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Int. J. Devl Neuroscience 23 (2005) 465–474

INTERNATIONAL JOURNAL of DEVELOPMENTAL NEUROSCIENCE

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Postnatal cerebellar defects in mice deficient in methylenetetrahydrofolate reductase

Zhoutao Chen [1], Bernd C. Schwahn [2], Qing Wu, Xinying He, Rima Rozen *

Departments of Human Genetics, Pediatrics and Biology, McGill University and Montreal Children's Hospital Research Institute, 4060 Ste. Catherine West, Room 200, Montreal, Que., Canada H3Z 2Z3

Received 6 January 2005; accepted 3 May 2005

Abstract

Patients with severe deficiency of methylenetetrahydrofolate reductase (MTHFR) suffer from a wide variety of neurological problems, which can begin in the neonatal period. MTHFR is a critical enzyme in folate metabolism; the product of the MTHFR reaction, 5-methyltetrahydrofolate, is required for homocysteine remethylation to methionine and synthesis of *S*-adenosylmethionine (SAM). To understand the mechanisms by which MTHFR deficiency leads to significant neuropathology, we examined early postnatal brain development in mice with a homozygous knockout of the *Mthfr* gene. These mice displayed a dramatically reduced size of the cerebellum and cerebral cortex, with enlarged lateral ventricles. *Mthfr* deficiency affected granule cell maturation, but not neurogenesis. Depletion of external granule cells and disorganization of Purkinje cells were mainly confined to the anterior lobules of mutant cerebella. Decreased cellular proliferation and increased cell death contributed to the granule cell loss. Reduced expression of Engrailed-2 (*En2*), Reelin (*Reln*) and inositol 1,4,5-triphosphate receptor type 1 (*Itpr1*) genes was observed in the cerebellum. Supplementation of $Mthfr^{+/-}$ dams with an alternate methyl donor, betaine, reduced cerebellar abnormalities in the $Mthfr^{-/-}$ pups. Our findings suggest that MTHFR plays a role in cerebellar patterning, possibly through effects on proliferation or apoptosis.

Keywords: MTHFR; Cerebellum; Apoptosis; Proliferation; Expression; Betaine

1. Introduction

Homocysteine is a neurotoxic sulphur amino acid derived from methionine through the transmethylation cycle (Rosenquist et al., 1996; Lipton et al., 1997). It can induce neuronal apoptosis and increase neuronal vulnerability to excitotoxicity (Kruman et al., 2000). Elevated plasma homocysteine is associated with an increased risk for stroke (Elkind and Sacco, 1998) and possibly Alzheimer's disease (Clarke et al., 1998; Miller, 1999).

Disruptions in homocysteine removal by transsulfuration or remethylation pathways increase homocysteine levels in body fluids and tissues. Nutritional deficiencies in certain vitamins (folate, cobalamin, or pyridoxine) and genetic defects in homocysteine-metabolizing enzymes can result in hyperhomocysteinemia or homocystinuria (Mudd et al., 2001; Rosenblatt and Fenton, 2001). The most frequent genetic cause of mild hyperhomocysteinemia is homozygosity for a common missense mutation (677C → T; A222V) in 5,10-methylenetetrahydrofolate reductase (MTHFR, E.C. 1.5.1.20) which results in moderately decreased enzyme activity (Frosst et al., 1995). MTHFR converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a methyl donor for homocysteine remethylation to methionine. Homozygosity for this variant is high (10–15%) in many North American and European populations, and has been reported to modify risk for several multifactorial diseases (Schwahn and Rozen, 2001). On the other hand, severe MTHFR deficiency with homocystinuria is a relatively rare inborn error of metabolism and is associated

* Corresponding author. Tel.: +1 514 412 4358; fax: +1 514 412 4331.
E-mail address: rima.rozen@mcgill.ca (R. Rozen).
[1] 454 Life Sciences, 20 Commercial St., Branford, CT 06405, USA.
[2] Clinic for General Pediatrics, University Children's Hospital, Düsseldorf, Germany.

0736-5748/$30.00
doi:10.1016/j.ijdevneu.2005.05.007

with much lower levels of MTHFR activity (usually less than 20%) (Rosenblatt and Fenton, 2001). Neurological symptoms in these patients include developmental delay, motor and gait disturbances, peripheral neuropathy, hypotonia, and seizures. Systematic studies on the neurological effects of severe MTHFR deficiency are few due to limited human material.

Homocysteine remethylation is critical for synthesis of methionine and *S*-adenosylmethionine (SAM), an important methyl donor for many cellular processes including DNA methylation, neurotransmitter synthesis and phospholipid metabolism. An alternate methyl donor for homocysteine remethylation is betaine, a metabolite of choline. The enzyme that catalyzes betaine-dependent homocysteine remethylation, betaine homocysteine methyltransferase (BHMT), is most abundant in liver and kidney (McKeever et al., 1999; Chadwick et al., 2000). Betaine supplementation has proven effective in ameliorating the biochemical abnormalities in homocystinuria due to severe MTHFR deficiency. It lowers the elevated homocysteine levels associated with this disease and increases plasma methionine concentrations (Wendel and Bremer, 1984; Holme et al., 1989). However, high-dose betaine treatment is unable to normalize homocysteine metabolism in homocystinuric patients, as indicated by plasma homocysteine levels that remain 5–10-fold elevated (Wendel and Bremer, 1984).

In earlier work, we cloned the cDNA/gene for MTHFR, identified mutations responsible for severe and mild MTHFR deficiency and generated an *Mthfr* knockout mouse (Goyette et al., 1994, 1995, 1996; Frosst et al., 1995; Chen et al., 2001). Both heterozygous and homozygous *Mthfr* knockout mice exhibit hyperhomocysteinemia and decreased methylation capacity due to decreased SAM or increased *S*-adenosylhomocysteine (SAH). In preliminary studies of $Mthfr^{-/-}$ mice, we reported the expected genotype distributions in offspring of $Mthfr^{+/-}$ matings, suggesting the absence of in utero losses of homozygous mutant animals. However, we observed considerable mortality in the first few weeks of life in the *Mthfr* −/− group. We also briefly described a diffuse internal granule cell layer (IGL) and a disorganized Purkinje cell layer in the anterior region of the cerebella, but not in the posterior region, in mutant mice of 4–5 weeks of age. Microarray analyses of brain RNA from 2-week-old $Mthfr^{-/-}$ mice had revealed numerous gene expression changes (Chen et al., 2002). One of the genes with significantly reduced expression, the gene encoding the inositol 1,4,5-triphosphate receptor type 1 (*Itpr1*), plays a role in Purkinje cell generation (Nordquist et al., 1998).

