain facilitates play in males and inhibits it in females, while septal AVP facilitates play in females and inhibits it in males (Veenema *et al.*, 2013b). An increase in AVP within the amygdala would presumably increase AVP within the LS, as cell bodies within the MeA project to the LS (Caffe *et al.*, 1987). Therefore, although these previous findings are complex, the effect on AVP within the LS is consistent with the data that we present here, where an increase in AVP within the female amygdala is coincident with increases in play. Alternatively, an increase in AVP mRNA within the female amygdala could increase social play by acting on V1 receptors within the amygdala (Ebner *et al.*, 2002; Salome *et al.*, 2006), which Veenema *et al.* 2013 did not examine. Therefore, although the relationship between AVP and juvenile social play is complex, the present data and previous data suggest that AVP within the MeA, BST, and LS facilitates juvenile social play in both males (Chapter 2), (Kurian *et al.*, 2008; Taylor *et al.*, 2012) and females (Veenema *et al.*, 2013b). However, it is also possible that other signaling molecules, such as opioids, endocannabinoids, dopamine, norepinephrine, serotonin, and GABA cause or influence the increase in play in females (Vanderschuren *et al.*, 1997; Auger and Olesen, 2009; Trezza *et al.*, 2010). Further studies are necessary to elucidate the relationship between PRs, juvenile social play, and AVP.

Social Recognition

The increase in AVP mRNA within the amygdala of females treated neonatally with CDB may also contribute to the improvement in social recognition in females. More than 20 years of research has established that AVP originating within the MeA and BST and acting on V1a receptors within the LS is necessary for social recognition in males (Dantzer *et al.*, 1988; Bielsky *et al.*, 2004; Bielsky *et al.*, 2005a). Although oxytocin appears to be the primary player for female recognition, AVP does facilitate recognition in females (Bluthe and Dantzer, 1990; Engelmann *et al.*, 1998). Furthermore, although social recognition in juvenile animals is less well understood, a recent study found that V1a receptors within the LS are important for social recognition in both juvenile males and females and that an injection of AVP into the LS improved social recognition in females, but not males (Veenema *et al.*, 2011). Alternatively, the increase in AVP in females could improve social recognition through acting on oxytocin receptors (Mouillac *et al.*, 1995; Chini *et al.*, 1996).

The mechanism for improved social recognition in CDB-treated males is unclear. Although AVP within the LS is necessary for normal social recognition in intact adult males, CDB treatment did not increase in AVP mRNA levels in males in our study. Studies have shown that there are other mechanisms that compensate for changes in AVP in order to maintain typical social recognition. Specifically, castration, which reduces AVP, only temporarily impairs social recognition and an AVP antagonist does not impair social recognition in these animals (Bluthe *et al.*, 1990). Although we did not see changes in AVP mRNA in CDB-treated males at PN35, it is clear that PRs suppress AVP levels within adult male brain (Auger and Vanzo, 2006). Therefore, it is possible that neonatal antagonism of PRs transiently influenced AVP expression. Similar to what is seen in castrated animals, this alteration of AVP during the neonatal period may have initiated a compensatory pathway that facilitates social recognition.

It is also possible that mechanisms other than AVP within the MeA influence social recognition in CDB-treated juvenile males and/or females. First, it is possible that neonatal CDB

treatment increased AVP within other brain regions that are important for social recognition, and this could contribute to the improvement. For example, AVP within hippocampus (van Wimersma Greidanus and Maigret, 1996) and olfactory bulb play a role in social recognition (Tobin *et al.*, 2010; Wacker and Ludwig, 2012). These brain regions were not assessed in the present study. Additionally, several other signaling molecules are involved in social recognition, including oxytocin, ERs, and ARs. Oxytocin is necessary for social recognition in rats and mice (Ferguson *et al.*, 2000; Ferguson *et al.*, 2001), particularly in females (Engelmann *et al.*, 1998), particularly within the MeA (Choleris *et al.*, 2006; Choleris *et al.*, 2007). Oxytocin within the LS, mPOA, and hippocampus appears to facilitate social recognition as well (Popik and Van Ree, 1991; Popik *et al.*, 1992; van Wimersma Greidanus and Maigret, 1996). Additionally, estrogens appear to facilitate social recognition primarily through acting on ERα, and are more important in females (Gabor *et al.*, 2012). The effects of estrogens may be through the regulation of oxytocin receptors (Young *et al.*, 1998). The role of androgens in social memory is complex, and seems to primarily influence social recognition in males by altering AVP levels (Bluthe *et al.*, 1990; Bluthe *et al.*, 1993). Therefore, although blocking neonatal PRs with the non-specific antagonist, RU-486, increases AR-ir within the male brain (Appendix B), it is unlikely that altered androgen activity accounts for the improvement in social recognition in males in the present study. Although the mechanism is unknown, neonatal CDB administration may increase the expression of one of these signaling molecules in males and/or females.

AVP mRNA

Although previous data have demonstrated that PRs regulate AVP expression in adult rats (Auger and Vanzo, 2006) and mice (Rood *et al.*, 2008), the present study is the first to show lasting effects on AVP expression following a transient neonatal manipulation of PRs. While Rood *et al.* 2008 found that PRKO male and females have increased AVP expression, males still expressed higher levels of AVP. However, as PR knockout mice lack PRs both during and

after development, this study did not distinguish between the organizational and regulatory effects of PRs.

As it is clear that PRs suppress AVP levels in male brain (Auger and Vanzo, 2006; Rood *et al.*, 2008), it is interesting that CDB did not increase AVP within the male amygdala. However, as stated previously, although we did not see changes in AVP expression in CDB-treated males at PN35, neonatal antagonism of PRs may have transiently influenced AVP expression. This would suggest that PRs regulate AVP expression in males, but play a role in the organization of AVP expression in females. Further studies are needed to test this hypothesis.

AVP Methylation

As females had higher methylation than males at two CpG sites and CDB treatment decreased methylation at these sites to male-typical levels, it appears that PRs play a role in sex-specific CpG methylation within the *Avp* gene promoter. Although the functional significance of the differential DNA methylation of the *Avp* promoter is unknown, the decrease in DNA methylation corresponds to an increase in AVP mRNA in females. These data are consistent with the general belief that methylation reduces gene transcription (Colot and Rossignol, 1999; Bird and Wolffe, 1999), and with data that suggests that the sex difference in AVP expression is mediated in part by differential DNA methylation (Chapter 3).

Mechanisms

PRs may be important for inhibiting AVP expression in the developing female brain. Testosterone and its metabolites are largely responsible for inducing male-typical AVP expression patterns, and although females are already resistant to the effects of testosterone because they do not have testes that release testosterone, perinatal ovarian production of estrogen is minimal, and they express an estrogen-sequestering protein during development (Germain *et al.*, 1978), the action of PRs may be another mechanism by which females are protected from a masculine phenotype. How this may occur remains unclear, and there are

several possibilities. First, progestins can downregulate ER in vitro (Read *et al.*, 1989; Alexander *et al.*, 1990; DonCarlos *et al.*, 1995), therefore it is conceivable that CDB blocks the ability of progestins to downregulate ER, thereby allowing for increased ER-mediated AVP expression in the developing female brain. Alternatively, PRs may directly inhibit AVP transcription by binding to a potentially inhibitory PRE site within the *Avp* promoter region.

Conclusions

Previous data suggest that PRs regulate AVP within the amygdala, but may not organize AVP expression (Auger and Vanzo, 2006; Rood *et al.*, 2008). The present study is the first to examine the specific role of PRs during early development on AVP and AVP-dependent behaviors. As neonatal antagonism of PRs increases AVP expression within the juvenile female amygdala, it appears that PRs play a role in protecting females from an induction of AVP expression. The mechanism of this protection and whether it is specific to AVP expression remain unclear.

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Figures

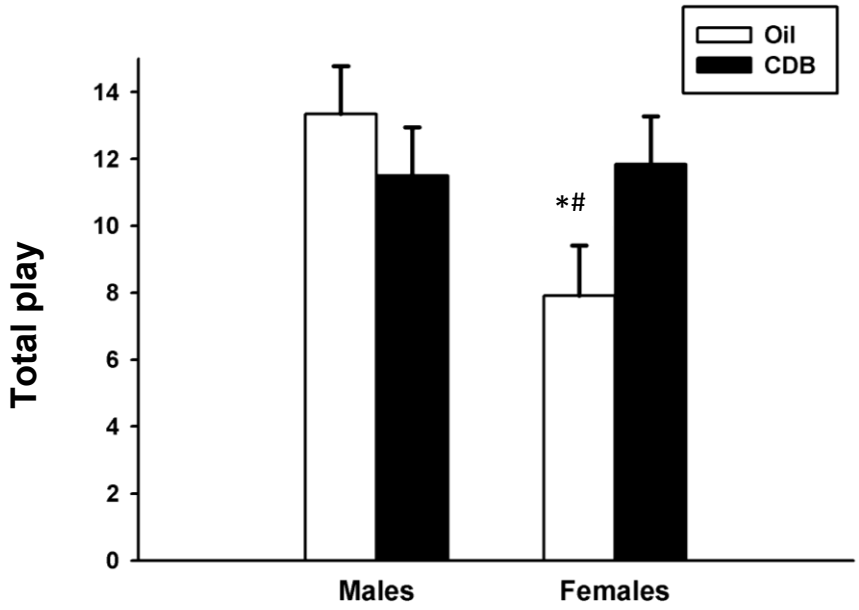

Figure 1. Social play in juvenile rats injected with CDB or control vehicle on PN0, 2, and 4. Post hoc comparisons indicate that control females initiated play less than control males (*p=0.01) and there was a trend of increased play initiation in females treated with CDB (#p=0.07). Each bar represents the mean total number of instances of play initiation. Two-way ANOVA. Error bars represent SEM.

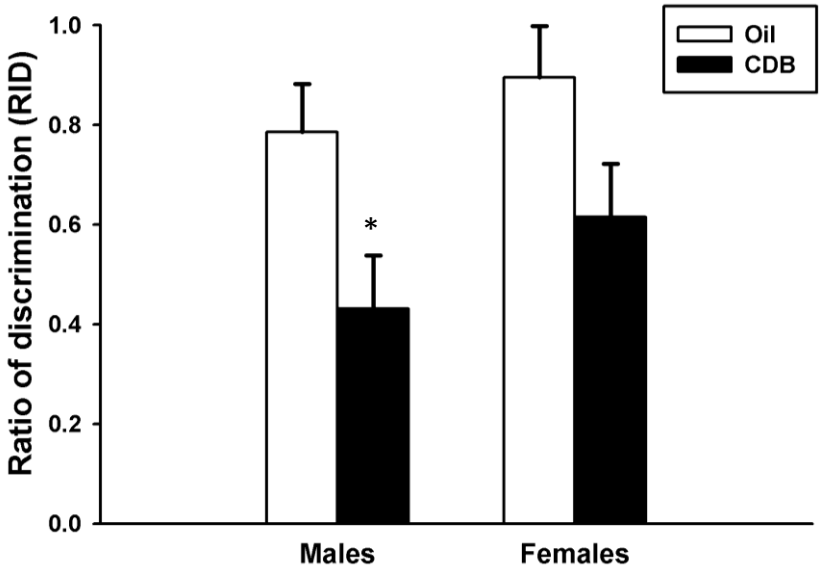

Figure 2. Social recognition in juvenile rats injected with CDB or control vehicle on PN0, 2, and 4. There was an overall effect of CDB treatment (p=0.01). Post hoc comparisons indicate that CDB treatment significantly increased social recognition in males (*p=0.03), but not females (p=0.1). Each bar represents the mean time spent investigating the familiar animal over the time spent investigating the novel animal (ratio of discrimination, RID). Smaller RID scores indicate social recognition. Two-way ANOVA. Error bars represent SEM.

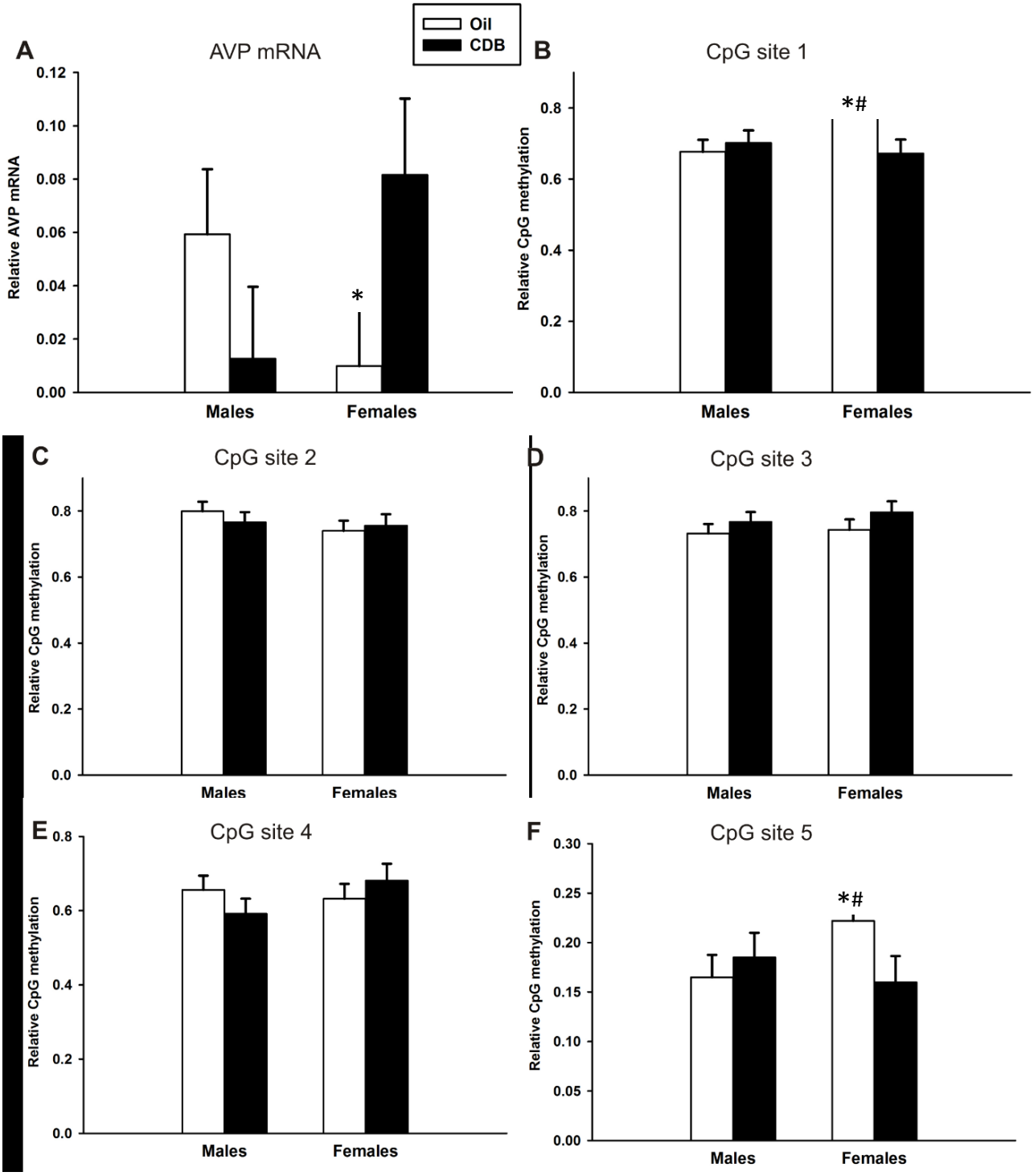

Figure 3. AVP mRNA and methylation of the *Avp* promoter region. (A) There was an interaction between sex and treatment on relative AVP mRNA (p=0.03). Post hoc comparisons indicate a trend of lower mRNA in oil-treated females compared to both CDB-treated females (*p=0.08) and oil-treated males (p=0.18). There was no effect in males (p=0.21). (B) There was a trend of an interaction between sex and treatment on methylation at CpG site 1 (p=0.06). Post hoc

comparisons indicate that oil-treated females had higher methylation than both oil-treated males (*p=0.03) and CDB-treated females (#p=0.04). (C, D, E) There was no effect of sex or treatment on methylation at CpG site 2, 3, or 4. (F) There was a trend of an interaction between sex and treatment on methylation at CpG site 1 (p=0.1). Post hoc comparisons indicate a trend of higher methylation in oil-treated females compared to both oil-treated males (*p=0.09) and CDB-treated females (#p=0.09). Two-way ANOVA. Error bars represent SEM.

