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DNA methylase and demethylase activities are modulated by one-carbon metabolism in Alzheimer's disease models☆,☆☆

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Abstract

Late-onset Alzheimer's disease seems to be a multi-factorial disease with both genetic and non-genetic, environmental, possible causes. Recently, epigenomics is achieving a major role in Alzheimer's research due to its involvement in different molecular pathways leading to neurodegeneration. Among the different epigenetic modifications, DNA methylation is one of the most relevant to the disease. We previously demonstrated that presenilin1 (*PSEN1*), a gene involved in amyloidogenesis, is modulated by DNA methylation in neuroblastoma cells and Alzheimer's mice in an experimental model of nutritionally altered one-carbon metabolism. This alteration, obtained by nutritional deficiency of B vitamins (folate, B12 and B6) hampered *S*-adenosylmethionine (SAM)-dependent methylation reactions. The aim of the present paper was to investigate the regulation of DNA methylation machinery in response to hypomethylating (B vitamin deficiency) and hypermethylating (SAM supplementation) alterations of the one-carbon metabolism. We found that DNA methylases (DNMT1, 3a and 3b) and a putative demethylase (MBD2) were differently modulated, in line with the previously observed changes of *PSEN1* methylation pattern in the same experimental conditions.

Keywords: DNA methylation; DNA demethylase; Alzheimer's Disease; One-carbon metabolism; S-adenosylmethionine; B vitamin deficiency

1. Introduction

Many human diseases present a complex pattern of different interconnected pathogenic, causative cofactors. In the recent years, a great attention was given to epigenetic factors and, among these, to DNA methylation in particular. It appears evident that DNA methylation could be, at least in part, at the basis of several human pathologies [1,2].

In previous studies we demonstrated that alterations of methylation metabolism were associated with the overexpression of presenilin1 (*PSEN1*), a gene involved in the production of amyloid β peptide whose deposits in intraneuronal spaces are one of the key features of Alzheimer's disease (AD) [3–6]. Our data are supported by other studies that evidenced how DNA methylation is prone to changes in AD and in aging in relation to time, brain region and genes [7–11]. These studies also stressed the observation that DNA methylation may undergo dynamic regulation also in a differentiated tissue. Moreover, a recent study evidenced that different factors involved in methylation maintenance are decreased in neurons in AD cases [12]. These data showed for the first time that homeostasis of methylation machinery (besides DNA methylation pattern) in AD brains, in areas more prone to AD features, could be altered in the direction of a methylation decrement.

DNA methyltransferases (DNMTs) use *S*-adenosylmethionine (SAM, which is the main methyl donor in eukaryotes) as substrate in a reaction that transfers the methyl group to a cytosine on DNA leading to *S*-adenosylhomocysteine (SAH) production. This molecule is a competitive inhibitor of DNMTs; for this reason, SAM/SAH ratio is considered an indicator of methylation potential of a biological system. Normally, SAH is quickly hydrolyzed to homocysteine (HCY) and adenosine; even if SAH synthesis would be thermodynamically favored, hydrolysis can go on since both adenosine and HCY are rapidly converted. HCY, in particular, can be remethylated to methionine (using vitamin B12 and folate as cofactors) or *trans*-sulfurated to cystathionine (using vitamin B6 as cofactor) [13,14]. The clearest evidence of one-carbon metabolism involvement with late-onset AD is the association of the disease with

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hyperhomocysteinemia, low B vitamins and impaired methylation [15–17]. On the basis of these data, we set up our nutritionally based protocol of altered one-carbon metabolism. The protocol, applied both to neuroblastoma SK-N-BE cell line and to TgCRND8 mice, consisted of a deficiency of folate, vitamin B12 and vitamin B6 [4,5] to hamper HCY transformation. Through this expedient, we were able to obtain HCY and SAH accumulation and SAM/SAH ratio impairment, with consequent decreased methylation ability.

In order to clarify the mechanistic insights of methylation-dependent PSEN1 up-regulation in our experimental system, we decided to study the regulation of the most known DNA methyltransferases (DNMT1, 3A and 3B) and one of the putative DNA demethylase (MBD2) [18,19]. DNMT1 is considered the maintenance DNA methyltransferase; it methylates CpG moieties with high specificity, with preference for hemimethylated DNA and in a processive reaction [20]. On the contrary, DNMT3a and 3b show no preference for hemimethylated DNA being, for this reason, indicated as *de novo* DNA methyltransferases [21]. Interestingly, DNMT3a and 3b have shown significant methylation activity on non-CpG sites [22–24]. Whereas DNA methyltransferases are well characterized on their biochemical and biological features, characterization of an enzyme able to actively demethylate DNA still has some aspects that deserve further clarification. Different enzymes with possible DNA demethylase activity were identified: 5-methylcytosine-DNA glycosylase, Gadd45a, MBD2, MBD2b, MBD3 and MBD4 [25–31]. It was also recently demonstrated that DNMTs could be associated with demethylase activity [28,32,33]; finally, it was recently demonstrated that MeCP2 phosphorylation can mediate demethylation/methylation of specific CpG sites [34]. In the present article, we decided to investigate MBD2 since it is well characterized and its association with one-carbon metabolism has already been evidenced [29–31]. Szyf et al. hypothesized that MBD2 can bind to methylated CpGs on DNA and remove the methyl group in presence of H_2O, leading to the formation of non-methylated CpG-bearing DNA (with no cytosine excision) and methanol [29]. We must underline that other authors disputed these findings and that the same authors do not exclude the possibility that MBD2 can interact with other proteins in the active demethylation reaction [19]. They also demonstrated that active DNA demethylation dependent on MBD2 activity was inhibited by SAM [31]. This indication, together with the knowledge that SAM and SAH availability plays a fundamental role in DNA methylation reactions [35], lead us to study the influence of one-carbon metabolism alterations on DNA methyltransferases and demethylase activity in our AD models.

2. Methods and materials

2.1. Cell cultures

Neuroblastoma SK-N-BE human cell line was maintained in F14 medium with 10% foetal calf serum (FCS) and shifted to complete differentiation medium (control medium, with 1% FCS plus 10 μM retinoic acid) or to differentiation medium deficient of folate, vitamin B12 and vitamin B6 (B deficient). B vitamin deficiency does not affect cell proliferation/differentiation as previously reported [3,4]. SAM 100 μM (*S*-adenosylmethionine disulphate *p*-toluensulfonate) was obtained by Gnosis (Desio, MI, Italy) and was added to media according to the experimental design. Cultures were re-fed every second day and stopped after 72 (methylation assays) or 96 h (gene and protein expression analyses); times indicated refer to medium shift as Day 0. Experiments were repeated at least three times.

2.2. Mice and diets

TgCRND8 (carrying human mutated APP gene) and wild-type 129Sv mice were maintained and assigned to control or B vitamin deficient diet as previously described [6]. SAM was administered to appropriate experimental groups (both in control and B vitamin deficient diet) by gavage-needle force-feeding at dosage of 400 μg/day (administrated as 800 μg every other day to limit animal stress). Analyses were carried out on a total of 48 animals; six (three females and three males) mice per group were fed for 60 days after weaning with either control or deficient diet, with or without SAM. Brains were collected and homogenized as previously described [5]; for bisulphite assay, two brain homogenates were grouped in pool for each experiment repetition.

