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Sex difference in the expression of DNA methyltransferase 3a (DNMT3a) in the rat amygdala during development

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Abstract

Sexual differentiation of the neonatal rat brain is regulated by dynamic processes occurring at the level of DNA, resulting in sexually dimorphic gene expression. Steroid hormone receptors act partly in the developing brain by recruiting co-activators, thereby increasing acetylation of histones and gene expression. Recent data indicate that sexual differentiation of the brain may also result, in part, from differences in promoter methylation patterns of some steroid responsive genes. Methylation of DNA is an epigenetic process that can decrease gene expression without altering the original DNA sequence. DNA cytosine-5-methyltransferases (DNMTs) 1 and 3a are two factors that induce methylation. We investigated whether sex differences in the expression of DNMT1 and DNMT3a were apparent in the amygdala, preoptic area, and medial basal hypothalamus at different time points during development. We found that females express significantly more DNMT3a mRNA and protein in the amygdala, but not within the preoptic area or the medial basal hypothalamus at postnatal day 1. There are no sex differences in DNMT3a mRNA or protein at postnatal day 10. Furthermore, no sex differences were observed in the expression of DNMT1 at either time point. As most sex differences in the brain are a result of a higher level of gonadal steroid hormone exposure in males at birth, we examined whether dihydrotestosterone or estradiol exposure would reduce DNMT3a expression in neonatal female rats. We found that both estradiol and dihydrotestosterone treatment significantly reduced DNMT3a, but not DNMT1, mRNA expression within the developing amygdala. Our results indicate that sex differences in DNMT3a within the developing amygdala are partly due to steroid exposure. This suggests that steroid hormone exposures may program lasting differences in amygdala function by altering the expression of the epigenetic factor, DNMT3a.

Keywords

Methyltransferase; development; epigenetics; sexual differentiation; amygdala; DNMT3a

Sexual differentiation of the brain during development is mediated by a number of mechanisms occurring at the level of DNA resulting in long-lasting sexually dimorphic gene expression. DNA methylation is a crucial epigenetic modification that partially regulates many cellular processes including genomic imprinting (1), x-inactivation (2), and transcription (3). Sex differences in cell volume (4–6) and cell morphology (7–9) have been observed in the preoptic area, ventromedial hypothalamus, and amygdala of adult rats. These areas are organized during early postnatal development (10–12) and modulate sexually dimorphic sexual and social behaviors into adulthood (13,14). How methylation influences sexual differentiation during early postnatal development is, as yet, largely unknown.

Methylation of promoter sites often precedes recruitment of methyl-binding proteins, such as methyl-CpG-binding protein 2 (MeCP2) (15), and co-repressors, such as nuclear receptor corepressor (NCoR) (16), which lead to deacetylation of histones and decreased gene expression (17). We have recently reported that female rats express significantly more MeCP2 (18) and NCoR (19) mRNA in the hypothalamus and amygdala at postnatal day 1, but not postnatal day 10. Additionally, transient decreases in the expression of MeCP2 and NCoR during development have been found to result in long-term consequences for sexually dimorphic behavior (19,20). As there are sex differences in these molecules that form co-repressor complexes, as well as DNA methylation patterns, it is possible that there are also sex differences in the methylating factors.

Co-repressor complexes form when DNA cytosine-5-methyltransferases (DNMTs) tag DNA promoters by catalyzing the transfer of a methyl group from S-adenosyl-L-methionine to the cytosine in CpG dinucleotides. DNMT1 is considered to be the main maintenance methyltransferase due to its high preference for hemimethylated DNA (21,22), whereas DNMT3a and DNMT3b are vital for *de novo* methylation (23,24). DNMT3a and DNMT3b are encoded by different genes mapping to different chromosomes (25). Many studies suggest that DNMT3a is the methyltransferase responsible for inducing methylation postnatally. DNMT3b is specifically expressed in embryonic stem cells, whereas DNMT3a is transiently expressed in both embryonic and postnatal tissues (26). Although DNMT3a protein expression increases during the first 3 weeks of postnatal brain development and then declines, DNMT3b protein expression is undetectable during this time period (27). Most interestingly, homozygous mouse knockouts of DNMT3a and DNMT3b show that DNMT3b is embryonically lethal, whereas DNMT3a results in death at approximately 4 weeks of age (28). These murine studies suggest that DNMT3a is the methyltransferase most likely responsible for *de novo* methylation in the brain during the early postnatal time period associated with sexual differentiation.

