

AGE-RELATED DISTURBANCES IN DNA (HYDROXY)METHYLATION IN APP/PS1 MICE

Abstract

Brain aging has been associated with aberrant DNA methylation patterns, and changes in the levels of DNA methylation and associated markers have been observed in the brains of Alzheimer's disease (AD) patients. DNA hydroxymethylation, however, has been sparsely investigated in aging and AD. We have previously reported robust decreases in 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC) in the hippocampus of AD patients compared to non-demented controls. In the present study, we investigated 3- and 9-month-old APPswe/PS1ΔE9 transgenic and wild-type mice for possible age-related alterations in 5-mC and 5-hmC levels in three hippocampal sub-regions using quantitative immunohistochemistry. While age-related increases in levels of both 5-mC and 5-hmC were found in wild-type mice, APPswe/PS1ΔE9 mice showed decreased levels of 5-mC at 9 months of age and no age-related changes in 5-hmC throughout the hippocampus. Altogether, these findings suggest that aberrant amyloid processing impact on the balance between DNA methylation and hydroxymethylation in the hippocampus during aging in mice.

Keywords

Aging • Alzheimer's Disease • APPswe/PS1ΔE9 • Epigenetics • DNA methylation • DNA hydroxymethylation

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Abbreviations

5-hmC, 5-hydroxymethylcytosine; 5-mC, 5-methylcytosine; A β , amyloid- β ; AD, Alzheimer's disease; APP, amyloid precursor protein; CA, cornu ammonis; DAB, 3,3'-diaminobenzidine tetrahydrochloride; DG, dentate gyrus; DNMT, DNA methyltransferase; HDAC, histone deacetylase; HDACi, HDAC inhibitors; IR, immunoreactivity; MBD, methyl-binding protein; MeCP, methyl-CpG binding protein; PS, presenilin; SPF, specified pathogen free; TET, ten-eleven translocation; WT, wild-type

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia and its neuropathology is


associated with altered gene expression, in a hippocampal subregion-specific manner [1,2]. Mutations in the genes encoding amyloid precursor protein (APP) and presenilin (PS) 1 have been identified in autosomal dominant cases of AD and are known to affect brain function by altering APP processing during aging, producing toxic amyloid beta (A β) protein species [3,4].

Accumulating evidence suggests that epigenetic regulation of gene expression is critically involved in the pathophysiology of AD [5,6]. Altered global levels of the DNA methylation marker 5-methylcytosine (5-mC) and changes in gene-specific methylation profiles have been observed in post-mortem brain material of AD patients, in peripheral lymphocytes of AD patients and also in animal and cell culture models of AD neuropathology

[7–14]. Further implication of epigenetic dysregulation in AD is supported by studies showing that treatment with HDAC inhibitors (HDACi), drugs that target the epigenetic machinery, improve behavioral outcomes in transgenic mouse models of AD [15,16].

The oxidized form of 5-mC, 5-hydroxymethylcytosine (5-hmC), has also been identified as an epigenetic regulator, that is particularly abundant in the brain [17,18]. Additionally, oxidation of 5-mC is thought to be the initial step of an active DNA demethylation pathway [19,20]. To date, still little is known about the role of DNA hydroxymethylation in AD [21]. Studies on the mouse hippocampus and cerebellum reported dynamic changes of 5-hmC during neurodevelopment and aging, such as an age-associated increase of hydroxymethylation of genes related to

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amyloid processing [22,23]. Interestingly, a genetic association between AD and one of the enzymes that catalyze the 5-mC to 5-hmC conversion, ten-eleven translocation (TET) 1, has been reported [24], suggesting that the conversion from 5-mC to 5-hmC might be particularly important in AD. Furthermore, we have reported a robust reduction of the DNA methylation marker 5-mC and the DNA methylation marker 5-hmC in the hippocampus of AD patients compared to matched controls, as well as an AD-associated decrease in 5-hmC in the hippocampus of a monozygotic twin pair discordant for AD [25]. Recently, the first epigenome-wide analyses of 5-hmC in relation to AD was published, identifying numerous sites with altered hydroxymethylation [26].

Aging is the most important risk factor for AD and age-related changes in epigenetic mechanisms in AD-vulnerable brain regions may thus be involved in setting the stage for the development of AD [27–29]. We have previously shown in large cohorts of wild-type (WT) mice that aging is associated with increased levels of 5-mC, as well as increased levels of 5-hmC in the hippocampus and cerebellum [30–32]. However, there is no conclusive evidence on whether age-related aberrations in APP and A β processing impact on the balance between 5mC and 5hmC in the brain. As such, it remains unknown whether the reports of global epigenetic changes in the AD brain are closely associated with aberrant amyloid processing.

Based on the postulated imbalance between DNA methylation and DNA hydroxymethylation in the pathophysiology of AD [33] and our findings in the hippocampus of AD patients and controls [25], the aim of the current study was to investigate age-related changes in hippocampal levels of DNA methylation and DNA hydroxymethylation, in a mouse model carrying familial mutations of AD. For that purpose, 5-mC and 5-hmC immunoreactivity (IR) was assessed in three hippocampal subregions, the dentate gyrus (DG), cornu ammonis (CA) 3, and CA1-2, of 3- and 9-month-old WT mice and transgenic APP^{swe}/PS1 Δ E9 mice. The transgenic APP^{swe}/PS1 Δ E9 mice that were used overexpress APP but overall are a relatively low expresser of APP

compared to other transgenic mice models of AD neuropathology such as the PDAPP and the Tg2576 [34]. The APP^{swe}/PS1 Δ E9 mice used develop AD-like amyloid pathology by 4 months of age due to a high ratio of A β 42/A β 40 ratio due to presence of the Presenilin mutation [35,36]. Hippocampal amyloid plaque loads were analyzed in these mice to verify A β deposition.