All the experiments were performed in such a way as to sacrifice the minimum number of animals required and were approved by the author's institution according to guidelines of Italian Ministry of Health (D.L. 92/116).

2.3. Real-time polymerase chain reaction analysis

RNA was extracted from homogenized brain with the RNeasy Lipid Tissue mini kit (Qiagen, Milano Italy); 1 μg of total RNA was used for cDNA synthesis, with 50 pmol of random examers and 50 U of transcriptor reverse transcriptase (Roche) at 50°C for 1 h, as indicated by the manufacturer. 1 μg of total cDNA was used for each real-time reaction; analyses were performed in triplicate for each sample as previously described [5] with an annealing temperature of 62°C. cDNA levels were standardized by normalizing them to the β-actin control and presented as the fold increase (ratio of the experimental gene value/actin gene value) over the control sample. Oligonucleotides used as primers in polymerase chain reaction (PCR) reactions were previously described (human β-actin [4]; human DNMT1, DNMT3a, DNMT3b [36]; human MBD2 [37]; mouse β-actin [5]; mouse DNMT1, DNMT3a, DNMT3b [38]) except for mouse MBD2 that were designed with the help of Primer3 software (http://frodo.wi.mit.edu/): MMMBD2L1 (5′-ACCTGGGAAATGCTGTTGAC-3′, forward) and MMMBD2R1 (5′-TGGCAATGTTGTGTTCAGGT-3′, reverse), giving an amplicon of 146 bp. Expression levels of interest genes were also normalized to other two reference genes, GAPDH and 18S, giving similar results (data not shown).

Expression of 5-MCDG was analyzed by semi-quantitative standard reverse transcriptase-PCR and ethidium bromide agarose gel electrophoresis using primers and conditions previously described [39].

2.4. Western blotting

Cultured cells and homogenized brains were lysed with 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.2 % Nonidet P-40, 1 % 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 2 mM EDTA, pheylmethylsulfonyl fluoride (PMSF, 200 μM), leupeptin (1 μM), pepstatin A (1 μM) and calpain inhibitor I (5 μM); 20–40 μg of protein extracts were run on 12% sodium dodecyl sulfate(SDS)-polyacrylamide gel electrophoresis, then blotted onto nitro-cellulose (Bio-Rad, Hercules, CA, USA). Western blot signals were acquired and analyzed by a Fluor-S densitometer and the Quantity One software (Bio-Rad); optical densities (O.D.) from at least three different experiments were calculated for each sample and normalized with the corresponding 14,3,3β and β-actin (not shown) signals O.D.; the O.D. ratios were then compared and expressed as the average fold increase, with 1 as the control (Tg or wild type, Diet A) value.

Primary antibodies characteristics are summarized in Table 1; secondary antibodies came from an ECL Western blotting reagents kit (GE Healthcare Europe); all the chemicals were from Sigma (St. Louis, MO, USA).

2.5. Preparation of DNA templates for methyltransferase/demethylase assays

DNA substrate for methyltransferase and demethylase assay was synthesized by PCR amplification of human and murine sequences of PSEN1 5′-flanking region previously analyzed by bisulphite method [6]. Human DNA was amplified using HSPS1BisF2 and HSPS1BisR2 primer pair, giving a fragment of 762 bp; mouse DNA was amplified by MMPS1BisF1 and MMPS1BisR2 primer pair, giving a fragment of 723 bp. Features of primers and of amplified fragments were previously described [6].

DNA from SK-N-BE cells and mice brain were extracted by classical phenol method. PCR amplifications were carried out on 50 ng of genomic DNA in a PTC-100 thermal cycle (MJ Research) performing 40 cycles (94°C×1 min, 62°C×1 min, 68°C×1.30 min), using a PlatinumTaq HiFi DNA polymerase (Invitrogen). Amplified fragments from four PCR replicates were run on 1.2% agarose gel; specific bands were removed from the gel and purified using a QiaQuick Gel Extraction Kit (Qiagen). DNA was quantified by A_{260}/A_{280} spectrophotometer analysis; sequence specificity of the amplified fragments was assessed through DNA sequencing (in service by PRIMM).

Twenty-five micrograms of DNA was incubated with 60 U of SssI methylase (New England Biolabs), SAM 3.2 mM, 50 mM EDTA for 4 h at 37°C. SAH was removed with Microcon 10 concentrator (Millipore), and the reaction was repeated with fresh 40 U of SssI and 3.2 mM SAM for further 4 h. DNA was recovered by phenol:chloroform:

Table 1
Characteristics of antibodies used for Western blotting

Protein	Antibody		Manufacturer	Band size	Dilution
DNMT1	sc-70981	Monoclonal	Santa Cruz Biotechnology	184 kDa	1:200
DNMT3a	sc-20703	Polyclonal	Santa Cruz, CA, USA	100–130 kDa	1:100
DNMT3b	sc-81252	Monoclonal		97 kDa	1:100
MBD2	sc-9397	Polyclonal		47 kDa	1:100
14-3-3β	sc-629	Polyclonal		30 kDa	1:200

All antibodies recognized both human and murine epitopes.

isoamylalcohol extraction and ethanol precipitation. Methylation was confirmed on DNA aliquots by MspI-HpaII endonucleases digestion assays. Hemi-methylated DNA for maintenance methylase assay was prepared by mixing an equal amount (5 μg) of methylated and non-methylated DNA, denatured at 95°C for 15 min and then re-annealed at room temperature.

Additionally, methylated DNA for radioactive demethylase assay were prepared as above described but with [^{3}H]-labeled SAM (Sigma).

2.6. DNA Methyltransferase activity assays

The assay was performed as previously described [40] with modifications. Briefly, for cells, 1×10^6 SK-N-BE cells were collected by trypsinization, washed in phosphate-buffered saline, resuspended in 75 μl lysis buffer and lysed by repeated freeze-taw; for tissues, 50 mg of brain homogenates were sonicated with 200 μl lysis buffer and passed through 25G syringe needle. Lysis buffer pH 7.8 contained: 50 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 0.01% sodium azide, 6 μg/ml PMSF, 10% glycerol, 1% Tween 80, 100 μg/ml RNAse A. Samples were centrifuged, and supernatant was quantified by Bradford method and used to measure enzymes activity.

Enzymes solution (20 μg of proteins) was incubated for 2 h at 37°C with 1 or 2 μCi [^{3}H]-SAM (Sigma, St. Louis, MO, USA), depending on the experimental design, and 2 μg of methylated or hemi-methylated DNA in a final volume of 40 μl with methylation buffer (20 mM Tris-HCl pH 7.4, 25% glycerol, 10 mM EDTA, 0.2 mM PMSF, 20 mM 2-mercaptoethanol). Reactions were stopped by incubation with 300 μl Stop solution (1% SDS, 2 mM EDTA, 3% 4-aminosalicylate, 5% butanol, 125 mM NaCl, 0.25 mg/ml salmon testes DNA, 1 mg/ml proteinase K) for 30 min. at 37°C. DNA was recovered by phenol:chloroform:isoamylalcohol extraction and ethanol precipitation. Precipitated DNA was filtered on Whatman GF/C filters, washed with 5% TCA followed by 70% ethanol and counted, with scintillation solution, on a Beckmann 6500 LS counter. Results were normalized as cpm/μg of proteins and expressed as relative variation versus values of control medium or diet. Positive (DNA methylated in vitro with SssI and radioactive SAM) and negative (demethylated DNA incubated with lysis buffer without nuclear extract) controls were used.

