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Sex-specific hippocampal 5-hydroxymethylcytosine is disrupted in response to acute stress

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ABSTRACT

Environmental stress is among the most important contributors to increased susceptibility to develop psychiatric disorders. While it is well known that acute environmental stress alters gene expression, the molecular mechanisms underlying these changes remain largely unknown. 5-hydroxymethylcytosine (5hmC) is a novel environmentally sensitive epigenetic modification that is highly enriched in neurons and is associated with active neuronal transcription. Recently, we reported a genome-wide disruption of hippocampal 5hmC in male mice following acute stress that was correlated to altered transcript levels of genes in known stress related pathways. Since sex-specific endocrine mechanisms respond to environmental stimulus by altering the neuronal epigenome, we examined the genome-wide profile of hippocampal 5hmC in female mice following exposure to acute stress and identified 363 differentially hydroxymethylated regions (DhMRs) linked to known (*e.g., Nr3c1* and *Ntrk2*) and potentially novel genes associated with stress response and psychiatric disorders. Integration of hippocampal expression data from the same female mice found stress-related hydroxymethylation correlated to altered transcript levels. Finally, characterization of stress-induced sex-specific 5hmC profiles in the hippocampus revealed 778 sex-specific acute stress-induced DhMRs some of which were correlated to altered transcript levels that produce sex-specific isoforms in response to stress. Together, the alterations in 5hmC presented here provide a possible molecular mechanism for the adaptive sex-specific response to stress that may augment the design of novel therapeutic agents that will have optimal effectiveness in each sex.

1. Introduction

Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis is the most common hallmark across all neuropsychiatric diseases (Bale, 2015; Martin et al., 2010). The HPA axis is a dynamic system that allows an organism to respond to environmental stress in a sex-specific manner by governing the activity of sex-specific endocrine mechanisms including the neuronal epigenome, which can effect gene expression (McCarthy et al., 2009; Meaney, 2001). Genomic analyses of human brain tissue showed that ~2.5% of genes are differentially expressed between males and females (Trabzuni et al., 2013). Thus, fundamental sex differences in the anatomy and the genetic regulatory network of the healthy brain likely underlie pronounced sex differences in susceptibility, progression, symptom severity, and pathology of neurological disorders (Cahill, 2006; Cosgrove et al., 2007; McCarthy et al., 2012). Consistent with this notion, females are more likely than males to develop depression, anxiety, and Alzheimer's disease, while males are more likely to be diagnosed with attention deficit hyperactivity disorder, autism, and Parkinson's disease (Balint et al., 2009; Gillberg et al., 2006; Hebert et al., 2013; Nolen-Hoeksema, 1987; Weissman et al., 1996; Wooten et al., 2004). The hippocampus is an important brain structure to study the effects of glucocorticoids and stress on gene expression as studies have shown that known stress-related genes undergo expression changes in the hippocampus following acute and chronic stress exposure (Cirelli et al., 2006; Gray et al., 2014; McGowan et al., 2009; Roth et al., 2009; Rubin et al., 2014). The negative feedback regulation of the HPA axis produced upon binding of glucocorticoids to their receptors in the hippocampus is critical for a healthy stress response. Understanding the biological and molecular underpinnings of sex differences in response to stress is likely to be a useful window into the mechanistic

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cause of mental illness in both men and women (Altemus et al., 2014; Rutter et al., 2003).

Alterations in environmentally sensitive epigenetic modifications are emerging as important factors in the long-term biological trajectories leading to stress-related psychiatric disorders (Hunter et al., 2009; Maccari et al., 2016). DNA methylation (5-methylcytosine (5mC)) is an epigenetic modification with important roles in chromatin remolding, gene silencing, embryonic development, cellular differentiation, and the maintenance of cellular identity (Chouliaras et al., 2013; Mellen et al., 2012; Tollervey and Lunyak, 2012). Moreover, changes in 5mC have been linked to neurological disorders as well as psychiatric disorders, including depression, anxiety, post-traumatic stress disorders (PTSD) and schizophrenia (Abdolmaleky et al., 2006; Collishaw et al., 2007; Kuratomi et al., 2008; Pidsley et al., 2014; Poulter et al., 2008; Robertson, 2005; Weaver et al., 2004). A recent study found differences in 5mC between males and females across all autosomes, some of which were associated with altered gene expression (Singmann et al., 2015), suggesting the potential for a sex-specific role for this DNA methylation modification. 5mC can be oxidized to 5-hydroxymethylcytosine (5hmC) following exposure to environmental stimuli (*e.g.,* oxidative stress). 5hmC is enriched in neuronal cells and is associated with the regulation of neuronal activity (Szulwach et al., 2011; Yao and Jin, 2014). Related to disease, 5hmC functions independently from 5mC in neurological disorders (*e.g.,* Rett syndrome and Autism) (Mellen et al., 2012; Papale et al., 2015; Zhubi et al., 2014) and neurodegenerative diseases (*e.g.,* Huntington's and Alzheimer's) (Chouliaras et al., 2013; Condliffe et al., 2014; Wang et al., 2013). We recently reported a genome-wide disruption of 5hmC in the hippocampus of male mice exposed to acute stress followed by a one-hour recovery (Li et al., 2016). The differential 5hmC pattern significantly resided in known stress-related genes, which serves to validate a role for 5hmC in response to stress and also reveals potentially novel stress-related genes. These findings prompted us to examine the genome-wide profile of hippocampal 5hmC in females following exposure to acute restraint stress to determine the molecular origins of sex-specific vulnerability to stress-related psychiatric disorders. Together, these studies provide new insight into sex-specific functional epigenetic contributions to a stress response and they are intended to serve as a conceptual basis that will facilitate the future study of cellular and brain regional dynamics of 5hmC, especially as it relates to stress and stress recovery. These results demonstrate the power of coupling methylome and transcriptome data to determine the molecular origins of stress-related psychiatric disorders, such as PTSD and anxiety disorders. Here we provide the first genome-wide map of 5hmC in the female mouse hippocampus following an acute stress, which reveals known and potentially novel genes contributing to the stress response in females. These findings establish a sex-specific role for 5hmC in acute stress and provide insights into the immediate genome-wide neuromolecular response to traumatic events.

