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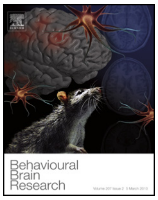

Research report

Global and gene-specific DNA methylation alterations in the adolescent amygdala and hippocampus in an animal model of caregiver maltreatment

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HIGHLIGHTS

- Maltreated males had higher 5-methylcytosine levels in the hippocampus.
- Maltreated males had lower 5-hydroxymethylcytosine levels in the amygdala.
- Maltreated females had greater *bdnf* DNA methylation in the amygdala and hippocampus.

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ABSTRACT

Epigenetic mechanisms such as DNA methylation are part of an emerging story on how early-life experiences can alter behavioral trajectories and lead to the development of disease and psychological disorders. Previous work from our laboratory has demonstrated alterations in methylation of DNA associated with the *brain-derived neurotrophic factor* (*bdnf*) gene within the amygdala and hippocampus of infant and adult rats that were repeatedly exposed to caregiver maltreatment outside the home cage during their first week of life. In the current study we examine changes in global levels of DNA methylation (5-mC) and hydroxymethylation (5-hmC), as well as gene-specific changes in methylation patterns of the candidate gene *bdnf* (at exons I and IV) within the adolescent amygdala and hippocampus resulting from exposure to maltreatment. While adolescent females exposed to maltreatment showed no significant alterations in global 5-mC or 5-hmC levels, examination of *bdnf* DNA methylation revealed that maltreated females had greater methylation of exon IV DNA in the amygdala and ventral hippocampus. While adolescent males exposed to maltreatment showed no significant alterations in *bdnf* DNA methylation, maltreated males had significantly higher 5-mC levels in the dorsal hippocampus and lower 5-hmC levels in the amygdala. These findings demonstrate that the effects of the early caregiving environment are detectable in the adolescent brain at the level of the epigenome, with brain-region specific and sexually-dimorphic epigenetic consequences that could have relevance to adolescent mental health and behavior.

1. Introduction

It is widely recognized that the early postnatal environment, specifically within the context of the caregiving relationship, moderates the development of behavior and disease with early, adverse experiences often conferring vulnerability to aberrant brain development, dysregulation of immune function, anxiety and mood disorders, deficits in learning and memory, and a myriad of other consequences that persist throughout the life of the organism [1–10]. While the consequences of disruption to the caregiving relationship are better known, the mechanisms by which these consequences occur are less understood. One proposed mechanism is DNA methylation, an epigenetic modification that occurs via the addition of methyl groups to cytosines (5-mC), typically within cytosine-guanine dinucleotides. This modification is generally associated with a decrease in gene transcription as it can both impede the binding of transcription factors and recruit repressor proteins that can condense chromatin (as reviewed in [11–14]). This is a dynamic process that varies by age, sex, and brain region, and one that is responsive to both pre- and postnatal adversity [11,15–19].

Recently, another modified form of cytosine has been gaining attention as an epigenetic modification that may play a significant role in development and behavior [20]. A derivative of 5-mC,

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Table 1
Nurturing and aversive behaviors and audible and ultrasonic vocalizations between infant conditions. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. maltreatment group. No significant differences were found between normal-care and cross-foster care. Data from [11].

Pup-directed maternal behavior	Normal-care (%)	Cross-foster (%)	Maltreatment (%)	*F*	*p*
Lick/groom	32.1^{**}	34.9^{***}	15.3	14.94	<0.001
Hover/Nurse	53.7^{***}	50.7^{***}	26.2	19.92	<0.001
Step on	5.2^{***}	6.6^{***}	17.9	21.39	<0.001
Drop	1.3^{***}	1.2^{***}	6.5	14.99	<0.001
Drag	3.7	3.2	5.0	2.29	0.112
Actively avoid	0.1^{***}	0.6	15.5	36.61	<0.001
Roughly handle	3.9^{***}	2.8^{***}	13.6	27.04	<0.001
Audible vocs	34.33^{*}	28.07^{**}	52.63	7.29	<0.01
Ultrasonic vocs	41.59^{***}	48.29^{***}	85.91	110.5	<0.001

5-hydroxymethylcytosine (5-hmC) occurs through an oxidative process that is catalyzed by the ten-eleven translocation (TET) family of proteins [21]. While it has been proposed that this oxidized form of methylcytosine is simply an intermediary in the process of demethylation, more recent evidence suggests it encompasses a larger role and may instead be a stable epigenetic modification [22–24]. For example, it is known to increase in concentration in neuronal cells as an organism ages [20], is responsive to changes in neural activity [23] including learning tasks such as fear extinction training [25], and is enriched in the brain and in genes related to synaptic function, implying a pivotal role in psychiatric disorders [26]. It has also been shown to inhibit methyl-CpG binding protein 2, a methyl binding protein important in the process of methylation, and is therefore posited to play a meaningful role in gene expression [27]. It is possible that 5-hmC functions in both capacities (as an intermediate and stable modification, both able to influence gene expression), and regardless of its exact role is a modification that requires more attention in developmental research.

