ORIGINAL ARTICLE

Insufficient DNA methylation affects healthy aging and promotes age-related health problems

Liang Liu · Thomas van Groen · Inga Kadish · Yuanyuan Li · Deli Wang · Smitha R. James · Adam R. Karpf · Trygve O. Tollefsbol

Abstract DNA methylation plays an integral role in development and aging through epigenetic regulation of genome function. DNA methyltransferase 1 (Dnmt1) is the most prevalent DNA methyltransferase that maintains genomic methylation stability. To further elucidate the function of Dnmt1 in aging and age-related diseases, we exploited the Dnmt1+/− mouse model to investigate how Dnmt1 haploinsufficiency impacts the aging process by assessing the changes of several major aging phenotypes. We confirmed that Dnmt1 haploinsufficiency indeed decreases DNA methylation as a result of reduced Dnmt1 expression. To assess the effect of Dnmt1 haploinsufficiency on general body composition, we performed dual-energy X-ray absorptiometry analysis and showed that reduced Dnmt1 activity decreased bone mineral density and body weight, but with no significant impact on mortality or body fat content. Using behavioral tests, we demonstrated that Dnmt1 haploinsufficiency impairs learning and memory functions in an age-dependent manner. Taken together, our findings point to the interesting likelihood that reduced genomic methylation activity adversely affects the healthy aging process without altering survival and mortality. Our studies demonstrated that cognitive functions of the central nervous system are modulated by Dnmt1 activity and genomic methylation, highlighting the significance of the original epigenetic hypothesis underlying memory coding and function.

Keyword DNA methylation · Healthy aging · Cognition · Epigenetics · Dnmt1

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Introduction

Aging epigenetics is an emerging research theme that bears the promise for unraveling the molecular causes of many age-related health problems in the future (Rando 2010). Epigenetic mechanisms represent the molecular interface mediating gene–environment interactions throughout the lifecycle. Aberrant epigenetic signaling plays a crucial role in tumorigenesis (Bjornsson et al. 2004), and it can also contribute to cellular senescence and organismal aging (Fraga and Esteller 2007). DNA methylation is a major epigenetic event and has been shown to play important roles in fundamental cellular processes such as differentiation, proliferation, and senescence by modulating both local and genome-wide gene transcription through the transfer of methyl groups to cytosines within CpG dinucleotides (Robertson 2005). The biological significance of DNA methylation has been clearly documented by the embryonic lethality in mice lacking major DNA methyltransferases (Dnmt1, 3a, and 3b; Li et al. 1992; Okano et al. 1999).

During early embryogenesis, DNA methylation patterns undergo dramatic changes (Santos et al. 2002). Upon completion of organogenesis, tissue-specific methylation profiles are established and maintained in a relatively stable pattern. DNA methylation is catalyzed by several Dnmts, including Dnmt1 that preferentially methylates hemimethylated DNA and two de novo methyltransferases, Dnmt3a and Dnmt3b, which have been shown to methylate previously unmethylated sequences (Okano et al. 1999). The great fidelity with which DNA methylation patterns in mammals are inherited after each cell division is ensured by the maintenance DNA methyltransferase Dnmt1. However, it has been shown that the aging cell undergoes a DNA methylation drift with generally an overall decrease in total genomic 5-methyl-2′-deoxycytidine (5-mdC) content in many tissue types (Mays-Hoopes et al. 1986; Rath and Kanungo 1989; Wang et al. 2008; Wilson and Jones 1983). It has been postulated that a passive demethylation of heterochromatic DNA may occur as a consequence of a progressive loss of Dnmt1 efficacy or erroneous targeting of the enzyme (Fraga and Esteller 2007). In addition, studies in cultured aging human fibroblasts have revealed that a gradually reduced Dnmt1 expression and activity occur with increased population doublings (an in vitro measurement of cellular age; Casillas et al. 2003). Despite the fact that many studies have demonstrated the critical role of Dnmt1 activity in regulating cancer cell growth and survival, Dnmt1 function with respect to aging studies awaits further investigations.

Understanding the molecular interface mediating aging and age-related health disorders has emerged as one of the major focuses in biomedical research. It is now well established that aberrant DNA methylation processes are often a causal factor in many forms of cancer as well as several major neurological disorders (Robertson 2005). In addition, it has been long hypothesized that the methylation pattern in neuronal DNA is an important regulator of cognitive functions such as learning and memory (Holliday 1999). The epigenetic component of cognitive function has gained experimental support from several important studies recently (Feng et al. 2007; Feng et al. 2010; Liu et al. 2009; Peleg et al. 2010; Reul et al. 2009). Several recent papers have also identified abnormal DNA methylation events in neuronal cells associated with aging and age-related neurodegenerative disorders such as Alzheimer's disease (Mastroeni et al. 2010; Siegmund et al. 2007; Silva et al. 2008). In addition, epigenetic contributions to metabolic syndromes ranging from insulin resistance to cardiovascular diseases are also beginning to be explored with great interest to provide mechanistic insights into the environmental causes of chronic human diseases (Gluckman et al. 2009; Sinclair et al. 2007; Stenvinkel et al. 2007). Here we focus on assessing the functional and biological significance of DNA methylation in healthy aging by investigating how Dnmt1 haploinsufficiency impacts general body composition, cognitive function and other health conditions during the aging process. Our results clearly support the likelihood that insufficient DNA methylation can lead to early onset of sub-optimal health conditions during the aging process including compromised cognitive function.

