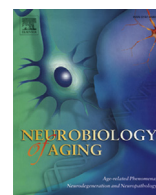


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## Brief communication

# Cross-region reduction in 5-hydroxymethylcytosine in Alzheimer's disease brain

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## ABSTRACT

Epigenetic processes play a key role in the central nervous system and altered levels of 5-methylcytosine have been associated with a number of neurologic phenotypes, including Alzheimer's disease (AD). Recently, 3 additional cytosine modifications have been identified (5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxylcytosine), which are thought to be intermediate steps in the demethylation of 5-methylcytosine to unmodified cytosine. Little is known about the frequency of these modifications in the human brain during health or disease. In this study, we used immunofluorescence to confirm the presence of each modification in human brain and investigate their cross-tissue abundance in AD patients and elderly control samples. We identify a significant AD-associated decrease in global 5-hydroxymethylcytosine in entorhinal cortex and cerebellum, and differences in 5-formylcytosine levels between brain regions. Our study further implicates a role for epigenetic alterations in AD.

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## 1. Introduction

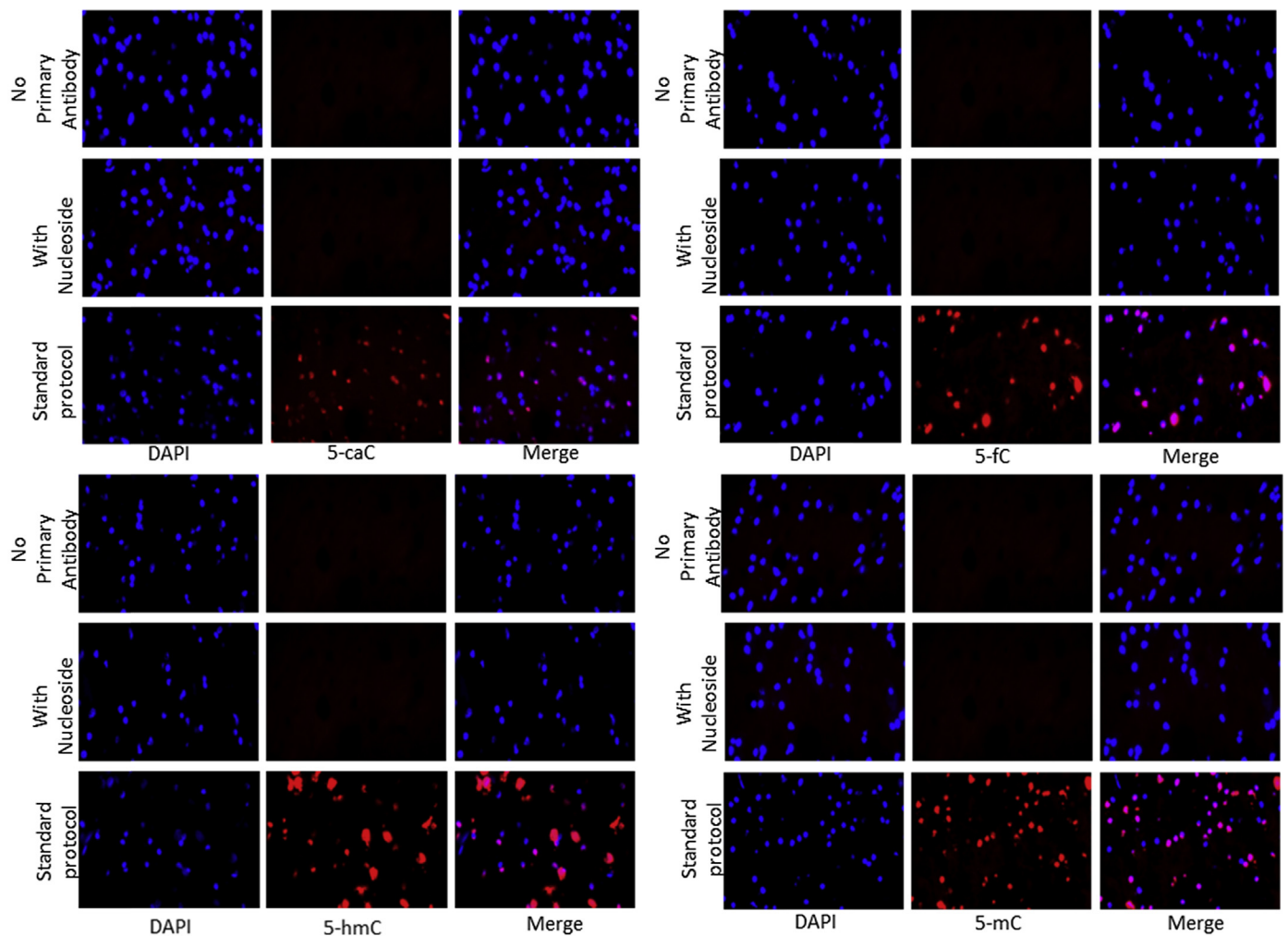
Alzheimer's disease (AD) is a chronic neurodegenerative disease affecting >5.4 million adults in the US and contributing significantly to the global burden of disease (Thies and Bleiler, 2011). Given the high heritability estimates for AD (Gatz et al., 2006), considerable effort has focused on understanding the role of genetic variation in disease etiology, although it has been recently speculated that epigenetic dysfunction is also likely to be important (Lunnon and Mill, 2013).