Previous studies have shown changes in DNMT expression due to hormonal exposure. Endocrine-disrupting chemicals, such as methoxychlor, have been found to increase DNMT3b expression in adult rat ovaries (29). Additionally, human endometrium shows significantly decreased levels of DNMT1 and DNMT3a during the mid-secretory phase than in the proliferative phase of the menstrual cycle (30). It has recently been found that the placentas of *in utero* female mice express significantly more DNMT1 than male placentas, while no sex difference is observed in DNMT3a. This sexually dimorphic expression pattern is correlated with sex differences in sensitivity to maternal stress during pregnancy (31). As females express higher levels of the methyl-binding protein MeCP2 and NCoR in the brain, we investigated the hypothesis that there are sex differences in the expression of DNMT3a and DNMT1 during neonatal rat brain development. Additionally, we examined whether neonatal hormone administration in females would influence DNMT3a and DNMT1 expression.

Materials and Methods

Tissue Collection

Adult female Sprague Dawley rats (Charles River Laboratories, Inc., Wilmington, Massachusetts) were mated and allowed to deliver normally. Cages were checked to determine date of birth. Animals were maintained on a 12-hr light, 12-hr dark cycle, and food and water were available *ad libitum*. Sex difference and hormone-treated animals were evenly distributed across multiple litters. Different sample sets were used for western immunoblotting and quantitation polymerase chain reaction (qPCR) experiments. Microdissections were utilized to isolate mRNA, DNA, and protein from the preoptic area (POA), amygdala, and medial basal hypothalamus (MBH), and brain regions were snap

frozen in isopentane on dry ice after rapid decapitation. Details of microdissections can be found in a previously published paper (19). These regions were frozen and stored at -80°C until homogenization for qPCR and western immunoblotting. This research was approved by the University of Wisconsin-Madison Animal Care and Use Committee.

Experiment 1: Sex differences in DNMT mRNA and protein expression

Males and females were collected via rapid decapitation at twenty-four hours (PN1) and ten days (PN10) after birth. Samples collected across multiple litters were used for qPCR experiments. DNMT3a and DNMT1 concentrations were quantified via qPCR for male and female rats at PN1 and PN10 in the amygdala (PN1: $n=9-10$ per sex; PN10: $n=4-6$ per sex), MBH (PN1: $n=7-10$ per sex; PN10: $n=4-7$ per sex), and POA (PN1: $n=7-11$ per sex; PN10: $n=4-6$ per sex). Separate animals across multiple litters were used for protein determination of DNMT3a using western immunoblotting. DNMT3a concentrations were quantified via western immunoblotting for male and female rats at PN1 and PN10 in the amygdala (PN1: $n=5-7$ per sex; PN10: $n=5-7$ per sex), MBH (PN1: $n=7$ per sex; PN10: $n=5-8$ per sex), and POA (PN1: $n=6-9$ per sex; PN10: $n=7$ per sex).

Experiment 2: Estradiol benzoate and dihydrotestosterone regulation of DNMT expression

Newborn female rats ($n=6-11$ per treatment) were given subcutaneous injections of 100 μg estradiol benzoate, 250 μg dihydrotestosterone, or oil. Selected drug dosages were based on previously published literature showing that these concentrations of hormone are required to pattern male-typical levels in the hypothalamus during development (32). Animals were pulsed with hormone or oil both immediately and 24 hours after birth. Females were collected forty-eight hours (PN2) after birth. DNMT3a and DNMT1 concentrations were quantified via qPCR for female rats at PN2 in the amygdala.

