THE JOURNAL OF COMPARATIVE NEUROLOGY 493:524–537 (2005)

Increased Cell Proliferation and Granule Cell Number in the Dentate Gyrus of Protein Repair-Deficient Mice

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ABSTRACT

Recent studies have demonstrated that mice lacking protein L-isoaspartate (D-aspartate) *O*-methyltransferase (*Pcmt1*–/– mice) have alterations in the insulin-like growth factor-I (IGF-I) and insulin receptor pathways within the hippocampal formation as well as other brain regions. However, the cellular localization of these changes and whether the alterations might be associated with an increase in cell number within proliferative regions, such as the dentate gyrus, were unknown. In this study, stereological methods were used to demonstrate that these mice have an increased number of granule cells in the granule cell layer and hilus of the dentate gyrus. The higher number of granule cells was accompanied by a greater number of cells undergoing mitosis in the dentate gyrus, suggesting that an increase in neuronal cell proliferation occurs in this neurogenic zone of adult *Pcmt1*–/– mice. In support of this, increased doublecortin labeling of immature neurons was detected in the subgranular zone of the dentate gyrus. In addition, double immunofluorescence studies demonstrated that phosphorylated IGF-I/insulin receptors in the subgranular zone were localized on immature neurons, suggesting that the increased activation of one or both of these receptors in *Pcmt1*–/– mice could contribute to the growth and survival of these cells. We propose that deficits in the repair of isoaspartyl protein damage leads to alterations in metabolic and growth-receptor pathways, and that this model may be particularly relevant for studies of neurogenesis that is stimulated by cellular damage. J. Comp. Neurol. 493: 524–537, 2005. © 2005 Wiley-Liss, Inc.

Indexing terms: isoaspartyl; neurogenesis; PCMT1; insulin receptor; IGF-I receptor; doublecortin

Protein L-isoaspartate (D-aspartate) *O*-methyltransferase (PCMT1) is an enzyme that is expressed in most living organisms and is found in all mammalian tissues, with the highest levels in the brain (Kim et al., 1997; Yamamoto et al., 1998). Functionally, it initiates the repair of protein damage due to the isomerization of aspartyl residues, a common protein degradation pathway in living systems (Johnson et al., 1987; McFadden and Clarke, 1987; Brennan et al., 1994; Ingrosso et al., 2000; Chavous et al., 2001; Clarke, 2003; Doyle et al., 2003; Lanthier and Desrosiers, 2004). Mice with a disrupted gene encoding this enzyme (*Pcmt1*–/– mice) accumulate higher levels of isoaspartyl-containing polypeptides in all tissues, especially the brain, when compared to levels in *Pcmt1*+/+ mice (Kim et al., 1997; Yamamoto et al., 1998; Lowenson et al., 2001). In addition, these mice develop

Grant sponsor: U.S. Department of Veterans Affairs (to C.R.H); Grant sponsor: National Institutes of Health; Grant number: NS046524 (to C.R.H.); Grant number: GM26020 (to S.G.C.); Grant number: AG18000 (to S.G.C.).

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Received 1 March 2005; Revised 20 June 2005; Accepted 21 July 2005
DOI 10.1002/cne.20780
Published online in Wiley InterScience (www.interscience.wiley.com).

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generalized seizures at ~30 days of age and usually die following a severe seizure episode at an average of 42 days of age (Kim et al., 1997, 1999; Yamamoto et al., 1998; Ikegaya et al., 2001; Farrar and Clarke, 2002). *Pcmt1–/–* mice also have a progressive enlargement of the brain (Yamamoto et al., 1998; Farrar et al., 2005). This suggests that complex, potentially compensatory changes are occurring in response to the lack of the protein repair enzyme.

Indeed, recent studies have revealed increased activation of the phosphatidylinositide 3-kinase (PI3K)/Akt signal transduction pathway in the brains of *Pcmt1–/–* mice (Farrar et al., 2005). Interestingly, increased activation of this pathway occurs in several other mouse models with regional or generalized brain enlargement, including mice lacking either tuberous sclerosis complex-1 (Uhlmann et al., 2002), phosphoinositide phosphatase (Groszer et al., 2001), caspase-9 (Kuida et al., 1998), or p27kip1 (Fero et al., 1996), and mice overexpressing either insulin-like growth factor-I (Carson et al., 1993) or β-catenin (Chenn and Walsh, 2002).

Further studies of the upstream elements of the PI3K/Akt pathway demonstrated increased activation of either the insulin-like growth factor-I (IGF-I) receptors, insulin receptors, or both in the hippocampus of *Pcmt1–/–* mice (Farrar et al., 2005). In addition, *Pcmt1–/–* mice were found to have a progressive increase in insulin receptor β-subunit protein levels in all brain regions and in multiple tissues from 20 to 50 days of age (Farrar et al., 2005). The higher levels of insulin receptor were even detectable in the brain tissue of *Pcmt1–/–* mice on the first postnatal day (P0), indicating that, aside from increased isoaspartyl-damage, an alteration of metabolic pathways may be one of the first phenotypes to manifest in these mice (Farrar et al., 2005). While insulin and IGF-I signaling pathways can be involved in tissue growth (Baker et al., 1993; Beck et al., 1995; Ish-Shalom et al., 1997; Anderson et al., 2002), it was unknown whether specific changes such as increased neuronal number and cell proliferation occur in this mouse model.

Although every region of the *Pcmt1–/–* mouse brain had altered levels of insulin and IGF-I signaling proteins compared to the corresponding regions of *Pcmt1+/+* mouse brain, these changes were especially striking in the hippocampal formation, particularly in the dentate gyrus (Farrar et al., 2005). This region is one in which neurogenesis persists into adulthood (Altman and Das, 1965; Kaplan and Bell, 1984) and one in which progenitor cell proliferation can increase under various conditions (for recent reviews, see Gould and Gross, 2002; Lie et al., 2004). Therefore, the present study was designed to identify possible changes in granule cell number and cell proliferation in the dentate gyrus, determine whether activated insulin-related receptors were present at higher levels in this neurogenic region, and ascertain if these receptors could be detected in immature neurons of adult *Pcmt1–/–* mice.

MATERIALS AND METHODS

Animals

Four male 40-day-old *Pcmt1–/–* mice and four sex- and age-matched littermate *Pcmt1+/+* mice were used for immunohistochemical and stereological studies. The *Pcmt1+/+* and *Pcmt1–/–* mice were generated as previously described (Kim et al., 1997; Farrar et al., 2005). By inbreeding mice that were heterozygous for the knockout mutation for many generations over 10 years, a congenic mutant line has been generated that is ~50% 129/svJae and 50% C57BL/6. Mice were weaned at 20 days of age, housed in a barrier facility with a 12-hour light/dark cycle, and had unlimited access to chow food (NIH-31 Modified Mouse/Rat Diet #7013) and fresh water. No behavioral seizures were observed in the *Pcmt1–/–* mice used in this study, but it is possible that they experienced seizures while not under direct observation.

For comparison with the *Pcmt1–/–* mice, tissue from six male C57Bl/6 mice (4 months of age) was included in a subgroup of the immunohistochemical studies as controls for the effects of seizure activity. Pilocarpine-induced status epilepticus had been induced in three of the mice 2 months earlier, and these mice had been experiencing frequent spontaneous seizures for several weeks prior to perfusion. The remaining three mice were included as age-matched controls and were perfused at the same time as the pilocarpine-treated mice. Protocols for pilocarpine treatment, care, and monitoring were identical to those described previously (Peng et al., 2004).

Mice were monitored by on-site veterinarians and all protocols were approved by the UCLA Animal Research Committee and conformed to National Institutes of Health guidelines.

Antisera

All antisera used in immunohistochemical experiments were obtained from commercial sources. Neurons were identified by using a mouse monoclonal antibody that recognizes the neuron-specific nuclear protein NeuN (MAB377, Chemicon International, Temecula, CA; diluted 1:1,000). This antibody was raised against purified cell nuclei from mouse brain and has been shown to recognize 2–3 bands at 46–48 kDa and possibly one at 66 kDa by Western blot (Chemicon International product datasheet; Mullen and Buck, 1992). It reacts with most neuronal cell types throughout the nervous system of mice and is primarily localized in the nucleus of the neurons with lighter staining in the cytoplasm. In this study, the NeuN antibody provided specific labeling of neurons throughout the hippocampal formation, and the staining pattern was very similar to that seen in other studies in which this antibody was used (e.g., Tang et al., 2005).

Dentate granule cells were labeled with a rabbit polyclonal antiserum (AB5475, Chemicon International; diluted 1:30,000) raised against a synthetic peptide corresponding to amino acids 722–737 of the C-terminus of the mouse Prox1 protein (Swiss-Prot protein sequence database, primary accession #P48437). This antiserum specifically labels Prox1-expressing cells of mouse, rat, and zebrafish origin and has been shown to label only differentiated dentate granule cells in the adult mouse brain (Bagri et al., 2002). In the current study the Prox-1 labeling in the hippocampal formation was specific for granule cells of the dentate gyrus and labeled no other cells in the brain regions examined, including hippocampus, cerebral cortex, and thalamus.

