Phenotypic Analysis of Seizure-prone Mice Lacking L-Isoaspartate (D-Aspartate) O-Methyltransferase*

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Within proteins and peptides, both L-asparaginyl and L-aspartyl residues spontaneously degrade, generating isomerized and racemized aspartyl residues. The enzyme protein L-isoaspartate (D-aspartate) O-methyltransferase (E.C. 2.1.1.77) initiates the conversion of l-isoaspartyl and D-aspartyl residues to normal L-aspartyl residues. This “repair” reaction helps to maintain proper protein conformation by preventing the accumulation of damaged proteins containing abnormal amino acid residues. Pcm1−/− mice manifest two key phenotypes: a fatal seizure disorder and retarded growth. In this study, we characterized both phenotypes and demonstrated that they are linked. Continuous electroencephalogram monitoring of Pcm1−/− mice revealed that abnormal cortical activity for ~50% of each 24-h period, even in mice that had no visible evidence of convulsions. The fatal seizure disorder in Pcm1−/− mice can be mitigated but not eliminated by antiepileptic drugs. Interestingly, antiepileptic therapy normalized the growth of Pcm1−/− mice, suggesting that the growth retardation is due to seizures rather than a global disturbance in growth at the cellular level. Consistent with this concept, the growth rate of Pcm1−/− fibroblasts was indistinguishable from that of wild-type fibroblasts.

Under physiological conditions, L-asparaginyl and L-aspartyl residues in polypeptides spontaneously degrade to L- and D-isoaspartyl and D-aspartyl residues with half-times ranging from a few hours to hundreds of days (1–5). These abnormal residues can affect both the structure and function of polypeptides and may underlie a portion of the aging-related loss of cellular and tissue function (6–9). However, many organisms have evolved a strategy for reversing at least some of this damage by an enzymatic methylation reaction that is targeted directly to peptides and proteins containing the major l-isoaspartyl degradation product. The widely distributed cystolic protein L-isoaspartate (D-aspartate) O-methyltransferase, also termed protein carboxyl methyltransferase 1 (Pcm1),1 can initiate the conversion of l-isoaspartyl residues to l-aspartyl residues by forming the methyl ester of the l-isoaspartyl residue (10). This ester is then converted, in a nonenzymatic reaction, to an l-succinimidyld residue. Spontaneous hydrolysis of the L-succinimidyld residue produces either an L-aspartyl or an L-isoaspartyl residue. If an L-isoaspartyl residue is produced, additional rounds of methyl esterification, succinimide formation, and hydrolysis eventually convert it to an L-aspartyl residue. This pathway has been experimentally demonstrated both in peptides (11–13) and in proteins (14, 15).

The methyltransferase-mediated protein-repair pathway is found in a wide variety of organisms, including bacteria, plants, nematodes, and vertebrates (16–18). In mammals, the enzyme is expressed in all tissues, with the highest levels of expression in the brain (19). The ubiquitous expression of this methyltransferase has led to the hypothesis that it might help to maintain cytosolic proteins in the proper conformation and prevent the accumulation of proteins with damaged amino acid residues. To test this hypothesis and to gain insights into the importance of this enzyme in higher organisms, we recently generated Pcm1 knockout mice (Pcm1−/− mice) (19). At the biochemical level, methyltransferase substrates (i.e., damaged proteins containing l-isoaspartyl residues) accumulated in all tissues of Pcm1−/− mice; the highest levels were found in the brain. At the whole-animal level, the Pcm1−/− mice manifested two unmistakable phenotypes: abnormally slow growth and sudden, premature death, beginning at about 22 days of age. Continuous videotape monitoring showed that the sudden death was caused by generalized seizures and that virtually all of the seizures were fatal. Very recently, the occurrence of fatal seizures in Pcm1 knockout mice was confirmed by another group of investigators (20).

The observation of fatal seizures in Pcm1−/− mice highlighted the importance of Pcm1 gene expression in the central nervous system but at the same time raised a number of new issues for investigation. One was whether the abnormal electrical activity in the brains of the Pcm1 knockout mice was episodic, as suggested by the continuous videotape monitoring, or pervasive, with the overt seizures simply representing the tip of the iceberg. A second issue was whether the fatal seizures might be mitigated or eliminated by antiepileptic drug therapy. A third issue was whether the absence of Pcm1 in the brain might lead to a significant abnormality in the metabolism of an abundant aspartate-containing dipeptide in the central nervous system, N-acetylaspartylglutamate (NAAG) (21). NAAG, a putative excitatory neurotransmitter that has been implicated in seizures, has recently been shown to exist in the brain in the succinimide form (22). The latter finding raised the possibility that N-acetylaspartylglutamate (β-NAAG) might also exist.