MTHFR is a cytoplasmic enzyme with ubiquitous expression, although the exact expression pattern in brain regions has not been examined. It is possible that MTHFR deficiency alters expression of some of the genes involved in cerebellar patterning. A study of mice with the Engrailed (En)-2/lacZ transgene demonstrated restricted expression of the transgene to the anterior lobe of the adult mouse cerebellum (Logan et al., 1993). Mice with a disruption of the very low density lipoprotein receptor (*Vldlr*) gene also showed more severe cerebellar defects in the anterior lobules than in posterior lobules (Trommsdorff et al., 1999). The VLDLR protein is known to function downstream of Reelin (*Reln*), as a component of a signaling pathway that governs cell positioning during brain development (Rice and Curran, 2001). However, these three genes (*En2*, *Vldlr* and *Reln)* did not show significant changes in expression in the aforementioned microarray analyses. This may have been due to the fact that RNA was isolated from whole brain, rather than cerebellum, or that the mice were relatively older (14 days) with respect to the critical timing for cerebellar patterning.

Here we report our investigation of early postnatal cerebellar development in $Mthfr^{-/-}$ mice to further characterize the cerebellar defects. We examined the expression of *En2*, *Vldlr*, *Reln* and *Itpr1* by RT-PCR, to determine if a disruption of *Mthfr* affected their expression in the cerebellum at an earlier stage of development. We also studied the effect of betaine supplementation of pregnant $Mthfr^{+/-}$ dams to determine the effect on cerebellar development in their $Mthfr^{-/-}$ pups.

2. Experimental procedures

2.1. Animal experimentation

All experiments were approved by the Animal Care Committee of the Montreal Children's Hospital and were conducted in accordance with the recommendations of the Canadian Council on Animal Care. Mice were progeny from matings of $Mthfr^{+/-}$ mice, that had been backcrossed five generations from 129/Sv-BALB/c F1 heterozygotes (Chen et al., 2001) to BALB/cAnNCrlBR (Charles River Canada, Saint Constant, Que.). Mice were fed laboratory rodent chow (Purina laboratory rodent diet 5001, Purina Mills). PCR-based genotyping was performed as described previously (Chen et al., 2001).

2.2. Histological examinations

Anaesthetized mice were perfused through the left ventricle with 10% neutral buffered formalin (Sigma–Aldrich Canada, Oakville, Ont.). Brains were excised, and fixed in formalin solution before embedding in paraffin for sectioning. Six-micrometre brain sagittal sections were mounted onto slides, and stained with haematoxylin and eosin (HE) for morphologic analyses.

2.3. BrdU incorporation assay

Postnatal littermates at postnatal day 5 (P5) from $Mthfr^{+/-}$ matings were injected intraperitoneally with diluted BrdU (50 μg/g) in 7 mM NaOH and killed 1 h later.

Brains were removed and fixed at room temperature by immersion in methanol/acetic acid (3:1) overnight, dehydrated, embedded in paraffin and sectioned (6 μm). Sections were prepared for BrdU immunohistochemistry as previously described (Przyborski et al., 1998). For colorimetric detection, sections were treated with 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma) and then counterstained with cresyl violet. Cells that incorporated BrdU displayed a dense brown precipitate in the nucleus.

2.4. Terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick end labeling (TUNEL)

TUNEL reactions were performed according to the manufacturer's instructions with some modifications (Roche Diagnostics, Laval, Que.). Briefly, deparaffinized sections were treated with 3% H_2O_2 for 5 min at room temperature. After rinsing with PBS, sections were incubated in TUNEL reaction mixture (TdT and fluorescein-labeled nucleotide mixture) for 30 min at 37 °C. Signal conversion was completed by incubating sections with anti-fluorescein antibody conjugated with horseradish peroxidase, followed by incubation with DAB substrate (Sigma–Aldrich Canada, Oakville, Ont.). Slides were counterstained with haematoxylin. Sections incubated with TUNEL reaction mixture without TdT were used as negative controls. Cell death was quantified by counting the brown DAB-stained nuclei within one field at a magnification of 400. Only strong-staining nuclei were counted in a total of 20 randomly chosen fields in at least two different sections per cerebellum, in three animals per genotype per age group.

2.5. Betaine supplementation

Female *Mthfr*$^{+/-}$ mice on laboratory rodent chow (Purina laboratory rodent diet 5001, Purina Mills) were supplemented with anhydrous betaine (Sigma–Aldrich Canada, Oakville, Ont.) at 2% (w/v) in drinking water before mating them to *Mthfr*$^{+/-}$ males; this dose of betaine was chosen because other work had demonstrated that it was successful in increasing survival of *Mthfr*$^{-/-}$ pups (Schwahn et al., 2004). Water intake was monitored. Betaine supplementation was continued until sacrifice of mice. Age-matched offspring from matings of untreated *Mthfr*$^{+/-}$ mice served as controls. Cages were checked daily. At postnatal day 9 (P9), mice were sacrificed. Brains were dissected, fixed by immersion in 10% formalin and processed for 5 μm paraffin sections and HE staining.

2.6. Gene expression studies

Cerebellar RNA was isolated from postnatal day 7 (P7) and P2 *Mthfr*$^{+/+}$ and *Mthfr*$^{-/-}$ mice using the TRIzol Reagent Total RNA Isolation Kit (Invitrogen). After DNaseI treatment, the A260/A280 ratio of isolated total RNA was 1.9–2.1. The reverse transcription (RT) reaction of 40 μl contained 7 μg total RNA, 160 ng random hexamers, 500 μM each of dATP, dCTP, dGTP, dTTP, 10 mM DTT, and 100 units SuperScript II Reverse Transcriptase, in 1× First-Strand Buffer supplied by the manufacturer. The reaction was conducted at 20 °C for 10 min, 42 °C for 50 min, and 70 °C for 15 min.

PCR reactions of 50 μl were performed for different cycle numbers, range = 18–26 cycles, under the following conditions (94 °C, 30 s; 62 °C, 30 s; 72 °C, 1 min) to determine the appropriate number of thermal cycles for the specific genes. The final cycle numbers for each gene were chosen to be as low as possible before reaching the amplification plateau, yet of sufficient intensity to be detectable by image analysis software (see below). 1.5–2 μl of cDNA from the RT reaction was used in each PCR, in a Biometra TGRADIENT machine. Distilled water, instead of cDNA, was used as a negative control. Amplified products were examined by electrophoresis in 9% polyacrylamide gels. The images of ethidium bromide stained bands were analyzed using Bio-Rad Quantity One software, relative to the intensity of the internal control, *Gapd*. RT-PCRs were performed for three animals per genotype per gene. Additional details (primer sequences, annealing temperature, sizes of PCR products and cycle numbers) are provided in Table 1.

3. Results

3.1. Underdeveloped brain with overexposed midbrain and enlarged lateral ventricle

The Mendelian ratio of *Mthfr*$^{+/+}$:*Mthfr*$^{+/-}$:*Mthfr*$^{-/-}$ mice after birth was close to the expected 1:2:1 ratio (data not shown), suggesting that the matings between *Mthfr*$^{+/-}$ mice did not result in any significant in utero losses of *Mthfr*$^{-/-}$ offspring. However, survival of *Mthfr*$^{-/-}$ mice was approximately 30% at the age of 5 weeks, with nearly 3/4 of the deaths occurring during the first two postnatal weeks. The actual cause of death has not been determined but the small size of the pups and/or abnormal cerebellar development (as discussed below) may affect muscle tone or coordination, and consequently decrease milk/food consumption and limit weight gain.