Neurons undergoing mitosis were labeled with a rabbit polyclonal antiserum that recognizes histone H3 phosphorylated at Ser10, identified as "mitosis marker" (#06-570, Upstate, Lake Placid, NY; diluted 1:400). This antiserum was raised against a synthetic serine-

phosphorylated peptide corresponding to amino acids 7–20 of human histone H3 (Swiss-Prot #P68431). This antiserum labels a single 17 kDa band by Western blot (Upstate certificate of analysis) and has been shown to specifically recognize mitotic cells of mammalian and non-mammalian origin by immunohistochemistry. In this study the mitotic marker labeling pattern, including cell morphology and localization in the subgranular zone of the dentate gyrus, was very similar to that seen in previous immunohistochemical studies that used phospho-H3 antibodies in adult rodent brain (Gould and Gross, 2002; Mandyam et al., 2004).

Immature and developing neurons in the subgranular zone were identified with either a goat antiserum (sc-8066, Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:4,000–10,000) directed against a synthetic doublecortin peptide corresponding to amino acids 385-402 at the C-terminus of human doublecortin (Swiss-Prot #O43911) or a guinea pig antiserum (AB5910, Chemicon International; diluted 1:4,000) directed against a synthetic doublecortin peptide corresponding to amino acids 350–365 of mouse doublecortin (Swiss-Prot #O88809). The doublecortin sc-8066 antiserum recognizes a 45-kDa band by Western blot (Santa Cruz Biotechnology product datasheet). It is specific for doublecortin of mouse, rat, and human origin by Western blotting, immunoprecipitation, and immunohistochemistry and is noncross-reactive with related protein KIAA0369. In the current study the staining pattern obtained with this doublecortin antiserum was the same as that seen in previous studies in which this antiserum was used (Kronenberg et al., 2003; Rao and Shetty, 2004; Couillard-Despres et al., 2005). The doublecortin antiserum AB5190 gave a virtually identical staining pattern to that seen with sc-8066.

IGF-I receptors phosphorylated at Tyr1131 (pIGF-IR) and insulin receptors phosphorylated at Tyr1146 (pIR) were detected with a rabbit polyclonal antiserum directed against a synthetic human pIGF-IR peptide (#3021 lots 3 and 4, Cell Signaling Technology, Beverly, MA; diluted 1:500) corresponding to amino acids 1151–1166 of human IGF-IR (Swiss-Prot #P08069). By Western blot, this antiserum recognizes a band at 90 kDa and detects endogenous levels of pIGF-IR (Tyr1131) and pIR (Tyr1146) proteins of mouse, rat, and human origin (Cell Signaling Technology product datasheet). This antiserum also cross-reacts with the activated form of other similar tyrosine kinase receptors, such as receptors for epidermal growth factor and fibroblast growth factor (Cell Signaling Technology). Therefore, we additionally used an antibody directed against activated insulin receptor substrate-1, which is downstream of only insulin, IGF-I, and interleukin-4 receptors. Insulin receptor substrate-1 phosphorylated at Tyr941 (pIRS-1) was localized with a rabbit polyclonal antiserum (sc-17199, Santa Cruz Biotechnology; diluted 1:2,000) directed against a synthetic human pIRS-1 peptide corresponding to amino acids 1229–1238 near the C-terminus of human IRS-1 (Swiss-Prot #P35568). This antibody is specific for pIRS-1 (Tyr941) protein of rat, mouse, or human origin (Santa Cruz Biotechnology product datasheet). Adsorption controls using the pIRS-1 blocking peptide (sc-17199 P, Santa-Cruz Biotechnology; diluted to 1 μg/ml) were used to confirm this antiserum's specificity. In addition, this antiserum was found to recognize a single major band at ~180 kDa in homogenized tissue from the hippocampus and cortex of *Pcmt1*+/+ and *Pcmt1*–/– mice. The staining pattern obtained with this antibody was very similar to that obtained with the pIGF-IR(Tyr1131)/pIR(Tyr1146) antiserum described above.

Dilution series analysis for each antiserum used in this study was performed on *Pcmt1*+/+ and *Pcmt1*–/– mouse tissue, and primary antiserum omission controls were used to further confirm the specificity of the immunohistochemical labeling.

Tissue preparation for immunohistochemistry

The mice were deeply anesthetized with sodium pentobarbital (90 mg/kg, i.p.) and perfused through the ascending aorta with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.3). After perfusion, the brains were maintained in situ at 4°C for 1 hour and then removed and postfixed in the same fixative for 1 hour. After thorough rinsing in phosphate buffer, the brains were cryoprotected in a 30% sucrose solution, blocked in the coronal plane, frozen on dry ice, and sectioned at 30 μm on a cryostat. Individual sections were stored in cryoprotectant solution at –20°C until processing.

Immunohistochemistry

Free-floating sections were processed for immunohistochemistry with standard avidin-biotin-peroxidase methods (Vectastain Elite ABC; Vector Laboratories, Burlingame, CA). Sections were incubated in 10% normal serum in 0.1 M Tris-buffered saline, pH 7.4 (TBS), containing 0.3% or 1% Triton X-100 for 1 hour. The sections were then incubated in the primary antiserum diluted with TBS containing 2% normal serum overnight at room temperature. After rinsing in TBS, the sections were incubated in biotinylated secondary antibody (diluted 1:1,000) at room temperature for 1 hour, rinsed in TBS, and incubated in ABC Elite solution (5 μl/ml) for 1 hour. After rinsing in 0.075 M sodium phosphate-buffered saline, pH 7.3 (PBS), the sections were processed with 0.06% 3,3′-diaminobenzidine-HCl and 0.006% H_2O_2 diluted in PBS for 5–15 minutes. After thorough rinsing, the sections were mounted on gelatin-coated slides, dehydrated, and coverslipped. In all experiments designed to compare the immunohistochemical labeling in *Pcmt1*–/– and *Pcmt1*+/+ mice, sections from the two groups of animals were processed identically and in parallel for each step of the immunohistochemical procedures. Likewise, sections from pilocarpine-treated and control mice were processed in parallel for immunohistochemical localization of doublecortin.

Quantitative analysis

Quantitative stereological analysis was performed blind to the experimental animal's genotype. The number of cells labeled for either Prox1 or the mitosis marker in the dentate gyrus of *Pcmt1*+/+ and *Pcmt1*–/– animals was estimated using standard stereological methods and a computer-assisted optical fractionator system (West et al., 1991; West, 1999) with Stereo Investigator software (MicroBrightField, Baltimore, MD). Stereological analysis was performed with a 100× objective (10,000× final magnification) for Prox1 and a 40× objective (4,000× final magnification) for mitosis marker. For the analysis of Prox1-labeled cells, every tenth section was analyzed

