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Diet-dependent survival of protein repair-deficient mice☆

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Abstract

Protein L-isoaspartyl (D-aspartyl) *O*-methyltransferase (PCMT1) is a protein-repair enzyme, and mice lacking this enzyme accumulate damaged proteins in multiple tissues, die at an early age from progressive epilepsy and have an increased *S*-adenosylmethionine (AdoMet) to *S*-adenosylhomocysteine (AdoHcy) ratio in brain tissue. It has been proposed that the alteration of AdoMet and AdoHcy levels might contribute to the seizure phenotype, particularly as AdoHcy has anticonvulsant properties. To investigate whether altered AdoMet and AdoHcy levels might contribute to the seizures and thus the survivability of the repair-deficient mice, a folate-deficient amino acid-based diet was administered to the mice in place of a standard chow diet. We found that the low-folate diet significantly decreases the AdoMet/AdoHcy ratio in brain tissue and results in an almost threefold extension of mean life span in the protein repair-deficient mice. These results indicate that the increased AdoMet/AdoHcy ratio may contribute to the lowered seizure threshold in young PCMT1-deficient mice. However, mean survival was also extended almost twofold for mice on a control folate-replete amino acid-based diet compared to mice on the standard chow diet. Survival after 40 days was similar in the mice on the low- and high-folate amino acid-based diets, suggesting that the survival of older PCMT1-deficient mice is not affected by the higher brain AdoMet/AdoHcy ratio. Additionally, the surviving older repair-deficient mice have a significant increase in body weight when compared to age-matched normal mice, independent of the type of diet. This weight increase was not accompanied by an increase in consumption levels, indicating that the repair-deficient mice may also have an altered metabolic state.

Keywords: Isoaspartyl; Folate; *S*-Adenosylmethionine; *S*-Adenosylhomocysteine; PCMT1; Mice

1. Introduction

Protein L-isoaspartyl (D-aspartyl) *O*-methyltransferase (PCMT1) is an enzyme that has been shown to repair proteins with isoaspartyl damage both in vitro and in vivo [1–7]. Mice lacking the enzyme (*Pcmt1*−/− mice) accumulate damaged proteins in multiple tissues, develop progressive epilepsy and die at an average of approximately 6 weeks of age [8,9]. The epilepsy appears to be the cause of the shortened life span as no abnormalities of other tissues have been noted and death occurs directly following a severe seizure episode. It has been proposed that the seizures may be due to the isoaspartyl damage of certain proteins, such as tubulin, calmodulin or synapsin [8–12]. However, a recent study demonstrated that the cofactor for the repair enzyme *S*-adenosylmethionine (AdoMet) and its metabolite *S*-adenosylhomocysteine (AdoHcy) is also altered in the brains of mice lacking the methyltransferase [13]. From these results, it was proposed that this alteration may contribute to the seizure phenotype, particularly as AdoHcy is a known anticonvulsant, and its reduction has been shown to lower the seizure threshold in the mammalian nervous system [14–16].

In order to examine whether altered AdoMet and AdoHcy levels might contribute to the seizures and thus the survivability of *Pcmt1*−/− mice, a method to adjust or correct the levels of these molecules in the brains of *Pcmt1*−/− mice was sought. In previous studies, injections of adenosine and homocysteine thiolactone were found to significantly increase cerebral AdoHcy levels and inhibit the occurrence of seizures in mice [16]. It was also shown that the brain AdoMet/AdoHcy ratio could be lowered through the administration of a folate-deficient diet [17]. For the present study, a low-folate, high-methionine diet was used to determine if AdoMet and AdoHcy levels could be altered in the brains of *Pcmt1*−/− mice, and if this could lead to increased survival in these mice. By this method, the folate insufficiency leads to a decrease in the AdoMet/AdoHcy ratio by promoting homocysteine accumulation via inhibition

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of methionine synthase [18]. High methionine levels are added to ensure the continued production of AdoMet, AdoHcy and homocysteine, and to limit the conversion of excess homocysteine to methionine via methionine synthase [19]. In addition, this diet includes an antibiotic to limit the substantial levels of folate provided by intestinal bacteria. We also included a control diet with normal levels of folate and methionine, as well as the chow diet that was fed to the mice used in the previous study to assess AdoMet and AdoHcy levels [13].

