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BRIEF COMMUNICATION

S-adenosylmethionine and *S*-adenosylhomocysteine levels in the aging brain of APP/PS1 Alzheimer mice

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Abstract Hyperhomocysteinemia and factors of homocysteine metabolism, *S*-adenosylhomocysteine (AdoHcy) and *S*-adenosylmethionine (AdoMet), may play a role in Alzheimer's disease (AD). With liquid-chromatography-tandem-mass-spectrometry AdoMet and AdoHcy were determined in brains of 8- and 15-month-old APP/PS1 Alzheimer mice, and their possible roles in AD brains investigated. The finding that AdoMet levels do not differ between the genotypes in (young) 8-month-old mice, but are different in (older) 15-month-old APP/PS1 mice compared to their wild-type littermates, suggests that alterations in AdoMet are a consequence of AD pathology rather than a cause. During aging, AdoMet levels decreased in the brains of wild-type mice, whereas AdoHcy levels diminished in both wild type and APP/PS1 mice. The finding that AdoMet levels in APP/PS1 mice are not decreased during aging (in contrast to wild-type mice), is probably related to less demand due to neurodegeneration. No effect of the omega-3 fatty acid docosahexaenoic acid (DHA) or cholesterol-enriched diets on AdoMet or AdoHcy levels were found.

Keywords *S*-adenosylmethionine · *S*-adenosylhomocysteine · Alzheimer's disease · APP/PS1 mice · Cholesterol · DHA

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Introduction

The most pronounced pathological features in the human Alzheimer (AD) brain are amyloid-β (Aβ) depositions, intracellular tangles, and neurodegeneration. Although the cause of the disease is largely unknown, the Aβ protein is seen as one of the major contributors [31].

Aβ is produced by cleavage of the amyloid precursor protein (APP) by the β and γ secretases, BACE, and Presenilin (PS). Missense mutations in either APP or the γ-secretase complex (PS1 and PS2), cause overproduction of Aβ, and early onset AD. Early onset AD represents only 5% of all AD cases and therefore explains only a small part of the cause of the disease. In addition, many studies show that Aβ depositions do not correlate well with neuronal damage and cognitive decline. Therefore, it is suggested that beside Aβ, other risk factors play an important role in the development of AD. Nowadays, more and more consensus is reached about vascular disorders being major risk factors for AD. Hyperhomocysteinemia for example, was until recently believed to be a risk factor for cardiovascular disorders [23] and associated with an increased risk of AD [20, 28]. It could therefore be suggested that hyperhomocysteinemia, cardiovascular disorders, and AD are interrelated with each other. However, this is currently under debate because of recent homocysteine-lowering intervention studies, showing no relation between cardiovascular

disease and hyperhomocysteinemia [4, 19]. In addition, it is unclear whether lowering of homocysteine (Hcy) by folic acid intake, can diminish AD prevalence or improve cognition [5, 21], because recent large randomized Hcy lowering intervention trials did not show beneficial effect of lowering Hcy with folate and vitamin B12 on cognitive function [21]. Among others, we therefore suggest that hyperhomocysteinemia itself is not causing AD, and that other factors of the Hcy metabolism, such as *S*-adenosylhomocysteine (AdoHcy) and *S*-adenosylmethionine (AdoMet), probably play a more important role in vascular disease [17] and AD development [2, 24, 32].

AdoMet is a biological methyl donor and is a product of the conversion of methionine and ATP. Transfer of the methylgroup from AdoMet leads to the formation of AdoHcy which in turn inhibits cellular methylation. Methylation is required in many essential biological processes, such as gene expression, regulation of protein function, and neurotransmitter synthesis, and altered methylation patterns might play a role in AD [2, 24, 32]. Subsequently, AdoHcy can be hydrolyzed to Hcy and adenosine by AdoHcy hydrolase. However, it is important to mention that this hydrolysis is a reversible reaction that favors the synthesis of AdoHcy. In vivo, the reaction proceeds in the direction of hydrolysis only if the products, adenosine and Hcy, are removed rapidly [6, 18, 22].