Decreased survival or obvious neurological abnormalities, as determined by preliminary histological examinations and simple neurobehavioral tests such as open field and footprint analyses, were not observed in the young *Mthfr*$^{+/-}$ mice (data not shown). All subsequent studies therefore focused on the *Mthfr*$^{-/-}$ mice.

Mthfr$^{-/-}$ mice were significantly smaller than their wild type littermates. Brain weight of postnatal day 14 (P14) *Mthfr*$^{-/-}$ mice ($n = 7$) was approximately 56% of that of wild type animals ($n = 9$); mean ± standard deviation = 0.23 ± 0.04 g versus 0.41 ± 0.04 g. The anterior and

Table 1
Primer sequences and amplification conditions

Gene	DNA strand	Sequences of primers	Annealing temperature (°C)	Size of PCR product (bp)	Thermal cycle number
En2	Sense Antisense	5′-GCTGAGTTTCAGACCAACAGGTA-3′ 5′-GCTTGTTCTGGAACCAAATCTT-3′	62	106	24
Reln	Sense Antisense	5′-AGTACAGTGTCAACAATGGCATCA-3′ 5′-CAGGGGGACGTTGTAAGACA-3′	62	98	22
Itpr1	Sense Antisense	5′-AGAACTAAAGGACCAGATGACAGAA-3′ 5′-GTTGACATTCATGTGAGGAGGAT-3′	62	85	24
Vldlr	Sense Antisense	5′-TAGTCGTGGCTATCAAATGGATCT-3′ 5′-TCCTGATGTCTCTTCGATTAGTGA-3′	62	95	24
Gapd	Sense Antisense	5′-CAGGAGCGAGACCCCACTAACAT-3′ 5′-AAGACACCAGTAGACTCCACGAC-3′	60	74	18

posterior colliculus of the midbrain in *Mthfr*$^{-/-}$ mice were overexposed compared to that of wild type animals at P14 (Fig. 1A). This is most likely due to the dramatically reduced size of the cerebellum and cerebral cortex. The overall brain structure in *Mthfr*$^{-/-}$ mice was intact, but the lateral ventricle in mutant mice was clearly enlarged (Fig. 1C).

3.2. Depletion of the external granule cell layer (EGL) in anterior cerebellum with decreased cellular proliferation

Since there were striking abnormalities in *Mthfr*$^{-/-}$ cerebellum in the early postnatal period, we investigated younger animals (P0) to determine whether development in utero had been compromised. However, the cerebella of mutant mice at P0 had similar laminar structures to those of wild type animals, and the thickness of the EGL also appeared to be quite similar in these two groups (Fig. 2A and B). These findings suggest that prenatal cerebellar development and early neurogenesis of the EGL are normal in *Mthfr*$^{-/-}$ mice.

At P5, however, the cerebellum of *Mthfr*$^{-/-}$ animals was reduced in size and foliation was severely retarded. Missing fissures and underdeveloped lobules were observed in mutant mice (Fig. 2G) as compared to age-matched wild type animals (Fig. 2C). At P5, instead of a distinct 4-layer laminar structure (EGL – molecular layer – Purkinje cell layer – IGL), as seen in normal mice (Fig. 2C and E), mutant mice possessed an EGL with dramatically reduced thickness and disorganized Purkinje cells in anterior lobules (Fig. 2G). In posterior lobules, these defects were also visible but to a much lesser degree (Fig. 2I). Furthermore, the BrdU incorporation assay revealed significantly decreased proliferation in cells from the anterior regions of mutant cerebella (Fig. 2H), but more normal proliferation in posterior regions (Fig. 2J).

3.3. Increased apoptosis in cerebellum

Depletion of the EGL and disruption of the laminar structure in young mutant cerebella could also reflect an increase in apoptosis, a process which has been well-documented in the normal developing mouse cerebellum (Wood et al., 1993). Cerebella from P11 and P14 mice were examined by TUNEL assay; a significant increase in TUNEL positive stained granule cells was found in the cerebella of *Mthfr*$^{-/-}$ mice at both ages (Fig. 3). The increases were observed in the EGL and the IGL, although the increase in the IGL of P11 mutant mice was not statistically significant. The numbers of TUNEL positive

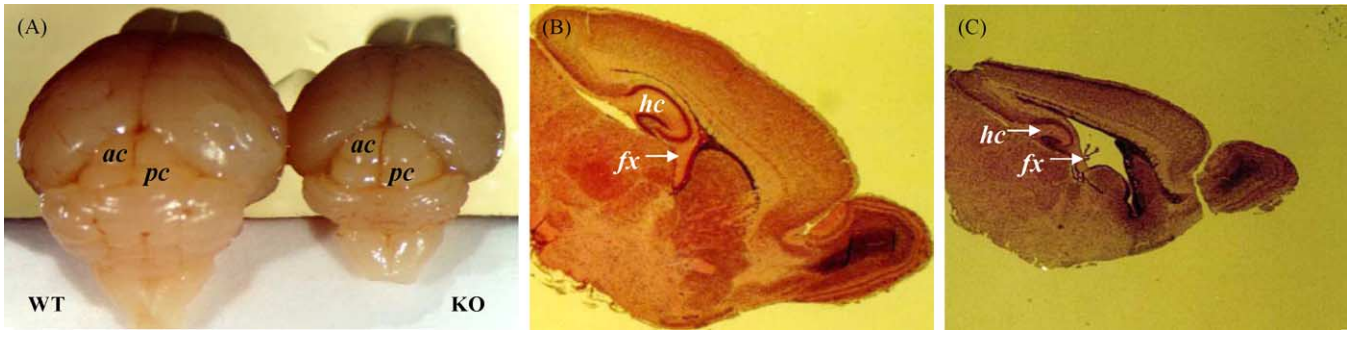

Fig. 1. Brain of *Mthfr*$^{-/-}$ mice. (A) Comparison of the brains of P14 *Mthfr*$^{-/-}$ and *Mthfr*$^{+/+}$ mice. *Mthfr*$^{-/-}$ brain (right) is smaller and the midbrain is overexposed compared to that of wild type (left). The cerebellum of the *Mthfr*$^{-/-}$ mouse is clearly underdeveloped. Parasagittal sections of P7 wild type mouse (B) and *Mthfr*$^{-/-}$ mouse (C). The lateral ventricle is abnormally enlarged in the mutant mouse. WT, wild type; KO, knockout; ac, anterior colliculus; pc, posterior colliculus; hc, hippocampus; fx, fornix.