Chapter 5

General Discussion

Over 30 years of research demonstrates that AVP is expressed at higher levels within the male MeA, BST, and their projection sites, and that this sex difference is largely organized and regulated by testosterone and its metabolites (e.g. De Vries *et al.*, 1981; De Vries and Buijs, 1983; De Vries *et al.*, 1983; Van Leeuwen *et al.*, 1985; De Vries and al-Shamma, 1990; Wang *et al.*, 1993; Han and De Vries, 2003). As previous research suggests that these differences are not likely caused by differential cell birth, death, or migration (al-Shamma and De Vries, 1996; Han and De Vries, 1999; De Vries *et al.*, 2008), another mechanism appears to establish phenotypic differences in expression levels. I hypothesized that steroid hormones organize sex differences in AVP expression within the rat amygdala through epigenetic modifications.

The data presented here examined the organization of AVP expression and AVP-dependent behaviors in the rat. In Chapter 2, I examined whether MeCP2 plays a role in the organization of AVP expression within the amygdala. I transiently disrupted MeCP2 expression by infusing MeCP2 or control siRNA into the amygdala during the first three days of postnatal life. AVP, galanin, and AR expression were measured in two-week old and adult animals. I found that a transient reduction in MeCP2 expression within the developing male amygdala decreases AVP, galanin, and AR expression, but only the effect on AVP lasts into adulthood. This highlights a unique role of MeCP2 in organizing sex differences in the brain. Furthermore, these data suggest that reductions in MeCP2 may impact male juvenile social behavior (Kurian *et al.*, 2008) by disrupting the organization of AVP within the developing amygdala.

Chapter 3 examined whether there are sex differences in DNA methylation of the *Avp* gene promoter region that correspond to the sex difference in AVP expression within the amygdala, and whether these sex differences are influenced by steroid hormones during development. Females had higher levels of methylation than males at two CpG sites within the *Avp* promoter region on PN10, and estradiol treatment in females decreased methylation at one of these sites and increased AVP mRNA. This is consistent with the conventional belief that methylation of CpG sites within a gene promoter region results in gene repression. Furthermore,

testosterone may organize the sex difference in AVP in part through decreasing CpG methylation of the *Avp* promoter region. The sex difference in methylation at these two CpG sites is interesting because both sites are near transcription factor response elements that are important for regulating *Avp*. CpG site 4 is near a GRE/PRE and CpG site 5 is near a CRE (Mohr and Richter, 1990; Shapiro *et al.*, 2000). Additionally, CpG site 4 is also near an AT rich region, which makes it a potential MeCP2 binding site (Klose *et al.*, 2005).

In Chapter 4, I examined whether PRs play a role in the organization of AVP-dependent behaviors and AVP expression within the amygdala. Rats were treated with CDB, a PR antagonist, during the first week of postnatal life. Neonatal PR antagonism had lasting consequences on different juvenile behaviors in males and females. While blocking PRs during development improved social recognition in both males and females, it increased social play in females to male-typical levels. This manipulation increased AVP mRNA within the female amygdala, but not the male amygdala, on PN35. Furthermore, while oil-treated females had higher methylation at CpG sites 1 and 5 than oil-treated males, blocking PRs decreased methylation of both of these sites. PRs may play a role in organizing sex differences in AVP expression through increasing methylation within the female amygdala.

Overall, these data suggest that testosterone and its metabolites organize AVP expression in part through epigenetic modifications. Furthermore, PRs not only regulate AVP expression, but play a role in its organization as well. This chapter will discuss some of the questions this research helped to answer, as well as some of the questions it raised that could be addressed in future studies.

Dynamic methylation corresponds to stable sex differences in expression

Although the sex difference in AVP expression is stable across development, the sex difference in CpG methylation at specific sites is not. On PN10, males have lower methylation at CpG sites 4 and 5, whereas on PN35 they have lower methylation at sites 1 and 5. Additionally,

preliminary data suggests that nine month-old males have lower levels of methylation only at CpG site 1 (Figure 1). While many studies have shown that some DNA methylation patterns appear to be somewhat stable, emerging data indicate that DNA methylation is dynamic in the developing and adult brain (Murgatroyd *et al.*, 2009; Schwarz *et al.*, 2010). The current data support the idea that relatively stable sex differences in expression levels of genes can be maintained by dynamic DNA methylation patterns. I find that each developmental time point is characterized by unique DNA methylation patterns on the *Avp* gene promoter region and these patterns are associated with stable sex differences in AVP mRNA levels (Figure 2). This suggests that different areas of the gene need to be made inaccessible at different developmental time points to maintain typical expression profiles.

While it is unclear why the methylation profile of the *Avp* promoter changes across development, this could be important for understanding the role of methylation as it pertains to the programming and then maintenance of the sex difference in AVP expression. The sex difference in methylation on PN10 was at two sites that are located near response elements, a GRE/PRE and a CRE. While on PN35 there was still a sex difference at the site near the CRE, in adulthood there was no longer a sex difference at any sites examined near response elements. Recent data indicate that methylation of the CpG sites near hormone response elements can fluctuate depending on the presence of circulating hormones (Auger *et al.*, 2011). One possible reason for the decrease in the number of methylated CpG sites near important response elements is that once AVP mRNA expression is organized, females may not need as much protection against the masculinizing effects of hormones at those sites. Interestingly, another study showed age-dependent hypomethylation of *Avp* without changes in expression within the PVN (Murgatroyd *et al.*, 2009). The site that is differentially methylated between males and females in adulthood may be situated near an unidentified consensus sequence binding site that is important for regulating AVP expression in the adult brain or that modifies the

efficacy of other important sites. Elucidating the function of methylation at this site may provide insights into the mechanisms by which sex differences are maintained in the adult brain.

It is possible that there are stable methyl marks on the *Avp* promoter region. While the present study examined 5 individual CpG sites, there are many more CpG sites within the promoter region as well as three other CpG islands within the *Avp* gene (Weber *et al.*, 2007). Additionally, early life stress results in hypomethylation of numerous CpG sites within the downsteam enhancer region, corresponding to an increase in AVP expression within the PVN, and differential methylation at some of these sites did not change with age (Murgatroyd *et al.*, 2009). This suggests that early life experiences can program stable methylation patterns at some CpG sites. Future research should examine whether stable sex differences in methylation occurs at CpG sites within the *Avp* gene that were not examined in the present research.

Hormones during the first week of postnatal life organize dynamic methylation patterns

It appears that both estradiol and PRs are involved in organizing sex differences in AVP expression within the amygdala via altering methylation patterns. However, although estradiol may induce AVP expression in part through altering methylation at CpG site 4, the sex difference in methylation at this site is not maintained through the juvenile or adult periods. Likewise, PRs may play a role in organizing sex differences in AVP expression through altering methylation at CpG sites 1 and 5, but only the sex difference in methylation at site 1 was maintained into adulthood. Although the sex difference at each of these sites appears to be regulated by hormone actions during the first week of life, the differential methylation at each site has a unique time course. Hormone action may induce transient differences in methylation at critical time points that are necessary for organizing lasting sex differences in AVP expression.

Although the sex difference in methylation at both sites 1 and 5 are influenced by PRs during early postnatal life, the former is present at PN35 but not at PN10. This provides a

possible example of how hormones during early life can set the brain up for sex-specific responses later in life. That is, it appears that the sex difference in methylation at site 1 is organized by PRs during the first week of life, but instead of altering methylation at that time point, the action of PRs causes the female brain to respond differently to some stimulus later in development. In this case, PRs within the first week of postnatal life may either prevent demethylation or induce methylation of CpG site 1 that occurs sometime between PN10 and PN35.

The organization and regulation of AVP depend on complex interactions.

The present data identify several factors involved in organizing AVP expression patterns within the amygdala. It appears that, while the actions of testosterone and its metabolites on ARs and ERs are important for inducing male-typical expression patterns, progestin action on PRs is important for preventing male-typical patterns. These systems may regulate AVP through independent mechanisms, for example by binding to their respective response element within the *Avp* gene promoter (Mohr and Richter, 1990; Iwasaki *et al.*, 1997; Shapiro *et al.*, 2000). While ERs have been shown to bind to an ERE to activate *Avp* transcription (Shapiro *et al.*, 2000), PRs have not yet been shown to bind to the GRE/PRE. These systems may also interact. For example, as discussed briefly in Chapter 4, progestins may down-regulate ER, as has been demonstrated *in vitro* (Read *et al.*, 1989; Alexander *et al.*, 1990; DonCarlos *et al.*, 1995), thereby inhibiting ER-mediated AVP expression within the female brain. If both direct gene regulation and this interaction between PRs and ERs occurs, this could explain why blocking PRs in neonatal females has such a large effect on AVP expression (Chapter 4). That is, if PRs inhibit male-typical AVP through both binding directly to the *Avp* promoter and inhibiting ERs, then blocking PRs could dramatically increase AVP expression in neonatal females, despite the lack of a surge in testosterone in females (Weisz and Ward, 1980; Rhoda *et al.*, 1984).

Additionally, estradiol can induce PRs (MacLusky and McEwen, 1978). Neonatal males express more PR within the mPOA and anteroventral periventricular nucleus than females (Wagner *et al.*, 1998; Quadros *et al.*, 2002), and the action of estradiol on ERα induces PRs within these brain regions (Wagner *et al.*, 2001; Quadros *et al.*, 2002). This also occurs in females. Endogenous estradiol induces PRs within the neonatal female mPOA (Jahagirdar *et al.*, 2008), and the induction of PRs within regions of the hypothalamus that control female reproduction is important for facilitating female sexual behavior (Brown *et al.*, 1987; Simerly *et al.*, 1996). However, the ability of estradiol to induce PRs is highly brain-region and age specific (Quadros and Wagner, 2008; Gonzales *et al.*, 2012), and it does not appear to occur within the amygdala in adult animals (Romano *et al.*, 1989). However, it is possible that induction of PRs via estradiol occurs within the neonatal female amygdala, and is important for inhibiting AVP expression (Chapter 4). Estradiol may induce PRs in females in order to directly compensate for the ability of estradiol to induce AVP. Although females do not experience a surge in testosterone during early life (Weisz and Ward, 1980; Rhoda *et al.*, 1984), they do have circulating testosterone from the placenta, maternal ovaries, and possibly synthesis within the brain. Females are also exposed to testosterone via their male siblings in utero (Slob and van der Schoot, 1982; vom Saal, 1989), although a recent study suggests that this may be a significant source of androgens, but not estradiol (Jahagirdar *et al.*, 2008). Regardless of the source of estradiol, it may be important to have a system that allows for stepwise compensation of its ability to induce AVP expression.

MeCP2 may also interact with hormones to alter AVP expression. Preliminary data suggest that hormones regulate MeCP2 expression. I have found that estradiol and DHT can increase or decrease MeCP2 mRNA and protein, depending on the time course of the injections (data now shown). Although these results are complex, they do present another mechanism by which hormones may indirectly regulate AVP expression. The role of estradiol, DHT, and progestins in regulating MeCP2 expression needs to be clarified in future studies.

It is important to note that other mechanisms may be involved in the organization and regulation of AVP, such as histone modifications and alterations to the polyA tail. Histone deacetylation could occur in conjunction with DNA methylation, either in coordination (Ghoshal *et al.*, 2002; Zhang *et al.*, 2005), or as a result of methylation (Nan *et al.*, 1998; Fuks *et al.*, 2003). Castration of adult male rats decreases the length of the AVP mRNA polyA tail within the BST, decreasing the stability of the mRNA and decreasing translation, while testosterone replacement restores the normal length (Carter and Murphy, 1993). However, it remains unclear whether this post-transcriptional modification plays a role in regulating sex differences in AVP expression. Much remains to be elucidated in regard to the roles of steroid hormones, their respective receptors, methyl-binding proteins, DNA methylation, and other factors in organizing and regulating sex differences in AVP expression.

MeCP2 may play distinct roles in the developing male and female brain.

It is interesting that a transient disruption in MeCP2 alters AVP expression in males but not females (Chapter 2). However, females, but not males, have increased GFAP expression following a transient reduction of MeCP2 during early postnatal life (Appendix A). This suggests that MeCP2 plays different roles in male and female rat brains. Specifically, MeCP2 may act to suppress gene expression in females and activate gene expression in males. Supporting this idea, there are sex differences in a number of epigenetic factors that are typically involved in gene repression and activation. Females express higher levels of MeCP2 (Kurian *et al.*, 2007), NCoR (Jessen *et al.*, 2010), and DNMT3a (Kolodkin and Auger, 2011) within the amygdala, which are typically associated with gene repression. Therefore, decreasing these factors may allow for a release of inhibition, for example, a decrease in MeCP2 increases GFAP expression in the female brain (Appendix A). In contrast, males express higher levels of phospohylated CREB (Auger *et al.*, 2001), CBP (Auger *et al.*, 2002), and SRC-1 (Auger, unpublished data) in several brain areas. It has been proposed that MeCP2 can interact with CREB and co-activators

at the genome to increase gene transcription (Chahrour *et al.*, 2008), therefore it follows that sex differences in the regulation of gene expression via MeCP2 might be the consequence of differential co-regulator expression patterns between males and females. That is, the higher levels of phosphorylated CREB and coactivator expression in the male brain may interact with MeCP2 to increase gene transcription; whereas the higher levels of co-repressors in the female brain may interact with MeCP2 to repress gene transcription (Figure 3). A future study should test this hypothesis. For example, it would be interesting to determine if MeCP2 binds directly to the *Avp* gene promoter, and whether it does so along with CREB in the male amygdala.

Some outcomes of a disruption in MeCP2 during development are lasting.

It is intriguing that while the effects of a transient reduction in MeCP2 are temporary in females (Appendix A), some of the effects are lasting in males (Chapter 2), (Kurian *et al.*, 2008). This suggests that males may be more sensitive to disruptions in MeCP2, which could be in part because they express lower levels of MeCP2 within some brain regions, including the amygdala (Kurian *et al.*, 2007). This is in agreement with *MECP2* mutations on the X-chromosome being generally lethal in males due to a lack of compensatory MeCP2 (Amir *et al.*, 1999). The role of MeCP2 within the female brain is largely understudied, primarily because the majority of knockout animals are male. Particularly because RTT occurs almost exclusively in females, further research should investigate whether there are lasting consequences in females following a disruption of MeCP2 during development.

Recent data indicate that there are no lasting outcomes following a loss of MeCP2 during development, as re-expression of MeCP2 in juvenile or adult mice restores many of the deficits induced by MeCP2 deletion (Guy *et al.*, 2007; Giacometti *et al.*, 2007). Our data challenge this idea by showing lasting alterations in AVP expression and juvenile social play (Chapter 2), (Kurian *et al.*, 2008). The previous studies measure severe symptoms that represent a rodent model of RTT, such as brain weight, hind-limb clasping, locomotor function,

and mortality. This highlights the importance of examining multiple phenotypes, and while it is significant that many of the severe RTT-related symptoms can be reversed, it is also important to investigate more subtle phenotypes that may be lasting.

As AVP within the amygdala is co-expressed with galanin (Miller *et al.*, 1993), and it is thought that AVP expression is induced in a subset of these neurons in a sexually dimorphic manner (Planas *et al.*, 1995; Han and De Vries, 1999), it is interesting that there was a lasting effect of a neonatal disruption in MeCP2 on AVP, but not galanin in the present study. Although some data suggests that MeCP2 regulates global gene expression (Nan *et al.*, 1997; Skene *et al.*, 2010; Singleton *et al.*, 2011), MeCP2 also regulates specific genes, such as BDNF and GFAP (Chen *et al.*, 2003; Martinowich *et al.*, 2003; Setoguchi *et al.*, 2006; Tsujimura *et al.*, 2009). The present data suggest that MeCP2 plays a role in regulating specific genes, and that it plays a role in organizing AVP expression (Chapter 2, Appendix A). It is possible that, while many of the outcomes related to global gene expression are reversed following a restoration in MeCP2, some of the alterations in the expression of specific genes are not.