Indeed, there are studies showing a role for AdoMet and AdoHcy in AD [2, 24, 30]. It is suggested that DNA methylation for example, is involved in APP processing and Aβ production through the regulation of Presenilin1 (PS1) expression. For example, AdoMet administration in human neuroblastoma cells down regulates PS1 gene expression and Aβ production [30]. Other studies from the same group showed that alteration in AdoMet/Hcy cycle (due to reduction of folate and vitamin B12 [10] or deprivation of folate and vitamin B12 and B6 [8, 9]) deregulates β and γ-secretases expression and Aβ production in both neuroblastoma cells and TgCRND8 Alzheimer mice [8–10].

Other studies have shown decreased AdoMet and AdoHcy levels in the cerebrospinal fluid (CSF) of AD patients compared to age-matched healthy volunteers [2, 24].

Until now, most studies determined AdoMet and AdoHcy levels in CSF of AD patients [3]. However, AdoMet and AdoHcy remain mostly intracellular and do not pass membranes easily, and it could therefore be possible that AdoMet and AdoHcy levels in brain tissue comprise a more reliable reflection of AD processes than CSF or serum levels which are a 100-fold lower.

There is only one study describing severely decreased AdoMet levels in brain tissue of AD patients [24]. Although the authors carefully matched with respect to postmortem time the results of this study might be difficult to interpret, since AdoMet levels decrease with more than 60% and AdoHcy levels increase with 80% within 15 h after death [25]. In addition, it is still unknown whether alterations in AdoMet and AdoHcy levels are cause or consequence of AD pathology.

In order to further investigate the role of AdoMet and AdoHcy in AD development we determined AdoMet and AdoHcy levels in brain tissue of 8- and 15-month-old wild type and APP/PS1 double transgenic Alzheimer mice. These two ages were chosen because in young (8-month-old) mice no neurodegeneration, almost no cognitive impairment, and very low levels of Aβ are present, whereas in old APP/PS1 mice (15-month-old) neurodegeneration in the hippocampus becomes clear, and cognitive impairment and Aβ deposition are quite severe [16].

In addition, we investigated the effect of aging and the effects of cholesterol- and the omega-3 fatty acid docosahexanoic acid (DHA) containing diets on AdoMet and AdoHcy levels. These nutritional components influence vascular health [11, 13, 14, 26] and the risk of developing AD [7, 15, 29]. In our previous studies we showed that long-term dietary interventions with cholesterol and or DHA also changed AD-like pathology and vascular factors in 8-and 15-month-old APP/PS1 mice. AdoMet and AdoHcy might also influence AD like pathology and vascular health [16] and it could be hypothesized that the above-mentioned nutritional components may influence and AdoMet and AdoHcy levels as well.

Materials and methods

Animals and diets

The APPswe/PS1dE9 founders were obtained from Johns Hopkins University, Baltimore, MD, USA (D. Borchelt and J. Jankowsky, Dept. of Pathology), and a breeding colony was established at the Radboud University Nijmegen Medical Centre, The Netherlands. Male APP/PS1 transgenic mice and their wild-type littermates were randomly assigned to different diet groups. The number of animals per group was decided on the basis of availability. The experimental diets differed with respect to the composition of the 5% fat in the diets [15] but are all isocaloric. Feeding the diets started at 2 months of age.

Transgenic mice and their wild-type littermates were fed either a Typical Western Diet (TWD), containing 1% cholesterol, a high percentage of saturated fatty acids (SFA = 53%), and long chain poly unsaturated fatty acid (LCPUFA) ratio of $n6/n3 = 22.5$, or a DHA diet containing 0.4% DHA, a low percentage SFA (20%), and a LCPUFA ratio of $n6/n3 = 2.5$, or a standard control diet (STD), with 38% SFA and LCPUFA ratio of $n6/n3 = 7.5$.