2. Methods

2.1. Stress paradigm, tissue acquisition, and DNA/RNA extraction

Mice were purchased from the Jackson laboratories (Bar Harbor, ME) and maintained for several generations on C57BL/6J background prior to experimentation. All mice were housed under uniform conditions in a pathogen-free mouse facility with a 12-hour light/dark cycle with food and water available *ad libitum*. All experiments were approved by the University of Wisconsin – Madison Institutional Animal Care and Use Committee (M02529). To minimize for the stress of animal handling, all of the following were conducted by a single researcher: animal colony maintenance; administration of the stress paradigm; and behavioral tests.

Notably, all experimental and control (male and female) mice were left undisturbed until weaning day and group housed with same sex littermates. The stress paradigm was administered on both sexes on the same day to ensure that identical procedures were conducted on both sexes. A description of the male mice was described previously (Li et al., 2016). For the female mice, on the day of the experiment seven-week old naïve virgin female C57BL/6 mice were randomly divided into experimental or control (naïve) groups ($N = 5$ and 3 per group, respectively). Following a published acute stress paradigm that resulted in alterations in epigenetic modifications (including 5hmC in males) and gene expression (Gray et al., 2014; Hunter et al., 2009; Li et al., 2016), the female experimental mice were restrained for 30 min (2 h after lights-on) head first into a 50 ml conical vial that has an 8 millimeter diameter hole at the tip to allow sufficient oxygen flow. Post restraint animals were individually housed in a clean cage for 1 h. After this recovery period, animals were briefly anesthetized (isoflurane) to minimize the stress of handling and sacrificed. At the same time that the experimental mice were taken from the cage to begin the stress paradigm, the naïve/control mice also were taken from the cage, briefly anesthetized (isoflurane) prior to sacrifice and tissue dissection. Finally, to minimize the effect of parent-to-offspring interaction per litter, a maximum of 2 pups/litter/sex were randomly selected for molecular analysis.

Whole brains were extracted and immediately flash frozen in 2-methylbutane and dry ice. Whole hippocampal tissue was excised by micropunch (−0.95 to −3.79 mm posterior to bregma) and approximately 30 mg of tissue was homogenized with glass beads (Sigma) and DNA and RNA were extracted using AllPrep DNA/RNA mini kit (Qiagen).

2.2. 5hmC enrichment of genomic DNA

Chemical labeling-based *5hmC* enrichment was described previously (Li et al., 2016; Song et al., 2011). Briefly, a total of 10 μg of hippocampal was sonicated to 300 bp fragments and incubated for 1 h at 37 °C in the following labeling reaction: 1.5 μl of N3-UDPG (2 mM); 1.5 μl β-GT (60uM); and 3 μl of 10× β-GT buffer, in a total of 30 μl. Biotin was added and the reaction was incubated at 37 °C for 2 h prior to capture on streptavidin-coupled dynabeads (Invitrogen, 65001). Enriched DNA was released from the beads during a 2-hour incubation at room temperature with 100 mM DTT (Invitrogen, 15508013), which was removed using a Bio-Rad column (Bio-Rad, 732–6227). Capture efficiency was approximately 5–7% for each sample.

2.3. Library preparation and high-throughput sequencing of genomic DNA

5hmC-enriched libraries were generated for all male and female enriched hippocampal DNA together using the NEBNext ChIP-Seq Library Prep Reagent Set for Illumina sequencing, according to the manufacturer's protocol. Briefly, *5hmC*-enriched DNA fragments were purified after the adapter ligation step using AMPure XP beads (Agencourt A63880). An Agilent 2100 BioAnalyzer was used to quantify the amplified library DNA and 20-pM of diluted libraries ($N = 6$) were used in each lane for sequencing, which yielded approximately 25 to 35 million uniquely mapped 50 bp reads from each library. 50-cycle single-end sequencing was performed by Beckman Coulter Genomics. Image processing and sequence extraction were done using the standard Illumina Pipeline.

2.4. Analysis of female 5hmC data: sequence alignment and peak identification

Alignment of sequence data was described previously (Li et al., 2016; Szulwach et al., 2011). Briefly, FastQ files from each sequenced library were separately aligned to mouse NCBI37v1/mm9 references using Bowtie 0.12.9. Each uniquely mapped read (.bed files), with no more than two mismatches in the first 25 bp, was concatenated to separately achieve experimental and control *5hmC* peaks of sequence reads. The MACS software was used to identify peaks of *5hmC* content using the default parameters, except for the following: effective genome