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‡ The abbreviations used are: Pcm1, protein carboxyl methyltransferase 1; NAAG, N-acetylaspartylglutamate; β-NAAG, N-acetylaspartylglutamate; EEG, electroencephalogram; AdoMet, S-adenosylmethionine; NMDA, N-methyl-D-aspartic acid.
and be a substrate for Pcm1. β-NAAG is an excitatory dipeptide that cannot be catabolized by the dipeptidase that normally cleaves NAAG (23, 24). Still another issue was whether the retarded growth of the Pcm1 knockout mice was due to inefficient growth of Pcm1-deficient tissues (perhaps because of an accumulation of damaged proteins within cells) or was simply due to the seizure disorder. In the current study, we have addressed each of these issues.

MATERIALS AND METHODS

Pcm1-deficient Mice—The generation of Pcm1 knockout mice has been described previously (19). For this study, homozygous Pcm1-deficient mice (Pcm1−/−) were produced by intercrossing heterozygous Pcm1-deficient mice (Pcm1+/−). All mice were of a mixed genetic background (approximately 50% C57BL/6J and 50% 129/SvJae) unless otherwise stated. Mice were weaned at 21 days of age, fed a chow diet containing 4.5% fat (Ralston Purina, St. Louis, MO), and housed in a full-barrier facility with a 12-h light/dark cycle. Mice were genotyped by Southern blot analysis of genomic DNA prepared from a tail biopsy (19).

Continuous Electroencephalographic Recordings—The mice were anesthetized with avertin (20 μg/g of body weight). A small midline incision was made in the abdomen, and a radiofrequency transmitter (PhysioTel Implant, model TA100A-F20, Data Sciences International, St. Paul, MN) was inserted into the peritoneal cavity. The peritoneum was then closed, and the two monopolar leads that extended from the transmitter were tunneled subcutaneously to a short cutaneous incision over the skin (extending from the base of the skin to the transverse suture). Two small apertures, located ~0.5 cm to the left and right of the sagittal suture and 0.5 cm caudal to the transverse suture, were made in the cranium with sharp forceps. The tips of the electrodes were placed within the epidural space on the surface of the cerebral cortex, and the leads were secured with Krazy Glue (Happ Controls, Oak Grove, IL). The skin incisions over the skull and abdomen were then closed with 5–0 silk sutures. Brain electrical potentials were recorded, either continuously or intermittently, with an MP100 system and AcqKnowledge software (Biopac Systems, Inc., Santa Barbara, CA). The skin incisions over the skull (extending from the base of the skull to the transverse suture that cannot be catabolized by the dipeptidase that normally cleaves NAAG (23, 24). Still another issue was whether the retarded growth of the Pcm1 knockout mice was due to inefficient growth of Pcm1-deficient tissues (perhaps because of an accumulation of damaged proteins within cells) or was simply due to the seizure disorder. In the current study, we have addressed each of these issues.

To analyze the effects of these interventions on the survival of Pcm1−/− mice, 21-day-old mice were given valproic acid alone (120 mg/kg) or in combination with 2 kg of mouse chow for 24 h, and then exhaustively dried in a fume hood. The average daily intake of valproic acid and clonazepam (Sigma) was 180 and 40 μg/g, respectively.

To analyze the growth characteristics of Pcm1−/− fibroblasts, we isolated fibroblasts from a 100-mm Petri dish. Cells were trypsinized every 3 days, counted, and replated at a density of 2 × 10^6 cells per 100-mm Petri dish. For 2- and 3-day growth experiments, 1 × 10^6 cells of each line (passage 2) were plated onto eight wells of duplicate 96-well plates. One of the plates was incubated for 12 h and the other plate for 72 h, after which the cell density for each fibroblast cell line was determined with a CellTiter 96 AQ One Solution cell proliferation assay kit (Promega, Madison, WI). The cell growth rate was calculated as the ratio of the 72-h cell density (average of eight wells) to the 12-h cell density (average of eight wells). The growth rates of the Pcm1+/− and Pcm1−/− fibroblasts were normalized to the growth rate observed in Pcm1+/− fibroblasts.