In total 82 mice were used; Table 1 describes the number of mice used in each experiment. The mice were selected upon availability from another existing study performed in our own lab (submitted). Throughout the experiments the animals were housed individually in a controlled environment (the mice were housed in the central animal facility with the temperature controlled at 21°C, and an artificial 12:12 h light:dark cycle (lights on at 07.00 am)), with some cage enrichment, consisting of an Iglo and some nesting material. Food and water were available *ad libitum.* The experiments were performed according to Dutch regulations of the Animal Experimentation Act and the EC Directive 86/609 and were ethically approved by the Ethical Review Committee of the Radboud University Nijmegen Medical Centre.

Brain sample collection and storage

Directly following anesthesia with Nembutal (60 mg/kg, i.p.) (Ceva Santa Animals BV, Maassluis), all mice were weighed and thereafter decapitated and the brains were removed from the skull. The entire brain, without the spinal cord was thereafter dissected into three smaller pieces, of which the frontal part of the brain was used for this experiment. All pieces were snap-frozen in liquid nitrogen and were kept frozen at −80°C until further processing.

A 40-mg tissue piece, containing the frontal part of the brain was prepared by sonification in 800 µl cold PBS, and thereafter centrifuged at 4°C at 14,000 rpm for 5 min. The supernatant was removed and diluted eight times. One part was used for HPLC tandem MS measurements, and another part for determination of the protein amount in the sample using the Lowry method [27].

S-adenosylmethionine (AdoMet) and *S*-adenosylhomocysteine (AdoHcy) measurements

As previously described by Gellekink et al. [12], AdoMet and AdoHcy levels in brain tissue were measured using liquid chromatography tandem mass spectrometry (LC-MS/MS).

In short, after addition of the internal standards AdoMet-d_3 and AdoHcy-d_5 to the pretreated tissue sample (see above), solid phase extraction (SPE) columns containing phenyl boronic acid (PBA) were used to bind AdoMet and AdoHcy and their internal standards and to deproteinize the samples. AdoMet and AdoHcy were eluted in 0.1 N formic acid and measured with the LC tandem mass spectrometer (MS/MS) (Quatro LC from Micromass), in the positive-ion mode.

Table 1 Number of male mice used in each diet group

Groups	8-month-old (*n*)	15-month-old (*n*)
STD (wt)	5	10
STD (tg)	6	8
TWD (wt)	6	9
TWD (tg)	6	8
DHA (wt)	5	9
DHA (tg)	7	3

STD standard diet, *TWD* Typical Western Diet, containing 1% cholesterol, *DHA* docosahexanoic acid diet, containing 0.4% of DHA. *Wt* wild-type mice, *Tg* transgenic APP/PS1 mice

Statistics

Linear regression analysis was used to verify the linearity of the calibration curves. In order to analyze the possible differences between the two genotypes and the different diet groups a Multivariate ANOVA was conducted with the between group factors diet and genotype. In order to investigate effects of aging in the two different genotypes a Multivariate ANOVA was used with the between group factors age and genotype.

Results

No changes in bodyweight were found between the genotypes, the two different age groups or diet groups.

Genotype effects

No changes in AdoMet and AdoHcy levels, and consequently in the ratio between AdoMet and AdoHcy levels (methylation index), were observed between 8-month-old wild type and APP/PS1 mice (Fig. 1a–c). In addition, also in 15-month-old mice no changes were observed in AdoHcy levels between the genotypes (Fig. 1b). However, we did observe a significant decrease in AdoMet levels in 15-month-old wild-type mice compared to APP/PS1 mice (Fig. 1a; $F(1,45) = 7.54$, $p < 0.01$). Consequently, the AdoMet/AdoHcy ratio is increased in the APP/PS1 mice (Fig. 1c; $((1,45) = 7.17$, $p < 0.01$).