Materials and methods

Mice breeding and genotyping

Normal aging C57BL/6 mice at three different ages (6, 12, and 18 months old, respectively) were purchased from the National Institute on Aging. Dnmt1+/− mice, bred onto a C57BL/6 background, were obtained from the Jackson Laboratory (Stock# 002198) at 2 months of age (Bar Harbor, ME). Female Dnmt1+/− mice were bred with male Dnmt1+/+ C57BL/6 mice. Tail DNA was extracted from the progenies for genotyping analysis using a PCR-based protocol provided by the Jackson Laboratory. Dnmt1+/− mice and Dnmt1+/+ littermate controls were maintained under identical conditions. Standard pellet mouse feed (Harlan Teklad 7012) and water were available ad libitum, and lighting cycle was the standard 12 h light/dark. All mice were housed in a specific pathogen-free environment at the University of Alabama at Birmingham Animal Care Facility. Sentinel mice were tested regularly for antibodies to murine viral pathogens, and all tests were negative throughout the experiment. All studies were performed with

approval from the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Body composition analysis using dual-energy X-ray absorptiometry

In vivo body composition (total body fat, soft-lean tissue, and bone mineral density) of mice was determined using a PIXImus densitometer (GE-Lunar PIXImus, Madison, WI) as previously described (Huffman et al. 2007; Nagy and Clair 2000). All body composition scans for both male and female mice were performed at 6 months of age. Mice were anesthetized with isoflurane (2%) and placed in a prostrate position on the imaging plate. A total body scan was done for approximately 5 min for each mouse. Body composition data were analyzed to determine fat and lean tissue masses as well as bone mineral density (BMD).

Mouse behavioral tests

To eliminate sex-dependent variations and potential hormonal impact on cognitive performance, we only focused on male mice in this study using several different cognitive tasks including the Morris water maze, the Barnes maze, and the eight-arm radial maze tests to determine learning and memory function, including long-term memory performance as previously reported (Liu et al. 2002). Briefly, in the water maze, mice at different ages were trained for four trials per day to swim from one of the four starting points of the pool to find a fixed, hidden platform, submerged below the water in one of the quadrants of the pool. After trial 20 on day 5, the fourth trial is immediately followed by a probe trial (no escape platform) for 1 min. Mice with intact memory function typically remain searching for a much longer time in the "correct" quadrant, whereas impaired mice would explore more evenly among the four quadrants. Learning of the task was evaluated by recording the latency to find the platform, the path length, and percentage of trials that each animal found the platform. We also employed the Barnes maze as an alternative cognitive test for mice that were subjected to the water maze test at an earlier age to avoid residual memory retained from previous swimming trainings (van Groen et al. 2002). The Barnes maze used in this study was adapted from the one developed for rats (Barnes 1979). The elevated round platform had a diameter of 140 cm, and the escape holes were distributed along the rim of the platform. Only one of them, the correct escape hole, has a modified home cage attached under it. Mice were trained to escape from the open platform (the start position is in the middle of the platform) to the escape cage; there were four trials per day for 5 days, similar to the water maze. The mice were removed from the maze after escaping or after 4 min had

passed, whichever came first. Learning of the task was evaluated by recording the latency to find the correct escape hole, the path length, and percentage of trials that each animal found the correct hole. Four trials were run per day. The learning of the maze took an average of 5 days. Additionally, a "wet" version of the eight-arm radial maze was used in the latest behavioral tests by placing an eight-arm radial water maze in a water pool according to the protocol reported previously (Shukitt-Hale et al. 2004). In this task, the mice were required to find the escape platform in the correct arm. They had four trials per day for 5 days. The mice started in four different arms each day, which were pseudo-randomly assigned. An entry into an incorrect arm was recorded a reference memory error, and an entry into an arm where it had been before was referred to as a working memory error. Following behavioral tests, the mice were sacrificed. The brain (divided into cortex and hippocampus) and liver were removed and used for gene expression analysis and also DNA methylation analysis as described below. Livers were collected from 22 months of mice for assessing tumor formations.

Hematoxylin and eosin staining

Mice livers were fixed in 10% formalin for over 24 h and embedded in paraffin prior to standard hematoxylin and eosin (H&E) staining. Deparaffinized sections (5 μm) were stained routinely with H&E for pathological evaluation by three independent observers who were blinded to the source of the tissues.

Gene expression analysis

Total tissue RNA was purified using the TriPure Isolation Reagent (Roche Applied Science). Extracted RNA was digested with RQ1 RNase-free DNase (Promega, Madison, WI) to remove residual DNA contamination. After digestion, DNase was removed by phenol–chloroform extraction. RT-PCR was done as described previously (Liu et al. 2004). RNA (~1 μg) was reverse transcribed in 20 μl of final reaction volume using the SuperScript preamplification system for first-strand cDNA synthesis (Invitrogen, Carlsbad, CA). cDNA was subjected to quantitative real-time PCR analysis using the ABI Prism 7900 Sequence Detection System (PE Applied Biosystems, Foster City, CA) and by following the Assays-on-Demand protocol. *Gapdh* was included as a control reference gene to normalize the amount and the quality of cDNA. Primers for *Dnmt1* and *Gapdh* were preoptimized and mixed with TaqMan MGB probe (FAM dye labeled, PE Applied Biosystems). Quantitative data were analyzed using the Sequence Detection System software version 2.1 (PE Applied Biosystems). This relative

quantification was based on the ratio of the mean value of the target gene (*Dnmt1*) to the mean value of the endogenous control (*Gapdh*) gene in each sample.

Genomic DNA methylation analysis

Genomic DNA was extracted using a Wizard genomic DNA extraction kit (Promega). 5-mdC levels were measured by liquid chromatography–mass spectrometry (LC-MS) analysis as reported previously (Song et al. 2005). Briefly, purified DNA was treated with RNase A/T1, followed by digestion with nuclease P1 and calf intestinal alkaline phosphatase to convert gDNA into component nucleosides. 5-mdC content was expressed as a ratio to deoxyguanosine as previously described (Song et al. 2005).

Statistical analysis

For DNA methylation analysis, differences of the means between the age groups were evaluated by using the analysis of variance (ANOVA, SAS 9.1) and considered significant if $p < 0.05$. The behavioral results were analyzed by using the Systat program and one-way ANOVA. For body composition studies, a two-sample t test or Wilcoxon rank sums test was used to compare mean differences or distribution differences between Dnmt1+/− mice and Dnmt1+/+ groups for variables weight, BMD, and fat that were stratified by gender variable using SAS 9.1.