2. Materials and methods

2.1 Animals

The present study used 11 male WT C57Bl6J and 9 male transgenic APP^{swe}/PS1 Δ E9 mice on a C57Bl6 background. The double transgenic APP^{swe}/PS1 Δ E9 line 85, originally described by [37], carries a co-integrate of 1) chimeric mouse/human APP695 carrying the Swedish mutation (K594M/N595L) and 2) human PS1 with deletion of exon 9 [35], each under control of a mouse prion protein promoter. Line 85 was back-crossed to a C57Bl6 background for more than seven generations. PCR amplification of genomic DNA, isolated from the mouse tails, was performed in order to determine the presence of the APP and PS1 mutations and verify the genotype of the mice used in the present study as described previously (van Tijn et al., 2012). All mice were housed in groups of two with *ad libitum* access to food and water, kept on a 12/12-hour light:dark cycle, and under standard temperature, humidity and specified pathogen free (SPF) conditions. All experiments were approved by the Animals Ethics Board of Maastricht University.

2.2 Experimental Design

Mice were sacrificed at ages of 3 and 9 months for immunohistochemical analysis. Thus, 4 experimental groups were generated based on age and genotype: 1) 3-month-old-WT mice (n = 5), 2) 9-month-old WT mice (n = 6), 3) 3-month-old APP^{swe}/PS1 Δ E9 mice (n = 4), and 4) 9-month-old APP^{swe}/PS1 Δ E9 mice (n = 5).

2.3 Tissue processing

The mice were anaesthetized and transcardially perfused with tyrode solution and fixative solution (4% paraformaldehyde, 0.9% NaCl, 1% acetic acid). Subsequently, the brains were

removed and stored in a 1% sodium azide TBS solution (at 4°C). They were then embedded in 10% gelatin and cut serially in 50 μ m-thick free-floating sagittal sections using a vibratome (VT 1200S, Leica, Wetzlar, Germany), yielding 10 sub-series of every 10th section. These were stored again in 1% sodium azide TBS solution at 4°C until further histological processing.

2.4 Immunohistochemical detection of 5-mC and 5-hmC

For each immunohistochemical procedure, a series of sections was stained using standard immunohistochemical protocols as previously described [30,31,38]. Mouse monoclonal anti-5-mC (dilution 1:500, Genway Biotech, San Diego, CA, USA) was used as a primary antibody for 5-mC, and a biotinylated donkey anti-mouse (dilution 1:200; Jackson Westgrove, PA, USA) as the secondary antibody. For the detection of 5-hmC, a rabbit polyclonal anti-5-hmC antiserum (dilution 1:25,000; Active Motif, Rixensart, Belgium) was used as a primary antibody and a biotinylated donkey anti-rabbit (dilution 1:200; Jackson Westgrove, PA, USA) as the secondary antibody. For each primary antibody, the sections were processed as a single batch to avoid differences in staining intensities between batches. The specificity of the commercially available primary antibodies has been confirmed in our previous work [25,39].

2.5 Semi-quantitative analysis of 5-mC and 5-hmC IR

Mean intensity and surface area of 5-mC and 5-hmC IR were analyzed. For each of the 2 markers, 2 images from the CA1-2 region, 2 images from the CA3 pyramidal layer and 4 images from the granule cell layer of the DG were taken (Figure 1) at 4 different lateral levels (2.525 mm, 1.95 mm, 1.35 mm and 0.675 mm according to the Allen Brain Atlas (<http://mouse.brain-map.org/>), using a 40x objective. Thus, a total of 32 images were taken for every animal for each marker, with a digital camera (F-view; Olympus, Tokyo, Japan) connected to an Olympus AX70 brightfield microscope (AnalySIS; Imaging System, Münster, Germany). Mean intensities and surface area measurements of each image were

obtained using the ImageJ software program (version 1.42q, Wayne Rasband, National Institutes of Health, Bethesda, Maryland, USA), after delineating the regions of interest and correcting for background variation by setting minimum thresholds as previously described in detail [30,31,38]. For statistical analyses the raw data derived from each image (intensity and surface area) were averaged for each animal. Surface area measurements might be affected by volume changes and could be corrected for that [38]. However, in the present study no significant hippocampal volume changes were detected (data not shown) and thus corrections for volumes were not performed. While the present analysis focused on semi-quantitative analysis of global 5-mC and 5-hmC distribution, previously published work from our group has shown quantitative validation of immunohistochemical findings using dot-blot [39].

2.6 Hippocampal A β plaque load IR

Hippocampal amyloid plaque loads were determined with a triple fluorescence immunohistochemical analysis in a series of sections from the same mice used for the 5-mC and 5-hmC staining. An antibody against the N-terminal of A β (Amyloid β [N] IBL international, Hamburg, Germany) was used to stain all A β , thioflavin S (Sigma Aldrich, Zwijndrecht, The Netherlands) to stain highly dense-core plaques, and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma Aldrich, Zwijndrecht, The Netherlands) was used as a counterstain. Briefly, antigen retrieval was performed with 10 mM citric buffer pH 6.0 at 95°C. Then, the sections were rinsed with TBS and TBS-T, incubated with H₂O₂ to quench endogenous peroxidase, washed again with TBS and TBS-T, and blocked with 3% NDS in TBS-T before overnight incubation with the primary antibody (diluted 1:1000 in 0.3% NDS). The next day, the sections were rinsed again with TBS and TBS-T, incubated with the secondary antibody (donkey anti-mouse Alexa 594 Invitrogen, Grand Island, NY, USA), rinsed again with TBS and TBS-T, incubated with thioflavin S (0.0075%) for 10 minutes, and then rinsed for 5 minutes in 70% ethanol. Subsequently, the sections were washed

