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The Impact of Environmental Factors on 5-Hydroxymethylcytosine in the Brain

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Abstract

Purpose of Review The aims of this review are to evaluate the methods used to measure 5-hydroxymethylcytosine (5-hmC), and then summarize the available data investigating the impact of environmental factors on 5-hydroxymethylcytosine (5-hmC) in the brain.

Recent Findings Recent research has shown that some environmental factors, including exposure to exogenous chemicals, stress, altered diet, and exercise, are all associated with 5-hmC variation in the brain. However, due to a lack of specificity in the methods used to generate a majority of the available data, it cannot be determined whether environment-induced changes in 5-hmC occur in specific biological pathways.

Summary Environment appears to shape 5-hmC levels in the brain, but the available literature is hampered by limitations in measurement methods. The field of neuroepigenetics needs to adopt new tools to increase the specificity of its data and enhance biological interpretation of exposure-related changes in 5-hmC. This will help improve understanding of the potential roles for environmental factors and 5-hmC in neurological disease.

Keywords 5-hydroxymethylcytosine · DNA hydroxymethylation · Environmental health · Toxicology · Epigenetics · Brain

Introduction

Previous research has suggested that gene-environment interactions play a role in the etiology of human neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), and Parkinson's disease (PD) [1–4]. However, the specific mechanisms by which gene-environment interactions modify neurodegenerative disease risk remain largely unclear. In an effort to address this knowledge gap, recent research has focused on the epigenome, which is one biological mechanism by which the environment can modify gene regulation. Epigenetic marks, which include chromatin modifications (e.g., histone acetylation), non-coding RNAs, and DNA modifications, not only help regulate gene expression throughout the lifespan, but are also sensitive to the environment [5–7]. In particular, previous research has shown that the epigenome changes in response to a variety of environmental cues, including stress, exercise, altered diet, and toxicant exposures [7–12]. As such, it has been proposed that the epigenome may be a mechanism by which the environment can modify neurodegenerative disease risk (Fig. 1) [13–15].

5-Methylcytosine (5-mC) is a well-characterized epigenetic mark that is defined as the addition of a methyl group to the 5′-carbon of cytosine in a cytosine-phospho-guanine (CpG) dinucleotide. Previous work has shown associations between 5-mC and transcriptional control [16–18], and there is growing interest in the DNA methylome as a potential mediator of the environment's role in the etiology of neurodegenerative disease [13–15]. Reflecting this idea, a number of recent studies have investigated associations between genome-wide DNA methylation and neurodegenerative diseases, including AD and PD [19–22]. While these previous studies have generated intriguing results, most of them relied on bisulfite treatment to measure 5-mC levels. This method has been used

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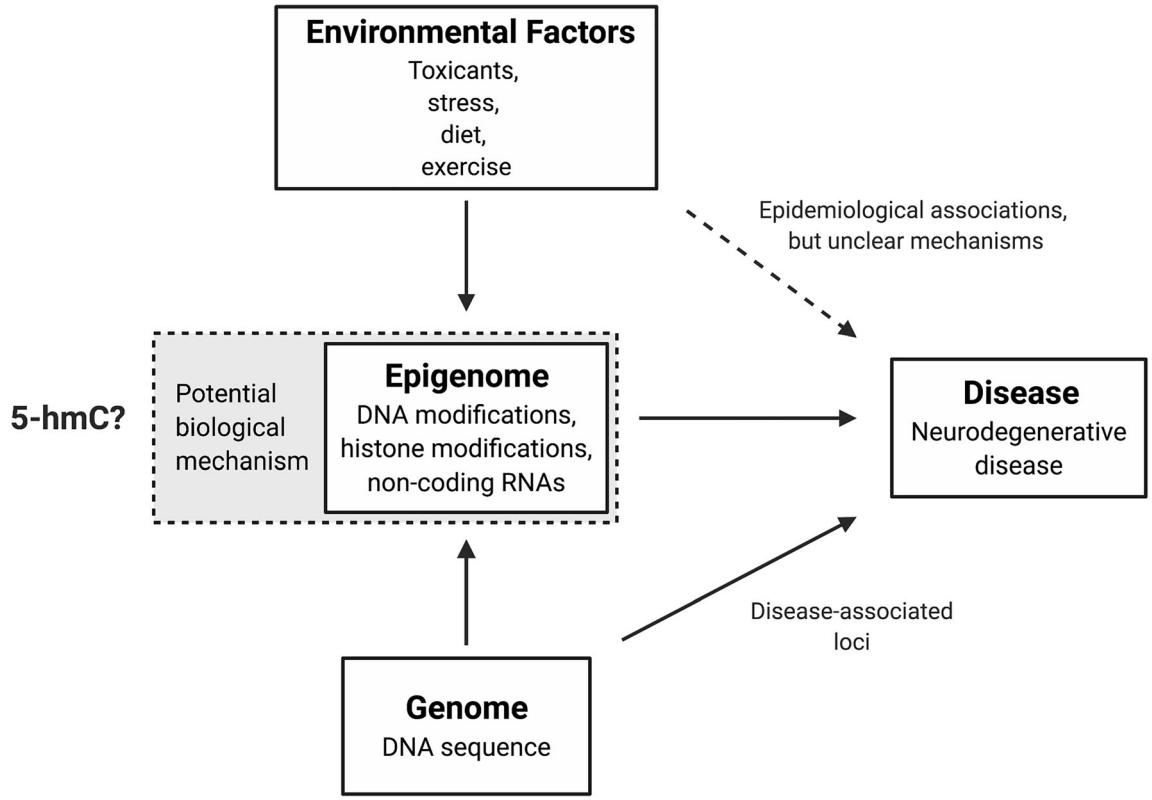

