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Altered Levels of *S*-Adenosylmethionine and *S*-Adenosylhomocysteine in the Brains of L-Isoaspartyl (D-Aspartyl) *O*-Methyltransferase-deficient Mice*

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L-Isoaspartyl (D-aspartyl) *O*-methyltransferase (PCMT1) is a protein repair enzyme that initiates the conversion of abnormal D-aspartyl and L-isoaspartyl residues to the normal 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 (*Pcmt1*−/−), as well as in their heterozygous (*Pcmt1*+/−) and wild type (*Pcmt1*+/+) littermates. Higher levels of AdoMet and lower levels of AdoHcy were found in the brains of *Pcmt1*−/− mice, and to a lesser extent in Pcmt1+/− mice, when compared with *Pcmt1*+/+ mice. In addition, these levels appear to be most significantly altered in the hippocampus of the *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 *Pcmt1*−/− mice.

One of the most common forms of protein damage *in vitro* and *in vivo* is deamidation and isoaspartyl formation caused by deprotonation of the peptide-bond nitrogen and its attack on the side chain carbonyl carbon of L-asparaginyl or 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 α-carbonyl group, the peptide bond is re-routed through the side chain carbonyl resulting in the formation of an L-isoaspartyl residue. The ring is also susceptible to racemization, allowing for the formation of D-aspartyl and D-isoaspartyl 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 isoaspartyl formation in specific peptides or proteins may be linked to certain disease processes. For example, the presence of isoaspartyl residues in the β-amyloid peptide contributes to its insolubility, and protein isomerization is elevated in β-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 isoaspartyl residues. This mechanism involves the enzyme L-isoaspartyl (D-aspartyl) *O*-methyltransferase (PCMT1).[1]

PCMT1 functions by transferring a methyl group from the molecule *S*-adenosylmethionine (AdoMet) to either the α-carboxyl group of the L-isoaspartyl residue or the β-carboxyl group of the 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 β-carbonyl, which typically occurs about 30% of the time at physiological pH, the residue returns to the normal 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 AdoMet 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

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[1] The abbreviations used are: PCMT1, L-isoaspartyl (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.

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(SAHH). 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 B_{12} 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*-methyltranferase, 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 ratios (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

Generation of Pcmt1−/−, Pcmt1+/−, and Pcmt1+/+ Mice—The *Pcmt1*+/+, *Pcmt1*+/−, and *Pcmt1*−/− mice were obtained as described previously (23, 27). By inbreeding mice that were heterozygous for the knockout mutation for over 8 generations, a congenic mutant line has been generated that is ~50% 129S4/SvJae and 50% C57BL/6. All *Pcmt1*−/− mice used in the following studies were compared with their *Pcmt1*+/+ and *Pcmt1*+/− littermates. Although both male and female mice were included in the same data sets, approximately the same number of each sex was used for each genotype. Mice were weaned at 21 days of age, housed in a barrier facility with a 12-h light/dark cycle, and had unlimited access to chow food and fresh water. Mice were monitored by on-site veterinarians, and all protocols were pre-approved by the UCLA Animal Research Committee.

Preparation of Brain Homogenates for Enzyme Assays—Mice were sacrificed by decapitation, and their tissues were extracted, weighed, and placed in ice-cold buffer (4 ml/g). The buffer consisted of 250 mM sucrose, 10 mM Tris-HCl, 1 mM disodium EDTA, pH 7.4, and protease inhibitor mixture (one "complete, mini, EDTA-free protease inhibitor mixture" tablet per 7 ml of buffer; Roche Diagnostics). The tissue was disrupted in a glass homogenization tube with a motor-driven Teflon-coated pestle rotating at 310 rpm for seven 10-s intervals. The extract was centrifuged at 20,800 × *g* for 10 min at 4 °C, and the resulting supernatant extract was stored at −80 °C until enzyme activity assays for PCMT1, MAT II, or SAHH were performed.

