

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

(Received for publication, February 11, 1999)

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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. *Pcmt1*<sup>-/-</sup> 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 *Pcmt1*<sup>-/-</sup> 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 *Pcmt1*<sup>-/-</sup> mice can be mitigated but not eliminated by antiepileptic drugs. Interestingly, antiepileptic therapy normalized the growth of *Pcmt1*<sup>-/-</sup> 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 *Pcmt1*<sup>-/-</sup> 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 cytosolic protein L-isoaspartate (D-aspartate) O-methyltransferase, also termed protein carboxyl methyltransferase 1 (*Pcmt1*),<sup>1</sup> can initiate the conversion of L-isoaspartyl residues to L-aspartyl res-

idues by forming the methyl ester of the L-isoaspartyl residue (10). This ester is then converted, in a nonenzymatic reaction, to an L-succinimidyl residue. Spontaneous hydrolysis of the L-succinimidyl 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 *Pcmt1* knockout mice (*Pcmt1*<sup>-/-</sup> mice) (19). At the biochemical level, methyltransferase substrates (*i.e.*, damaged proteins containing L-isoaspartyl residues) accumulated in all tissues of *Pcmt1*<sup>-/-</sup> mice; the highest levels were found in the brain. At the whole-animal level, the *Pcmt1*<sup>-/-</sup> 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 *Pcmt1* knockout mice was confirmed by another group of investigators (20).

The observation of fatal seizures in *Pcmt1*<sup>-/-</sup> mice highlighted the importance of *Pcmt1* 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 *Pcmt1* 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 *Pcmt1* 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*-acetylisoaspartylglutamate ( $\beta$ -NAAG) might also exist

partylglutamate; EEG, electroencephalogram; AdoMet, *S*-adenosylmethionine; NMDA, *N*-methyl-D-aspartic acid.

\* This work was supported by National Institutes of Health Grants HL47660 and AG15451-01 (to S. G. Y.) and GM26020 (to S. C.). 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.

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<sup>1</sup> The abbreviations used are: *Pcmt1*, protein carboxyl methyltransferase 1; NAAG, *N*-acetylaspartylglutamate;  $\beta$ -NAAG, *N*-acetylisoas-

and be a substrate for *Pcmt1*.  $\beta$ -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 *Pcmt1* knockout mice was due to inefficient growth of *Pcmt1*-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

***Pcmt1*-deficient Mice**—The generation of *Pcmt1* knockout mice has been described previously (19). For this study, homozygous *Pcmt1*-deficient mice (*Pcmt1*<sup>-/-</sup>) were produced by intercrossing heterozygous *Pcmt1*-deficient mice (*Pcmt1*<sup>+/-</sup>). 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  $\mu$ g/g of body weight). A small midline incision was made in the abdomen, and a radiofrequency transmitter (PhysioTel Implant, model TA10EA-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 skull (extending from the base of the skull 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 III software (Biopac Systems, Inc., Santa Barbara, CA).

**Treatment of Mice with Antiepileptic Drug Therapy**—Antiepileptic drugs were administered by adding them to the chow or to the water. The dosage was based on the fact that mice consume approximately 0.13 g of chow and 0.20 ml of water per gram of body weight per day (25). In some experiments, valproic acid (Sigma) was added to the water bottle (final concentrations of either 0.6 or 0.9 mg/ml), so that the average daily intake of valproic acid was predicted to be either 120 or 180  $\mu$ g/g. In other experiments, both valproic acid and clonazepam (Sigma) were added to the chow at 1.35 mg and 1.5  $\mu$ g per gram of chow, respectively. The drugs were dissolved in 1.5 liters of ethanol, mixed 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 was 180 and 0.2  $\mu$ g/g, respectively.

To assess the effects of these interventions on the survival of *Pcmt1*<sup>-/-</sup> mice, 21-day-old mice were given valproic acid alone (120 ( $n = 20$ ) or 180  $\mu$ g/g ( $n = 11$ )), a combination of valproic acid and clonazepam ( $n = 12$ ), or no treatment ( $n = 11$ ). The food and water in each cage were changed every 3 days.

**Analyzing the Growth of *Pcmt1*-deficient Cells in Culture**—To examine the growth characteristics of *Pcmt1*-deficient cells, we isolated fibroblasts from *Pcmt1*<sup>+/+</sup>, *Pcmt1*<sup>+/-</sup>, and *Pcmt1*<sup>-/-</sup> embryos. *Pcmt1*<sup>+/-</sup> mice were intercrossed, and pregnant females were sacrificed at 12.5 days post coitus. Embryos were dissected from the uterus and placenta, and yolk sacs were retained for genotyping. Embryos were incubated in 5 ml of 0.25% trypsin-EDTA (Life Technologies, Inc.) for 8 h at 4 °C and then for 20 min at 37 °C. The trypsin solution was removed, and the embryos were mechanically disrupted by repeated pipetting in 5 ml of cell culture medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (v/v), L-glutamine, nonessential amino acids, penicillin-streptomycin (all from Life Technologies), and 2-mercaptoethanol (Sigma; final concentration, 100  $\mu$ M)). After the debris had settled, the cell suspension was removed, diluted to 1  $\times$  10<sup>7</sup> ml with medium, and plated in T75 flasks. Approximately 1  $\times$  10<sup>7</sup> cells were obtained from each embryo. The cells were grown in an incubator with 7% CO<sub>2</sub> at 37 °C.

The fibroblasts were passaged according to a 3T6 protocol (26). Cells were trypsinized every 3 days, counted, and replated at a density of 2  $\times$  10<sup>6</sup> cells per 100-mm Petri dish. For 2- and 3-day growth experiments, 1  $\times$  10<sup>4</sup> 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 *Pcmt1*<sup>+/-</sup> and *Pcmt1*<sup>-/-</sup> fibroblasts were normalized to the growth rate observed in *Pcmt1*<sup>+/+</sup> fibroblasts.

Next we examined the survival characteristics of *Pcmt1*<sup>-/-</sup> fibroblasts. Each fibroblast cell line (passage 4) was plated onto 96-well plates at a density of 1  $\times$  10<sup>4</sup> 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 *Pcmt1*<sup>-/-</sup> 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 *Pcmt1*<sup>-/-</sup> 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 BioImaging analyzer, BAS 1000 with MacBAS, Fuji Medical Systems, USA, Inc., Stamford, CT).