Some behaviors are more resilient to neonatal disruptions.

While some behaviors are fairly sensitive to neonatal disruptions, others are resilient. For example, juvenile social play appears to be extremely sensitive to several types of disruptions. A reduction in MeCP2 within the neonatal male amygdala reduces juvenile social play to female-typical levels (Kurian *et al.*, 2008), a systemic disruption of PRs in neonatal females increases play to male-typical levels (Chapter 4), and prenatal exposure to lipopolysachride reduces play in males to female-typical levels (Taylor *et al.*, 2012). This is consistent with data that demonstrate that the first week of postnatal life is a critical period of development, and disruptions during this time can result in lasting changes in the brain and behavior (Meaney and Stewart, 1981; Beatty *et al.*, 1981; De Vries *et al.*, 1983; Wang *et al.*, 1993; e.g.Meaney and Szyf, 2005; Kurian *et al.*, 2008; Kurian *et al.*, 2010; Edelmann and Auger,

2011; Edelmann *et al.*, 2013). On the other hand, some behaviors are resilient to these same disruptions. Although a neonatal disruption in MeCP2 reduced play behavior in male rats, it did not disrupt anxiety-like behavior or sociability (Kurian *et al.*, 2008), despite the fact that this behavior is also highly influenced by AVP (Caldwell, 2012). Likewise, a neonatal disruption of PRs did not alter anxiety-like behavior and improved social recognition in males and females (Chapter 4). There are several reasons why some behaviors may be more sensitive than others. One possibility is the difference in evolutionary importance of the system. Some behaviors may not only be more important for survival than others, but they may also have a narrower range that is considered normal functioning.

Although play is a rewarding and "joyful" behavior in both males and females (Calcagnetti and Schechter, 1992; Knutson *et al.*, 1998; Knutson *et al.*, 2002), it may be particularly important for the development of some male-typical behaviors. Social play is thought to be important for the development of male sexual behavior, normal levels of male aggression, and the ability to establish dominance (Auger and Olesen, 2009). However, studies have demonstrated that males allowed just a short amount of play per day have normal development of aggressive, sexual, and social behaviors (van den Berg *et al.*, 1999). Although there is a sex difference in the frequency of social play, a reduction in play in males to female-typical levels or an increase in play in females to male-typical levels may not be evolutionarily maladaptive. That is, there may be a wide range in the amount of social play that allows for normal development, and the alterations in play behavior in the present research (Chapter 2 and 4) are still within the range of normal functioning.

I would argue that play itself is not necessary for survival, while normal anxiety-like behavior, sociability, and social recognition are. Sociability, which is the preference to spend time with another animal, is a measure of general social functioning in both males and females, and is an important trait in social species such as rats. Although increased anxiety-like behavior is typically used as a model of human anxiety disorders, a rats typical performance in these

tests is an appropriate and adaptive demonstration of fear (Ohl, 2005). Social recognition is an evolutionarily important behavior, as it is critical for reproduction, kin recognition, territorial defense, and the establishment of dominance hierarchies (Ferguson *et al.*, 2002). Despite having altered AVP expression, the animals in these studies had normal or improved functioning in regard to these evolutionarily adaptive behaviors (Chapter 2 and 4). It is possible that additional or compensatory be mechanisms exist to maintain normal functioning in regards to these critical behaviors. Additionally, sex differences in these behaviors are not typically found (Markham and Juraska, 2007; Simpson and Kelly, 2012), suggesting that there may be a narrower range in normal functioning in regards to these behaviors compared to social play.

Some of these findings may be relevant to sexual differentiation of AVP within the BST.

With the exception of Chapter 2, the data presented here examined the regulation of AVP only within the amygdala. Whether these findings apply to other brain regions, specifically to sexually dimorphic AVP within the BST, is unknown. The BST and CMA are highly interconnected (De Olmos and Ingram, 1972; Krettek and Price, 1978; Swanson and Cowan, 1979; De Olmos and Heimer, 1999; Dong *et al.*, 2001; Cooke and Simerly, 2005), these brain regions have been collectively called the extended amygdala, in part due to their shared autonomic and endocrine functions and neurochemical specializations (Alheid and Heimer, 1988; Alheid *et al.*, 1998; De Olmos and Heimer, 1999).

However, although the organization and regulation of sexually dimorphic AVP within these two regions is generally the same (De Vries *et al.*, 1994; Wang and De Vries, 1995), there are differences in the responsiveness of the two regions to circulating hormones. Within the MeA, there is a sex difference in the number of AVP cells, but not in the amount of AVP mRNA per cell (Wang and De Vries, 1995); within the BST, there is a sex difference in both (De Vries *et al.*, 1994). This suggests that individual cells within the male BST that have the potential to express AVP are more responsive to circulating hormones than they are within the female BST,

while individual cells within the male and female MeA have the same ability to express AVP in response to circulating hormones. Therefore, although sex differences in DNA methylation may contribute to the sex difference in AVP expression within the BST, it is possible that the differential methylation occurs at distinct CpG sites. On the other hand, as PRs regulate AVP expression within both the MeA and the BST in adult males (Auger and Vanzo, 2006), I would predict that neonatal PR antagonism would also increase AVP expression within the BST. Further studies should examine the role of epigenetic modifications in the organization and regulation of AVP within the BST.

Implications.

This research helps to elucidate the role of epigenetic modifications in organizing sex differences in the brain and behavior, as well as how alterations in the typical hormonal milieu can result in atypical development. Furthermore, understanding epigenetic phenomena can help to elucidate how environmental perturbations during early life can result in lasting changes in the brain and behavior. As there are sex differences in the risk of numerous neurodevelopmental disorders, it is important to elucidate the factors that contribute to the development of the male and female brain.

Numerous studies on many species have demonstrated that the action of hormones during early life can result in lasting changes in behavior. For example, androgens increase juvenile social play in females, (Meaney and Stewart, 1981; Beatty *et al.*, 1981), glucocorticoids decrease play in males, (Meaney *et al.*, 1982), and neonatal antagonism of PRs alters juvenile social behaviors (Chapter 4). Recent studies have begun to elucidate a mechanism for these effects, by demonstrating that hormones during the first week of postnatal life can alter DNA methylation patterns (Chapter 3 and 4), (Kurian *et al.*, 2010; Schwarz *et al.*, 2010). This research is important for understanding the consequences of early hormone exposure in humans. Exposure to steroid hormones in utero can change the physiology, brain, and behavior

of humans (Cohen-Bendahan *et al.*, 2005; Berenbaum and Beltz, 2011). This exposure can be due to a clinical condition, such as congenital adrenal hyperplasia; natural variations in the prenatal environment; low-dose exposure to hormones and endocrine disruptors in the external environment (Vandenberg *et al.*, 2012), and administration to prevent or mediate a clinical condition, such as premature birth. For example, progesterone is often given during pregnancy to prevent preterm birth (Keirse, 1990; Mackenzie *et al.*, 2006) and corticosteroids are often given for a variety of therapeutic reasons (Purdy *et al.*, 2008). Although the benefits may typically outweigh any potential risks, particularly for the use of progesterone, which is widely believe to be safe for the developing fetus (Norwitz and Caughey, 2011), it is important to elucidate the long term effects of these treatments.

Additionally, hormone levels can be altered via environmental perturbations. For example, stressors in rats such as foot shock (Andersen *et al.*, 2004) and sleep deprivation (Andersen *et al.*, 2005) can increase progesterone. In pregnant females, these hormones can then cross the placenta, altering the development of the offspring (Wagner *et al.*, 1998). In other cases, the environment can alter gene expression directly through epigenetic modifications, without an apparent change in hormone levels. For example, early life stress can change MeCP2 activity, thereby changing *Avp* methylation and expression (Murgatroyd *et al.*, 2009). Furthermore, simulated maternal grooming increases ERα mRNA and decreases DNA methylation in the female rat amygdala (Edelmann and Auger, 2011). In order to understand the consequences of these environmental perturbations, it is important to understand the role and the regulation of the molecules they affect within the developing brain.

This research also has implications for understanding the development of typical and atypical social behaviors. As previously mentioned, social play in rats during the juvenile period is particularly sensitive to neonatal manipulations, which is not surprising considering that the amygdala plays an important role in social play (Meaney *et al.*, 1981; Meaney and McEwen, 1986). For example, MeCP2 within the neonatal male amygdala is important for the

development of male-typical juvenile social play (Kurian *et al.*, 2008) and AVP expression (Chapter 2). Interestingly, atypical amygdala functioning is implicated in autism (Amaral *et al.*, 2003), and studies have found altered social play in children with autism (Corbett *et al.*, 2013). Furthermore, MeCP2 expression is reduced in individuals with autism (Shibayama *et al.*, 2004) and this corresponds with increased methylation of the *MECP2* promoter (Nagarajan *et al.*, 2006), and AVP is also implicated in autism (Lukas and Neumann, 2013). It is possible that altered MeCP2 and/or AVP expression may also contribute to alterations in play behavior in children with autism. Elucidating the biological basis for the development of social play behavior can help to understand and potentially develop therapies for individuals with autism. However, much work remains to be done in untangling the relationship between MeCP2, AVP, and other factors within the brain.

MeCP2 also plays a role in RTT, X-linked mental retardation, and severe neonatal encephalopathy (Gonzales and LaSalle, 2010), and AVP is also implicated in other disorders, such as schizophrenia and affective disorders (Frank and Landgraf, 2008; Surget and Belzung, 2008). There are sex differences in the prevalence of many of these disorders (Hagberg, 1985; Tang *et al.*, 1996; Aleman *et al.*, 2003; American Psychiatric Association, 2013), suggesting that risk and resilience for these disorders is influenced by sex. As there are sex differences in the expression of both MeCP2 and AVP within the rat brain (De Vries *et al.*, 1981; De Vries and Buijs, 1983; Van Leeuwen *et al.*, 1985; Kurian *et al.*, 2007), elucidating the sex-specific functions and regulation of these molecules can lead to a better understanding of sex differences in risk and resilience for developing neurological disorders.

Conclusions.

The present data demonstrate that epigenetic modifications play an important role in organizing sex differences in AVP within the amygdala and some AVP-dependent behaviors. First, these data reveal a unique function of MeCP2 in sexual differentiation of the brain by

demonstrating that MeCP2 plays a critical role in the organization of male-typical AVP expression. While recent data suggest that MeCP2 does not play a unique role during development (Guy *et al.*, 2007; Giacometti *et al.*, 2007), the present data challenge this concept. Second, I found that dynamic DNA methylation patterns within the *Avp* gene promoter region correspond to stable sex differences in expression, and one of these marks is organized by estradiol during the first week of postnatal life. While the traditional view is that DNA methylation is stable across development, emerging data indicate that DNA methylation is dynamic in the developing and adult brain (Murgatroyd *et al.*, 2009; Schwarz *et al.*, 2010). This highlights the importance of assessing methylation at multiple developmental time points, as it cannot be assumed that these marks are permanently programmed. Lastly, although previous data demonstrate that PRs regulate AVP expression in adults (Auger and Vanzo, 2006; Rood *et al.*, 2008), these data are the first to demonstrate that PRs play a role in the organization of sex differences in AVP and AVP-dependent behaviors. While the actions of testosterone and its metabolites on ARs and ERs are important for inducing male-typical AVP expression (De Vries *et al.*, 1986; De Vries *et al.*, 1994; Zhou *et al.*, 1994; Wang and De Vries, 1995; Han and De Vries, 2003), progestins acting on PRs may be important for preventing male-typical expression, in part through altering DNA methylation patterns. This research further elucidates the role of epigenetic modifications in organizing sex differences in the brain and behavior, and has implications for understanding sex differences in risk and resilience for developing neurological disorders.

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Figures

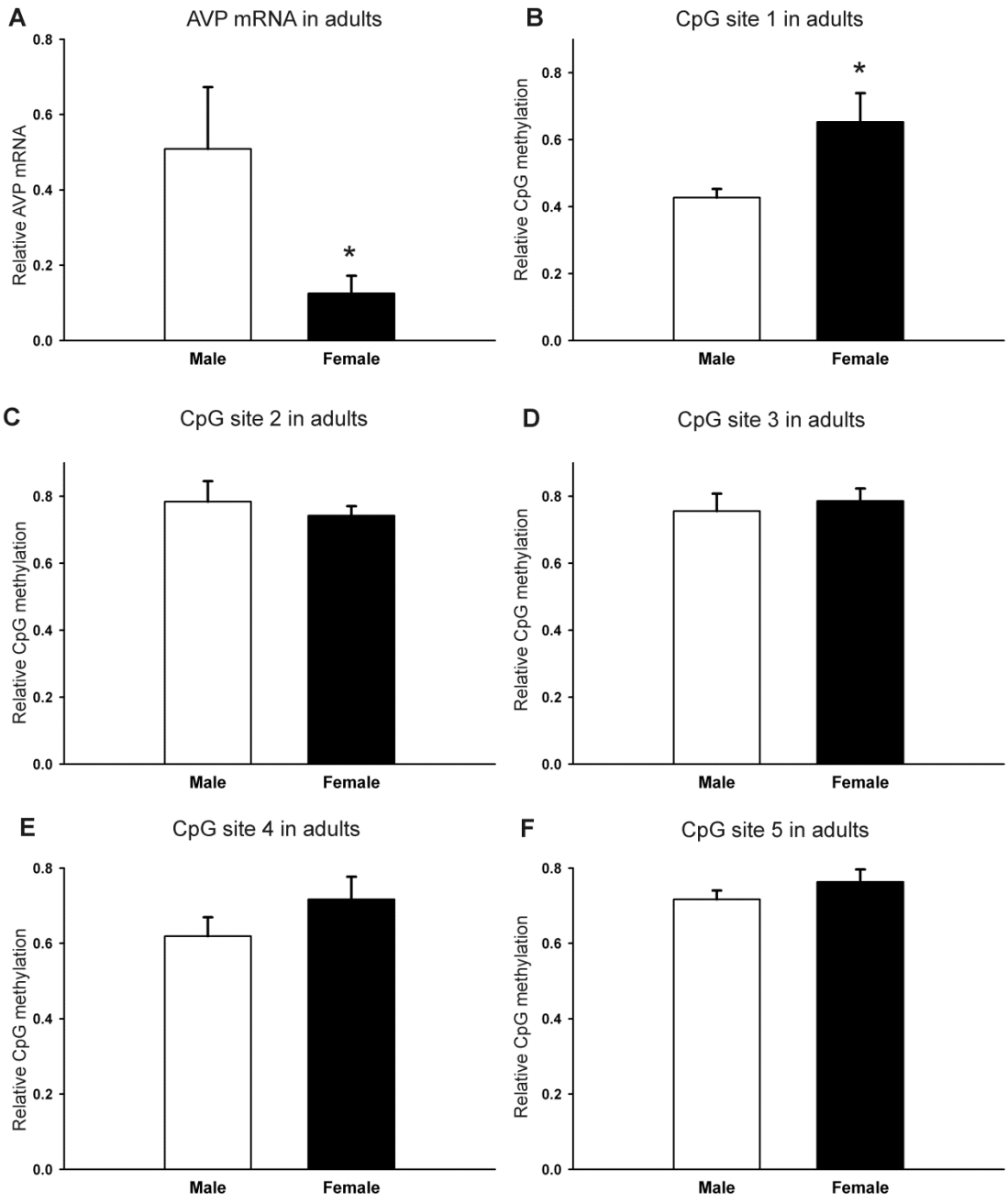

Figure 1. AVP mRNA and methylation of the promoter region within the adult amygdala. Males expressed higher levels of relative AVP mRNA than females (A, $p < .05$). Females had higher levels of methylation at CpG site 1 (B, $p=0.04$), but there was no sex difference in methylation of CpG site 2 (C, $p=0.53$), CpG site 3 (D, $p=0.64$), CpG site 4 (E, $p=0.25$), or CpG site 5 (F, $p=0.30$).

