Altered Levels of S-Adenosylmethionine and S-Adenosylhomocysteine in the Brains of \( L - \text{Aspartyl} \) O-Methyltransferase-deficient Mice*

Christine Farrar and Steven Clarke‡

From the Department of Chemistry and Biochemistry and the Molecular Biology Institute, UCLA, Los Angeles, California 90095-1569

\( L - \text{Aspartyl} \) (d-aspartyl) \( O \)-methyltransferase (PCMT1) is a protein repair enzyme that initiates the conversion of abnormal \( \text{d-aspartyl} \) and \( \text{l-aspartyl} \) residues to the normal \( \text{l-aspartyl} \) form. In the course of this reaction, PCMT1 converts the methyl donor S-adenosylmethionine (AdoMet) to S-adenosylhomocysteine (AdoHcy). Due to the high level of activity of this enzyme, particularly in the brain, it seemed of interest to investigate whether the lack of PCMT1 activity might alter the concentrations of these small molecules. AdoMet and AdoHcy were measured in mice lacking PCMT1 \( \text{Pcmt1}^{-/-} \), as well as in their heterozygous \( \text{Pcmt1}^{+/-} \) and wild type \( \text{Pcmt1}^{+/+} \) littersmates. Higher levels of AdoMet and lower levels of AdoHcy were found in the brains of \( \text{Pcmt1}^{-/-} \) mice, and to a lesser extent in \( \text{Pcmt1}^{+/-} \) mice, when compared with \( \text{Pcmt1}^{+/+} \) mice. In addition, these levels appear to be most significantly altered in the hippocampus of the \( \text{Pcmt1}^{-/-} \) mice. The changes in the AdoMet/AdoHcy ratio could not be attributed to increases in the activities of methionine adenosyltransferase II or S-adenosylhomocysteine hydrolase in the brain tissue of these mice. Because changes in the AdoMet/AdoHcy ratio could potentially alter the overall excitatory state of the brain, this effect may play a role in the progressive epilepsy seen in the \( \text{Pcmt1}^{-/-} \) mice.

One of the most common forms of protein damage in vitro and in vivo is deamination and isopropyl form conversion caused by deprotonation of the peptide-bond nitrogen and its attack on the side chain carbonyl carbon of \( \text{l-asparaginyl} \) or \( \text{l-aspartyl} \) residues (1, 2). This spontaneous reaction results in the loss of ammonia (from asparaginyl residues) or water (from aspartyl residues) and the formation of the side chain into an intermediate succinimidyl ring. If this ring is hydrolyzed at the \( \text{d-carboxyl} \) group, the peptide bond is re-routed through the side chain carbonyl resulting in the formation of an \( \text{l-aspartyl} \) residue. The ring is also susceptible to racemization, allowing the formation of \( \text{d-aspartyl} \) and \( \text{l-aspartyl} \) residues, as well. These aberrant residues can cause drastic conformational changes in their proteins, resulting in loss of biological activity (1), increased propensity to aggregate (2), and/or increased immunogenicity (3). Recent studies have shown that isopropyl formation in specific peptides or proteins may be linked to certain disease processes. For example, the presence of isopropyl residues in the \( \beta \)-amyloid peptide contributes to its insolubility, and protein isomerization is elevated in \( \beta \)-amyloid peptides and paired helical filaments purified from Alzheimer's disease brains (4–6). However, just as cells have mechanisms to combat other forms of damage, they have at least one to restrict the accumulation of isopropyl residues. This mechanism involves the enzyme \( \text{l-isopropyl (d-aspartyl} \) O-methyltransferase (PCMT1).1

PCMT1 functions by transferring a methyl group from the molecule S-adenosylmethionine (AdoMet) to either the \( \alpha \)-carboxyl group of the \( \text{l-isopropyl} \) residue or the \( \beta \)-carboxyl group of the \( \text{d-aspartyl} \) residue (7–9). The resulting methyl ester is then susceptible to spontaneous hydrolysis leading to the loss of methanol and the re-formation of the succinimidyl ring. If the intermediate ring structure then hydrolyzes at the \( \beta \)-carboxyl, which typically occurs about 30% of the time at physiological pH, the residue returns to the normal \( \text{l-aspartyl} \) form (10, 11). However, 70% of the time, the cyclic imide returns to one of the aberrant forms, which can be re-methylated by PCMT1 until the peptide or protein in which it resides is no longer a substrate for the enzyme. Therefore, one side effect of this repair mechanism may be a potentially large consumption of the methyl donor, AdoMet.