**Measuring Protein L-Isoaspartate (D-Aspartate) O-Methyltransferase Activity**—Fibroblasts from a 100-mm plate were lysed in 2 ml of a hypotonic solution (0.1 $\times$  phosphate-buffered saline) and then disrupted with a Dounce homogenizer. Methyltransferase activity in the cytosolic fraction was assessed by measuring the ability of the extracts to transfer [<sup>14</sup>C]methyl groups from *S*-adenosyl-L-[methyl-<sup>14</sup>C]methionine ([<sup>14</sup>C]AdoMet) to L-isoaspartyl residues in chicken ovalbumin. Briefly, 4  $\mu$ l of the cytosolic fraction was incubated with 0.8 mg of ovalbumin (Sigma, grade V) and [<sup>14</sup>C]AdoMet (Amersham Pharmacia Biotech, 55 mCi/mmol; final concentration, 10  $\mu$ M) in 31  $\mu$ l 0.2 M BIS-TRIS, pH 6.0, for 15 min at 37 °C. After this incubation, 70  $\mu$ l of 0.2 M sodium hydroxide was added to quench the reaction and to convert the base-labile [<sup>14</sup>C]methyl esters to [<sup>14</sup>C]methanol. The base-treated mixture was then spotted onto a 2  $\times$  8-cm piece of filter paper (Bio-Rad, no. 165–090) and immediately wedged into the neck of a 20-ml scintillation vial containing 5 ml of Safety-Solve II counting fluor (Research Products International). The vial was capped and allowed to equilibrate at room temperature for 2 h, during which the [<sup>14</sup>C]methanol diffuses into the fluor and the nonvolatile [<sup>14</sup>C]AdoMet remains on the filter paper. The filter paper was then removed, and the radioactivity in the vials was counted. In parallel, the [<sup>14</sup>C]methanol radioactivity was measured in reactions lacking ovalbumin, and that amount of radioactivity was considered to be the background of the assay and was subtracted from the tubes containing ovalbumin. A unit of methyltransferase activity was defined as 1.0 pmol of methyl groups transferred per minute under these conditions. The amount of protein in the cytosol preparations was determined with a Lowry protein assay (27).

**Quantitation of the Damaged Amino Acid Residues**—To measure the amount of damaged amino acid residues within cytosol preparations, we used recombinant human L-isoaspartate (D-aspartate) O-methyltransferase to transfer [<sup>14</sup>C]methyl groups from [<sup>14</sup>C]AdoMet to damaged aspartyl and asparaginyl residues within cytosolic extracts. The extent of methylation of the cytosolic proteins reflects the extent of the damaged amino acid residues. In its basic format, this assay is similar to the methylation assay described above, except that the proteins in 10  $\mu$ l of fibroblast cytosol were used as the methyl-accepting substrates instead of ovalbumin. In addition, 44.0 units of recombinant human protein L-isoaspartate (D-aspartate) O-methyltransferase (28) were added to the cytosolic extracts to ensure complete methylation of the high affinity L-isoaspartyl/D-aspartyl residues. The methylation reaction was allowed to proceed for 120 min, after which the [<sup>14</sup>C]methyl esters were quantitated as described above.

**Methylation of Aspartyl-containing Neurotransmitters**—*N*-acetyl-L-aspartic acid (NAA), NAAG,  $\beta$ -NAAG, *N*-methyl-D-aspartic acid (NMDA), and  $\gamma$ -aminobutyric acid were obtained from Sigma. The NAAG preparation from Sigma contained an average of 6.5 mol of isopropanol and 1.7 mol of acetone per mol of NAAG. Because those reagents interfere with the methylation assay, they were removed by dissolving the compound in water and then lyophilizing the solution. The ability of each compound to be methylated by human protein L-isoaspartate (D-aspartate) O-methyltransferase was initially assayed for 1 h with recombinant methyltransferase (44.0 units) and [<sup>14</sup>C]AdoMet, according to the procedures described above. To determine the  $K_m$  values of those compounds shown to be substrates in the

initial assay, various concentrations of  $\beta$ -NAAG (0.025–3.2 mM) and NAA (3.3 mM) were methylated as described above, except that 5.5 units of enzyme were used and the incubation time was 11 min in a 1.0 M sodium HEPES buffer, pH 7.5. The kinetic constants for the methylation of these substrates were determined by fitting the data points to the Michaelis-Menten equation with DeltaGraph 4.0 (DeltaPoint, Bellevue, WA).

**Analysis of NAAG and  $\beta$ -NAAG in Mouse Brain and Urine—***Pcmt1*<sup>-/-</sup> and *Pcmt1*<sup>+/+</sup> mice were euthanized by cervical dislocation, and the brains were removed rapidly. Cytosolic extracts were prepared as described previously (19). Urine was also collected from groups of four *Pcmt1*<sup>-/-</sup> and four *Pcmt1*<sup>+/+</sup> mice housed in metabolic cages. NAAG and  $\beta$ -NAAG were isolated with a high performance liquid chromatography system (two model 510 pumps with a U6K injector and a model 441 absorbance detector set to 214 nm), according to published procedures (29). Aliquots of brain cytosol (100  $\mu$ l, 0.99–2.14 mg/ml) and urine (100  $\mu$ l) were injected onto an anion-exchange column (Partisil SAX; particle size, 10  $\mu$ m; 250  $\times$  4.6 mm; Alltech) equilibrated in 60 mM monopotassium phosphate, pH 4.5, and the peptides were eluted isocratically at 1.0 ml/min. The peptides were identified and quantified by comparison with synthetic NAAG and  $\beta$ -NAAG (15 nmol each), which eluted from the column after 23 and 28 min, respectively.