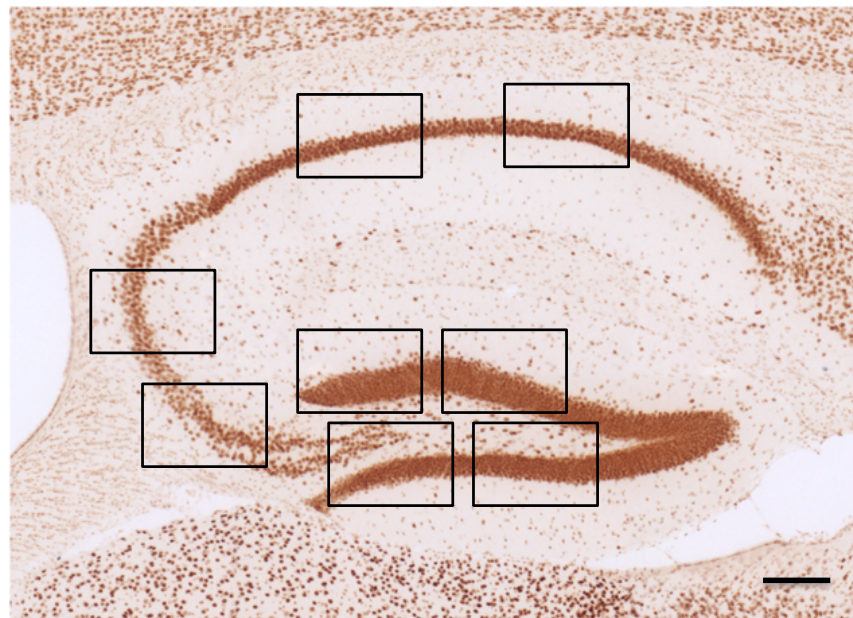


Figure 1. Hippocampal 5-hydroxymethylcytosine (5-hmC) immunoreactivity (IR). Representative photomicrograph of a hippocampal section stained for 5-hmC (lateral level 1.35 mm). The black boxes indicate where the high-magnification photomicrographs were taken for both the 5-hmC and the 5-methylcytosine (5-mC) analysis. A total of 32 photomicrographs per animal (4 photomicrographs in the dentate gyrus, 2 in the cornu ammonis [CA] 3, and 2 in the CA1-2 regions, at 4 different lateral levels) were taken for each staining (see text for more details). Scale bar = 200 μ m.

again with TBS and TBS-T and counterstained with DAPI. The sections were then mounted on gelatin-coated microscope glasses, taken through Sudan black (Sigma Aldrich, St Louis, MO, USA) to reduce autofluorescence, and coverslipped using 80% glycerol in TBS.

2.7 Analysis of Hippocampal A β plaque load densities

All measurements were performed using a modified BX50 bright field microscope (Olympus, Tokyo, Japan), Olympus UPlanApo objectives (Olympus), a three-axis high-accuracy computer-controlled stepping motor specimen stage for automatic sampling (4x4 Grid Encoded Stage; Ludl Electronics, Hawthorne, NY, USA), a linear z-axis position encoder (Ludl), a MBF-CX9000 CCD color camera (1200x1800 pixels; CX9000; MBF Bioscience, Williston, VT, USA), and controlling software (Stereo Investigator, MBF Bioscience). Plaque density analysis was performed in all the hippocampal sections stained for A β . Delineations of the total hippocampus and the cell layers of the DG, the CA3 and the CA1-2 were

performed using the 20x objective. Within the delineated areas, the plaque area was measured with the Area Fraction Fractionator probe of the Stereo Investigator software using a 40x objective (oil, NA=1.00). Thioflavin S staining indicated the presence of dense-core plaques, while antibody A β [N] could demonstrate the less fibrillar plaque load. The plaque area was compared to the total hippocampal area of that respective section to obtain an estimate of the plaque load (Supplementary Figure 1).

2.8 Statistical Analysis

All data are presented as mean and standard error of the mean (SEM). The general linear model univariate analysis of variance (GLM) was used for comparisons between groups, accounting for the main and interactive effects of age and genotype. Statistical significance was set at $\alpha = 0.05$ and corrected total degrees of freedom (df)=19 for all main comparisons. Pairwise comparisons were performed with a Bonferroni post-hoc test. In the absence of a significant interaction, main effects of age were analyzed through an additional stratified

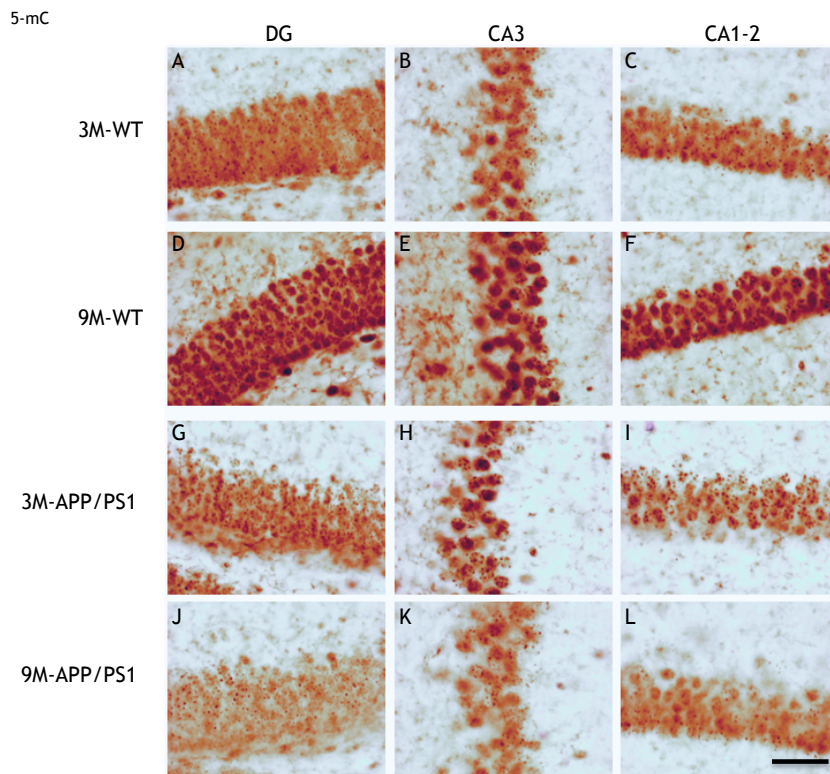


Figure 2. Representative photomicrographs of 5-methylcytosine (5-mC) immunoreactivity (IR). High-magnification representative fields of the hippocampal dentate gyrus (DG), cornu ammonis (CA) 3, and CA1-2 regions are shown. A-C represent a 3-month-old wild-type (WT) mouse, D-F represent a 9-month-old WT mouse. G-I, represent a 3-month-old APPswe/PS1 Δ E9 mouse, and J-L a 9-month-old APPswe/PS1 Δ E9 mouse. Note that an increase in 5-mC IR is observed from 3 to 9 months in WT mice, while a loss of 5-mC IR is observed in from 3 to 9 months in APPswe/PS1 Δ E9 mice in all three hippocampal subregions. The photomicrographs were taken with a 40x objective. Scale bar = 50 μ m.

analysis per genotype, to assess whether overall effects of age were more pronounced in a particular genotype group (corrected total $df=10$ for WT groups and $df=8$ for APPswe/PS1 Δ E9). Correlation analyses between 5-mC and 5-hmC intensity measurements were carried out by calculating the Pearson's correlation coefficient (r_p). All statistical calculations were performed using the Statistical Package for the Social Sciences, (SPSS 17, SPSS Inc., Chicago, IL, USA). Graphs were built in GraphPad Prism (Version 4, GraphPad Software, San Diego, USA).