Measurement of PCMT1 Activity in Brain Homogenates—The supernatant fractions from homogenized tissue (described above) were thawed, and 5 μl (~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*-^{14}C]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 hydrolyze the [^{14}C]methyl esters formed on L-isoaspartyl residues in ovalbumin to [^{14}C]methanol. The reaction mixture was immediately spotted onto an accordion-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 [^{14}C]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 [^{14}C]methanol production. Incubations containing only *S*-adenosyl-L-[*methyl*-^{14}C]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 L-isoaspartyl (D-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)[2]) in 0.2 M BisTris-HCl buffer, pH 6.0, containing 10 μM *S*-adenosyl-L-[*methyl*-^{14}C]methionine in a 30-μl volume at 37 °C for 2 h. After base hydrolysis, [^{14}C]methanol production was measured as described above to quantify L-isoaspartyl and D-aspartyl methyl-accepting sites in cellular proteins. Incubations containing only *S*-adenosyl-L-[*methyl*-^{14}C]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 damaged aspartyl residue levels.

Measurement of AdoMet and AdoHcy Levels in Deproteinized Tissue Homogenates—Mice were decapitated, and their tissues were immediately submerged 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 $HClO_4$ (2 ml/g wet weight) at 4 °C in a glass homogenization tube with a motor-driven Teflon-coated pestle rotating at 310 rpm for seven 10-s intervals. The homogenates were centrifuged at 10,000 × *g* for 20 min at 4 °C. The resulting deproteinized supernatant fractions were neutralized with KOH. The precipitated $KClO_4$ 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 C_{18} 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% HPLC grade acetonitrile (Fisher). The samples were eluted at room temperature with a linear gradient of 0–16% eluent B from 0 to 16 min, then 16–21% eluent B from 16 to 25 min, and 21–95% eluent B from 25 to 28 min. The column was washed with 95% eluent B from 28 to 33 min and re-equilibrated with 100% eluent A from 33 to 60 min. The flow rate was maintained at 1 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 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 (including pons and midbrain), cerebellum, hippocampus, thalamus (including hypothalamus), and cortex (including basal ganglia). Each section was weighed and homogenized at 4 °C in 0.4 M $HClO_4$. 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.

extract (described above) was incubated for 60 min at 37 °C in a reaction mixture (100 μl) containing 70 mM Tris-HCl, pH 8.0, 35 mM KCl, 35 mM $MgCl_2$, 1.4 mM dithiothreitol, 10 mM ATP (Sigma), and 1 mM L-methionine (Sigma). SAHH activity was assayed by measuring the reverse reaction, namely the synthesis of AdoHcy from adenosine and homocysteine using a procedure adapted from that of Poulton and Butt (17). In short, 20 μl of brain extract was incubated for 60 min at 37 °C in a reaction mixture (100 μl) containing 70 mM Tris, pH 8.0, 35 mM KCl, 35 mM $MgCl_2$, 1.4 mM dithiothreitol, 10 mM DL-homocysteine (Sigma), and 1 mM adenosine (Sigma). The reactions for both enzymes were stopped with 10 μl of 4 M $HClO_4$, and the samples were frozen at −20 °C until the level of AdoMet or AdoHcy was assayed by HPLC as described above. Before analysis, the samples were thawed and centrifuged at 20,800 × *g* for 10 min, and the supernatants were neutralized with KOH and re-centrifuged at 20,800 × *g* for 10 min to clear the precipitated $KClO_4$.

Protein Assay—Protein concentrations for the extracts used in measuring enzyme activities were determined by a modification of the Lowry procedure (30) using bovine serum albumin (Sigma) as a standard.

Statistical Analysis—The results are expressed as means with the S.D. from *n* mice. Tests of significance were conducted for data within each age group using the unpaired, two-tailed Student's *t* test.

RESULTS

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. 1*A*). 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. 1, *B* 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. 1*C*). 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 (31). 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. 2*A*). The specified "AdoHcy" 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. 2*B*).