## RESULTS

**Analysis of Seizure Activity in *Pcmt1*<sup>-/-</sup> Mice—**We previously reported that *Pcmt1*<sup>-/-</sup> 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 episodic seizures or was more pervasive, we performed continuous electroencephalographic monitoring in *Pcmt1*<sup>-/-</sup> mice and in littermate controls (*Pcmt1*<sup>+/+</sup> mice). Abnormal cortical electrical activity, consisting of high amplitude spikes and waves, was present during ~50% of each 24-h period for the *Pcmt1*<sup>-/-</sup> mice; controls showed no abnormal activity (Fig. 1). Remarkably, the abnormal electrical activity was present in the *Pcmt1*<sup>-/-</sup> 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 *Pcmt1*<sup>-/-</sup> Mice with Antiepileptic Drug Therapy—**To determine whether antiepileptic drug therapy might prevent or mitigate the lethal seizure disorder, groups of *Pcmt1*<sup>-/-</sup> mice were treated with valproic acid or a combination of both valproic acid and clonazepam. A low dose of valproic acid (120  $\mu$ g/g/day) extended the survival of *Pcmt1*<sup>-/-</sup> mice, but by only 10–15 days (Fig. 3). A higher dose of valproic acid (180  $\mu$ g/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 *Pcmt1*<sup>-/-</sup> Mice—***Pcmt1*<sup>-/-</sup> mice grew more slowly than *Pcmt1*<sup>+/+</sup> littermate controls. At 7 weeks of age, both male and female *Pcmt1*<sup>-/-</sup> 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 *Pcmt1* defi-

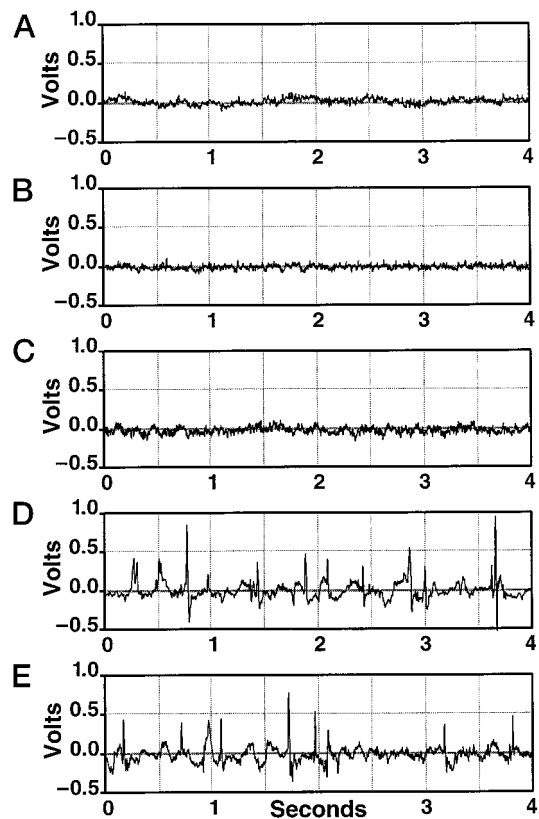


FIG. 1. **Representative EEG tracings.** A and B, representative EEG tracings from *Pcmt1*<sup>+/+</sup> mice. C, an EEG from a *Pcmt1*<sup>-/-</sup> mouse that appears to be normal. D and E, abnormal EEG recordings from the same *Pcmt1*<sup>-/-</sup> mouse a few minutes later. Abnormal cortical electrical activity in *Pcmt1*<sup>-/-</sup> mice, as illustrated in D and E, was present for approximately 50% of each 24-h period.

ciency and of the accumulation of damaged proteins containing L-isoaspartyl residues. However, the pervasive EEG abnormalities in *Pcmt1*<sup>-/-</sup> 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 *Pcmt1*<sup>-/-</sup> mice and their littermates would be reduced or eliminated by antiepileptic drug therapy. To test this possibility, we placed male and female *Pcmt1*<sup>-/-</sup> and *Pcmt1*<sup>+/+</sup> mice on valproic acid (180  $\mu$ g/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 *Pcmt1*<sup>+/+</sup> mice at 7 weeks of age, but it significantly increased the body weight of *Pcmt1*<sup>-/-</sup> mice and eliminated the differences in body weight between *Pcmt1*<sup>-/-</sup> and *Pcmt1*<sup>+/+</sup> mice.

We also found that the mating behavior was abnormal in *Pcmt1*<sup>-/-</sup> mice. We did not observe mounting of *Pcmt1*<sup>-/-</sup> females by wild-type males or mounting of wild-type females by *Pcmt1*<sup>-/-</sup> 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 *Pcmt1*<sup>-/-</sup>, *Pcmt1*<sup>+/-</sup>, and *Pcmt1*<sup>+/+</sup> mice in which all of the mice were fed chow containing the combination of clonazepam and valproic acid described above. Ten 6-week-old *Pcmt1*<sup>+/-</sup> or *Pcmt1*<sup>+/+</sup> female mice were housed with five 6-week-old *Pcmt1*<sup>-/-</sup> 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 *Pcmt1*<sup>-/-</sup> females were housed with four *Pcmt1*<sup>+/-</sup> or *Pcmt1*<sup>+/+</sup> males for 4 weeks (one or two females and one male per cage), only one of