2. Methods and materials

2.1. Generation of *Pcmt1*−/− and *Pcmt1*+/+ mice

The *Pcmt1*+/+ and *Pcmt1*−/− mice were obtained as previously described [8]. By inbreeding mice that were heterozygous for the knockout mutation for many generations, a congenic mutant line has been generated that is approximately 50% 129/svJae and 50% C57BL/6. All *Pcmt1*−/− mice used in the following studies were compared with their *Pcmt1*+/+ littermates. Although both male and female mice were included in the study, approximately the same number of each sex was used for each data set. Mice were weaned at 20 days of age, housed in a barrier facility with 12-h light/dark cycle and had unlimited access to food and fresh water. Mice were monitored by on-site veterinarians and all protocols were preapproved by the UCLA Animal Research Committee.

2.2. Experimental diets

From the point of weaning at 20 days of age, *Pcmt1*−/− and *Pcmt1*+/+ mice were fed either NIH-31 Modified Open Formula Mouse/Rat Sterilizable Diet # 7013 (NIH-7013 diet; Harlan Teklad, Madison, WI), Modified Clifford L-Amino Acid Rodent Diet with 2 mg/kg folate, 3 g/kg L-methionine with 1% succinyl sulfathiazole modified for pelleting #517851 (high-folate diet; Dyets, Bethlehem, PA) or Modified Clifford L-Amino Acid Rodent Diet with 0.2 mg/kg folate, 8.2 g/kg L-methionine with 1% succinyl sulfathiazole modified for pelleting #517894 (low-folate diet; Dyets). Antibiotics were added to the low-folate diet as a significant amount of folate can be supplied through bacterial populations present in the gut of mice. The same antibiotic was also added to the high-folate diet to control for any advantage of antibiotics unrelated to folate reduction in the low-folate diet. A comparison of the contents of the diets is presented in Table 1.

2.3. Measurement of AdoMet and AdoHcy levels in deproteinized brain homogenates

Levels of AdoMet and AdoHcy in tissue samples were determined by ion pairing reverse-phase high-performance liquid chromatography (HPLC) as described previously [13]. Mice were decapitated and their tissues were immediately submerged in liquid nitrogen and stored at −80°C until analysis was performed. Frozen tissue was extracted, weighed and homogenized in 0.4 M $HClO_4$ (4 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 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, Deerfield, IL) 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 Scientific, Fair Lawn, NJ). 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 reequilibrated with 100% eluent A from 33 to 60 min. The flow-rate was maintained at 1 ml/min and the UV absorbance 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.

2.4. Body weight and consumption rate measurements

Mice were weighed using a calibrated GSI-200 portable electronic balance (Acculab, West Chester, PA) every 5–10 days. Food was weighed before distributing to the mice and weighed again every 5–10 days, at which time, fresh preweighed food was provided. Consumption rates were calculated by the subtraction of before and after weights of food and divided by the number of days between feedings.

2.5. Statistical analysis

The results are expressed as means±S.E.M. from *n* mice. Tests of significance were conducted for data within each age group using the unpaired two-tailed Student *t* test.

3. Results

3.1. Levels of AdoMet and AdoHcy are altered in brain tissue of *Pcmt1*−/− mice on a low-folate diet

The levels of AdoMet and AdoHcy were measured in homogenized brain tissue from 50-day-old mice on the three experimental diets. In *Pcmt1*−/− mice on the NIH-7013 diet, levels of AdoMet are about 25% higher (Fig. 1A), levels of AdoHcy are about 40% lower (Fig. 1B) and