Replicate assays were also performed on demethylated DNA using 100 μM nonlabeled SAM; after recovery DNA was analyzed by bisulphite modification with the Epitect Bisulphite Kit (Qiagen); subsequent cloning and sequencing was performed as previously described [6].

2.7. DNA Demethylase activity assays

The assay was performed as previously described [41] with modifications. Briefly, 20 μl of the enzyme solution obtained as per DNA methyltransferase assay were incubated with 20 μl of buffer L (10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$) and 2.5 ng of [^{3}H]-labeled methylated DNA for 24 h at 37°C. Reaction was stopped by adding of 100 μl Tris-EDTA pH 8.0, 10 mg salmon testes DNA, 10 μl NaCl 5 M. In some cases, as described in the Results section, 0.1 μg of MBD2 (or β-actin) antibodies were added to the reaction to assess demethylase activity concomitantly with MBD2 protein inhibition. DNA was precipitated and filtered, and residual radioactivity was counted as described above. Values are represented as semiquantitative analysis, with the cpm/μg of proteins of a [^{3}H]-methylated fragment incubated with only 40 μl of buffer L assumed as 1 and the other values normalized to control and represented as the inverse value to evidence the changes in enzymatic activity (i.e., a decrement of 50% in radioactive counts is expressed as a twofold increase in demethylase activity). Negative controls (DNA methylated in vitro with non-radioactive SAM) were also used.

Parallel assays were also performed on 1 μg of DNA methylated with nonlabeled SAM; after recovery, DNA was analyzed by bisulphite modification as above described. DNA aliquots recovered from demethylase assay and from controls incubated for the demethylase assay but without nuclear extracts were assayed by quantitative real-time PCR in order to verify that the loss of radioactivity or methylation was not due to DNA degradation (data not shown). Moreover, to have further control of the assay, we performed the "standard" demethylase assay [41] with neuroblastoma control and B deficient; briefly, the "standard" assay takes advantage of a completely methylated ^{32}P-labeled [methyl-CpG]n double strand oligomer cleaved by micrococcal nuclease after incubation with the nuclear extracts whose methyl-cytidinemonophosphate (methyl-CMP) and non-methyl-CMP products are separated by thin layer chromatography (TLC).

2.8. Statistical analysis

One-way analysis of variance was computed and Bonferroni posttest was used to evaluate any significant ($P<.05$) difference reported in this article. All assays were repeated at least three times; all histograms show the mean value±S.E.M. Asterisks in figures evidence the statistically significant differences; differences lacking remarks are to be considered non-significant.

To assess the correlation between quantitative variables, we computed the linear correlation coefficient r (Pearson's) with the corresponding significance level. Specifically, when examining the relationship between protein levels and methylase/demethylase activities, the average of values obtained on cell cultures/subjects used for each experimental condition were considered.

3. Results

3.1. mRNA and protein levels of DNMT1, 3a, 3b and MBD2

mRNA expression of DNMT1, DNMT3a, DNMT3b and MBD2 in SK-N-BE cells and in wild-type (129Sv) and transgenic (TgCRND8) mice brain was assessed by semiquantitative real-time PCR. Only mRNA coding for the putative demethylase MBD2 showed evident and similar modulation in SK-N-BE cells and in wild type (129Sv) and transgenic (TgCRND8) mice brain (Fig. 1A–C). Indeed, both in cells and in mice brain, MBD2 mRNA was up-regulated in B vitamin deficient conditions versus control and was down-regulated by SAM treatment versus control and B vitamin deficient conditions without SAM.

mRNA expression of 5-MCDG was performed on SK-N-BE human neuroblastoma cell cultures by classical PCR and failed to show any modulation in the experimental conditions here analyzed (data not shown).

DNMT1, DNMT3a, DNMT3b and MBD2 protein levels were assessed in SK-N-BE cells and in wild-type (129Sv) and transgenic (TgCRND8) mice brain by Western blotting. Fig. 1D–F, shows representative results obtained for these proteins in neuroblastoma cells and in mice brain. Semiquantitative analysis evidenced that DNMT1 protein was not modulated, whereas DNMT3a and 3b were down-regulated by B vitamin deficiency both in cells and mice brain, unlike observed for mRNAs (DNMT3a: SK-N-BE, 0.73±0.08; 129Sv, 0.78±0.08; TgCRND8, 0.70±0.09; DNMT3b: SK-N-BE, 0.74±0.1; 129Sv, 0.09±0.08; TgCRND8, 0.68±0.09; $P<.05$ vs. controls); SAM supplementation did not alter DNMTs protein levels (semi-quantitative data not shown). MBD2 was up-regulated by B vitamin deficiency and down-regulated by SAM supplementation, showing a protein expression pattern similar to that observed for mRNA expression; protein up-regulation in B vitamin-deficient conditions was even higher than mRNA up-regulation in the same conditions (Fig. 1G–I).

3.2. DNA methyltransferase activity

DNA methylase activity was evaluated as incorporation of tritiated $-CH_3$ in the substrate DNA sequence. Fig. 2 and Supplementary Fig. 1 show results concerning maintenance (hemimethylated target DNA; upper panels A-C) and de novo (unmethylated target DNA; lower panels D-F) DNA methylase activity. Maintenance methylation appeared more modulated in neuroblastoma cells than in mice brain. Indeed, nuclear extracts from SK-N-BE cells showed decreased $-CH_3$ incorporation in samples from B vitamin deficient medium and increased incorporation in SAM-treated samples (Fig. 2A). On the contrary, nuclear extracts from mice brain only showed a light (thought significant) decrease of maintenance methylase activity in B vitamin deficient samples (Fig. 2B and C). Unlike maintenance methylation, de novo activity resulted modulated in similar way both in neuroblastoma cells and in mice brain; tritiated $-CH_3$ incorporation appeared decreased in B vitamin deficiency conditions and increased in SAM-treated samples (Fig. 2D–F).