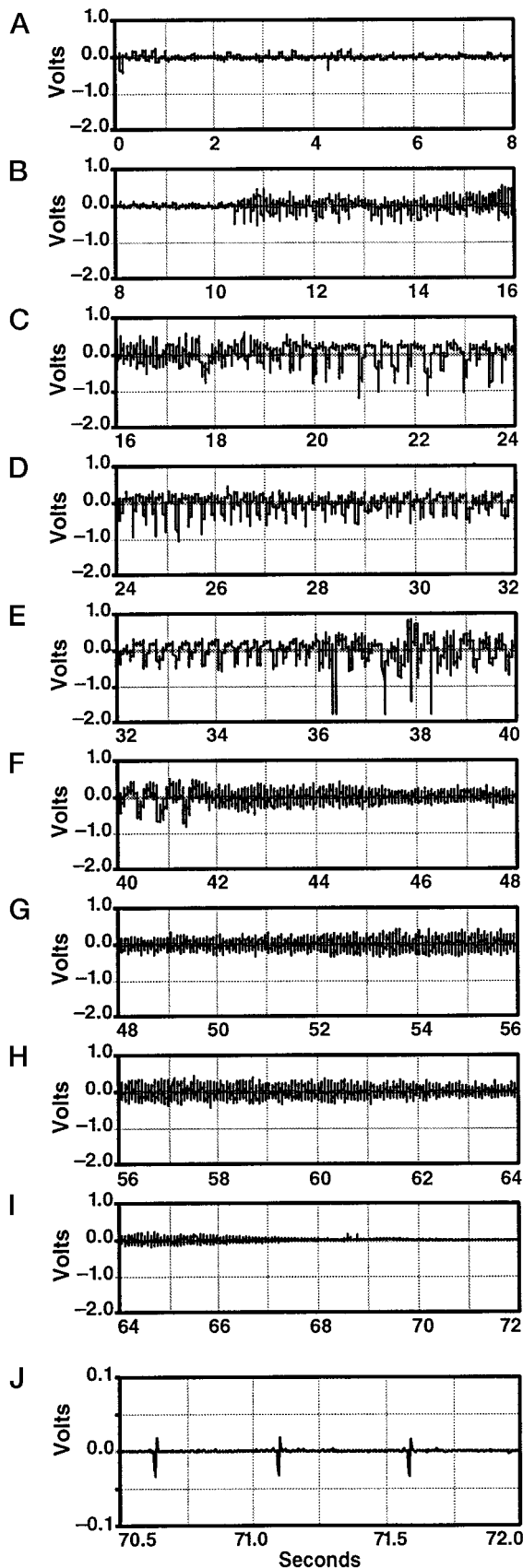


FIG. 2. An EEG recording of a fatal seizure in a *Pcmt1*<sup>-/-</sup> mouse. The fatal seizure began with high amplitude spikes and waves, followed by diminished cortical activity in the postictal period. A–I show consecutive 8-s intervals throughout the course of the seizure. J shows a 1.5-s interval at the conclusion of the seizure, with the voltage range adjusted to show from 0 to 0.1 volts. At that point, the only detectable electrical potentials were cardiac depolarizations.

the *Pcmt1*<sup>-/-</sup> females was noted to have a vaginal plug, and that mouse did not become pregnant. At the same time, eight *Pcmt1*<sup>+/-</sup> and *Pcmt1*<sup>+/+</sup> males were housed with 16 *Pcmt1*<sup>+/-</sup> and *Pcmt1*<sup>+/+</sup> females (two females and one male per cage). Despite the anticonvulsant therapy, nine of the 16 females were observed to have a vaginal plug, and all nine became pregnant.

**Effects of *Pcmt1* Deficiency on the Growth of Primary Embryonic Fibroblasts**—The fact that antiepileptic drugs eliminated the differences in the growth in *Pcmt1*<sup>-/-</sup> and *Pcmt1*<sup>+/+</sup> mice suggested that central nervous system dysfunction might underlie the retarded growth of *Pcmt1*<sup>-/-</sup> 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*<sup>-/-</sup> embryos and compared their growth and survival in culture with fibroblasts from *Pcmt1*<sup>+/-</sup> and *Pcmt1*<sup>+/+</sup> embryos. First, to determine whether cultured fibroblasts normally express *Pcmt1*, we tested fibroblast cytosolic extracts for their capacity to transfer methyl groups from [<sup>14</sup>C]AdoMet to ovalbumin, which contains L-isoaspartyl residues. Methyltransferase activity in fibroblast cellular extracts from *Pcmt1*<sup>+/+</sup> mice ( $13.75 \pm 3.49$  units/mg of protein) was almost as high as that in the cytosolic extracts of brains of *Pcmt1*<sup>+/+</sup> mice ( $22.30 \pm 1.46$  units/mg of protein). As expected, *Pcmt1* activity was at background levels ( $0.71 \pm 0.22$  units/mg of protein) in *Pcmt1*<sup>-/-</sup> fibroblasts.

To determine whether *Pcmt1* deficiency affects the growth rate of cells in culture, early passage *Pcmt1*<sup>-/-</sup>, *Pcmt1*<sup>+/-</sup>, and *Pcmt1*<sup>+/+</sup> primary embryonic fibroblasts were plated at equal densities, and 72-h growth rates were measured. The growth rates for *Pcmt1*<sup>-/-</sup>, *Pcmt1*<sup>+/-</sup>, and *Pcmt1*<sup>+/+</sup> cells were essentially identical; the growth rates for *Pcmt1*<sup>-/-</sup> cells ( $n = 6$ ) and *Pcmt1*<sup>+/-</sup> cells ( $n = 17$ ) were  $104.7 \pm 5.0\%$  and  $106.5 \pm 3.7\%$ , 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 [<sup>14</sup>C]AdoMet were used to methylate damaged amino acids within fibroblast cytosolic extracts. Not surprisingly, the *Pcmt1*<sup>-/-</sup> cells had nearly 3-fold higher levels of damaged cytosolic proteins (i.e. an increase in methyl-accepting protein substrates) than the *Pcmt1*<sup>+/+</sup> 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 immortaliza-

FIG. 3. Survival of *Pcmt1*<sup>-/-</sup> mice receiving three different antiepileptic drug regimens. Groups of *Pcmt1*<sup>-/-</sup> mice were treated with valproic acid or a combination of valproic acid and clonazepam, as indicated.

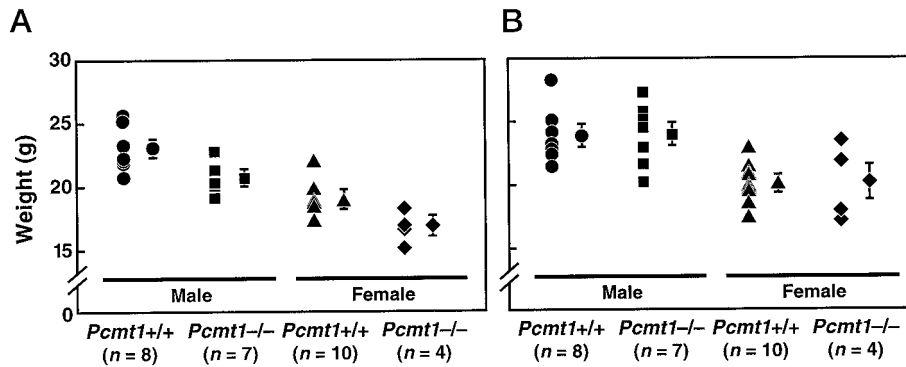
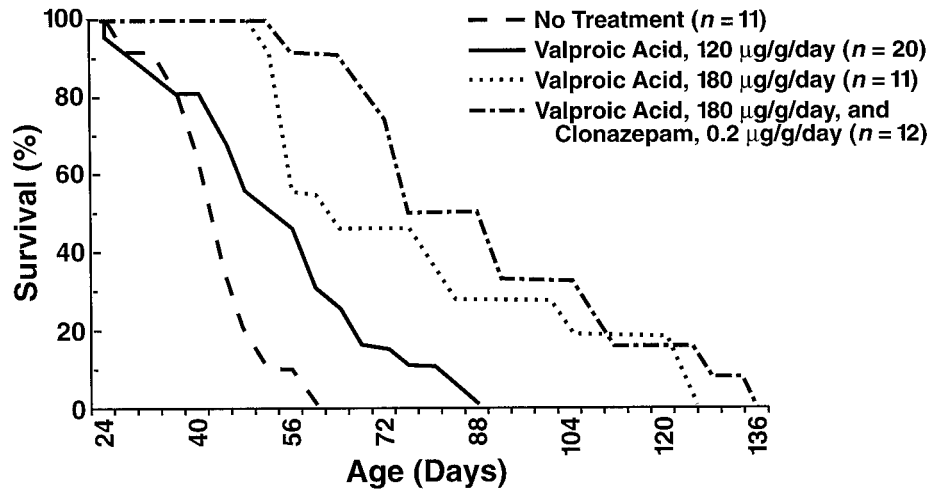


