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Catherine Cai April 17, 2012

Effects of Aging and Enriched Environment On 5-Hydroxymethylcytosine (5-hmC) Levels in Mice

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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By Catherine Cai

The study of epigenetics has gained particular attention and interest in recent years as increasing numbers of studies have demonstrated the importance of epigenetic regulation in governing normal processes as well as its involvement in various disease states. Epigenetic changes concern modifications to the genome that influence gene expression in a cell- or tissue-specific manner, but do not change the specific base sequence of the DNA. Recently, it was discovered that the common epigenetic marker 5-methylcytosine (5-mC) can be further modified by the Tet family of enzymes to yield 5-hydroxymethylcytosine (5-hmC). In this study, we characterize 5-hmC as a novel epigenetic mark and examine the effect of exposure to an enriched environment, and more specifically the effect of voluntary exercise, on levels of 5-hm in various tissues. In previous experiments performed in our lab, it was discovered that the levels of 5-hmC are higher in younger animals and decrease as a result of exposure to an enriched environment (Irier HA and Jin P., 2012). In this study, we explore the effects of an enriched environment on the 5-hmC levels of tissue from other major organs, including the heart and the lungs. The study found that the heart and lung tissue share the brain's age-related decline in 5-hmC levels, but in contrast with the brain, exposure to an enriched environment increases the level of 5-hmC in the heart tissue.

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As I reviewed my experience in the honors biology program, I noted that it certainly succeeded in its basic goals. It improved my understanding of concepts in biology, it built my confidence in the laboratory and it prodded me toward achieving greater independence as a thinker.

But perhaps more importantly, it also led me to develop a sense of appreciation for the mystery and complexity of science, and the beauty of living organisms. This is something that I will remember from this year, and something that I will carry with me long after I have graduated from Emory.

My deepest thanks.

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Introduction

The study of epigenetics

Over the past few decades, evidence has mounted that epigenetic regulation of the genome plays a large role in controlling gene expression. The study of epigenetics concerns alterations to the genome that influence gene expression, but do not change the specific base sequence of the DNA. This definition includes modifications of the DNA nucleobases, modifications of the associated histone proteins, and long noncoding microRNAs, which can together alter gene expression in a cell- or tissue-specific manner (Bonasio et al., 2010).

The molecular changes induced through epigenetic regulation often result in chromatin remodeling. In the traditional model of DNA, the strands of DNA are tightly wrapped around an octamer of histone proteins. In order to initiate transcription, the first step in the process of gene expression, the DNA must first transiently disassociate from the histone proteins so that the transcription machinery may access the genetic material. Epigenetic modifications to the genome, which include histone acetylation, methylation and carboxylation and DNA methylation among others, "repackage" the chromatin structure in a similar way (Cheng X and Robert MB, 2011; Casadesús J and David L., 2006). These modifications result in charge disruption and steric hindrance that induce chromatin remodeling, which gives rise to altered levels of gene expression (Dai et al., 2005).

Additionally, epigenetic modifications are dynamic and can change throughout an organism's lifetime. Various environmental factors, including diet, infection, oxidative stress and drug exposure have all been cited to impact the occurrence and frequency of

different epigenetic changes (Zawia et al., 2009). Nevertheless, studies have suggested that certain epigenetic modifications may still be heritable and have a "transgenerational legacy" (Kubota et al., 2012).

Epigenetics and disease

A wealth of literature compiled over the decades has demonstrated the wide-reaching importance of regulated epigenetic modifications in normal processes as well as the devastating diseases that can occur when epigenetic modifications go awry. Regulated epigenetic changes are largely cited as the primary players in the processes of X-chromosome inactivation, neuronal differentiation, important steps in development and embryogenesis, as well as in several other normal processes (Heard et al., 1997; Gräff et al., 2011). On the other hand, undesirable epigenetic alterations have been heavily implicated in a number of disorders, including cancer, depression, neurodegenerative disorders like Alzheimer's Disease and imprinting diseases like Prader-Willi Syndrome and Angelman Syndrome (Zunyan et al., 2005; Klein A and H Glaesmer, 2012; Zawia et al., 2009; Chamberlain et al., 2010).

An understanding of how epigenetic regulation functions in the control of gene expression in both normal and disease states holds promise for novel therapeutic options that use epigenetic marks as pharmacological targets. For example, a recent clinical trial at the NIH observed the effects of a pro-methylation dietary supplement on the cognitive function and global methlylation levels of patients with Angelman's syndrome (Bird et al, 2011). With the emergence of these types of experimental drugs, which use epigenetic marks as pharmacological targets, research that seeks to elucidate the functional

significance of newly discovered markers has become of great interest to the scientific community.

DNA methylation

One of the most well studied epigenetic modifications is the methylation of the cyclic 5-Carbon on the Cytosine nucleotide, which gives rise to a 5-methylcytosine (5-mC). This epigenetically marked nucleobase is often referred to as "the 5th base of the genome" (Matarese et al., 2011). This modification is catalyzed by methyltransferases enzymes (DNMT1, DNMT3a, DNMT3b), and it commonly occurs in the Cytosines of CpG dinucleotides. While CpG dinucleotides are generally rare in the genome, they are found at greater frequencies in regions of the genome known as CpG islands (Du et al., 2012). These islands typically occur in the upstream 5' regions of genes, and as much as 70-80% of the Cytosines in CpG dinucleotides are methylated in adult human somatic cells (Colot & Rossignol, 1999).

DNA methylation appears to be an evolutionarily conserved process, as it is widely found in both prokaryotes and eukaryotes, including protists, plants, fungi and even higher animals (Ponger L and WH Li, 2005). It has also been postulated to have varying functions depending on the region of the genome and the organism in which it occurs. For example, the hypermethylation of the promoter region of genes is associated with inhibition of transcript initiation and thus gene silencing (Colot & Rossignol, 1999). Similarly, while the active X-chromosome is completely unmethylated, methylation has been attributed to be the primary reason that the inactive X-chromosome remains stable in this state in the somatic cells (Li et al., 1997).