Results

Dnmt1 haploinsufficiency reduces genomic 5-mdC content and adversely impacts cognitive performance

We obtained Dnmt1+/− heterozygous knockout mice from the Jackson Laboratory for all experiments in this study. Following continuous breeding, Dnmt1+/− mice colonies and corresponding Dnmt1+/+ littermate control colonies were maintained under identical conditions. To measure genomic 5-mdC content, DNA was extracted and subjected to LC-MS analysis as reported previously (Song et al. 2005). We first showed that 5-mdC content decreased with age in the liver, hippocampus, and brain cortex in normal C57BL/6 mice at 7, 13, and 19 months of age, respectively, which is consistent with previous observations (Mays-Hoopes et al. 1986; Wilson and Jones 1983; Fig. 1a). An age-related reduction in learning and memory in mice has been demonstrated previously using the water maze test. Our eight-arm radial

Fig. 1 **a** Changes in genomic content of 5-mdC in brain cortex, hippocampus, and liver during normal aging ($n=4$). **b** Comparisons of mRNA levels between age-matched Dnmt1+/− mice and Dnmt1+/+ mice at three different ages, respectively. *$p < 0.05$
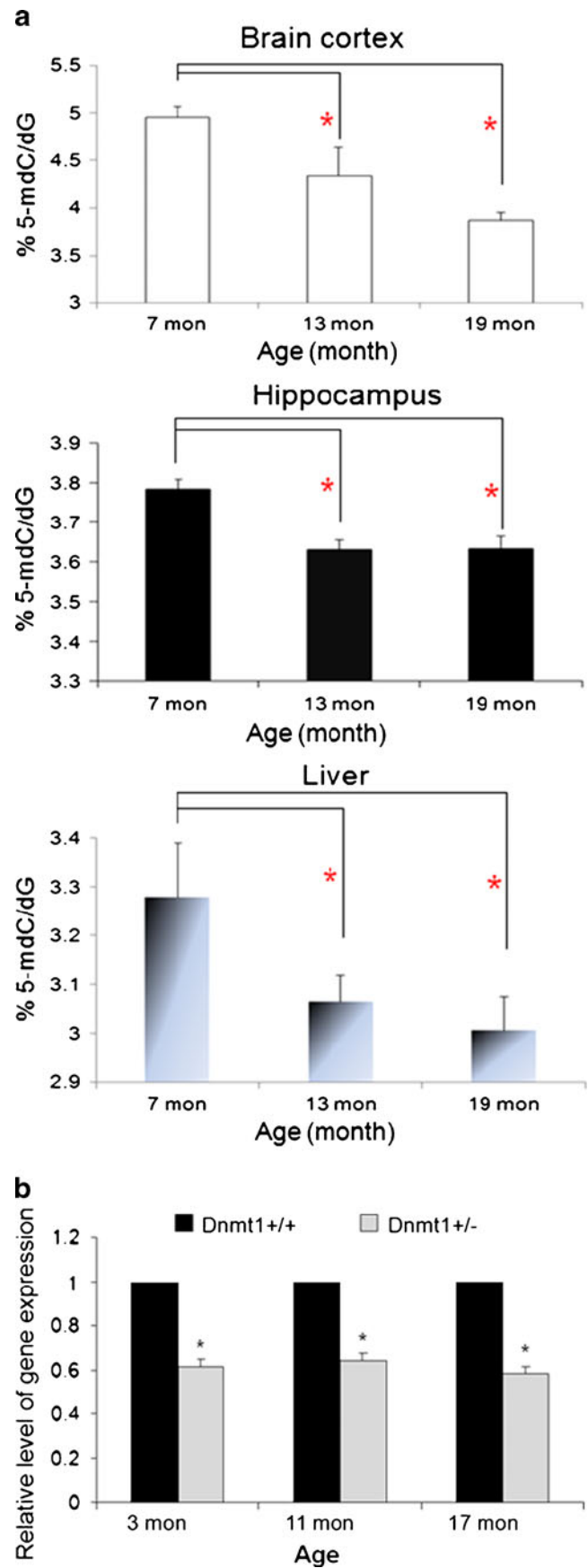
maze memory test also revealed a steady age-dependent decline in learning and memory functions as indicated by the decreasing percentage of mice that passed this cognitive test with increasing age (summarized in Table 1), which confirms that there exists a functional correlation between genomic DNA methylation levels and cognitive performance during the aging process. To determine whether Dnmt1 haploinsufficiency reduced genomic methylation levels, hippocampus and brain cortex gDNA from both the Dnmt1+/− mice and Dnmt1+/+ littermate controls were subjected to similar analysis. As summarized in Table 2, Dnmt1 haploinsufficiency did not cause a corresponding 50% decrease in genomic 5-mdC content, but an obvious reduction in 5-mdC level was consistently seen among the three age groups examined. The age-dependent loss of genomic 5-mdC in the brain cortex of Dnmt1+/+ mice in Table 2 was also consistent with the pattern of methylation changes presented in Fig. 1a, and a similar trend was observed in the hippocampus but at a smaller scale. Compared with age-matched control mice, Dnmt1 expression in Dnmt1+/− mice was also dramatically reduced at the mRNA level in three different age groups, as determined by real-time RT-PCR analysis (Fig. 1b).

Impact of Dnmt1 haploinsufficiency on body compositions and general health conditions

As a first approach to assessing the role of Dnmt1 in healthy aging, age- and gender-matched mice between these two genotypes were used for comparative analysis of changes in body composition. To evaluate how Dnmt1 haploinsufficiency affects overall body composition, we applied dual-energy X-ray absorptiometry analysis to measure BMD and body fat deposition in Dnmt1+/− mice and Dnmt1+/+ littermate controls. We used a two-sample t test or Wilcoxon rank sums test to compare mean differences or distribution differences between Dnmt1+/− mice and Dnmt1+/+ groups for the variables weight, BMD, and

Table 1 Age-related memory function changes in normal mice tested by eight-arm radial maze assay

Age group (month)	Percentage of mice learned the task by the end of the experiment (%)
7	100
13	83.3
19	33.3

Each mouse was subjected to this test and was considered to be competent in learning and memory function if they learned to retrieve the food pellet accurately from the eight-arm device by the end of the 21-day period of the experiment. Twelve mice were included in each age group. The results were based on the percentage of mice that passed the test criteria and no statistical analysis was applied

Table 2 Comparative analysis of genomic 5-mdC levels between Dnmt1+/+ and Dnmt1+/− mice

Age (month)	Percentage of genomic 5-mdC	
	Dnmt1+/+	Dnmt1+/−
Brain cortex		
3	4.62	4.52
11	4.32	4.07
17	4.24	4.1
Brain hippocampus		
3	4.08	3.98
11	3.97	3.84
17	3.85	3.87