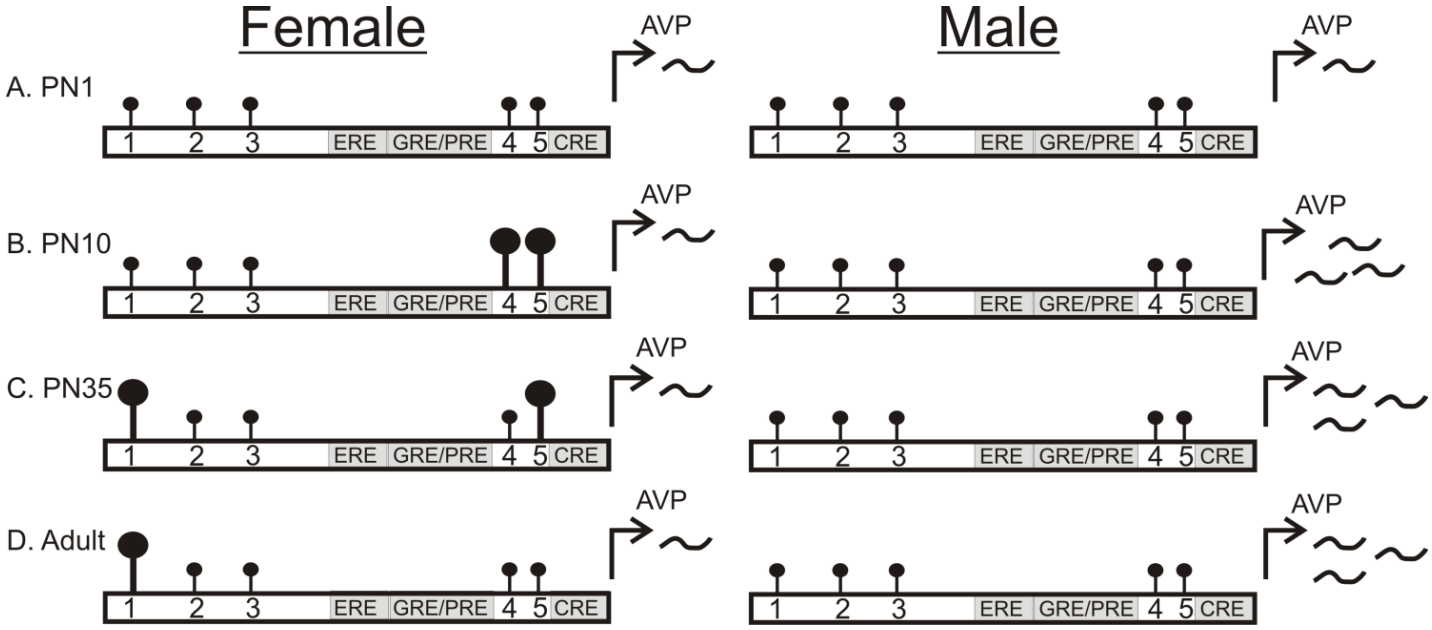

Figure 2. Schematic of dynamic methylation patterns across the lifespan. (A) On PN1, there is no difference in methylation patterns or AVP mRNA levels. (B) On PN10, a sex difference in AVP mRNA levels emerges, as well as a sex difference in CpG methylation at sites 4 and 5, near responsive elements. (C) On PN35, the sex difference in AVP mRNA levels and CpG methylation at site 5 remain, and a sex difference at CpG site 1 emerges, but there is no longer a sex difference at CpG site 4 (D). The sex difference in AVP mRNA levels is maintained into adulthood, but the sex difference in methylation is now only at CpG site 1.

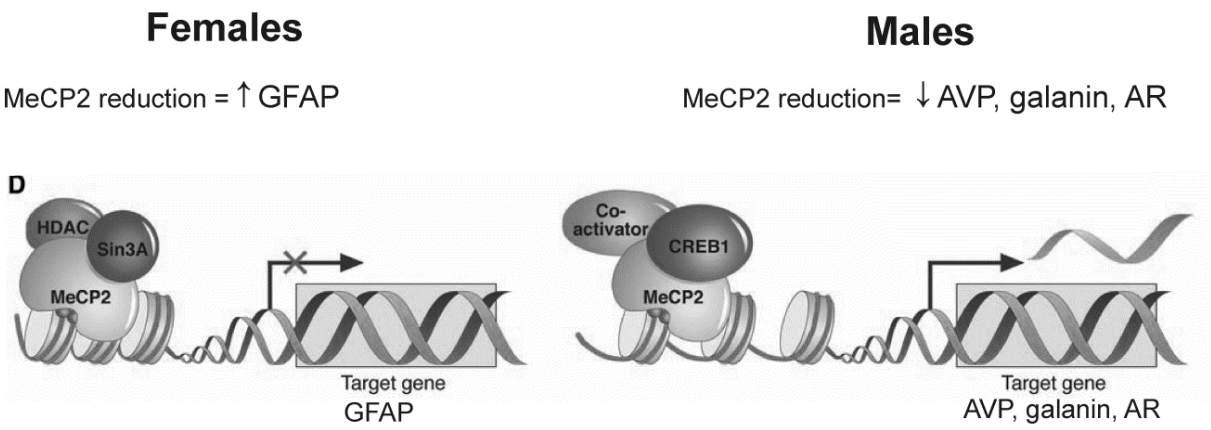

Figure 3. MeCP2 may play distinct roles within the male and female brains. In the female brain, the higher levels of co-repressors may interact with MeCP2 to repress gene transcription. A transient reduction in MeCP2 within the developing amygdala and hypothalamus increases GFAP expression within the female brain (Appendix A). In the male brain, the higher levels of phosphorylated CREB and coactivator expression may interact with MeCP2 to increase gene transcription. A transient reduction in MeCP2 within the developing amygdala decreases AVP, galanin, and AR expression within the male brain (Chapter 2).

Appendix A

MeCP2 regulates GFAP expression within the developing brain

RM Forbes-Lorman, JR Kurian, AP Auger (2014) Brain Research 1543:151-8

Abstract

Mutations in *MECP2* cause Rett syndrome (RTT), an X-linked neurodevelopmental disorder that primarily affects females. Individuals with RTT have increased glial fibrillary acidic protein (GFAP) expression in the brain. GFAP is an intermediate filament protein that is expressed predominately within astrocytes in the CNS. MeCP2 binds to methylated regions of the GFAP promoter region and suppresses GFAP expression *in vitro*. Therefore, we wanted to determine if transiently reducing MeCP2 expression would increase GFAP expression in the developing rat brain. Male and female rats received infusions of either MeCP2 or control siRNA targeting the amygdala during the first 3 days of postnatal life. Brains were collected 6 hours or two-weeks following the last infusion. MeCP2 siRNA increased GFAP mRNA and protein within the female, but not the male, amygdala on postnatal day (PN) 2. Two weeks following the infusion, levels returned to normal. MeCP2 siRNA targeting the hypothalamus also increases GFAP mRNA within the female hypothalamus on PN2, suggesting the regulation is not brain region-specific. It appears that MeCP2 does not regulate all astrocyte markers in the developing female brain, but specifically regulates GFAP expression, as levels of S100β and vimentin were not altered in the female amygdala at either time point. These data contribute to the idea that the role of MeCP2 differs in the developing male versus female brain. Further elucidating the regulation and function of GFAP can contribute to our understanding of MeCP2 function and perhaps RTT etiology.

Introduction

Mutations in *MECP2* cause Rett syndrome (RTT), an X-linked neurodevelopmental disorder that primarily affects females (Amir *et al.*, 1999). While numerous data have demonstrated the importance of MeCP2 (methyl-CpG-binding 2) function in neurons (Chen *et al.*, 2001; Kishi and Macklis, 2004; Luikenhuis *et al.*, 2004; Fyffe *et al.*, 2008; Samaco *et al.*, 2009), functional MeCP2 is also necessary in glial cells. For example, restoration of MeCP2 in glial cells can reverse some RTT symptoms in mice that have a mutation in MeCP2 (Lioy *et al.*, 2011). Furthermore, individuals with RTT have increased expression of glial fibrillary acidic protein (GFAP) (Colantuoni *et al.*, 2001), an intermediate filament protein family that is predominantly expressed in astrocytes within the CNS (Eng *et al.*, 1971; Eng, 1985), suggesting that MeCP2 suppresses GFAP expression. Indeed, MeCP2 directly regulates GFAP by binding to methylated regions of the *GFAP* promoter region to suppress its expression *in vitro* (Setoguchi *et al.*, 2006). While it is clear that GFAP within astrocytes plays an important structural role, it is also becoming clear that GFAP is also important for synaptic plasticity, vesicular transport of neurochemicals, and responses to injury (Middeldorp and Hol, 2011). Therefore, GFAP expression within the developing brain likely impacts a wide variety of cellular processes. More importantly, atypical expression of GFAP has been observed in numerous mental health diseases and increased expression of GFAP in mice causes astrocyte dysfunction and is lethal (Messing *et al.*, 1998). Taken together, these data suggest that tight control of GFAP expression may be necessary for typical brain development and function.

Interestingly, we have previously reported that a transient reduction in MeCP2 expression within the developing amygdala differentially alters the brain and behavior of male and female rats. Specifically, play behavior and arginine vasopressin expression within the amygdala were decreased in males but not females (Kurian *et al.*, 2008; Forbes-Lorman *et al.*, 2012). There are also sex differences in astrocyte morphology, including within subregions of the amygdala, which are in part organized by hormones during the first week of postnatal life

(Mong *et al.*, 1996; Mong *et al.*, 1999; Rasia-Filho *et al.*, 2002; Johnson *et al.*, 2012). Therefore, we hypothesized that MeCP2 differentially regulates GFAP expression in the developing amygdala of male and female rats. To test this, we examined GFAP expression within the amygdala following a transient reduction in MeCP2 using siRNA during the first three days of postnatal life, an important time period for astrocyte differentiation (Wang and Bordey, 2008).

Methods

Subjects. Sprague-Dawley rats supplied by Charles River were bred in our animal facility. Ten dams were allowed to deliver normally and were checked daily to determine the day of birth. The rats were housed under a 12:12 light/dark cycle with food and water available ad lib. This research was approved by the University of Wisconsin Institutional Animal Care and Use Committee.

MeCP2 disruption with siRNA. MeCP2 siRNA was used to transiently reduce MeCP2 expression as previously described (Kurian *et al.*, 2008; Forbes-Lorman *et al.*, 2012). Briefly, MeCP2 (sc-35893, Santa Cruz Biotechnology) and control siRNA (sc-37007, Santa Cruz Biotechnology) were resuspended to 100μM in the supplied siRNA diluent with Lipofectamine LTX reagent (#15338100, Invitrogen). After cold anesthetization, the amygdala (males and females on postnatal day (PN) 2 and PN14) or hypothalamus (females on PN2) was bilaterally infused with 100pmol of either MeCP2 or control siRNA. Each animal received three infusions 24 h apart on PN0–PN2, when astrocyte differentiation occurs (Wang and Bordey, 2008). Either 6 hours after the last infusion on PN2 or on PN14, animals were sacrificed by rapid decapitation and the amygdala or hypothalamus were microdissected and snap-frozen in methylbutane. Final numbers were 10 control siRNA males, 12 MeCP2 siRNA males, 11 control siRNA females, and 13 MeCP2 siRNA females collected on PN2 and 5 control siRNA males, 7 MeCP2 siRNA males, 7 control siRNA females, and 5 MeCP2 siRNA females collected on PN14. Each dam contributed approximately equal numbers of sex and treatment.

Real-time PCR. Total RNA was isolated from approximately half of the amygdala samples collected on PN2 (5 control siRNA males, 5 MeCP2 siRNA males, 4 control siRNA females, and 6 MeCP2 siRNA females), the hypothalamus samples collected on PN2 (6 control siRNA females, 6 MeCP2 siRNA females), and all of the samples collected on PN14 using the AllPrep DNA/RNA Mini Kit (#80204, Qiagen). RNA concentrations were determined using a Qubit® Fluorometer (Invitrogen) and a Qubit® RNA assay (Q32855, Invitrogen). cDNA was generated using ImProm-II™ Reverse Transcription System (A3800, Promega) in an Eppendorf MasterCycler Personal PCR machine and stored at -80˚C until used in Real-time PCR. Real-time PCR was used to quantify GFAP, vimentin, and S100β mRNA using a Stratagene Mx3000P™ real-time PCR system as previously described (Forbes-Lorman *et al.*, 2012). Primers designed to target GFAP, vimentin, S100β, and HPRT (Table 1) were run in separate reactions in duplicate. Efficiency of all primers was between 90-110%. Relative cDNA levels were calculated using the ΔΔCT method.

Western Immunoblotting. The amygdala from approximately half of the samples collected on PN2 (5 control siRNA males, 7 MeCP2 siRNA males, 7 control siRNA females, and 7 MeCP2 siRNA females) were homogenized in 300µl of lysis buffer. To remove cellular debris and nuclei, the samples were centrifuged and the supernatant was collected. For the samples collected on PN14, protein was isolated from the samples following RNA extraction according to an adaptation of the Qiagen supplementary protocol RY22 and a previously published method (Nolan and Teller, 2006). Briefly, protein was precipitated in 100% ethanol overnight, washed in acetone, and dissolved in 250µL of 2% DEA in 50mM NaCl with 0.3µL Protease Inhibitor Cocktail (P8340, Sigma), and 0.3µL Phosphatase Inhibitor Cocktail (P2850, Sigma). Samples were centrifuged at 12,000xg for 10min at 4°C, the supernatant was collected, and 0.1M Tris-HCl, pH 6.8 was added to neutralize the samples. For both sample sets, total protein concentration was determined by a Pierce™ BCA Protein Assay (#23225, Thermo Fisher).

Western immunoblotting was carried out as previously described (Kurian *et al.*, 2007; Kurian *et al.*, 2008). Briefly, a precast SDS-PAGE 4–20% Tris-glycine gel (#58645, Cambrex Bio Science) was used and transferred to a polyvinyl difluoride membrane (Immobilon-P, #IPVH20200, Millipore). Membranes were blocked for 1 h in 0.1 M TBS containing 5% nonfat dry milk and then incubated overnight at 4°C with agitation in TBS containing 0.05% Tween-20 (TBS-T) and 2% nonfat dry milk with an antibody that recognizes GFAP (1:10,000, mouse monoclonal, G3893, Sigma). The next day, the membranes were washed and then incubated in a goat anti-rabbit horseradish peroxidase-linked secondary antibody (1:3000, #7074, Cell Signaling Technology) and horseradish peroxidase-conjugated anti-biotin antibody (1:5000, #7075, Cell Signaling Technology) for 30 min at room temperature with agitation. Membranes were washed one final time and immunoreactive bands were detected using a chemiluminescence kit (#7003, Cell Signaling Technology) and exposed to x-ray film. To correct for total protein concentration, membranes were incubated in Ponceau S solution (P7170, Sigma) for 5 min at room temperature with agitation and then washed in distilled water. Ponceau S staining has been shown to correlate well with blotting for β-actin and allows for accurate quantification of the amount of total protein bound to a membrane (Klein *et al.*, 1995). Because the protein band appearing at 45 kDa contains all proteins potentially ranging from 43 to 47 kDa, normalizing to this band takes into account a multitude of proteins. The analysis of multiple proteins reduces the possibility of confounds attributable to sex differences in glyceraldehyde-3- phosphate dehydrogenase (GAPDH) (Perrot-Sinal *et al.*, 2001), actin (Stanley and Fink, 1986), or hormonal regulation of GAPDH (Funabashi *et al.*, 1994) and β-actin (Matsumoto, 1997). Ponceau-S-stained membranes and the immunoblot films were digitized using a scanner and analyzed using the macro gel plot 2 within the Scion Image program. Ratios of GFAP immunoreactive protein to the nonspecific protein band were obtained for each animal.

Statistical analyses. All data was analyzed with a two-tailed Student's *t* test using Sigma Stat statistical software for Windows version 3.5 (Systat Software, Inc.). Outliers were determined using the Grubbs' test by GraphPad Software, Inc. (http://www.graphpad.com). Effects with a p value of < 0.05 were considered statistically significant.