Quantitative PCR

Total RNA was isolated from snap frozen sections using the GenElute Mammalian Total RNA Miniprep Kit (Sigma, St. Louis, Missouri; catalog item sc-RTN-70), and mRNA concentrations were determined using the ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, Delaware). RNA was converted to cDNA with the Enhanced Avian RT First Strand Synthesis Kit (Sigma, St. Louis, Missouri; catalog item sc-STR1-1KT) and an Eppendorf MasterCycler Personal PCR machine. cDNA concentrations were determined via spectrophotometry. Samples containing cDNA, DNMT3a or DNMT1 primers and HPRT primers, 50 mM MgCl_2 , Rox reference dye, and Platinum Quantitative PCR SuperMix-UDG kit (Invitrogen, Carlsbad, California; catalog item sc-11730-017), underwent qPCR in a Stratagene Mx3000TM real-time PCR system. Multiplexing occurred by including JOE-labeled (6-carboxy-40, 50-dichloro-20, 70-dimethyloxyfluorescein) HPRT1 primers (Invitrogen, Carlsbad, California) for housekeeping, and FAM-labeled (60-carboxy-fluorescein) DNMT3a or DNMT1 primers (Invitrogen, Carlsbad, California) within each well. Therefore, each individual sample was normalized to its own internal control (HPRT1). DNMT3a (accession number NM_001003958.1.) primer sequences were: forward- CGGTAGCGCCTCTTCTTTGAGTTCTAC [FAM] G, reverse- GCGATCATCTCCCTCCTTGG. DNMT1 (accession number NM_053354.2.) primer sequences were: forward- CGGCGGAGGTGTCCTAACTTGGC [FAM] G, reverse- GGGTGACGGCAACTCTGGTA. HPRT1 has been found to be an ideal reference gene, and particularly suited to hormonal studies (33,34). In the current study, gene expression of HPRT1 did not differ by sex, and no significant difference in HPRT1 values was seen after hormone treatment. The amplification protocol was as follows: an initial UDG carryover prevention step at 50°C for two minutes and an initial UDG inactivation/denaturing step at 95°C for two minutes were followed by 50 cycles of a 95°C melting step for 15 seconds, 55°C annealing step for 30 seconds, and a 72°C elongation step for 30 seconds. A

dissociation curve was obtained following amplification to assess primer specificity. Amplification occurred around 25–30 cycles for both DNMT3a and hprt1. Resulting amplification curves were analyzed using a non-adaptive baseline setting, and relative cDNA levels were calculated using the $\Delta\Delta CT$ method (35).

Western immunoblotting

The snap frozen dissected brain regions were homogenized after adding 300 μ L of lysis buffer composed of 50 mM Tris-HCL, 1% Na-deoxycholate, 0.25% Nonidet P-40, 150 mM NaCl, 2 mM EDTA, protease inhibitor and phosphatase inhibitor cocktail. Samples were centrifuged at 14,000 r.p.m. for ten minutes at 4°C. Supernatant was collected and protein concentrations were ascertained with a BSA protein assay kit (Thermo Scientific, Rockford, Illinois; catalog item sc-23225). 10 μ g of sample from each animal was combined with 10 μ g of diluted Laemmli Sample Buffer (Bio-Rad, Hercules, California; catalog item sc-161-0737), and 15 μ g of the mixture was added to a precast SDS-PAGE 4–20% Tris Glycine gel (Lonza, Rockland, Maine; catalog item sc-58545) for gel electrophoresis. Samples were transferred to a Immobilon-P polyvinyl difluoride membrane, and blocked for one hour in 0.1 M TBS containing 5% non-fat dry milk with constant agitation at room temperature. Membranes were then incubated overnight at 4°C with agitation in TBS containing 0.05% Tween-20 (TBS-Tween), 2% non-fat dry milk, and a rabbit polyclonal primary antibody raised against amino acids 1–295 of human Dnmt3a, a sequence conserved in both rat and mouse (Santa Cruz Biotechnology, Inc., Santa Cruz, California; catalog item sc-20703). The following day membranes were given three quick washes followed by four five minute washes in TBS-Tween with concurrent agitation. Following washes, membranes were incubated in an anti-rabbit IgG, HRP-linked secondary antibody (Cell Signaling Technology, Inc., Danvers, Massachusetts; catalog item sc-7074) and anti-biotin HRP-linked antibody (Cell Signaling Technology, Inc., Danvers, Massachusetts; catalog item sc-7075) for 30 minutes at room temperature with agitation. Membranes were then washed again three times for five minutes with TBS-Tween and twice for five minutes with TBS. Immunoreactive bands were detected using a Lumiglo kit (Cell Signaling Technology, Inc., Danvers, Massachusetts; catalog item sc-7003) and exposed to HyBlot LC autoradiography film (Denville Scientific, Inc., Metuchen, New Jersey). Membranes were incubated in Ponceau S solution (Sigma, St. Louis, Missouri; catalog item sc-P3504) for five minutes at room temperature with agitation to normalize for protein concentration, as published previously (18,36). Membranes were then washed twice for one minute in distilled water and dried. Ponceau S solution-stained membranes and films were analyzed using the western blot macro gel plot 2 within the Scion Image program. Ratios of DNMT3a immunoreactive protein to the nonspecific protein band found with Ponceau S staining.

Statistical Analysis

Statistical comparisons were made between sexes for the POA, MBH, and amygdala at PN1 and PN10 with the Student's two-tailed t-test application within the Sigma Stat v 3.11 program (SPSS Inc., Chicago, Illinois). A one-way ANOVA, Tukey post-hoc test was utilized for statistical comparisons of hormone-treated females within the amygdala.