The % value of 5-mdC is expressed as a ratio of 5-mdC to all deoxyguanosine (dG). Each value represents the mean of the results from two different mice, and no statistical analysis was applied

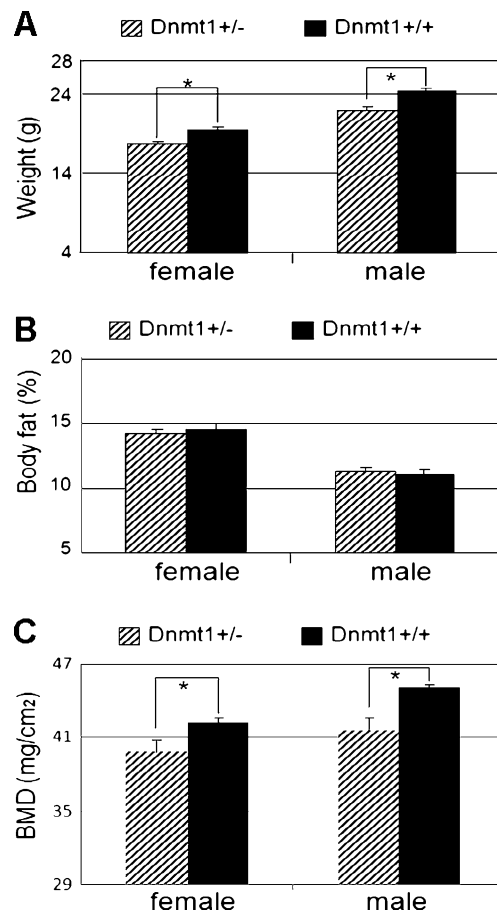fat that were stratified by gender variable. As summarized in Fig. 2, we found that the body weight of Dnmt1+/+ mice

Fig. 2 Comparative analysis of body weight (g), BMD (mg/cm^2), and fat deposition (%) between Dnmt1+/+ and Dnmt1+/− mice. Total number of mice used in the analysis was: male Dnmt1+/+ ($n=16$), male Dnmt1+/− ($n=16$), female Dnmt1+/+ ($n=13$), and male Dnmt1+/− ($n=19$). *$p<0.05$

was significantly higher than that of Dnmt1+/− mice both for the female group ($p=0.0012$) and the male group ($p=0.0069$). Significant differences were also observed for BMD between the two genotype groups both for the female mice ($p=0.04$) and the male mice ($p=0.03$). However, no significant differences were observed in body fat content between these two genotypes for either the female mice ($p=0.19$) or the male mice ($p=0.38$). Interestingly, the female mice tend to have more body fat content and lower BMD than their genotype-matched male counterparts (Fig. 2). Additionally, the pathological examination of sacrificed mice upon tissue collection revealed that sporadic forms of health abnormalities (liver and lung tumors, cataract, testicular inflammation, etc.) were present in old Dnmt1+/− mice but not in the Dnmt1+/+ mice (Table 3 and Fig. 3). Apart from these phenotypes, we found no differences in the overall survival or mortality rate between the Dnmt1+/+ and Dnmt1+/− groups up to 22 months of age, suggesting that optimal Dnmt1 activity is selectively important for maintaining the proper conditions of specific physiological functions.

Cognitive impairment in Dnmt1+/− mice increased with age

A genome-wide decline in DNA methylation occurs in the brain during normal aging that coincides with a functional decline in learning and memory with age (Liu et al. 2009). It has long been speculated that DNA methylation in neurons might be involved in memory encoding (Holliday 1999). To assess the impact of Dnmt1 haploinsufficiency on cognition, both Dnmt1+/− mice and Dnmt1+/+ littermate controls were tested in the water maze test to determine their cognitive status. Our preliminary studies based on a group of age- and gender-matched Dnmt1+/− and Dnmt1+/+ mice (five mice in each group) indicated to us a significant impairment of learning and memory function at 12 months of age due to Dnmt1 haploinsufficiency ($p<0.05$; Fig. 4). We followed up this intriguing observation by including more mice (15 mice in each group) and by starting at an earlier age (i.e., first cognitive assessment at 6 months of age). The water maze test results presented in Fig. 4, however, indicated that no significant differences were present in

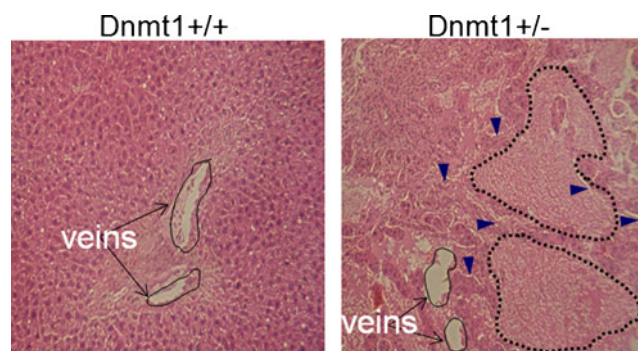**Fig. 3** Histological comparisons of the liver from Dnmt1+/+ and Dnmt1+/− mice at 22 months of age assessed by H&E staining. The histological structure of the liver from Dnmt1+/+ mice (*left*) is clear and the *solid line* represents normal central vein in hepatic lobule. The structure of the hepatic lobule from Dnmt1+/− mice (*right*) was destructed by invasive tumor. *Dotted lines* indicate representative necrosis area and *small arrowheads* point to tumor bulks in the liver. Representative pictures were taken using a Nikon Eclipse E400 inverted microscope and DXM1200 digital camera

the learning and memory abilities (i.e., the learning curves) between these two groups at 6 months of age throughout the trials over 8 days of training. The swimming speeds were not significantly different between the two groups of mice (19.0 ± 2.1 and 17.8 ± 2.2 m/min, for Dnmt1+/− and Dnmt1+/+, respectively). The only significant difference was seen in the reversal training at the end of the experiment (day 8) showing that the Dnmt1+/− mice did significantly better than the control Dnmt1+/+ mice in this part of the task (Fig. 5), which might be related to the possibility that the normal mice spent more time to explore around the previously correct quadrant whereas the Dnmt1+/− mice explored the quadrants randomly to find the new correct escape quadrant more rapidly.