Aging effects

AdoHcy levels significantly decrease during aging in both wild-type and APP/PS1 mice ($F(1,79) = 9.50$, $p < 0.01$). Due to an interaction between genotype and age in AdoMet levels and the ratio between AdoMet and AdoHcy levels (methylation index), the effects of aging in wild type and APP/PS1 mice were determined separately for these parameters.

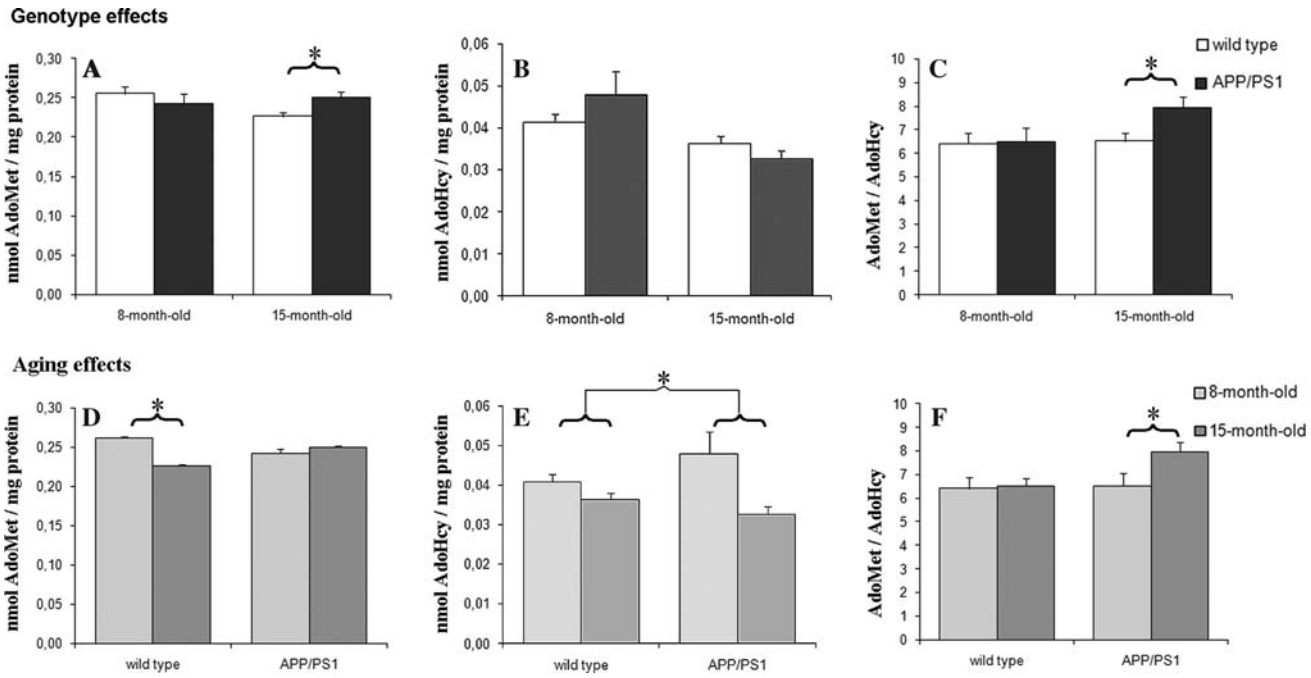

Fig. 1 Levels of *S*-adensylmethionine (AdoMet; **a**, **d**) and *S*-adenosylhomocysteine (AdoHcy; **b**, **e**) and the methylation index (AdoMet/AdoHcy; **c**, **f**) in brain tissue of 8- and 15-month-old APP/PS1 mice and their wild-type littermates. 8-month-old wild-type mice $n = 16$, 8-month-old APP/PS1 mice $n = 19$ 15-month-old wild-type mice $n = 28$, 15-month-old APP/PS1 mice $n = 19$. *Values* represent mean and SEM. * $p < 0.01$

A significant decrease in AdoMet levels during aging was only found in wild-type mice ($F(1,42) = 8.09$, $p < 0.01$) and not in APP/PS1 mice.