When AdoMet donates its methyl group it becomes S-adenosylhomocysteine (AdoHcy). The concentration of AdoHcy compared with that of AdoHcy is often used as an index for the activity of the AdoMet-dependent methyl transfer system in living organisms (12–14). For example, if the AdoMet/AdoHcy ratio were lowered, it might indicate greater AdoMet “consumption” through increased methyltransferase activity. In rat studies, the administration of 3-(3,4-dihydroxyphenyl)-l-alanine (l-dopa) results in a significant increase in the activity of catecholamine-O-methyltransferase causing a reduction in the brain concentration of AdoMet, with a concomitant elevation in the level of AdoHcy, thereby lowering the AdoMet/AdoHcy ratio (15). However, if 3-(3,4-dihydroxyphenyl)-l-alanine continues to be administered, the level of AdoMet returns to normal due to the feedback regulation of methionine adenosyltransferase II (MAT II), the enzyme that produces AdoMet in the brain (16). Another enzyme that is important in maintaining a proper AdoMet/AdoHcy ratio is S-adenosylhomocysteine hydrolase.

* This work was supported by National Institutes of Health Grants AG15451, AG18000, and GM26020. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: 640 Paul D. Boyer Hall, 611 Charles E. Young Drive East, Los Angeles, CA 90095-1570. Tel.: 310-825-8754; Fax: 310-825-1968; E-mail: clarke@mbi.ucla.edu.

† The abbreviations used are: PCMT1, L-isopropyl (d-aspartyl) O-methyltransferase; AdoMet, S-adenosyl-l-methionine; AdoHcy, S-adenosyl-l-homocysteine; MTa, 5'-deoxy-5'-[methylthio]adenosine; MAT II, methionine adenosyltransferase II; SAHH, S-adenosyl-l-homocysteine hydrolase; BisTris, 2,2-bis(hydroxymethyl)-2,2',2''-nitrilotriethanol; HPLC, high performance liquid chromatography.

† The abbreviations used are: PCMT1, L-isopropyl (d-aspartyl) O-methyltransferase; AdoMet, S-adenosyl-l-methionine; AdoHcy, S-adenosyl-l-homocysteine; MTa, 5'-deoxy-5'-[methylthio]adenosine; MAT II, methionine adenosyltransferase II; SAHH, S-adenosyl-l-homocysteine hydrolase; BisTris, 2,2-bis(hydroxymethyl)-2,2',2''-nitrilotriethanol; HPLC, high performance liquid chromatography.
Altered AdoMet Metabolism in Brains of PCMT1-deficient Mice

(SAHII). AdoHcy must be kept from accumulating because many methyltransferases have a higher affinity for AdoHcy than AdoMet, making AdoHcy a potent inhibitor of many methylation reactions (17–20, 55). Although the levels of AdoMet and AdoHcy are normally well regulated, there are conditions under which the levels of these small molecules can be altered for prolonged periods, and they are often accompanied by severe physiological complications. For example, deficiencies in folic acid or vitamin B12 can cause a considerable decrease in the AdoMet/AdoHcy ratio (21). These deficiencies lead to severe neurological disorders, such as demyelination of the spinal cord characterized by vacuolar myelopathic lesions. It has been suggested that the demyelination is caused by the defect in methylation (21). This hypothesis is supported by animal studies showing that similar lesions are produced in mice treated with cycloleucine, an inhibitor of methionine adenosyltransferase (21). In the case of deficiencies in glycine N-methyltransferase, a liver enzyme that is believed to serve as an alternate pathway for the conversion of AdoMet to AdoHcy, there is a significant increase in plasma AdoMet/AdoHcy (22). In humans, this condition has been associated with enlargement of the liver and elevation of serum liver transaminases (22). Because PCMT1 may have the capacity to use relatively large amounts of AdoMet (as suggested above), it seemed of interest to investigate whether the lack of this methyltransferase results in alterations in the AdoMet and AdoHcy levels in the recently developed PCMT1-deficient mouse.

