

Folate deficiency increases mtDNA and D-1 mtDNA deletion in aged brain of mice lacking uracil-DNA glycosylase

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ARTICLE INFO

Article history:

Received 6 November 2010

Revised 8 January 2011

Accepted 16 January 2011

Available online 28 January 2011

Keywords:

Neurodegeneration

Folate

DNA repair

Mitochondrial DNA

Uracil

Mitochondrial DNA deletion

ABSTRACT

Strong epidemiological and experimental evidence links folate deficiency and resultant hyperhomocysteinemia with cognitive decline and neurodegeneration. Here, we tested the hypothesis that uracil misincorporation contributes to mitochondrial pathology in aged brain following folate deprivation. In a 2×2 design, 14-month-old mice lacking uracil DNA glycosylase (*Ung*−/−) versus wild-type controls were subjected to a folate-deficient versus a regular diet for six weeks. Folate-deficient feeding significantly enhanced mtDNA content and overall abundance of the D-1 mtDNA deletion in brain of *Ung*−/−, but not of wild-type mice. Independent of folate status, the frequency of the D-1 mtDNA deletion in mtDNA was significantly increased in *Ung*−/− mice. The rate of mitochondrial biogenesis as assessed at six weeks of the experimental diet by mRNA expression levels of transcriptional coactivator peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α and of mitochondrial transcription factor A (Tfam) was not affected by either *Ung*−/− genotype or short-term folate deficiency. Similarly, citrate synthase (CS) activity in the brain did not differ across experimental groups. By contrast, independent of genotype, lactate dehydrogenase (LDH) activity was significantly reduced in folate-deficient animals. Our results suggest that impaired uracil excision repair causes an increase in mitochondrial mutagenesis in aged brain along with a compensatory increase in mtDNA content in response to low folate status. Folate deficiency may contribute to neurodegeneration via mtDNA damage.

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Introduction

Folic acid plays a crucial role in neuroplasticity and in the maintenance of neuronal integrity. Folate deficiency and subsequent hyperhomocysteinemia have been linked epidemiologically and experimentally with neuropsychiatric and neurodegenerative disease (D'Anci and Rosenberg, 2004; Mattson and Shea, 2003). In humans, an association is emerging between low folate status and reduced hippocampal and amygdalar volumes as well as global brain atrophy (den Heijer et al., 2003; Scott et al., 2004; Yang et al., 2007). Furthermore, an inverse correlation between cerebral N-acetyl aspartate (NAA) levels and total plasma homocysteine has been demonstrated using ¹H magnetic resonance spectroscopy (Bisschops et al., 2004; Engelbrecht et al., 1997). Importantly, NAA is synthesized

in neuronal mitochondria and is used as a spectroscopic marker of neuronal viability and function. Preclinical experimental research also points to the special role of folate deficiency in brain structural and metabolic abnormalities associated with many neurological and neuropsychiatric disorders such as Alzheimer's disease (Kruman et al., 2002), Parkinson's disease (Duan et al., 2002; Ekstrand et al., 2007) and stroke (Endres et al., 2005).

Folic acid plays an essential role in one-carbon metabolism: it is required both in the remethylation of homocysteine to methionine and in the synthesis of S-adenosyl-methionine, the principal methyl donor in numerous methylation reactions (e.g. creatine synthesis). Lack of folate and resultant hyperhomocysteinemia therefore exert complex effects on CNS function on many different levels. Because of its free thiol group, homocysteine is a highly reactive compound that acts as a pro-oxidant (Ueland et al., 1996).

Folate is also required for DNA synthesis, specifically for the synthesis of thymidine from uracil. Lack of folate leads to a reduction of dTTP ("methyl trap") while levels of dUTP increase (Goulian et al., 1980). Elevated levels of dUTP promote uracil misincorporation into

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DNA during S-phase or DNA repair as an A:U mismatch (Courtemanche et al., 2004). Furthermore, folate deficiency may also increase uracil in double-stranded DNA through cytosine deamination (Shen et al., 1992).