Epigenetics refers to the reversible regulation of various genomic functions occurring independently of DNA sequence, with cytosine methylation being the best-understood and most stable epigenetic modification modulating the transcription of mammalian genomes. Recent studies have identified global- and site-specific alterations in 5-methylcytosine (5-mC) levels in AD brain (Bakulski et al., 2012; Mastroeni et al., 2009, 2010; Rao et al., 2012).

A number of additional cytosine modifications have recently been described. 5-hydroxymethylcytosine (5-hmC) has been shown to be enriched in brain (Khare et al., 2012), suggesting it may play an important role in neurobiological phenotypes and disease. Importantly, current approaches based on sodium bisulfite converted DNA are unable to distinguish between 5-mC and 5-hmC (Nestor et al., 2010). 5-hmC is believed to be an intermediate step in the demethylation of 5-mC to unmodified cytosine by the oxidation of 5-mC by ten eleven translocation

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**Fig. 1.** Immunofluorescence demonstrating the specificity of the antibodies used in this study. At the concentrations used no staining was observed in either the no primary antibody control, or the nucleoside control. Staining of tissue (in absence of controls) was co-localized with 4',6-diamidino-2-phenylindole (DAPI) demonstrating nuclear staining.

proteins (Tahiliani et al., 2009). 5-hmC is thought to play a specific role in transcriptional regulation as it is recognized by key proteins that do not recognize 5-mC (Jin et al., 2010), has a distinct genomic distribution to 5-mC (being predominantly found in gene promoters and gene bodies and rarely in intergenic regions [Jin et al., 2011; Stroud et al., 2011]), is more abundant in constitutive exons than alternatively spliced ones (Khare et al., 2012) and has a lower affinity to methyl-binding proteins than 5-mC (Hashimoto et al., 2012). 5-hmC appears to be present in all tissues, although at differing levels, with the highest levels observed in brain (Li and Liu, 2011) with enrichment in genes involved in synapse-related functions (Khare et al., 2012); in contrast the distribution of 5-mC appears to be relatively uniform across tissues (Globisch et al., 2010). It has been suggested that although some hydroxymethylated-CpG loci are stable during aging, others are more dynamically altered (Szulwach et al., 2011).

In 2011, two additional cytosine modifications were described in mouse embryonic stem cells and somatic tissues (Inoue et al., 2011; Ito et al., 2011). 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC) were found to result from the further oxidation of 5-hmC by ten eleven translocation proteins (Ito et al., 2011), suggesting that these modifications represent further steps along the