Mice lacking PCMT1 (Pcmt1–/– mice) have been generated independently by two groups in the last 5 years and exhibit the same major phenotype in each case (23, 24). This phenotype is the development of progressive epilepsy that leads to an early death at an average of 6 weeks of age. It has been proposed that the severe neurological manifestations of these mice are due to the accumulation of one or more isoaspartyl-damaged proteins (such as tubulin, calmodulin, or synapsin) (23–26). Another theory proposes that the metabolism of a small molecule or neurotransmitter such as N-acetylaspartylglutamate may be affected by the lack of PCMT1 in these mice (27). Although both theories are plausible, in this study we explore yet another possibility. Specifically, we ask if the deficiency of PCMT1 causes a change in the overall AdoMet methylation flux and whether this may be contributing to the seizure phenotype seen in the Pcmt1–/– mouse.

EXPERIMENTAL PROCEDURES

Preparation of Brain Homogenates for Enzyme Assays—Mice were decapitated, and their tissues were immediately submersed in liquid nitrogen and stored at −80 °C until analysis was performed. Frozen tissue (either brain or testes) was extracted, weighed, and homogenized in 0.4 M HClO4 (2 ml/g wet weight) at 4 °C for 10 min at 4°C. After base hydrolysis, 14C-methionine production was measured as described above to quantify isoaspartyl and n-aspartyl methyl-accepting sites in cellular proteins. Incubations containing only S-adenosyl-l-[methyl-14C]methionine, tissue homogenate, and buffer constituted the blank for each sample; the radioactivity in these tubes was subtracted from total counts in the determination of enzyme activity.

Measurement of Damaged Aspartyl Residues in Brain Homogenates—Tissue homogenates (5 μl each), the same as those used for enzyme activity assays, were incubated with 1 μg of recombinant human t-isoaspartyl (n-aspartyl) O-methyltransferase (specific activity, 20,000 pmol of methyl esters/min/mg of protein), made by a modification of a method described previously (28). The mixture was centrifuged at 20,800  ×  g for 10 min at 4°C. The supernatant extract was stored at −110°C.

Measurement of PCMT1 Activity in Brain Homogenates—The supernatant fractions from homogenized tissue (described above) were thawed, and 5 μl (between 50 μg of protein) of each was incubated with 0.6 mg of ovalbumin (Sigma, grade V) in 0.2 M BisTris-HCl, pH 6.8, containing 10 μM S-adenosyl-l-[methyl-14C]methionine (53 mCi/mmol; Amersham Biosciences) in a 30-μl volume at 37 °C for 15 min. Sodium hydroxide (70 μl of a 0.2 M solution) was added to stop the reactions and to neutralize the 14C-methionine formed. The tissue extract was thawed to 1°C and was incubated with 0.05 ml of 14C-methanol. The reaction mixture was immediately spotted onto an accordi-folded 8 × 2-cm piece of filter paper and incubated above 5 ml of Safety-Solve scintillation fluid (Research Products International) in the neck of a sealed 20-ml scintillation vial at room temperature for 2 h to allow 14C-methanol to diffuse into the scintillation fluid. The filter paper was then removed, and the radioactivity in the scintillation fluid was counted. Enzyme activity was determined as a function of [14C]methanol production. Incubations containing only S-adenosyl-l-[methyl-14C]methionine, tissue homogenate, and buffer constituted the blank for each sample; the radioactivity in these tubes was subtracted from total counts in the determination of enzyme activity.