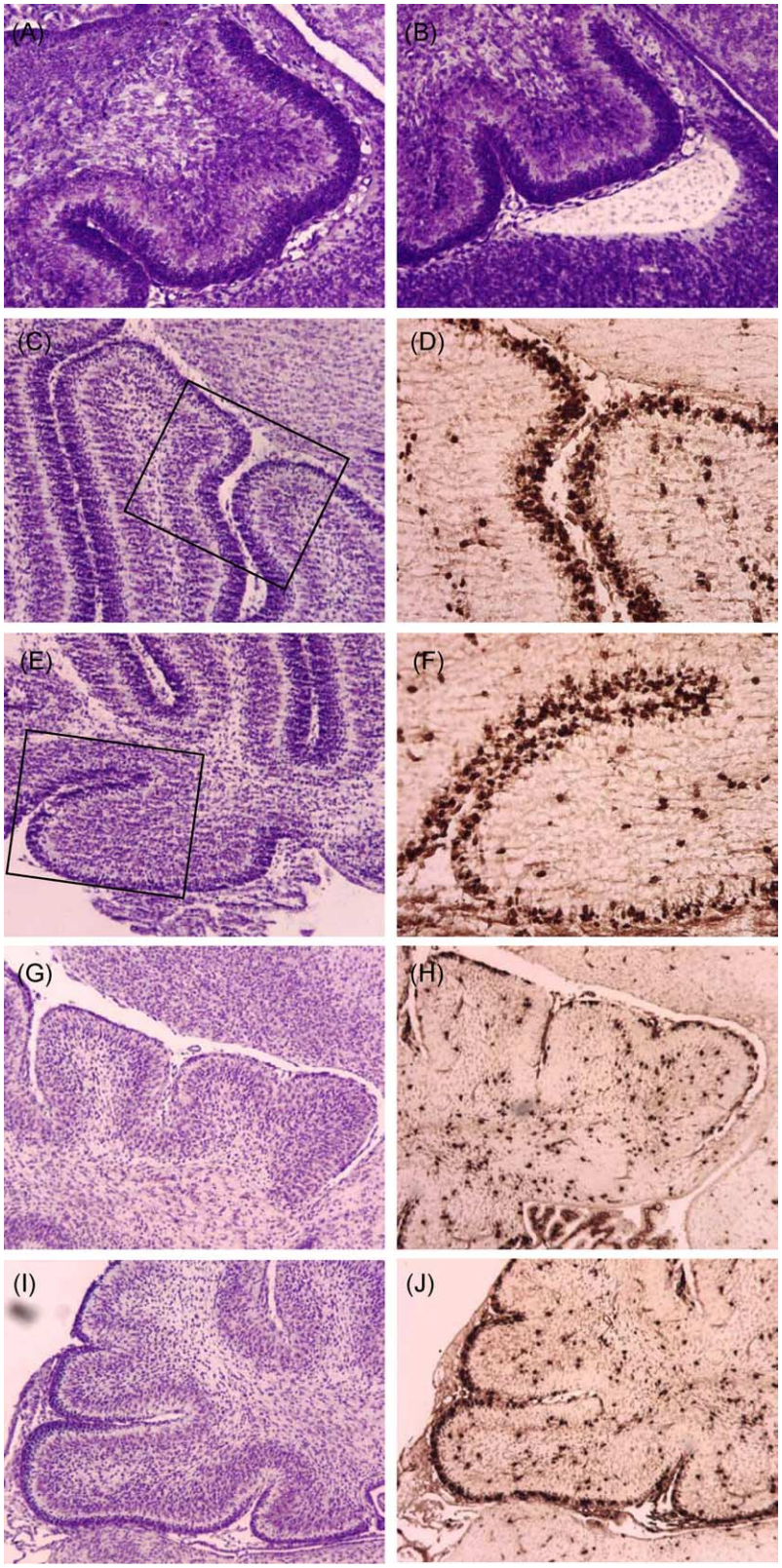

Fig. 2. Anterior cerebellar defects in *Mthfr*$^{-/-}$ mice are associated with decreased cell proliferation. Haematoxylin and eosin stained sagittal sections of anterior cerebella of wild type (A) and *Mthfr*$^{-/-}$ mice (B) at P0. Sagittal sections of cerebella of P5 wild type (C–F); and mutant mice (G–J). Depletion of the EGL and disorganized Purkinje cells were seen in the anterior cerebellum of mutant mice (G). Milder defects were observed in the posterior cerebellum (I). D, F, H and J are BrdU immuno-stained sections. D and F are sections from wild type mice corresponding to the rectangular regions in C and E, respectively. H and J are BrdU immuno-stained anterior (H) and posterior (J) cerebella of *Mthfr*$^{-/-}$ mice. Original magnification, ×200 (A, B, D and F) and ×100 (others).