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. 3, *A* 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. 3, *A* and *B*). The levels of AdoMet and AdoHcy in *Pcmt1−/−* and *Pcmt1+/−* mice

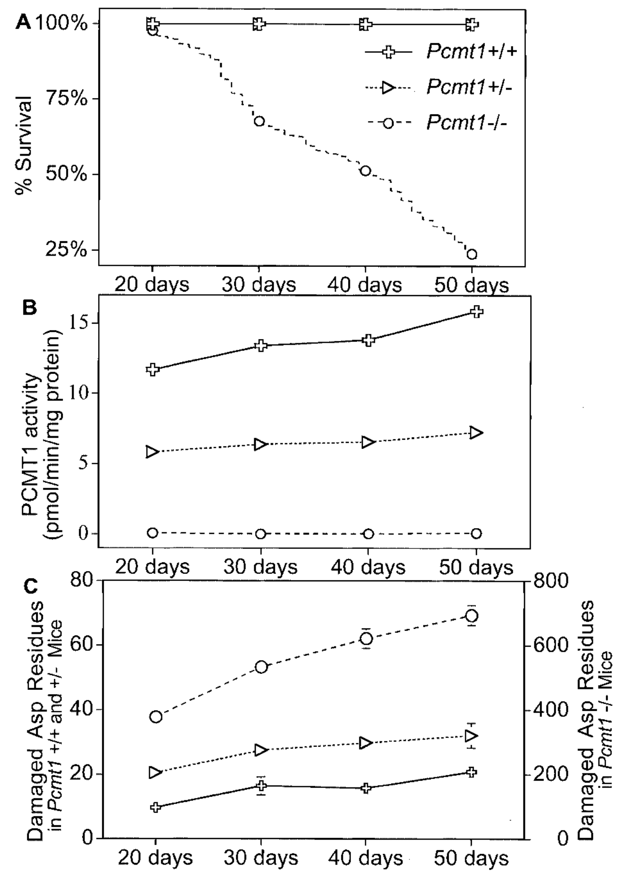

FIG. 1. **Survival, PCMT1 activity, and isoaspartyl-damaged proteins in the brains of *Pcmt1+/+*, *Pcmt1+/−*, and *Pcmt1−/−* mice.** *A*, survival data for *Pcmt1+/+*, *Pcmt1+/−*, and *Pcmt1−/−* mice between the ages of 20 and 50 days. The average age of death for the *Pcmt1−/−* mice ($n = 100$) was 43 days. The average age of death for the *Pcmt1+/+* mice ($n = 20$) and the *Pcmt1+/−* mice ($n = 32$) was 745 and 649 days, respectively (data not shown). These data represent only mice that died spontaneously. *B*, level of PCMT1 activity in the brains of *Pcmt1+/+*, *Pcmt1+/−*, and *Pcmt1−/−* mice. Endogenous brain PCMT1 activity was quantitated by its ability to transfer a methyl group from AdoMet to the damaged aspartyl residues in ovalbumin and is expressed as pmol of sites methylated per min per mg of brain homogenate protein. Data are means with the S.D. ($n = 4$ for each data point). *C*, damaged aspartyl levels in the brains of *Pcmt1+/+*, *Pcmt1+/−*, and *Pcmt1−/−* mice. Recombinant human L-isoaspartyl (D-aspartyl) *O*-methyltransferase was used to quantitate damaged aspartyl residues in polypeptides of brain homogenates and are expressed as pmol of methylatable sites per mg of protein. Data are means with the S.D. ($n = 4$ for each data point). Note the 10-fold enlargement in the scale for the *Pcmt1−/−* mouse data. Although *error bars* indicating the S.D. of the mean are included for all data points in *B* and *C*, most are smaller than the plot symbols.

were also compared with those of their *Pcmt1+/+* littermates 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. 3*A*). 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. 3*B*). 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