Results

MeCP2 siRNA was infused into the rat amygdala on PN0-2. Importantly, we have previously shown that MeCP2 protein levels are reduced in both the male and female amygdala six hours following the last infusion on PN2 (Kurian *et al.*, 2008) and that levels return to control levels by PN10 (Kurian *et al.*, 2008; Forbes-Lorman *et al.*, 2012). In the present study, MeCP2 siRNA increased relative GFAP mRNA ($t(8)=2.7$, $p=0.03$, Fig 1A) and relative GFAP protein levels ($t(12)=3.9$, $p=0.002$, Fig 1B) in the female amygdala on PN2. There was no effect of MeCP2 siRNA on relative GFAP mRNA ($t(7)=0.6$, $p=0.6$, Fig 2A) or relative GFAP protein levels ($t(10)=1.4$, $p=0.2$, Fig 2B) in the male amygdala on PN2.

In order to determine whether the effects were specific to the amygdala, MeCP2 siRNA was infused into the developing female hypothalamus. This increased relative GFAP mRNA expression on PN2 ($t(10)=3.0$, $p=0.01$, Fig 3). To determine the specificity of a MeCP2 disruption on the expression of astrocyte markers, we also measured S100β, a glial-specific marker that is expressed primarily in mature astrocytes (Raponi *et al.*, 2007), and vimentin, a marker of immature astrocytes (Pixley and de, 1984). There was no significant effect of MeCP2 siRNA on relative S100β mRNA ($t(8)=0.8$, $p=0.5$, Fig 4A) or vimentin mRNA ($t(7)=1.6$, $p=0.1$, Fig 4B) in the female amygdala on PN2.

In order to determine whether the increase in GFAP expression in females was lasting, GFAP mRNA and protein levels were measured in the amygdala on PN14. There was no effect of MeCP2 siRNA on relative GFAP mRNA ($t(9)=1.1$, $p=0.3$, Fig 5A) or relative GFAP protein levels ($t(10=1.8$, $p=0.1$, Fig 5B) in the female amygdala on PN14. As expected, there was no

effect of MeCP2 siRNA on relative GFAP mRNA ($t(10)=1.9$, $p=0.08$, Fig 6A) or relative GFAP protein levels ($t(10)=0.7$, $p=0.5$, Fig 6B) in the male amygdala on PN14. There was also no effect of MeCP2 siRNA on relative S100β mRNA ($t(10)=0.6$, $p=0.6$, Fig 7A) or vimentin mRNA ($t(10)=0.4$, $p=0.7$, Fig 7B) in the female amygdala on PN14.

Discussion

In the present study, we report that a transient reduction in MeCP2 within the developing amygdala increased GFAP expression on PN2 within the female, but not male, amygdala. This effect is not specific to the amygdala, as MeCP2 siRNA infused into the female hypothalamus also increased GFAP mRNA within this region. Our *in vivo* findings are consistent with previous data indicating that MeCP2 suppresses GFAP expression *in vitro* (Setoguchi *et al.*, 2006). Interestingly, there were also no significant changes in the levels of vimentin or S100β mRNA on PN2 or PN14, indicating that MeCP2 regulates GFAP expression but not all markers of astrocytes. Furthermore, the increase in GFAP expression was transient, with levels returning to control levels by PN14. To note, there was also no lasting effect in an additional adult sample set that was examined using immunocytochemistry (unpublished data). Therefore, while MeCP2 regulates GFAP expression within the developing female brain, these effects appear to be transient and do not result in lasting alterations in GFAP expression. These data are consistent with previous findings that report the reversal of some developmental abnormalities in *Mecp2*-deficient mice after re-introduction of a functional MeCP2 (Guy *et al.*, 2007; Giacometti *et al.*, 2007).

While the present data indicate that MeCP2 regulates GFAP expression, it is less clear if this is accomplished via direct binding of MeCP2 to the *GFAP* gene or via intermediate signaling pathways. Previous data demonstrates that MeCP2 binds directly to the *GFAP* promoter region of neural stem cells (Setoguchi *et al.*, 2006), suggesting that MeCP2 directly regulates GFAP within these cells. Additional supporting data indicate that MeCP2 couples with SIN3A to bind

the GFAP promoter and recruit HDACs, which deacetylate histones to repress GFAP expression (Cheng *et al.*, 2011). Combined, these data support the idea that MeCP2 may participate in the direct repression of GFAP expression at its promoter. However, as the mechanism was not examined in the present study, indirect regulation, such as the modulation of the JAK/STAT pathway, hormonal responses, or other growth factors, cannot be ruled out.

As far as we know, the present study is the first to demonstrate that MeCP2 regulates GFAP expression within the developing brain. However, the lasting consequences of transiently elevated GFAP expression remain unclear. Previous data indicate that MeCP2 plays a role in neuronal maturation but not differentiation *in vivo.* Specifically, the number of neurons and glia was not altered in *Mecp2*-deficient mice (Kishi and Macklis, 2004; Smrt *et al.*, 2007). This is also consistent with data demonstrating redundant functions of methyl-binding proteins in neural stem cell differentiation in the postnatal mouse brain (Setoguchi *et al.*, 2006; Martin Caballero *et al.*, 2009). The present data are somewhat consistent with the idea that a disruption in MeCP2 during early postnatal development may not have lasting consequences on astrocyte differentiation, as we found a transient increase in GFAP expression and no alterations in other markers of astrocytes. However, it is possible that a transient disruption in MeCP2 accelerated differentiation of glia without altering glial cell number in the end, as previous data shows early gliogenesis in *Mecp2*-null cells (Okabe *et al.*, 2010). Additionally, while the regulation of GFAP is transient, GFAP participates in a wide variety of cellular processes and it is possible that altered GFAP expression during brain development results in lasting alterations in neuronal function.

A lack of a lasting effect in GFAP expression in the female amygdala might not account for the changes in astrocyte morphology known to accompany typical sexual differentiation (Mong *et al.*, 1996; Mong *et al.*, 1999). Female rats have higher GFAP-immunoreactivity in the adult medial amygdala (Rasia-Filho *et al.*, 2002) and astrocyte morphology is organized by steroid hormones during early postnatal life in the hypothalamus (Mong *et al.*, 1996; Mong *et al.*,

1999) and the amygdala (Johnson *et al.*, 2012). MeCP2 may play a role in the organization of these sex differences. It is possible that the transient reduction in MeCP2 in the present study resulted in lasting alterations in astrocyte morphology but not GFAP mRNA and protein levels in females. Interestingly, previous data found lasting alterations in gene expression and behavior in males, but not females, infused with MeCP2 siRNA (Forbes-Lorman *et al.*, 2012). Although it is unclear why these effects differ between males and females, the present data provide additional evidence that responses to MeCP2 disruption within the developing brain may be influenced by sex.

While a transient reduction in MeCP2 increased GFAP expression in females, a lasting decrease in arginine vasopressin expression and juvenile social play behavior was found in males following this same treatment (Forbes-Lorman *et al.*, 2012). This supports an intriguing idea that MeCP2 may partly act to suppress gene expression in females and activate gene expression in males. Supporting this idea, there are sex differences in a number of epigenetic factors that are typically involved in gene repression and activation. Females express higher levels of MeCP2 (Kurian *et al.*, 2007), NCoR (Jessen *et al.*, 2010), and DNMT3a (Kolodkin and Auger, 2011) within the amygdala, which are typically associated with gene repression. Therefore, decreasing these factors may allow for released inhibition. In contrast, males express higher levels of phospohylated CREB (Auger *et al.*, 2001), CBP (Auger *et al.*, 2002), and SRC-1 (Auger, unpublished data) in several brain areas. It has been proposed that MeCP2 can interact with CREB and co-activators at the genome to increase gene transcription (Chahrour *et al.*, 2008), whereas MeCP2 also interacts with co-repressors to repress gene transcription (Nan *et al.*, 1998). It follows that sex differences in the regulation of gene expression via MeCP2 might be the consequence of differential co-regulator expression patterns between males and females. That is, the higher levels of phosphorylated CREB and coactivator expression in the male brain may interact with MeCP2 to increase gene transcription; whereas the higher levels of co-repressors in the female brain may interact with MeCP2 to repress gene transcription.

It is intriguing that while the effects of a transient reduction in MeCP2 are temporary in females in the present study, there are some lasting effects on the brain and behavior in males (Kurian *et al.*, 2008; Forbes-Lorman *et al.*, 2012). This suggests that males may be more sensitive to disruptions in MeCP2. This is in agreement with *MECP2* mutations on the X-chromosome being generally lethal in males due to a lack of compensatory MeCP2 (Amir *et al.*, 1999). Furthermore, although some outcomes following a transient disruption in MeCP2 are lasting (Kurian *et al.*, 2008; Forbes-Lorman *et al.*, 2012), the current data, along with others (Guy *et al.*, 2007; Giacometti *et al.*, 2007), indicate that not all consequences of MeCP2 disruption during development are lasting.

In summary, the present data indicate that MeCP2 regulates GFAP expression in the female rat brain and the effects of a transient disruption in MeCP2 are temporary. These data are consistent with others reporting that some outcomes of MeCP2 disruption are not lasting (Guy *et al.*, 2007; Giacometti *et al.*, 2007). As individuals with RTT have increased expression of GFAP (Colantuoni *et al.*, 2001), elucidating the regulation and functional significance of this gene can improve our understanding of RTT. Further research must be done on the role of MeCP2 within the developing brain in order to further our understanding of sex differences in the etiology and symptomology of MeCP2-related neurodevelopmental disorders

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Tables

Gene	Accession #	Forward sequence	Reverse sequence	Product size
GFAP	NM_017009	GGTGTGGAGTGCCTTCGTAT	CGATGTCCTGGGAAAAGG	139 bp
Vimentin	NM_031140	CTGCACGATGAAGAGATCCA	CACCTGTCTCCGGTATTCGT	261 bp
S100β	NM_013191	TGCTTGTCCTCTGTGCAAAC	TCCTATGGGGACAATGGTGT	272 bp
HPRT	NM_012583	GCAGACTTTGCTTTCCTTGG	CCGCTGTCTTTTAGGCTTTG	239 bp

Table 1. Primers used for real-time PCR

Figures

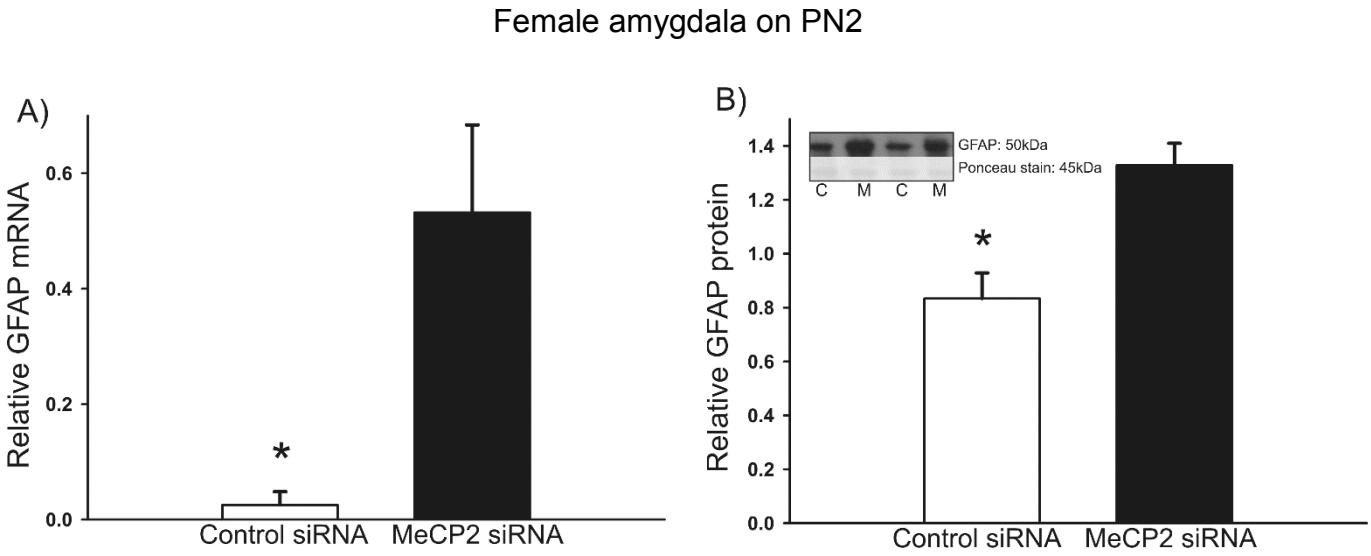

Figure 1. GFAP expression in the female amygdala on PN2. A) MeCP2 siRNA increased relative GFAP mRNA in the female amygdala on PN2 ($t(8)=2.7$, $p=0.03$). Each bar represents the mean relative mRNA expression (2^-ΔΔCT). B) MeCP2 siRNA increased relative GFAP protein levels in the female amygdala on PN2 ($t(12)=3.9$, $p=0.002$). Each bar represents the mean relative protein expression (GFAP/Ponceau S band optical density). Student's two-tailed t-test. Error bars represent SEM.

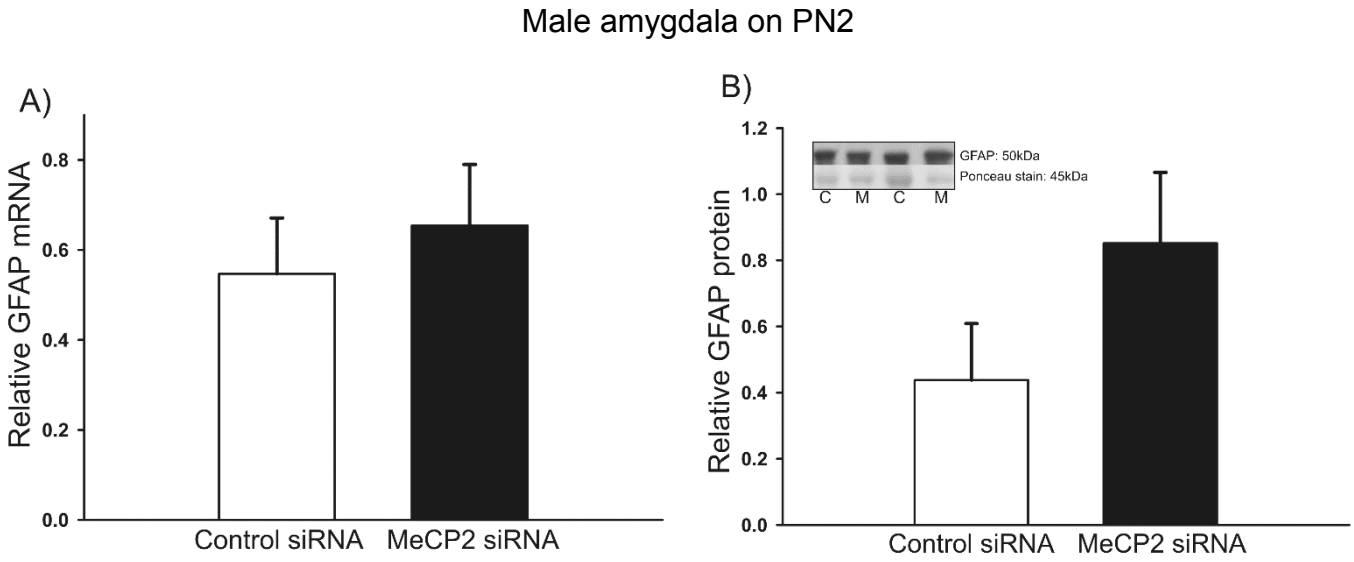

Figure 2. GFAP expression in the male amygdala on PN2. A) There was no effect of MeCP2 siRNA on relative GFAP mRNA in the male amygdala on PN2 ($t(7)=0.6$, $p=0.6$). Each bar represents the mean relative mRNA expression (2^-ΔΔCT). B) There was no effect of MeCP2 siRNA on relative GFAP protein levels in the male amygdala on PN2 ($t(10)=1.4$, $p=0.2$). Each bar represents the mean relative protein expression (GFAP/Ponceau S band optical density). Student's two-tailed t-test. Error bars represent SEM.