Fig. 1 Conceptual framework for the role of environmental factors in altering the epigenome, thereby affecting neurodegenerative disease risk. The epigenome, which is sensitive to the environment and helps regulate gene expression, represents a potential mechanism by which the environment can alter neurodegenerative disease risk. However, the contribution of 5-hmC to the relationship between environment, epigenome, and disease remains largely unclear

extensively in the field, but it does not distinguish between 5-mC and 5-hydroxymethylcytosine (5-hmC), an alternative epigenetic DNA modification [23].

5-mC is oxidized to 5-hmC through the activity of ten-eleven translocation (Tet) enzymes [24]. Early research recognized 5-hmC as an oxidized intermediate on the DNA demethylation pathway, but more recent studies have shown that 5-hmC is stable and may act as an independent epigenetic mark [25, 26]. In both human and animal tissues, 5-hmC has its highest levels in the brain, where it is thought to play a role in neuron development and maintenance [27–30]. In particular, past research has shown that 5-hmC is acquired during neuronal development [31, 32], maintained throughout adulthood [29], and enriched at genic regions, distal regulatory elements, and exon–intron boundaries [33–35]. In contrast to 5-mC, which is depleted at active genes in neuronal tissue, 5-hmC is enriched in transcriptionally active gene bodies in the nervous system [36]. Furthermore, 5-mC and 5-hmC preferentially recruit distinct sets of DNA binding proteins and differ in their genomic distribution in the brain [29, 37], suggesting a unique regulatory role for 5-hmC in the brain. Supporting this idea, recent work has identified links between 5-hmC and neurodegenerative diseases, including Alzheimer's and Parkinson's disease [28, 38]. Taken together, the available data emphasize the need to measure 5-hmC in neuroepigenetic studies.

Over the last decade, new methods have been developed to measure 5-hmC, and researchers are beginning to use these methods to distinguish the separate effects of environmental factors on 5-mC and 5-hmC. Here, we evaluate the most widely used methods to measure 5-hmC, and then summarize relevant studies from the last 5 years that have used these methods to investigate associations between environmental factors and 5-hydroxymethylcytosine levels in the brain. From this data, we conclude that environmental factors are associated with altered 5-hmC levels in various brain regions, but emphasize the major gaps that still exist in the literature. In particular, a majority of the available studies utilize global measures of 5-hmC, which provide a broad view of associations between environmental factors and 5-hmC, but have limited interpretability. Future studies should move beyond measuring global 5-hmC, and instead leverage regional and single base-pair resolution methods to better characterize how the environment shapes 5-hmC in the brain. This will help improve understanding of whether 5-hmC plays a role in the gene-environment interactions that mediate neurological disease.

Critical Evaluation of Methods Used to Generate 5-hmC Data

Recent work has developed a number of methods to measure 5-hmC levels from genomic DNA [39]. In general, these methods measure 5-hmC at three different genomic resolutions:

1. *Global*–Average 5-hmC levels across the entire genome.
   a. Only broad-scale levels; cannot be paired with next-generation sequencing.

2. *Regional*–5-hmC enrichment (e.g., peaks) at target gene regions.
   a. Can be paired with next-generation sequencing for genome-wide scale.

3. *Base-pair resolution*–5-hmC quantification (e.g., beta values) at individual CpG sites
   a. Can be paired with next-generation sequencing for genome-wide scale.

Global methods measure the total hydroxymethyl-CpG content in genomic DNA. Methods to measure global 5-hmC levels include immunohistochemistry (IHC) [40], tandem liquid chromatography-mass spectrometry (LC-MS) [41], and antibody-based enzyme-linked immunosorbent assay (ELISA) kits. While these methods all provide an average level of global 5-hmC as their output, they have their own strengths and weaknesses. For example, IHC has the benefit of providing tissue localization information, but requires optimized staining and microscopy protocols. Meanwhile, ELISA is affordable and can be run in any plate reader that measures absorbance, but lacks sensitivity at low target levels. Lastly, LC-MS is more sensitive than ELISA, but requires expensive, specialized equipment. Regardless of the selected method, these three approaches have several major benefits to researchers interested in 5-hmC. First, they do not require enzymatic treatment or bisulfite conversion, which can diminish DNA quality. Second, they are low cost and do not require a sequencer. Third, they produce data that are human readable and do not require specialized software or complicated bioinformatics analysis. As a result of these advantages, global measures of 5-hmC have seen widespread use. Despite their ubiquity, however, these global 5-hmC methods only provide an average of 5-hmC across the entire genome and are limited to detecting large-scale changes in 5-hmC. This ignores the gene- and CpG-level specificity of epigenetic changes and does not provide information about where environment-induced changes in 5-hmC levels occur in the genome. As a result, the data produced using global methods fail to inform follow-up, mechanistic analyses at the gene or CpG level.