3. Results

3.1 Qualitative analysis of 5-mC and 5-hmC IR

Nuclear IR of both 5-mC and 5-hmC was

observed in the hippocampal subregions (Figures 2 and 3). 5-mC IR consisted mainly of compact condensations, while 5-hmC IR was spread throughout the nucleus. Qualitative microscopic inspection indicated marked increases of both 5-mC and 5-hmC IR in all three hippocampal subregions of 9-month-old as compared to 3-month-old WT mice (Figures 2A-F and 3A-F). In contrast, an age-related decrease of 5-mC IR (Figure 2G-L) was observed in the 9-month-old as compared to 3-month-old APPswe/PS1 Δ E9, while no apparent differences in 5-hmC IR were observed in these same mice (Figure 3G-L).

3.2 5-mC intensity

GLM revealed a main effect of genotype in the DG ($p < 0.001$, $F=22.2$) and CA1-2 ($p = 0.008$, $F=8.9$) regions, and a main effect of age in the

DG ($p = 0.02$, $F=6.7$; Figure 4A-C). Furthermore, statistically significant age \times genotype interactions were observed in all hippocampal subregions ($p = 0.001$, $F=17.3$ for DG, $p = 0.002$, $F=14.2$ for CA3, and $p = 0.004$, $F=11.1$ for CA1-2; Figure 4A-C). Pairwise Bonferroni post-hoc comparisons showed that age was associated with a significant increase in the intensity of 5-mC IR in the DG (+8.6%, $p = 0.001$) and the CA3 (+6.5 %, $p = 0.044$) regions of WT mice. Moreover, Bonferroni post-hoc comparisons revealed a decrease of 5-mC IR intensity in the 9-month-old APPswe/PS1 Δ E9 mice compared to 9-month-old WT mice in all hippocampal subregions (-11.3%, $p < 0.001$ for DG; -9.1%, $p = 0.004$ for CA3; and -10.8%, $p = 0.001$ for CA1-2). Thus, age was associated with significantly higher levels of 5-mC IR in WT mice, whereas the APPswe/PS1 Δ E9 genotype with lower levels of 5-mC IR in 9-month-old mice.

3.3 5-mC surface area

GLM for surface area measurements of 5-mC IR showed a significant age \times genotype interaction in the DG ($p = 0.048$, $F=4.6$) (Figure 4D), but not in CA3 ($p = 0.079$) or CA1-2 ($p = 0.104$). Pairwise post-hoc comparisons using the Bonferroni correction showed that the observed changes were not specific for any individual experimental groups.

3.4 5-hmC intensity

GLM revealed non-significant effects for the main effect of age in the DG ($p = 0.053$) and the CA3 ($p = 0.099$) subregions, and no effect in the CA1-2 region ($p = 0.149$). Stratified analyses per genotype showed an increase of mean intensity of 5-hmC IR associated with aging from 3- to 9-month-old mice in all hippocampal subregions ($p = 0.022$, $F=7.6$ for DG, $p = 0.016$, $F=8.64$ for CA3, and $p = 0.044$, $F=5.5$ for CA1-2) in the WT mice, but not in the APPswe/PS1 Δ E9 mice ($p = 0.565$ for DG, $p = 0.688$ for CA3, and $p = 0.726$ for CA1-2) (Figure 5A-C).

3.5 5-hmC surface area

GLM revealed non-significant trends for increased 5-hmC surface area IR by age in the DG ($p = 0.074$) and the CA3 ($p = 0.054$) subregions, and no effect in CA1-2 ($p = 0.249$; Figure 5D-F).

3.6 5-mC and 5-hmC correlation

Correlation analyses between 5-mC and 5-hmC IR revealed non significant positive correlations between 5-mC and 5-hmC IR in the DG ($r_p = 0.542$, $p = 0.085$) and the CA3 ($r_p = 0.576$, $p = 0.064$) regions of WT mice, and no effects in the CA1-2 ($r_p = 0.211$, $p = 0.534$). No correlations between 5-mC and 5-hmC IR were observed in the APP^{swe}/PS1 Δ E9 transgenic mice (Figure 6A-F).

3.7 Hippocampal A β plaque load densities

Amyloid plaque analysis demonstrated an age-dependent deposition from 3- to 9-month old mice (Supplementary Figure 1).

4. Discussion

Qualitative and quantitative assessment of the various hippocampal subregions revealed that aging in WT mice was associated with increased levels of 5-mC and 5-hmC. APP^{swe}/PS1 Δ E9 mice showed, however, lower levels of 5-mC IR at 9 months of age and no significant alterations in 5-hmC levels. Altogether, these findings indicate that mutations in APP and/or PS1 impact on the balance between DNA methylation and hydroxymethylation in the aging hippocampus.