Next we examined the survival characteristics of Pcm1−/− fibroblasts. Each fibroblast cell line (passage 4) was plated onto 96-well plates at a density of 1 × 10^5 cells per well (eight wells per cell line). The plates were then grown under standard conditions, except that the cell culture medium contained a low concentration of fetal bovine serum (0.5%). The medium was changed every 4 days, and cell survival at various time points from 12 h to 16 days was assessed with the CellTiter 96 AQ kit. The data were expressed as the cells surviving at each time point, relative to the number of cells at the 12-h time point.

In several experiments, equal numbers of wild-type and Pcm1−/− fibroblasts (passage 3) were mixed and then passaged according to a 3T6 protocol. DNA was isolated from the mixtures at several passages, both before and after immortalization. The ratio of wild-type to Pcm1−/− fibroblasts in the mixtures at various time points was determined by performing Southern blots of genomic DNA and then analyzing the blots with a phosphor imager (Fuji Biomaging analyzer, BAS 1000 with MacBAS, Fuji Medical Systems, USA, Inc., Stamford, CT).

Analysis of Pcm1-deficient Mice—The fibroblasts were passaged according to a 3T6 protocol. Cells were trypsinized every 3 days, counted, and replated at a density of 2 × 10^6 cells per 100-mm Petri dish. For 2- and 3-day growth experiments, 1 × 10^6 cells of each line (passage 2) were plated onto eight wells of duplicate 96-well plates. One of the plates was incubated for 12 h and the other plate for 72 h, after which the cell density for each fibroblast cell line was determined with a CellTiter 96 AQ One Solution cell proliferation assay kit (Promega, Madison, WI). The cell growth rate was calculated as the ratio of the 72-h cell density (average of eight wells) to the 12-h cell density (average of eight wells). The growth rates of the Pcm1+/− and Pcm1−/− fibroblasts were normalized to the growth rate observed in Pcm1+/− fibroblasts.

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Analysis of Pcm1-deficient Mice

RESULTS

Analysis of Seizure Activity in Pcm1−/− Mice—We previously reported that Pcm1−/− mice die from generalized seizures and that most of the seizures recorded by continuous videotape monitoring were fatal (19). An analysis of multiple videotaped seizures revealed a characteristic seizure phenotype. First, there were jerky movements for a mean period of 10 s, followed by a period of grooming and ataxia for 10 s. Next, there was a period of vigorous jumping and running for a mean of 4 s, followed by tonic/clonic movements for a mean of 4 s. After the tonic/clonic activity, the mice were motionless and without spontaneous respirations.

To determine whether abnormal brain electrical activity was present during the epileptic seizures or was more pervasive, we performed continuous electroencephalographic monitoring in Pcm1−/− mice and in littermate controls (Pcm1+/+ mice). Abnormal cortical electrical activity, consisting of high amplitude spikes and waves, was present during ~50% of each 24-h period for the Pcm1−/− mice; controls showed no abnormal activity (Fig. 1). Remarkably, the abnormal electrical activity was present in the Pcm1−/− mice at the same time that they appeared overtly healthy and active in their cages. Fatal seizures were characterized by high amplitude spikes and waves, followed by diminished cortical activity in the postictal period (Fig. 2). During the postictal period, spontaneous respiratory activity was absent, and within a short time, the only detectable electrical potentials were cardiac depolarizations.

Treatment of Pcm1−/− Mice with Antiepileptic Drug Therapy—To determine whether antiepileptic drug therapy might prevent or mitigate the lethal seizure disorder, groups of Pcm1−/− mice were treated with valproic acid or a combination of both valproic acid and clonazepam. A low dose of valproic acid (120 μg/g/day) extended the survival of Pcm1−/− mice, but by only 10–15 days (Fig. 3). A higher dose of valproic acid (180 μg/g/day) was more effective in prolonging life span, but the majority of mice still died by 65–75 days of age. The combination of valproic acid (180 mg/kg/day) and clonazepam (0.2 mg/kg/day) was the most effective in prolonging survival, but even with that regimen, more than 50% of mice died before 85 days and fewer than 25% survived more than 110 days.