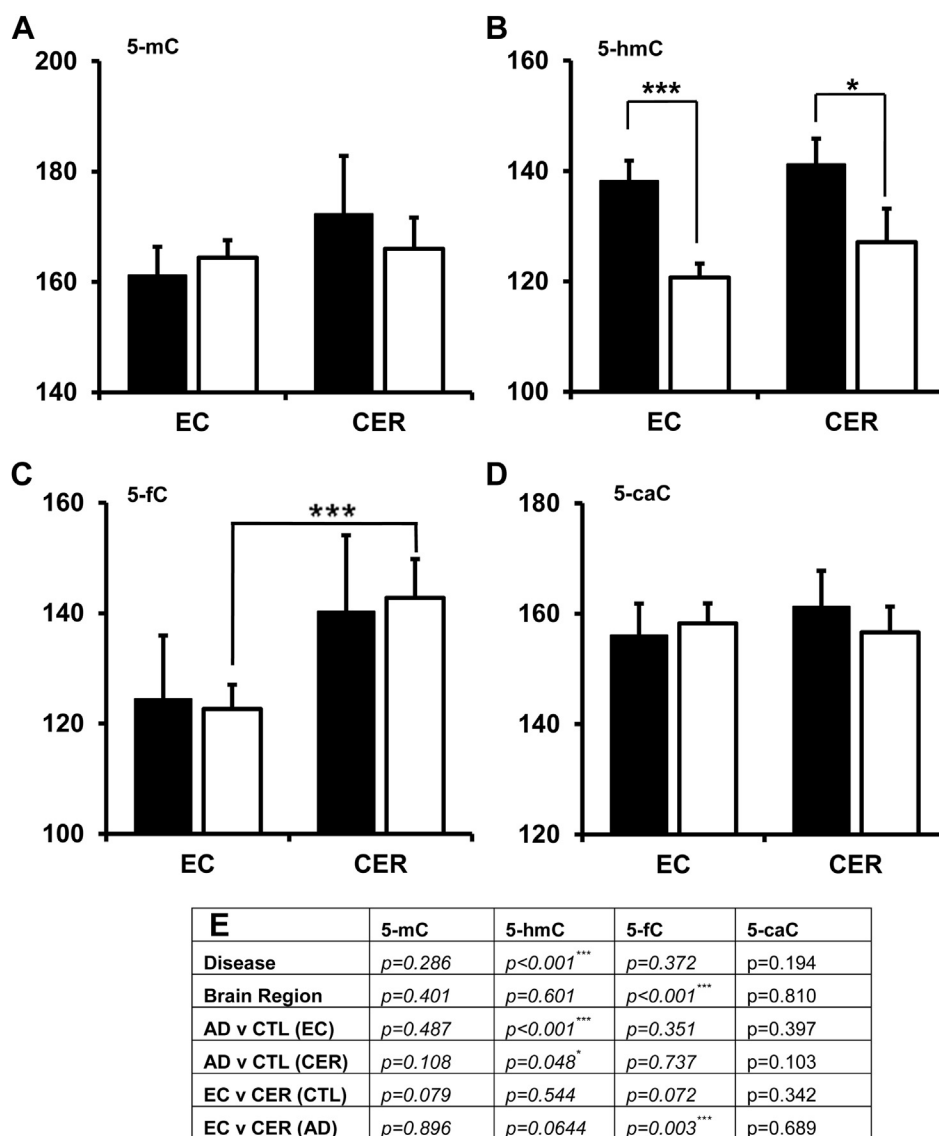
demethylation pathway (Inoue et al., 2011). Although little is known about their functional role and prevalence in the healthy genome, recent studies have mapped 5-fC to gene regulatory elements, namely poised enhancers and CpG island promoters (Raiber et al., 2012; Song et al., 2013).

The aim of the present study was to investigate the relative abundance of the four described cytosine modifications across two distinct anatomic brain regions (entorhinal cortex [EC] and cerebellum [CER]) in tissue obtained from AD cases and elderly controls.

## 2. Methods

### 2.1. Subjects and sample preparation

Formalin-fixed tissue punches from the EC and CER were obtained from AD cases ( $n = 13$ ) and cognitively normal elderly control (CTL) subjects ( $n = 8$ ) from the MRC London Neurodegenerative Diseases Brain Bank (<http://www.kcl.ac.uk/iop/depts/cn/research/MRC-London-Neurodegenerative-Diseases-Brain-Bank/MRC-London-Neurodegenerative-Diseases-Brain-Bank.aspx>). Sample demographics are shown in Supplementary Table 1.