Given that our initial study based on five mice indicated a significant impairment of cognitive function at 12 months of age, we reasoned that there might be an age-dependent cognitive impairment due to Dnmt1 haploinsufficiency. Thus, to test the possibility that the impairment of cognitive function due to Dnmt1 haploinsufficiency may manifest later during the aging process, we decided to test the two 15-mice groups at 12 months of age again. Since mice generally do better in subsequent water maze tests following previous swimming trainings (van Groen et al., unpublished observations), we employed the Barnes maze as a novel cognitive test for these groups of mice at 12 months of age. Interestingly, the results from this new cognitive test revealed that Dnmt1+/− mice had slightly, but significantly ($p=0.038$), impaired cognitive functions compared to the Dnmt1+/+ mice (Fig. 6), suggesting that their learning and memory abilities were indeed affected by Dnmt1 haploinsufficiency at 12 months of age, which is consistent with our earlier results (shown in Fig. 4). No

Table 3 Comparative analysis of age-related health problems between Dnmt1+/+ and Dnmt1+/− mice at the age of 22 months

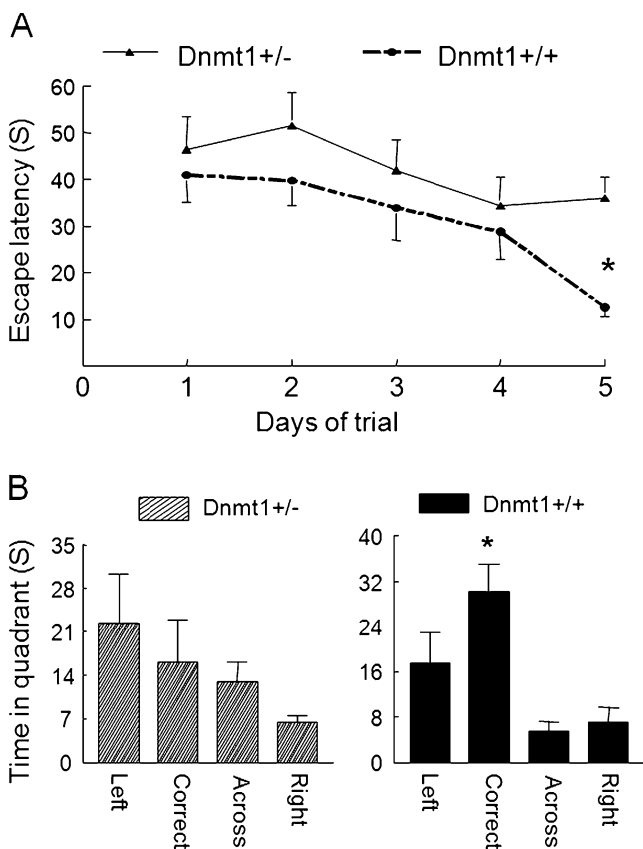| Abnormality	Dnmt1+/+ ($n=7$)	Dnmt1+/− ($n=8$)
Severe inflammation	0	2
Cataract	0	1
Liver tumor	0	3
Lung tumor	0	1
Mortality	0	0

Fig. 4 Assessment and comparison of cognitive functions using the water maze test between Dnmt1+/+ and Dnmt1+/− mice at 12 months of age. Five mice in each genotype group were tested. Statistical analyses of the escape latency differences between these two groups were performed using the Systat program by ANOVA. By the end of the 5-day trial period, Dnmt1+/− mice displayed a significantly delayed escape latency time than the age- and gender-matched Dnmt1+/+ mice (i.e., on the last day of training *$p=0.026$). **a** Improvement of escape latency with the number of trial days. **b** Bar graph illustrating the time that each mouse group spent in one of the four quadrants during the probe trial. Note that the Dnmt1+/+ mice spent significantly more time on the correct quadrant than the rest ($p=0.003$), whereas the Dnmt1+/− mice explored the four quadrants randomly
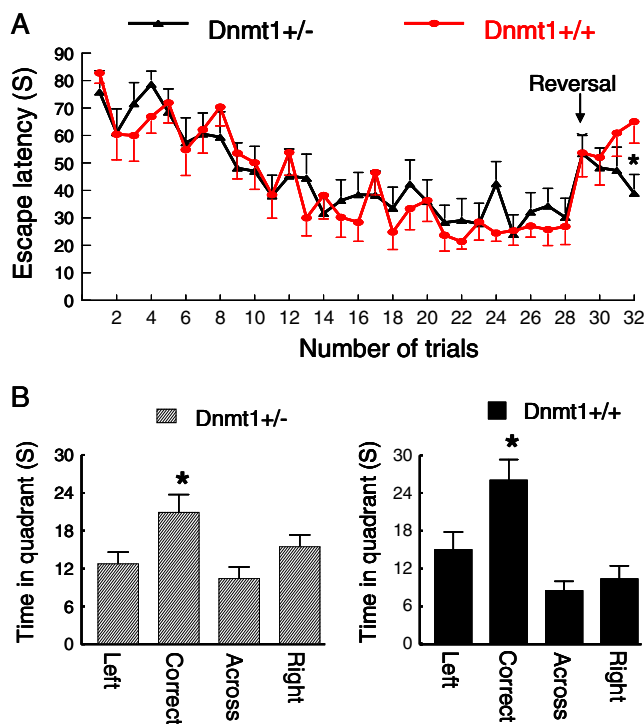