Additionally, the level of methylation can vary throughout the lifetime of an organism. In fact, active demethylation is necessary during various normal processes, including tissue differentiation and embryogenesis (Brandeis et al, 1993). The necessity and significance of DNA methylation was further underlined in a study that demonstrated that a mutation in the major methyltransferase genes in mouse embryos invariably results in fatality (Heard et al, 1997).

DNA hydroxymethylation

Recently, another epigenetically modified base, 5-hydroxymethylcytosine (5-hmC), was discovered in mammalian genomes (Münzel et al., 2011). The conversion of 5-mC to 5-hmC is an oxidation reaction mediated by ten-eleven translocase (Tet1, Tet2, Tet3*)* enzymes [Figure 1]. Depending on the TET enzyme involved, a 5-hmC can be generated *de novo* or from a hemimethylated Cytosine (Irier HA and Jin P., 2012). Studies have speculated that 5-hmC marks 5-mC and signals for active demethylation (Wossidlo et al., 2011), although others have suggested that 5-hmC itself is a stable epigenetic modification rather than an intermediate state (Wu H and Y Zhang, 2011). Unlike 5-mC, the distribution of 5-hmC in the genome is more tissue specific, and the modification is found in highest levels in cells of the central nervous system, including in the brain (Münzel et al., 2011). 5-hmC may affect the differentiation potential of cells, thus playing an essential role in regulating the pluripotency of embryonic stem cells (Szulwach et al.). However, there is still little information concerning the functional significance of 5-hmC.

Objectives of this study

Here, we characterize 5-hmC as a new epigenetic mark that may be involved in the regulation of gene expression. Because it has long been known that gene-environment interaction can alter the epigenetic state of an animal's genome, we hypothesized that the levels of 5-hmC in various organs of individual organisms would differ as a result of exposure to an enriched environment [Figure 2]. The enriched environment provides opportunities for exercise, which has been shown to cause improved cognitive function and potentially delay the onset of Alzheimer's Disease (Adler et al., 2005). Although previous studies had characterized the relative differences in 5-hmC levels in different organs of mice, the literature offered little information concerning the effects of environment.

Preliminary experiments performed in our lab have suggested that the levels of 5-hmC are higher in the brain tissue of younger animals compared to aged animals, and that the levels of 5-hmC in the hippocampus and cortex decrease with exposure to an enriched environment, which allows the animals to engage in voluntary exercise. In this study, we examine the effects of an enriched environment on the 5-hmC levels in the tissue of other major organs, including the heart and the lungs, and attempt to elucidate a potential role for 5-hmC in gene-environment interactions.

Methods

Experimental Animals

A total of 32 male mice (C57BL6) were purchased for the initial enriched environment experiments of this study. Twelve young mice (approximately 3 months) were purchased from The Jackson Laboratory in Bar Harbor, Maine, and 20 aged mice (16 to 18 months) were purchased from the National Institute of Aging. The animals within each of the aged and young cohorts were randomly assigned to be either control or enriched environment (EE) mice, so that the control and EE groups contained an equal number of animals. In summary, the study analyzed mice in four groups: 10 aged EE mice, 10 aged Control mice, 6 young EE mice and 6 young Control mice.

Enriched Environment treatment

The 32 animals were randomly assigned to either enriched or standard environmental conditions, so that each group contained 16 animals, 8 young and 8 aged [Figure 3]. All animals were housed in the animal facilities of the Whitehead Biomedical Research Building of Emory University in groups of 4, in standard condition cages with food (5-10 pellets of standard rodent diet), corncob bedding and access to water in a temperature-controlled room kept at 22°C. Prior to sacrifice, the EE animals were subjected to EE treatment sessions for 3 hours a day during a 12-hour light period, every day for 4 weeks, before being returned to the standard condition cages. The EE sessions consisted of introducing the EE animals to a large, transparent Rubbermaid container

(56.5 cm long x 41.5 cm wide x 22 cm high) that served as the enriched environment chamber (EE chamber) [Figure 4].

In addition to providing food, bedding and water, the EE cage offered a larger space with access to a running wheel, allowing for voluntary exercise. The EE cage also contained numerous other structures of varying color, shape and texture purchased from pet stores, including tubes, balls and boxes. These structures were replaced every three days to ensure that the animals were being exposed to novel objects in the EE setting. To ensure proper ventilation, 70-80 small holes 3 mm in diameter were drilled into the cover and the upper sides of the container, out of reach of the animals.

The animals were closely monitored during the initial days of EE treatment for aggressive behavior and fighting pairs. For the remainder of the EE sessions, the animals were monitored every 30 minutes. Between EE sessions, the container and the structures were cleaned with soap and water and disinfected with Virkon-S solution and 70% ethanol. The Control animals were never exposed to this EE setting and were continually housed in the standard condition cages prior to sacrifice.

Morris Water Navigation Task

After the 16 EE animals (10 aged EE mice, 6 young EE mice) received a total of 4 weeks of daily EE treatment, during which the 16 Control animals (10 aged Control mice, 6 young Control mice) remained under standard conditions, the animals were subjected to behavioral tests. The primary behavioral task utilized in this study was the Morris Water Navigation Task, which assesses hippocampus-dependent spatial memory and the learning ability of individual animals [Figure 5].

The task involves a large, circular basin of water (52 inches in diameter) marked by visual extra maze cues that contains a safety platform located in a specific location 1 cm below the surface of the water. During the initial training sessions, the mice were introduced to the basin with their paws touching the wall from 4 different starting positions (N, S, E, W). If the animal failed to reach the platform in 60 seconds, it would be manually guided to the platform.