Effects of Antiepileptic Drug Therapy on the Growth and Mating Behavior of Pcm1−/− Mice—Pcm1−/− mice grew more slowly than Pcm1+/+ littermate controls. At 7 weeks of age, both male and female Pcm1−/− mice were 2–3 g smaller than their littermates (Fig. 4A). Initially, we hypothesized that this growth disturbance was due to a global disturbance in cellular metabolism in many tissues, a consequence of the Pcm1 deficiency and of the accumulation of damaged proteins containing 1-isoaspartyl residues. However, the pervasive EEG abnormalities in Pcm1−/− mice led us to consider an alternative hypothesis: that the retarded growth was due to abnormal brain electrical activity. If that alternative hypothesis were correct, we reasoned that the size difference between Pcm1−/− mice and their littermates would be reduced or eliminated by antiepileptic drug therapy. To test this possibility, we placed male and female Pcm1−/− and Pcm1+/+ mice on valproic acid (180 μg/g/day) at 21 days of age and weighed them 4 weeks later (Fig. 4, A and B). Interestingly, antiepileptic drug treatment had no effect on the body weight of Pcm1−/− mice at 7 weeks of age, but it significantly increased the body weight of Pcm1−/− mice and eliminated the differences in body weight between Pcm1−/− and Pcm1+/+ mice.

We also found that the mating behavior was abnormal in Pcm1−/− mice. We did not observe mounting of Pcm1−/− females by wild-type males or mounting of wild-type females by Pcm1−/− males. This was the case even when the mice were treated with the three regimens of anticonvulsant drugs described above. We then performed a mating experiment involving Pcm1−/−, Pcm1+/−, and Pcm1+/+ mice in which all of the mice were fed chow containing the combination of clonazepam and valproic acid described above. Ten 6-week-old Pcm1+/+ or Pcm1+/+ female mice were housed with five 6-week-old Pcm1−/− males for 4 weeks (two females and one male per cage). No vaginal plugs were observed (inspections were performed daily), and none of the female mice became pregnant. Similarly, when seven 6-week-old Pcm1−/− females were housed with four Pcm1+/− or Pcm1+/+ males for 4 weeks (one or two females and one male per cage), only one of
Effects of Pcmt1 Deficiency on the Growth of Primary Embryonic Fibroblasts—The fact that antiepileptic drugs eliminated the differences in the growth in Pcmt1+/– and Pcmt1+/+ mice suggested that central nervous system dysfunction might underlie the retarded growth of Pcmt1+/– mice. However, because Pcmt1 gene expression has been observed in all tissues that have been examined, and because its absence results in an accumulation of damaged intracellular proteins (19, 20), we thought that it was important to directly test the possibility that Pcmt1-deficiency might adversely affect growth at the cellular level.

We isolated fibroblast cell lines from Pcmt1+/– embryos and compared their growth and survival in culture with fibroblasts from Pcmt1+/+ and Pcmt1+/+ embryos. First, to determine whether cultured fibroblasts normally express Pcmt1, we tested fibroblast cytosolic extracts for their capacity to transfer methyl groups from [14C]AdoMet to ovalbumin, which contains L-isoaspartyl residues. Methyltransferase activity in fibroblast cellular extracts from Pcmt1+/+ mice (13.75 ± 3.49 units/mg of protein) was almost as high as that in the cytosolic extracts of brains of Pcmt1+/+ mice (22.30 ± 1.46 units/mg of protein). As expected, Pcmt1 activity was at background levels (0.71 ± 0.22 units/mg of protein) in Pcmt1+/– fibroblasts.

To determine whether Pcmt1 deficiency affects the growth rate of cells in culture, early passage Pcmt1+/–, Pcmt1+/+ and Pcmt1+/- primary embryonic fibroblasts were plated at equal densities, and 72-h growth rates were measured. The growth rates for Pcmt1+/–, Pcmt1+/-, and Pcmt1+/- cells were essentially identical; the growth rates for Pcmt1+/– cells (n = 6) and Pcmt1+/- cells (n = 17) were 104.7 ± 5.0% and 106.5 ± 3.7%, respectively, of the growth rate of wild-type cells (n = 5). In addition, Pcmt1 deficiency had no detectable effect on the survival of early passage cells grown under minimal growth conditions (e.g. 0.5% fetal bovine serum) (Fig. 5). Thus, Pcmt1 deficiency did not affect either the short-term growth or the survival of fibroblasts. In one of the latter experiments, the accumulation of damaged amino acid residues was measured in cytosolic extracts from fibroblasts grown under minimal growth conditions. In this assay, the recombinant human Pcmt1 methyltransferase and [14C]AdoMet were used to methylate damaged amino acids within fibroblast cytosolic extracts. Not surprisingly, the Pcmt1+/– cells had nearly 3-fold higher levels of damaged cytosolic proteins (i.e. an increase in methyl-accepting protein substrates) than the Pcmt1+/- cells (data not shown).