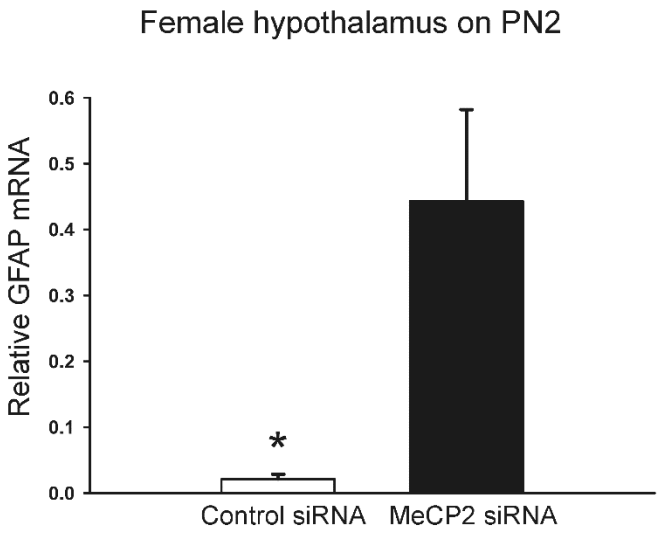

Figure 3. GFAP expression in the female hypothalamus on PN2. MeCP2 siRNA increased relative GFAP mRNA in the female hypothalamus on PN2 (*t*(10)=3.0, *p*=0.01). Each bar represents the mean relative mRNA expression (2^-ΔΔCT). Student's two-tailed t-test. Error bars represent SEM.

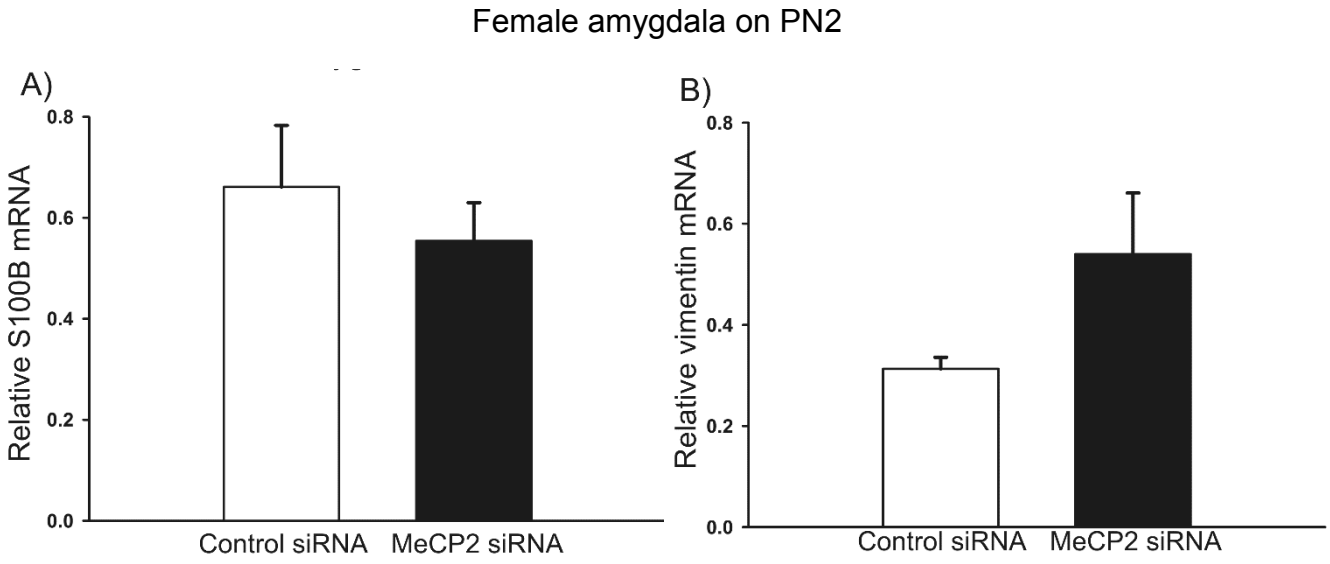

Figure 4. S100B and vimentin expression in the female amygdala on PN2. A) There was no effect of MeCP2 siRNA on relative S100B mRNA in the female amygdala on PN2 (*t*(8)=0.8, *p*=0.5). B) There was no effect of MeCP2 siRNA on relative vimentin mRNA in the female amygdala on PN2 (*t*(7)=1.6, *p*=0.1). Each bar represents the mean relative mRNA expression (2^-ΔΔCT). Student's two-tailed t-test. Error bars represent SEM.

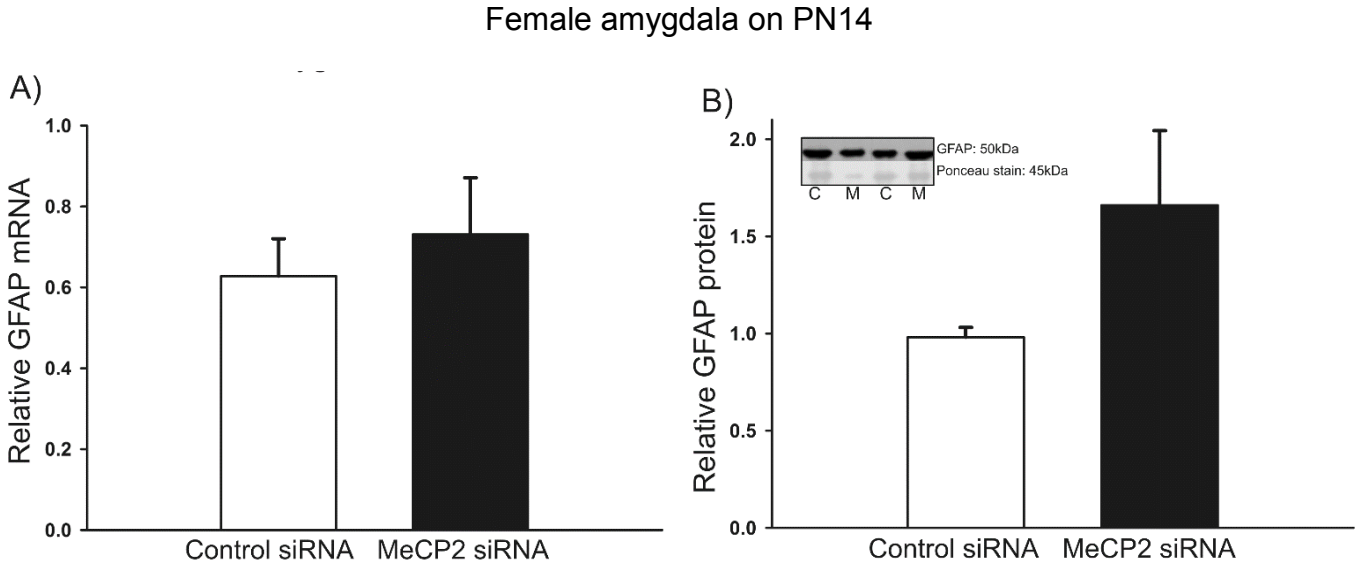

Figure 5. GFAP expression in the female amygdala on PN14. A) There was no effect of MeCP2 siRNA on relative GFAP mRNA in the female amygdala on PN14 ($t(9)=1.1$, $p=0.3$). Each bar represents the mean relative mRNA expression (2^-ΔΔCT). B) There was no effect of MeCP2 siRNA on relative GFAP protein levels in the female amygdala on PN14 ($t(10=1.8$, $p=0.1$). Each bar represents the mean relative protein expression (GFAP/Ponceau S band optical density). Student's two-tailed t-test. Error bars represent SEM.

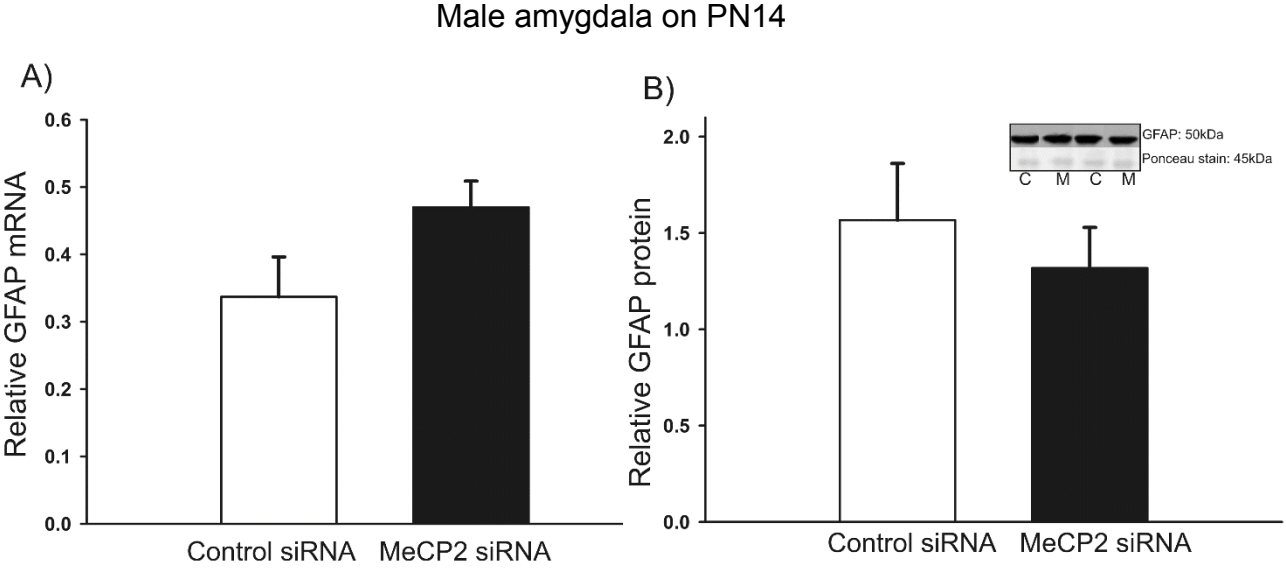

Figure 6. GFAP expression in the male amygdala on PN14. A) There was no effect of MeCP2 siRNA on relative GFAP mRNA in the male amygdala on PN14 ($t(10)=1.9$, $p=0.08$). Each bar represents the mean relative mRNA expression (2^-ΔΔCT). B) There was no effect of MeCP2 siRNA on relative GFAP protein levels in the male amygdala on PN14 ($t(10)=0.7$, $p=0.5$) Each bar represents the mean relative protein expression (GFAP/Ponceau S band optical density). Student's two-tailed t-test. Error bars represent SEM.

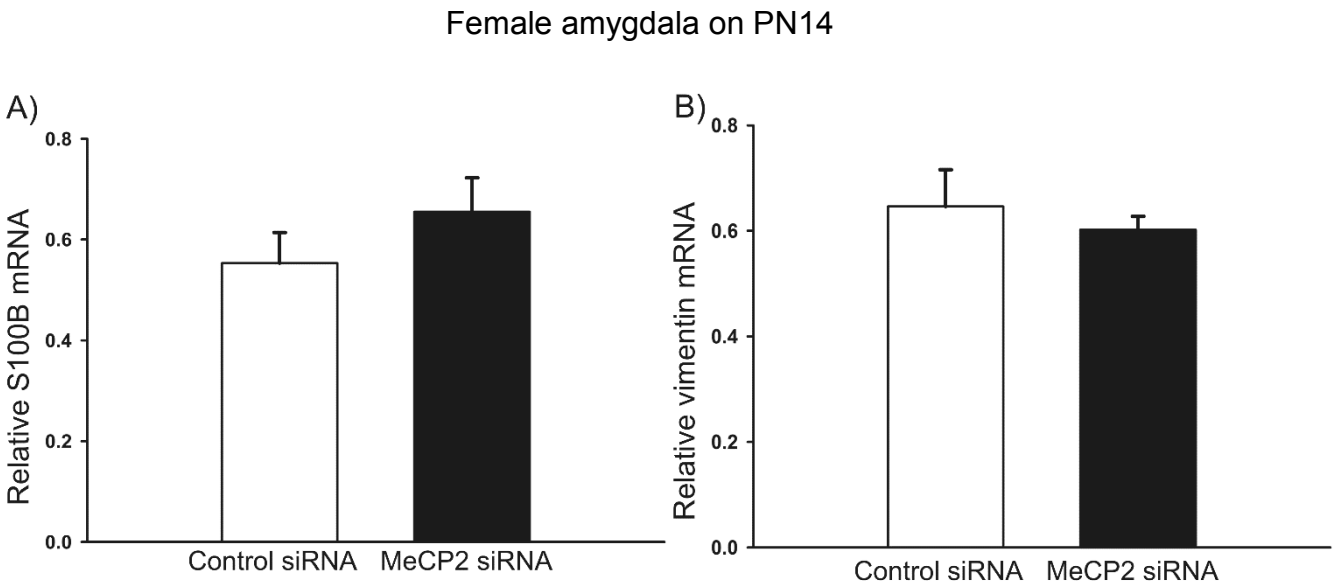

Figure 7. S100B and vimentin expression in the female amygdala on PN14. A) There was no effect of MeCP2 siRNA on relative S100B mRNA in the female amygdala on PN14 ($t(10)=0.6$, $p=0.6$). B) There was no effect of MeCP2 siRNA on relative vimentin mRNA in the female amygdala on PN14 ($t(10)=0.4$, $p=0.7$). Each bar represents the mean relative mRNA expression (2^-ΔΔCT). Student's two-tailed t-test. Error bars represent SEM.

Appendix B

Neonatal RU-486 (Mifepristone) Exposure Increases Androgen Receptor Immunoreactivity and Sexual Behavior in Male Rats

RM Forbes-Lorman, AP Auger, CJ Auger (2013) Brain Research 1543:143-50

Abstract

Progesterone and progestin receptors (PRs) are known to play a role in the development of brain physiology and behavior in many different species. The distribution and regulation of PRs within the developing brain suggest that they likely contribute to the organization of the brain and behavior in a sex-specific manner. We examined the role of PR signaling during development on the organization of adult sexual behavior and androgen receptor (AR) expression in the brain. We administered the PR antagonist, RU-486, subcutaneously to male and female rats on postnatal days 1-7 (0 = day of birth) and examined adult sexual behavior and AR-immunoreactivity (AR-ir) in the adult brain. A typical sex difference in lordosis quotient was observed and neonatal RU-486 treatment did not alter this behavior. In contrast, neonatal RU-486 treatment increased adult male sexual behavior and AR-ir in several brain areas in males. These data indicate that a transient disruption in PR signaling during development can have lasting consequences on the male brain and may increase male sexual behavior in part by increasing AR expression, and therefore androgen sensitivity, in adulthood.

Introduction

While testosterone and its metabolites are well established to be important in the development of the male brain (MacLusky and Naftolin, 1981; Cooke *et al.*, 1998), several lines of evidence suggest that progesterone and progestin receptors (PRs) are as well. Both male and female neonatal rats have circulating progesterone (about 500pg-2.0 ng/ml; (Dohler and Wuttke, 1974; Weisz and Ward, 1980), which is synthesized in the adrenal glands and locally in the brain (Shapiro *et al.*, 1976; Kohchi *et al.*, 1998; Zwain and Yen, 1999). Progesterone in neonates also has a maternal origin, as maternal progesterone crosses the placenta and is present in milk (Grota and Eik-Nes, 1967; Sanyal, 1978). Furthermore, males express PRs as early as embryonic day 20 in many brain areas, including throughout the bed nucleus of the stria terminalis (BST), amygdala, hypothalamus, and preoptic area (Quadros *et al.*, 2007), and express more PRs than females within the medial preoptic area (mPOA) and anteroventral periventricular nucleus during early postnatal life (Wagner *et al.*, 1998; Quadros *et al.*, 2002). As this sex difference in neonatal PR expression is no longer observed by about postnatal day (PN) 10, PRs during this critical period of development may be important for the organization of the male-typical brain.