Upon reaching the safety platform, the animal was allowed to remain there for 5 seconds in order to promote the observation of spatial clues in the environment, which would aid in future navigation trials. Each animal was given 4 trials a day for 5 days, with 15-minute intervals between each trial. The temperature of the water ranged from 22°C to 25°C during the testing, and animals were moved to a dry plastic holding cage lined with paper towels. The animals were monitored at all steps.

After the initial 5 days of task acquisition training, the safety platform was removed and the animal was re-introduced to the pool. Various parameters, such as the amount of time spent in the quadrant of the basin that formerly contained the platform and the distance swam to reach that quadrant were measured over 60 seconds. All trials were videotaped and each individual animal's performance was analyzed using MazeScan (Clever Sys, Inc.).

Genomic DNA isolation

After collecting data on each individual animal's performance on the behavioral task, we sacrificed the animals and collected multiple organs for DNA isolation for later experiments. The heart, lung and liver were removed as entire organs and stored in at

-80°C. The brain was further subdivided into the hippocampus, cerebellum and cortex before being stored.

To isolate genomic DNA (gDNA), we thawed the tissue and cut approximately a 25 mg sample from the same region of the organ using a surgical blade and added the piece to a mortar containing 600 μL of lysis buffer. The tissue was homogenized using a 2 mL mortar and pestle, and the homogenate in the lysis buffer was transferred to a new centrifuge tube. At this step, 150 μL of the homogenate was added to 800 μL of TriZol Reagent (Invitrogen, Cat# 15590-018) and stored in -80°C for later RNA isolation. 17.5 μL of 900 u/ml Proteinase K solution (Fermentas, Cat#) was added to the remaining homogenate for DNA isolation, and the homogenate was left to incubate at 55°C overnight.

After incubation, the homogenate was cooled to room temperature and 3 μL of RNAse H solution was added. The homogenate was incubated at 37°C for 15 minutes. The tubes were spun down at 16,000x g at 4°C in a centrifuge machine for 5 minutes. The supernatant was removed and added to a new centrifuge tube containing 600 μL of isopropanol. The tube was inverted multiple times to allow DNA to precipitate and then spun down at 16,000x g at 4°C for 1 minute. The supernatant was removed using a pipette, and the pellet was washed with 750 μL of 70% ethanol. The tube was again spun down at 8,000x g for 5 minutes, and the supernatant was removed.

The ethanol was evaporated, and the remaining pellet was re-suspended in 50 μL of 10 mM Tris HCl pH 8 buffer. Concentrations of the gDNA were determined using a spectrophotometer (NanoDrop 1000, Thermo Scientific), and the DNA suspensions were stored at -20°C.

Dotblot analysis of 5-hmC level

Dotblot analysis was used to analyze the levels of 5-hmC present within the gDNA of various tissues of individual animals. A 50-ng/μL aliquot of gDNA was created from the stock DNA suspensions, and the concentration was verified using a NanoDrop machine. A Hyabond-N+ membrane (Amersham Biosciences, #RPN303B) was cut to fit the Bio-Dot Apparatus loading chamber (#170-6545, BIO-RAD) and rinsed in 6X SSC for 15 minutes. 10 μL of the 50-ng/μL aliquot of gDNA samples were loaded over the wells and onto the membrane to create a "dot" of 500 ng of gDNA in total. Each gDNA sample was loaded three times in order to create triplicates.

After the membrane was loaded, it was baked in an oven at 80°C for 1 hour. The dried membrane was then blocked with PBS containing 5% dry milk and 0.1% Triton X-100 for 1 hour at room temperature. A primary rabbit anti-5-hydroxymethylcytosine antibody (1:10,000, #39769, Active motif) was applied to the membrane and incubated overnight at 4°C. Controls for 5-meC and Cytosine were used in two initial dotblots to ensure the fidelity of the antibody, and standard DNA templates (D5405, ZYMO) were included in final amounts of 2 ng, 1 ng, 0.5 ng and 0.25 ng on every blot.

The next day, the membrane was washed with 5% milk solution in 1x PBST for 10 minutes three times before it was incubated with HRP-conjugated anti-rabbit secondary antibody (1:5,000, #A-0545, Sigma) for 1 hour at room temperature. The membrane was washed again with 5% milk solution in 1x PBST for 10 minutes three times before being covered with a chemoluminescent spray for 2 minutes. The membrane was then transferred to a film tray and covered with a plastic sheet. An autoradiography film was laid over the membrane for 30 seconds and developed in a dark room to capture

the fluorescence emitted from each dot. The film was scanned and saved. The membrane was then stained with a methyl blue solution overnight, rinsed with deionized water and scanned to create an image that would serve as the loading control. The membrane was stored in a -4°C in 1x PBST.

ImageJ quantification analysis

ImageJ, an image-processing program, was used to quantify differences in intensity between dots. The program generates a curve representing the intensity of each dot on the image of the scanned film, so that the area underneath the curve can be taken to represent and determine the amount of 5-hmC originally present in the gDNA loaded onto the membrane. The same analysis was performed for the image of the methyl blue stained membrane to account for initial loading differences. The intensity of each dot was normalized to the loading control by subtracting the area under the loading control curve from the area under the fluorescence intensity curve.

A linear function giving the absolute percentage of 5-hmC in the original loading was created from the areas under the curves taken for the 5-hmC standards, for which the original input volume and absolute amount of 5-hmC was known. The areas under the curves representing the fluorescence intensity of each dot were converted to absolute percentages of 5-hmC using this linear function. The average value for each category was taken for each category (e.g. an average value for aged control heart tissue), and ratios were used to represent the fold difference and relative differences between various categories (e.g. aged control heart tissue compared to aged control lung tissue).