We next assessed whether Pcmt1 deficiency might perturb the long-term growth of cultured fibroblasts. Todaro and Green (26) described the long-term growth of mouse primary embryonic fibroblasts when passaged according to 3T3, 3T6, and 3T12 protocols. Over the first 10 passages, the growth rate of these cells steadily declines. The cells then undergo a “crisis,” during which the growth rate reaches a nadir. This nadir is analogous to a Hayflick limit (30, 31) for senescent human fibroblast cell lines. Unlike senescent human cells, however, senescent mouse fibroblasts are susceptible to spontaneous immortalization, and their growth rate increases several passages after the crisis stage. We were interested in determining whether Pcmt1 deficiency would lead to a premature crisis, a prolonged crisis, or even preclude spontaneous immortalization.
tion. When the cells were grown according to a 3T6 protocol, there were minor differences in the growth rates of fibroblasts isolated from Pcm1+/− embryos (n = 5) and Pcm1+/+ embryos (n = 6) (Fig. 6). However, plotting of the growth rates for all 11 cell lines showed that Pcm1 deficiency had no detectable adverse effect on cell growth.

We also tested the hypothesis that we might be able to uncover a subtle disturbance in the growth of Pcm1+/− cells, relative to Pcm1+/+ cells, by mixing the cells together and allowing them to compete for growth in the same cell culture dish. For these experiments, equal numbers of early passage fibroblast cell lines were mixed, plated, and then passaged according to a 3T6 protocol. Southern blot analyses were performed on genomic DNA at a number of different passages, and the intensities of the wild-type and knockout bands were measured with a phosphor imager. The ratio of the wild-type and knockout bands were measured between wild-type and knockout cells in each culture before, during, and after the crisis. After 28 passages, the ratio of wild-type and knockout cells varied. However, the number of wild-type/knockout mixes containing predominantly wild-type cells equaled those containing predominantly Pcm1-deficient cells (Fig. 7). These experiments did not support a competitive disadvantage for Pcm1-deficient cells, compared with wild-type cells.

Assessing Whether N-Acetylaspartylglutamate Is a Substrate for the Pcm1-Methyltransferase—One way in which the absence of the methyltransferase deficiency might cause seizures in Pcm1+/− mice is by altering the levels of excitatory or inhibitory neurotransmitters. Although the absence of protein repair could diminish the activity of specific proteins involved in neurotransmitter metabolism, Pcm1 might also be involved more directly, perhaps through methylation of a neurotransmitter. Of interest in this regard is NAAG, an abundant brain dipeptide that is thought to be a neurotransmitter recognized more directly, perhaps through methylation. Of potential relevance to these studies, first, it is active as -NAAG by spontaneous hydrolysis. N-NAAG, might also be present because the -NAAG has two proper

![FIG. 3. Survival of Pcm1+/− mice receiving three different antiepileptic drug regimens. Groups of Pcm1+/− mice were treated with valproic acid or a combination of valproic acid and clonazepam, as indicated.](image)

![FIG. 4. Body weights in male and female Pcm1+/− and Pcm1+/+ mice. A, body weights of Pcm1+/− and Pcm1+/+ mice fed an ad libitum chow diet in the absence of drug therapy. Mice were weaned onto a normal chow diet at 21 days of age, and body weights were measured at 7 weeks of age. Male Pcm1+/+ mice were heavier than male Pcm1+/− mice (23.07 ± 1.804 g (n = 9) versus 20.64 ± 1.732 g (n = 5); p = 0.029). Female Pcm1+/+ mice were also heavier than female Pcm1+/− mice (19.01 ± 1.322 g (n = 9) versus 16.78 ± 0.936 g (n = 5); p = 0.006). B, body weights of Pcm1+/− and Pcm1+/+ mice treated with valproic acid 180 μg/g/day. Mice were weaned onto a normal chow diet at 21 days of age, and drug therapy was initiated. Body weights were measured at 7 weeks of age. During drug therapy, there were no significant weight differences between male Pcm1+/− and male Pcm1+/+ mice (23.643 ± 2.427 g (n = 7) versus 23.6 ± 2.131 g (n = 8); p = 0.9715); similarly, there were no weight differences between female Pcm1+/− and female Pcm1+/+ mice (19.925 ± 2.738 g (n = 4) versus 19.88 ± 1.474 g (n = 10); p = 0.9683). The addition of valproic acid to the chow did not affect the weight of male Pcm1+/− mice (p = 0.5844) or female Pcm1+/− mice (p = 0.1959). However, valproic acid caused an increase in the weight of both male Pcm1+/− mice (p = 0.0327) and female Pcm1+/− mice (p = 0.0452). All statistical comparisons were made with a two-tailed t test.