Regional methods measure the enrichment of 5-hmC content on DNA across large genomic regions (i.e., > 250 bp). While there are a number of methods to measure regional 5-hmC enrichment [39], the most commonly used approaches are T4 bacteriophage β-glucosyltransferase chemical tagging or hydroxymethylated DNA immunoprecipitation (hMeDIP) [42, 43]. Often, these methods are paired with either quantitative real-time PCR or next-generation sequencing to measure 5-hmC at specific gene regions or across the entire genome, respectively [43, 44]. By measuring 5-hmC enrichment at the gene level, the regional methods can identify associations between environmental factors and 5-hmC at genetic loci of particular biological interest. Furthermore, when performed at a genome-wide scale, lists of genes with differentially hydroxymethylated regions can be combined with pathway analysis to identify biological processes that may be epigenetically altered by the environment. As such, this method provides greater specificity and interpretability than global measures of 5-hmC. However, regional measures of 5-hmC enrichment do not quantify 5-hmC at individual CpG sites, and these methods cannot be used to interrogate the effects of an environmental variable on 5-hmC at specific CpG sites. This limits the adaptability of regional methods to downstream mechanistic studies, which may aim to induce targeted changes in 5-hmC at individual CpG sites and locations within a gene.

Base-pair resolution methods quantify 5-hmC at individual CpG sites in the genome. Widely used methods to measure base-pair level 5-hmC levels include Tet-assisted bisulfite sequencing (TAB-seq) and paired oxidative bisulfite and bisulfite treatment sequencing (oxBS/BS-seq) [45, 46]. These methods can be paired with either targeted sequencing methods (i.e., pyrosequencing) or whole-genome sequencing, depending on the experimental question. Compared to the global and regional measures of 5-hmC, these methods provide the greatest specificity and can be used to identify individual CpG sites that show differential 5-hmC in response to an environmental variable. These differentially hydroxymethylated CpG sites can then be annotated to genes, thereby informing both downstream pathway analyses and follow-up mechanistic studies. From a data interpretation standpoint, base-pair level 5-hmC data are ideal and provide the most biological information. However, both the TAB-seq and oxBS/BS-seq methods are also highly expensive, require large amounts of input DNA, and involve complicated wet lab workflows. In addition, the generation of large of amounts of data restricts statistical power to detect differences. For these reasons, neuroepigenetic studies have continued to largely rely on global, non-specific methods to investigate the effects of environmental factors on 5-hmC levels.

While global 5-hmC data can provide some context regarding large-scale shifts in average 5-hmC, they fail to inform whether the measured changes in 5-hmC occur at genes in

particular biological pathways. As such, global 5-hmC data have limited use to determine whether environment-induced changes in 5-hmC are involved in neurological disease development. To better characterize the role of 5-hmC in disease development, the field should transition to the methods outlined above that capture 5-hmC at base-pair resolution. These methods have already been utilized in a number of human epigenome-wide association studies, but have not been adopted in well-controlled animal model studies. This is at least partially driven by the lack of low-cost epigenome-wide arrays (i.e., Illumina BeadChip) for animal model species. In lieu of arrays, studies in animal models are restricted to larger-scale methods, including whole-genome oxBS-seq or TAB-seq [45, 46]. While these methods present their own challenges, they provide a profound increase in sensitivity compared to global measures of 5-hmC and should be considered by researchers in the field of neuroepigenetics. In addition, many groups are working to develop new techniques for base-pair resolution mapping of 5-mC and 5-hmC that may address some of the challenges in existing methods. Using large-scale datasets generated from base-pair level methods, it will be possible to identify environmentally sensitive target genes for follow-up in hypothesis-driven studies. Furthermore, it will be possible to better characterize 5-hmC's potential role in neurological diseases.

Effects of Environmental Factors on 5-Hydroxymethylcytosine in the Brain

To identify relevant references for this review, we queried PubMed for articles published from January 1, 2014 to October 10, 2019 (search date), using the terms "5-hydroxymethylcytosine" or "DNA hydroxymethylation" or "hydroxymethylation" or "5-hmC" AND "brain"; in combination with the terms "exposure," "environment," "toxicant," "chemical," "stress," "diet," "exercise," or "physical activity." After reviewing the abstracts produced in our PubMed query, we excluded any studies that used in vitro models; this additional exclusion criterion was instituted because cell culture has been shown to rapidly reprogram 5-hmC levels in mammalian cell lines [47], and comparability of changes in these models to in vivo studies remains unclear. In total, our search identified 20 publications to include in "Effects of Environmental Factors on 5-Hydroxymethylcytosine in the Brain" of this review.

Toxicant Exposures

In the last 5 years, a small number of studies have investigated the effects of toxicant exposures on 5-hmC in the brain (Table 1). These studies have utilized a number of different exposure paradigms, experimental models, and methods to measure 5-hmC. In general, the available literature suggests that some environmental toxicant exposures can alter 5-hmC levels in the brain, but the direction, scale, and magnitude of these changes vary in an exposure-specific manner. This is not altogether unexpected, as different toxicants likely affect the brain epigenome through different mechanisms of action. Furthermore, the included studies utilized inconsistent timing for both toxicant exposure and 5-hmC measurement, making it difficult to determine whether toxicant-induced changes in 5-hmC were the result of sensitivity during a specific period of life. Below, we summarize the available data and evaluate the interpretability of the results.