Fig. 5 Assessment of cognitive functions of Dnmt1+/+ and Dnmt1+/− mice at 6 months of age by using the water maze test. Tests and statistical analyses were performed as described in Fig. 2 except that the testing lasted for 8 days, with a reversal of platform position on the last day (day 8) of training. **a** Improvement of escape latency with the number of trials. No statistically significant differences in the escape latency time were present between the Dnmt1+/+ and Dnmt1+/− mouse groups. However, the latencies of the reversal trials on day 8 were significantly different ($p=0.034$) between the groups. The Dnmt1+/− group learned the reversal on day 8 and showed a significant preference for the correct quadrant ($p=0.04$), whereas the Dnmt1+/+ mice did not. **b** Bar graphs illustrating the time that each group spent in one of the four quadrants. Note that both Dnmt1+/+ mice and Dnmt1+/− mice spent significantly more time ($p=0.003$) in the correct quadrant during the probe trial on day 7, indicating intact learning and memory functions at the tested age

significant differences were present in the walking speeds (5.5 ± 1.2 and 6.1 ± 1.2 m/min, for Dnmt1+/− and Dnmt1+/+, respectively) and in the number of errors between the groups (not shown). To further validate this observation, the same groups of mice were retested again for their cognitive functioning at 18 months of age using the wet version of an eight-arm radial maze. Similarly, we noticed that Dnmt1+/− mice exhibited significantly impaired cognitive functions as compared to the Dnmt1+/+ control mice (Fig. 7). It is worthwhile to point out that the poorer performance by Dnmt1+/− mice was obvious throughout the experimental periods ($p<0.001$; 5 days in total), but that Dnmt1+/+ mice started their performance at a significantly lower escape latency than the Dnmt1+/− mice (Fig. 7), confirming a preexisting difference in long-term memory (i.e., cognitive

functioning) between these two groups prior to the test. Swimming speeds were similar between the two groups, 12.0 ± 1.7 and 11.9 ± 1.5 m/min for Dnmt1+/− and Dnmt1+/+, respectively. But it should be noted that even if they were significantly impaired compared to the Dnmt1+/+ mice, the Dnmt1+/− mice did learn the task at the end of the training (significant improvement in the escape latency, $p<0.001$). Taken together, these studies suggest that Dnmt1 haploinsufficiency had more pronounced detrimental effects on cognitive function at later ages (i.e., 12 months and later) but not at an early age (6 months).

Discussion

In this study, we examined the functional impact of Dnmt1 haploinsufficiency and genomic DNA methylation alterations
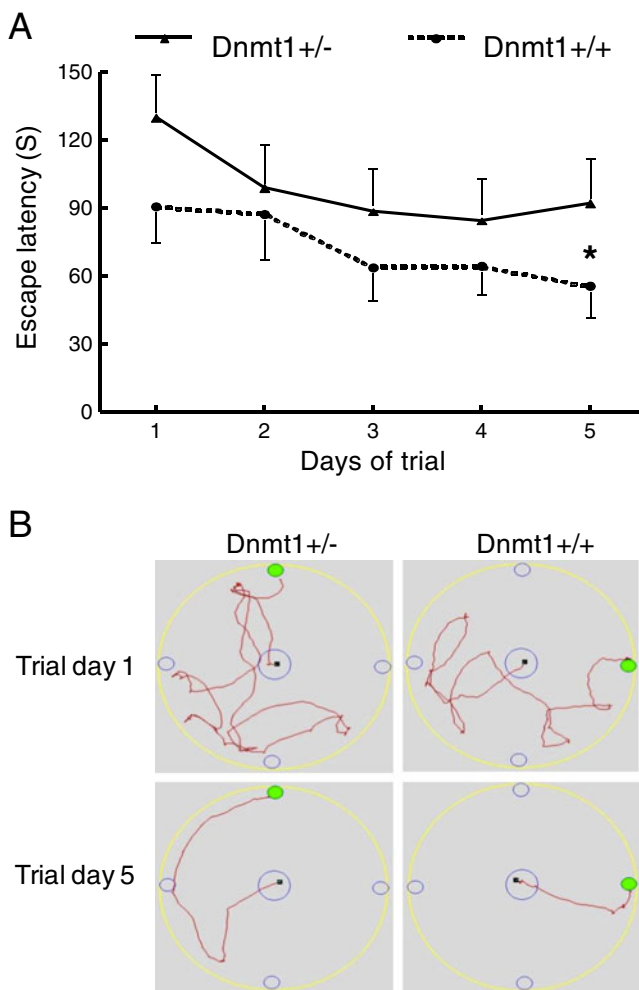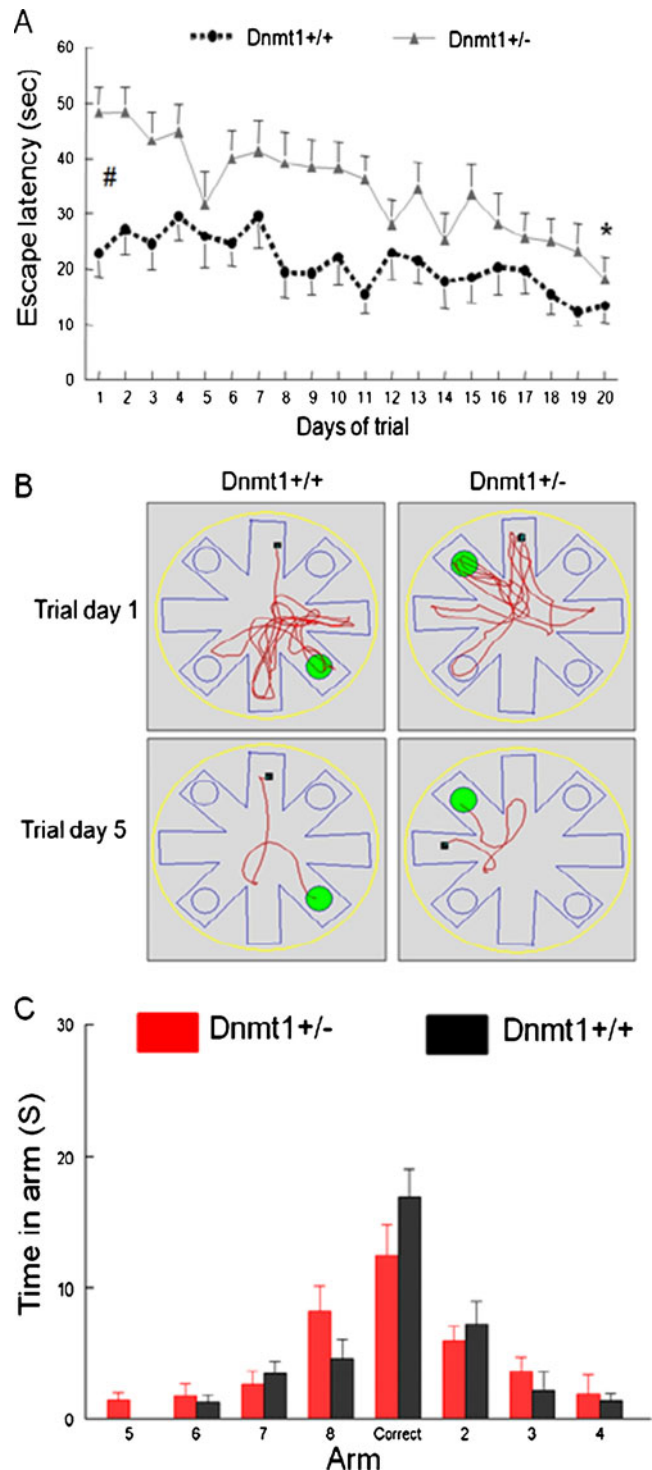
on healthy aging outcome by monitoring several major aging phenotypes including longevity, body composition changes, and cognitive functions. The results suggested that reduction in global DNA methylation, due to a heterozygous knockout of Dnmt1, had adverse health effects during the aging process. The significant decrease in BMD in Dnmt1+/− mice as