If β-NAAG is normally methylated by the methyltransferase in a pathway leading to its conversion to NAAG, it is conceivable that β-NAAG would accumulate in the brains of Pcm1+/− mice. Such an accumulation would be expected to disrupt the balance of excitatory and inhibitory neurotransmitters. To de-
determine whether this is in fact the case, we measured NAAG and \( \beta \)-NAAG levels in brain cytosolic extracts by high performance liquid chromatography and anion-exchange chromatography (Fig. 8). No significant differences in NAAG levels in brain extracts were found in brain extracts of wild-type and knockout mice, and little or no \( \beta \)-NAAG was detected in either extract. Urine samples from \( \text{Pcmt1}/+ \) and \( \text{Pcmt1}/– \) mice were also examined. Interestingly, only \( \beta \)-NAAG was detectable in the urine, and the levels were similar in the \( \text{Pcmt1}/+ \) and \( \text{Pcmt1}/– \) mice.

Although the overall levels of the NAAG in whole brains did not appear to be different in the wild-type and knockout mice, these results do not exclude the possibility that \( \text{Pcmt1} \) is involved in NAAG metabolism within a specific cellular compartment. Although the latter issue is difficult to address definitively, we reasoned that it would at least be possible to determine whether \( \beta \)-NAAG is a substrate for the methyltransferase in vitro. Using recombinant human \( \text{Pcmt1} \) and \([^{14}\text{C}]\text{AdoMet} \), we found that \( \beta \)-NAAG is methylated by the enzyme with a \( V_{\text{max}} \) of \(-2300 \text{ pmol/min/mg} \) of protein (about 20% of that for ovalbumin (33)) and a \( K_m \) of 1.53 \text{ mM}. We also tested N-acetylaspartate, a breakdown product of NAAG, and found that it was a very low affinity substrate for the methyltransferase, with a \( V_{\text{max}} \) of only 17% that of ovalbumin and a \( K_m \) of 57 \text{ mM}. No methylation was detected with 5 \text{ mM} NAAG or with other related carboxylic acids, such as 5 \text{ mM} \gamma\text{-aminobutyric acid} (an inhibitory neurotransmitter), 2 \text{ mM} NMDA (the synthetic glutamate receptor agonist), 64 \text{ mM} \text{L-aspartic acid}, or 64 \text{ mM} \text{L-asparagine}.

The fact that \( \beta \)-NAAG was recognized and methylated by \( \text{Pcmt1} \) (albeit with a relatively low affinity) raised the possibility that \( \text{Pcmt1} \) might modulate \( \beta \)-NAAG levels in vivo. To further assess this possibility, we performed computer modeling of the spontaneous and enzymatic reactions involved in \( \beta \)-NAAG formation and repair (34). We assumed that the amount of NAAG that can be extracted from \( \text{Pcmt1}/+ \) mouse brain (determined in this study as 3.06 \text{ nmol/mg} of soluble protein) corresponds to a concentration of 89 \text{ pM}. The rate constant for the spontaneous formation of the NAAG succinimide intermediate was assumed to be similar to that measured for the peptide VYPDAA (\( t_{\frac{1}{2}} = 263 \text{ days} \) at pH 7.4 and 37 °C) (2). The rates of succinimide hydrolysis and racemization were assumed to be the same as those measured for the peptide VYP-Asu-GA, where Asu denotes the aspartyl succinimide (1). The enzymatic methylation of \( \beta \)-NAAG was represented on the Michaelis-Menten equation, using the \( K_m \) (1.53 \text{ mM}) measured in this study; and the velocity of the reaction (81.2 \text{ nm/min}) was calculated from the \( V_{\text{max}} \) measured here and the amount of \( \text{Pcmt1} \) activity in wild-type mouse brain (19). The rate of spontaneous demethylation of the \( \beta \)-NAAG-methyl ester was estimated to be similar to that of VYP-(isoAsp-methyl ester)-AA (\( t_{\frac{1}{2}} = 43 \text{ min} \)) (2). The change in each component of the pathway
but could not prevent, the fatal seizure disorder.