The earliest study identified from our PubMed query investigated the effects of perinatal exposure to a polybrominated diphenyl ether congener, BDE-47, on global 5-hmC levels in the frontal lobe of adult male and female rats. In this study, the authors used ELISA kits to measure global 5-hmC and showed that there was no significant effect of perinatal BDE-47 exposure on global 5-hmC in the frontal lobe [48]. While these results were negative, the sample size was small ($n = 8$ per exposure group; $n = 4$ male, $n = 4$ female), and it is not clear whether the authors controlled for sex in their linear regression analysis. In addition, this study only measured global 5-hmC, which will miss any small, locus-specific changes that could occur with environmental exposures. Based on these considerations, it is not possible to determine whether this study's 5-hmC data represent a true negative finding.

In addition to the BDE-47 study, we also found a single study that examined the effects of simulated Gulf War Illness (GWI) on 5-hmC in the brain. For this study, GWI was simulated using a 28-day exposure regimen of pyridostigmine bromide (1.3 mg/kg/day, oral in water), DEET (N,N-diethyl-3methylbenzamide; 40 mg/kg/day, dermal in 70% ethanol), permethrin (0.13 mg/kg/day, dermal in 70% ethanol), and mild stress (5-min restraint once per day) [49]. One year after exposure, global 5-hmC was measured in the rat cerebellum, cortex, and hippocampus using an ELISA kit. Combined exposure of mild stress and GWI-related chemicals was associated with no change in 5-hmC in the hippocampus, decreased global 5-hmC in the cortex, and increased global 5-hmC in the cerebellum 1 year after exposure [49]. This study provides initial evidence that a complex, simulated disease state can alter 5-hmC in specific brain regions, which could be the result of different baseline levels of genome-wide 5-hmC across distinct brain regions, as has been shown in humans and mice [50, 51]. However, the use of global 5-hmC techniques in this GWI study hampers further interpretation and follow-up studies.

We also identified two studies from the same group that examined the effects of proton irradiation on genome-wide 5-hmC levels in hippocampal DNA from adult male mice. These studies measured enrichment of 5-hmC across the

Table 1 Summary of studies that tested associations between toxicant exposures and 5-hmC levels in the brain

Study Author (citation)	Exposure	Model organism	Tissue	Sex	Method to measure 5-hmC	Direction of differential 5-hmC	Genomic scale of differential 5-hmC	Differentially hydroxymethylated gene IDs
Byun et al. 2015 [40]	BDE-47	Wistar rats	Frontal lobe	Male and female	ELISA	No significant change	Global	N/A
Pierce et al. 2016 [41]	Pyridostigmine bromide, DEET, permethrin, and mild stress (GWI)	Sprague-Dawley rats	Cerebellum, cortex, and hippocampus	Male	ELISA	Increased (cerebellum), decreased (cortex)	Global	N/A
Impey et al. 2016 [42]	proton irradiation	C57Bl6/J mice	Hippocampus	Male	hMeDIP-sequencing	Bidirectional (increased and decreased)	Genome-wide	Thousands of genes with hyper- and hypo-DhMRs; exact number unclear in manuscript
Impey et al. 2017 [43]	Proton irradiation	C57Bl6/J mice	Hippocampus	Male	hMeDIP-sequencing	Bidirectional (increased and decreased)	Genome-wide	1709 and 1628 genes with hyper- and hypo-DhMRs; 677 genes with bidirectional DhMRs.
Acharya et al. 2017 [44]	^{28}Si particle irradiation	C57Bl6/J mice	Hippocampus	Male	Immunofluorescence	Increased	Global	N/A
Ozturk et al. 2017 [45]	Ethanol	C57BL/6 mouse embryo (E17)	Frontal cortex (subventricular zone and ventricular zone)	N/A	Immunocytochemistry	Decreased	Global	N/A
Du et al. 2018 [46]	Arsenic trioxide	Sprague Dawley rats	Cortex and hippocampus	Male	Liquid chromatography-mass spectrometry	Decreased	Global	N/A
Bordoni et al. 2019 [47]	Permethrin	Wistar rats	Substantia nigra pars compacta and striatum nucleus	Male and female	ELISA	Increased (male), decreased (female)	Global	N/A
Malloy et al. 2019 [48]	Bisphenol A	A^{vy} (viable yellow agouti) mice	Cortex and midbrain	Male and female	oxBS-pyrosequencing (*Kcnq1* gene)	No significant change	CpG-level	N/A

In the last 5 years, a small number of studies have investigated the effects of toxicant exposures on 5-hmC in the brain using animal/cell models. Here, we summarize the results of these studies, and also provide information regarding the specific exposure, model organism, tissue, sex, and methods used to measure 5-hmC. Most of the available data relies on global measures of 5-hmC, which do not provide the specificity necessary to truly understand whether gene-level changes in 5-hmC modify disease risk. Only one study by Malloy et al. [48] measured 5-hmC at the CpG-level

genome using hMeDIP-sequencing. Using this method, both studies showed widespread, bidirectional changes in genome-wide 5-hmC in the hippocampus [52, 53]. In total, both of these studies identified thousands of differentially hydroxymethylated regions (DHMRs). In an effort to better interpret these data, the authors used gene ontology (GO) analyses to determine whether the identified DHMRs from these two exposure studies are within genes that fall into known biological pathways. From their analyses, the authors determined that their DHMRs are in genes related to a number of brain-related GO terms, including neuron projection and synapse. In addition, the authors showed significant overlap between identified DHMRs and regions of differential 5-mC, indicating that environmental factors can modify both 5-hmC and 5-mC at specific gene regions. These data provide some initial evidence that environmental exposure to proton irradiation can alter 5-hmC at brain-related genes, but the hMeDIP method does not provide base-pair resolution, so it is difficult to hypothesize a specific regulatory role for the identified DHMRs. Future work should expand on these discovery studies by examining identified DHMRs in target genes using targeted, base pair-specific sequencing methods.