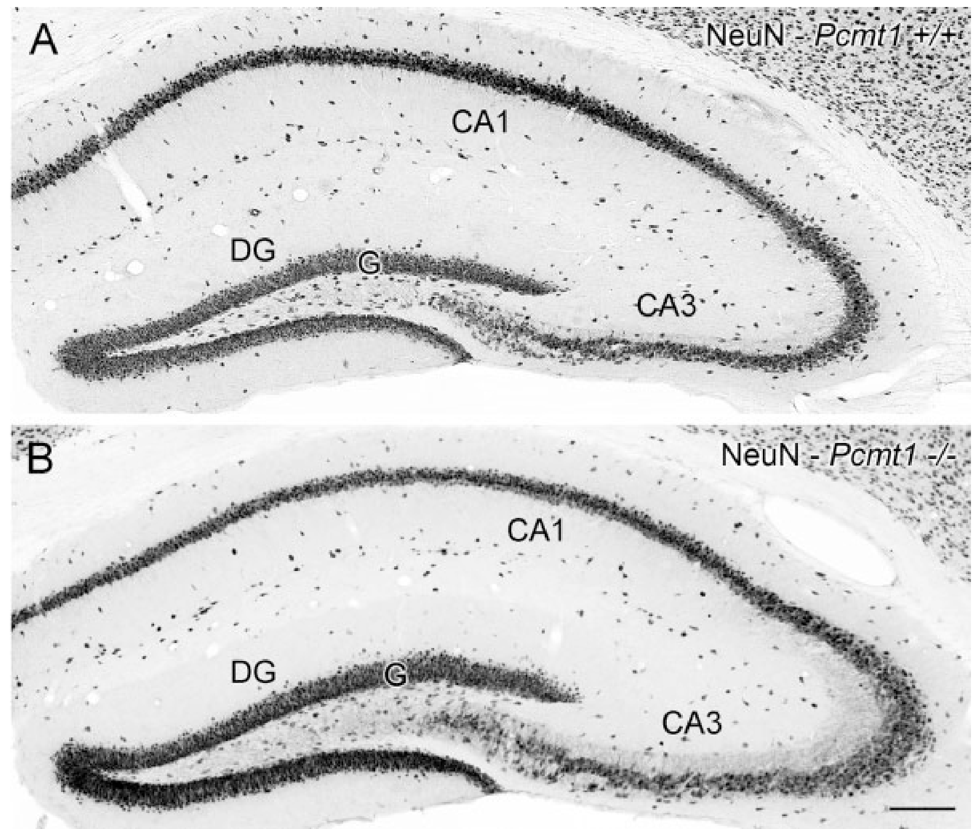

Fig. 1. NeuN immunolabeling of neurons in coronal sections through the hippocampal formation of *Pcmt1+/+* and *Pcmt1–/–* mice. The distribution and density of neurons in the hippocampal formation of the *Pcmt1–/–* mouse (**B**) are very similar to those of the *Pcmt1+/+* mouse (**A**). However, the size of the hippocampal formation in the *Pcmt1–/–* mouse (B) appears to be larger, and the granule cell layer (G) of the dentate gyrus (DG) is somewhat thicker than that seen in the *Pcmt1+/+* mouse (A). Scale bar = 200 μm in B (applies to A,B).

starting from the rostral end of the hippocampus and proceeding caudally until no Prox1-labeled cells could be detected. An average of ~5% of the total granule cell layer per section was sampled systematically and randomly with a counting frame of 25 × 25 μm. Total section thickness was used for dissector height, and only nuclei within the counting frame or overlapping the right or superior border of the counting frame, and which came into focus while focusing down through the dissector height, were counted.

For the analysis of mitosis marker-labeled cells, every seventh section was analyzed starting from the rostral end of the hippocampus and proceeding caudally until no cells of the subgranular zone could be detected. The subgranular zone was sampled in each section with a counting frame of 75 × 75 μm. The region of the subgranular zone was delineated by a contour line drawn 12 μm above and below the inner border of the granule cell layer.

The hilar area was sampled in each section with a counting frame of 75 × 75 μm for Prox1- and mitosis marker-labeled cells. Cells in the hilus were considered those within the area between the upper and lower blades of the dentate gyrus, excluding the 12 μm region below the granule cell layer, and not within CA3. The total numbers of neurons labeled for Prox1 and the mitosis marker in the defined regions were estimated and the average number of neurons ±SEM was calculated for both groups of *Pcmt1+/+* and *Pcmt1–/–* mice. The data were analyzed statistically with Student's *t*-test to determine significant differences in the number of neurons between groups (*Pcmt1+/+* and *Pcmt1–/–* mice) in each region; $P < 0.05$ was considered significant.

Double-labeling and confocal microscopy

Free-floating coronal sections from the same animals described above were incubated in 10% normal donkey serum in TBS containing 0.3% Triton X-100 for 2 hours. The sections were then placed in primary antisera (1:5,000 goat anti-doublecortin and either 1:4,000 rabbit anti-pIRS-1 or 1:1,000 rabbit anti-pIGF-IR/pIR) diluted with TBS containing 2% normal donkey serum and incubated for 3 days at room temperature. After thorough rinsing in TBS, sections were incubated in a mixture of donkey antigoat IgG conjugated to Alexa Fluor 488 and donkey antirabbit IgG labeled with Alexa Fluor 555 (both 1:500; Molecular Probes, Eugene, OR) at room temperature for 2 hours. Sections were then rinsed in TBS for at least 20 minutes, mounted on slides, and coverslipped with Prolong antifade medium (Molecular Probes). Sections were analyzed with a Zeiss (Thornwood, NY) LSM 510 confocal microscope. Images compared in figures were adjusted identically for brightness and contrast.

RESULTS

Enlarged hippocampal formation but relatively normal histology in *Pcmt1–/–* mice

In NeuN-labeled sections the *Pcmt1–/–* mice demonstrated relatively normal neuronal morphology in the hippocampal formation, with no macroscopic tissue abnormalities aside from an enlarged appearance (Fig. 1A,B). Throughout the dentate gyrus of the *Pcmt1–/–* mice the granule cell layer often appeared longer and wider than that of the *Pcmt1+/+* mice (Fig. 1A,B).

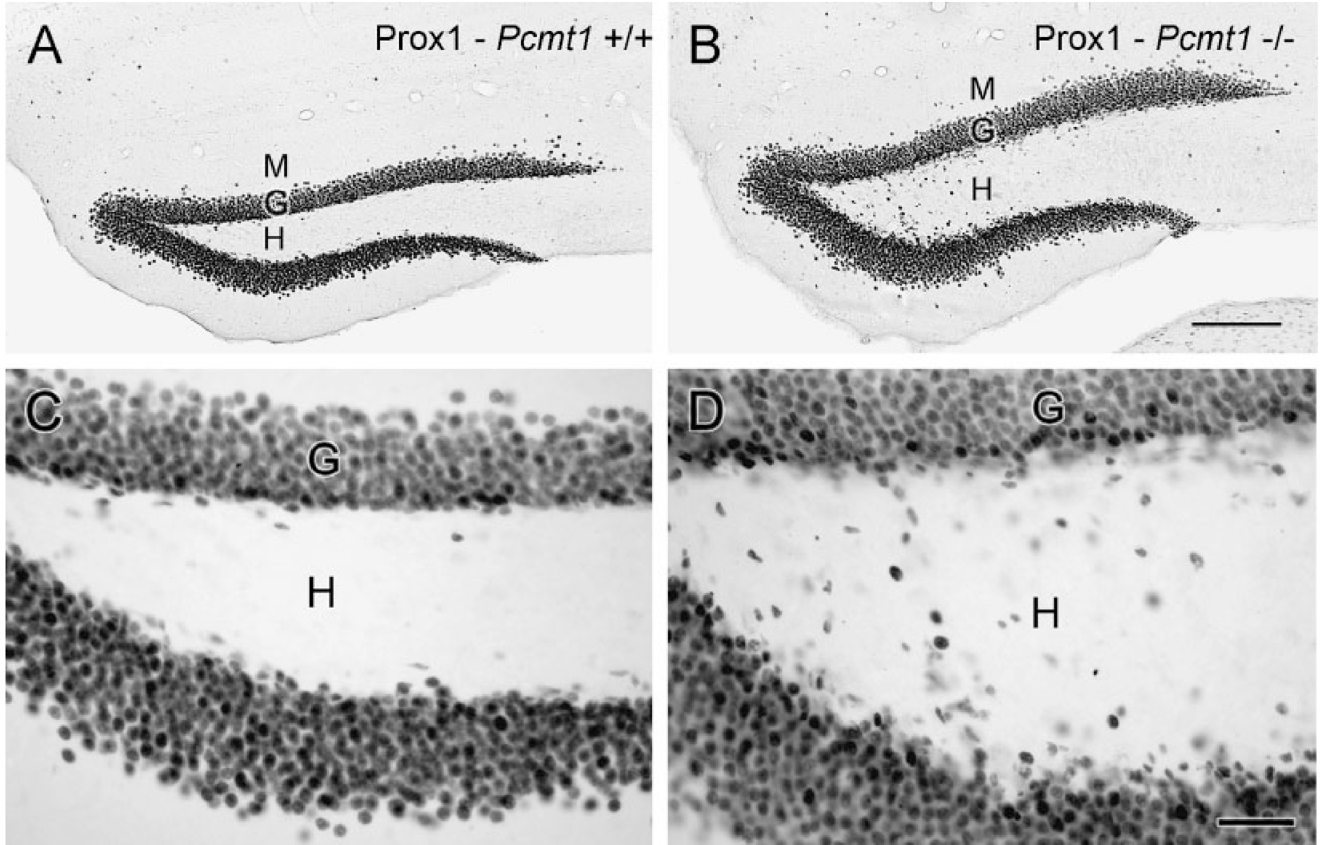

Fig. 2. Prox1 immunolabeling of granule cells in the dentate gyrus of *Pcmt1*+/+ and *Pcmt1*–/– mice. **A:** In the *Pcmt1*+/+ mouse, granule cells in the dentate gyrus are confined mainly to the granule cell layer (G) with a few scattered neurons within the molecular layer (M) and hilus (H). **B:** In the *Pcmt1*–/– mouse, the granule cell layer appears slightly thicker than that of the *Pcmt1*+/+ mouse (A), and more granule cells are evident in the molecular layer and hilus. **C:** Higher magnification of the hilus in the *Pcmt1*+/+ mouse shows relatively few granule cells in this region. **D:** Higher magnification of the hilus in the *Pcmt1*–/– mouse shows many more granule cells in this region than in the *Pcmt1*+/+ mouse (C). Scale bars = 200 μm in B (applies to A,B); 25 μm in D (applies to C,D).