4.1 Age-related increase of hippocampal 5-mC and 5-hmC IR in WT mice

In the present study, 5-mC IR was increased in 9-month-old compared to 3-month-old WT mice. As the anti-5-mC antibody detects CpG-rich loci with higher sensitivity, the observed increase of 5-mC IR likely reflects increased methylation of CpG islands [31]. These findings are in agreement with studies reporting that aging is associated with increases in global DNA methylation in brains of rodents [40,41], as well as hypermethylation of CpG islands and loss of methylation of loci outside CpG islands [27,42,43]. Our current findings of increased 5-mC IR from 3- to 9-month-old WT mice are in accordance with our earlier findings of age-associated increases in levels of 5-mC [31] and DNA methylation-associated markers, like Dnmt3a and HDAC2 in C57Bl6

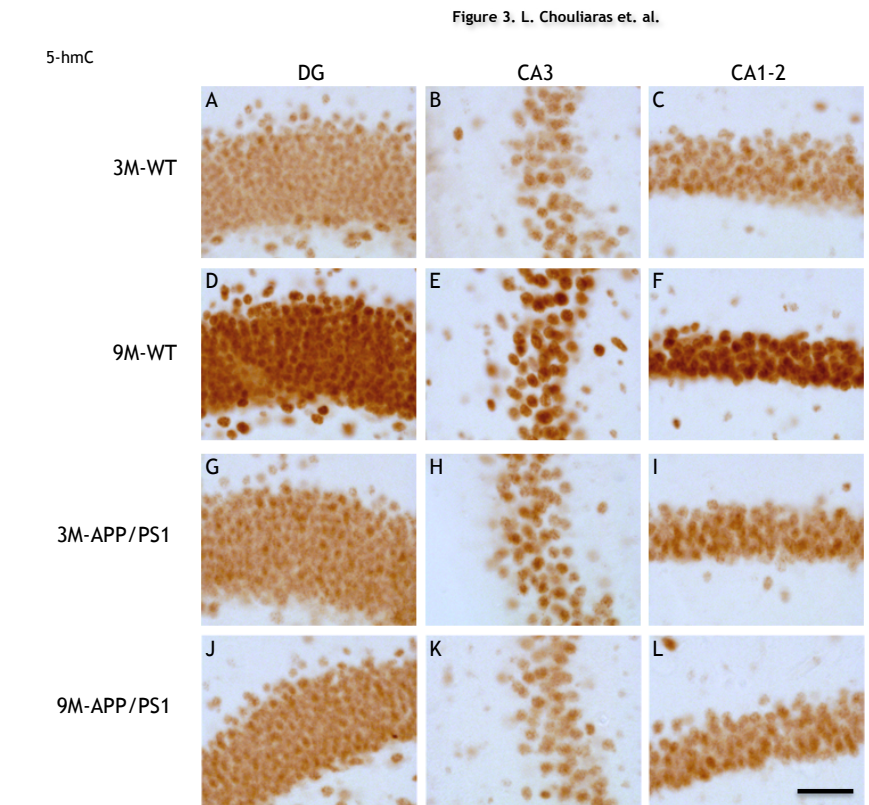


Figure 3. Representative photomicrographs of 5-hydroxymethylcytosine (5-hmC) immunoreactivity (IR). High magnification representative photomicrographs of the hippocampal dentate gyrus (DG), cornu ammonis (CA) 3, and CA1-2 regions are shown. A-C represent a 3-month-old wild-type (WT) mouse, D-F represent a 9-month-old WT mouse. G-I, represent a 3-month-old APP^{swe}/PS1 Δ E9 mouse, and J-L a 9-month-old APP^{swe}/PS1 Δ E9 mouse. Note that an increase in 5-hmC IR is observed from 3 to 9 months in WT mice, while no apparent age-related differences are observed in the APP^{swe}/PS1 Δ E9 mice in all three hippocampal sub-regions. The images were taken with a 40x objective. Scale bar = 50 μ m.

mice [38,44]. Similar to the findings on 5-mC IR in WT mice, 5-hmC IR in hippocampal subregions showed an age-related increase from 3- to 9-month-old in WT mice. 5-hmC has recently gained increased attention as it was described to be particularly abundant in the brain [45,46]. A number of studies have shown that 5-hmC, which is an oxidized form of 5-mC, has a different role in the regulation of gene expression when compared to 5-mC [47–50]. Our finding of an age-associated increase of 5-hmC IR in WT mice parallels a report showing increases in 5-hmC in the aging mouse cerebellum [22]. Interestingly, the increases in DNA hydroxymethylation in that study were particularly pronounced in genes related to neurodegeneration, including those encoding presenilins and secretases. The current findings are also in agreement with our previous

observations of increasing hippocampal 5-hmC IR with age in mice [30]. We have furthermore observed correlations between 5-mC and 5-hmC in WT mice, but considering the significant main effects of age, it is difficult to ascertain whether the correlations observed in these cases are independent of age.

4.2 Disturbed age-related alterations of 5-mC and 5-hmC in APP^{swe}/PS1 Δ E9 mice

Noticeable differences were observed in the temporal patterns of age-related changes in 5-mC IR in WT as compared with APP^{swe}/PS1 Δ E9 mice: 5-mC IR increased with aging from 3 to 9 months of age in WT mice, while aging from 3 to 9 months of age in APP^{swe}/PS1 Δ E9 mice was associated with lower levels of 5-mC IR. A similar, but less pronounced,