We found a single study that investigated the effects of ^{28}Si particle irradiation on global 5-hmC in the brain using immunofluorescence. This study, which was also in mice, showed that ^{28}Si particle irradiation was associated with increased global 5-hmC levels in the hippocampus [54]. While this work provides some evidence that ^{28}Si particle irradiation could affect 5-hmC in the brain, it also relied on global 5-hmC data, which limits biological interpretation.

We identified a single study that investigated the effect of ethanol exposure on global 5-hmC levels in the brain. In this study, global 5-hmC levels were measured in the subventricular zone and ventricular zone (SVZ/VZ) of embryonic day 17 (E17) frontal cortex samples using immunocytochemistry. The authors showed that prenatal alcohol exposure in mice was associated with reduced global 5-hmC in the SVZ/VZ [55]. This study provides preliminary evidence that ethanol exposure affects 5-hmC levels in a particular region of the frontal cortex, but further biological interpretation is not possible from global 5-hmC data.

A single study investigated the effects of developmental arsenic trioxide exposure on global 5-hmC in the brain using tandem liquid chromatography-mass spectrometry (LC-MS). In this study, developmental arsenic trioxide exposure via drinking water was associated with decreased global 5-hmC in rat cortex and hippocampus [56]. Supporting this finding, the authors also found an arsenic-induced decrease in *Tet1* and *Tet3* gene expression, suggesting that the arsenic exposure affects 5-hmC levels by disrupting epigenetic machinery. However, as above, the global scale of this study's 5-hmC results makes it difficult to interpret the data, and it cannot be determined whether arsenic exposure alters 5-hmC at genes related to neurological disease.

We also identified a single study that examined the effects of the insecticide permethrin on global 5-hmC in specific brain using 5-hmC ELISA kits. For this study, early-life exposure (postnatal day 6 to postnatal day 21) to the insecticide permethrin was associated with altered global 5-hmC in the substantia nigra pars compacta and striatum nucleus of adult rats [57]. Of note, the identified permethrin-related changes in 5-hmC were sex-specific, with exposed adult male rats showing increased global 5-hmC, and exposed adult females showing decreased global 5-hmC in the substantia nigra pars compacta and striatum nucleus. These sex-specific results are consistent with other work showing sex-specific effects of environmental exposures on DNA modifications in the brain [58–61]; however, the global scale of the permethrin data once again limits interpretability.

Finally, we found a single study that examined the effects of perinatal bisphenol A (BPA) exposure on 5-hmC on a specific subset of genes in the brain. In this study, BPA exposure had a non-significant effect on 5-hmC levels at the imprinted *Kcnq1* locus in mouse cortex or midbrain, but the sample size was only $n = 6$ for the control and BPA-exposed brains [62]. Of note, this study examined multiple regions of the brain and measured 5-hmC at the base-pair level using oxidative bisulfite treatment paired with pyrosequencing. The authors found no significant effect of BPA on mean 5-hmC in either cortex or midbrain samples, but 5-hmC levels across the investigated locus decreased in midbrain and increased in the cortex, suggesting that the effect of BPA on 5-hmC may vary in a tissue-specific manner. Future work should follow up on these results with a larger sample size.

Stress

In addition to toxicant exposure, several recent studies have investigated the effects of chronic and acute stress on 5-hmC in the brain (Table 2). Unlike the toxicant exposure literature, the available stress studies rarely incorporated global measures of 5-hmC into their design, instead relying largely on targeted or genome-wide 5-hmC analysis. To a certain extent, this aids in data comparison across studies, but there are still enough differences in stress paradigm, selected sex, and methodology to limit comparability of the available data.

We found a single study that investigated the effect of chronic restraint stress on 5-hmC in the brains of adult mice. For this first study, global 5-hmC was measured using dot plot analysis of genomic DNA, and genome-wide 5-hmC enrichment was measured using a selective chemical labeling method that tags 5-hmC with the T4 bacteriophage β-glucosyltransferase. Using these methods, the authors showed that chronic stress was associated with decreased global 5-hmC and widespread bidirectional changes in genome-wide

Table 2 Summary of studies that tested associations between stressors and 5-hmC levels in the brain