Thus, AdoHcy decreased in both wild type and APP/PS1 mice during aging, whereas AdoMet only decreased in wild-type mice. Consequently, the AdoMet/AdoHcy ratio (methylation index) in wild-type mice did not alter due to aging, but was significantly increased in APP/PS1 mice with aging (Fig. 1f; $F(1,36) = 6.61$, $p = 0.01$).

Diet effects

Neither a cholesterol- nor a DHA-containing diet did alter AdoMet or AdoHcy levels in brain tissue of 8- and 15-month-old APP/PS1 and wild-type mice as compared to the standard diet (data not shown).

Discussion

In this study we showed that AdoMet tissue levels did not differ between 8-month-old APP/PS1 mice and wild-type littermates, but are decreased in 15-month-old wild-type mice compared to APP/PS1 mice. Moreover, tissue levels of AdoHcy decreased in both wild type and APP/PS1 mice during aging, whereas AdoMet only decreases in wild-type mice. Because AdoMet and AdoHcy brain levels in (young) 8-month-old mice are unchanged, in contrast to the (old) 15-month-old mice, these results indicate that alterations in AdoMet may be a consequence of AD-like pathology in the APP/PS1 mice rather than a cause. In addition, we showed that cholesterol-containing, or DHA-enriched diets did not affect AdoMet or AdoHcy levels in brain tissue of APP/PS1 and wild-type mice.

This is the first study describing AdoMet and AdoHcy levels in brain tissue of aging mice. Stramentinoli et al. found decreased AdoMet levels in brain tissue of aging rats [33]. In addition they measured the synthesis of AdoMet by adenosyl transferase and AdoMet utilization by COMT, and concluded that the decrease in AdoMet levels was a consequence of increased utilization during aging. The decrease in AdoMet and AdoHcy found in our wild-type mice could also be the consequence of increased utilization. It could be suggested that AdoMet levels in the brain tissue of the APP/PS1 mice, used in this study, do not decrease during aging because of less demand caused by neurodegeneration. This seems to be confirmed by the hippocampal atrophy and increased Aβ deposition we observed in the hippocampus and frontal cortex of the 15-month-old APP/PS1 mice compared to 8-month-old APP/PS1 mice [16]. If the above-mentioned hypothesis is valid, and AdoMet remains unaltered in aged APP/PS1 mice compared to young APP/PS1 mice, because of less demand for methylation due to neurodegeneration, AdoHcy levels

will also be expected to be decreased or unchanged. Indeed, AdoHcy levels are similar in the 15-month-old wild-type mice and APP/PS1 mice. In Fig. 2, the above-mentioned findings and hypothesis are schematically depicted.

Although we have hypothesized that AdoMet levels remain high because of less demand due to neurodegeneration, we do not have proof for that yet. Because of the complexity of factors that are involved in AdoMet metabolism in AD, other factors in the methylation cycle or for example oxidative stress, could be involved and should be investigated before we can validate our statement. Bernardo shows [1] that 16–18-month-old Tg2576 AD mice have higher Hcy levels than wild-type mice. This could have explained the high-AdoMet levels; however, the AdoMet levels in the 8-month-old APP/PS1 mice in our study, should have been increased compared to 8-month-old wild types, and that was not the case. In addition, high Hcy levels are known to inhibit AdoHcy hydrolase, which results in increase in AdoHcy levels [32], and therefore higher AdoHcy levels would have been expected in our APP/PS1 mice compared to wild types which was also not the case.

The finding that AdoMet levels significantly differ only in 15-month-old APP/PS1 mice compared to their wild-type littermates, but not in 8-month-old mice, also indicates that alterations in AdoMet levels are a consequence of AD pathology rather than a cause. The difference in AdoMet levels between wild type and AD mice is also found by Fuso et al. [9]. They show a concentration of 158 nmol/g AdoMet in TgCRND8 mice compared to 52 nmol/g AdoMet in their control mice. It could be argued that these mice are much younger and should not be compared with our mice; however, the 3-month-old TgCRND8 animals show early and age-related Aβ deposition and cognitive impairment what is comparable to our 15-month-old APP/PS1 mice. In contrast, our 8-month-old APP/PS1 mice show almost no Aβ plaque deposition and very mild cognitive impairment.