The assay for determination of methylation activity is based on the incubation of nuclear extracts with tritiated SAM. This protocol could, in part, minimize differences between endogenous activities, due to the activation effect exerted by exogenous SAM on methylase activity [24]. We repeated the methylation assays with a lower amount of tritiated SAM (Fig. 2, light gray columns): 1 μCi [^{3}H]-SAM instead of the standard amount of 2 μCi [^{3}H]-SAM. The highest SAM concentration is consistent with the common protocols. The lowest concentration, giving a reduced signal in all the conditions tested, was used in order to emphasize the role of SAM supplemented with culture medium or mice diet. Aim of this experiment was to demonstrate that supplemented SAM was able to induce DNA methyltransferase activity, independently on the radioactive SAM used in the assay. As

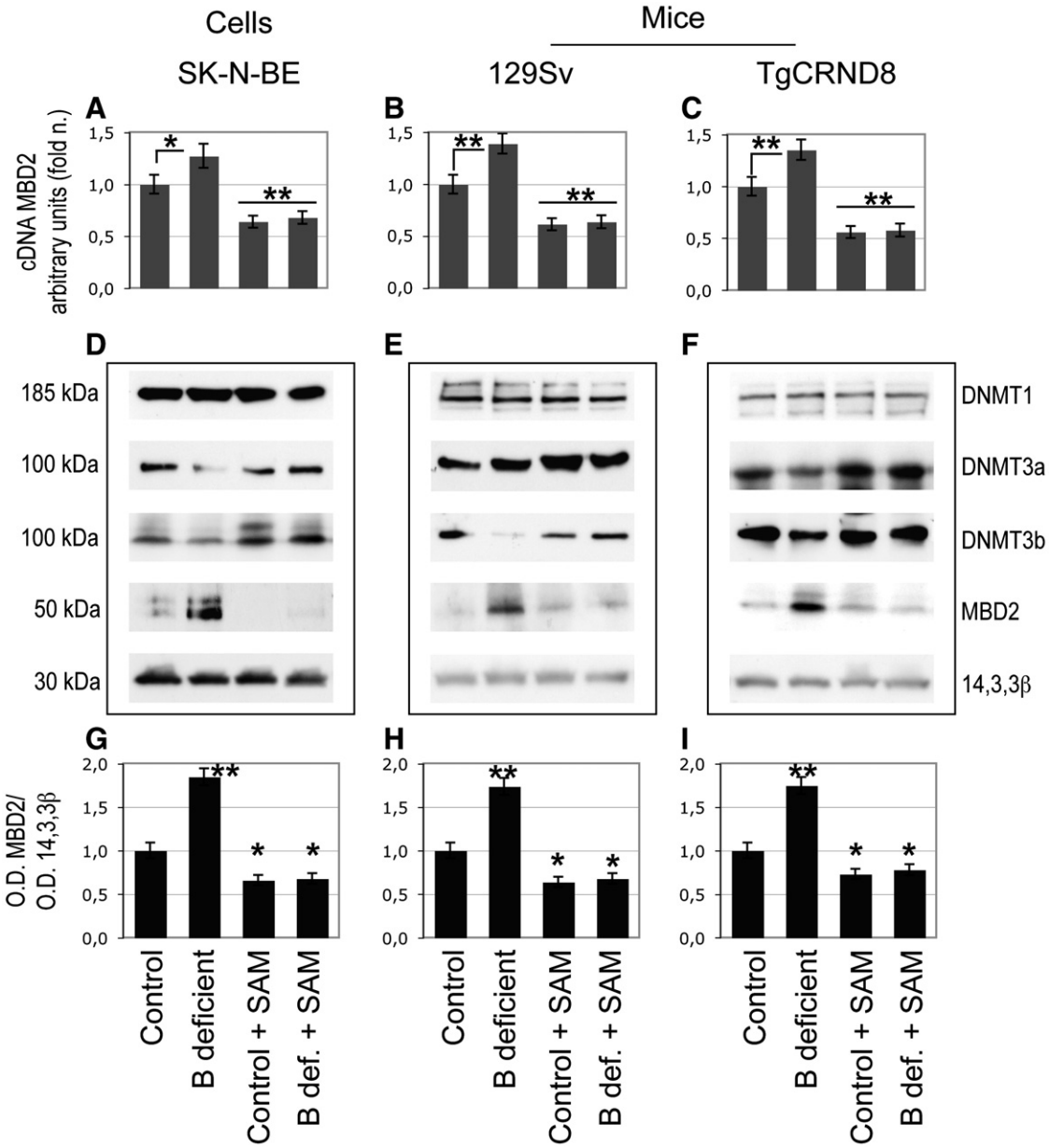

Fig. 1. (A–C) Real-time PCR analysis of MBD2 mRNA in SK-N-BE cells and in wt and TgCRND8 mice respectively. (D–E) Representative Western blotting assays of DNMT1, DNMT3a, DNMT3b, MBD2 and 14-3-3 β proteins on SK-N-BE cells and on wt and TgCRND8 mice respectively. (G–I) MBD2 densitometric quantification of repeated experiments summarized in D–E, obtained by O.D. of MBD2 signals normalized with O.D. of 14-3-3β signals. Units are expressed as "fold increase" with control indicated as 1±S.E.M.; *P<.05; **P<.01; cells, n=3; mice, n=6. "B-deficient" indicates B vitamin-deficient cell culture medium or rodent diet; "SAM" indicates supplementation with 100 μM SAM for cell cultures and 400 μg/day SAM for mice.

shown by histograms, lowering concentration of tritiated SAM resulted in weaker scintillation counts (less than one half of the respective conditions with 2 μCi [^{3}H]-SAM), but also in greater differences between conditions. In particular, SAM-treated samples showed a significantly higher increase of de novo DNA methylase activity (P<.01 vs. control and B def.) both in neuroblastoma cells and in mice brain (Fig. 2D–F). Maintenance methylase activity also was increased in neuroblastoma cells and in mice brain although maintaining the same p values calculated for the assay with standard SAM concentration.

3.3. DNA demethylase activity

Demethylase activity was evaluated as residual tritiated $-CH_3$ in substrate DNA previously methylated with SssI methylase in presence of [^{3}H]-SAM; histograms in Fig. 3 were obtained by plotting the inverse of residual radioactivity after normalization to the value of fully methylated substrate DNA (light gray column), in order to easily visualize the increase/decrease of activity. Histograms show that both in neuroblastoma cells (A) and in wild-type (B) and transgenic (C) mice, demethylase activity was significantly increased in B vitamin deficient conditions. On the contrary, SAM supplementation significantly inhibited demethylase activity both in control and B vitamin deficient conditions. The highest differences in demethylase activity were evident in transgenic mice. Initial radioactivity of methylated DNA samples was comparable for human (1875±21 cpm) and mouse fragments (1790±21 cpm); after incubation with water or with buffer L alone, we cannot observe any significant decrement in measured radioactivity. The counts of substrates in control sample, assumed as 1 in histograms, corresponded to an initial radioactivity of