Study author (citation)	Stressor	Model organism/ cell line	Tissue	Sex	Method to measure 5-hmC	Direction of differential 5-hmC	Genomic scale of differential 5-hmC	Differentially hydroxymethylated gene IDs
Cheng et al. 2018 [49]	Chronic restraint stress	C57BL/6 WT mice	Prefrontal cortex	Male	Dot plot (global), T4 bacteriophage β-glucosyltransferase chemical tagging (genome-wide)	Decreased (global), Bidirectional (genome-wide)	Global, genome-wide	1251 and 3239 genes with hyper- and hypo-DhMRs
Li et al. 2015 [50]	Acute restraint stress	C57BL/6J mice	Hippocampus	Male	Immunohistochemistry and LC-MS (global), tet-assisted bisulfite sequencing (CpG-level)	No effect (global), Increased (CpG-level)	Global, CpG-level	*Nr3c1*
Li et al. 2016 [51]	Acute restraint stress	C57BL/6J mice	Hippocampus	Male and female	T4 bacteriophage β-glucosyltransferase chemical tagging (genome-wide)	Bidirectional (increased and decreased)	Genome-wide	*Notch1, Notch2, Notch3, Irs2, Crebbp,* etc. (470 and 166 genes with hyper- and hypo-DhMRs)
Papale et al. 2016 [52]	Acute restraint stress	C57BL/6J mice	Hippocampus	Male and female	T4 bacteriophage β-glucosyltransferase chemical tagging (genome-wide)	Bidirectional (increased and decreased)	Genome-wide	*Nr3c1, Ntrk2,* etc. (241 and 128 genes with hyper- and hypo-DhMRs)
Dong et al. 2015 [53]	Prenatal restraint stress	Swiss albino ND4 mice	Frontal cortex and hippocampus	Male	hMeDIP-qPCR	Increased	Gene-level	*Bdnf*
Dong et al. 2016 [54]	Prenatal restraint stress	Swiss albino ND4 mice	Frontal cortex	Male	hMeDIP-qPCR	Increased	Gene-level	*Gad1*, *Reln*, and *Bdnf*
Papale et al. 2017 [55]	Variable, mild early-life stressors (e.g., novel noise, saturated bedding, 5-min restraint, etc.)	C57BL/6J mice	Hypothalamus	Female	T4 bacteriophage β-glucosyltransferase chemical tagging (genome-wide)	Bidirectional (increased and decreased)	Genome-wide	474 genes and 482 genes with hyper- and hypo-DhMRs

In the last 5 years, a small number of studies have investigated the effects of chronic and acute stressors on 5-hmC in the brain using animal models. Here, we summarize the results of these studies, and also provide information regarding the specific exposure, model organism, tissue, sex, and methods used to measure 5-hmC. As shown in the eighth column (from the left), much of the available data from stress studies measured genome-wide 5-hmC using enrichment-based methods, which allow investigators to determine whether stress leads to gene-level changes in 5-hmC. However, these enrichment-based methods do not provide base-pair resolution 5-hmC data. Only one study by Li et al. 2015 [50] measured 5-hmC at the CpG-level

5-hmC in the prefrontal cortex of 7-to-8-week-old male mice [63]. Of particular interest, most of the identified differentially hydroxymethyated regions were located in exons, introns, and intergenic regions. These data fit with previous work in human brain showing enrichment for 5-hmC in genic regions and distal regulatory elements [35] and suggest that 5-hmC levels in these genomic features may be environmentally sensitive. Further backing up the functional relevance of the chronic restraint stress study's findings, the authors examined the effects of Tet1/2 knockout on their regions of genome-wide differential 5-hmC. Of note, they showed that the Tet1 and Tet2 enzymes were responsible for gain of 5-hmC and loss of 5-hmC in stressed animals, respectively [63]. These data suggest that Tet enzyme activity can respond to environmental cues, leading to dynamic shifts in 5-hmC levels at specific regions across the genome. While the data from this single study cannot be used to determine the effect of chronic stress on 5-hmC at the base-pair level, they do show that chronic stress alters 5-hmC levels at a large number of intergenic regions across the genome.

In addition, we identified three studies from a single group that investigated the effect of acute restraint stress on 5-hmC in the brain of adult mice. In the first of these studies, global 5-hmC in the hippocampus was measured using both immunohistochemistry and LC-MS, and region-specific 5-hmC at the *Nr3c1* gene was measured using locus-specific tet-assisted bisulfite sequencing (TAB-seq). Using these methods, the authors showed that a single, 30-min exposure to acute restraint stress did not alter global hippocampal 5-hmC levels in 7-week-old male mice, but there was a stress-related increased in 5-hmC levels at the 3′ UTR of the *Nr3c1* gene, which encodes the glucocorticoid receptor [64]. This finding underscores that global methods may miss CpG-level changes of potential biological importance.

In a pair of follow-up studies from the same group, acute stress exposure was associated with sex-specific, genome-wide differential 5-hmC in the hippocampus of adult mice [65, 66]. In both of these follow-up studies, genome-wide 5-hmC enrichment was measured using the T4 bacteriophage β-glucosyltransferase selective chemical labeling method. Of note, the authors identified female-specific differentially hydroxymethylated regions (DhMRs) at genes associated with stress response and psychiatric disorders, including *Nr3c1* and *Ntrk2* [66]. These data provide evidence that stress may have differential effects on 5-hmC in male and female mice. Future studies should follow up on these sex-specific results using methods that measure CpG-level changes in 5-hmC at the identified target genes.