The finding that cholesterol- or DHA-containing diets do not alter AdoMet or AdoHcy levels in brain tissue of either wild type or APP/PS1 mice, but in contrast do have effects on AD pathology and cerebral hemodynamics [7, 15, 29], suggests that cholesterol- or DHA-containing diets do not influence cerebral hemodynamics or AD pathology via the methylation cycle. Thus, AdoMet and AdoHcy are no important key players in the mechanisms explaining the effects of cholesterol and DHA in AD pathology.

Main conclusions

We measured AdoMet and AdoHcy levels in brain tissue of 8- and 15-month-old wild type and APP/PS1 mice, to investigate whether there is a role for these metabolites of methylation in the AD brain. The finding that AdoMet levels do not differ between the genotypes in (young) 8-month-old mice, but do differ in (older) 15-month-old APP/PS1 mice compared to their wild type littermates,

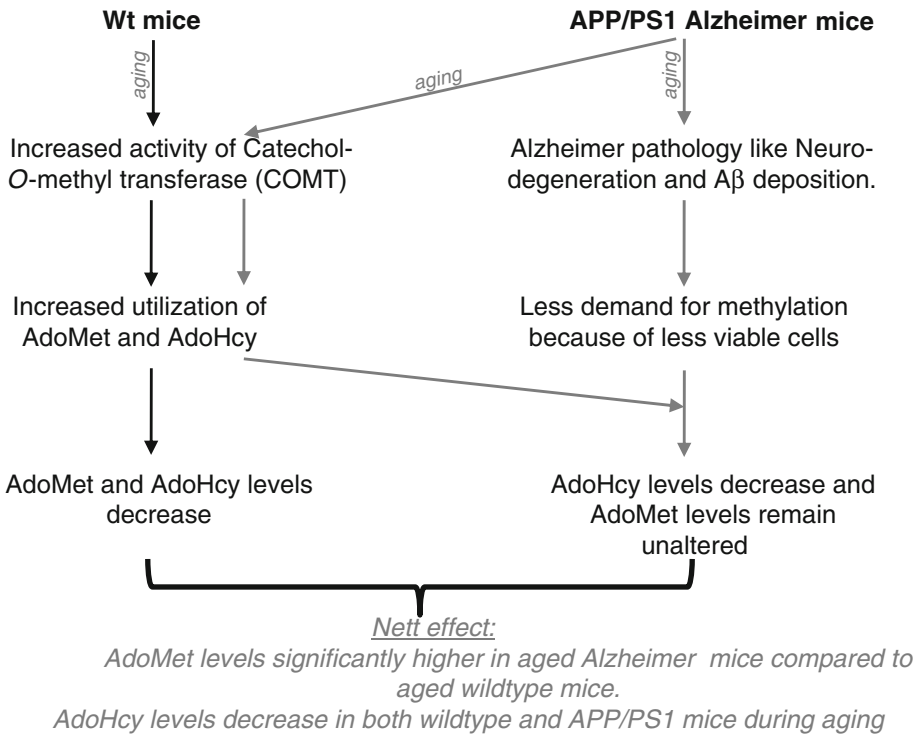

Fig. 2 Concept model of the hypothetical effects of aging on *S*-adensylmethionine (AdoMet) and *S*-adenosylhomocysteine (AdoHcy) levels in wild type and APP/PS1 mice

suggests that alterations in AdoMet are a consequence of AD pathology rather than a cause, and it could be suggested that it is the result of neurodegeneration during aging (Fig. 2). However, because of a lack of brain tissue we only measured AdoMet and AdoHcy levels in this experiment. It would, however, be of great value to measure more components of the methylation cycle in future studies to validate our conclusions.

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