Table 1
Components of experimental diets

	NIH-7013	High folate	Low folate
Amino acids			
Methionine (g/kg)	3.5	3.0	8.2
Arginine (g/kg)	12.7	11.2	11.2
Cystine (g/kg)	3.2	3.5	3.5
Histidine (g/kg)	4.1	3.3	3.3
Isoleucine (g/kg)	9.0	8.2	8.2
Leucine (g/kg)	15.1	11.1	11.1
Lysine (g/kg)	9.6	18.0	18.0
Phenylalanine + tyrosine (g/kg)	15.4	15.1	15.1
Threonine (g/kg)	7.1	8.2	8.2
Tryptophan (g/kg)	2.2	1.7	1.7
Valine (g/kg)	9.1	8.2	8.2
Nonessential amino acids (g/kg)	91.0	79.0	79.0
Vitamins			
Folic acid (mg/kg)	1.5	2.0	0.2
Vitamin A (U/g)	24.6	4.0	4.0
Vitamin D3 (U/g)	4.2	1.0	1.0
Vitamin E (U/kg)	44.7	50.0	50.0
Choline (mg/g)	1.9	2.0	2.0
Niacin (mg/kg)	86.6	30.0	30.0
Pantothenic acid (mg/kg)	36.9	16.0	16.0
Pyridoxine (vitamin B6) (mg/kg)	9.5	7.0	7.0
Riboflavin (vitamin B2) (mg/kg)	7.4	7.0	7.0
Thiamine (vitamin B1) (mg/kg)	76.3	6.0	6.0
Menadione (vitamin K3) (mg/kg)	22.7	50.0	50.0
Biotin (mg/kg)	0.3	0.2	0.2
Vitamin B12 (μg/kg)	62.0	50.0	50.0
Minerals			
Calcium (g/kg)	11.9	5.9	5.9
Phosphorus (g/kg)	9.2	3.1	3.1
Sodium (g/kg)	3.1	4.9	4.9
Chlorine (g/kg)	4.8	7.5	7.5
Potassium (g/kg)	6.3	7.7	7.7
Magnesium (g/kg)	2.0	0.5	0.5
Iron (mg/kg)	336.0	142.7	142.7
Manganese (mg/kg)	155.9	59.2	59.2
Zinc (mg/kg)	48.2	34.2	34.2
Copper (mg/kg)	13.2	31.0	31.0
Iodine (mg/kg)	2.0	0.2	0.2
Selenium (mg/kg)	0.3	0.9	0.9
Cobalt (mg/kg)	0.5	–	–
Molybdate (mg/kg)	–	0.8	0.8
Fluoride (mg/kg)	–	2.1	2.1
Chromium (mg/kg)	–	2.0	2.0
Other additives			
Linoleic acid (%)	2.6	–	–
Ash (%)	6.0	–	–
Sucrose (%)	–	19.8	19.8
Dextrin (%)	–	36.6	36.6
Sodium bicarbonate[a] (%)		0.8	0.8
Succinyl sulfathiazole[b] (%)		1.0	1.0
Nutritional totals			
Protein (%)	18.2	17.6	17.1
Fat[c] (%)	6.3	10.0	10.0
Fiber[d] (%)	4.5	5.0	5.0
Carbohydrate[e] (%)	53.8	59.4	59.4
Metabolizable energy (kcal/g)	3.13	3.92	3.92

AdoMet/AdoHcy ratio is about 2.4-fold higher (Fig. 1C) than those in *Pcmt1*+/+ mice on the same diet. These results are similar to those seen previously with *Pcmt1*−/− and *Pcmt1*+/+ mice on this diet [13]. In *Pcmt1*−/− mice on the high-folate diet, the AdoMet/AdoHcy ratio is also about twofold higher than that in *Pcmt1*+/+ mice on this diet (Fig. 1C). However, in *Pcmt1*−/− mice on the low-folate diet, AdoMet levels, AdoHcy levels and AdoMet/AdoHcy ratios are similar to those seen in *Pcmt1*+/+ mice on the same diet (Fig. 1A–C). In comparing *Pcmt1*−/− mice on the low-folate diet to those on the high-folate diet, AdoMet levels are lower (Fig. 1A), AdoHcy levels are significantly higher (Fig. 1B), and the AdoMet/AdoHcy ratio is significantly lower (Fig. 1C). In a previous study of mice on a folate-deficient diet, only brain AdoMet levels were found to be significantly altered, and not AdoHcy levels [17]. However, in our study, we show that the brain AdoHcy levels are even more significantly effected by the diet than the AdoMet levels (Fig. 1A, B). Also, because the AdoMet/AdoHcy ratio in *Pcmt1*−/− mice on the low-folate diet is reduced to similar levels seen in *Pcmt1*+/+ mice on the same diet, this diet appears to have fulfilled its intended purpose of decreasing the AdoMet/AdoHcy ratio in the brains of *Pcmt1*−/− mice. Additionally, results showing that the AdoMet/AdoHcy ratio is higher in *Pcmt1*−/− compared to *Pcmt1*+/+ mice on the high-folate diet indicate that this diet is sufficient to serve as a control for the low-folate diet in the investigation of whether this ratio effects the survival of the *Pcmt1*−/− mice.