Building on research in adult mice, three recent studies have examined the effects of early-life stress on 5-hmC levels in adulthood. In the first two of these studies, which are from the same lab, prenatal stress was induced via restraint for 45-min intervals three times per day (seventh day of pregnancy to delivery), and promoter-specific 5-hmC levels at a small set of target genes were measured by hMeDIP-qPCR. Using this experimental paradigm, the authors first showed that prenatal stress was associated with increased 5-hmC at the brain-derived neurotrophic factor (*Bdnf)* promoter in frontal cortex and hippocampus of adult male mice [67]. Supporting the functional relevance of the stress-related increase in 5-hmC levels at the *Bdnf* promoter, the authors also showed that *Bdnf* mRNA levels decreased with stress in both the frontal cortex and hippocampus. Next, in a follow-up study, they replicated the *Bdnf* result in the hippocampus and further showed prenatal stress-related increases in 5-hmC at the glutamic acid decarboxylase 67 (*Gad1*) and reelin (*Reln*) promoters in the hippocampus of adult male mice [68]. Meanwhile, in a third study from a separate group, early-life (postnatal days 12–18) exposure to variable, mild stressors (e.g., novel noise, saturated bedding, 5-min restraint) was associated with bidirectional changes in genome-wide hypothalamic 5-hmC in adult female mice [69]. For this third study, genome-wide 5-hmC enrichment was measured using the T4 bacteriophage β-glucosyltransferase selective chemical labeling method. Together, these studies provide evidence that prenatal stress may alter programming of 5-hmC at specific genes in the brain and that these effects can persist through adulthood. While both of these studies provide target genes for follow-up in future studies, neither of them generated data at the base-pair level, which limits the mechanistic interpretability of the results. Future work should expand on these data by examining base-pair resolution 5-hmC at specific CpGs within regulatory regions of the identified target genes.

Diet

In addition to stress and environmental exposures, two recent studies have examined the effects of diet on 5-hmC levels in the brain. In the first of these studies, gene-level 5-hmC was measured at the *Sirt1* locus using hMeDIP-qPCR, and global 5-hmC levels were measured with tandem liquid chromatography-mass spectrometry (LC-MS). Using these methods, the authors showed that high-fat diet (HFD)-induced obesity had no significant effect on global 5-hmC levels, but that HFD was associated with decreased 5-hmC levels at the *Sirt1* gene promoter in hippocampus of male C57BL/6J mice [70]. These data again underscore that global measures of 5-hmC are not able to capture environment-induced changes in 5-hmC at specific genes. Further supporting the idea that diet can alter 5-hmC in the brain, a second study showed that aging-associated increases in 5-hmC in mouse cerebellar Purkinje cells were mitigated by caloric restriction [71]. These data suggest that caloric restriction may slow epigenetic aging in the mouse brain, a result that matches previous reports that caloric restriction delays the biological aging process in mice [72]. For this study, global 5-hmC was measured

using immunohistochemistry with an antibody for 5-hmC. Taken together, these initial studies indicate that both high-fat diet and caloric restriction can modify 5-hmC levels in the murine brain. Further work should be done to investigate the specific effects of these types of dietary exposures on genome-wide 5-hmC levels, especially in the context of caloric restriction-induced changes in aging.

Exercise

Two recent studies have also examined the effects of exercise on 5-hmC in the brain. In the first of these studies, researchers investigated the effects of exercise on both regional 5-hmC at a single target gene (*miR-137*) and global 5-hmC using hMeDIP-qPCR and an ELISA kit, respectively [73]. The authors of this first study showed that exercise was associated with a moderate, but significant decrease in global 5-hmC in hippocampus of both young and old mice [73]. Furthermore, exercise was associated with increased hippocampal 5-hmC at the *miR-137* gene. This locus encodes miR-137, a microRNA that helps regulate nervous system development and differentiation [74]. As such, this initial study provides evidence that exercise may increase 5-hmC at brain-related genes. In contrast, in a second study, researchers showed that voluntary physical activity did not affect region- or CpG-level 5-hmC at the *VegfA* promoter in the rat hippocampus, despite clear 5-hmC enrichment in the measured region [75]. This second study measured both 5-mC and 5-hmC in the *VegfA* promoter using paired oxidative and standard bisulfite pyrosequencing. While these two studies show seemingly divergent effects of exercise on 5-hmC, the data were collected at different loci, which suggests that exercise may alter hippocampal 5-hmC in a locus-specific manner.

Future Directions

Using our selected search terms in PubMed, we identified 20 publications that met our inclusion criteria. The few available studies provide foundational evidence for an effect of environmental factors on 5-hmC in the brain, but much more work needs to be done to fully understand how the environment shapes this emerging DNA modification. In particular, the available evidence suggests that directionality of changes in 5-hmC are locus-specific, underscoring the weakness of global assays of 5-hmC, which miss CpG- or region-level changes in 5-hmC. Based on this weakness, future studies should move beyond global measures of 5-hmC, instead focusing on environment-induced changes in 5-hmC at specific genomic loci across the life course.

Over the last decade, a number of new methods have been developed to measure 5-hmC at base-pair resolution. The two most widely adopted methods—Tet-assisted bisulfite sequencing (TAB-seq) and paired oxidative bisulfite and bisulfite treatment sequencing (oxBS/BS-seq) [45, 46]—are tweaks on the bisulfite treatment method that has long been used to measure DNA methylation. TAB-seq and oxBS/BS-seq both provide reliable 5-hmC data, but they are reliant on sodium bisulfite deamination, which introduces strand breaks and reduces DNA integrity. As a result, these methods have high DNA input requirements and cannot be used for small amounts of DNA, as might be isolated from sorted cell populations or micro-dissected tissues. To get around this weakness, multiple research groups have proposed new methods to measure base-pair resolution 5-hmC, including Jump-seq, chemical-assisted mismatch sequencing (CAM-seq), TET-assisted pyridine borane sequencing (TAPS), and APOBEC-coupled epigenetic sequencing (ACE-seq) [76–80]. While none of these methods have yet been widely adopted, they all focus on replacing bisulfite treatment with alternative approaches. For example, the (ACE-seq) method uses apolipoprotein B mRNA-editing catalytic polypeptide-like (APOBEC) enzymes, a family of proteins that can deaminate cytosines [79, 80]. While these enzymes work well for measuring 5-hmC, they are not able to distinguish between unmodified cytosine and 5-mC, which means that their utility in measuring multiple DNA modifications is limited. To address this weakness in the APOBEC-based methods, a recent bioRxiv preprint combined selective enzymatic protection of 5-mC and/or 5-hmC, enzymatic deamination by APOBEC3A, and long-read sequencing technologies (i.e., PacBio and Nanopore) to measure 5-mC and 5-hmC at base pair resolution without compromising DNA integrity [81]. This type of modern approach, which integrates multiple methodological advances into a single workflow, will help the field of neuroepigenetics better understand the impact of environmental factors on base-pair resolution 5-hmC levels.