Greater number of Prox1-labeled cells in the dentate gyrus and hilus of *Pcmt1–/–* mice

The mouse homolog of the *Drosophila* gene *prospero, prox-1*, is a divergent homeobox gene expressed almost exclusively in dentate granule cells in the postnatal rodent brain (Oliver et al., 1993; Liu et al., 2000; Pleasure et al., 2000). Antiserum against Prox1 was used to label granule cells of the dentate gyrus in both *Pcmt1*+/+ and *Pcmt1*–/– mice (Fig. 2A,B). Labeled cells within the granule cell layer and hilus were counted separately using stereological methods.

Pcmt1–/– mice demonstrated a 22% increase in the number of cells within the granule cell layer over that in *Pcmt1*+/+ mice (Table 1; Fig. 2A,B). As the volume of this

TABLE 1. Number of Prox1- and Mitosis Marker-immunoreactive Cells per Region of the Dentate Gyrus in *Pcmt1*+/+ and *Pcmt1*−/− Mice

	Measurement	*Pcmt1*+/+ Mean[1]	SEM[2]	*Pcmt1*−/− Mean[1]	SEM[2]	*Pcmt1*−/− value/ *Pcmt1*+/+ value	*P* value
Prox1-immunoreactive neurons	Cells in the granule cell layer	538,978	17,651	658,426	9,092	122%	**0.003
	Granule cell layer volume (mm^3)	206	28	260	29	126%	*0.021
	Granule cell layer cell density (cell/mm^3)	2,737	292	2,635	312	96%	0.428
	Cells in the hilus	5,586	214	14,499	1,568	260%	*0.010
	Hilar volume (mm^3)	200	11	304	20	152%	**0.008
	Hilar cell density (cell/mm^3)	28	2	47	2	167%	**0.001
Mitosis marker-immunoreactive cells	Cells in the subgranular zone	2,487	233	5,810	317	234%	**0.005
	Cells in the hilus	865	117	1,999	219	231%	**0.004

[1]Mean value of four mice.
[2]SEM is the standard error of the mean corresponding to the preceding value.
*$P < 0.05$.
**$P < 0.01$.

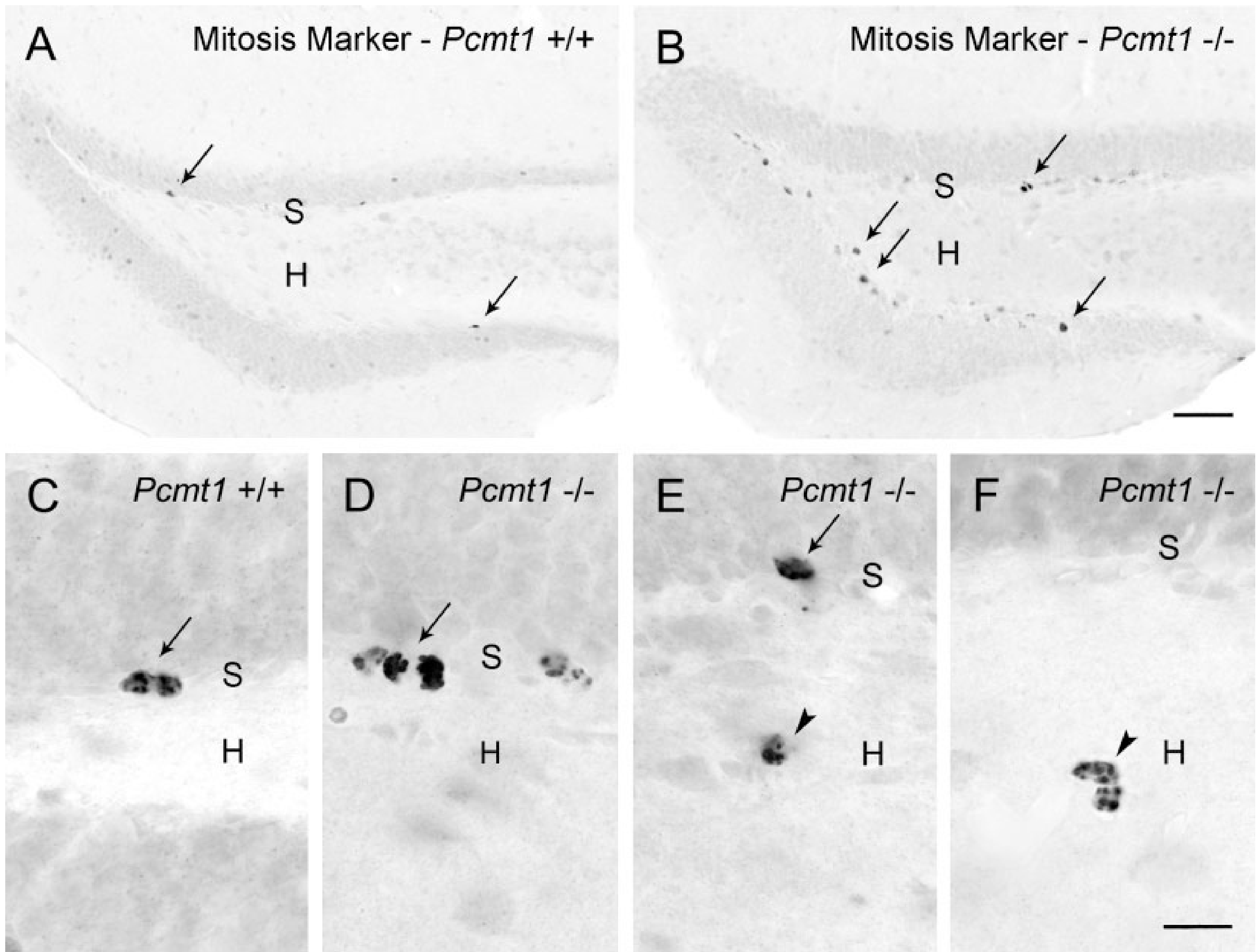

Fig. 3. Mitosis marker immunolabeling in the dentate gyrus of *Pcmt1*+/+ and *Pcmt1*–/– mice. **A:** In the *Pcmt1*+/+ mouse a few cells undergoing mitosis (examples at arrows) can be seen within the subgranular zone (S) of the dentate gyrus and sometimes in the hilus (H). **B:** In the *Pcmt1*–/– mouse many more mitotic cells can be seen within the subgranular zone and hilus than within these regions in the *Pcmt1*+/+ mouse (A). **C–F:** Higher magnification of mitotic cells in the subgranular zone (arrows) and hilus (arrowheads) of *Pcmt1*+/+ (C) and *Pcmt1*–/– (D–F) mice. The labeling of the mitotic cells often appears as a group of dots (C,F), and these cells can sometimes be found alone (E), in pairs (C,F), or in clusters (D). Scale bars = 50 μm in B (applies to A,B); 10 μm in F (applies to C–F).

cell layer was also increased by ~26%, the cell density was not significantly different from that of *Pcmt1*+/+ mice (Table 1). These findings were consistent with the general observation of similar sizes of granule cells in *Pcmt1*–/– and *Pcmt1*+/+ mice.

In the hilus of *Pcmt1*–/– mice, the number of granule cells was ~160% greater than that seen in *Pcmt1*+/+ mice, and the region was ~52% greater in volume (Table 1; Fig. 2C,D). The estimated number of granule cells in both regions was greater in all *Pcmt1*–/– mice compared to their *Pcmt1*+/+ littermates.