3.2. Survival of *Pcmt1*−/− mice on low- and high-folate diets is increased

For *Pcmt1*−/− mice on the NIH-7013 diet, 50% of the mice died by approximately 40 days of age (Fig. 2A, B). For *Pcmt1*−/− mice on the high-folate diet, 50% of the mice died by approximately 80 days of age, and for those on the low-folate diet, 50% of the mice died by approximately 120 days of age (Fig. 2A, B). These results indicate that both the low- and high-folate diets benefit the survival of *Pcmt1*−/− mice. In older *Pcmt1*−/− mice, the high- and low-folate diets seem to have a similar benefit on survival as the slope of the curve is similar after approximately 40 days of age (Fig. 2A). However, in mice under approximately 40 days of age, the low-folate diet seems to have the most beneficial effect on their survival (Fig. 2B). This result indicates that the lowering of the AdoMet/AdoHcy ratio may have an effect on the survival of young *Pcmt1*−/− mice, but that some other component or components of the high- and low-

Notes to Table 1:
[a] Added for pelleting in high and low folate diets.
[b] Antibiotic in high and low folate diets.
[c] Corn oil in high and low folate diets.
[d] Cellulose in high and low folate diets.
[e] Nitrogen-free extract for NIH-7013 diet; sucrose & dextrin for high and low folate diets.

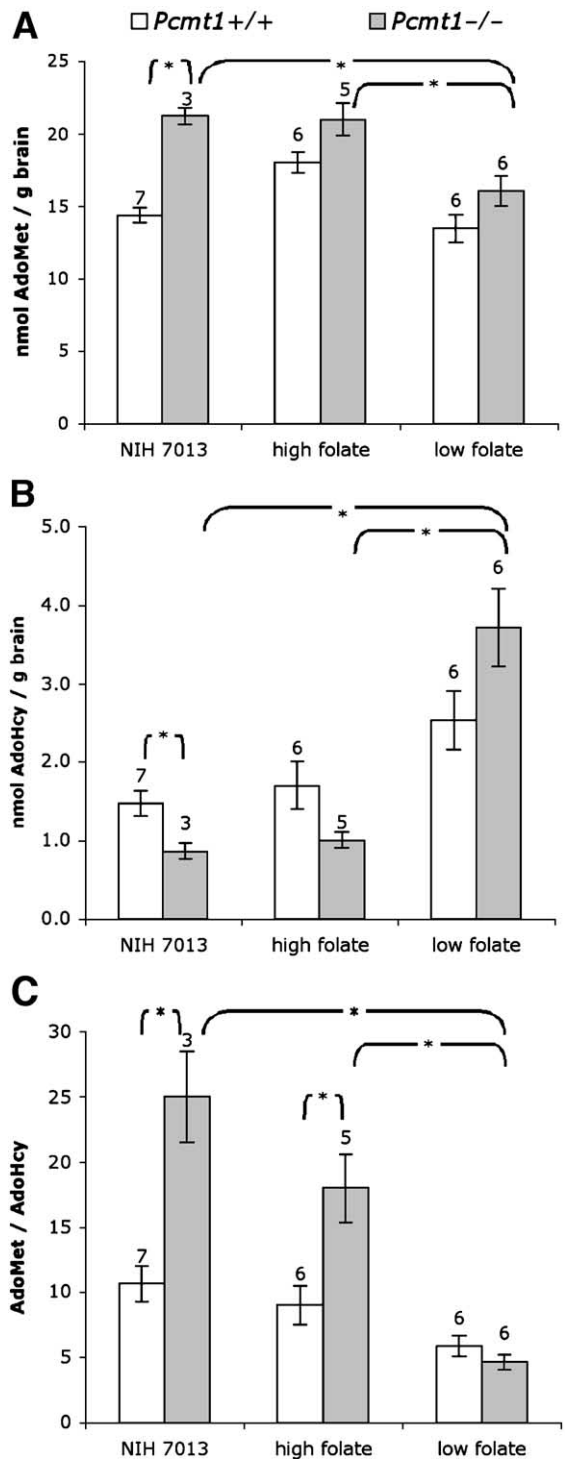

Fig. 1. Average nanomole of AdoMet/g brain tissue (A), average nanomole of AdoHcy/g brain tissue (B) and average AdoMet/AdoHcy ratio (C) in brain tissue of *Pcmt1*−/− and *Pcmt1*+/+ mice on experimental diets. The results are presented as means±S.E.M.; the number of mice included in each data set is indicated above each bar, **P*<.05.

folate diets may have a beneficial effect on the survival of older *Pcmt1*−/− mice. It is unknown if the deaths of older *Pcmt1*−/− mice were caused by seizures as their deaths were not witnessed. Also, no *Pcmt1*+/+ mice died spontaneously either by natural cause or disease on any of the experimental diets.