Results

Experiment 1: Sex differences in DNMT mRNA and protein expression

Expression of DNMT3a mRNA was quantified using qPCR within the POA, MBH, and amygdala at PN1 and PN10 in male and female rats. Females expressed significantly more DNMT3a in the amygdala at PN1 ($p = 0.040$, t-test; Figure 1). This sexually dimorphic expression was not seen in the POA or the MBH. No sex differences were seen in expression levels of DNMT3a at PN10 (Figure 2).

Expression of DNMT1 was quantified within the POA, amygdala, and MBH at PN1 (Figure 1) and PN10 (Figure 2) in male and female rats. There were no sex differences at either time point.

Expression of DNMT3a protein was measured in the POA, amygdala, and MBH at PN1 and PN10 in male and female rats using Western immunoblotting. Confirming the sex difference in DNMT3a mRNA levels, females expressed significantly more DNMT3a protein in the amygdala at PN1 ($p = 0.047$, t-test; Figure 3). This sexually dimorphic expression was not seen in the POA or the MBH. No sex difference was seen in expression levels of DNMT3a at PN10 (Figure 3).

Experiment 2: Estradiol benzoate and dihydrotestosterone regulation of DNMT expression

Expression of DNMT3a and DNMT1 was quantified in the amygdala at PN2 in female rats treated with estradiol benzoate (EB), dihydrotestosterone (DHT), or oil (O). O-treated females expressed significantly more DNMT3a in the amygdala at PN2 than EB-treated ($P = 0.032$, ANOVA; Figure 4) and DHT-treated females ($p < 0.001$, ANOVA; Figure 4), suggesting that both EB and DHT decreases DNMT3a levels. No differences were observed in DNMT1 expression.

Discussion

We questioned whether there were significant sex differences in the expression of DNA methyltransferases DNMT1 and DNMT3a during the neonatal period, and whether these differences were regulated by hormones. We have found that females express significantly more *de novo* methyltransferase DNMT3a mRNA and protein at postnatal day 1 (PN1) in the amygdala, but not the preoptic area or medial basal hypothalamus, and that this sex difference is eliminated by PN10. The sex difference in expression levels of DNMT3a are likely due to differential exposure to hormones, as both estradiol benzoate (EB) and dihydrotestosterone (DHT) treatment at PN0 and PN1 significantly decreased DNMT3a expression in the amygdala of females. Thus, hormonally-induced sexual dimorphisms in DNMT3a expression are both regionally and temporally specific during neonatal brain development.

Our results suggest sexually dimorphic gene expression of DNMT3a does not occur concurrently with DNMT1. Although it is not yet clear how regulation of DNMT1 or DNMT3a expression alters cell fate, temporally distinct regulation of these genes has previously been found. DNMT1 and DNMT3a are expressed at discrete time points during olfactory neuron development. DNMT1 expression is induced in progenitor cells and persists in post-mitotic neurons, whereas DNMT3a levels are high only in post-mitotic immature neurons prior to terminal maturation into olfactory receptor neurons (37). DNMT1 is considered to be the main maintenance methyltransferase due to its high preference for hemimethylated DNA (21,22), and DNMT3a is vital for *de novo* methylation (23,24). The maintenance role of DNMT1 is supported by evidence that DNMT1 levels vary during cell cycle checkpoint steps, an important step in mitosis, whereas little change is seen with DNMT3a. As sexually dimorphic DNMT3a expression correlates with a critical developmental time period, our findings suggest DNMT3a acts as a *de novo* DNA methyltransferase to facilitate changes in gene expression associated with sexual differentiation.

The differential expression of DNMT3a in the amygdala at PN1 follows a surge in testicular testosterone release occurring at PN0 in male rats (38). It is well known that hormonal surges during neonatal critical periods have an organizational effect on sexually dimorphic brain regions and induce long-lasting changes at the cellular and molecular level (39). Brain

regions organized by early hormone exposure include the amygdala, preoptic area, and medial basal hypothalamus; however, sexually dimorphic DNMT3a expression during development appears to be region-specific as differences in DNMT3a levels are seen only in the amygdala. It is not currently clear why there is only a sex difference observed in DNMT3a levels within the amygdala and not in the other steroid hormone sensitive brain regions.