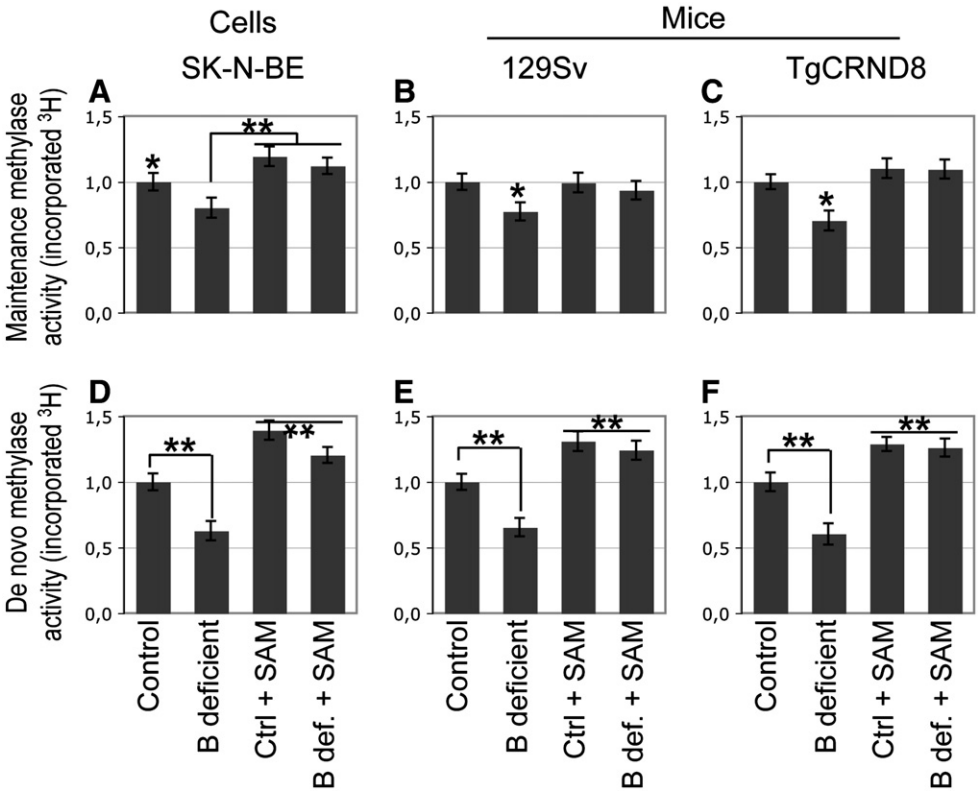

Fig. 2. In vitro DNA methyltransferase activity assay. Maintenance (A–C) and de novo (D–F) DNA methylase activity was evaluated as incorporation of tritiated $-CH_3$ in the substrate DNA sequence (hemimethylated and demethylated, respectively) after incubation with nuclear extracts of SK-N-BE cells (A, D) and wild-type (B, E) and TgCRND8 (C, F) mice in presence of 2 μCi [^{3}H]-SAM. Radioactivity (cpm) values are expressed as "fold increase" versus control. Units, statistics and abbreviations as in Fig. 1.

93.75±2.11 cpm per μg of protein for human DNA assays and to 80.72 ±3.25 cpm per μg of protein for mice DNA assays.

We checked the amount of DNA after incubation by quantitative real-time PCR to verify that loss of radioactivity (used to quantify DNA demethylation) was not due to DNA degradation. We also compared the results of our demethylase assay with one commonly used assay [37] (here defined as "standard assay") in neuroblastoma cells in control and B deficient conditions, obtaining completely overlapping results (Supplementary Fig. 2).

In order to assess demethylase activity when MBD2 protein was inhibited, we performed demethylase assay in presence of anti-MBD2 antibody in control and B vitamin deficiency conditions. Results evidence that the presence of anti-MBD2 antibody was able to inhibit, at least in part, demethylase activity observed in both experimental conditions, in cells and in mice (Fig. 3 D–F). Incubation with anti-β-actin antibody did not cause inhibition of demethylase activity; specificity of MBD2 immunoprecipitation in these assays was assessed by western blotting (data not shown).

3.4. Correlation of protein levels with methylase/demethylase activities

Since the different assays were performed on extracts from the same cell cultures and mice brains, it was possible to evaluate the correlation between DNMTs and MBD2 protein levels and DNA methylase/demethylase activities by Pearson's "*r*" analysis (Table 2). This analysis confirms, both for SK-N-BE cells and for mice brain, the existence of (i) direct correlation between MBD2 protein levels and demethylase activity; (ii) direct correlation between DNMT3a/b protein levels and de novo methylase activity; (iii) inverse correlation between DNMT3a/b protein levels and demethylase activity. A direct correlation between DNMT1 protein levels and maintenance methylase activity was found only in SK-N-BE cells. We also verified a direct correlation between MBD2 mRNA and protein levels and, consequently, between MBD2 mRNA levels and demethylase activity (data not shown).

3.5. Sequence-specific in vitro methylation/demethylation

Features of human and mouse 5′-flanking regions analyzed by bisulphite modification after in vitro methylation/demethylation assays were as previously reported [6]. Briefly, a 762-bp region of human PSEN1 promoter and a 723-bp region of mouse PSEN1 promoter were analyzed. These sequences included 150 cytosine residues in human and 122 cytosine residues in mouse PSEN1 5′-flanking region; among these, CpG moieties were 21 in human and 11 in mouse.

Bisulphite analysis of human and mouse (both transgenic and wild-type) sequences after in vitro methylation with 100 μM SAM revealed that both hemimethylated and unmethylated DNA substrates were methylated without any evident sequence preference (data not shown). Analysis of DNA incubated with nuclear extracts deriving from SAM-treated samples evidenced a detectable degree of non-CpG methylation, similarly to what previously demonstrated in SK-N-BE cells and mice treated with SAM. Results obtained with SK-N-BE extracts are shown in Supplementary Fig. 3; results obtained with TgCRND8 mice brain are shown in Supplementary Fig. 4 (results for wild-type mice were similar to transgenic and are not shown). Reliability of bisulphite reactions and sequencing (in particular for non-CpG methylation data) was assessed by analyzing positive and negative controls and by digestion with methylation-sensitive and