3.3. Older *Pcmt1*−/− mice weigh more than age-matched *Pcmt1*+/+ mice

It was previously reported that *Pcmt1*−/− mice younger than 30 days of age generally weighed less than their *Pcmt1*+/+ littermates [8,9]. Here, our results indicate that although some of the *Pcmt1*−/− mice weighed less than *Pcmt1*+/+ mice at 20 days of age, they quickly caught up and surpassed the weights of *Pcmt1*+/+ mice by 50 days of age. In mice on the NIH-7013 diet, this trend continued in surviving male *Pcmt1*−/− mice (Fig. 3A, B). In mice on the high- and low-folate diets, which have a higher caloric content and glycemic index (Table 1), the weights of older *Pcmt1*−/− mice were higher in both male and female mice when compared to age- and sex-matched *Pcmt1*+/+ mice (Fig. 3A, B). The increase in weight did not seem to be accompanied by an increase in bone length as determined by comparisons of femur bones from *Pcmt1*+/+ and *Pcmt1*−/− mice (data not shown). In addition, the heavier *Pcmt1*−/− mice appeared to have more adipose tissue, as opposed to

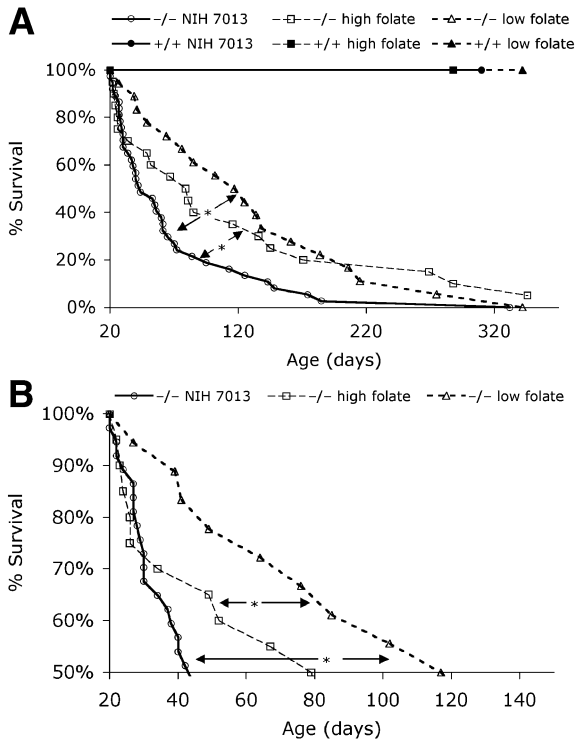

Fig. 2. Survival data for *Pcmt1*−/− or *Pcmt1*+/+ mice fed NIH-7013, high-folate or low-folate diets starting at 20 days of age (A) and survival data for *Pcmt1*−/− mice on experimental diets under the age at which 50% of the mice in each group have died (B); $n=37$, 20 and 18 for *Pcmt1*−/− mice on the NIH-7013, high-folate and low-folate diets, respectively; $n=15$, 6 and 6 for *Pcmt1*+/+ mice on the NIH-7013, high-folate and low-folate diets, respectively. Approximately equal proportions of sexes were represented between data sets: **P*<.05.