Uracil-DNA N-glycosylase (UNG) is the most widely distributed glycosylase that removes uracil residues from DNA (Kavli et al., 2002). Using uracil-DNA glycosylase deficient mice (*Ung*^{−/−}), we have previously demonstrated the important role of efficient uracil excision repair for recovery after transient brain ischemia (Endres et al., 2004) and for sustaining CNS structural and functional integrity during prolonged periods of folate deprivation (Kronenberg et al., 2008; Endres et al., 2005). The *Ung* gene encodes both nuclear (UNG2) and mitochondrial (UNG1) isoforms of UNG (Nilsen et al., 2000; Caradonna and Muller-Weeks, 2002). Challenges such as folate deficiency or brain ischemia lead to an increase in UNG activity (Cabelof et al., 2004; Endres et al., 2004). Importantly, the most significant increase of uracil-excising activity after brain damage is seen in mitochondrial/cytosolic extracts and may be exclusively caused by UNG1 (Endres et al., 2004).

In this study, we employed folate deficiency to promote uracil misincorporation into DNA while the *Ung* knockout was intended to prevent the repair of uracil-containing nucleotides. We demonstrate an increase in mtDNA and in the abundance of the D-1 mtDNA deletion in folate-deprived *Ung*^{−/−} mice.

Materials and methods

Animals and treatments

All experimental procedures conformed to institutional guidelines and were approved by local authorities. The generation of mice deficient in UNG has been described elsewhere (Endres et al., 2004). Folate deficiency was induced as described previously (Endres et al., 2005). Briefly, for selective intestinal decontamination, experimental diets (Altromin special diet C1027 lacking folic acid and Altromin control diet C1000; Altromin, Lage, Germany) were supplemented with 1% succinylsulfathiazole (Sigma-Aldrich). *Ung*^{−/−} and littermate *Ung*^{+/+} mice of 14 months of age were subjected to folate deficiency for six weeks. All animals were kept on a 12-hour light/dark schedule with ad libitum access to their respective diet and water. All animals were deeply anesthetized with ketamine and decapitated. Brains were quickly removed en bloc, snap frozen in liquid N₂ and stored at −80 °C. Serum homocysteine levels were determined by high performance liquid chromatography using a homocysteine reagent kit for HPLC in serum (Chromsystems, Munich, Germany).

DNA and RNA isolation

Hemispheres were homogenized and total DNA and RNA extracted using TRIZOL Reagent (Invitrogen, Karlsruhe, Germany). Purification of extracts was performed according to Invitrogen protocols. RNA was digested with RQ1 RNase-free DNase (Promega, Mannheim), following phenol–chloroform extraction and ethanol precipitation. Reverse transcription was performed with 2 µg of total RNA, random primers and MMLV-RT (Promega).

Real-time PCR

For PCR amplification, we used gene-specific primers (Table 1) and Light Cycler FastStart DNA Master SYBR GreenI (Roche Diagnostics, Mannheim). PCR conditions were as follows: preincubation 95°, 10 min; 95°, 15 s, primer specific annealing temperature, 10 s, 72°, 15 s (45 cycles). Crossing points of amplified products were determined using the Second Derivative Maximum Method (Light Cycler Version 3.5, Roche). Quantification was relative to Gelsolin promoter (*Gsn Prom*) as housekeeping gene for mtDNA and D-1 mtDNA deletion and to β-actin (*rod Act*) for cDNA, respectively.

Table 1
Primer sequences.

Gene	Forward	Reverse
mt control	CATTCTAGCCTCGTACCAACAC	AGTATTCTGAAGCTTGGAGGATG
D-1 deletion	CATTCTAGCCTCGTACCAACAC	GATTTCGTATGCTGTACATAGCTG
<i>Gsn Prom</i>	GAACCCAGATGTCTCAGAGAT	CCGCGCTCAGACACCCGAC
PGC-1	CACGCAGCCCTATTCAITGTTTCG	GCTTCTCGTCTCTTTGCGGTAT
Tfam	CTTCGATTTCCACAGAACAGC	CTTTGTATGCTTTCACTCAGC
<i>rod Act</i>	ACCCACACTGTGCCCATCTA	GCCACAGGATTCATACCCA

Total mtDNA was measured by amplifying a 238 bp product from mtDNA (control). MtDNA with a D-1 deletion yields a product of 233 bp. Specificity of PCR products was checked using melting curve analysis and electrophoresis in a 1.5% agarose gel.