Indeed, some evidence suggests that progesterone and PRs play a role in the organization of male-typical behavior. For example, progesterone administration during early life decreases male sexual behavior in rats (Hull *et al.*, 1980; Hull, 1981) and guinea pigs (Regestein *et al.*, 1975). However, the impact of blocking PR activity during the neonatal period on male sexual behavior is less clear. Treating neonatal rats with RU-486 has been reported to either increase (Csaba and Karabelyos, 2001) or decrease male sexual behavior in adulthood (van der Schoot and Baumgarten, 1990; Lonstein *et al.*, 2001; Breton *et al.*, 2012). Importantly, the development of adult male sexual behavior is highly dependent upon androgen receptor (AR) expression, as mice containing a mutation in AR have disrupted sexual behavior in

adulthood (Sato et al., 2004) and increases in male sexual behavior are coincident with increases in the expression of AR (Sachs and Meisel, 1994). In adulthood, RU-486 treatment increases the number of AR-immunoreactive (ir) cells in the endometrium of both macaques and humans (Slayden *et al.*, 2001; Narvekar *et al.*, 2004). Moreover, AR expression, as well as male sexual behavior, is increased in the brains of adult male PR knockout (PRKO) mice (Schneider *et al.*, 2005). Despite the complexity of the results, previous data suggest that PRs influence AR expression and AR-sensitive behaviors, such as male sexual behavior.

However, it is not clear if transiently blocking PRs during development alters AR expression or AR-dependent sexual behavior. The present study examines whether blocking PR signaling by administration of RU-486 during early postnatal life influences AR expression and aspects of sexual behavior in adulthood. AR-ir in adulthood was analyzed in brain areas that were chosen based on their PR content during development or the presents of PR-containing afferent projections. In addition, the areas chosen are involved in appetitive or consummatory aspects of sexual behavior or receive projections from these areas. Lastly, all areas chosen for analysis contain well-characterized AR expression levels in adulthood. Based on the previous data, we predicted that blocking PRs during early postnatal life would increase sexual behavior in adult males. Additionally, because male sexual behavior is highly dependent on ARs (Sachs and Meisel, 1994; Sato *et al.*, 2004), and PRs play a role in regulating AR expression (Slayden *et al.*, 2001; Narvekar *et al.*, 2004; Schneider *et al.*, 2005), we predicted that increases in male sexual behavior might also be accompanied by an increase in AR expression.

Methods

Subjects and Treatment. Sprague-Dawley rats obtained from Charles River Laboratories were bred in our animal facility. Dams were allowed to deliver normally and were checked daily to determine the day of birth. Twenty male and 20 female pups were pooled from 5 different

litters and assigned to a treatment group. Pups were foot-marked with India ink and placed back with 4 dams in litters of mixed sex and treatment. Pups were treated subcutaneously with either 20μg/0.01cc/g body weight of the PR antagonist RU-486 (dissolved in a 0.2% benzyl alcohol, 0.6% benzyl benzoate, and sesame oil vehicle) or 0.01cc/g body weight of vehicle (0.2% benzyl alcohol, 0.6% benzyl benzoate, and sesame oil) from PN1 through PN7 (day of birth = PN0). The weight of the pups ranged from 5-16 grams over the 8 days of treatment. We used a dose of RU-486 that is approximately equivalent to the dose used to block PR-induced sexual behavior in adult guinea pigs and rats (Brown and Blaustein, 1984; Brown and Blaustein, 1986; Auger *et al.*, 1997), adjusted to pup size. This dose has also been demonstrated to act on the progestin but not the glucocorticoid systems regulating female sexual behavior(Brown and Blaustein, 1984). All pups remained with dams until weaning at PN22, at which time they were separated into cages of six animals, with at least one animal per treatment group and sex per cage. On PN33, the animals were separated into same-sexed, mixed-treatment groups of two animals per cage. The final number of rats per group used was 8 vehicle males, 8 RU-486 males, 8 vehicle females, and 12 RU-486 females. The rats were housed under a 12:12 light/dark cycle with food and water available *ad lib*. This research was approved by the *University of Wisconsin Institutional Animal Care and Use Committee*.

Lordosis Quotient (LQ). All rats were gonadectomized on PN55 and tested for female sexual behavior 3 weeks later. Rats received 5μg estradiol benzoate in 0.1mL of sesame oil 48 hours prior to testing and 500μg progesterone in 0.1mL of sesame oil 4 hours prior to testing. One aspect of female sexual behavior was measured as previously described (Auger *et al.*, 2002). Manual stimulation was used in order to control for the amount of somatosensory stimulation received by each animal, as mating stimulation provided by a male rat can facilitate sexual behavior (Auger *et al.*, 1997). Before testing, the flanks and perineum of each rat was manually stimulated for 3 seconds to facilitate sexual behavior and to accustom the animal to stimulation.

Lordosis was scored in real time by an experimenter blind to the treatment groups. LQ ([# lordosis / 10 stimulation bouts] × 100), was recorded for each animal.

Male Sexual Behavior. Following female sexual behavior testing, all rats were implanted with Silastic® implants (2.5cm long, 1.5mm inner diameter, 2.4mm outer diameter, Dow Corning Corp.) filled with testosterone (Sigma-Aldrich, Inc) and tested for male sexual behavior 5 weeks later. Animals were tested three times, each a week apart, and the data from the third session were used. This was done in order to test sexually experienced animals, as previous data suggests that sexual experience is necessary in order to show reliable mounting behavior (Beach and Holz-Tucker, 1949). The protocol used to test male sexual behavior is similar to what we have published previously (Auger *et al.*, 2002). Each animal was placed in a testing chamber and allowed to acclimate for 5 min. After the acclimation period, a sexually receptive female was placed into the testing chamber with the experimental animal. Sexually receptive females were treated with 5μg estradiol benzoate in 0.1mL of sesame oil 48 hours prior to testing and 500μg progesterone in 0.1mL of sesame oil 4 hours prior to testing. During a 20 min period, latency to first mount, number of mounts, and intromissions were recorded.

Tissue processing and AR Immunocytochemistry. Approximately 1 week after male sexual behavior testing, trunk blood and brains were collected. Rats were deeply anaesthetized with isoflurane and rapidly decapitated. The brains were fixed by immediate immersion in 5% acrolein (Sigma-Aldrich, Inc.) in 0.1M Tris-buffered saline (TBS) for 24 hours followed by 72 hours in 30% sucrose dissolved in TBS. Following fixation, the brains were rapidly frozen in methylbutane on dry ice and stored at -80°C until tissue processing. Brains were cut into 40μm sections using a cryostat and stored at -20°C in cryprotectant (60% sucrose, 30% ethylene glycol, 2% polyvinylpyrrolidone). Immunocytochemistry was carried out on every fourth section of the brain as previously described (Forbes-Lorman *et al.*, 2012). The tissue was incubated for 18 hours with rabbit anti-AR, PG-21 primary antibody (06-680, Millipore) diluted at a

concentration of 1:2000 in a solution of 2% goat serum in 0.3% TBS-Triton X-100. A control was done to ensure the specificity of the primary antibody. A pre-incubation was done overnight using an AR peptide (ab45603, Abcam), which corresponds to amino acids 2-16 of AR (EVQLGLGRVYPRPPS). Anti-AR PG-21 corresponds to amino acids 1-21 (MEVQLGLGRVYPRPPSKTYRGC). Pre-incubation of both 100ng and 1μg of anti-AR eliminated staining.

Immunocytochemical Analysis. The following areas were chosen for analysis based on their PR content during development (e.g. mPOA) or PR containing afferent projections (e.g. LSV). In addition, the areas chosen for examination are involved in appetitive or consummatory aspects of sexual behavior or receive projections from these areas. Lastly, all areas chosen for analysis contain well-characterized AR expression levels in adulthood. The Atlas of Paxinos and Watson (Paxinos and Watson, 1986) was used to match a bilateral plate, for each animal containing the Pir, corresponding to plate 17; LSV, corresponding to plate 18; BSTMP, corresponding to plate 21; mPOA, corresponding to plate 22; VMHdm, corresponding to plate 30; and MeA, corresponding to plate 31. These areas were inspected under bright-field illumination using an Olympus BX-61 microscope fitted with a 20x objective and an Olympus FV II digital camera. The number of AR-ir cells was determined in the same sized area for each animal. The software used for analysis was the Olympus MicroSuite (Soft Imaging Corp.). The threshold to detect foreground was determined automatically by the computer software. The computer-determined background was based on 3x the standard deviation of the grey value mean of the background staining. Microscope light intensity was kept constant throughout the imaging session for each brain region analyzed to ensure consistent measurements across all brains.

Testosterone EIA. At least 1ml of trunk blood was collected from each animal, immediately placed on ice following collection, and subsequently centrifuged for 10 min at 10,000g's. The serum was removed and stored in a clean tube at -20°C until used in testosterone EIA (582707, Cayman Chemical), as previously described (Forbes-Lorman *et al.*, 2012).

Statistical analyses. All statistical comparisons were carried out using Sigma Stat statistical software for Windows version 3.11 (Systat Software, Inc.). All data were compared with Two-way ANOVAs. Main effects and interactions with a p value of < 0.05 were considered statistically significant. Fisher LSD post-hoc comparisons were run when the main effect or interaction was statistically significant.

Results

Male Sexual Behavior. There was a main effect of sex ($F(1,25)=24.20$, $p<0.001$), no main effect of treatment ($F(1,25)=2.57$, $p=0.12$), but a trend of an interaction of sex and treatment ($F(1,25)=2.96$, $p=0.09$) on the number of mounts (Fig 1A). Post-hoc tests revealed that vehicle males mounted more than vehicle females ($p=0.04$) and RU-486 males mounted more than RU-486 females ($p<0.001$). In addition, RU-486 males mounted more than vehicle males ($p=0.03$) while RU-486 treatment had no effect on mounting in females ($p=0.93$).

There was a main effect of sex ($F(1,26)=10.17$, $p=0.004$) and a main effect of treatment ($F(1,26)=4.59$, $p=0.04$) on the latency to first mount (Fig 1B). Post-hoc tests revealed that vehicle males had a shorter latency to mount than vehicle females ($p<0.05$) and RU-486 males had a shorter latency to mount than RU-486 females ($p=0.02$). However, post-hoc tests did not reveal a significant effect of RU-486 treatment on latency to first mount within males ($p=0.15$) or females ($p=0.13$) alone.

There was a main effect of sex ($F(1,26)=39.47$, $p<0.001$), a trend of a main effect of treatment ($F(1,26)=3.05$, $p=0.09$), and an interaction of sex and treatment ($F(1,26)=4.86$, $p=0.04$) on the number of intromissions (Fig 1C). Post-hoc tests revealed that vehicle males had more intromissions than vehicle females ($p=0.01$) and RU-486 males had more intromissions than RU-486 females ($p<0.001$). In addition, RU-486 males had more intromissions than vehicle males ($p=0.01$) while RU-486 treatment had no effect on intromissions in females ($p=0.73$).

Lordosis Quotient. There was a main effect of sex ($F(1,30)=12.19$, $p=0.002$) and no main effect of treatment ($F(1,30)=0.15$, $p=0.70$) on LQ (Fig 1D). Post-hoc tests revealed that, as expected, females had a higher LQ than males ($p=0.002$).

AR Immunocytochemistry. AR staining was restricted to the cell nucleus throughout the majority of the brain and in all of the areas analyzed. There was a main effect of sex in all brain areas examined, as well as either a main effect of treatment or an interaction between sex and treatment. Furthermore, post-hoc tests revealed that RU-486 males had more AR-ir cells compared to both vehicle males and RU-486 females within all brain regions examined.

In the piriform cortex (Pir), there was a main effect of sex ($F(1,25)=12.7$, $p=0.002$), no main effect of treatment ($F(1,25)=1.57$, $p=0.22$), but an interaction of sex and treatment ($F(1,25)=7.43$, $p=0.01$) on AR-ir cell number (Fig 2A). Post-hoc tests revealed that RU-486 males had more AR-ir cells compared to both vehicle males ($p=0.006$) and RU-486 females ($p<0.001$) within the Pir. In the ventral lateral septum (LSV) there was a main effect of sex ($F(1,27)=6.27$, $p=0.02$) and a main effect of treatment ($F(1,27)=5.96$, $p=0.02$) on AR-ir cell number (Fig 2B & 3A). Post-hoc tests revealed that RU-486 males had more AR-ir cells compared to both vehicle males ($p=0.004$) and RU-486 females ($p=0.003$) in the LSV. In the medial posterior division of the bed nucleus of the stria terminalis (BSTMP), there was a main effect of sex ($F(1,23)=6.47$, $p=0.02$) and a main effect of treatment ($F(1,23)=4.47$, $p<0.05$) on AR-ir cell number (Fig 2C). Post-hoc tests revealed that RU-486 males had more AR-ir cells compared to both vehicle males ($p=0.02$) and RU-486 females ($p=0.007$) in the BSTMP. In the mPOA, there was a main effect of sex ($F(1,27)=6.14$, $p=0.02$) and a main effect of treatment ($F(1,27)=4.39$, $p<0.05$) on AR-ir cell number (Fig 2D & 3B). Post-hoc tests revealed that RU-486 males had more AR-ir cells compared to both vehicle males ($p=0.006$) and RU-486 females ($p=0.002$) in the mPOA. In the dorsomedial ventromedial hypothalamus (VMHdm), there was a main effect of sex ($F(1,27)=4.39$, $p<0.05$), no main effect of treatment ($F(1,27)=3.00$, $p=0.10$), and an interaction of sex and treatment ($F(1,27)=4.60$, $p=0.04$) on AR-ir cell number (Fig 2E &

3C). Post-hoc tests revealed that RU-486 males had more AR-ir cells compared to both vehicle males (p=0.009) and RU-486 females (p=0.004) in the VMHdm. In the medial amygdala (MeA), there was a main effect of sex (F(1,26)=5.51, p=0.03), no main effect of treatment (F(1,26)=1.45, p=0.24), and an interaction of sex and treatment (F(1,26)=4.26, p<0.05) on AR-ir cell number (Fig 2F & 3D). Post-hoc tests revealed that RU-486 males had more AR-ir cells compared to both vehicle males (p=0.02) and RU-486 females (p=0.003) in the MeA.

The number of AR-ir cells did not differ between RU-486 females and vehicle females. Although there was an overall sex difference in the number of AR-ir cells within all of the brain areas examined, this was driven by the substantial increase of AR-ir in RU-486 males and there were no sex differences in AR-ir cell number in vehicle rats. This is as expected since the rats were gonadectomized and testosterone-implanted (see testosterone EIA results for serum testosterone levels).

Testosterone EIA. As all rats were implanted with testosterone-capsules, there was no effect of sex (F(1,21)=0.005, p=0.94) or treatment (F(1,21)=0.774, p=0.39) on serum testosterone level. Average testosterone levels were 1.89±1.39 ng/mL in vehicle females, 2.14±1.85 ng/mL in vehicle males, 3.59±1.31 ng/mL in RU-486 females, and 3.12±1.51 ng/mL in RU-486 males. These levels fall within the range or slightly higher than typical adult male levels of 1.25 - 2.15 ng/mL (Dohler and Wuttke, 1974; Lee *et al.*, 1975).

Discussion

Our data demonstrate that transient neonatal PR antagonism with RU-486 increases aspects of sexual behavior in adult male rats. This increase is accompanied by an increase in the number of AR-ir cells in several brain areas important for sexual behavior. Interestingly, there is no effect of RU-486 treatment on behavior or AR-ir in females. This strengthens the notion that the male brain is sensitive to PR antagonism during development and provides

further evidence for the idea that PR function is important for development of masculine behavior.

In agreement with our results, previous data found that a single dose of 100μg RU-486 within 24 hours of birth increased male sexual behavior in adulthood (Csaba and Karabelyos, 2001). It is important to note that several studies have shown a decrease in adult male sexual behavior following neonatal RU-486 treatment (van der Schoot and Baumgarten, 1990; Lonstein *et al.*, 2001). However, the dose, timing, and/or route of administration differed in these studies compared to the current study. The dose of RU-486 used in these studies was 2-5 times higher than the one used in the present study. The dose we used was adapted from studies using a dose of 5mg of RU-486 to block PR-dependent female sexual behavior in guinea pigs and rats (Brown and Blaustein, 1984; Auger *et al.*, 1997), adjusted for body weight in neonates. Our data are also consistent with data showing increased male sexual behavior in intact sexually experienced PRKO mice (Schneider *et al.*, 2005). Interestingly, another study reported that naive male PRKO mice displayed decreased sexual behavior (Phelps *et al.*, 1998) compared to wild-type controls. Also, they found in this study that aspects of sexual behavior decreased more precipitously in sexually experienced PRKO mice after castration compared to wild-type controls. While these two studies do point toward the importance of sexual experience in males, they were each performed differently, (e.g., hormonal manipulation and time of day in which behavior testing took place) making the conclusions difficult to compare. Additionally, and perhaps more importantly for the current experiment, these mice have disrupted PR throughout development and in adulthood, making the role of PRs in only the organizational aspects of adult male sexual behavior unclear.