An obvious phenotype of the Pcm1 knockout mice is retarded growth (19). Initially, we hypothesized that the retarded growth in the Pcm1 knockout mice was due to inefficient growth at the cellular level, caused by the widespread accumulation of damaged intracellular proteins (19, 20). However, the current findings support the viewpoint that the growth disturbance is likely a consequence of the seizure disorder. First, extensive studies of cellular growth in Pcm1-deficient fibroblasts uncovered no growth abnormality, despite the fact that wild-type fibroblasts express a high level of the enzyme activity and accumulate damaged proteins in its absence. Second, there was no detectable difference in the weights of Pcm1+/+ and Pcm1−/− mice when they were treated with valproic acid. In those experiments, valproic acid did not measurably affect the weight of Pcm1+/+ mice, but it increased the weight of the Pcm1−/− mice. Thus, it is reasonable to conclude that the increased weight of valproic acid-treated Pcm1−/− mice was due to a suppression of seizure activity rather than to an appetite-stimulating effect of the drug.

Another obvious phenotype of the Pcm1 knockout mice is that the homozygous animals could not mate or reproduce, even when they were treated with the combination of clonazepam and valproic acid. Although it is conceivable that Pcm1 deficiency in the gonads or endocrine tissues might contribute to the apparent sterility of these animals, several recent observations suggest that the abnormal mating behavior is simply another manifestation of central nervous system dysfunction. First, we recently rescued the lethal seizure disorder in Pcm1−/− mice with a Pcm1 transgene under the control of a neuron-specific promoter, and we have documented that the abnormal mating behavior in the rescued Pcm1 knockout mice is significantly improved. Second, we have noted that spermatogenesis from male Pcm1−/− mice have normal morphology, motility, and ability to fertilize mouse eggs.

At this point, the mechanism for the seizures in Pcm1-deficient mice remains unclear. As soon as we recognized seizures to be a hallmark of Pcm1 deficiency, we immediately considered the possibility that an inherited form of human epilepsy might map to the PCMT1 locus. We were initially quite intrigued by the fact that Lafayette’s epilepsy mapped to human chromosome 6q23–25 (35) within a 17-centimorgan region that overlapped with the location of the human PCMT1 gene (6q22.3–24 region) (36). Although the tissues of Pcm1-deficient mice did not reveal the characteristic intracellular Lafora inclusion bodies (found in tissues of humans with this form of epilepsy), it seemed conceivable that the Pcm1-deficient mice simply died before the characteristic pathology developed. To explore this issue, we examined PCMT1 activity levels and PCMT1 heat stability in erythrocytes from human Lafora patients (kindly provided by Dr. Antonio Delgado-Escueta, University of California, Los Angeles, CA), and sequenced the exons of the PCMT1 gene from Lafora disease patients. Neither of these approaches uncovered abnormalities. Subsequent fine mapping studies revealed that the PCMT1 gene maps at least 1.7 megabases telomeric of the D6S311 marker, which represents the telomeric border of the Lafora disease gene (35). Together, these studies exclude the PCMT1 gene as a culprit in Lafayette’s epilepsy.

We have also considered the possibility that Pcm1 might cause epilepsy by directly affecting neurotransmitter metabolism in the brain. We were particularly interested in the dipeptide NAAG, which is abundant in the brain, because it contains

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**Fig. 3. Identification and quantification of NAAG in brain cell cytosol from Pcm1+/+ and Pcm1−/− mice by anion-exchange high performance liquid chromatography.** The UV absorbance of the eluent was monitored at 214 nm. The elution positions of NAAG and β-NAAG at 23 and 28 min, respectively, were determined with synthetic standards. The top two curves show representative chromatograms of brain cytosol from two Pcm1+/+ mice; the bottom two curves show chromatograms from two Pcm1−/− mice. We estimated an average content of NAAG to be 3.0 pmol/mg of protein for brain cytosol extracts from two Pcm1+/+ mice and 2.7 pmol/mg of protein for extracts from two Pcm1−/− mice. Using similar methods, we determined that the concentration of β-NAAG in pooled urine samples was 0.31 μmol/mg of creatinine/creatinine for the Pcm1+/+ mice and 0.41 μmol/mg of creatinine/creatinine for the Pcm1−/− mice.