Measurement of AdoMet and AdoHcy Levels in Deproteinized Homogenates—Deproteinized supernatant fractions were neutralized with KOH. The precipitated KClO4 was removed from the samples by centrifugation at 10,000  ×  g for 20 min at 4°C. The final supernatant fractions were stored at −80 °C until analysis by high performance liquid chromatography (HPLC). The HPLC system included two Waters model 510 pumps, a model 680 automatic gradient controller, a model 411 UV absorbance detector, and a model U6K sample injector. The samples were thawed, and 200 μl were loaded onto an Econosphere C18 column (particle size 5 μm, 25 × 0.46 cm; Alltech) equilibrated with mobile phase A (50 mM sodium phosphate, 10 mM sodium heptane sulfonic acid, 4% acetonitrile, final pH 3.2). Mobile phase B consisted of 100% acetonitrile. The HPLC system was operated at 100°C (column temperature was monitored with a thermistor in the column heater). A Waters model 248B UV detector was used at 254 nm. The mobile phase was delivered at a flow rate of 0.6 ml/min, and the UV absorbance was monitored at 254 nm. Concentrations were calculated by converting the peak area to moles based on a molar extinction coefficient for AdoMet and AdoHcy of 15,400 at 254 nm. Calculations were verified using AdoMet and AdoHcy standards of known concentrations.

Measurement of AdoMet and AdoHcy Levels in Deproteinized Homogenates of Dissected Brains—Mice were decapitated, and the brain tissue was immediately chilled, extracted, and weighed. Dissection of the brain was performed on ice and completed within 5 min. The brains were dissected into five sections as follows: brain stem (includingpons and midbrain), cerebellum, hippocampus, thalamus (including thalamus), and cortex (including basal ganglia). Each section was weighed and homogenized at 4°C in 0.4 M HClO4. The homogenates were then centrifuged, neutralized, and analyzed by HPLC as described above.

Measurement of MAT II and SAHH Activity in Brain Homogenates—MAT II activity was assayed by measuring the formation of AdoMet from ATP and l-methionine, modified from the method described previously by Oden and Clarke (29). Briefly, 20 μl of homogenized brain

2 C. E. Farrar and S. G. Clarke, unpublished data.
Altered AdoMet Metabolism in Brains of PCMT1-deficient Mice

The survival of Pcmt1−/− mice is severely affected between the ages of 20 and 50 days. Between the ages of 20 and 50 days, the survival of the Pcmt1−/− mice drops from 98 to 23% (Fig. 1A). Because this time period appears to be the most critical in the development of fatal seizures in the Pcmt1−/− mice, the following studies focus on the analysis of brain tissue from mice that are between these ages. The activity of PCMT1 in the brains of Pcmt1+/+ and Pcmt1+/− mice increases slightly during this time period, as does their level of damaged aspartyl residues (Fig. 1B and C). Due to the lack of the enzyme, the Pcmt1−/− mice have ∼20 times more damaged aspartyl residues in their brains than the Pcmt1+/+ and Pcmt1+/− mice. Although the level of isoaspartyl damage is high in Pcmt1−/− mice, the rate of damaged aspartyl residue accumulation actually decreases slightly after 30 days of age (Fig. 1C). Therefore, although it is possible that isoaspartyl-damaged substrates may be responsible for the limited survival seen in the Pcmt1−/− mice, there is no evidence of a drastic increase in the accumulation of total substrate levels between the critical ages of 20 and 50 days.

Levels of AdoMet and AdoHcy are altered in Pcmt1−/− and Pcmt1+/+ mice—Levels of AdoMet and AdoHcy in tissue samples were determined by ion pairing reverse phase HPLC. To confirm the identification and purity of the chromatogram peak specified as “AdoMet” in homogenized brain sample, the breakdown of AdoMet to MTA was examined (51). After boiling the brain sample for 10 min at pH 3, the peak that co-eluted with an AdoMet standard was eliminated, while the peak that co-eluted with an MTA standard was increased by an equal magnitude (Fig. 2A). The specified “AdoMet” peak was confirmed by monitoring its breakdown by SAHH. This was accomplished by incubating homogenized brain sample with an excess of SAHH and adenosine deaminase at 37 °C for 60 min and comparing its HPLC profile with that of a control sample lacking excess SAHH and adenosine deaminase (Fig. 2B).