FIG. 4. Body weights in male and female *Pcmt1*<sup>-/-</sup> and *Pcmt1*<sup>+/+</sup> mice. A, body weights of *Pcmt1*<sup>-/-</sup> and *Pcmt1*<sup>+/+</sup> mice fed an *ad libitum* chow diet in the absence of drug therapy. Mice were weaned onto a normal chow diet at 21 days of age, and body weights were measured at 7 weeks of age. Male *Pcmt1*<sup>+/+</sup> mice were heavier than male *Pcmt1*<sup>-/-</sup> mice ( $23.07 \pm 1.804$  g ( $n = 9$ ) versus  $20.64 \pm 1.372$  g ( $n = 5$ );  $p = 0.023$ ). Female *Pcmt1*<sup>+/+</sup> mice were also heavier than female *Pcmt1*<sup>-/-</sup> mice ( $19.01 \pm 1.322$  g ( $n = 9$ ) versus  $16.78 \pm 0.936$  g ( $n = 5$ );  $p = 0.006$ ). B, body weights of *Pcmt1*<sup>-/-</sup> and *Pcmt1*<sup>+/+</sup> mice treated with valproic acid  $180 \mu\text{g/g/day}$ . 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 *Pcmt1*<sup>-/-</sup> and male *Pcmt1*<sup>+/+</sup> mice ( $23.643 \pm 2.427$  g ( $n = 7$ ) versus  $23.6 \pm 2.131$  g ( $n = 8$ );  $p = 0.9715$ ); similarly, there were no weight differences between female *Pcmt1*<sup>-/-</sup> and female *Pcmt1*<sup>+/+</sup> mice ( $19.925 \pm 2.738$  g ( $n = 4$ ) versus  $19.88 \pm 1.474$  g ( $n = 10$ );  $p = 0.9683$ ). The addition of valproic acid to the chow did not affect the weight of male *Pcmt1*<sup>+/+</sup> mice ( $p = 0.5844$ ) or female *Pcmt1*<sup>+/+</sup> mice ( $p = 0.1959$ ). However, valproic acid caused an increase in the weight of both male *Pcmt1*<sup>-/-</sup> mice ( $p = 0.0327$ ) and female *Pcmt1*<sup>-/-</sup> mice ( $p = 0.0452$ ). All statistical comparisons were made with a two-tailed *t* test.

tion. When the cells were grown according to a 3T6 protocol, there were minor differences in the growth rates of fibroblasts isolated from *Pcmt1*<sup>-/-</sup> embryos ( $n = 5$ ) and *Pcmt1*<sup>+/+</sup> embryos ( $n = 6$ ) (Fig. 6). However, plotting of the growth rates for all 11 cell lines showed that *Pcmt1* 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 *Pcmt1*<sup>-/-</sup> cells, relative to *Pcmt1*<sup>+/+</sup> 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 was used to gauge the relative numbers of 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 *Pcmt1*-deficient cells (Fig. 7). These experiments did not support a competitive disadvantage for *Pcmt1*-deficient cells, compared with wild-type cells.

*Assessing Whether N-Acetylisopartylglutamate Is a Substrate for the Pcmt1-methyltransferase*—One way in which the absence of the methyltransferase deficiency might cause seizures in *Pcmt1*<sup>-/-</sup> 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, *Pcmt1* also might 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 by NMDA receptors, a class of glutamate receptors implicated in the development of seizures (24, 32). Recently, the succinimidyl form of NAAG has been identified in rat brain (22), a finding that strongly suggested that the *L*-isopartyl-containing form of NAAG,  $\beta$ -NAAG, might also be present because the succinimide would be expected to form both NAAG and  $\beta$ -NAAG by spontaneous hydrolysis.  $\beta$ -NAAG has two properties of potential relevance to these studies. First, it is active as an excitatory neurotransmitter (24). Second, it cannot be cleaved by the dipeptidase that hydrolyzes NAAG (23, 24).

If  $\beta$ -NAAG is normally methylated by the methyltransferase in a pathway leading to its conversion to NAAG, it is conceivable that  $\beta$ -NAAG would accumulate in the brains of *Pcmt1*<sup>-/-</sup> mice. Such an accumulation would be expected to disrupt the balance of excitatory and inhibitory neurotransmitters. To de-