Greater numbers of mitosis marker-labeled cells in the subgranular zone and hilus of *Pcmt1–/–* mice

An increase in cell number could signify an increase in cell proliferation or a decrease in cell death. Therefore, to investigate the level of cell proliferation occurring in the dentate gyrus of *Pcmt1*–/– compared to *Pcmt1*+/+ mice, mitotic cells were localized with an antiserum against the endogenous marker, histone H3 phosphorylated at Ser10. The appearance of this marker coincides with mitotic chromosome condensation and is only present in cells that are actively dividing (Hendzel et al., 1997), as opposed to those undergoing DNA repair or those with altered cellular uptake, as can sometimes occur with bromodeoxyuridine (BrdU) labeling (Cooper-Kuhn and Kuhn, 2002; Gould and Gross, 2002; Rakic, 2002). Although BrdU labeling might have provided an additional measurement of cell proliferation, *Pcmt1*–/– mice are particularly sensitive to handling at this age, and such experiments could be complicated by the increased occurrence of seizures and death in these mice. In *Pcmt1*+/+ and *Pcmt1*–/– mice, immunolabeling for the mitosis marker was observed in cells of both the subgranular zone and the hilus (Fig. 3A–F), but labeled cells in both regions appeared more

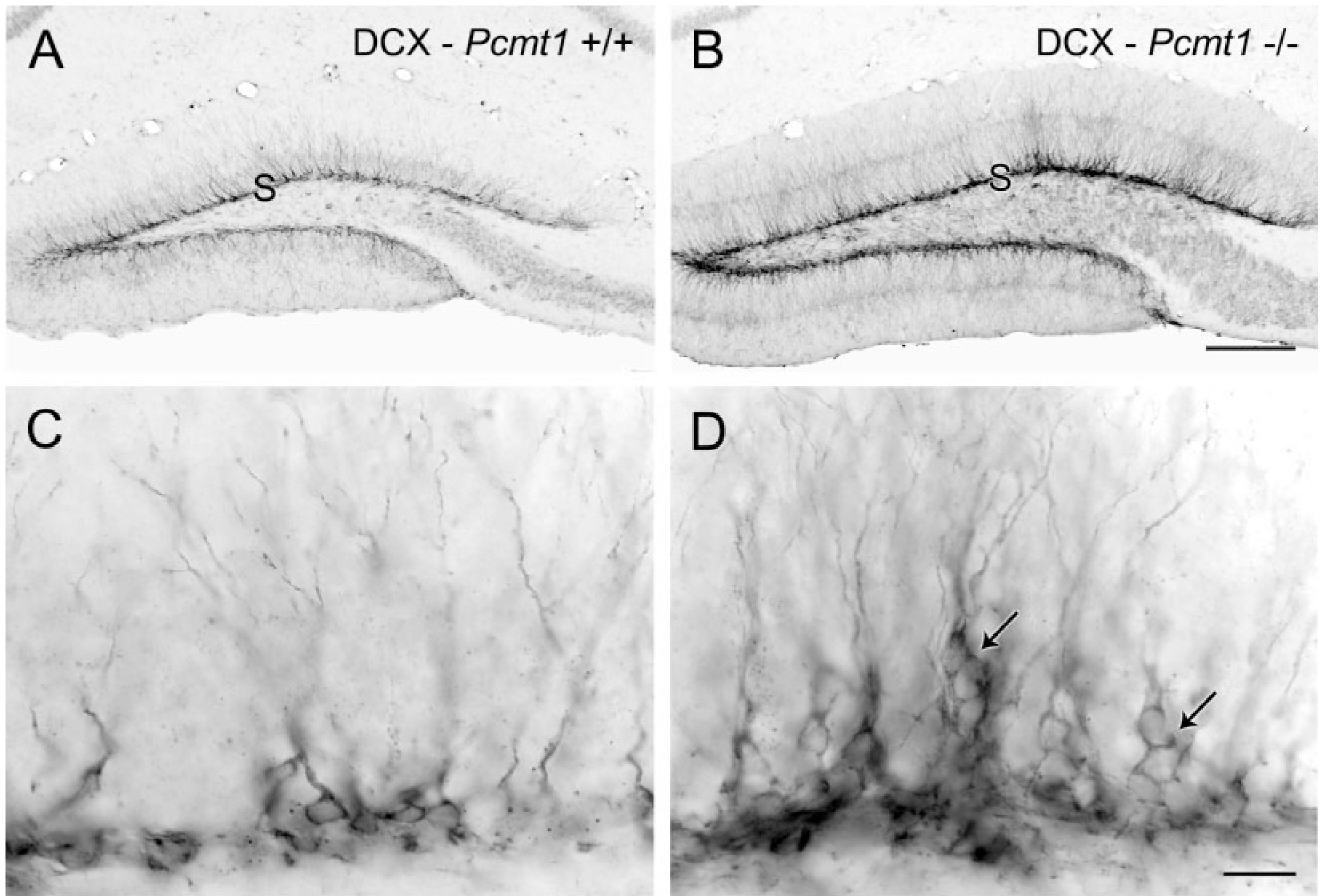

Fig. 4. Doublecortin (DCX) immunolabeling in the dentate gyrus of *Pcmt1*+/+ and *Pcmt1*–/– mice. **A,B:** In *Pcmt1*+/+ (A) and *Pcmt1*–/– (B) mice, doublecortin labeling is visible along the subgranular zone (S) of the dentate gyrus, but the labeling is much stronger in the *Pcmt1*–/– mouse (B). Also, labeling of dendrites is more extensive in the granule cell and molecular layers of the *Pcmt1*–/– mouse (B) than in the *Pcmt1*+/+ mouse (A). **C,D:** Further magnification shows a greater number of labeled cell bodies in the subgranular zone in the *Pcmt1*–/– (D) compared to the *Pcmt1*+/+ (C) mouse. The immature neurons in the *Pcmt1*–/– mouse are more often found clustered and extending as rows into the granule cell layer (D; examples at arrows). Scale bars = 200 μm in B (applies to A,B); 10 μm in D (applies to C,D).

abundant in the *Pcmt1*–/– mice (Fig. 3A,B). Using stereological methods to estimate the numbers of mitosis marker-labeled cells, *Pcmt1*–/– mice were found to have a 134% increase in the number of actively dividing cells in the subgranular zone over that in *Pcmt1*+/+ mice (Table 1). In the hilus of *Pcmt1*–/– mice, the number of mitosis marker-labeled cells was 131% higher than that of *Pcmt1*+/+ mice (Table 1). The labeling of mitotic cells was greater in all of the *Pcmt1*–/– mice compared to their *Pcmt1*+/+ littermates.

Although no quantitative studies were conducted, the subventricular zone was examined qualitatively and no evidence for increased cell proliferation was found in the current group of animals at 40 days of age.

Increased labeling of doublecortin in the subgranular zone of *Pcmt1–/–* mice

Doublecortin is a microtubule binding protein that is transiently expressed in proliferating progenitor cells and the newly generated progeny of adult neural progenitor cells (Francis et al., 1999; Brown et al., 2003). In this study, doublecortin immunoreactivity was substantially increased in the subgranular zone of *Pcmt1*–/– mice (Fig. 4B) compared with *Pcmt1*+/+ mice (Fig. 4A). The increase in labeling in the subgranular zone of *Pcmt1*–/– mice appeared to be due to both a greater number of labeled neuronal cell bodies and increased labeling of cellular processes (Fig. 4A,B). In addition, the labeling of apical dendrites extended further into the molecular layer in *Pcmt1*–/– mice (Fig. 4B) compared to that in *Pcmt1*+/+ mice (Fig. 4A). Labeling in *Pcmt1*+/+ mice was generally in cell bodies aligned in a single row along the base of the granule cell layer (Fig. 4C). In *Pcmt1*–/– mice, however, more doublecortin-labeled cell bodies could be seen along the base and extending as rows into the granule cell layer itself (Fig. 4D). Increased doublecortin labeling was observed in all *Pcmt1*–/– mice compared to their *Pcmt1*+/+ littermates.

To determine if increased doublecortin labeling could be related to seizure activity, independent of the *Pcmt1* mutation, control experiments were conducted in pilocarpine-treated mice during the chronic stage when the animals