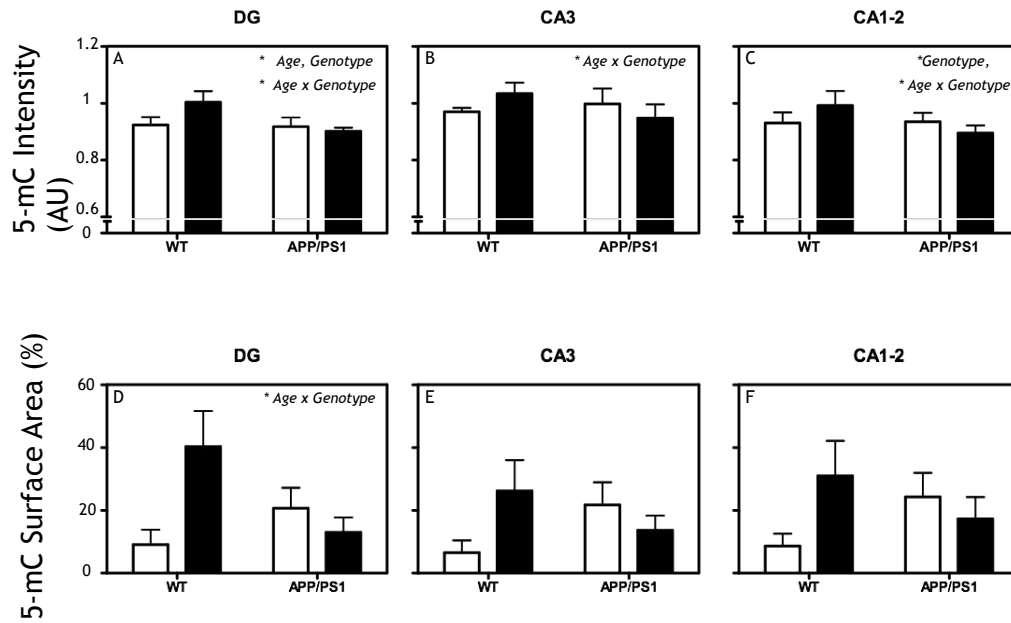


Figure 4. 5-methylcytosine (5-mC) intensity and surface area. Mean and standard error of the mean intensity value measurements of 5-mC immunoreactivity (IR; A-F). Pooled data from the 4 groups of 3-month-old (white bars) and 9-month-old wild-type (WT) and APPswe/PS1ΔE9 mice (black bars) are represented separately for the dentate gyrus (DG; A, D), cornu ammonis (CA) 3 (B, E), and CA1-2 (C, F). Statistically significant effects from the univariate analysis of variance including age and genotype are displayed in the upper right corner of the graphs. In case of a significant age x genotype interaction no main effects are shown. Statistically significant differences between the age and genotype groups, as determined through Bonferroni-corrected post-hoc comparisons, are also indicated in the graphs. AU, arbitrary units; **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

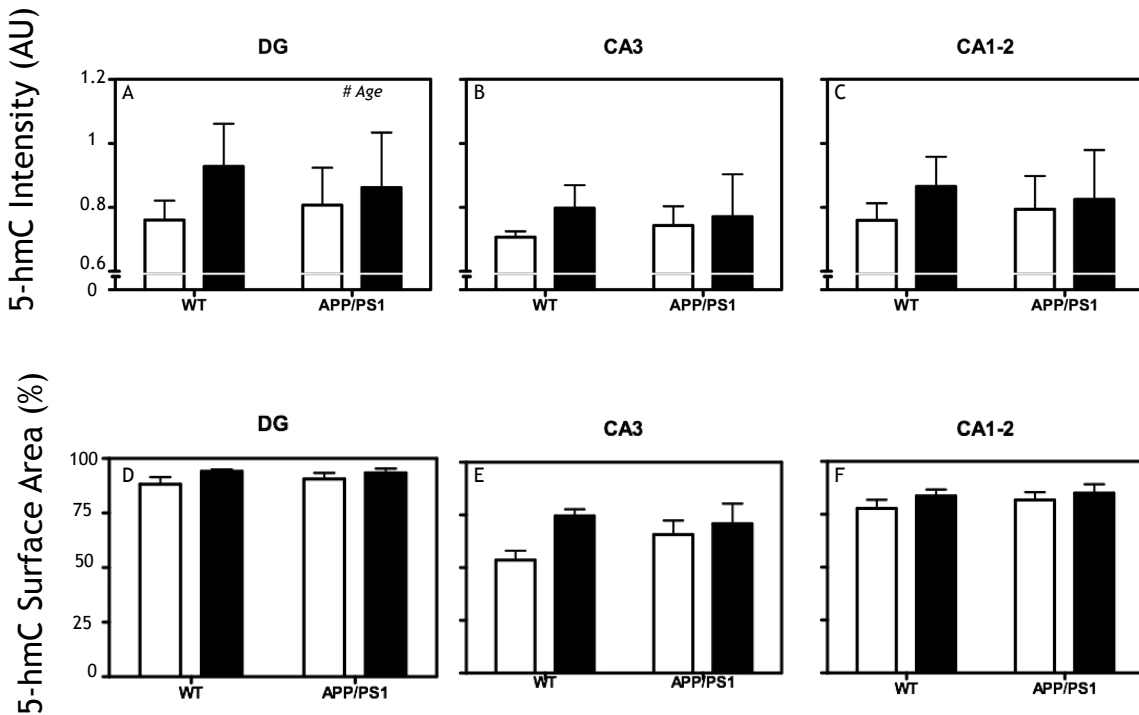


Figure 5. 5-hydroxymethylcytosine (5-hmC) intensity and surface area. Mean and standard error of the mean intensity value measurements of 5-hmC immunoreactivity (IR; A-F). Pooled data from the 4 groups of 3-month-old (white bars) and 9-month-old (black bars) wild-type (WT) and APPswe/PS1ΔE9 mice are represented separately for the dentate gyrus (DG; A, D), cornu ammonis (CA) 3 (B, E), and CA1-2 (C, F). No statistically significant effects of age or genotype were found with a univariate analysis of variance, however a stratified analysis per genotype showed age-related increases of 5-hmC intensity in the WT mice, as shown in the graphs. AU, arbitrary units; **p* < 0.05.