The levels of AdoMet and AdoHcy were measured in homogenized brain tissue from mice at the ages of 20, 30, 40, and 50 days. Between 20 and 50 days there is an overall decrease in the level of AdoMet in the brains of Pcmt1+/+/ mice (p < 0.001 when comparing 20 and 30 day values to 40 and 50 day values) with no significant change in their AdoHcy levels (Fig. 3A and B). On the other hand, Pcmt1−/− mice demonstrate an overall decrease in their AdoHcy levels (p < 0.0001 when comparing 20- and 30-day values to 40- and 50-day values) with no significant change in their AdoMet levels (Fig. 3A and B). The levels of AdoMet and AdoHcy in Pcmt1−/− and Pcmt1+/+ mice were also compared with those of their Pcmt1+/+ littersmates at each individual age. At 20 days of age, the Pcmt1−/− mice have significantly higher levels of AdoMet in their brains than the Pcmt1+/+ mice of the same age (Fig. 3A). This trend continues and becomes even more pronounced by 50 days of age. The Pcmt1+/+ mice appear to have slightly higher values of AdoMet in their brains than the Pcmt1+/+ mice in each age group; however, the values are not significantly different from the Pcmt1+/+ values until 50 days of age.

The AdoHcy values in the 20-day-old mice are not significantly altered between Pcmt1−/−, Pcmt1+/−, and Pcmt1+/+ mice (Fig. 3B). However, by 30 days of age, the AdoHcy values of Pcmt1−/− mice do appear slightly lower than Pcmt1+/+ and Pcmt1+/− values, and by 40 days the decrease becomes quite significant. Although the Pcmt1+/− mice appear to have slightly lower levels of AdoHcy at 50 days of age compared with
the Pcmt1+/+ mice, the difference is not statistically significant. The level of AdoHcy for Pcmt1+/− mice is very similar to the levels of AdoHcy for Pcmt1+/+ mice in all the other age groups.

Because the AdoMet/AdoHcy ratio is often used as an indicator for the activity of the AdoMet-dependent methyl transfer system (12–14), this ratio was calculated for each brain sample. In Pcmt1+/+ mice, the ratio was found to decrease slightly from 20 to 30 days and then remain relatively constant from 30 to 50 days (Fig. 3C). The Pcmt1+/− mice follow the same trend in their AdoMet/AdoHcy ratios as the Pcmt1+/+ mice until 50 days of age when the ratio is slightly but significantly raised ($p < 0.05$). However, for the Pcmt1−/− mice, there is a dramatic increase in the AdoMet/AdoHcy ratios compared with those of the Pcmt1+/+ mice. Although the values at 20 days are similar, by 30 days the ratio for Pcmt1−/− compared with Pcmt1+/+ mice is about 1.6-fold greater, and by 40 days the Pcmt1−/− ratio is about 2.3-fold greater than the Pcmt1+/+ ratio. By 50 days the ratio has not changed much from the 40-day value. It is possible that by 50 days, we may be selecting for Pcmt1−/− mice that have less of an elevation in their AdoMet/AdoHcy ratios, if this value is any indication of the survivability of these mice.

The levels of PCMT1 in adult Pcmt1+/+ mice are much...
higher in the brain than in any other tissue except for the testes which seem to have levels comparable with those in the brain (24, 32). Therefore, if the altered levels of AdoMet and AdoHcy are caused by the lack of PCMT1, one might expect to see a difference in the testes of the Pcmt1+/− mice similar to that seen in their brains. After measuring the levels of AdoMet and AdoHcy in the testes of mice between 20 and 50 days of age, we found a slight but significant increase in testicular AdoMet levels of 40- and 50-day-old Pcmt1+/− mice compared with Pcmt1+/+ mice (p < 0.1 and 0.05, respectively); however, there did not appear to be a significant difference in the AdoMet/AdoHcy ratios for any of the ages tested (data not shown).2

Levels of MAT II Activity in Brain Homogenates Are Lower in Pcmt1+/− and Pcmt1+/+ Mice—An increase in the AdoMet/AdoHcy ratio in the brain might result from the decreased consumption of AdoMet caused by lowered AdoMet-dependent methyltransferase activity. However, it might also result from higher levels of AdoMet production and AdoHcy metabolism. To determine whether the latter was occurring in the Pcmt1+/− mice, the activity of the enzymes that catalyze AdoMet production and AdoHcy metabolism in the brain, namely MAT II and SAHH, was measured at the different ages in the Pcmt1+/+, Pcmt1+/−, and Pcmt1−/− mice.