**Fig. 2.** Differences in nuclear fluorescence intensity for each cytosine modification in tissue from control (black) and AD (white) brains. Data are represented as the mean fluorescence per positive cell across all samples in the group ( $\pm$ SEM). (A) There was no difference in 5-mC levels in AD samples, or across brain regions. (B) There was an overall effect of disease on 5-hmC levels ( $p < 0.001$ ), with a decrease in 5-hmC in AD samples compared with control in both the EC ( $p < 0.001$ ) and CER ( $p = 0.0476$ ). (C) There was a significant effect of brain region on 5-fC levels ( $p < 0.001$ ), with a significant difference in 5-fC in EC compared with CER in AD ( $p = 0.0026$ ). (D) There was no difference in 5-caC in AD samples, or across brain regions. (E) Between groups  $p$ -values are tabulated. Abbreviations: AD, Alzheimer's disease; 5-caC, 5-carboxylcytosine; CER, cerebellum; EC, entorhinal cortex; 5-fC, 5-formylcytosine; 5-hmC, 5-hydroxymethylcytosine; 5-mC, 5-methylcytosine; SEM, standard error of the mean.

## 2.2. Immunofluorescence

Samples were cryosectioned at 10  $\mu$ m on a freezing Cryostat before immunostaining with antibodies purchased from Active Motif (CA, US) against 5-mC (Cat# 39649), 5-hmC (Cat# 39791), 5-caC (Cat# 61225), and 5-fC (Cat# 61223). Briefly following antigen retrieval (10 mM sodium citrate, 0.05% tween, pH 6.0, 80  $^{\circ}$ C), quenching of endogenous immunofluorescence (300 mM glycine), and membrane permeabilization (Triton-X), nonspecific binding was blocked with 10% normal goat serum (Vector Laboratories) for 30 minutes before the addition of primary antibody (1:2000 dilution in 5% normal goat serum for 16 hours at 4  $^{\circ}$ C). Slides were incubated with biotinylated goat secondary antibody (Vector Laboratories, Peterborough, UK) at 1:200 dilution for 1 hour, followed by streptavidin-AF596 (Life Technologies, CA, USA) for 1 hour. To minimize auto-fluorescence, samples were incubated

with 0.3% Sudan Black for 30 minutes then mounted on coverslips with Prolong-Gold with 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies, California, US).

## 2.3. Microscopy and analysis

Appropriate controls to demonstrate the specificity of antibodies were included for every sample, including a primary antibody control (incubation with the relevant nucleoside [Inoue et al., 2011] before staining) and a secondary antibody control (incubation in the absence of primary antibody). Immunofluorescence images were acquired on a Zeiss Axio Imager II at 40 $\times$  magnification, with all camera and microscope settings constant for all images taken within each antibody. The two control sections were checked by eye for each slide followed by the capture of 10 images from nonoverlapping fields of view taken from each

of the two stained sections. Images were analyzed using an ImageJ script to determine the level of fluorescence per positive cell that applied a threshold pixel value to the red (AF546) channel to create a binary mask image, which was then used as a template for measuring the red fluorescence. A binary mask was used as it allowed the measurement of fluorescence only within the nuclei and removed any nonspecific background staining from the analysis. We then calculated the intensity within each nuclei staining positive in a given image and generated an average per cell by dividing the total fluorescence intensity in the binary mask of a given image by the number of nuclei staining positive in that image. This was then averaged across the 10 images. Data were assessed for normality and analyzed first using mixed effects modeling in STATA (StataCorp) to identify differences between AD cases and controls across each brain region, while controlling for age and gender. When an association with disease was observed we used linear modeling to look for differences associated with Braak score, a measure of disease stage (Braak and Braak, 1991) while controlling for age and gender.

### 3. Results and discussion

#### 3.1. Each cytosine modification is present in adult human brain

Initially, we sought to investigate whether each of the four-cytosine modifications could be detected in adult human brain. Because two of the antibodies (5-fC and 5-caC) had yet to be tested in human samples, we first stained sections of human EC with antibodies specific to each modification (Fig. 1). Staining of all four antibodies co-localized with DAPI confirming nuclear staining. To test for nonspecific staining, each antibody was incubated with its complimentary nucleoside before staining to act as a negative control. As expected no staining was observed, demonstrating the specificity of the antibodies. Similarly, no staining was observed in the absence of the primary antibody. Once we had established the specificity of the antibodies and the presence of each cytosine modification in human brain we compared their relative abundance in two distinct anatomic brain regions and whether different levels are present in AD cases compared with controls (Fig. 2).