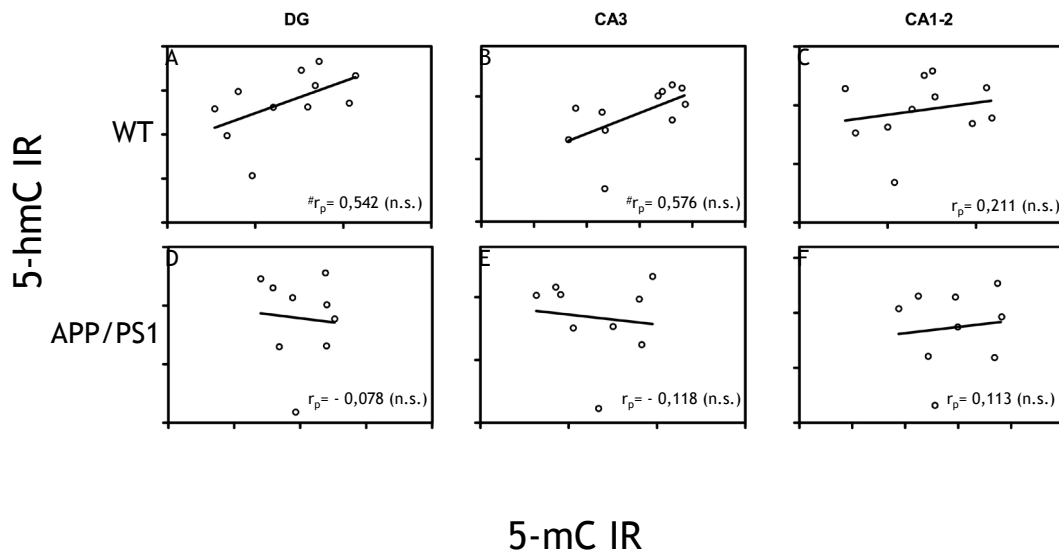


Figure 6. 5-hydroxymethylcytosine (5-hmC) and 5-methylcytosine (5-mC) correlation analysis. Correlation analysis of 5-hmC and 5-mC immunoreactivity (IR) for wild-type (WT; A-C) and APP^{swE}/PS1 Δ E9 (D-F) mice are represented separately for the dentate gyrus (DG; A, D), cornu ammonis (CA) 3 (B, E) and CA1-2 (C, F). Spearman's correlation coefficients (r_p) are noted in the bottom right of each graph. ns, non-significant ($p > 0.05$).

opposition of age-related patterns was found for 5-hmC IR in the hippocampus: 5-hmC IR increased with aging from 3 to 9 months of age in WT mice, but did not show significant age-related changes in APP^{swE}/PS1 Δ E9 mice. Thus, our findings indicate that mutant APP and/or PS1 induce alterations in the molecular cascade of DNA methylation and hydroxymethylation during aging in the mouse hippocampus, and suggest that aberrant amyloid processing may be associated with DNA (hydroxy)methylation changes also observed in the human AD brain [25,51].

The age-related disturbances in 5-mC and 5-hmC in the APP^{swE}/PS1 Δ E9 from 3 to 9 months of age coincide with the development of AD-like pathology and behavioral deficits in this model [52–54]. Although our findings indicate that mutant APP and/or PS1 impact on age-related changes of DNA methylation and hydroxymethylation, it remains to be answered whether age-related changes of DNA methylation and hydroxymethylation are primarily causal or compensatory in the development of AD-associated pathology [21]. In this context, other studies have documented that intervening in the DNA methylation processes has consequences on amyloid

processing. For example, dietary disturbances of the one-carbon metabolism, which regulates the methylation potential, induced promoter hypomethylation of the PS1 gene and increased amyloid plaque deposition in mice [11,55,56], while dietary supplementation with the methyl-donor S-adenosylmethionine was found to ameliorate these alterations [57].

A recent study on epigenetic changes with age in various animal models of AD did not find an age-related decrease in hippocampal 5-mC or 5-hmC in APP/PS1 Δ E9 mice, but did find an age-related decrease in hippocampal 5-mC in J20 mice, expressing human mutant APP [58]. An important distinction with the present study, however, is that we compared 5-mC and 5-hmC levels before and after plaque development, while the youngest APP/PS1 Δ E9 mice included in the study by Lardenoije et al. were 6 months of age, which coincides with the first appearance of plaques. It could thus be said that while the present study detects epigenetic changes after plaque deposition, the study by Lardenoije et al. would detect age-related epigenetic changes under the increasing presence of plaques. Given their observation of no further age-related increase in 5-mC and 5-hmC in APP/PS1 Δ E9,

it is possible that, in these mice, levels of the epigenetic markers drop quickly after the first plaque deposition, but do not significantly drop during further aging. Potentially, the age-related increases in 5-mC and 5-hmC observed in WT mice and the decreases in these markers in relation to the development of AD pathology are independent, and cancel each other out at advanced ages when looking at global levels of these markers. Indeed, when comparing the three AD mouse models included in the Lardenoije et al. study, the model with the least amount of plaque deposition, the 3xTg-AD model, shows age-related increases in hippocampal DNA methylation (age-related epigenetic processes mainly drive global changes), the model with intermediate plaque deposition, the APP/PS1 Δ E9 model, shows no significant age-related changes (age- and AD-related epigenetic processes equally drive global changes), and the model with the most severe plaque load, the J20 model, shows age-related decreases (AD-related epigenetic processes mainly drive global changes).

Compared to our findings, Cadena-del-Castillo et al. [59] have found an age-dependent decline in global 5-mC and an age-dependent increase in global 5-hmC in the cortex of

3xTg-AD mice, which carry mutations of APP, PS1, and Tau [60]. This discrepancy could be explained by methodological differences, such as the different brain areas examined (hippocampus vs. cortex), tissue processing or alternatively directly linked to the differences in the mutations and neuropathology characteristics between the 3xTg-AD and the APP/PS1 Δ E9 mice [61,62]. More specifically the 3xTg-AD mice develop tau pathology in addition to amyloid pathology. Therefore, it could be speculated that the presence of tau pathology induces alterations in 5-hmC that were not captured in our study. To support this notion studies in AD patients and transgenic AD drosophila have specifically linked 5-hmC changes with tau-mediated neurotoxicity [63]. Clearly, the interrelations between aging, amyloid processing, and DNA (hydroxy) methylation are complex and further work in this area is needed.

Overall, the present findings can be linked to our previous observations of decreased 5-mC and 5-hmC in the hippocampus of AD patients and controls [25] and suggest that the presence of amyloid pathology is closely related to robust changes in global DNA methylation. In line with our present findings, various reports have shown alterations in DNA methylation in certain brain regions as well as in lymphocytes of AD patients when compared to controls (for review see: [5]). For example, Mastroeni and colleagues [6] have shown a robust loss of 5-mC IR and DNA methylation-stabilizing factors, like Dnmt1, Methyl-CpG Binding Protein (MeCP) 2 and HDAC2 in the entorhinal cortex of AD patients when compared to controls, and in a monozygotic twin pair discordant for AD [64]. Studies focusing on methylation changes in specific genes, repetitive elements and ribosomal DNA have furthermore reported significant changes in AD cases as compared to controls [7,13,14,65–68].

It is important to mention that many of the current techniques used for the detection of DNA methylation either cannot detect 5-hmC or cannot discriminate it reliably from 5-mC [69–71]. Thus, the importance of 5-hmC might be underestimated in current epigenetics research on aging and AD [33]. Interestingly our group has shown an increase of 5-hmC in a specific layer of the sub-ventricular zone of patients with AD which is associated with neurogenesis and proliferation in that area [39].