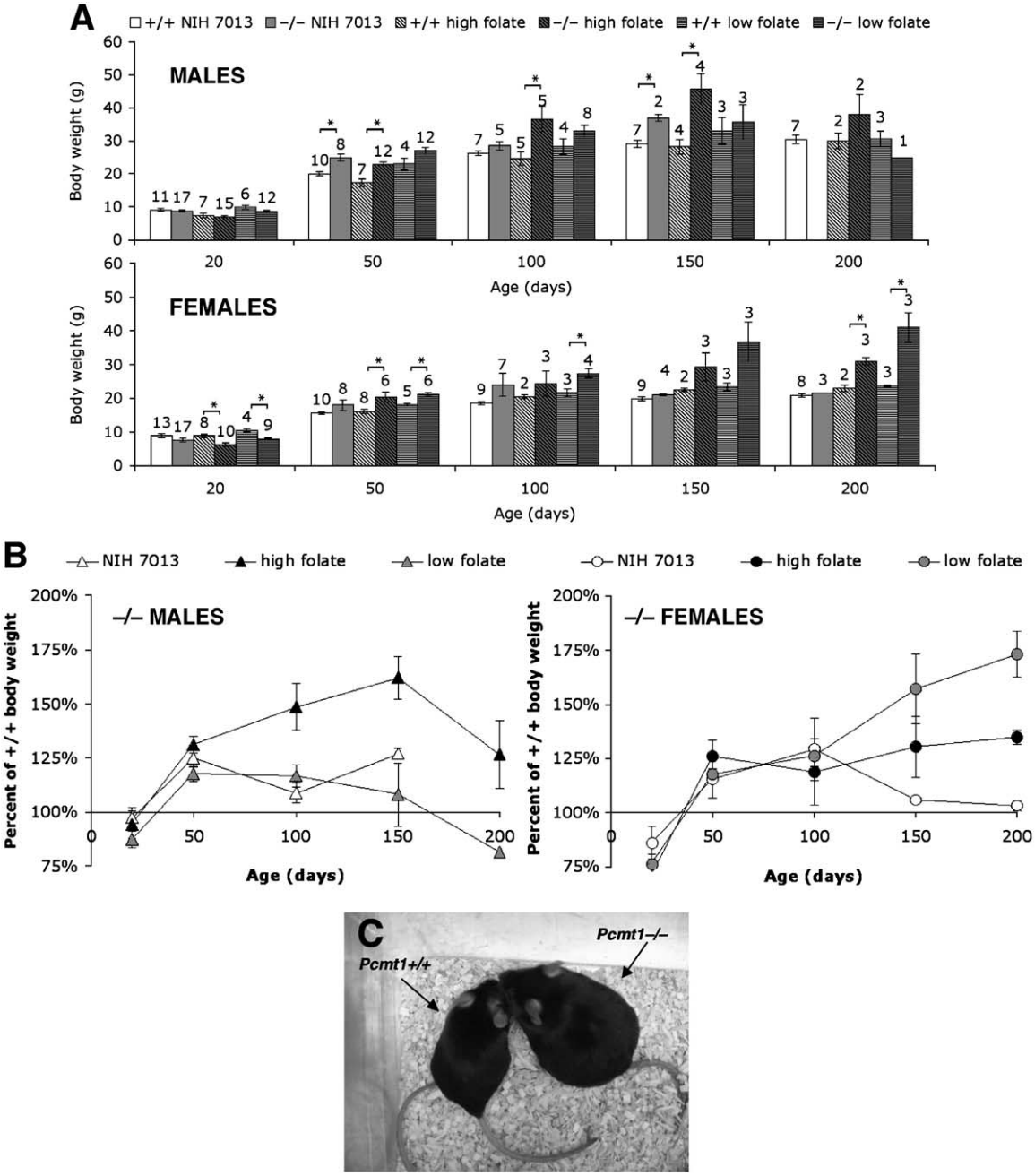

Fig. 3. Average whole-body weights of male and female *Pcmt1*−/− and *Pcmt1*+/+ mice on NIH-7013, high folate or low-folate diets at 20, 50, 100, 150 and 200 days of age (A). Data are presented as means±S.E.M.; the number of mice included in each data set is indicated above each bar, $*P<.05$. Scatter graph of percentage of average whole-body weights of *Pcmt1*−/− mice over that of *Pcmt1*+/+ mice on either NIH-7013 diet or high- and low-folate diets from 20 to 200 days of age (B); the number of mice in each data set of panel *B* is the same as for panel *A*. Photograph of 150-day-old *Pcmt1*−/− and *Pcmt1*+/+ male littermates on high-folate diet showing the increased size of a *Pcmt1*−/− compared to a *Pcmt1*+/+ mouse (C).

muscle tissue (Fig. 3C), although direct quantification of these tissues has not yet been made.