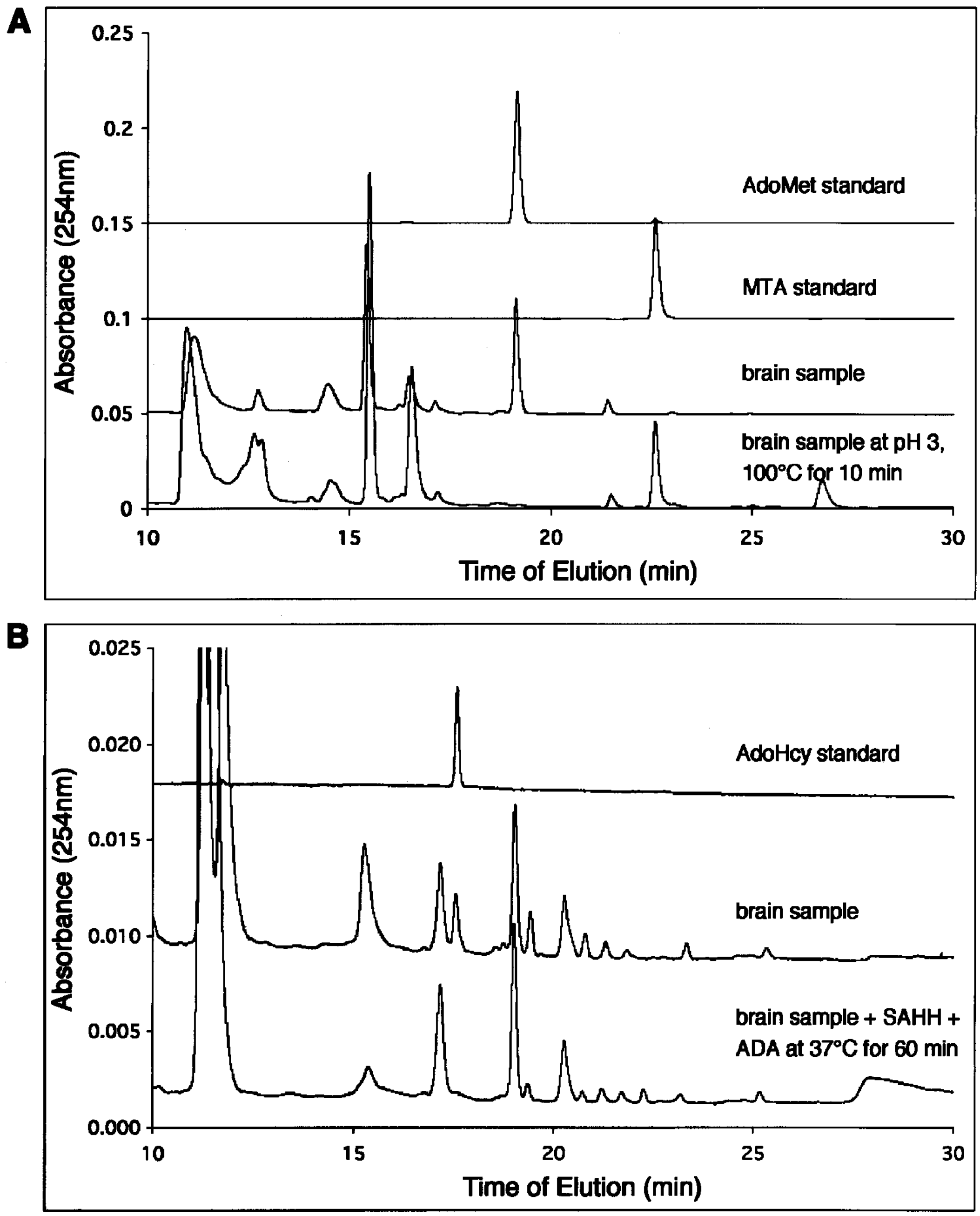

FIG. 2. **Verification of AdoMet and AdoHcy peaks in reverse phase HPLC chromatograms of deproteinized brain homogenate.** *A*, reverse phase HPLC chromatograms of 1 nmol of AdoMet, 1 nmol of MTA, deproteinized whole brain homogenate (brain), and deproteinized whole brain homogenate that has been boiled at pH 3 for 10 min. The volume of each brain sample was 100 μl, representing a homogenate of 49 mg of brain tissue. The column eluent was monitored at an absorbance of 254 nm. *B*, reverse phase HPLC chromatograms of 50 pmol of AdoHcy, deproteinized brain homogenate, and deproteinized brain homogenate that was incubated with *S*-adenosylhomocysteine hydrolase and adenosine deaminase for 60 min at 37 °C as described under "Experimental Procedures." The volume of each brain sample was 50 μl, representing a homogenate of 18 mg of brain tissue.