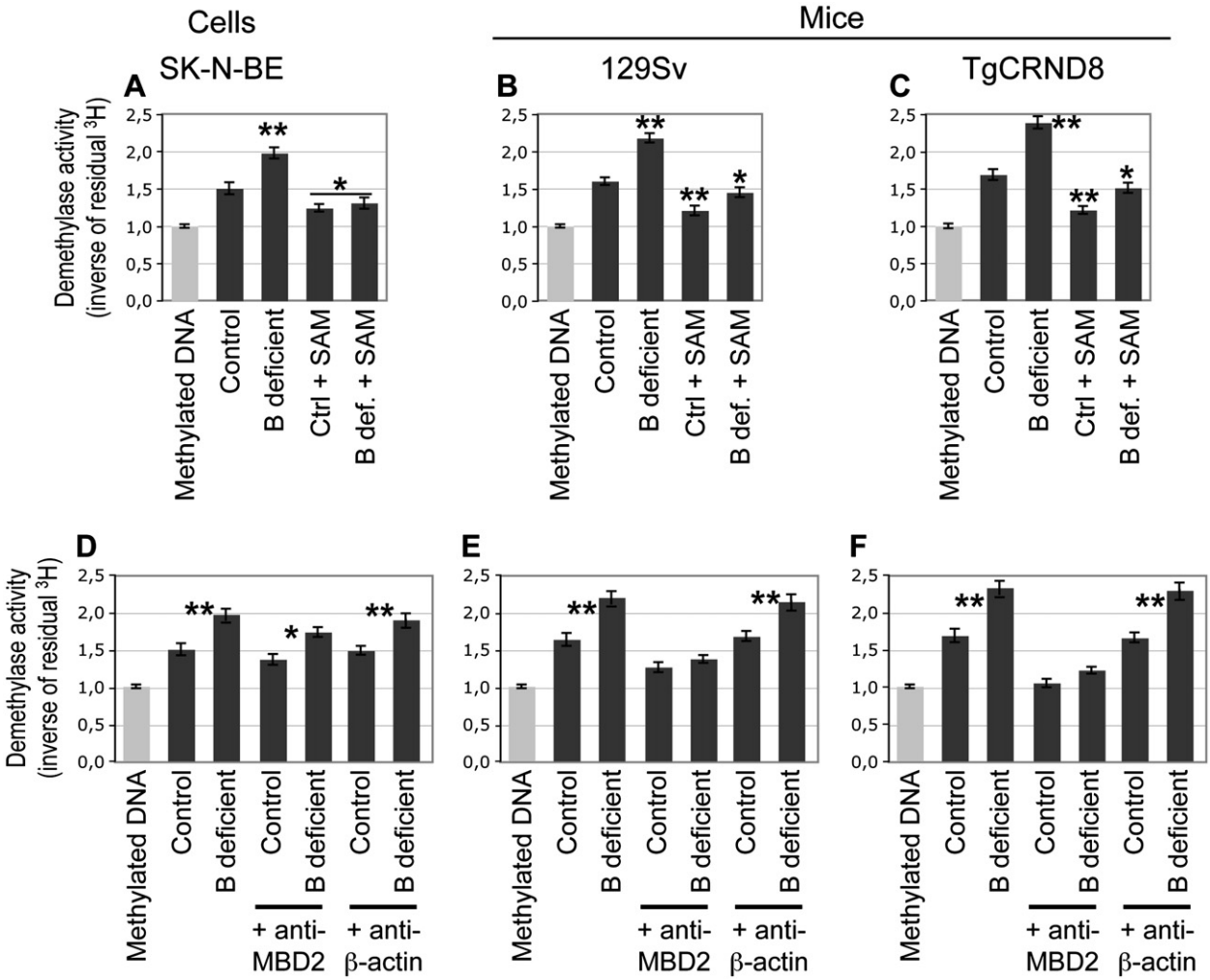

Fig. 3. In vitro DNA demethylase activity assay. Demethylase activity was evaluated as residual tritiated $-CH_3$ in substrate DNA previously methylated with SssI methylase in presence of [^{3}H]-SAM, after incubation with nuclear extracts of SK-N-BE cells (A) and wild-type (B) and TgCRND8 (C) mice. Values are represented as the inverse of residual radioactivity and normalized to value of full methylated substrate DNA. Demethylase activity was also evaluated in nuclear extracts of control and B vitamin deficient conditions in SK-N-BE cells (D) and wild-type (E) and TgCRND8 (F) mice in presence of anti-MBD2 antibody. Anti-β-actin antibody was used as control. Asterisks in panels D, E and F are referred to the difference between each couple of Control and B-deficient conditions. Inhibition of demethylase activity in presence of anti-MBD2 in brain extracts (E, F) is significant for control and B deficient conditions versus no-antibody and anti-b-Actin assays (P<.001). Units, statistics and abbreviations as in Fig. 1.

methylation-insensitive restriction endonucleases, as previously described [6].

Bisulphite analysis was also performed on SssI methylated DNA with non-radioactive SAM on SK-N-BE (Fig. 4 A–D) and TgCRND8 mice brain extracts (Fig. 4 E–H); results on wild type mice (data not shown) were substantially similar to TgCRND8 mice. Demethylation activity analysis through bisulphite sequencing revealed that the same CpG moieties previously found modulated in PSEN1 promoter became preferentially demethylated in B vitamin deficiency conditions, both in human neuroblastoma and mice brain extracts (Fig. 4B and F, respectively, highlighted by black frames).

4. Discussion

The present work was aimed to investigate if the modulation of the methylation machinery could be the mechanistic link connecting

Table 2
Pearson's "*r*" values and associated *P* values for the correlation between protein levels and methylases/demethylase activity

	DNMT1 vs. maintenance	DNMT3a vs. de novo	DNMT3b vs. de novo	DNMT3a vs. demethylase	DNMT3b vs. demethylase	MBD2 vs. demethylase
SK-N-BE						
r	0.81	0.95	0.93	−0.99	−0.97	0.99
P<	.05	.001	.001	.001	.001	.001
Sv129 mice						
r	0.46	0.88	0.79	−0.93	−0.97	0.98
P<	n.s.	.01	.05	.001	.01	.001
TgCRND8 mice						
r	−0.12	0.89	0.99	−0.96	−0.97	0.97
P<	n.s.	.01	.001	.001	.001	.001

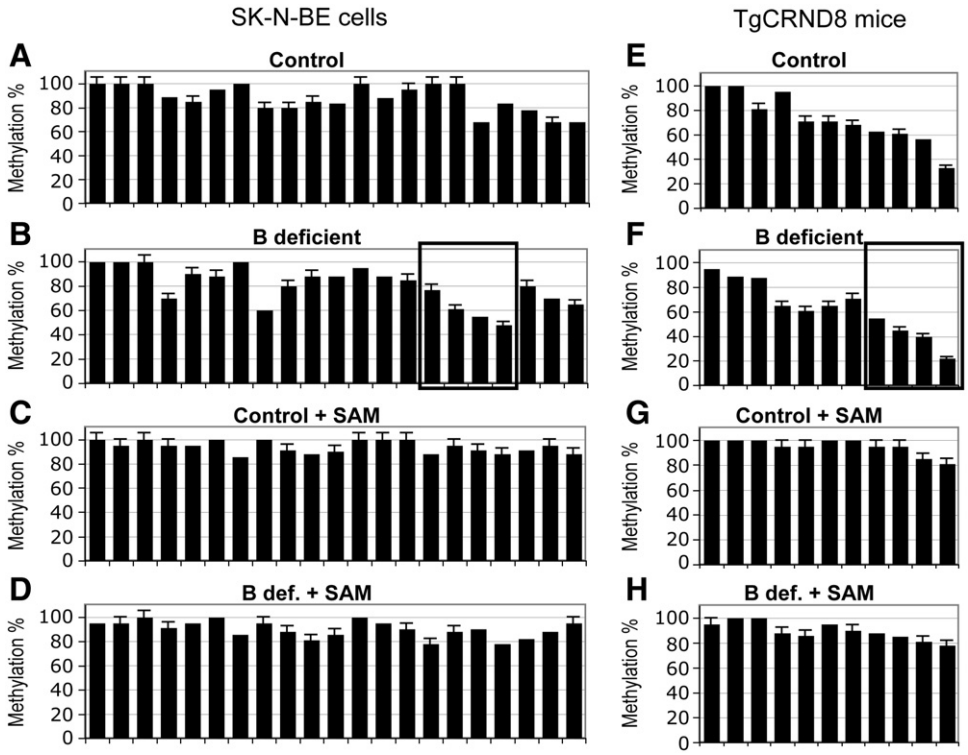

Fig. 4. In vitro assay of site-specific demethylation. Human and murine PSEN1 promoter sequence were methylated in vitro with SssI methylase and non-radioactive SAM, then incubated with neuroblastoma cells (A–D) and Tg mice brain (E–H) extracts. Histograms show results of bisulphite treatment and sequencing on DNA recovered after in vitro demethylase assay and are expressed as percent of DNA methylation for each analyzed CpG moiety. Black frames highlight the four CpG moieties previously found demethylated in B vitamin deficient conditions [6]. For mice brain: $n=3$ (3 pools of 2 brains each). Units, statistics and abbreviations as in Fig. 1.

one-carbon metabolism to PSEN1 regulation. B vitamin deficiency was used as condition leading to HCY and SAH accumulation and to inhibition of methyltransferase reactions; SAM supplementation was used as promoter of methylation reactions and to revert B vitamin deficiency effects on methylation. We demonstrated that methylase and demethylase activity are modulated by nutritionally induced alterations of one-carbon metabolism. It is already known that diet can affect epigenome modulation [19]. It is also known that accumulation of HCY and SAH is able to inactivate DNA methyltransferase(s) [42] and that SAM is able to inhibit DNA demethylase activity [31]. Finally, direct evidence that a "methyl-deficiency" can alter the expression of methyltransferases and methyl binding proteins was obtained in rat liver [43].