Often, studies addressing the link between maternal behavior and/or adverse early environments and epigenetic modifications have focused on gene-specific changes in methylation. In this study, however, we chose to begin our investigation at a global level. While a handful of studies have examined global 5-mC levels following various forms of early-life stress [28–31], the results have been inconsistent and the most commonly examined time point has been in adulthood. For these reasons, we deemed it necessary to determine global alterations of both 5-mC and 5-hmC in the adolescent brain occurring in response to our maltreatment regimen.

As stated, a more heavily used approach has been examination of gene-specific DNA methylation [15–17,32–40]. This includes methylation of the *brain-derived neurotrophic factor* (*bdnf*) gene, a critical player in development and synaptic plasticity [41,42] that is known to exhibit environmentally-driven epigenetic changes, particularly in response to stress or quality of caregiving [19,37,43,44]. While previous work from our laboratory has uncovered alterations in methylation levels of DNA associated with this gene in both infants and adults following exposure to caregiver maltreatment [11,18,37], one age point that remains to be investigated is adolescence, particularly in the amygdala and hippocampus. Our previous data also support the idea that exposure to maltreatment produces alterations in CNS DNA methylation that either persist or change with development (i.e. disappear or emerge with maturation). These changes are intriguing from a mechanistic point of view as the brain regions investigated are known players in the development and regulation of fear- and anxiety-related behaviors [45,46], and their functions are known to be affected by stress [47]. These changes are also relevant in terms of psychiatric disorders considering that *bdnf* disturbances have been implicated in the pathology of anxiety-related disorders [48], depression [49], and PTSD [44,50]. However, before we can confidently move forward in linking epigenetic alterations with behavioral trajectories, we must first understand precisely when and where these alterations are occurring—information that we currently lack for the adolescent brain in our model. Beyond extending our understanding of maltreatment-induced epigenetic alterations, investigation of the biological effects of early-life stress on the adolescent brain and the role those effects play in adolescent mental health is crucial, as it is estimated that one in five adolescents are affected by some type of psychiatric disorder that they will carry into adulthood [51].

Therefore, the current study examines global alterations in 5-mC and 5-hmC as well as gene-specific DNA methylation alterations in the adolescent rat amygdala and hippocampus in response to brief and repeated exposure to an adverse caregiving environment during infancy. We also compared patterns in both males and females, as there is increasing evidence of sexually-dimorphic epigenetic changes in response to early environmental factors [11,15,18,52,53].

2. Methods

2.1. Subjects and caregiving manipulations

Male and female outbred Long-Evans rats were housed in polypropylene cages in a temperature-controlled room on a 12-h light/dark cycle with lights on at 6:00 am. All rats were given plenty of bedding and ad libitum access to food and water. Rats were bred and the day of parturition was designated Postnatal Day (PN) 0. All dams had given birth at least once prior to beginning the experiment in order to ensure that no first-time mothers were used. On PN1, litters were culled to 5–6 males and 5–6 females and split into three groups using a within-litter design. Beginning on PN1 and ending on PN7, groups (up to 4 pups—ideally 2 males and 2 females) were exposed to the maltreatment condition, the cross-foster care condition, or the normal-care condition. Pups in the maltreatment condition were exposed to a dam in a novel environment wherein the dam was given little nesting material and little time (5 min) to habituate to the environment. Pups in the cross-foster care condition were exposed to a dam in a novel environment wherein the dam was given plenty of nesting material and ample time (1 h) to habituate to the environment. Pups in the normal-care condition were only exposed to the home cage and caregiving from their biological mother.

All sessions were live-scored by a trained observer and then scored again from video playback by a second trained observer. Both nurturing caregiving behaviors (nursing, licking and grooming) and aversive caregiving behaviors (stepping on, dropping, dragging, roughly handling, or actively avoiding the pups) were tallied in 5 min time bins (marking the occurrence or not), averaged across the 7 exposure days, and then an average of the two observers' scores was taken for statistical analysis (Table 1). Pup vocalizations (both audible and ultrasonic (40 kHz)) were also recorded during each session and two trained individuals subsequently marked the occurrence (or not) of a vocalization during

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