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. 3*C*). 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

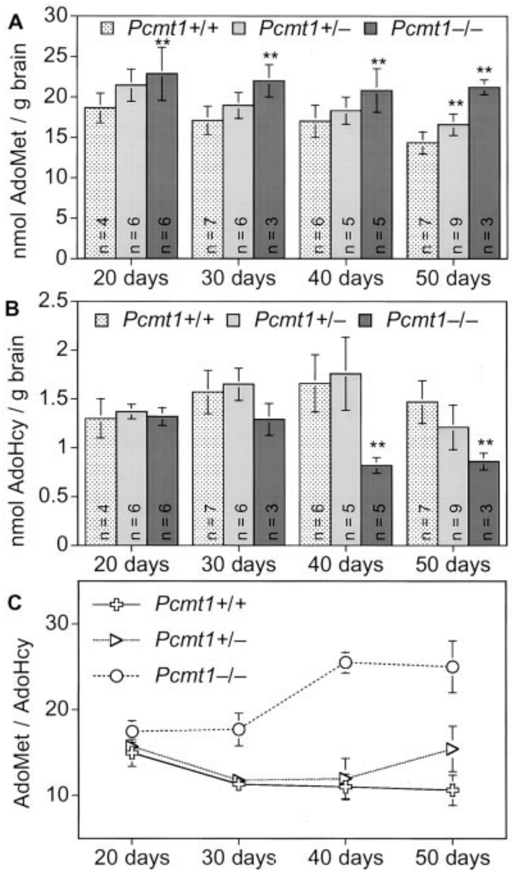

FIG. 3. **AdoMet and AdoHcy levels in deproteinized whole brain homogenates.** *A*, average nmol of AdoMet/g brain for *Pcmt1*+/+, *Pcmt1*+/−, and *Pcmt1*−/− mice at 20, 30, 40, and 50 days of age as measured by reverse phase HPLC. *B*, average nmol of AdoHcy/g brain for *Pcmt1*+/+, *Pcmt1*+/−, and *Pcmt1*−/− mice at 20, 30, 40, and 50 days of age as measured by HPLC. *A* and *B*, the number of mice analyzed is indicated at the *bottom* of each *bar*. *C*, average AdoMet/AdoHcy ratios in brain homogenates of *Pcmt1*+/+, *Pcmt1*+/−, and *Pcmt1*−/− mice at 20, 30, 40, and 50 days of age. Each ratio is based on the AdoMet and AdoHcy values from each brain homogenate; therefore, the number of mice represented by each plot symbol in *C* is the same number as indicated in *A* and *B*. *A*–*C*, the *error bars* indicate S.D. of the mean. **, $p < 0.05$ *versus Pcmt1*+/+ values.

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.