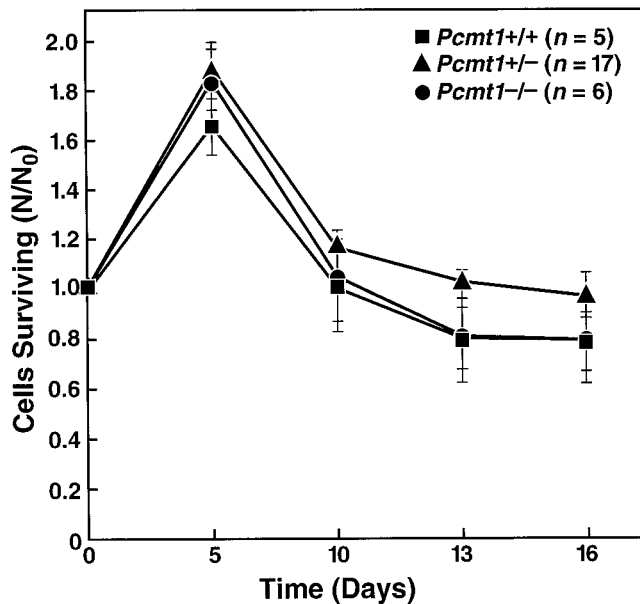


FIG. 5. Survival of *Pcmt1*<sup>+/+</sup>, *Pcmt1*<sup>+/-</sup>, and *Pcmt1*<sup>-/-</sup> cells in the presence of a low concentration of fetal bovine serum. Early passage primary embryonic fibroblasts were grown in 10% fetal bovine serum and then transferred into cell culture medium containing 0.5% fetal bovine serum. Survival was then assessed for the next 16 days.

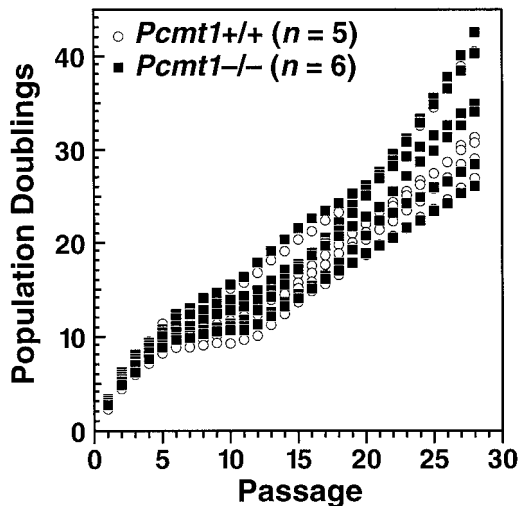


FIG. 6. Growth rates of fibroblasts isolated from *Pcmt1*<sup>-/-</sup> (n = 5) and *Pcmt1*<sup>+/+</sup> (n = 6) embryos. Cells were grown for 28 passages (3 days/passage) according to a 3T6 protocol.

termine 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 *Pcmt1*<sup>+/+</sup> and *Pcmt1*<sup>-/-</sup> mice, and little or no  $\beta$ -NAAG was detected in either extract. Urine samples from *Pcmt1*<sup>+/+</sup> and *Pcmt1*<sup>-/-</sup> mice were also examined. Interestingly, only  $\beta$ -NAAG was detectable in the urine, and the levels were similar in the *Pcmt1*<sup>+/+</sup> and *Pcmt1*<sup>-/-</sup> 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 *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 methyltrans-

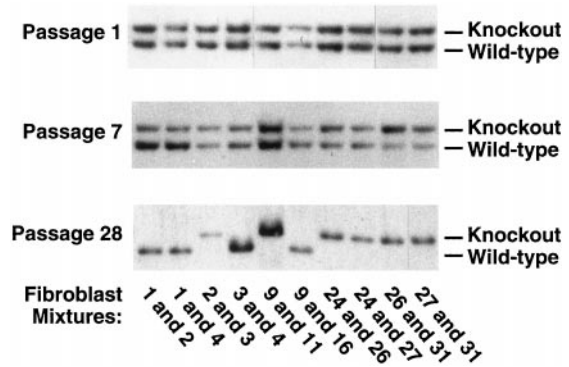


FIG. 7. The growth of *Pcmt1*<sup>-/-</sup> and *Pcmt1*<sup>+/+</sup> fibroblasts competing for growth in the same cell culture dish. Equal numbers of early passage fibroblast cell lines were mixed and passaged according to a 3T6 protocol. Southern blots were performed on the genomic DNA prepared from the mixture of cells at multiple time points. This figure shows Southern blots of the genomic DNA at passages 1, 7, and 28. For these experiments, primary embryonic fibroblasts were prepared from embryos from three different *Pcmt1*<sup>+/-</sup> intercrosses. Embryos 1, 3, 11, 24, and 31 were homozygous for the wild-type allele, whereas embryos 2, 4, 9, 16, 26, and 27 were homozygous for the knockout allele. It is clear that as early as passage 7, some of the *Pcmt1*<sup>-/-</sup> fibroblasts were in the process of overtaking the *Pcmt1*<sup>+/+</sup> fibroblasts, by virtue of their more rapid growth (see Southern blots for mixtures 26–31 and 27–31 at passage 7). On the other hand, some of the *Pcmt1*<sup>+/+</sup> fibroblasts were in the process of overtaking the *Pcmt1*<sup>-/-</sup> cells (see Southern blots for mixtures 1–2 and 1–4 at passage 7). By the time of passage 28, all of the mixtures were overgrown by either the *Pcmt1*<sup>+/+</sup> cells or the *Pcmt1*<sup>-/-</sup> cells. There was no indication of a competitive growth advantage for the *Pcmt1*<sup>+/+</sup> fibroblasts; approximately one-half of the mixtures were overgrown by *Pcmt1*<sup>+/+</sup> cells, and one-half were overgrown by *Pcmt1*<sup>-/-</sup> cells.

ferase reaction *in vitro*. Using recombinant human *Pcmt1* and [<sup>14</sup>C]AdoMet, we found that  $\beta$ -NAAG is methylated by the enzyme with a  $V_{max}$  of ~2300 pmol/min/mg of protein (about 20% of that for ovalbumin (33)) and a  $K_m$  of 1.53 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  $V_{max}$  of only 17% that of ovalbumin and a  $K_m$  of 87 mM. No methylation was detected with 5 mM NAAG or with other related carboxylic acids, such as 5 mM  $\gamma$ -aminobutyric acid (an inhibitory neurotransmitter), 2 mM NMDA (the synthetic glutamate receptor agonist), 64 mM L-aspartic acid, or 64 mM L-asparagine.