MBD2 up-regulation by B vitamin deficiency and down-regulation by SAM supplementation, are consistent with the general methylation/expression paradigm and with the previously observed gene regulation in consequence of one-carbon metabolism alterations. MBD2 up-regulation in hypomethylating conditions and down-regulation in hypermethylating conditions is perfectly fitting with its putative molecular and physiological role, of active DNA demethylase, in cellular homeostasis. Although it has been shown that MBD2 possesses demethylase activity, and although we found MBD2 modulation coherent with demethylase activity modulation, a direct demonstration should require the use of a different model, like a MBD2 knock-out. However, we tried to answer to this point by assaying the demethylase activity in presence of an anti-MBD2 antibody, showing that MBD2 inhibition could, at least in part, decrease DNA demethylase activity. The observation that MBD2 inhibition caused a dramatic down-regulation of demethylase activity in mice brain extracts and a slighter inhibition in neuroblastoma cell extracts is of particular interest; this observation seems coherent with the proliferating status of neuroblastoma cells where passive demethylation could have a major role than in brains. On this regard, we must underline that, as previously observed [3,4], SK-N-BE cells continue to proliferate also in low serum, despite of a slight differentiation, at least up to the time experiments were stopped. Of course, it should be borne in mind that other enzymes, were suggested to have DNA demethylase activity. 5-Methyl-citosine-DNA-glycosylase (5-MCDG) activity is also well characterized [25], but we found that in our system it is not modulated. Moreover, demethylase activity of 5-MCDG seems to be exerted mainly on hemimethylated sites [44], which are characteristic of proliferating cells and appears, for this reason, not a good candidate for DNA demethylation in adult brain. As for Gadd45a, the supposed demethylase activity of this enzyme [29] was recently disconfirmed [45,46]. More interest has to be addressed to putative demethylase activity of DNMT3 [28,32] and of DNMTs in general [33]. These papers have demonstrated that DNA methyltransferases are involved in dynamical processes of methylation/demethylation; however, a direct evidence that DNMTs possess active demethylase activity themselves is still to be fully ascertained. Levenson et al. evidence different caveats on the specificity of DNMT inhibitors used in the experiments and on the direct role of DNMTs in demethylation [33]. On the other hand, the work by Métivier et al. evidence that DNMT3a/b have roles in both methylation and demethylation, but they hypothesize that this last activity could be mediated by the association among DNMT3a/b, TDG (thymine DNA glycosylase) and p68, (TDG retaining demethylase activity or being these factors responsible for the recruitment of an as yet unidentified deaminase) [28,32]. We cannot exclude that DNMTs are involved in active demethylation; even if DNMTs regulation in our system appears opposite to the hypothetical regulation of a demethylase (i.e., decreased expression in demethylating conditions) it could be possible to hypothesize that decreased maintenance and de novo

activities observed in B-deficient conditions could at least play a common role in determining DNA demethylation. These observations are reinforced by the significant positive correlation we found between demethylase activity and MBD2 up-regulation on the one hand and the inverse correlation with DNMT3a/b levels on the other. If we must leave open the possibility that MBD2 could not be the active demethylase, but just be associated with demethylase activity, the same caution should be used for the role of DNMTs since data, so far, still failed to demonstrate an identity of methyltransferase with demethylase. In conclusion, the possibility exists that DNMTs down-regulation and MBD2 up-regulation could be part of a larger process responsible for active demethylation. However, our results strongly stress the hypothesis that MBD2 is one of the major protagonists in active DNA demethylation mechanisms. A further indication of the mechanistic insides studied in this paper could be offered by the analysis of MBD2 gene promoter methylation dynamics, with the attempt to investigate the regulation of MBD2 expression; unfortunately, the sequence of MBD2 gene promoter is, at present, not published. After the submission of this manuscript, a paper was published reporting that MeCP2 is involved in dynamic demethylation and remethylation of specific CpG sites mediated by CaM-kinaseII phosphorylation [34]; these results evidence a role for MeCP2 as a central player in regulation of DNA methylation patterns and mark a new area of investigation in search of DNA demethylase, besides the other above-discussed proteins.

Lack of modulation of DNMT1 together with its lower levels in mice brain than in cells, is consistent with its role of maintenance DNA methyltransferase. It seems reasonable that both in proliferating (neuroblastoma, still proliferating in differentiation medium at the analyzed time point) and in non-proliferating (brain) cells, maintenance methylation is constant but with lower levels of expression in brain, that do not require maintenance methylation. Similar observation was made for mRNA expression levels in mice brain and in neuroblastoma cells (data not shown) and is reinforced by the significant correlation of DNMT1 levels with maintenance methylase activity only in neuroblastoma cells [32–34].

Analysis of DNA methylase activity (both maintenance and *de novo*) was performed using two different concentrations of tritiated SAM added to the nuclear extract. The higher SAM concentration is in line with the common protocols applied for the study of DNA methyltransferase activity. The lower concentration, giving a reduced signal in all the conditions tested, was used in order to emphasize the contribution of non-marked SAM supplemented with culture medium or mice diet. Supplemented SAM was uptaken by cells and mice as previously described [3–5]; the aim of this experiment was to demonstrate that supplemented SAM was able to induce DNA methyltransferase activity independently on the radioactive SAM used in the assay. In fact, the use of the same high activity of radioactive SAM in all samples could minimize possible differences between nuclear extracts deriving from samples undergone different alteration of one-carbon metabolism. As expected, in the assay performed with the lower activity of radioactive SAM, it is possible to evidence higher differences in DNA methyltransferases activity, even if the statistical significance of differences between experimental conditions remains the same. Both in wild type and transgenic mice, it is only possible to observe significant but moderate reduction of maintenance methyltransferase activity in B vitamin deficient diet. On the contrary, de novo DNA methyltransferase activity is strongly reduced by B vitamin deficiency and also strongly induced by SAM supplementation. These results stress the hypothesis that de novo DNA methylation (i.e., DNMT3a and 3b) could be ascribed to inducible enzymes and are in agreement with the suggestion that DNMT3a and 3b enzymes show more complex regulation respect to DNMT1 [18]. Moreover, these data also evidence that alteration of one-carbon metabolism can directly affect DNA methylation through SAM and SAH levels and methyltransferase activity regulation.

DNA demethylase activity resulted modulated in opposite manner respect to methyltransferases activities: indeed, it was increased by B vitamin deficiency and decreased by SAM supplementation. It was already demonstrated that active DNA demethylase could be down-regulated by SAM in vitro [31]; however, as far as we know, these results are the first evidence that SAM levels could modulate demethylase activity in whole cells or tissues. In particular, we demonstrated that hypomethylating conditions, induced by B vitamin deficiency (i.e., SAH levels increase), could improve demethylase activity. A recent paper [12] evidenced MBD2 decrement in AD brains. Since in the present work we used neuroblastoma cells and mice brain under conditions of altered one-carbon metabolism, it is not possible to infer any comparison of the respective results. However, it will be interesting to extend our data in the future with the analysis of specific brain area in transgenic mice.