At 20 days of the age, the Pcmt1−/− mice appear to have slightly but significantly lower levels of MAT II activity than the Pcmt1+/+ mice of the same age (Fig. 4A). At 30 and 40 days of age there does not appear to be a significant change in activity levels for any of the genotypes. However, at 50 days of age, not only do the Pcmt1−/− mice have lower MAT II activity but the Pcmt1+/− mice have lower levels as well. From these results, it can be presumed that the higher AdoMet levels in the brains of the Pcmt1−/− and Pcmt1+/− mice are not caused by increased production of AdoMet but more likely by its lowered consumption. In fact, the lowered activity of MAT II may result from the higher concentrations of AdoMet in the Pcmt1−/− and Pcmt1+/− mice, as MAT II is feedback inhibitory to physiological concentrations of AdoMet (33, 34).

Levels of SAHH Activity Are Slightly Lower in Pcmt1−/− Mice—As mentioned previously, the relative concentration of AdoHcy is very important in the regulation of methylation reactions, as many methyltransferases that use AdoMet are extremely sensitive to competitive inhibition by AdoHcy (17–20, 55). To sustain proper methylation levels in most cells, it is necessary to remove AdoHcy by the action of SAHH. Although the action of SAHH is reversible, it is believed that in vivo and under normal conditions the reaction is favored in the direction of AdoHcy hydrolysis by the rapid removal of adenosine and homocysteine (17). Therefore, one reason for lowered AdoHcy levels, like those observed in the Pcmt1−/− mice, would be an increase in SAHH activity. However, upon measuring the activity levels of SAHH in the Pcmt1−/− mice, it appears that, if
 anything, SAHH activity is actually decreased. The Pcmt1−/− mice have slightly lower levels of SAHH activity at 20, 30, and 40 days of age compared with those of the Pcmt1+/+ mice (Fig. 4B). However, by 50 days, there does not appear to be a significant difference between any of the three genotypes. In any case, it does not appear that there is increased metabolism of AdoHcy by SAHH in the Pcmt1−/− mice between 20 and 50 days of age.

The AdoMet/AdoHcy Ratio Is Most Significantly Altered in the Hippocampus of Pcmt1−/− Mice—Although PCMT1 is widely distributed throughout the brain, it is apparent from immunolocalization data that it is more highly localized in certain brain regions and less in others. For example, in rat brain PCMT1 appears to be most highly expressed in neurons of the hippocampus, cortex, and basal ganglia, followed by those in the cerebellum, brain stem, and hypothalamus (35–37). We find a similar distribution of PCMT1 expression in mouse brain.3 If the altered levels of AdoMet and AdoHcy resulted from the absence of PCMT1, one might expect to find the regions with the greatest alterations of these molecules in the Pcmt1−/− mice are the same as those with the highest levels of PCMT1 expression in the Pcmt1+/+ mice. After measuring the AdoMet and AdoHcy levels in the different brain regions of 50-day-old mice, it was determined that Pcmt1−/− mice have higher levels of AdoMet in all the regions compared with those of the Pcmt1+/+ mice (Fig. 5A). Also, the Pcmt1−/− mice have lower levels of AdoHcy in every brain region except the thalamus (Fig. 5B). Finally, in comparing the AdoMet/AdoHcy ratios between the Pcmt1−/− and Pcmt1+/+ mice, the most significant difference appears to occur in the hippocampus (Fig. 5C). The ratios in the cortex, cerebellum, and brainstem are also significantly higher; however, the ratio in the thalamus is relatively unchanged.