Interestingly, Schneider et al. (2005) also found an increase in AR expression within the mPOA and BST in PRKO mice. AR expression is important for the development of adult male sexual behavior, as mice containing a mutation in AR have disrupted sexual behavior in adulthood (Sato *et al.*, 2004), also increases in male sexual behavior are coincident with

increases in the expression of AR (Sachs and Meisel, 1994). In the present study, transient PR antagonism during development also increased the number of adult AR-ir cells within several brain regions involved in male sexual behavior, including the mPOA and BSTMP. These results are also consistent with previous data showing that acute RU-486 treatment increases AR expression in the endometrium of adult macaques and humans (Slayden *et al.*, 2001; Narvekar *et al.*, 2004). Taken together, these data suggest that transient manipulation of PRs results in a lasting increase in AR expression, which may be behaviorally manifested as an enhancement of male sexual behavior.

Although RU-486 inhibits PR activity by blocking the conformational changes that are required for transcriptional activity (Edwards *et al.*, 1995), the mechanism by which a decrease in PR activity during development increases AR expression in adulthood is unclear. One possible mechanism might involve RU-486 directly influencing AR activity. Data suggest that RU-486 can increase AR activity (Kemppainen *et al.*, 1992). Importantly, the activation of neonatal ARs may have lasting consequences on the expression of ARs; for example, early testosterone administration has a lasting effect of increasing AR expression in adult sheep (Ortega *et al.*, 2009). In the present study, RU-486 may permanently increase AR expression through altering the epigenetic programming of ARs during development. This is consistent with the idea that epigenetic perturbations during development can influence gene expression patterns in adulthood (Auger and Auger, 2013). For example, perturbations of factors that act on DNA during development can have lasting effects on gene expression levels in adult brain in a sex-specific manner (Forbes-Lorman *et al.*, 2012).

A more likely mechanism involves the regulation of ARs indirectly through estrogen-mediated mechanisms. For example, estrogen acts on estrogen receptors (ERs) to increase ARs in castrated rats (Handa *et al.*, 1996) and progestins can down regulate ER in vitro (Read *et al.*, 1989; Alexander *et al.*, 1990; DonCarlos *et al.*, 1995). Taken together, it is conceivable that RU-486 blocks the ability of progestins to down regulate ER, thereby allowing for increased

ER mediated AR expression in the developing male brain. This increase in ERs, and subsequently increased AR expression, could result in hypermasculinization of the brain in response to androgens. Accordingly, the lasting increases in AR expression that we see in adult male brain are consistent with the notion that hormonal treatment and potentially receptor activation early in development can result in lasting changes in steroid receptor profiles in adulthood (Ortega *et al.*, 2009). Further studies are necessary to elucidate this potential mechanism.

In summary, our data demonstrate that RU-486 treatment during the first week of postnatal life permanently alters male-typical behavior and AR expression. While it is likely that the increase in AR-ir expression underlies the increase in male sexual behavior, it is important to note that the lasting increase in adult AR expression may influence other non-sexual AR-dependent behaviors, such as aggression. Although the specific mechanism for the regulation of ARs is unknown, our data suggest that the organization of typical AR-dependent behavior and AR expression in the adult brain is accomplished, at least in part, through mechanisms related to neonatal PR function.

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Figures

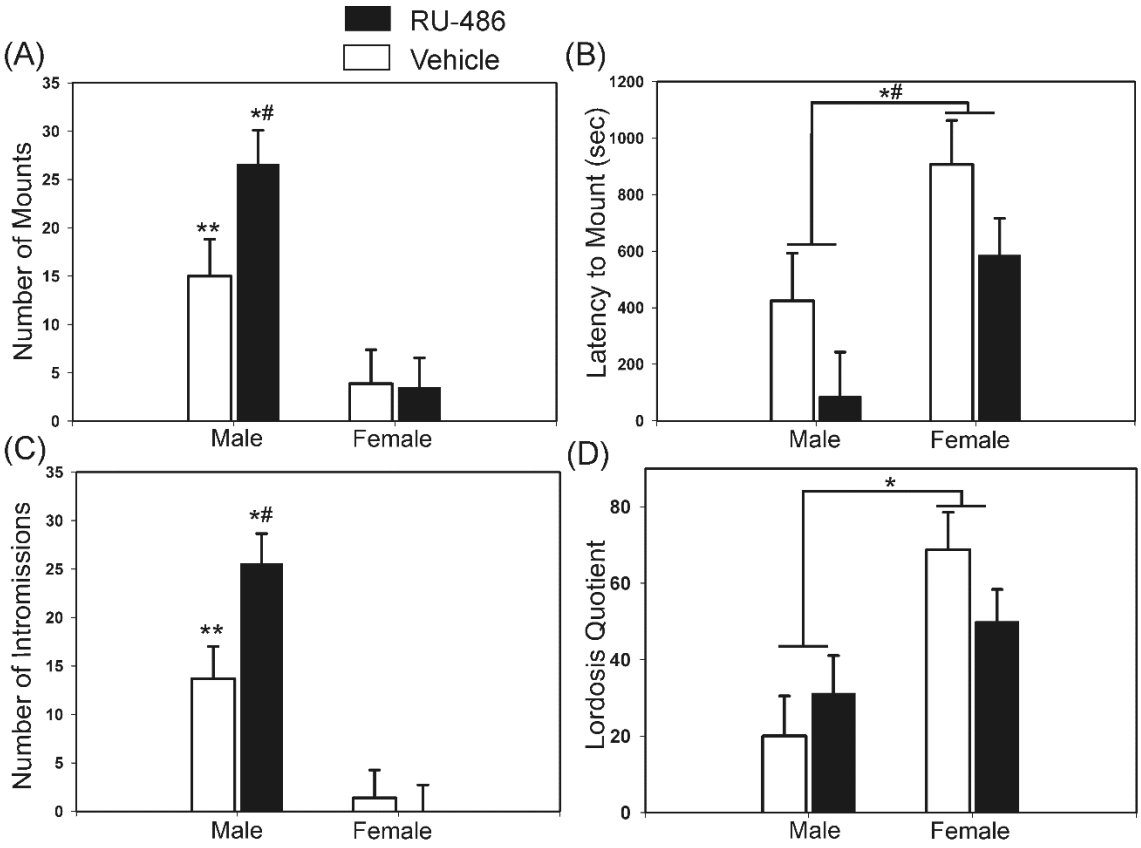

Figure 1. (A) Males mounted more than females (ANOVA, $p<0.001$). Post-hoc tests indicated that vehicle males mounted more than vehicle females (**$p=0.04$) and RU-486 males mounted more than RU-486 females (*$p<0.001$). Additionally, RU-486 males mounted more than vehicle males (#$p=0.03$). (B) Males had a shorter latency to first mount than females (ANOVA, *$p=0.004$) and RU-486 treatment reduced the latency to first mount (ANOVA, #$p=0.04$). (C) Males had more intromissions than females (ANOVA, $p<0.001$). Post-hoc tests indicated that vehicle males had more intromissions than vehicle females (**$p=0.01$) and RU-486 males had more intromissions than RU-486 females (*$p<0.001$). Additionally, RU-486 males had more intromissions than vehicle males (#$p=0.01$). (D) Females had a higher LQ than males (ANOVA, *$p=0.002$) and there was no effect of treatment.

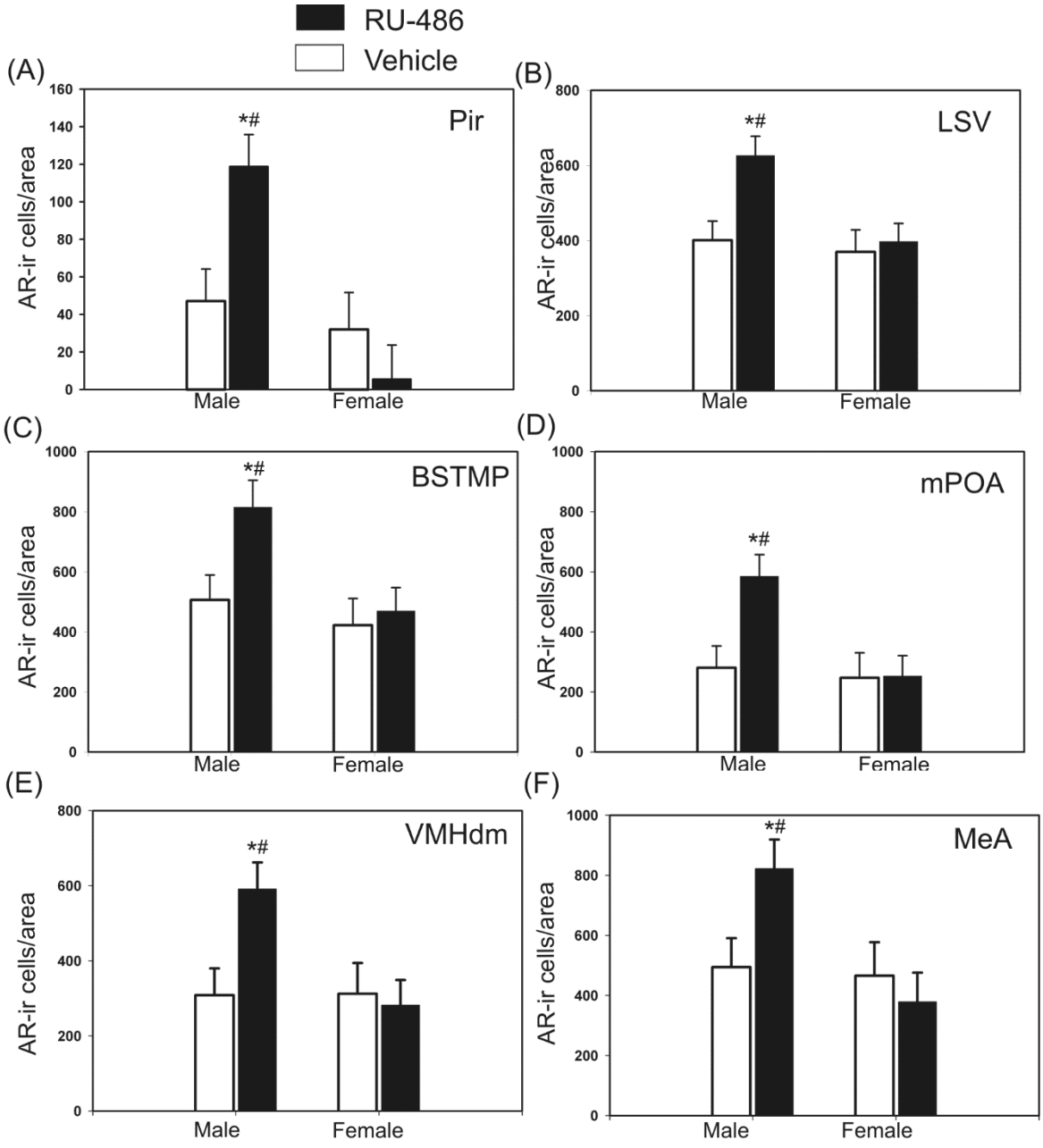

Figure 2. A Two-way ANOVA revealed a main effect of sex and either a main effect of treatment or an interaction between sex and treatment in all brain areas examined. Post-hoc tests indicate that RU-486 treatment increased the number of AR-ir cells in males, but not females, in all of the brain areas examined. RU-486 males had a higher number of AR-ir cells compared to vehicle males (*) and RU-486 females (#) within the (A) Pir (*p=0.006, #p<0.001) (B) LSV (*p=0.004, #p=0.003), (C) BSTMP (*p=0.02, #p=0.007), (D) mPOA (*p=0.006, #p=0.002), (E) VMHdm (*p=0.009, #p=0.004) and (F) MeA (*p=0.02, #p=0.003).

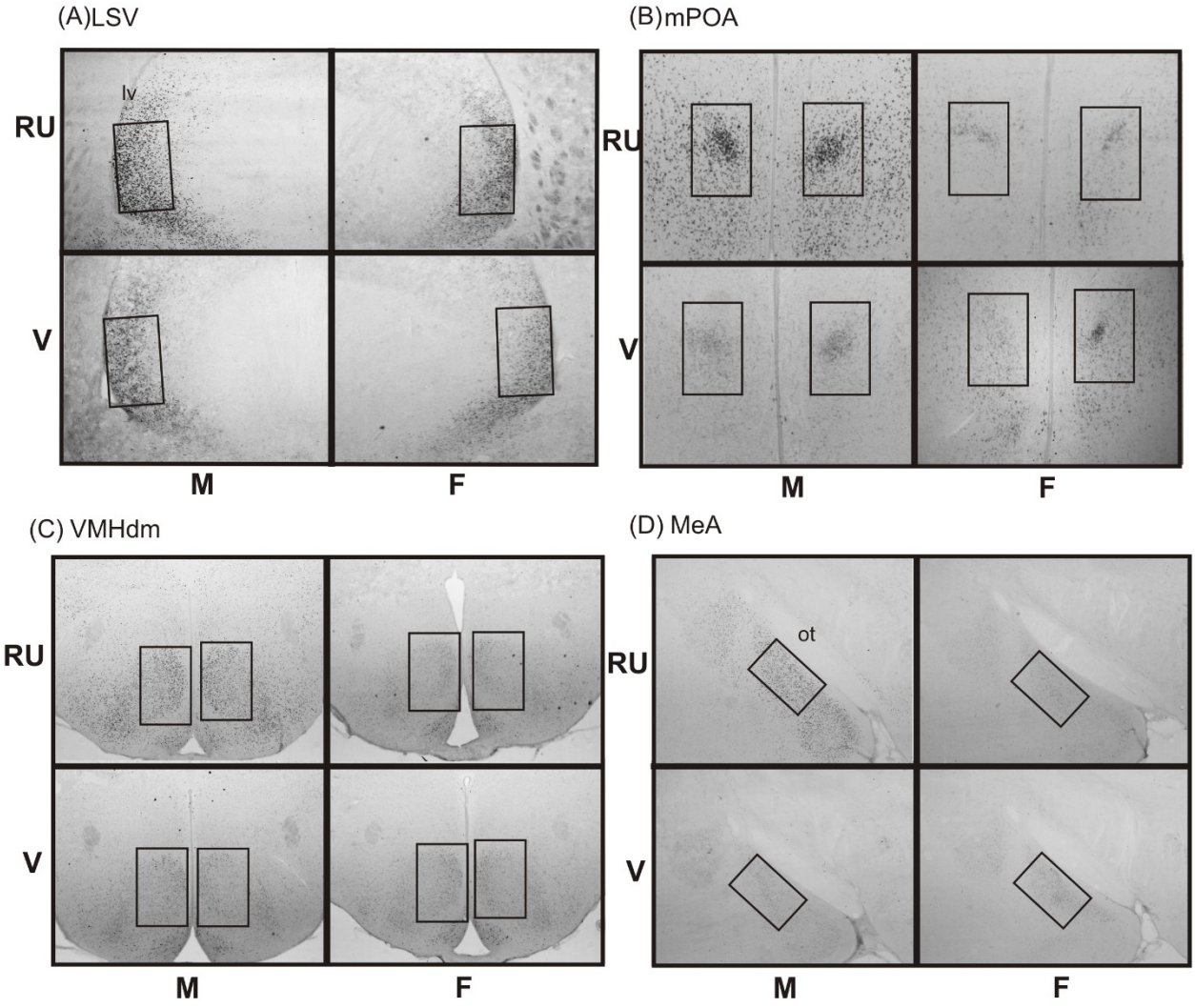

Figure 3. Photomicrograph of AR-ir cells in the (A) LSV, (B) mPOA, (C) VMHdm, and (D) MeA of male and female rats treated with RU-486 or vehicle. Box represents approximate area analyzed. Abbreviations: M, male; F, female; RU, RU-486 treated; V, vehicle treated; lv, lateral ventricle; ot, optic tract.