**DISCUSSION**

The cytosolic protein L-isoaspartate (d-aspartate) O-methyltransferase, or protein carboxyl methyltransferase, can convert isomerized and racemized aspartyl residues to normal L-aspartyl residues (10–15, 34). In vitro, the Pcm1 “repair” reaction actually improves the functional properties of proteins (14, 15). Mice lacking Pcm1 are small but otherwise appear grossly normal. Damaged proteins accumulate in their tissues, most notably in the brain, and the mice die suddenly from generalized seizures, typically by 40–45 days of age (19). In the current study, we demonstrate that the abnormal brain electrical activity in these mice is not simply a rare stochastic event, as one might have reasonably suspected from the overt phenotype of the mice, but instead is pervasive, occurring for ~50% of each 24-h period. Antiepileptic drug therapy with valproic acid or a combination of valproic acid and clonazepam mitigated, 

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*3 C. G. DeVry and S. Clarke, unpublished observations.*

*2 E. Kim, unpublished observations.*
an aspartyl residue that we suspected could isomerize, leading to the formation of the isoaspartyl-containing dipeptide β-NAAG. NAAG activates both an ionotropic glutamate receptor (NMDA-type) (32) and the metabotropic glutamate receptor 3 and can be hydrolyzed by a cell-surface dipeptidase (glutamate carboxypeptidase II) into N-acetylaspartyl and glutamate, a neurotransmitter with broader specificity and stronger excitatory activity than NAAG (32, 37). In experimental animals, high local concentrations of NAAG in the brain cause seizures (38). In vitro, β-NAAG also behaves as a glutamate receptor agonist (21). Unlike NAAG, however, β-NAAG cannot be hydrolyzed by the dipeptidase and, in fact, competitively inhibits NAAG catabolism (23). Finally, the studies presented in this paper indicate that Pcmt1 does methylate β-NAAG, and this methylation reaction would be expected to lead to the conversion of β-NAAG to NAAG. These considerations made it appear plausible that Pcmt1 deficiency might lead to an accumulation of β-NAAG, which in turn might have a significant impact on brain neurotransmitter metabolism.

However, despite the intuitive attractiveness of a NAAG/β-NAAG-Pcmt1 connection, our current studies suggest that we should be extremely cautious about the physiologic importance of such a connection. First, both NAAG levels in the brain and β-NAAG levels in the urine were quite similar in wild-type and knockout mice. Second, the Vmax of the methyltransferase reaction was relatively low when β-NAAG was the substrate, and computer modeling predicted that the Pcmt1-mediated reaction would have only a small impact on the steady-state.

The fact that Pcmt1−/− mice die from fatal seizures indicates that Pcmt1 plays a critical role in the brain. In the initial description of Pcmt1 knockout mice (19), we demonstrated that the absence of Pcmt1 leads to high levels of substrates for the methyltransferase (i.e., proteins containing isoaspartyl residues) in brain cytosol extracts. Given the accumulation of abnormal proteins in the brain and the ability of isoaspartyl residues within proteins to impair protein function (14, 15), it seemed reasonable to hypothesize that the seizures might be caused by a defective brain protein—either diminished function of an enzyme, or perhaps a structurally altered protein with a “gain of function.” Our current studies do not exclude that scenario. However, it is important to note: 1) that the central nervous system phenotype caused by Pcmt1 deficiency is severe and lethal; 2) that Pcmt1-deficient fibroblasts grow normally, despite the absence of the methyltransferase; and 3) that Pcmt1 deficiency does not cause detectable pathology outside of the central nervous system (at least in young mice), even though there is a substantial accumulation of damaged cytosolic proteins in many tissues. These findings have led us to believe that it may be time to consider the possibility that this protein methyltransferase plays a role in the brain unrelated to protein repair, such as a role in the biosynthesis of a small molecule, perhaps a neurotransmitter, or in the degradation of a small, bioactive compound. As noted earlier, we have doubts that β-NAAG is that mysterious small molecule, even though the isomerized version of this molecule is methylated by Pcmt1. In the future, however, we believe that it will be important to continue the search for small molecule substrates for this methyltransferase in the central nervous system. For these studies, the Pcmt1-deficient mice will be a valuable research tool.

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