Tissue homogenization for metabolic enzyme assays

Brain tissue from mice regardless of genotype and/or diet was homogenized in homogenization buffer (50 mM HEPES, pH 7.4, 2 mM EDTA, 0.2% Triton X-100). Prior to homogenization PMSF was added to the buffer to a final concentration of 1 mM. Tissue homogenization was performed at 4 °C with a PowerGen 125 homogenizer (Fisher Scientific, Ottawa, Canada) on full speed for 10 s for 3 cycles. Samples were centrifuged for 10 min at 500g (4 °C). Protein concentrations of supernatants were measured using the Bradford technique with a BioRad protein kit. Homogenates were stored at −80 °C.

Citrate synthase (CS), lactate dehydrogenase (LDH) and cytochrome c oxidase (Complex IV) activities

Metabolic enzyme assays were performed essentially as in Stuart et al. (2005). Briefly, all assays were performed at 30 °C, using a Varian Cary 100 Bio UV-Visible Spectrophotometer. Conditions for CS activity were: 0.5 mM 5,5'-dithiobis(2-nitrobenzoic) acid, 0.1 mM acetyl-CoenzymeA, 0.05% Triton X-100, 50 mM Tris (pH 8.0). 10 µg of tissue homogenate was added to obtain a background absorbance. The reaction was initiated with the addition of 0.5 mM oxaloacetate, and a change in absorbance was measured at 412 nm. LDH activity was measured in a solution containing 20 mM HEPES buffer (pH 7.3), 0.2 mM NADH, and 30 µg of tissue homogenates. A background change in absorbance was measured before the addition of 10 mM pyruvate after which a change in absorbance was measured at 340 nm. Conditions for the complex IV activity assay were: 25 mM potassium phosphate buffer with 0.5% Tween 20 (pH 7.2). Five micrograms of tissue homogenate was added to obtain a background rate of absorbance change before the reaction was initiated with the addition of 50 µM fully reduced cytochrome c and the instantaneous change in absorbance was measured at 550 nm. Reduced cytochrome c was prepared by adding sodium dithionite to an aqueous solution of cytochrome c until fully reduced, then passing the solution through a Sephadex G-25 (Sigma-Aldrich) column to remove the excess dithionite.

Statistical analysis

Statistical comparisons were performed by two-way ANOVA with level of significance set at 0.05 and two-tailed *p* values. Post hoc testing was performed where appropriate. Values are presented as means ± SEM.

Results

In order to ascertain the effectiveness of the folate-deficient diet (FD) versus the regular diet (ND), we assessed serum homocysteine levels. Regardless of genotype, homocysteine levels [in µM] were

moderately increased in those animals that had been fed the folate-deficient chow (10.6 ± 2.1 versus 5.1 ± 0.6 ; two-way ANOVA for factor treatment: $F_{1,18} = 6.0$, $p = 0.03$).

Mitochondrial biogenesis and quantification of D-1 mtDNA deletions

Direct repeat sequences are often unstable and can lead to looping of the DNA followed by excision of the intervening sequence. Thus, they are good markers for sites of potential deletion events. Several such sites have been described. Importantly, one of these sites is not nested, i.e. PCR interpretation is not confounded by overlapping deletion events. This so-called D-1 deletion is also large enough that one can design PCR primers to a short flanking sequence which, in a short (15 s) PCR extension cycle, only yields a product when the intervening DNA is absent—i.e. in the case of a deletion (Tanhauser and Laipis, 1995). As is illustrated in Fig. 1, two 15 bp direct repeats (yellow boxes) flank a portion of the mouse mitochondrial genome. In the case of a deletion event, a DNA fragment between the repeats (including one copy of the repeat itself) is excised. Whereas the common-fwd and control-rev primers yield a 238 bp product from normal, undeleted DNA, the product from the common-fwd and Δ -rev primers is too long to be amplified in the short PCR cycle used. However, when DNA with a D-1 deletion is present, the control-rev binding site is no longer there and the common-fwd yields a 233 bp product with the Δ -rev primer (Fig. 1B). The following genes and tRNAs are located within this deletion interval (9089–12962): cytochrome c oxidase subunit III, NADH dehydrogenase subunits 3, 4, 4L, 5, tRNA-Gly, tRNA-Arg, tRNA-His, tRNA-Ser and tRNA-Leu.