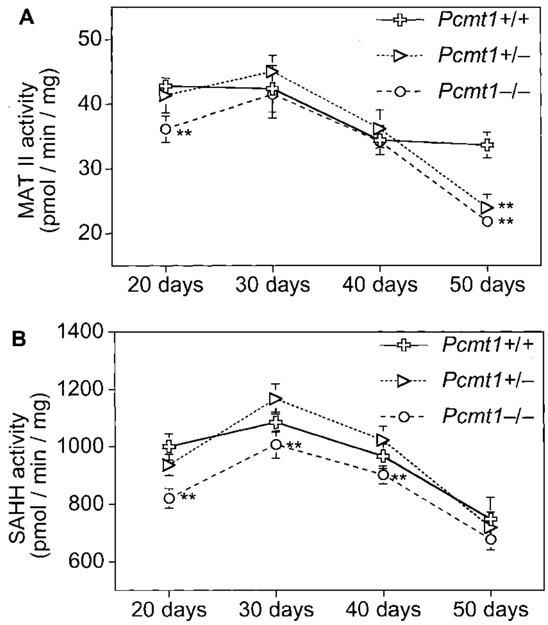

FIG. 4. **MAT II and SAHH activity in whole brain homogenates.** *A*, MAT activity (pmol/min/mg) in brain homogenates of *Pcmt1*+/+, *Pcmt1*+/−, and *Pcmt1*−/− mice at 20, 30, 40, and 50 days of age. MAT II activity was measured by the formation of AdoMet during the incubation of brain homogenates with an excess of ATP and methionine for 60 min at 37 °C, as described under "Experimental Procedures" (n = 6, 4, 4, and 4 for *Pcmt1*+/+ mice at 20, 30, 40, and 50 days respectively; n = 6, 4, 4, and 5 for *Pcmt1*+/− mice at 20, 30, 40, and 50 days respectively; n = 6, 4, 4 and 5 for *Pcmt1*−/− mice at 20, 30, 40, and 50 days respectively). *B*, SAHH activity (pmol/min/mg) in brain homogenates of *Pcmt1*+/+, *Pcmt1*+/−, and *Pcmt1*−/− mice at 20, 30, 40, and 50 days of age. SAHH was measured by the formation of AdoHcy during the incubation of brain homogenates with an excess of homocysteine and adenosine for 60 min at 37 °C (n = 4, 4, 4, and 4 for *Pcmt1*+/+ mice at 20, 30, 40, and 50 days, respectively; n = 4, 3, 4, and 5 for *Pcmt1*+/− mice at 20, 30, 40, and 50 days, respectively; n = 4, 4, 4, and 5 for *Pcmt1*−/− mice at 20, 30, 40, and 50 days, respectively). *A* and *B*, *error bars* indicate S.D. of the mean. **, $p < 0.05$ *versus Pcmt1*+/+ values.

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. 4*A*). 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 inhibited by 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. 4*B*). 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 that 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. 5*A*). Also, the *Pcmt1*−/− mice have lower levels of AdoHcy in every brain region except the thalamus (Fig. 5*B*). Finally, in comparing the AdoMet/AdoHcy ratios between the *Pcmt1*−/−and *Pcmt1*+/+ mice, the most significant difference appears to occur in the hippocampus (Fig. 5*C*). 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.