PCMT1 activity was also measured in dissected and homogenized Pcmt1+/+ brain tissue using the same procedure described for whole brain homogenates (data not shown). These data did not demonstrate the variability in the regional distribution of PCMT1 that is readily seen by immunolocalization data. The reason for this inconsistency is believed to be a result of the loss of PCMT1 following homogenization and centrifugation of the brain tissue as some of the protein has been shown to associate with the membrane fraction (38–41). In addition, it has yet to be determined whether this association is variable between the different brain regions. Therefore, the comparison of the AdoMet/AdoHcy ratios to PCMT1 levels in the various regions of the brain was made with intact brain tissue as opposed to dissected and homogenized tissue.

DISCUSSION

In rat brain, there is a significant decrease in the AdoMet/AdoHcy ratio between 1 and 4 weeks of age with a more gradual decrease during maturation (13). This trend is consistent with our results from Pcmt1+/+ mice between the ages of 20 and 50 days. Because this time period also appears to be the most critical in the development of fatal seizures in the Pcmt1−/− mice, we were interested in examining the AdoMet/AdoHcy ratio in Pcmt1−/− mice between these ages. Our results show that these mice, and to a limited extent their Pcmt1+/− littermates, exhibit a progressive elevation in their AdoMet/AdoHcy ratios when compared with Pcmt1+/+ mice. This elevation can either be interpreted as a decrease in the methylation flux or as an increase in AdoMet production and/or AdoHcy metabolism. However, after measuring the activities of the enzymes that regulate AdoMet production and AdoHcy metabolism in the brains of these mice, they were found to be altered in a way that would actually lead to a decrease in the AdoMet/AdoHcy ratio, not an increase. For example, in the brains of 50-day-old Pcmt1−/− and Pcmt1+/+ mice, SAHH activity was either unchanged or slightly lowered, and MAT II activity was significantly reduced, perhaps due to feedback inhibition by the elevated levels of AdoMet. These findings indicate that the increase in the AdoMet/AdoHcy ratio is most likely a result of a decrease in the consumption of AdoMet as opposed to an increase in its production.

One of the objectives of this study was to determine whether there was a direct cause and effect relationship between the lack of PCMT1 and the increase in the AdoMet/AdoHcy ratio. One way we attempted to accomplish this was to see if the AdoMet/AdoHcy ratio was altered in the testes of Pcmt1−/− mice, another tissue in which PCMT1 is highly expressed in Pcmt1+/+ mice. Whereas AdoMet levels were higher in the testes of Pcmt1−/− mice at 40 and 50 days of age, we were unable to find significantly altered AdoMet/AdoHcy ratios for the ages studied here. Another way we attempted to determine whether there was a direct cause and effect relationship was to compare the localization of PCMT1 expression in the Pcmt1+/+ mouse brain with the localization of altered levels of the AdoMet/AdoHcy ratio seen in the Pcmt1−/− mouse brain. When comparing the AdoMet/AdoHcy ratio from Pcmt1−/− mice to Pcmt1+/+ mice, the most significant elevation occurs in the hippocampus and cortex, and the lowest

---

3 C. E. Farrar, S. G. Clarke, and C. R. Houser, unpublished data.
Altered AdoMet Metabolism in Brains of PCMT1-deficient Mice

alteration occurs in the region of the thalamus. This correlates well with immunolocalization of the enzyme in rat (35–37) and mouse brain.3 These data suggest that the lack of the PCMT1 enzyme may be the primary cause of the altered AdoMet/AdoHcy ratios in Pcm1−/− and Pcm1+/+– mice; however, the extent of this effect may be limited to brain tissue.

In Fig. 6, we depict a model for the condition of the Pcm1−/− mouse in which the lack of PCMT1 decreases the overall methylation flux in the brain. This deficit results in a build up of AdoMet, a diminishment of AdoHcy, an accumulation of isoaspartyl-damaged proteins (X-isoAsp), and a potential increase in the methylation of other AdoMet-dependent methyltransferase substrates (X-CH3).