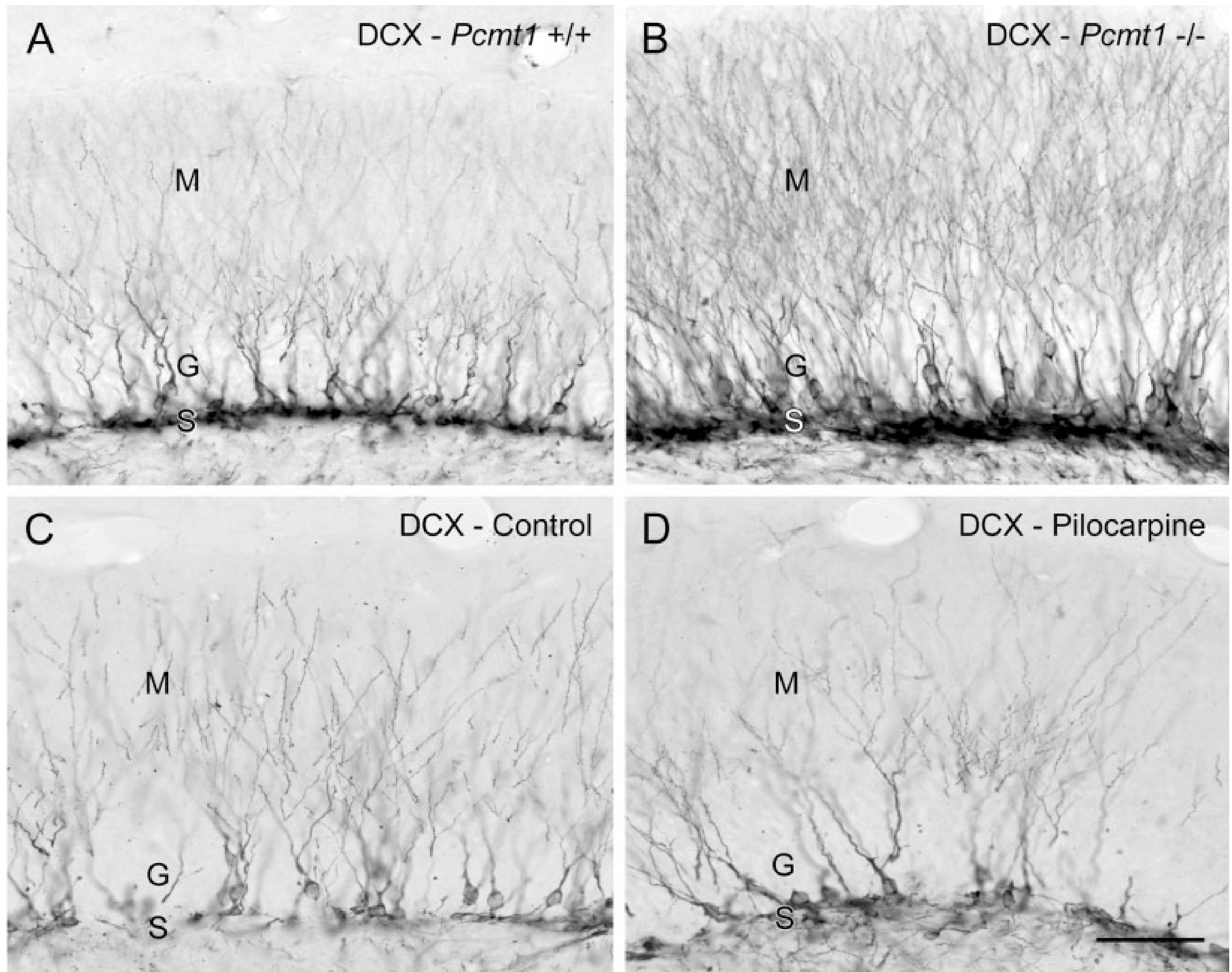

Fig. 5. Comparison of doublecortin (DCX) labeling in the dentate gyrus of *Pcmt1*+/+ (**A**) and *Pcmt1*–/– (**B**) mice with control (**C**) and pilocarpine-treated (**D**) mice. A,B: Doublecortin labeling of cell bodies in the subgranular region (S) and dendritic processes in the molecular layer (M) is substantially increased in the *Pcmt1*–/– mouse (B) as compared to that in the *Pcmt1*+/+ mouse (A). Labeled neurons in the granule cell layer (G) are also more numerous in the *Pcmt1*–/– mouse (B). C,D: In contrast, doublecortin labeling is not increased in a chronic pilocarpine-treated mouse (D) that was having frequent spontaneous seizures when compared with that in an age-matched (4-month-old) control mouse (C). The more extensive labeling in the *Pcmt1*+/+ mouse (A) than in the control C57Bl/6 mouse (C) is likely due to the younger age of the *Pcmt1* mice. Scale bar = 25 μm in D (applies to A–D).

were experiencing frequent spontaneous seizures. No increase in doublecortin labeling was observed in these mice at 2 months following pilocarpine-induced status epilepticus when compared to age-matched control mice (Fig. 5C,D). However, in the same immunohistochemical experiment, doublecortin labeling was substantially greater in *Pcmt1*–/– mice (Fig. 5B) compared to that in *Pcmt1*+/+ mice (Fig. 5A), consistent with the previous descriptions. Doublecortin labeling was greater in both *Pcmt1*+/+ and *Pcmt1*–/– mice than in the control and pilocarpine-treated mice (compare Fig. 5A,B to 5C,D). This difference is presumably related to the normally greater neurogenesis in young animals than in older animals. (*Pcmt1* mice were only 40 days of age, whereas the control and pilocarpine-treated mice were 4 months of age at the time of perfusion.)

Increased labeling of insulin-related pathway components in the subgranular zone of *Pcmt1–/–* mice

Type I insulin-like growth factor receptor (IGF-IR) and insulin receptor (IR) are transmembrane tyrosine kinases that share significant similarity in both structure and function. Upon binding of their individual ligands, autophosphorylation of the receptors' beta subunits occurs. The triple tyrosine cluster (Tyr1131, Tyr1135, and Tyr1136 for IGF-IR and Tyr1146, Tyr1150, and Tyr1151 for IR) within the kinase domain is the earliest major site of autophosphorylation for both receptors (Hernandez-Sanchez et al., 1995) and is necessary for their activation (White et al., 1985, 1988; Baserga, 1999; Lopaczynski et al., 2000). Using an antibody that detects both IGF-IR

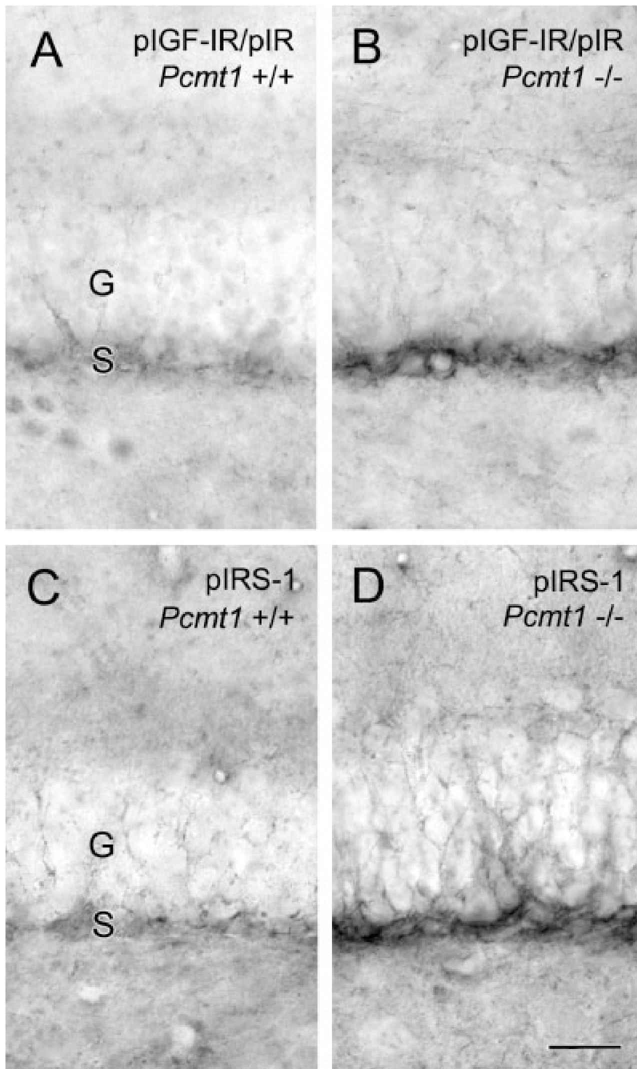

Fig. 6. Phosphoinsulin-like growth factor-I receptor/phospho-insulin receptor (pIGF-IR/pIR) and phospho-insulin receptor substrate-1 (pIRS-1) immunolabeling in the subgranular zone of *Pcmt1*+/+ and *Pcmt1*–/– mice. **A:** In the *Pcmt1*+/+ mouse, pIGF-IR/pIR immunolabeling is evident in some cell bodies in the subgranular zone (S) and their proximal dendrites that extend into the granule cell layer (G). **B:** In the *Pcmt1*–/– mouse, the labeling for pIGF-IR/pIR is visible in cell bodies and dendrites of the same region, but at a much higher level. **C:** Labeling for pIRS-1 in the subgranular zone of the *Pcmt1*+/+ mouse is similar to that seen for pIGF-IR/pIR in this mouse (A). **D:** In the *Pcmt1*–/– mouse, labeling for pIRS-1 is increased in the cell bodies of the subgranular zone as well as their proximal dendrites when compared to the *Pcmt1*+/+ mouse (C). Scale bar = 10 μm in D (applies to A–D).

phosphorylated at Tyr1131 and IR phosphorylated at Tyr1146, increased immunoreactivity was found in the subgranular zone of all *Pcmt1*–/– mice (Fig. 6B) compared to that of their *Pcmt1*+/+ littermates (Fig. 6A). The increased labeling of pIGF-IR/pIR in *Pcmt1*–/– mice was evident in multiple regions of both the hippocampus and cortex but was especially prominent within the subgranular zone of the dentate gyrus. The increased labeling of this region appeared to be within both cell bodies and the processes that extended into the granule cell layer.

One of the major substrates for both IR and IGF-IR is insulin receptor substrate-1 (IRS-1) (Sun et al., 1991; White and Yenush, 1998), which appears to provide a link between these receptors and downstream pathways essential for DNA synthesis and cellular proliferation (Sun et al., 1993). IRS-1 is phosphorylated at tyrosine-941 by IGF-I and insulin receptors and is one of the main binding sites for PI3K (Xu et al., 1995). In this study, a similar increase in immunoreactivity was seen for pIRS-1 (Fig. 6D) as was seen for pIGF-IR/pIR in the subgranular zone of all *Pcmt1*–/– mice when compared to their *Pcmt1*+/+ littermates (Fig. 6C). These results are consistent with those obtained previously in 50-day-old *Pcmt1*–/– and *Pcmt1*+/+ mice (Farrar et al., 2005).