3.4. Consumption rates of *Pcmt1*−/− mice on the experimental diets are not increased

Despite the increased weight of *Pcmt1*−/− mice, neither male nor female mice appeared to consume more food than age- and sex-matched *Pcmt1*+/+ mice (Fig. 4). In fact, in male mice between 150 and 200 days of age, *Pcmt1*−/− mice on the high-folate diet appeared to consume less food than *Pcmt1*+/+ mice. This may have been due to a deterioration in the health of male *Pcmt1*−/− mice between 150 and 200 days of age as half of the surviving male *Pcmt1*−/− mice died during this period. However, these results indicate that the increased weight of *Pcmt1*−/− mice is not due to an increased consumption of food, but may rather be due to an altered metabolic state. While it is possible that the difference could also be attributed to a

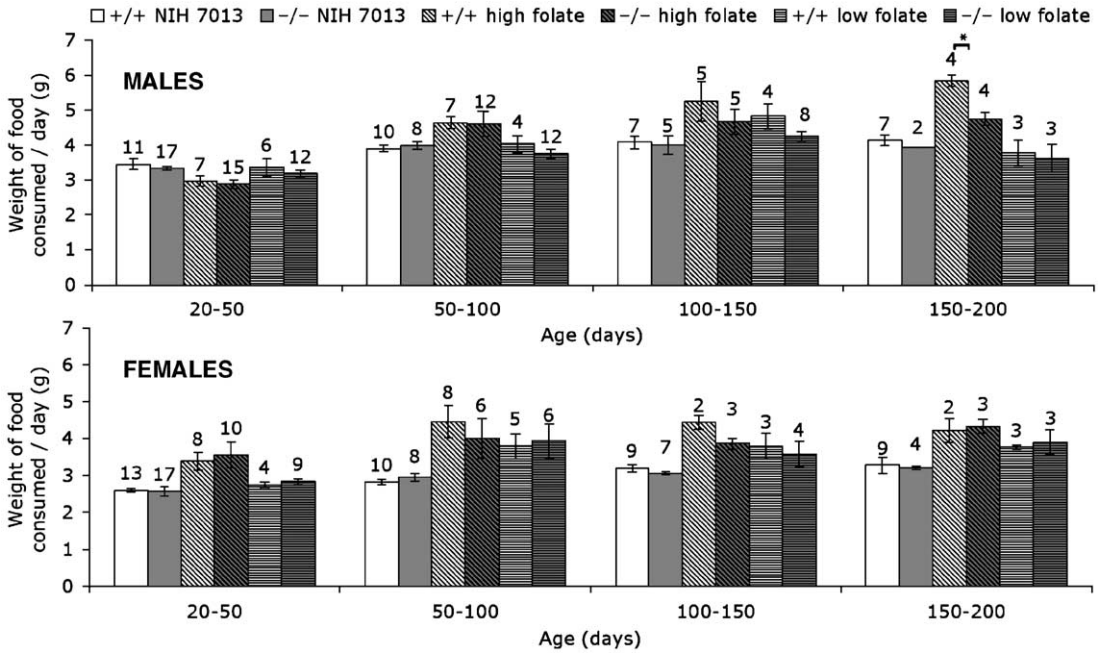

Fig. 4. Weight of food consumed per day by *Pcmt1*−/− and *Pcmt1*+/+ mice on experimental diets between the ages of 20 and 50, 50 and 100, 100 and 150, and 150 and 200 days. Consumption levels of male mice are indicated in the top graph and those of female mice are indicated in the bottom graph. Results are presented as means±S.E.M. for all values; the number of mice included in each data set is indicated above each bar, $*P<.05$.

decrease in caloric usage by the *Pcmt1*−/− mice through daily activity, we did not observed a difference in roaming, sleeping, or grooming behaviors.

4. Discussion

Previous studies have demonstrated that elevated intracellular AdoHcy levels are associated with human pathologies such as peripheral arterial occlusive disease [20], end-stage renal failure [21] and uremia [22,23]. Such elevated levels may result in unbalanced methylation events as AdoHcy is a powerful competitive inhibitor of AdoMet-dependent methyltransferases [24]. Other studies have shown that young rats with elevated AdoHcy levels have higher levels of damaged L-isoaspartyl residues in hepatic proteins, suggesting that one of the methyltransferases effected by the higher intracellular AdoHcy levels in young mammals is the protein repair L-isoaspartyl (D-aspartyl) *O*-methyltransferase [25]. In addition, we previously demonstrated that mice lacking this methyltransferase had an increased brain AdoMet/AdoHcy ratio, which was reflective of both higher AdoMet levels and lower AdoHcy levels [13]. It was proposed that these differences might be attributed to the lack of the methyltransferase, which is highly active in the brains of normal animals. Therefore, it is possible that the levels of AdoMet and AdoHcy and the activity of PCMT1 may be particularly interdependent in young mammals.