Statistical analysis

Data were analyzed and saved as Microsoft Excel spreadsheets. Statistical analysis was performed using T-tests to a p-value of 0.05. Graphs were created using PRISM.

Comparison to in-house raised mice

After the initial EE/Control animal study, we sacrificed mice that were raised within the Whitehead Biomedical Research Building to compare any potential differences between the 5-hmC levels of the animals raised within our own facilities and the animals purchased from The Jackson Laboratories and NIA. This group of animals was not exposed to EE treatment. It comprised nine animals, including three aged mice (12 months), three young mice (2 months), as well as two mice transgenic for the amyloid precursor gene (APP) that served as animal models for Alzheimer's Disease.

Results

Morris Water Navigation Task

Previous experiments in our lab have shown that aged control mice take a statistically significantly longer time to mount the platform than younger mice during task acquisition after 3 days of training, suggesting a deficit in the spatial memory and learning ability of aged control mice. Additionally, it was discovered that within the aged cohort, the EE mice demonstrated better spatial memory, as measured by the percentage of time the animals spent in the quadrant of the navigation basin that originally contained the safety platform. Among the young mouse cohort, young EE animals also spent more time in the correct quadrant than their young control counterparts. The data suggest that prolonged exposure to an enriched environment reduced the learning deficit of the aged mice (Irier HA and Jin P., 2012) [Figure 6].

5-hmC levels in brain tissue

Previous work in our lab also showed a statistically significant difference between the level of 5-hmC in the hippocampus and cortex of aged control animals as compared to young control animals. The levels of 5-hmC were higher in both tissues in the young animals. Additionally, it was found that among both young and aged animals, the level of 5-hmC was higher in the cortex and hippocampus tissue of control animals as compared to EE animals, suggesting that exposure to an enriched environment decreased 5-hmC levels in these tissues [Figure 7].

The absolute amount of 5-hmC as a percentage of the total genomic DNA ranged from approximately 0.05% to 0.5% in the hippocampus, approximately 0.3% to 0.85% in the cortex, and 0.6% to 0.8% in the cerebellum (Irier HA and Jin P., 2012).

5-hmC levels in heart and lung tissue

In the enriched environment study that compared 12 young animals aged 3 months and 20 aged animals aged 16-18 months, a statistically significant difference was discovered between the levels of 5-hmC in both heart and lung tissue of aged versus young animals [Figure 8]. The level of 5-hmC was significantly higher in young animals for both heart and lung tissue. In young animals, 5-hmC constituted approximately 0.85% of the total genomic DNA on average in the heart tissue and approximately 1.0% of total genomic DNA in the lung tissue. In contrast, in aged animals, 5-hmC only constituted on average about 0.75% of total genomic DNA in heart tissue and 0.13% of total genomic DNA in lung tissue.

Additionally, it was noted that despite having experienced the same type of environmental setting and despite being of the same approximate age, the animals within each of the four cohorts studied exhibited a degree of individual variation [Figure 9].

Effects of enriched environment

It was also discovered that exposure to an enriched environment increased the level of 5-hmC in the heart tissue of young mice. The EE treatment correlated with more than a 2.5-fold increase in the level of 5-hmC in the heart tissue of young EE animals as compared to young control animals. However, no statistically significant effect could be

discerned for either the EE treatment on the heart tissue of aged animals, or the lung tissue of either aged or young animals.

In-House Raised Animals

The mice that were raised within the Whitehead Biomedical Research Building did not exhibit the same patterns of age-dependent genomic 5-hmC level differences as the mice purchased from The Jackson Laboratory and NIA. In a comparison of 4 animals, 2 mice aged 12 months and 2 mice aged 2 months, it was found that the aged animals had higher levels of 5-hmC in both the heart and lung tissue. While the amount of 5-hmC was often several fold greater in the heart and lung tissue of the young animals purchased from Jackson Laboratory, the levels of 5-hmC in the heart and lung tissue of animals raised within our own facilities were much more similar [Figure 10].

Discussion

Amounts of 5-hmC

The relative levels of 5-hmC of different organ types found in this study were consistent with the findings in previous studies. We found that 5-hmC levels were highest in the brain, with lower levels found in the heart and lung tissue; the relative amounts of 5-hmC by organ type confirm previous studies that found similar trends. The tissue-specific levels of 5-hmC could be related to function of the cells in that type of tissue. For example, because the neurons of the adult brain are terminally differentiated, it is not surprising that a different hydroxymethylation pattern than the heart or lung tissue is observed. Because oxygen is required for the enzymatic activity of Tet proteins, another factor that could account for different levels of 5-hmC between tissue types could be different levels of oxygenation.

The absolute amounts of 5-hmC as a percentage of total genomic DNA in aged mice ranged from approximately 0.02% to 2%, which was also consistent with values found in existing literature (Dahl et al., 2012). However, it is often difficult to assess the absolute amount of 5-hmC present due to the slight variation in figures that results from using different methodologies. The techniques themselves also pose certain constraints; for example, it was difficult to detect very low levels of 5-hmC due to the limited sensitivity of the dotblot. Other techniques, including immunohistochemistry and isotope-based liquid chromatography mass spectrometry may prove to be more effective in analyzing the absolute levels of 5-hmC. Additionally, the low number of animals (3 young, 3 aged) used to study levels of 5-hmC in animals raised in our own facilities also posed certain limitations.

It is also interesting to note that a certain degree of variability existed between individual mice in each of the 4 cohorts of animals. This seems consistent with the processes of epigenetic modifications. Concerning epigenetic changes like 5-hmC, it is not uncommon for different animals to respond to the same treatment and environments to differing degrees.