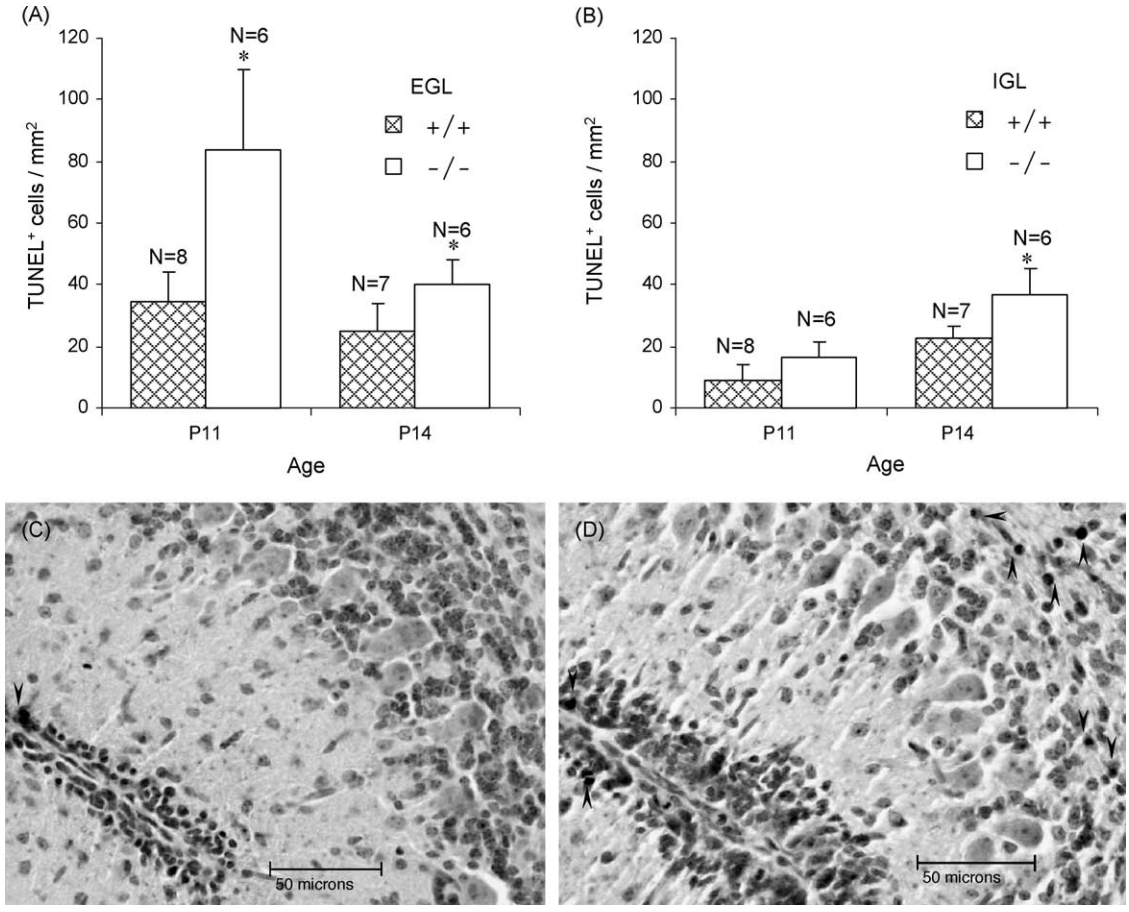

Fig. 3. Increased apoptosis in mutant cerebellum. (A and B) Quantification of TUNEL-positive cells in the EGL and IGL of the cerebella of wild type (+/+) and homozygous mutant (−/−) mice at P11 and P14. (*) Student's *t*-test of mutant compared with wild type, $P < 0.03$. Examples of TUNEL staining of cerebellar sections from wild type (C) and mutant (D) mice at P14. Arrowheads point to TUNEL-positive cells. Bars, 50 μm; *N*, number of samples.

cells in anterior and posterior regions of mutant cerebella appeared similar (data not shown). Despite the overall increase of apoptotic granule cells in mutants, both wild type and mutant animals demonstrated decreased cell death in the EGL from P11 to P14, and increased cell death in the IGL from P11 to P14. These observations suggest that the disruption of the *Mthfr* gene affects survival of granule neurons during postnatal development, but the overall apoptotic pattern in cerebellum is not changed.

3.4. Decreased expression of genes involved in cerebellar patterning, neuronal migration and growth

The deficit in the EGL and the disruption of the laminar structure in *Mthfr*$^{-/-}$ mice was largely confined to the anterior region of the cerebellum. We speculated that MTHFR deficiency might alter expression of genes involved in cerebellar patterning, possibly through effects on DNA methylation. We therefore examined expression of *En2*, *Vldlr*, *Reln* and *Itpr1* in the cerebellum of mice at P2 and P7 by RT-PCR. We did not observe any changes in gene expression at P2 (data not shown). However, at P7, we identified decreased gene expression of *En2*, *Reln* and *Itpr1* in the cerebella of *Mthfr*$^{-/-}$ mice (Fig. 4); expression of *Vldlr* did not appear to be altered by MTHFR deficiency. The average expression level of three mice per genotype (with range) of *En2*, *Reln*, *Itpr1* and *Vldlr* in wild type, compared to mutant, cerebella, after adjustment for *Gapd* expression levels, were 3.0 (2.7–3.2), 1.9 (1.6–2.5), 2.7 (0.9–5.6) and 1.03 (1.0–1.1), respectively.

3.5. Effect of maternal betaine supplementation on cerebellar development

Body and brain weights of 9-day-old *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ pups from betaine-supplemented dams were not different from those of untreated pups. However, *Mthfr*$^{-/-}$ pups with maternal betaine supplementation had significantly higher body and brain weights at P9 than those of untreated *Mthfr*$^{-/-}$ pups (data not shown).

At P9, the brain structures of *Mthfr*$^{+/+}$ mice with or without maternal betaine supplementation were very similar. Gross anatomy of brains of *Mthfr*$^{-/-}$ mice revealed proportionately smaller cerebella (Fig. 5B). However, pups from betaine-supplemented dams had a much larger cerebellar size (Fig. 5C) compared to that in mice from

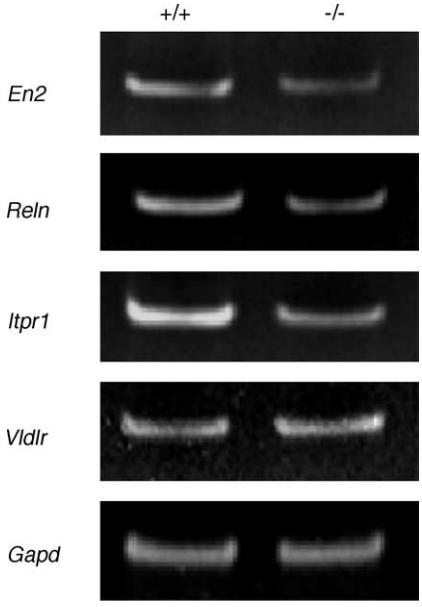

Fig. 4. Representative RT-PCR results of cerebellar RNA in P7 mice (three mice per genotype). The average expression levels of *En2*, *Reln*, *Itpr* and *Vldlr* in wild type (+/+) cerebella compared to mutant, after adjustment for *Gapd* levels, were 3.0, 1.9, 2.7 and 1.0, respectively.

untreated dams (Fig. 5B). Untreated *Mthfr*$^{-/-}$ mice exhibited the same types of abnormalities reported above. Foliation and differentiation of the cerebellar cortex at P9 (Fig. 5B and E) appeared immature compared to *Mthfr*$^{+/+}$ mice. There was no well-formed internal granule cell layer (IGL) and no distinguishable single Purkinje cell layer. The defects were observed in both anterior and posterior lobules, but were more severe in the anterior lobules. Losses of external granule cells were restricted mainly to the anterior region of the cerebellum. In comparison, maternal betaine supplementation improved cerebellar development of *Mthfr*$^{-/-}$ mice (Fig. 5C and F). Most defects were restricted to only one or two anterior lobules and the lamination disruption in these anterior lobules was less severe. EGL – molecular layer – Purkinje cell layer – IGL laminar structure was clearly visible, although Purkinje cells were still disorganized in the molecular layer and did not form a single cell layer (Fig. 5F). The EGL in these lobules appeared normal.

4. Discussion

The effects of severe MTHFR deficiency on brain development are not well-characterized, largely due to limited clinical material. The creation of *Mthfr* homozygous knockout mice provides a unique opportunity to investigate the role of *Mthfr* in CNS development.