Taken together, data from the available recent studies suggest that some environmental factors can modify 5-hmC levels in the brain. However, the effect of different factors on 5-hmC varies in its direction by exposure/treatment (Table 1 and Table 2), indicating that the various tested factors may alter 5-hmC levels through distinct biological pathways. Furthermore, there is some evidence, particularly from the GWI and BPA exposure studies, that exposure-induced changes in 5-hmC may be brain region-specific. This region specificity could be driven by the different baseline levels of genome-wide 5-hmC that occur in distinct brain regions, as has been shown in humans and mice [50, 51], or by the characteristic cell type compositions of separate brain regions. In this second scenario, it is possible that exposure-induced changes in region-specific 5-hmC levels reflect particular sensitivity of individual cell types. However, it is also possible that these exposure-induced changes reflect shifts in specific cell populations across tissues. These ideas need to be further investigated in studies using DNA from enriched or sorted neuronal cell populations.

Further adding to the complexity of measuring brain 5-hmC, most of the available studies only investigated 5-hmC in male animals, but there is some preliminary evidence that exposure-induced changes 5-hmC are sex-specific [57–61]. To build on the available data, future studies should test the effects of environmental exposures on enriched cell populations from multiple regions of the brain in both male and female animals. While this type of study design is complex, it will allow researchers to determine which brain structures show the greatest epigenetic sensitivity in both sexes and will provide greater insights into the effect of exposures on 5-hmC in the brain.

As the field generates large-scale 5-hmC datasets, there is a need for appropriate statistical methods to analyze the effects of environment on 5-hmC at a genome-wide scale. So far, several studies have addressed this issue by developing methods to better estimate 5-hmC levels from oxBS/BS-seq data using maximum likelihood estimation [82, 83]. Even with these improved estimation methods, however, there remains a lack of clarity regarding best practice for analyzing 5-hmC levels in response to a modified environment. Unlike 5-mC, 5-hmC has a zero-inflated beta value distribution, which means that the genome-wide analysis methods established for DNA methylation may not be appropriate for this secondary epigenetic mark. Furthermore, 5-mC and 5-hmC levels are biologically related, and not truly independent. This complicates interpretation of 5-hmC data, particularly when 5-mC levels are not also measured. To address these concerns, we recently proposed a novel application of mixed effects beta regression models to co-analyze 5-mC and 5-hmC base-pair resolution data as "repeated" measures [84]. This method provides an informative supplement to the traditional approach of analyzing 5-mC and 5-hmC data as separate datasets and should be incorporated into future neuroepigenetics studies that measure base pair resolution 5-mC and 5-hmC levels.

Conclusions

Available data indicate that some environmental factors, including exposure to exogenous chemicals, stress, altered diet, and exercise are all associated with changes in 5-hydroxymethylcytosine levels in different brain regions. Taken together, these results suggest that 5-hmC may play an important role in mediating the effects of environmental exposures in the brain. In this way, exposure-induced changes in 5-hmC could have significant effects on gene regulation and may alter susceptibility to neurological disease. While this is an attractive hypothesis, the available data are limited and are not yet sufficient to support this type of in-depth biological conclusion. Of particular concern, most of the existing studies that investigated the effects of environmental factors on 5-hmC relied upon global measures of 5-hmC. Only a few studies in the brain examined the effects of environmental factors on regional 5-hmC, and even fewer interrogated base pair-level 5-hmC. This lack of base pair-resolution is likely driven by a dearth of affordable tools and methods to investigate 5-hmC levels in animal models. Whereas some recent human studies have utilized a combination of BS/oxBS treatment and Illumina BeadChip arrays to co-measure 5-mC and 5-hmC in the brain, these types of curated arrays do not yet exist for animal models. As a result, only more expensive options, like WGBS, TAB-seq, or hMeDIP-seq, are available in well-controlled animal experiments. Further disincentivizing genome-wide 5-hmC data generation, it remains unclear whether the identified exposure-induced changes in 5-hmC actually reflect shifts in cell populations. In addition, there is no gold standard statistical method to co-analyze the effects of environmental factors on genome-wide 5-mC and 5-hmC levels in the brain. Recent advances have attempted to address this issue, but novel methodologies and statistical tools are only now becoming available to co-analyze paired 5-mC and 5-hmC in a single analysis pipeline [81, 84]. Despite these challenges, the available data provide a weight of evidence that 5-hmC is sensitive to environmental cues, and emphasize the need to assess this often-overlooked DNA modification in future neuroepigenetics studies.

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