Effects of Enriched Environment

Our findings that enriched environment can have an effect on the 5-hmC levels of heart and lung tissues in C57 BL6 male mice is a novel discovery. While the level of 5-hmC in heart and lung tissue decreased with age, a trend that was similar to what was found in the brain, the EE treatment had a different effect on the heart tissue than it did on the brain. The level of 5-hmC in the heart tissue of young EE animals was higher than the level in young control animals, suggesting that the EE treatment caused an increase in 5-hmC levels in the genomic DNA of heart cells. In contrast, the level of 5-hmC in the hippocampus and cortex of young EE animals decreased, suggesting that EE treatment results in a decrease in 5-hmC levels in certain regions of the brain. These differences could be due to varying roles of 5-hmC. It is possible that in addition to the level of 5-hmC, the distribution of 5-hmC in the genome is also important for determining the function of the modification. Like methylation patterns, hydroxymethylation could have different functions depending on the type of tissue in which it occurs. Future sequencing efforts using Next-Generation Sequencing should help determine the specific loci modified in this context.

Additionally, the primary variable we focused on in the EE treatment was the inclusion of an exercise wheel, which allowed animals to engage in voluntary exercise. However, it is difficult to assess to what degree individual animals participated in this voluntary exercise. In future experiments, it may be useful to consider using an animal treadmill, an apparatus that would allow for more standardized measurements of exercise activity levels.

In-House Raised Mice

It is interesting to note that in addition to a certain degree of individual variability within each of the 4 cohorts of animals, there were notable differences between the levels and patterns of 5-hmC occurrence in the heart and lung tissue of animals purchased from The Jackson Laboratory and NIA, and the animals raised within our own facilities. This could be a result of inevitable differences in the rearing conditions between the different facilities. It is possible that the animals purchased from The Jackson Laboratory and NIA experienced a higher degree of stress than the animals raised in our own facilities, largely due to the transportation of the purchased animals across several states. Differences in the 5-hmC levels of purchased versus in-house raised mice could be used to support the underlying theme of this study that varying environmental experiences will affect epigenetic states.

Future Directions

Although this study established that 5-hmC levels vary depending on age and tissue type, and that exposure to an enriched environment can alter these levels in some

tissue types, it did not elucidate the functional significance of this epigenetic mark. A more gene-centered approach could be valuable toward this aim. Previous studies have shown that knockdown of Dnmt1 and Dnmt3a, two genes involved in DNA methylation, results in lower levels of neuronal methylation in the mice and poorer performances on the Morris Water Navigation Task, suggesting decreased spatial memory and learning ability (Feng et al., 2010). Studying conditionally mutant mice knockdown for the Tet gene could generate similar information concerning the role of hydroxymethylation and the 5-hmC epigenetic marker in spatial memory and learning.

Additionally, total RNAs were also isolated from each of the animal subjects during the process of DNA isolation. Another useful future step would be to create cDNA libraries and to perform rt-PCR experiments to assess the transcription levels of a few candidate genes, such as those known to play a role in aging or Alzheimer's Disease. Exon array could also be used to determine the overall mRNA expression. Western blot analysis should follow these experiments to determine the level of translation for these particular mRNAs. The data from these experiments taken together could potentially determine a functional role of 5-hmC.

Figures and Graphs

Epigenetic Changes

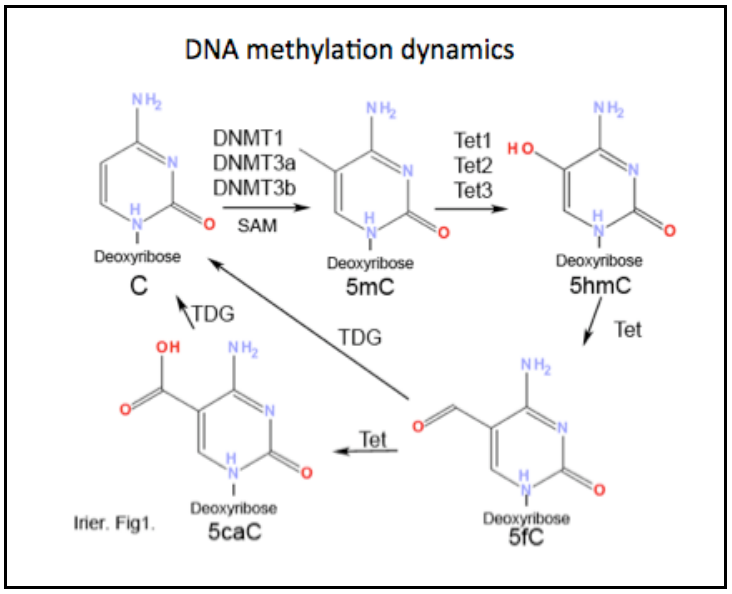

Figure 1. The cyclic 5th carbon of the Cytosine nucleotide can be methylated by methyltransferase proteins to yield 5-methylcytosine (5-mC). 5-mC can be oxidized to 5-hydroxymethylcytosine (5-hmC) (Irier HA and Jin P., 2012).