#### 3.2. Cross-tissue differences in 5-mC in AD

Recent studies have demonstrated differential DNA methylation at specific loci between EC and CER (Davies et al., 2012) and between AD and controls (Bakulski et al., 2012). Here, we found no significant difference in global 5-mC level between brain regions ( $p = 0.286$ ) nor in disease ( $p = 0.401$ ) (Fig. 2A). Similarly, two previous studies have also demonstrated no difference in 5-mC in CER in AD (Mastroeni et al., 2009, 2010), however, one of these studies showed decreased 5-mC in AD EC (Mastroeni et al., 2010). Discrepancies between that study and our own could be because of a number of technical issues, including different reagents, fewer samples within our study, different sample cohorts and perimortem differences such as postmortem delay and brain pH, where their influence on DNA methylation are currently unknown (Pidsley and Mill, 2011).

#### 3.3. Cross-tissue differences in 5-hmC in AD

We found an overall significant effect of disease status ( $p < 0.001$ ) but not brain region ( $p = 0.061$ ) on 5-hmC levels, with a significant decrease in 5-hmC in AD compared with control in both EC ( $p < 0.001$ ) and CER ( $p = 0.0476$ ) (Fig. 2B). We also observed a significant negative correlation between global 5-hmC and Braak

score, a measure of AD neuropathology and disease stage, in both the EC ( $r = 0.6927$ ,  $p < 0.001$ ) and CER ( $r = 0.2795$ ,  $p = 0.029$ ). Our findings indicate that there is a global reduction in 5-hmC in AD brain that is present in both regions of primary neuropathology (EC) and regions largely devoid of neuropathology (CER), suggesting a potential global response to the disease process. Our results also concur with a recent study by Chouliaras et al., 2013 that identified AD- and neuropathology-associated reductions in glial 5-hmC in the hippocampus and in the affected sib of a monozygotic twin pair discordant for AD.

#### 3.4. Cross-tissue differences in 5-fC and 5-caC in AD

We found an overall significant effect of brain area ( $p < 0.001$ ), but not of disease ( $p = 0.372$ ) on 5-fC abundance, with significantly less 5-fC in EC than CER in AD ( $p = 0.0026$ ), and a near-significant reduction in controls ( $p = 0.072$ ) (Fig. 2C). There was no significant effect of brain area ( $p = 0.810$ ), nor disease ( $p = 0.194$ ) on 5-caC levels (Fig. 2D). Both 5-fC and 5-caC are reported to be present at 10 to 1000-fold lower levels than 5-hmC in primordial germ cells (Vincent et al., 2013), accounting for between 3 and 20 parts per million cytosines across the genome (Song et al., 2012).

### 4. Conclusions

This study confirmed that all four cytosine modifications are present in adult human brain and that there is a significant reduction in 5-hmC in AD, across two different anatomic regions of the brain. These data concur with previous reports of an AD-associated reduction in 5-hmC in the hippocampus (Chouliaras et al., 2013). Although there was a regional difference in 5-fC levels, no AD-associated changes in 5-mC, 5-fC, or 5-caC were observed, although previous reports have shown decrements in 5-mC in AD EC and hippocampus (Chouliaras et al., 2013; Mastroeni et al., 2010). Given the relatively small numbers of samples assessed in this study, further research investigating the specificity of the differences in 5-hmC and 5-fC, we report, particularly across different neuron and glia subtypes, will be of particular interest to the field. Our observation has implications for current efforts to map site-specific changes in 5-mC in AD using bisulfite-based approaches that cannot distinguish between 5-mC and 5-hmC and suggests that disease-associated differentially methylated regions should be interrogated using approaches that can specifically target these modifications (e.g., oxidative-bisulfite sequencing [Booth et al., 2012, 2013]).

#### Disclosure statement

The authors declare that they have no conflicts of interest in regard to this work.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.neurobiolaging.2014.02.002>.

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