To some degree, an increase in mitochondrial volume density may compensate for mitochondrial respiratory dysfunction. Mitochondrial biogenesis and the amount of D-1 deletions in brain were quantified after six weeks on the experimental diets (Fig. 2). The combination of folate deficiency and of *Ung* null genotype led to a significant increase in the amount of mtDNA in brain (Fig. 2A). Similarly, the abundance of the D-1 deletion as normalized to nuclear-encoded gelsolin promoter was significantly increased in *Ung*^{−/−} mice subjected to the folate-free chow (Fig. 2B). Further analysis yielded a significant effect of

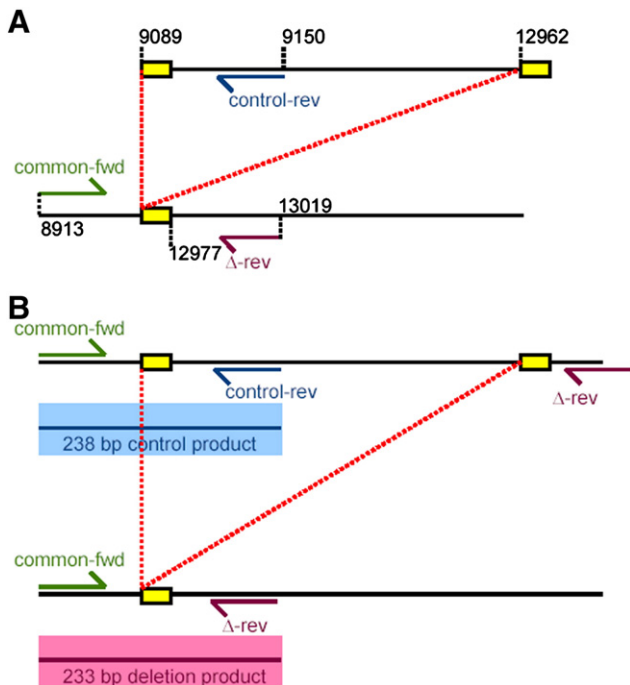


Fig. 1. Detection of D-1 deletion in mtDNA. For a detailed explanation of the PCR strategy used in this study, see main text.