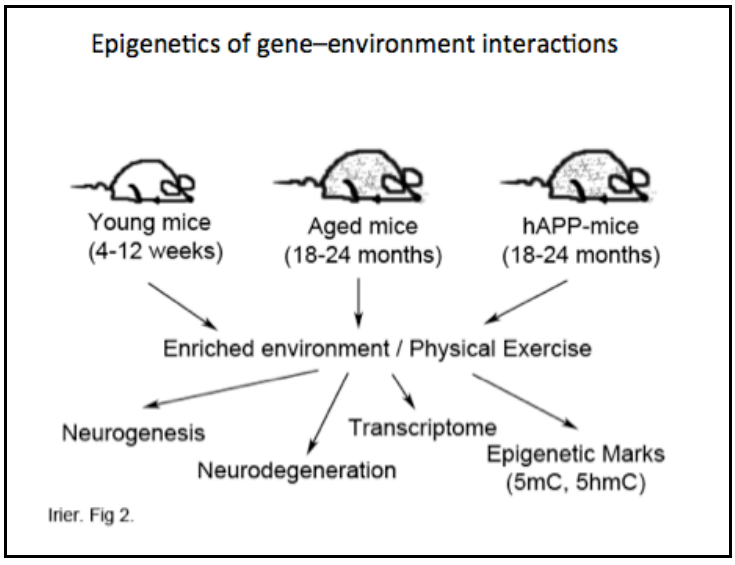

Figure 2. Exposure to enriched environments and participation in physical exercise can alter levels of epigenetic marks like 5-mC and 5-hmC (Irier HA and Jin P., 2012).

Enriched Environment Treatment

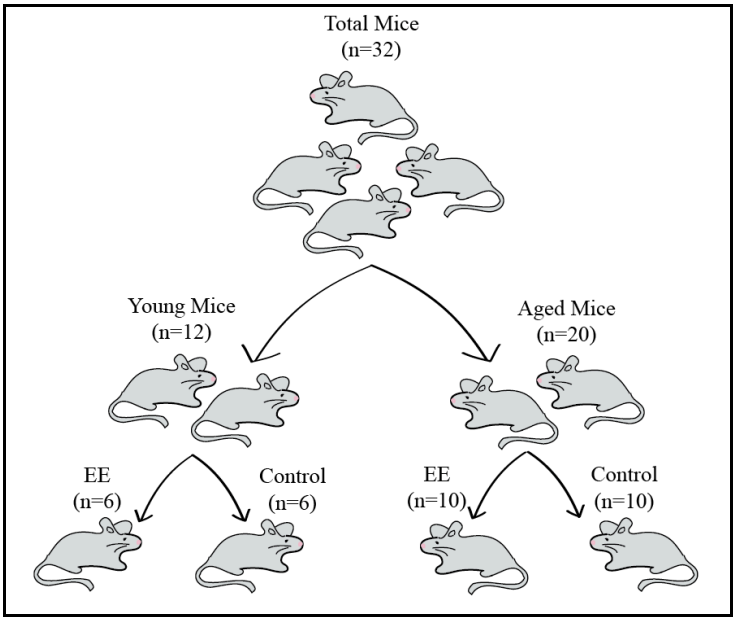

Figure 3. The animals in young groups purchased from The Jackson Laboratory, and animals in aged group were purchased from NIA (National Institute of Aging). They were randomly assigned to either control or enriched environments.

Figure 4. The EE chamber (left) included an exercise wheel, which allowed for voluntary exercise, as well as numerous novel objects and structures of varied color and texture. The control environment (right) provided only food, bedding and water.

Morris Water Navigation Task

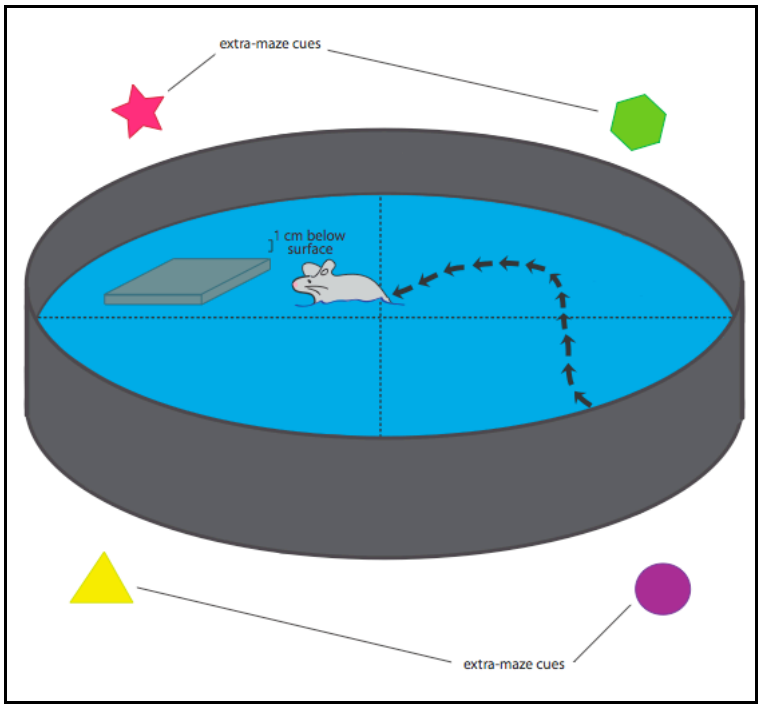

Figure 5. After either control or EE treatment, animals were subjected to the Morris Water Navigation task, which assesses spatial memory and learning.