DNA methyltransferase and demethylase activities in vitro were also studied through bisulphite analysis to evidence sequence-specific differences in methylation pattern of *PSEN1* gene [6]. Despite some technical trouble (i.e., low recovery of DNA after in vitro treatment and bisulphite reaction, probably due to DNA degradation associate with bisulphite treatment), we found some interesting results. Firstly, unmethylated and hemimethylated substrates showed similar in vitro methylation levels, with no differences in patterns, indicating that maintenance and de novo activities had no sequence specificity in our models. Moreover, CpG methylation levels were generally lower in samples with B vitamin deficiency and generally higher in samples with SAM supplementation, confirming that alteration of one-carbon metabolism modulates methyltransferases activity. Low differences between B vitamin deficient and SAM supplemented samples could probably be ascribed to the use of elevated SAM concentration for in vitro reactions; the use of a lower SAM concentration failed to show significant methylation levels with this technique (data not shown). Another very interesting result was the observation that SAM-supplemented samples showed detectable levels of non-CpG methylation, as previously demonstrated in whole neuroblastoma cells and mice brain. This finding was obtained both in unmethylated and hemimethylated substrates, the lasts carrying, obviously, unmethylated non-CpG cytosine moieties. This indicates that non-CpG methyltransferase activity is independent on surrounding CpG methylation pattern. Non-CpG methylation was found more evident in neuroblastoma cells, indicating that this cell line could be more prone to non-CpG methylation (probably because of its cancer nature) as postulated in our previous paper [6]. Interestingly, DNMT3a and 3b have shown significant methylation activity on non-CpG sites [22]. Finally, we observed that the same CpG moieties previously found demethylated in B vitamin-deficient conditions [6] undergo demethylation also when in vitro-methylated PSEN1 DNA was incubated with B vitamin-deficient derived nuclear extracts for site-specific analysis of DNA demethylation. This result confirms that: (i) active demethylase activity should be involved in PSEN1 promoter demethylation in vivo; (ii) one-carbon metabolism alteration in general (besides SAM supplementation) can influence DNA demethylase activity; (iii) site-specific DNA demethylation is maintained also in the in vitro assay as similarly observed in whole cells and brains. Regulation of active demethylation is a particularly interesting topic since MBD2 biochemistry has still to be fully ascertained and since other enzymes could contribute to DNA methylation.

In conclusion, all these results demonstrate that alterations of one-carbon metabolism, both in the sense of methylation impairment (i.e., B vitamin deficiency) and in the sense of methylation improvement (i.e., SAM supplementation) are able to modulate DNA methylation reactions through the regulation of DNA methyltransferase(s) and demethylase activity and evidence that nutritional alteration could

result in functional modulation of such a complex biochemical pattern. Interestingly, a preliminary experiment on neuroblastoma cells treated in control medium with Deazaaristeromicin (not shown), a potent inhibitor of methyltransferases, showed reduced methyltransferase activity but normal demethylase activity and failed to show site-specific demethylation (although maintaining general demethylation) reinforcing the hypothesis that active demethylase is primarily involved on the observed DNA demethylation.

Besides giving more insides into the complex regulation of DNA methyltransferases and demethylases, and give a direct evidence that nutritional alteration could result in the functional modulation of a complex biochemical pattern such as the one-carbon metabolism, this study adds another piece to the jigsaw representing the connection between one-carbon metabolism and Alzheimer's Disease. In fact, these experiments moved from our previous research on molecular relationship between B vitamin deficiency and amyloid plaques deposition [3,5]. In the light of these last results, we can formulate a consequential model describing our hypothesis on the sequence of the biomolecular and biochemical events leading to amyloid deposition (Fig. 5). According to this hypothesis, B vitamin deficiency can induce HCY and SAH accumulation causing the hampering of methyltransferases activity and the improvement of demethylase activity. Methylation/demethylation imbalance leads to PSEN1 promoter site-specific demethylation, PSEN1 over-expression and γ-secretase activity up-regulation. Finally, we can observe the overproduction of amyloid β-peptide [5]. Exogenous SAM seems able to contrast this mechanism by rescuing methylation/demethylation imbalance. Effects of SAM on amyloid processing were partly described in neuroblastoma cells [4], whereas a paper with data on mice is in preparation. Obviously, further investigations are needed in order to clarify the role of oxidative homeostasis consequent to one-carbon metabolism alteration.

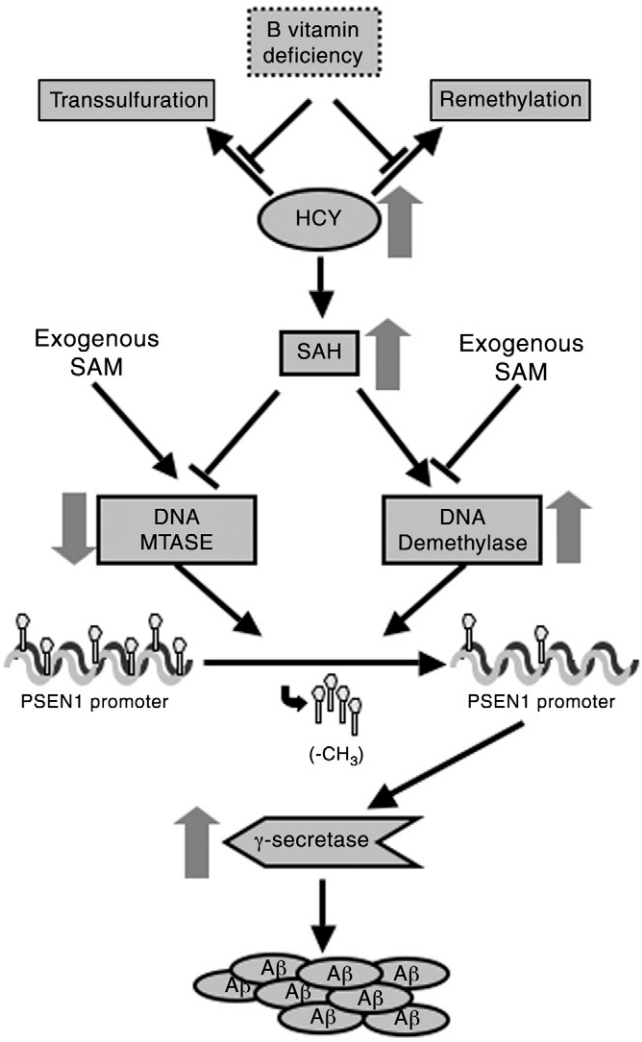

Fig. 5. Schematic representation of the hypothesized pathway connecting one-carbon metabolism to Alzheimer's disease. Black arrows indicate the pattern caused by B vitamin deficiency; gray arrows indicate consequent alterations of metabolite or enzyme activity. MET, methionine; GSH, glutathione; DNA MTASE, DNA methyltransferases; $-CH_3$, methyl groups; Aβ, amyloid plaques.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2010.01.010.

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