Although all three regions are important for mediating sex behavior (13,14), the amygdala is also known to be important for the control of sexually dimorphic social behaviors like juvenile social play (40), aggression (41), and anxiety (42,43). Metabolism of testosterone into estradiol and dihydrotestosterone (DHT), and subsequent binding to estrogen and androgen receptors, respectively, largely contributes to sexual differentiation of the developing male brain (44). While neonatal administration of either estradiol (45) or DHT (46) to females masculinizes later juvenile social play, testosterone-bearing cannulae implanted into the amygdala of neonatal females is sufficient to masculinize social play behavior (47). These data indicate the importance of steroid hormone action within the developing amygdala on the programming of juvenile social play behavior. Our current data suggests that epigenetic factors, such as DNMT3a, are possible contributors to these lasting outcomes of a brief neonatal exposure to steroid hormones. While the role of DNMT3a on programming sex differences in the amygdala has not been tested, other epigenetic factors within the developing amygdala have been shown to be important for organizing juvenile social play patterns.

Our laboratory has found that the amygdala exhibits sex differences in multiple epigenetic factors during development, and that these factors may program later juvenile social play. Specifically, we found that neonatal females express significantly more MeCP2, NCoR, and DNMT3a than males within the amygdala. We have also found that transiently decreasing the expression of genes that are recruited to methylated DNA, such as MeCP2 and NCoR, within the amygdala during the neonatal period results in altered social play behaviors later in life (19,20). Interestingly, reductions in MeCP2 decreased social play in males to female levels, while reductions in NCoR hyper-masculinized play in males. These results suggest that different epigenetic factors or combinations induce divergent phenotypes.

Recent studies report a role for DNMTs in regulating emotional behavior, synapse function, and DNA methylation patterns (48,49). This suggests that changes in DNMTs levels can dramatically alter brain development, function and behavior. It has been postulated that activation of estrogen receptors induces both an increase and decrease of transcripts that are vital for proper masculinization of the brain during the neonatal period (50). An intriguing possibility is that estrogen receptors organize long-term sexually dimorphic phenotypes through complex regulation of epigenetic genes, in turn leading to proper masculinization. Our results show that EB reduces DNMT3a expression. As DNMT3a activity is one of the earlier steps leading to recruitment of methyl-binding proteins and co-repressors, such as MeCP2 and NCoR, respectively, it could potentially be an important molecule mediating estradiol-induced brain differentiation. Future studies will help elucidate the potential contribution of hormonally-sensitive, sexually dimorphic DNMT3a expression on sexual differentiation.

Sexual differentiation of the neonatal brain is associated with dynamic changes in DNA expression that can organize behaviors seen throughout the life of the animal. Changes seen in DNMT3a, but not DNMT1, expression during the early postnatal period suggest that DNMT3a may be a key factor mediating changes in methylation patterns at specific promoter sites during brain development. It is intriguing that females express significantly more DNMT3a, NCoR, and MeCP2 within the amygdala as this suggests a region-specific

concurrent regulation of multiple factors involved in silencing gene expression. Thus, hormonal regulation of genes vital for silencing expression during a critical time period of development is likely to be an important mechanism mediating sexual differentiation of the amygdala through organization at the level of the DNA.

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Figure 1.

Quantitative PCR results for relative mean expression levels of DNMT3a and DNMT1 at postnatal day 1 (PN1) in the preoptic area, medial basal hypothalamus, and amygdala of male and female rats. Females express significantly more DNMT3a, but not DNMT1, mRNA in the amygdala compared to males at PN1. Each sample was normalized to its own internal control, HPRT1. Error bars represent S.E.M. (*, $P < 0.05$)

Figure 2.

Quantitative PCR results for relative mean expression levels of DNMT3a and DNMT1 at postnatal day 10 in the preoptic area, medial basal hypothalamus, and amygdala of male and female rats. Sex differences in DNMT3a mRNA expression at PN1 are no longer present at PN10. Each sample was normalized to its own internal control, HPRT1. Error bars represent S.E.M.

Figure 3.

Representative western immunoblot bands and mean western immunoblot quantifications of DNMT3a after normalization to a Ponceau stained band in the amygdala, medial basal hypothalamus, and preoptic area at PN1 and PN10. Females have more DNMT3a, but not DNMT1, protein in the amygdala compared to males at PN1. Error bars represent S.E.M. (*, $P < 0.05$)

Figure 4. Quantitative PCR results for relative mean expression levels of DNMT3a and DNMT1 at postnatal day 2 in the amygdala of females treated with vehicle (VEH), estradiol benzoate (EB), or dihydrotestosterone (DHT). EB- and DHT-treated females have decreased levels of DNMT3a, but not DNMT1, compared to controls. Each sample was normalized to its own internal control, HPRT1. Error bars represent S.E.M. (*, $P < 0.05$, **, $P < 0.001$)