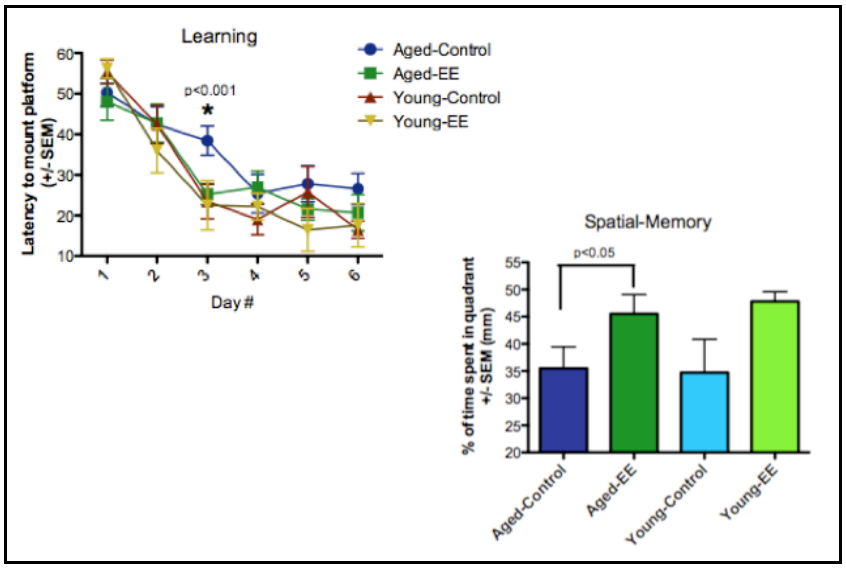

Figure 6. At Day 3 of task acquisition, aged control animals exhibited a significant deficit in learning. EE animals on average spent more time in the correct quadrant than their Control counterparts (Irier HA and Jin P., 2012).

Effects of Enriched Environment in the Brain

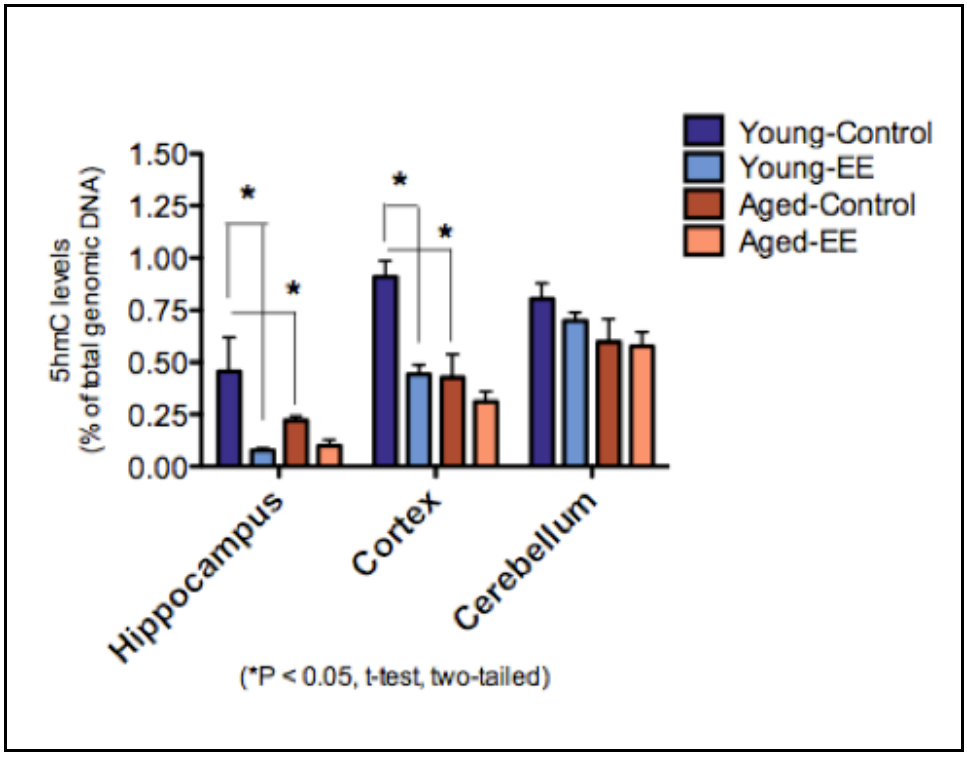

Figure 7. Young animals that were exposed to an enriched environment had lower levels of 5-hmC in the hippocampus and cortex than young control animals (Irier HA and Jin P., 2012).