Localization of doublecortin and insulin-related pathway components in the same cells of the dentate gyrus

Although doublecortin and the insulin-related pathway markers, pIGF-IR/pIR and pIRS-1, appeared to be increased in the same region, it was unknown whether they were expressed on the same cells. In order to investigate this, double immunofluorescence labeling for doublecortin and the insulin-related pathway markers was performed. As in the single-labeling studies, doublecortin labeling of neurons in the subgranular zone and their dendrites that extended into the granule cell layer was increased in *Pcmt1*–/– (Fig. 7D,G) compared to *Pcmt1*+/+ mice (Fig. 7A). Likewise, the intensity of pIRS-1 and pIGF-IR/pIR immunofluorescence labeling was higher in the *Pcmt1*–/– mice (Fig. 7E,H) and was seen in a greater number of neurons and their processes in the subgranular zone when compared to that in the *Pcmt1*+/+ mice (Fig. 7B).

Localization of doublecortin and pIGF-IR/pIR was observed in many of the same neurons and processes of both *Pcmt1*+/+ (Fig. 7C) and *Pcmt1*–/– mice (Fig. 7F). Localization of doublecortin and pIRS-1 was also observed in the same cells of both *Pcmt1*+/+ (not shown) and

Fig. 7. Double immunofluorescence labeling of doublecortin and insulin-related proteins in the granule cell layer of *Pcmt1*+/+ and *Pcmt1*–/– mice. **A–C:** In the *Pcmt1*+/+ mouse, doublecortin (DCX) labeling (A) is visible in the immature neurons of the subgranular zone (S) and proximal dendrites that extend into the granule cell layer (G). The pIGF-IR/pIR immunolabeling (B) in this mouse is also seen in the subgranular zone neurons and their proximal dendrites. In the merged image (C), the same cells that are labeled for doublecortin are also labeled for pIGF-IR/pIR. **D–F:** In the *Pcmt1*–/– mouse, doublecortin (D) is visible in the immature neurons of the subgranular zone, but at a higher level than that seen in the *Pcmt1*+/+ mouse (A). Immunolabeling for pIGF-IR/pIR (E) is also increased in the neurons and dendrites of the subgranular zone when compared to the *Pcmt1*+/+ mouse (B). In the merged image (F), both doublecortin and pIGF-IR/pIR are localized in the same neurons of the *Pcmt1*–/– mouse. **G–I:** Doublecortin labeling (G) and pIRS-1 labeling (H) in the *Pcmt1*–/– mouse closely resemble the labeling patterns for doublecortin and the insulin-related receptors (D–F). Likewise, doublecortin and pIRS-1 are localized in many of the same neurons of the subgranular zone (I). **J–L:** At higher magnification, doublecortin labeling of dendrites in the granule cell layer of the *Pcmt1*–/– mouse has a smooth, continuous appearance (J). In contrast, the pIGF-IR/pIR immunolabeling in the same region has a punctate appearance (K). In the merged image, the punctate labeling of pIGF-IR/pIR appears to surround the smooth doublecortin labeling in microtubules of the dendrites, consistent with the location of the receptors on the dendritic surface. Scale bars = 10 μm in C (applies to A–I); 5 μm in L (applies to J–L).

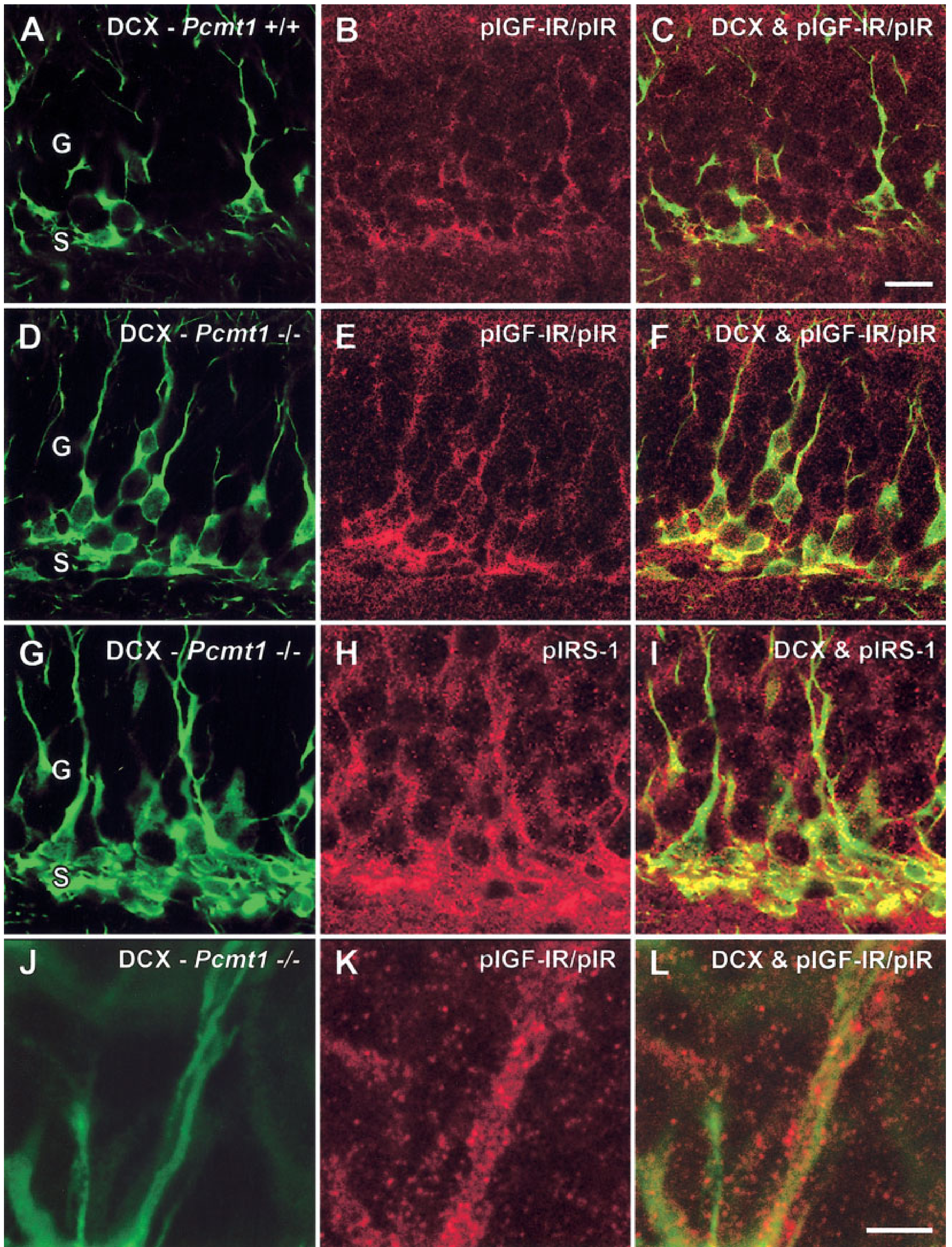

Figure 7

Pcmt1–/– mice (Fig. 7I). Much of the pIGF-IR/pIR and pIRS-1 labeling had a punctate appearance (Fig. 7B,E,H,K) that contrasted with the more uniform labeling of the microtubule-associated doublecortin protein (Fig. 7A,D,G,J). The difference in labeling patterns was particularly obvious at higher magnification (compare Fig. 7J and 7K). An increase in the punctate labeling of pIGF-IR/pIR and pIRS-1 was not confined to the doublecortin-labeled neurons but was also evident throughout the granule cell and molecular layers of the dentate gyrus. The subcellular localization of the punctate structures is unknown. However, such labeling, particularly in the mature neurons, would be consistent with previous reports showing these receptors in certain synaptic locations (Garcia-Segura et al., 1997; Abbott et al., 1999).

DISCUSSION

Evidence for increased neurogenesis and involvement of insulin-related receptor pathways in *Pcmt1–/–* mice

Mice lacking a key protein repair enzyme, PCMT1, have an apparent increase in activation of the IGF-I/insulin receptor pathway (Farrar et al., 2005). In this study, *Pcmt1*–/– mice were also found to have an increase in the number of granule cells in the granule cell layer and hilus of the dentate gyrus. In addition, they had an increased abundance of immature neurons in the subgranular zone, indicating either an increase in neuron proliferation or an inhibition of apoptosis in the progeny of adult neural progenitor cells (Åberg et al., 2000; D'Ercole et al., 2002). Using a mitotic marker to examine active cell proliferation, a dramatic increase in the number of proliferating cells was found in the dentate gyrus of the *Pcmt1*–/– compared to *Pcmt1*+/+ mice. These findings strongly suggest that in the *Pcmt1*–/– mice the greater number of granule cells is a result of increased cell production.