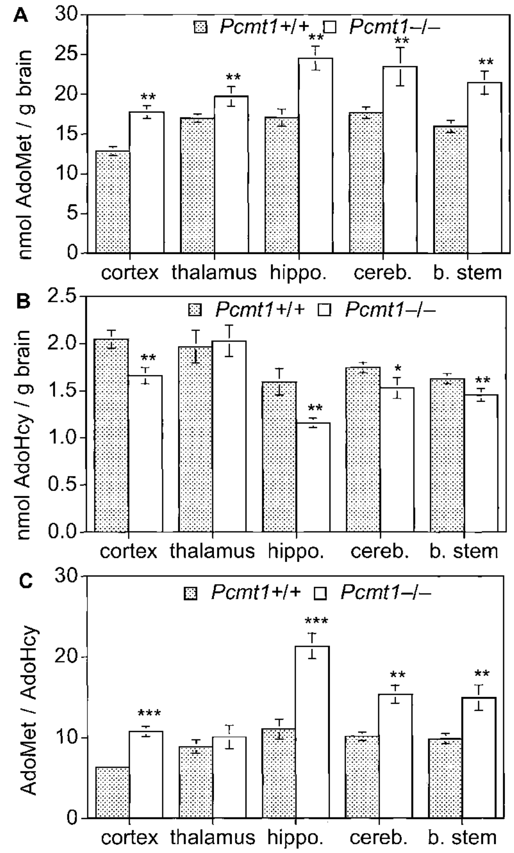

FIG. 5. **AdoMet and AdoHcy levels in deproteinized homogenates of dissected brains from 50-day-old *Pcmt1*+/+ and *Pcmt1*−/− mice.** *A*, average nmol of AdoMet/g brain section for *Pcmt1*+/+ and *Pcmt1*−/− mice at 50 days of age as measured by HPLC. *B*, average nmol of AdoHcy/g brain section for *Pcmt1*+/+ and *Pcmt1*−/− mice at 50 days of age as measured by HPLC. *C*, average values of the AdoMet/AdoHcy ratio in dissected brain homogenates of *Pcmt1*+/+ and *Pcmt1*−/− mice at 50 days of age. *A–C*, the *error bars* indicate S.D. of the mean (n = 6 for each *bar*). ***, $p < 0.01$ *versus* *Pcmt1*+/+ values. **, $p < 0.05$ *versus* *Pcmt1*+/+ values. *, $p < 0.1$ *versus* *Pcmt1*+/+ values.

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.

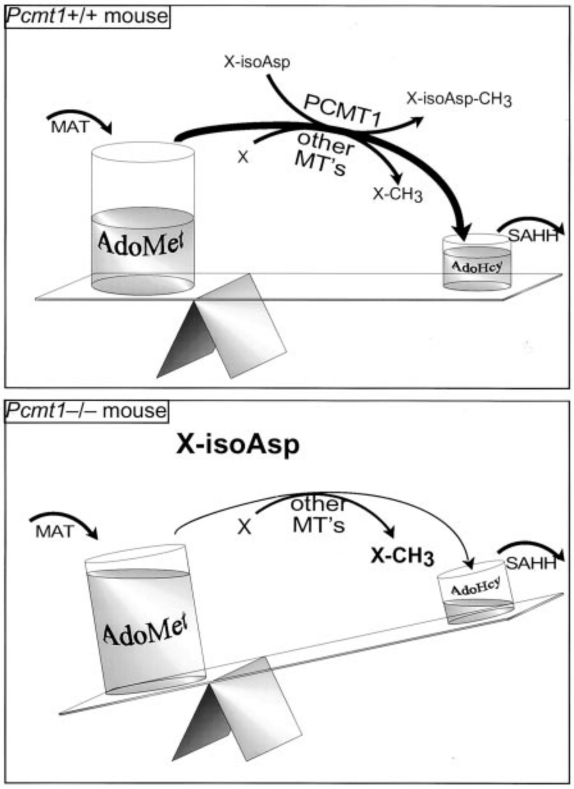

FIG. 6. **Disruption of methylation flux in *Pcmt1*−/− mouse brain compared with *Pcmt1*+/+ mouse brain.** In the *Pcmt1*+/+ mouse, the levels of AdoMet and AdoHcy are kept in balance through the production of AdoMet, conversion of AdoMet to AdoHcy, and metabolism of AdoHcy. The lack of PCMT1 decreases the overall methylation flux in the brain of the *Pcmt1*−/− 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*-CH_3).

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 *Pcmt1*−/− and *Pcmt1*+/− 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 *Pcmt1*−/− 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*-CH_3). Any one of these effects could potentially lower the seizure threshold in the *Pcmt1*−/− mice. The altered AdoMet/AdoHcy ratio could affect the activity of one or more of the many other AdoMet-dependent methyltransferases, such as those involved in neurotransmitter metabolism, receptor transduction, DNA expression, or phospholipid methylation (55). For example, in several studies it has been suggested that the transduction of receptor-mediated events could be altered through membrane lipid methylation (42). In studies where reticulocyte ghosts were incubated with varying concentrations of AdoMet, the number of β-adrenergic receptor sites increased with a paralleled increase in membrane fluidity (43). Aside from altering the activities of other AdoMet-dependent methyltransferases, it was also possible that higher levels of AdoMet could lead to greater synthesis of polyamines through the action of AdoMet decarboxylase. Polyamines have been shown to have excitatory properties when 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 *Pcmt1*−/− 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 *Pcmt1*−/− mice or if they are secondary to them. However, if the altered levels of these small molecules is influencing the seizure threshold in the *Pcmt1*−/− mice, there may be ways to rescue this phenotype. If we are able to prolong their lives, perhaps older *Pcmt1*−/− 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.

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