Hydrocephalus internus of ventricles has been reported in two patients with MTHFR deficiency (Baethmann et al., 2000). We also observed enlarged lateral ventricles in the brain of *Mthfr*$^{-/-}$ mice. In addition, MTHFR deficiency in mice delayed brain development and caused severe cerebellar abnormalities, with effects on granule cell development and neuronal organization. It is not clear why the cerebellum displayed such significant effects, although an imbalanced distribution of homocysteine in the brain could be a contributor. A previous study reported that

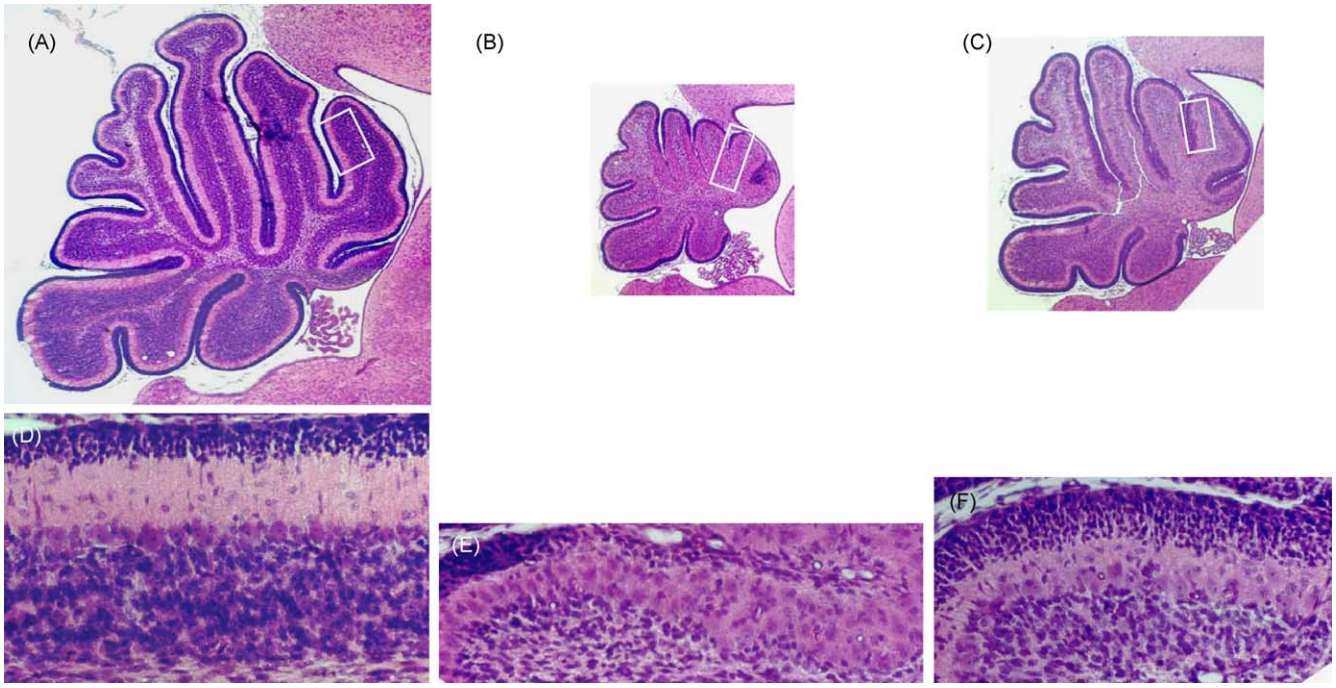

Fig. 5. HE stained parasagittal cerebellar sections of mice at P9 with (A, C, D and F) or without (B and E) maternal betaine supplementation. (A–C) Overview of the cerebellum. (D–F) Magnified rectangular regions in the anterior lobule. (A and D) were from *Mthfr*$^{+/+}$ mice. B, C, E and F were from *Mthfr*$^{-/-}$ mice.

the amount of homocysteine in mouse cerebellum was two-fold higher than that in other brain regions (Broch and Ueland, 1984). Dysfunction of MTHFR could therefore push homocysteine levels into a particularly toxic level in the cerebellum. However, homocysteine is not likely to be the only cause of the cerebellar defects in the *Mthfr*$^{-/-}$ mice. Mice deficient in cystathionine beta-synthase (CBS) do not show obvious cerebellar defects although their plasma homocysteine levels are also quite high (Watanabe et al., 1995). In contrast to MTHFR deficiency, CBS deficiency does not affect synthesis of methionine, an important nutrient and precursor of *S*-adenosylmethionine for methylation reactions; this additional disturbance in *Mthfr*$^{-/-}$ pups may have an impact on cerebellar development.

Cerebellar development in the postnatal period is considerable; since *Mthfr*$^{-/-}$ mice are not subject to intrauterine loss and have relatively normal cerebella at P0, it is likely that disruptions in brain development might be more severe in regions that develop postnatally, compared to those that develop prenatally. Maternal folate levels presumably protect the fetal brain in utero, but the increased requirement for folate in the early postnatal period might be a particular challenge for *Mthfr*$^{-/-}$ mice.

Consist with our previous study of older *Mthfr*-deficient mice (Chen et al., 2001), the deficit in the EGL and the disruption of the laminar structure in younger *Mthfr*$^{-/-}$ mice were largely confined to the anterior region, and were more severe in the vermis than in the hemispheres. A similar discrete patterning defect has been seen in the *meander tail* mutant and *leaner* mutant (Ross et al., 1989; Herrup and Wilcyzynski, 1982). The *leaner* mutation lies in an alpha 1A voltage-sensitive calcium channel gene, which is uniformly expressed in the cerebellum (Fletcher et al., 1996), and the *meander tail* (mea) gene has not yet been cloned. It is unlikely that disruption of *Mthfr* directly results in this compartmental defect, but indirect effects on expression of other genes could be contributory. It is possible that *Mthfr* alters gene expression by modifying DNA methylation, which in turn could affect brain development. DNA methylation changes have been demonstrated in these *Mthfr*-deficient mice (Chen et al., 2001) and in clinical studies of the milder form of MTHFR deficiency (Friso et al., 2002).

Through RT-PCR, we observed decreased expression of *En2*, *Reln* and *Itpr1* in P7 *Mthfr*$^{-/-}$ mice. *En2* expression is restricted to the anterior cerebellum (Logan et al., 1993); reduced *En2* expression could contribute to the compartmental defect in *Mthfr*$^{-/-}$ cerebellum. Although we did not observe a change in the level of expression of *Vldlr*, expression of *Reln*, an upstream component of *Vldlr* in the Reelin signaling pathway, was altered. Reelin signaling regulates the migration of neurons along the radial glial fiber network (Curran and D'Arcangelo, 1998); decreased *Reln* expression could contribute to the defects in cerebellar lamination and granule cell migration. ITPR1 acts as a calcium release channel. Acute localized loss of function of ITPR1 leads to growth arrest and neurite retraction (Takei et al., 1998); decreased *Itpr1* expression could contribute to the increased apoptosis in mutant cerebella since there are ample precedents for calcium involvement in neuronal cell death (Koh and Cotman, 1992; Choi, 1995). Interestingly, we did not see any obvious alterations in the expression levels of *En2*, *Reln*, *Itpr1* and *Vldlr* at postnatal day 2. An immediate question is whether the decreased expression of *En2*, *Reln* and *Itpr1* in P7 *Mthfr*$^{-/-}$ cerebellum is a cause or result of the cell losses and cerebellar defects in mutant mice. We observed similar expression levels of *Vldlr* and *Gapd* at P7; nonetheless, it is still possible that the expression changes in *En2*, *Reln* and *Itpr1* are secondary to the cell losses (due to increased apoptosis and decreased proliferation) if these genes are expressed in a cell-specific manner. Additional studies are necessary to address this question.