Fig. 6 Assessment of cognitive functions between Dnmt1+/+ and Dnmt1+/− mice by Barnes maze at 12 months of age. The same groups of mice as in Fig. 3 were used for this test, and statistical analyses were performed as described in Fig 2. **a** Improvement of escape latency with the number of trial days. Dnmt1+/− mice had worse cognitive performance at the beginning of the test as well as at the end of the test ($p=0.038$), although their learning ability and relative short-term memories were not severely impaired. **b** Graphs illustrating the typical path of a mouse to escape from the maze on days 1 and 5 of the experiment. The Dnmt1+/+ mice escaped more efficiently from the maze than Dnmt1+/− mice on both days

Fig. 7 Confirmation of impaired cognitive functions in Dnmt1+/− mice ▶ vs. Dnmt1+/+ mice by using eight-arm radial water maze at 18 months of age. The same groups of mice as in Fig. 3 were used for this test, and statistical analyses were performed as described in Fig 2. **a** Improvement of escape latency over the five trial days. Dnmt1+/− mice have a much worse cognitive performance at the beginning of the test as well as at the end of the test ($\#p<0.001$; $*p<0.003$), although their learning ability and short-term memory were not impaired. **b** Graphs illustrating the typical path for the mice to escape to the correct arm (containing the escape platform) on days 1 and 5 of the experiment. The Dnmt1+/+ mice escaped faster from the maze than Dnmt1+/− mice. **c** Comparison of the time spent in each arm between the Dnmt1+/+ mice and Dnmt1+/− mice in the probe trial at the end of the test (i.e., day 5). Note that Dnmt1+/− mice spent significantly ($p<0.03$) less time in the correct arm than the Dnmt1+/+ mice did

compared with age- and gender-matched Dnmt1+/+ mice suggests that Dnmt1 haploinsufficiency could cause premature aging effects. This is further evidenced by the relatively poor cognitive function in the Dnmt1+/− mice as compared with the Dnmt1+/+ mice in the learning and memory tests. The elevated incidence of tumor formation in old Dnmt1+/− mice is consistent with previous observations that mice carrying a hypomorphic Dnmt1 allele display severe genomic hypomethylation and are prone to the development of aggressive T cell lymphomas at early ages (Gaudet et al. 2003). In contrast, Dnmt1 haploinsufficiency seemed to have no significant impact on survival or mortality based on the relatively small number of mice in our study for up to 22 months of age. Nevertheless, these observations are consistent with previously published findings (Ray et al. 2006). As most of the mice were used in our longitudinal aging experiments at different ages in this study, we were limited to the availability of mice at a specific age for tissue collection and gene expression analysis. So there are slight variations in the age of mice used in behavioral test, 5-mdC measurement, and gene expression analysis. Given that aging is a continuous process, changes in both DNA methylation and Dnmt1 gene expression are likely to follow a consistent trend, which is also supported by our results. Therefore, we do not think that these slight variations in age should have any significant impact on the conclusions drawn from these experiments. Taken together, our results highlight the role of Dnmt1 in regulating the healthy aging process through genomic methylation changes, and also provide direct in vivo evidence in support of the reduced Dnmt1 expression as a major factor that leads to the early onset of age-related health problems such as decreased BMD and cognition decline.

BMD is known to decline in middle-aged and elderly individuals as a result of the normal aging process. Age-related bone loss (osteopenia), as measured by an overall reduction in total BMD, is a natural physiological process but can eventually lead to osteoporosis under pathological conditions. Although osteoporosis can occur in men, it is most common in women especially during post-menopausal stage. A similar gender-specific difference in age-related bone loss has also been reported in rodents (Banu et al. 2002). Our results show that Dnmt1 haploinsufficiency leads to an early onset of reduction in BMD in both male and female mice, suggesting that DNA methylation may function as an essential player in bone biology. However, the mechanism by which this occurs is not clear. Given that bone turnover is generally affected by the balance between bone resorption (mediated by osteoclasts) and bone formation (mediated by osteoblasts), it is reasonable to assume that insufficient DNA methylation activity weakens osteoblastic activity or enhances osteoclastic activity. It will be of great interest to identify the key genes that regulate these two opposing processes whose expressions are modulated by DNA methylation. Although we have no clear evidence about what causes the reduced body weight in Dnmt1+/− mice, it is possible that insufficient methylation activity may adversely impact tissue stem cell renewal and thus tissue growth rate (Schlessinger and Van Zant 2001), which will be investigated in our future studies.