4.3 Limitations, future prospects and conclusions

The current study design involved WT and double transgenic APP^{swe}/PS1 Δ E9 mice. As such, it cannot be explained whether the findings of this study are due to genetic variation in the APP gene, the PS1 gene or the combination of both. While the observed epigenetic changes might be associated with the presence of AD pathology in the transgenic mice and the stochastic events that occur with aging, it should be noted that epigenetic mechanisms are dynamic processes that are subject to environmental exposures and thus the effects of the environment and its impact on the epigenome should be more thoroughly investigated in the context of AD.

While the present study focused solely on the hippocampus of male mice, investigation of potential sex effects and examination of other brain regions that are either heavily affected by aging and AD or are resilient are of equally high importance. The fact that 5-mC but not 5-hmC decreases in correspondence with A β plaque deposition in these mice suggests that mutant APP and/or PS1 disturb the balance between these epigenetic regulators during aging. Clearly, the exact implications of this disturbance and its possible role in the pathophysiology of AD remain to be elucidated. The current immunohistochemical approach

allows for the detection of hippocampal sub-region-specific changes and gives an estimate of total levels of 5-mC and 5-hmC. It would be interesting to investigate gene-specific alterations of the two epigenetic markers and identify potential target pathways that are specifically affected by aging and by APP and PS1. Evidence from AD case-control post-mortem studies employing a genome wide approach points towards a widespread AD-associated dysregulation of the DNA methylome and have identified novel targets and pathways that maybe involved in the pathophysiology of AD [13,14], however, apart from a recent report on the DNA hydroxymethylation profile of AD [26] little is known with regards to the involvement of gene-specific 5-hmC changes in AD. Evidently, the appropriate methods for the precise detection of each modification should be selected. Furthermore, the functional impact of epigenetic changes in DNA (hydroxy) methylation on gene expression, associated AD-like pathology, and behavioral correlates should be further examined.

In conclusion, the present findings reveal differential age-associated changes of DNA methylation and hydroxymethylation in the hippocampus of WT and transgenic APP^{swe}/PS1 Δ E9 mice and suggest that an altered DNA methylation/hydroxymethylation balance is involved in the pathophysiology of AD.

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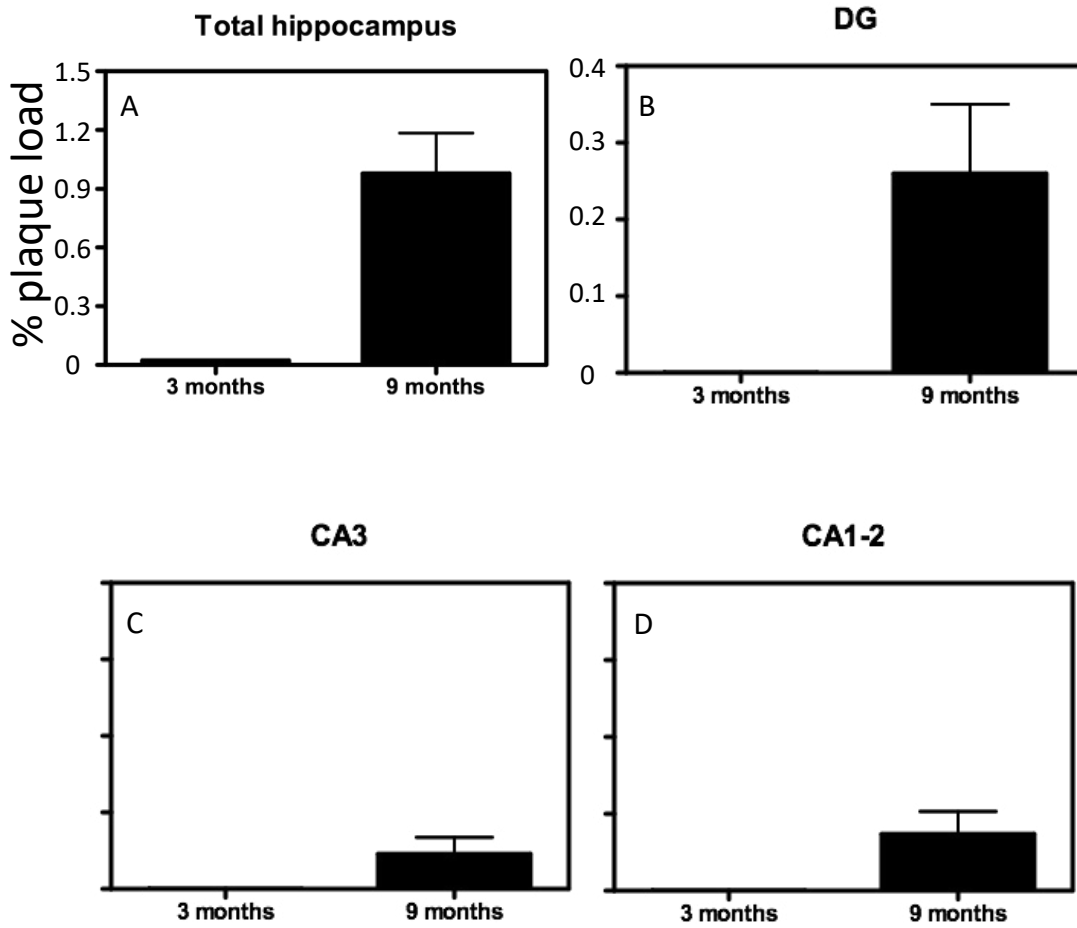
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Supplementary Figure 1. Hippocampal plaque load in 3- and 9-month-old APPswe/PS1 Δ E9 mice. Mean and standard error of the mean of hippocampal plaque loads (percentage of area covered by plaques compared to total area) in the whole hippocampus (A) and the three hippocampal subregions (dentate gyrus [DG], cornu ammonis [CA] 3, and CA1-2; B, C, and D respectively) of transgenic APPswe/PS1 Δ E9 mice. Note that a clear increase in plaque load is observed from 3- to 9-month-old mice. These measurements were performed to validate the age-dependent hippocampal amyloid- β plaque deposition in the mice used.