5-hmC in Heart and Lung Tissue

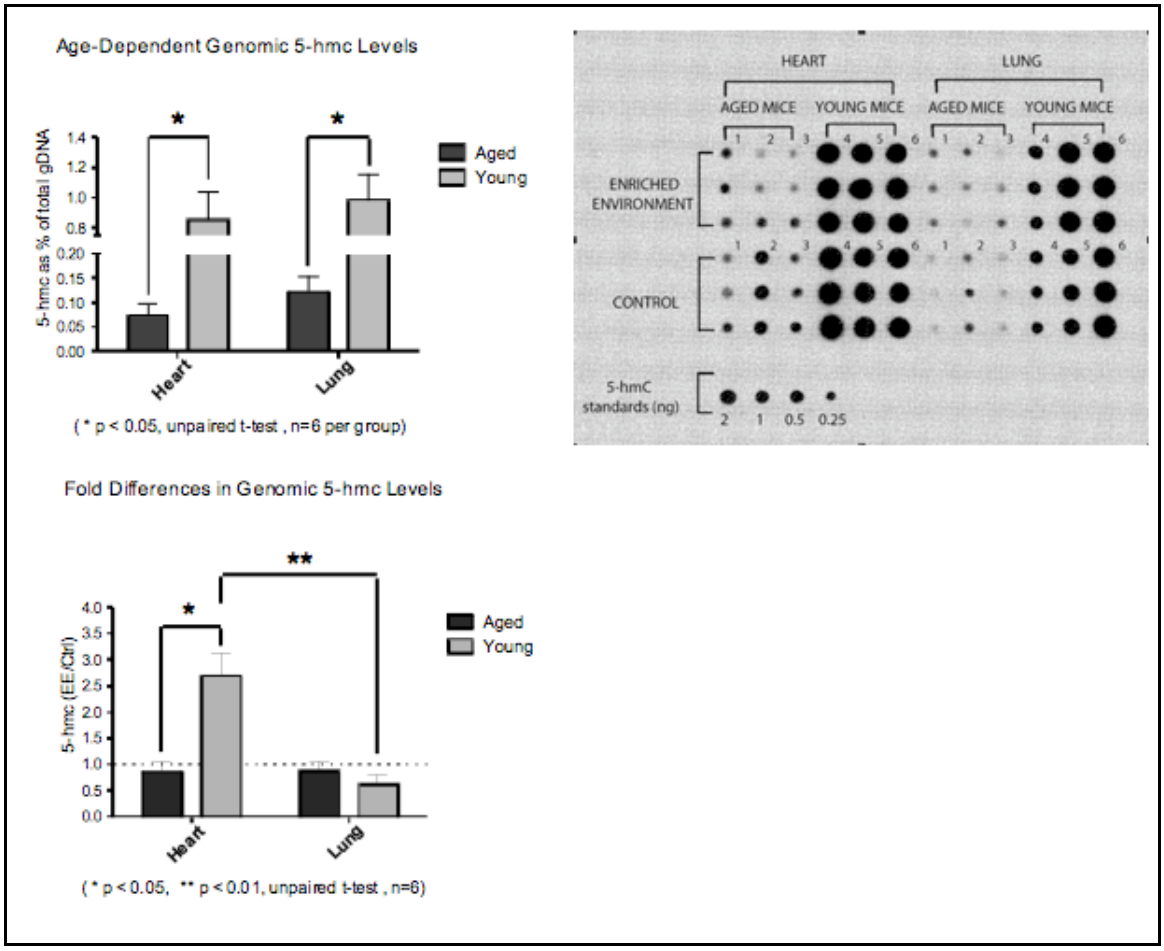

Figure 8. Levels of 5-hmC were higher in the heart and lung tissue of young animals compared to aged animals. Young animals subjected to EE treatment had higher levels of 5-hmC in heart tissue than young control animals.

Variations

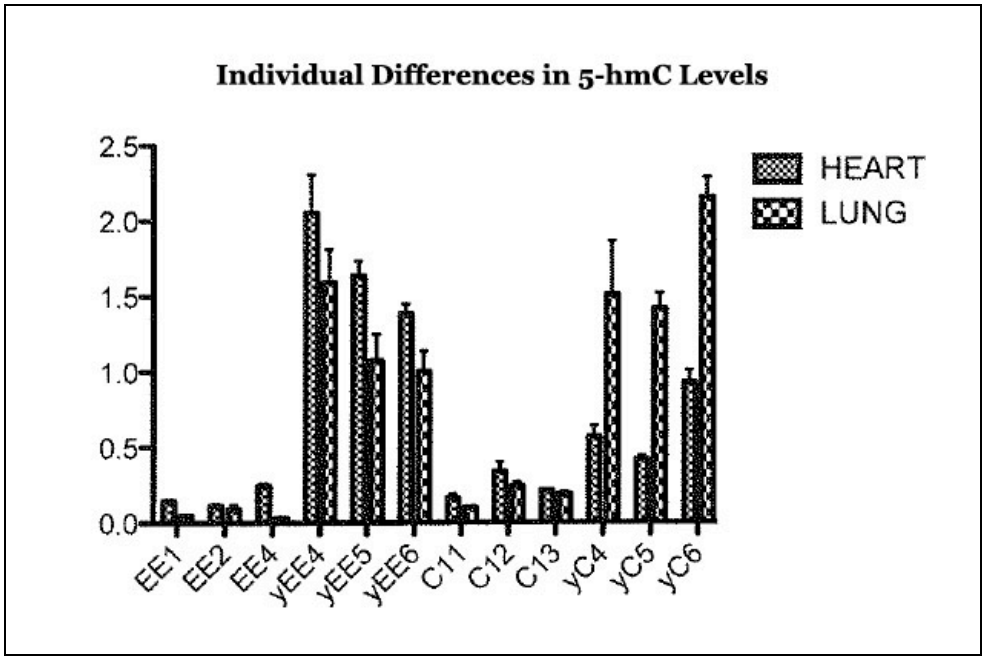

Figure 9. The individual animals within each of the 4 cohorts exhibited variation in levels of 5-hmC in heart and lung tissue and as a function of EE exposure.

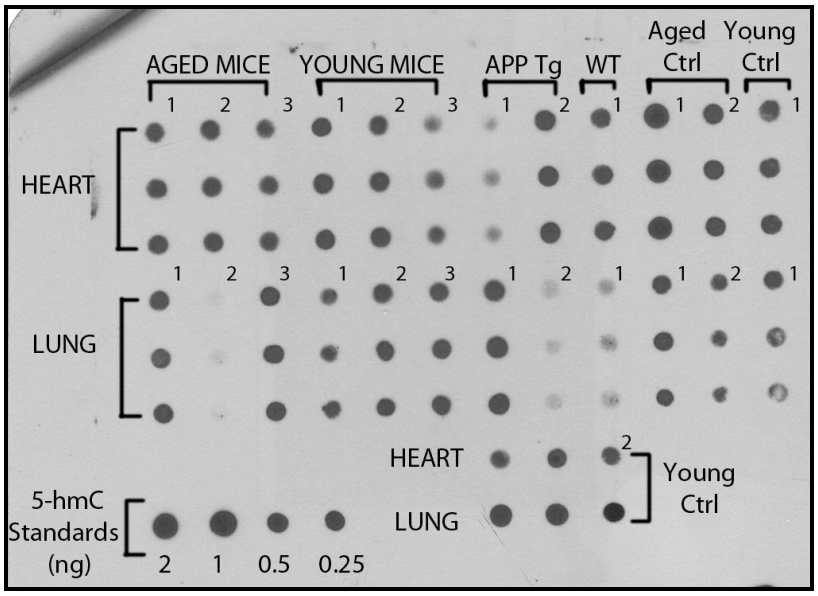

Figure 10. Animals raised in our facilities did not exhibit the same age-dependent decline in 5-hmC levels as mice purchased from The Jackson Laboratory.

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