An increased abundance of immature neurons in the *Pcmt1*–/– mice was detected with antibodies against doublecortin, a microtubule protein specific to the recent progeny of neural progenitor cells (Francis et al., 1999; Brown et al., 2003) and one that is considered a reliable and specific indicator of adult neurogenesis and its modulation (Couillard-Despres et al., 2005). A striking increase in doublecortin labeling was not only detected in the cell bodies of immature neurons, but also in the dendritic processes that extend into the granule cell and molecular layers of the dentate gyrus. Previous studies of *Pcmt1*–/– mice have demonstrated increased labeling for polysialic acid-enriched neural cell adhesion molecule (PSA-NCAM) in processes that perforated perpendicularly through the granule cell layer (Ikegaya et al., 2001), but the significance of this labeling was not addressed. It is very likely that the PSA-NCAM-labeled processes described in the earlier studies are the same as those labeled for doublecortin in this study, as both markers have been observed in immature neurons of this region in normal adult animals (Seki and Arai, 1993; Seri et al., 2004).

Another major finding of this study was the localization of activated components of the IGF-I/insulin receptor pathways on the immature neurons of *Pcmt1*–/– mice. Evidence from multiple lines of animals with genetic alterations in the IGF-I pathway (O'Kusky et al., 2000; D'Ercole et al., 2002; Bondy and Cheng, 2004) and animals in which IGF-I was administered systemically (Åberg et al., 2000; Anderson et al., 2002) indicates that this pathway promotes neurogenesis, neuronal survival, process growth, and synaptogenesis. Although IGF-I receptors have been localized on progenitor cells in hippocampal cell cultures (Åberg et al., 2003), this appears to be the first demonstration of activated insulin or IGF-I receptors on immature neurons in the subgranular zone of the dentate gyrus. The increased activation of the insulin or IGF-I pathways in this region of the *Pcmt1*–/– mice could potentially increase the growth and survival of newly generated granule cells and their developing axons.

Potential relationship of neurogenesis, insulin-related pathways, and seizures in *Pcmt1–/–* mice

Pcmt1–/– mice develop generalized seizures that usually appear after 30 days of age (Kim et al., 1997, 1999; Yamamoto et al., 1998; Ikegaya et al., 2001; Farrar and Clarke, 2002). Therefore, it remains possible that the seizures in the *Pcmt1*–/– mice could be stimulating the neurogenesis (Bengzon et al., 1997; Parent et al., 1997; Gray and Sundstrom, 1998; Covolan et al., 2000; Sankar et al., 2000; Scharfman et al., 2000), as well as the alterations observed in insulin-related receptor pathways, such as occurs in the trkB receptor pathway of kindled mice (He et al., 2002). Future studies, possibly involving the suppression of seizures in *Pcmt1*–/– mice, will be necessary to determine the contribution of the seizure phenotype to the level of neurogenesis in these mice. However, the current finding that pilocarpine-treated mice with frequent spontaneous seizures did not show an increase in doublecortin-labeled neurons suggests that brief spontaneous seizures alone are not enough to stimulate a substantial increase in neurogenesis. Considering these findings, it appears unlikely that the marked increase in doublecortin labeling in the *Pcmt1*–/– mice is related primarily to seizure activity.

In addition, increased levels of the insulin receptor β-subunit are found in the brain tissue of very immature (P0) *Pcmt1*–/– mice (Farrar et al., 2005), indicating that insulin-related growth pathways are altered in these mice before the development of generalized seizures. In addition, the accumulation of damaged proteins is also present in the brain tissue of *Pcmt1*–/– mice before the development of seizures (Lowenson et al., 2001), suggesting that the accumulation of damaged proteins, and not the seizures, precedes and possibly causes the increased activation of insulin-related growth pathways.

Interestingly, despite the greater number of granule cells and the occurrence of seizures in *Pcmt1*–/– mice, there is no evidence of mossy fiber sprouting into the inner molecular layer of the dentate gyrus (Ikegaya et al., 2001). Likewise, no hippocampal cell loss has been detected in *Pcmt1*–/– mice under 50 days of age and only very rarely in those over this age. (In a small number of *Pcmt1*–/– mice over 50 days of age, some cell loss was present in patterns that resembled seizure-induced damage; Farrar and Houser, unpubl. findings.) However, the lack of cell loss and mossy fiber sprouting in *Pcmt1*–/– mice does not preclude the occurrence of other types of aberrant neuronal growth and reorganization. The increased activation of growth and survival pathways could lead to multiple changes in granule cell morphology that might include

dendritic, as well as axonal growth. If abnormal growth of neuronal processes occurs in *Pcmt1–/–* mice, this, along with the increased granule cell number, could lead to the formation of aberrant and excessive connections. Such changes could contribute to the hyperexcitability of the mossy fiber path in *Pcmt1–/–* mice, as described previously (Ikegaya et al., 2001), and potentially contribute to their recurrent seizures.

In this study, increased numbers of granule cells were found not only in the subgranular zone but also in the hilus. Similar increases in hilar granule cells have been observed in other animal models following episodes of induced status epilepticus (Parent et al., 1997; Scharfman et al., 2000; Shapiro and Ribak, 2005). This increase in granule cells within the hilus has generally been attributed to errors in cell migration. However, during certain periods of normal development, granule cells are produced in the hilus and subsequently migrate to the granule cell layer (Altman and Bayer, 1990). The present findings of increased numbers of mitotic cells and Prox1-labeled granule cells in the hilus of *Pcmt1–/–* mice suggest that, under certain conditions, increased generation of granule cells can occur in the hilus, as well as in the more commonly recognized subgranular zone, in adult animals.

Implications and future directions

Adult neurogenesis in the dentate gyrus is stimulated by numerous factors, including hormones (Gould et al., 1992; Tanapat et al., 1999), neurotransmitter levels (Gould et al., 1994; Brezun and Daszuta, 1999), growth factors (Anderson et al., 2002), enriched environments (Kempermann et al., 1997), and running (van Praag et al., 1999). While many of these influences may be considered positive, such as enriched environment and running, accumulating evidence indicates that various brain insults, such as ischemia (Liu et al., 1998) and a severe seizure episode (for recent review, see Parent, 2003) also promote neurogenesis. This study raises the possibility that the accumulation of isoaspartyl-damaged proteins may be another type of brain insult that stimulates neurogenesis. This is an especially intriguing possibility considering recent studies showing increased expression of immature neuronal markers in the dentate gyrus of patients with Alzheimer's disease, another condition in which damaged proteins are known to accumulate (Jin et al., 2004).

The increased brain size of the *Pcmt1–/–* mice is another interesting finding that remains unexplained. Although increased neurogenesis of dentate granule cells cannot account for this change, the increased activation of insulin receptor-related pathways could stimulate additional growth-related changes in the brain, such as neuronal hypertrophy, increased proliferation and survival of glia, and reduced apoptosis (see D'Ercole et al., 2002, for review). Future studies will be needed to determine the contributions of these factors to the enlargement of the hippocampus, as well as other brain regions, in the *Pcmt1–/–* mouse (Farrar et al., 2005).

The cause of alterations in the insulin and/or IGF-I pathways in these protein repair-deficient mice remains unknown. Aside from the possibility that either single or multiple components of these pathways accumulate isoaspartyl-damage, the possibility that a general accumulation of damaged proteins may alter tissue metabolism and growth must be considered. One piece of evidence that the *Pcmt1–/–* mice may possess an altered metabolic state is the increased expression of insulin receptors from the first postnatal day and in multiple tissues (Farrar et al., 2005). Interestingly, unusually high numbers of insulin receptors have been found in the brain tissue of patients who died of Alzheimer's disease, and the investigators interpreted these changes as the brain's attempt to compensate for the receptors' decreased activity (Frolich et al., 1998). Whatever the cause for the altered insulin receptor levels in *Pcmt1–/–* mice, one consequence may be alterations in the IGF-I pathway, which may have a prominent role in promoting cellular growth and survival, particularly in the brain (Bondy and Cheng, 2004). Future studies will be necessary to determine the mechanisms involved in the increased cellular proliferation and alterations in metabolic and growth pathways in the *Pcmt1–/–* mouse brain. Such studies could prove to be particularly useful for determining the relationship between cellular damage and growth-receptor pathways, especially in the context of neurogenesis during such conditions as epilepsy or the accumulation of protein damage.

ACKNOWLEDGMENTS

We thank Dr. Monique Esclapez for assistance with stereological methods and helpful discussions and Dr. Zechun Peng for assistance with double immunofluorescence methods and confocal microscopy.

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