The fact that  $\beta$ -NAAG was recognized and methylated by *Pcmt1* (albeit with a relatively low affinity) raised the possibility that *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 *Pcmt1*<sup>+/+</sup> mouse brain (determined in this study as 3.06 nmol/mg of soluble protein) corresponds to a concentration of 89  $\mu$ M. The rate constant for the spontaneous formation of the NAAG succinimide intermediate was assumed to be similar to that measured for the peptide VYPDAA ( $t_{1/2}$  = 266 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 by the Michaelis-Menten equation, using the  $K_m$  (1.53 mM) measured in this study; and the velocity of the reaction (81.2 nM/min) was calculated from the  $V_{max}$  measured here and the amount of *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 ( $t_{1/2}$  = 43 min) (2). The change in each component of the pathway

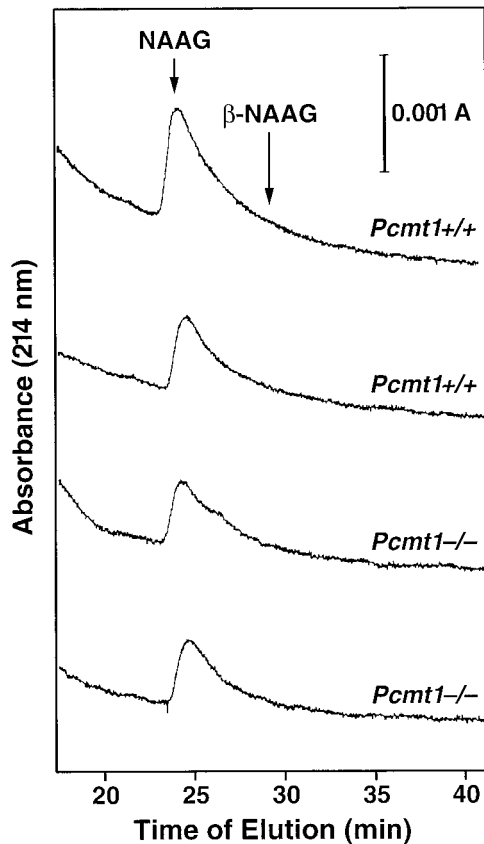


FIG. 8. Identification and quantification of NAAG in brain cell cytosol from *Pcmt1*<sup>+/+</sup> and *Pcmt1*<sup>-/-</sup> mice by anion-exchange high performance liquid chromatography. The UV absorbance of the eluent was monitored at 214 nm. The elution positions of NAAG and  $\beta$ -NAAG at 23 and 28 min, respectively, were determined with synthetic standards. The top two curves show representative chromatographs of brain cytosol from two *Pcmt1*<sup>+/+</sup> mice; the bottom two curves show chromatographs from two *Pcmt1*<sup>-/-</sup> mice. We estimated an average content of NAAG to be 3.0 pmol/mg of protein for brain cytosol extracts from two *Pcmt1*<sup>+/+</sup> mice and 2.7 pmol/mg of protein for extracts from two *Pcmt1*<sup>-/-</sup> mice. Using similar methods, we determined that the concentration of  $\beta$ -NAAG in pooled urine samples was 0.31  $\mu$ mol/mg of creatinine/creatinine for the *Pcmt1*<sup>+/+</sup> mice and 0.41  $\mu$ mol/mg of creatinine/creatinine for the *Pcmt1*<sup>-/-</sup> mice.

from its initial concentration was calculated from the above rate constants for each minute and iterated to give the cumulative changes over the 50 h of simulated time. After 50 h, the levels of  $\beta$ -NAAG in the brains of *Pcmt1* knockout mice would be predicted to be only about 3% higher than in the wild-type mice, but this estimate is highly dependent on the assumed values in the simulation.

#### DISCUSSION

The cytosolic protein L-isoadipate (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 *Pcmt1* “repair” reaction actually improves the functional properties of proteins (14, 15). Mice lacking *Pcmt1* 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,

but could not prevent, the fatal seizure disorder.

An obvious phenotype of the *Pcmt1* knockout mice is retarded growth (19). Initially, we hypothesized that the retarded growth in the *Pcmt1* 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 *Pcmt1*-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 *Pcmt1*<sup>+/+</sup> and *Pcmt1*<sup>-/-</sup> mice when they were treated with valproic acid. In those experiments, valproic acid did not measurably affect the weight of *Pcmt1*<sup>+/+</sup> mice, but it increased the weight of the *Pcmt1*<sup>-/-</sup> mice. Thus, it is reasonable to conclude that the increased weight of valproic acid-treated *Pcmt1*<sup>-/-</sup> mice was due to a suppression of seizure activity rather than to an appetite-stimulating effect of the drug.

Another obvious phenotype of the *Pcmt1* 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 *Pcmt1* 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 *Pcmt1*<sup>-/-</sup> mice with a *Pcmt1* transgene under the control of a neuron-specific promoter,<sup>2</sup> and we have documented that the abnormal mating behavior in the rescued *Pcmt1* knockout mice is significantly improved. Second, we have noted that spermatozoa from male *Pcmt1*<sup>-/-</sup> mice have normal morphology, motility, and ability to fertilize mouse eggs.<sup>2</sup>

At this point, the mechanism for the seizures in *Pcmt1*-deficient mice remains unclear. As soon as we recognized seizures to be a hallmark of *Pcmt1* 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 Lafora’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 *Pcmt1*-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 *Pcmt1*-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.<sup>3</sup> Subsequent fine mapping studies revealed that the PCMT1 gene maps at least 1.7 megabases telomeric of the D6S311 marker,<sup>3</sup> which represents the telomeric border of the Lafora disease gene (35). Together, these studies exclude the PCMT1 gene as a culprit in Lafora’s epilepsy.

We have also considered the possibility that *Pcmt1* 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

<sup>2</sup> E. Kim, unpublished observations.

<sup>3</sup> C. G. DeVry and S. Clarke, unpublished observations.

an aspartyl residue that we suspected could isomerize, leading to the formation of the isoaspartyl-containing dipeptide  $\beta$ -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*,  $\beta$ -NAAG also behaves as a glutamate receptor agonist (21). Unlike NAAG, however,  $\beta$ -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  $\beta$ -NAAG, and this methylation reaction would be expected to lead to the conversion of  $\beta$ -NAAG to NAAG. These considerations made it appear plausible that *Pcmt1* deficiency might lead to an accumulation of  $\beta$ -NAAG, which in turn might have a significant impact on brain neurotransmitter metabolism.