![Diagram](Image 65x432 to 281x728)

**FIG. 6.** Disruption of methylation flux in Pcm1−/− mouse brain compared with Pcm1+/+ mouse brain. In the Pcm1+/+ mouse, the levels of AdoMet and AdoHcy are kept in balance through the production of AdoMet, conversion of AdoMet to AdoHcy, and metabolism of AdoHcy. The lack of PCMT1 decreases the overall methylation flux in the brain of the Pcm1−/− mouse. This alteration results in a build up of AdoMet, a diminishment of AdoHcy, an accumulation of isoaspartyl-damaged proteins (X-isoAsp), and a potential increase in the methylation of other AdoMet-dependent methyltransferase substrates (X-CH3).

injected intraventricularly into mice (44, 45). In these studies, it was demonstrated that even a small amount of spermine could cause extreme hyperexcitability in the mice, and convulsions could be precipitated by the slightest sound or touch.

It is also possible that lower AdoHcy levels alone might affect the seizure threshold. For example, AdoHcy has been shown to have anticonvulsant properties in rabbit, rat, and cat (46). These studies demonstrated that AdoHcy administration decreased epileptiform discharges after hippocampal stimulation and decreased the incidence of pentetrazol convulsions. AdoHcy has also been proposed as a candidate ligand for the benzodiazepine receptor based on its capacity to inhibit flunitrazepam binding to the benzodiazepine recognition site of the γ-aminobutyric acid, type A, receptor (47). Probably the most convincing study on the anticonvulsant action of AdoHcy involves the induction of seizures in mice by L-methionine-dl-sulfoximine and the inhibition of these seizures by the co-administration of adenosine and homocysteine thiolactone (48). In these studies, it was determined that the most effective anticonvulsant action of this treatment occurred when cerebral AdoHcy levels were at their highest.

While it is possible that an increase in the AdoMet/AdoHcy ratio may be responsible for lowering the seizure threshold in these mice, there is no evidence that externally administered AdoMet, which does not appear to be able to cross the plasma membrane (54), would produce the same effect. In fact, in a study of the use of AdoMet as an antidepressant for patients with chronic epilepsy, it was found that daily intravenous administration of AdoMet had no adverse effect on seizure frequency (49). On the other hand, the administration of folate, the levels of which are correlated with intracellular AdoMet levels in rat brain (53), can greatly aggravate seizure control in epilepsy patients (50) and in experimental animals (51, 52). Thus, orally (or intravenously) administered AdoMet would probably not mimic the effect we observe in the Pcm1−/− mice, which is most likely an intracellular alteration of AdoMet/AdoHcy levels.

Up to this point, the study of PCMT1 has usually been associated with aging research due to its firmly established role in protein repair (1, 25). Before the generation of the PCMT1-deficient mouse, it was thought that a knockout of this gene in a mammalian system might provide an advanced aging model. Due to the strong expression of the enzyme in the central nervous system, it was also anticipated that the knockout mouse might show signs of advanced neurological aging, such as neuronal degeneration or the formation of neuritic plaques. However, when the mice were finally generated, it was unanticipated how very limited their survival would turn out to be (23, 24). Trying to make a connection between the deficiency of the methyltransferase and the development of fatal epilepsy seen in this short-lived mouse has been a challenge, especially considering the diversity and number of potential substrates for the enzyme. Theories involving the damage or alteration of various proteins, peptides, and small molecule substrates have been proposed (23–27). However, one previously overlooked substrate was the methyltransferase’s own cofactor, AdoMet. From the studies mentioned above, we have determined that the levels of AdoMet and AdoHcy are indeed altered by the lack of PCMT1, although we do not yet know if these alterations are causing the seizures seen in the Pcm1−/− mice or if they are secondary to them. However, if the altered levels of these small molecules is influencing the seizure threshold in the Pcm1−/− mice, there may be ways to rescue this phenotype. If we are able to prolong their lives, perhaps older Pcm1−/− mice would exhibit phenotypes directly related to the aging of
specific protein substrates, and perhaps even provide a model of advanced aging in the nervous system.

Acknowledgments—We thank Dr. Carolyn R. Houser (Department of Neurobiology and Brain Research Institute, UCLA) for guidance in brain dissections and helpful discussions. We also thank Dr. S. Harvey Mudd (National Institute of Mental Health) for helpful discussions.

REFERENCES