Loss of genomic DNA methylation with age has been observed in different tissues and cell types by assessing the methylation status of various specific repetitive DNA elements (Wilson and Jones 1983; Mays-Hoopes et al. 1986; Rath and Kanungo 1989). Our methylation analysis by LC-MS allows the assessment of total genomic 5-mdC content, which reveals that DNA methylation decreases with normal aging and is also lower in Dnmt1+/− mouse brain tissues than that in Dnmt1+/+ mouse brain tissues. However, we have not characterized which genomic regions or genes are preferentially affected. Further efforts will be directed to identify such DNA elements and target genes to provide more specific explanations to the functional impact of Dnmt1 haploinsufficiency as reported in this study. A recent study in elderly people reveals a gradual age-dependent loss of genomic DNA methylation within the same individual over an 8-year span (Bollati et al. 2009). This longitudinal study provides important in vivo evidence in humans supporting that loss of DNA methylation indeed occurs with age under normal physiological conditions. However, this loss of DNA methylation appears to be consistently seen only in the Alu repetitive regions but not in the LINE regions (Bollati et al. 2009). In a previous study, total methylation levels in T cells were shown to decrease in early aging but then increase with late aging in heterozygous Dnmt1-deficient mice that demonstrated a delayed development of autoimmunity (Yung et al. 2001). It is intriguing to see such a dynamic change in methylation patterns despite the loss of expression of one copy of the Dnmt1 allele. In the brain cortex and hippocampus, as examined in our study, DNA methylation levels in Dnmt1+/− mice are clearly lower than that in age-matched Dnmt1+/+ mice. Concomitantly, DNA methylation levels decrease with age in both Dnmt1+/− and Dnmt1+/+ mice (Table 2). The discrepancy between our results and previous findings may be due to different tissue types studied as well as the differences in methods used to analyze DNA methylation. It is interesting to note that, however, the heterozygous Dnmt1-deficient mice were shown to develop jejunal apolipoprotein AII amyloidosis, confirming that Dnmt1 haploinsufficiency is indeed detrimental to healthy aging due to the risk of amyloid deposition with age that might eventually lead to late-onset Alzheimer's disease (Ray et al. 2006).

Aging in humans, as well as in experimental animals, is associated with a slow deterioration of cognitive performance especially in memory, reaction time, and reasoning abilities (Gallagher and Rapp 1997; Grady and Craik 2000). Analysis of DNA methylation patterns across 12 Alzheimer's disease (AD) susceptibility loci in post-mortem brain samples from late-onset AD patients revealed a pronounced difference in DNA methylation from the control that further increases with age, supporting a role of aberrant DNA methylation drift in AD (Wang et al. 2008). Our results show that reduced global DNA methylation in the brain cortex and hippocampus is associated with impaired memory function in the Dnmt1+/− mice compared to the Dnmt1+/+ mice. This effect is, however, more readily detectable in older animals (12-month and older) than in young animals (6 months of age; Figs. 4 and 5). This age-dependent effect on long-term memory may be attributed to the fact that a steep decline in brain cortex DNA methylation did not occur in Dnmt1+/− mice until a relatively late age (7 months, Table 2). It should be noted that short-term memory was still intact in the old Dnmt1+/− mice, i.e., they could still learn the task. Although we have not directly addressed how DNA methylation loss contributes to cognitive decline, it is possible that a decreased DNA methylation activity could compromise adult neurogenesis activity during aging and therefore slows the generation of new functional neurons in the central nervous system (Zhao et al. 2003). Although Dnmt1 is generally highly expressed during S-phase in mitotic cells and is downregulated in resting cells, post-mitotic neurons and glia in the perinatal and adult brain are shown to have a high level of Dnmt1 expression (Brooks et al. 1996; Goto et al. 1994; Inano et al. 2000). It is proposed that Dnmt1 might be required to facilitate specific forms of DNA repair in post-mitotic brain cells or required for the methylation of mitotic precursor cells and their daughter cells (Feng et al. 2007). Thus, a decreased DNA methylation activity in the brain might lead to improper methylation modification of the genome of the newly generated neurons, and thus results in their poor survival, integration, or inability to form efficient connections with surrounding neurons. Consistent with this idea, Dnmt1 deficiency in mitotic CNS precursor cells resulted in DNA hypomethylation in daughter cells that leads to their functional elimination from the CNS (Fan et al. 2001). These findings provide important cellular evidence to support the role of DNA methylation in modulating the CNS cognitive function during the aging process.

It is interesting to note that at 6 months of age, the Dnmt1+/− mice learned the reversal task more quickly than the Dnmt1+/+ mice following the four training sessions as demonstrated by the probe trial data (Fig. 5). The Dnmt1+/+ mice kept on searching near the previously correct position,

whereas the Dnmt1+/− mice changed their strategy and searched near the new correct location, indicating differences in interactions between long-term and short-term memory between these two groups of mice. Furthermore, both the Dnmt1+/+ and the Dnmt1+/− mice learned the water maze task equally well, indicating intact long-term memory of the platform position. Dnmt1+/− mice did learn the new position in four trials in 1 day, suggesting that they also have intact short-term memory at 6 months of age. The interaction between long-term ("old" platform position) and short-term memory ("new" platform position) is, however, clearly different between Dnmt1+/+ and Dnmt1+/− mice. At 12 months of age and later, however, the Dnmt1+/+ mice are performing significantly better than the Dnmt1+/− mice, indicating impaired memory function in Dnmt1+/− mice. Interestingly, in the eight-arm water maze task at 18 months of age, the Dnmt1+/+ mice appeared to remember the task, i.e., they could find the escape platform quickly, whereas the Dnmt1+/− mice did not remember this (note the significant difference in the performance by these two groups on the first day of training). This indicates that there is a pronounced difference in the retention of long-term memory encoding (i.e., the task of swimming to find an escape platform) between these two groups of mice at old ages. On the other hand, it should be noted that the Dnmt1+/− mice improved in the task by the end of the experiment, and the probe trial data indicate that both groups learned the position of the escape platform and searched primarily at the "correct" position. Therefore, long-term memory formation (for at least a 24-h period) for the escape platform position is still present in these mice. It is of interest to note that both groups of mice showed an age-related decrease in swimming speed, i.e., from approximately 19 cm/s to approximately 12 cm/s.

In summary, the results from our study suggest that the level of Dnmt1 activity is intimately involved in regulating genomic methylation levels and age-related physiological health conditions. We provide direct in vivo evidence showing that an appropriate level and activity of Dnmt1 are required to sustain a better health condition and cognitive function. Although age-related cognitive decline is considered a normal biological process, individuals with mild cognitive impairment have a much higher risk of developing Alzheimer's disease than the general population (Liu et al. 2003). Our findings shed new insights into one of the important mechanisms underlying such major age-related disease etiology, and thus pave the way for further mechanistic studies and molecular interventions in the future. Based on these findings, it is tempting to explore the possibility of slowing or even reversing age-related health deterioration by enhancing Dnmt1 bioactivity through pharmacological or dietary intervention.

Conflict of interest The authors declare that they have no conflict of interest.

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