However, despite the intuitive attractiveness of a NAAG/ $\beta$ -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  $\beta$ -NAAG levels in the urine were quite similar in wild-type and knockout mice. Second, the  $V_{\max}$  of the methyltransferase reaction was relatively low when  $\beta$ -NAAG was the substrate, and computer modeling predicted that the *Pcmt1*-mediated repair reaction would have only a small impact on the steady-state.

The fact that *Pcmt1*<sup>-/-</sup> 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  $\beta$ -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.

**Acknowledgments**—We thank Christine Farrar for analyzing the videotape records of the mouse seizures, Christopher DeVry for providing us with the mapping data for PCMT1, Antonio Delgado-Escueta for providing materials from Lafora disease patients and for helpful discussions, and Stephen Ordway and Gary Howard for editorial assistance.

#### REFERENCES

- Geiger, T., and Clarke, S. (1987) *J. Biol. Chem.* **262**, 785–794
- Stephenson, R. C., and Clarke, S. (1989) *J. Biol. Chem.* **264**, 6164–6170
- Capasso, S., Mazzarella, L., and Zagari, A. (1991) *Pept. Res.* **4**, 234–238
- Oliyai, C., and Borchardt, R. T. (1994) *Pharm. Res.* **11**, 751–758
- Tyler-Cross, R., and Schirch, V. (1991) *J. Biol. Chem.* **266**, 22549–22556
- Visick, J. E., and Clarke, S. (1995) *Mol. Microbiol.* **16**, 835–845
- Noguchi, S., Miyawaki, K., and Satow, Y. (1998) *J. Mol. Biol.* **278**, 231–238
- Catanzano, F., Graziano, G., Capasso, S., and Barone, G. (1997) *Protein Sci.* **6**, 1682–1693
- Fujii, N., Satoh, K., Harada, K., and Ishibashi, Y. (1994) *J. Biochem.* **116**, 663–669
- Lowenson, J. D., and Clarke, S. (1995) in *Deamidation and Isoaspartate Formation in Peptides and Proteins* (Aswad, D. W., ed) pp. 47–64, CRC Press, Boca Raton, FL
- McFadden, P. N., and Clarke, S. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 2595–2599
- Johnson, B. A., Murray, E. D., Jr., Clarke, S., Glass, D. B., and Aswad, D. W. (1987) *J. Biol. Chem.* **262**, 5622–5629
- Galletti, P., Ciardiello, A., Ingresso, D., Di Donato, A., and D'Alessio, G. (1988) *Biochemistry* **27**, 1752–1757
- Johnson, B. A., Langmack, E. L., and Aswad, D. W. (1987) *J. Biol. Chem.* **262**, 12283–12287
- Brennan, T. V., Anderson, J. W., Jia, Z., Waygood, E. B., and Clarke, S. (1994) *J. Biol. Chem.* **269**, 24586–24595
- Kagan, R. M., and Clarke, S. (1995) *Biochemistry* **34**, 10794–10806
- Kagan, R. M., McFadden, H. J., McFadden, P. N., O'Connor, C., and Clarke, S. (1997) *Comp. Biochem. Physiol.* **117B**, 379–385
- Ichikawa, J. K., and Clarke, S. (1998) *Arch. Biochem. Biophys.* **358**, 222–231
- Kim, E., Lowenson, J. D., MacLaren, D. C., Clarke, S., and Young, S. G. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 6132–6137
- Yamamoto, A., Takagi, H., Kitamura, D., Tatsuoka, H., Nakano, H., Kawano, H., Kuroyanagi, H., Yahagi, Y.-I., Kobayashi, S.-I., Koizumi, K.-I., Sakai, T., Saito, K.-I., Chiba, T., Kawamura, K., Suzuki, K., Watanabe, T., Mori, H., and Shirasawa, T. (1998) *J. Neurosci.* **18**, 2063–2074
- Koenig, M. L., Rothbard, P. M., DeCoster, M. A., and Meyerhoff, J. L. (1994) *Neuroreport* **5**, 1063–1068
- Brovia, V., Costa, A., and Barbeito, L. (1996) *J. Neurochem.* **67**, 382–388
- Serval, V., Barbeito, L., Pittaluga, A., Cheramy, A., Lavielle, S., and Glowinski, J. (1990) *J. Neurochem.* **55**, 39–46
- Coyle, J. T. (1997) *Neurobiol. Dis.* **4**, 231–238
- Wassarman, P. M., and DePamphilis, M. L. (1993) in *Guide to Techniques in Mouse Development* (Wassarman, P. M., and DePamphilis, M. L., eds) pp. xxxv, Academic Press, San Diego, CA
- Todaro, G. J., and Green, H. (1963) *J. Cell Biol.* **17**, 299–313
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- MacLaren, D. C., and Clarke, S. (1995) *Protein Expression Purif.* **6**, 99–108
- Molinar-Rode, R., and Pasik, P. (1992) *Exp. Brain Res.* **89**, 40–48
- Hayflick, L., and Moorhead, P. S. (1961) *Exp. Cell Res.* **25**, 585–621
- Hayflick, L. (1965) *Exp. Cell Res.* **37**, 614–636
- Valivullah, H. M., Lancaster, J., Sweetnam, P. M., and Neale, J. H. (1994) *J. Neurochem.* **63**, 1714–1719
- Lowenson, J. D., and Clarke, S. (1991) *J. Biol. Chem.* **266**, 19396–19406
- Lowenson, J. D., and Clarke, S. (1992) *J. Biol. Chem.* **267**, 5985–5995
- Sainz, J., Minassian, B. A., Serratos, J. M., Gee, M. N., Sakamoto, L. M., Iranmanesh, R., Bohlega, S., Baumann, R. J., Ryan, S., Sparkes, R. S., and Delgado-Escueta, A. V. (1997) *Am. J. Hum. Genet.* **61**, 1205–1209
- MacLaren, D. C., O'Connor, C. M., Xia, Y.-R., Mehrabian, M., Klisak, I., Sparkes, R. S., Clarke, S., and Luskis, A. J. (1992) *Genomics* **14**, 852–856
- Luthi-Carter, R., Barezak, A. K., Speno, H., and Coyle, J. T. (1998) *Brain Res.* **795**, 341–348
- Zaczek, R., Koller, K., Cotter, R., Heller, D., and Coyle, J. T. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80**, 1116–1119