Early neurogenesis of EGL cells appeared normal in the P0 *Mthfr*$^{-/-}$ mice. However, after birth, MTHFR deficiency might affect granule cell maturation and radial migration. BrdU incorporation assays revealed decreased cell proliferation, mostly in the anterior lobules of mutant cerebella, which could be responsible for the decreased number of EGL cells in this region. In the posterior lobules, the lesser degree of depletion of EGL cells in mutant mice was associated with nearly normal cellular proliferation. Increased granule cell death, as observed in P11 and P14 cerebellum of *Mthfr*$^{-/-}$ mice, could also have contributed to decreased number of cells. However, the increased cell death in the *Mthfr*$^{-/-}$ cerebella did not show a compartmental pattern, suggesting that it might be regulated by different mechanisms. Highly elevated plasma homocysteine in mutant mice may directly contribute to cell loss since homocysteine has been shown to induce neuronal apoptosis and increase neuronal vulnerability to excitotoxicity (Kruman et al., 2000).

Betaine supplementation showed a beneficial effect on cerebellar development of the pups. It reduced the severity of lamination disruption in the cerebellum and limited the defects to only one or two anterior lobules. However, the compartmental defects were still evident in the *Mthfr*$^{-/-}$ pups with maternal betaine supplementation. Betaine is an effective therapeutic agent in disorders of homocysteine remethylation (Wendel and Bremer, 1984). Although betaine can cross the blood brain barrier, homocysteine remethylation to methionine, catalyzed by BHMT, occurs mainly in liver. *S*-adenosylmethionine is released from liver and can also cross the blood brain barrier, with partial restoration of the decreased methyl donor pool in brain caused by MTHFR deficiency. This phenomenon may explain why there is still a high level of homocysteine in brain, despite the increase in homocysteine remethylation to methionine in liver following betaine administration (Schwahn et al., 2003, 2004).

Survival of *Mthfr*$^{-/-}$ mice on the BALB/c background was lower than that seen in mutants on the more mixed

genetic background of our original colony (Chen et al., 2001). This is not surprising since genetic background of knockout mice has been shown to affect survival (Threadgill et al., 1995). Nonetheless, it is not clear why *Mthfr*$^{-/-}$ mice in this study displayed different phenotypic severity despite their similar genetic background. One explanation for this variability is differential access to maternal milk, which contains 5-methylTHF, the product of the MTHFR reaction.

In summary, MTHFR deficiency in newborn mice is associated with normal neurogenesis, presumably due to maternal supply of 5-methyltetrahydrofolate. However, MTHFR deficiency in the early postnatal period affects cerebellar patterning, and results in a greater disturbance in anterior, compared to posterior, lobules in the cerebellum. Both decreased cellular proliferation and increased cell death may contribute to the EGL cell loss observed in mutant mice. Betaine supplementation can improve early cerebellar development and reduce the severity of the defects. Investigation of the genes which contribute to these defects may help in elucidating the regulatory role of MTHFR in development of the cerebellum.

Acknowledgments

We are grateful to Dr. Susan Ackerman (Jackson Laboratory, Bar Harbor, Maine) for her assistance with the BrdU incorporation assay and critical comments on the manuscript. We thank Liyuan Deng and Daniel Leclerc for technical support. This work was supported by the Canadian Institutes for Health Research (CIHR). RR is a Senior Scientist of the CIHR.

References

Baethmann, M., Wendel, U., Hoffmann, G.F., Gohlich-Ratmann, G., Kleinlein, B., Seiffert, P., Blom, H., Voit, T., 2000. Hydrocephalus internus in two patients with 5,10-methylenetetrahydrofolate reductase deficiency. Neuropediatrics 31, 314–317.

Broch, O.J., Ueland, P.M., 1984. Regional distribution of homocysteine in the mammalian brain. J. Neurochem. 43, 1755–1757.

Chadwick, L.H., McCandless, S.E., Silverman, G.L., Schwartz, S., Westaway, D., Nadeau, J.H., 2000. Betaine-homocysteine methyltransferase-2: cDNA cloning, gene sequence, physical mapping, and expression of the human and mouse genes. Genomics 70, 66–73.

Chen, Z., Karaplis, A.C., Ackerman, S.L., Pogribny, I.P., Melnyk, S., Lussier-Cacan, S., Chen, M.F., Pai, A., John, S.W.M., Smith, R.S., Bottiglier, T., Bagley, P., Selhub, J., Rudnicki, M.A., James, S.J., Rozen, R., 2001. Mice deficient in methylenetetrahydrofolate reductase exhibit hyperhomocysteinemia and decreased methylation capacity, with neuropathology and aortic lipid deposition. Hum. Mol. Genet. 10, 433–443.

Chen, Z., Ge, B., Hudson, T.J., Rozen, R., 2002. Microarray analysis of brain RNA in mice with methylenetetrahydrofolate reductase deficiency and hyperhomocysteinemia. Gene Expression Patterns 1, 89–93.

Choi, D.W., 1995. Calcium: still center-stage in hypoxic-ischemic neuronal death. Trends Neurosci. 18, 58–60.

Clarke, R., Smith, A.D., Jobst, K.A., Refsum, H., Sutton, L., Ueland, P.M., 1998. Folate, vitamin B12, and serum total homocysteine levels in confirmed Alzheimer disease. Arch. Neurol. 55, 1449–1455.

Curran, T., D'Arcangelo, G., 1998. Role of reelin in the control of brain development. Brain Res. Brain Res. Rev. 26, 285–294.

Elkind, M.S., Sacco, R.L., 1998. Stroke risk factors and stroke prevention. Semin. Neurol. 18, 429–440.

Fletcher, C.F., Lutz, C.M., O'Sullivan, T.N., Shaughnessy Jr., J.D., Hawkes, R., Frankel, W.N., Copeland, N.G., Jenkins, N.A., 1996. Absence epilepsy in tottering mutant mice is associated with calcium channel defects. Cell 87, 607–617.

Friso, S., Choi, S.W., Girelli, D., Mason, J.B., Dolnikowski, G.G., Bagley, P.J., Olivieri, O., Jacques, P.F., Rosenberg, I.H., Corrocher, R., Selhub, J., 2002. A common mutation in the 5,10-methylenetetrahydrofolate reductase gene affects genomic DNA methylation through an interaction with folate status. Proc. Natl. Acad. Sci. U.S.A. 99, 5605–5611.

Frosst, P., Blom, H.J., Milos, R., Goyette, P., Sheppard, C.A., Matthews, R.G., Boers, G.J., den Heijer, M., Kluijtmans, L.A., van den Heuvel, L.P., Rozen, R., 1995. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nature Genet. 10, 111–113.