It was also suggested that the altered levels of AdoMet and AdoHcy in the brains of *Pcmt1*−/− mice might contribute to the lower seizure threshold, as AdoHcy is also a known anticonvulsant. To test this theory, an experimental diet in which the folate and methionine levels are altered causing a decrease in the cellular AdoMet/AdoHcy ratio was fed to the mice. Because there are many differences between the NIH-7013 chow diet (used in the previous study to assess AdoMet and AdoHcy levels in mice) and the designed low-folate diet, a high-folate diet that only differed from the low-folate diet in its levels of folate and methionine was used as a control. AdoMet and AdoHcy measurements in mice on the three experimental diets demonstrated that the low-folate diet was effective in lowering the AdoMet/AdoHcy ratio in the brain tissue of *Pcmt1*−/− mice and that the high-folate diet showed a similar ratio to that seen with the NIH-7013 diet.

Survival rates of the mice on the three experimental diets demonstrated that the low-folate diet was effective in increasing the survival of *Pcmt1*−/− mice between the ages of 20–40 days compared to *Pcmt1*−/− mice on either the high-folate or the NIH-7013 diets. These results indicate that alterations of the AdoMet/AdoHcy ratio in the brain may indeed have an effect on the seizure threshold in young *Pcmt1*−/− mice, possibly through the anticonvulsant effect of AdoHcy. However, this treatment does not restore a normal life span to the *Pcmt1*−/− mice, indicating that while alterations in the AdoMet and/or AdoHcy levels may be contributing factors to the survival in these mice, they are not the only cause of seizure development in the *Pcmt1*−/− mice. However, it was not expected that any experimental diet would completely restore a normal life span to the *Pcmt1*−/− mice, as they have high levels of isoaspartyl damage in their cellular

proteins, even at 20 days of age. In fact, the ultimate purpose of the experimental diets was to extend the life span of the *Pcmt1*−/− mice in order to further study the "long-term" effects of the accumulation of this type of protein damage in the brain and other tissues. In this sense, the low-folate diet was successful in increasing the average life span of *Pcmt1*−/− mice from approximately 40 days of age to approximately 120 days of age.

In older *Pcmt1*−/− mice, there appeared to be a beneficial effect on survival for mice on both the high- and low-folate diets. The increased survival of the older *Pcmt1*−/− mice on these diets is most likely attributable to some component or components other than folate or methionine, as these components are present at similar levels in both the high-folate and NIH-7013 diets. There are many differences between the high- and low-folate diets and the NIH-7013 diet. However, one of the most notable changes is the difference in fat content (10% *versus* 6.3%). It is well known that a ketogenic diet can benefit people with seizures [26], and it is possible that the higher fat content of the low- and high-folate diets could lower the seizure threshold in the *Pcmt1*−/− mice, although none of the diets used here would be expected to be ketogenic.

Another major difference in the high- and low-folate diets is the presence of 19.8% sucrose and 36.6% dextrin. In addition, the *Pcmt1*−/− mice on these diets showed a significant increase in weight in comparison to *Pcmt1*+/+ mice on the same diets without an increase in food consumption. The presence of these high-glycemic index carbohydrates may amplify any alteration in the metabolic state that may exist in the *Pcmt1*−/− mice, which may help to explain their dramatic increase in weight. Aside from the accumulation of isoaspartyl-related damage of proteins in peripheral tissues, the increase in weight is the only other phenotype noted in the *Pcmt1*−/− mice that does not necessarily relate directly to alterations in the brain. In future studies, it will be important to obtain data on body composition, such as the accumulation of adipose tissue, as the increase in body weight may be an important clue in determining the long-term effects of the lack of PCMT1 in the mammalian system.

Aside from the differences in fat and sucrose content, it is also possible that the addition of antibiotics or any one of the many other minor alterations in the diets could be the cause of the increased survival for the *Pcmt1*−/− mice. It will be important in future studies to determine which component or components of these diets contributes the most to the increased survival of the *Pcmt1*−/− mice.

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