Goyette, P., Sumner, J.S., Milos, R., Duncan, A.M.V., Rosenblatt, D.S., Matthews, R.G., Rozen, R., 1994. Human methylenetetrahydrofolate reductase: isolation of cDNA, mapping and mutation identification. Nature Genet. 7, 195–200.

Goyette, P., Frosst, P., Rosenblatt, D.S., Rozen, R., 1995. Seven novel mutations in the methylenetetrahydrofolate reductase gene and genotype/phenotype correlations in severe methylenetetrahydrofolate reductase deficiency. Am. J. Hum. Genet. 56, 1052–1059.

Goyette, P., Christensen, B., Rosenblatt, D.S., Rozen, R., 1996. Severe and mild mutations in *cis* for the methylenetetrahydrofolate reductase (MTHFR) gene, and description of five novel mutations in MTHFR. Am. J. Hum. Genet. 59, 1268–1275.

Herrup, K., Wilcyzynski, S.L., 1982. Cerebellar degeneration in *leaner* mutant mice. Neuroscience 7, 2185–2196.

Holme, E., Kjellman, B., Ronge, E., 1989. Betaine for the treatment of homocystinuria caused by methylenetetrahydrofolate reductase. Arch. Dis. Child 64, 1061–1064.

Koh, J.Y., Cotman, C.W., 1992. Programmed cell death: its possible contribution to neurotoxicity mediated by calcium channel antagonists. Brain Res. 587, 233–240.

Kruman, I.I., Culmsee, C., Chan, S.L., Kruman, Y., Guo, Z., Penix, L., Mattson, M.P., 2000. Homocysteine elicits a DNA damage response in neurons that promotes apoptosis and hypersensitivity to excitotoxicity. J. Neurosci. 20, 6920–6926.

Lipton, S.A., Kim, W.K., Choi, Y.B., Kumar, S., D'Emilia, D.M., Rayudu, P.V., Arnelle, D.R., Stamler, J.S., 1997. Neurotoxicity associated with dual actions of homocysteine at the *N*-methyl-D-aspartate receptor. Proc. Natl. Acad. Sci. U.S.A. 94, 5923–5928.

Logan, C., Khoo, W.K., Cado, D., Joyner, A.L., 1993. Two enhancer regions in the mouse *en-2* locus direct expression to the mid-hindbrain region and mandibular myobalsts. Development 119, 905–916.

McKeever, M.P., Weir, D.G., Molloy, A., Scott, J.M., 1999. Betaine-homocysteine methyltransferase: organ distribution in man, pig and rat and subcellular distribution in rat. Clin. Sci. 81, 551–556.

Miller, J.W., 1999. Homocysteine and Alzheimer's disease. Nutr. Rev. 57, 126–129.

Mudd, S.H., Levy, H.L., Kraus, J.P., 2001. Disorders of transsulfuration. In: Scriver, C.R., Beaudet, A.L., Sly, W.S., Valle, D. (Eds.), The Metabolic and Molecular Bases of Inherited Disease. eighth ed. McGraw-Hill, New York, pp. 2007–2056.

Nordquist, D.T., Kozak, C.A., Orr, H.T., 1998. cDNA cloning and characterization of three genes uniquely expressed in cerebellum by Purkinje neurons. J. Neurosci. 12, 4780–4789.

Przyborski, S.A., Knowles, B.B., Ackerman, S.L., 1998. Embryonic phenotype of Unc5h3mutant mice suggests chemorepulsion during the formation of the rostral cerebellar boundary. Development 125, 41–50.

Rice, D.S., Curran, T., 2001. Role of the reelin signaling pathway in central nervous system development. Annu. Rev. Neurosci. 24, 1005–1039.

Rosenblatt, D.S., Fenton, W., 2001. Inherited disorders of folate and cobalamin transport and metabolism. In: Scriver, C.R., Beaudet, A.L., Sly, W.S., Valle, D. (Eds.), The Metabolic and Molecular Bases of Inherited Disease. eighth ed. McGraw-Hill, New York, pp. 3897–3933.

Rosenquist, T.H., Ratashak, S.A., Selhub, J., 1996. Homocysteine induces congenital defects of the heart and neural tube: effect of folic acid. Proc. Natl. Acad. Sci. U.S.A. 93, 15227–15232.

Ross, M.E., Fletcher, C., Mason, C.A., Hatten, M.E., Heintz, N., 1989. Meander tail reveals a developmental unit in mouse cerebellum. Proc. Natl. Acad. Sci. U.S.A. 87, 4189–4192.

Schwahn, B., Rozen, R., 2001. Polymorphisms in the methylenetetrahydrofolate reductase gene: clinical consequences. Am. J. Pharmacogenom. 1, 189–201.

Schwahn, B.C., Chen, Z., Laryea, M.D., Wendel, U., Lussier-Cacan, S., Genest Jr., J., Mar, M.-H., Zeisel, S.H., Castro, C., Garrow, T., Rozen, R., 2003. Homocysteine-betaine interactions in a murine model of 5,10-methylenetetrahydrofolate reductase deficiency. FASEB J. Express 17, 512–514.

Schwahn, B.C., Laryea, M.D., Chen, Z., Melnyk, S., Pogribny, I., Garrow, T., James, S.J., Rozen, R., 2004. Betaine rescue of an animal model with methylenetetrahydrofolate reductase deficiency. Biochem. J. 382, 831–840.

Takei, K., Shin, R.M., Inoue, T., Kato, K., Mikoshiba, K., 1998. Regulation of nerve growth mediated by inositol 1,4,5-trisphosphate receptors in growth cones. Science 282, 1705–1708.

Threadgill, D.W., Dlugosz, A.A., Hansen, L.A., Tenenbaum, T., Lichti, U., Tee, D., Lamantia, C., Mourton, T., Herrup, K., Harris, R.C., Barnard, J.A., Yuspa, S.H., Coffey, R.J., Magnuson, T., 1995. Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. Science 269, 230–234.

Trommsdorff, M., Gotthardt, M., Hiesberger, T., Shelton, J., Stockinger, W., Nimpf, J., Hammer, R.E., Richardson, J.A., Herz, J., 1999. Reeler/disabled-like disruption of neuronal migration in the knockout mice lacking the VLDL receptor and ApoE receptor 2. Cell 97, 689–701.

Watanabe, M., Osada, J., Aratani, Y., Kluckman, K., Reddick, R., Malinow, M.R., Maeda, N., 1995. Mice deficient in cystathionine beta-synthase: animal models for mild and severe homocyst(e)inemia. Proc. Natl. Acad. Sci. U.S.A. 92, 1585–1589.

Wendel, U., Bremer, H.J., 1984. Betaine in the treatment of homocystinuria due to 5,10-methylenetetrahydrofolate reductase. Eur. J. Pediatr. 142, 147–150.

Wood, K.A., Dipasquale, B., Youle, R.J., 1993. In situ labeling of granule cells for apoptosis-associated DNA fragmentation reveals different mechanisms of cell loss in developing cerebellum. Neuron 11, 621–632.