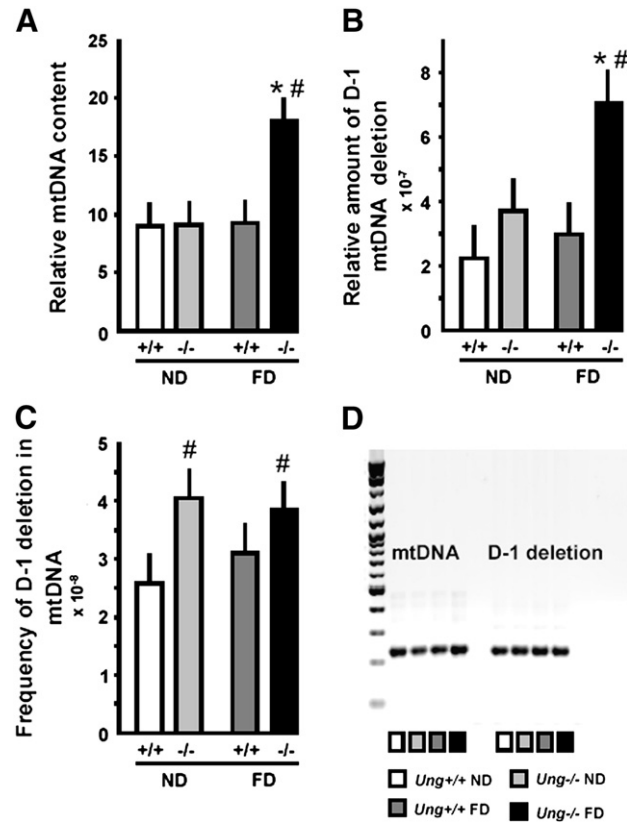


Fig. 2. Measurement of total mtDNA and of D-1 mtDNA deletion in brain DNA. A. Abundance of mtDNA (D-loop) and B. the amount of D-1 deletion in mtDNA were assessed in aged *Ung*^{+/+} and *Ung*^{−/−} mice after six weeks on the experimental diet. Quantification is relative to nuclear encoded gelsolin promoter. C. Analysis of the frequency of D-1 deletion in mtDNA revealed a significant effect of genotype (two-way ANOVA for factor diet $p = 0.76$; for factor genotype $p = 0.04$). D. Detection of 238 bp product from mitochondrial DNA (left) and of 233 bp product amplified from deletion D-1 in mtDNA (right) by gel electrophoresis. Lanes 5 and 10 are control PCRs (no DNA template). * $p < 0.05$, ND versus FD; # $p < 0.05$, *Ung*^{+/+} versus *Ung*^{−/−}. $n = 4$ –7 animals per group.

impaired uracil excising activity on the frequency of the D-1 deletion in mtDNA with no additional effect of the experimental diet (Fig. 2C).

The transcriptional coactivator peroxisome proliferator-activated receptor- γ coactivator (PGC)-1 α and mitochondrial transcription factor A (Tfam) play crucial roles in mitochondrial DNA replication and mitochondrial biogenesis in brain (Onyango et al., 2010; Puigserver et al., 1998; Virbasius & Scarpulla, 1994). mRNA expression levels of both of these factors did not differ significantly between experimental groups (Fig. 3). Citrate synthase (CS) is a nuclear-encoded mitochondrial matrix protein that serves as a common marker of mitochondrial volume density (e.g. Nishigaki et al., 2003). CS activity also did not differ significantly between experimental groups (Fig. 4A).

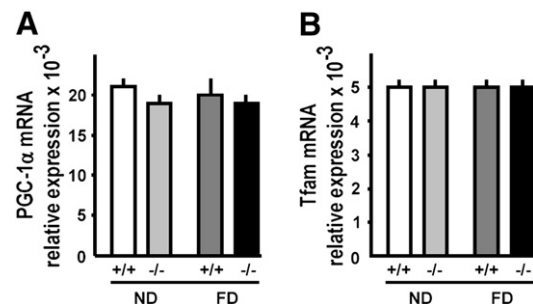


Fig. 3. Transcriptional regulation of mitochondrial biogenesis. Neither PGC-1 α (A) nor Tfam (B) mRNA expression levels differed significantly between experimental groups. Quantification is relative to β -actin. $n = 4$ –7 animals per group.

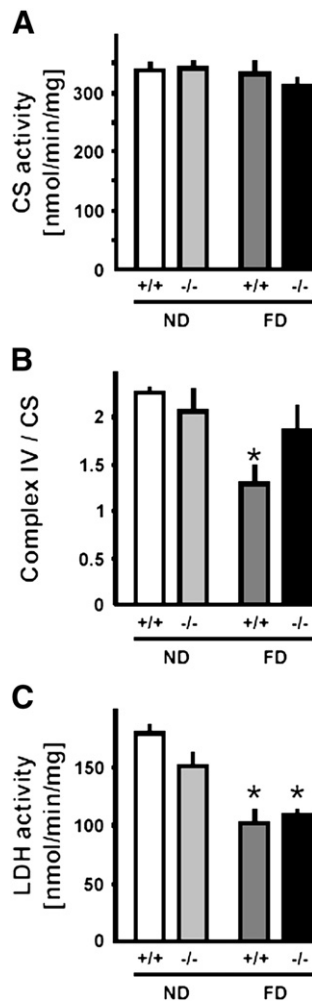


Fig. 4. Metabolic enzyme activities were assessed at six weeks on the respective experimental diets. A. Activity of mitochondrial marker protein citrate synthase (CS) did not differ significantly across experimental groups. B. Complex IV activity. C. Lactate dehydrogenase (LDH) activity. * $p < 0.05$, normal versus folate-deficient diet. $n = 4$ –9 animals per group.

Cytochrome *c* oxidase is the terminal electron acceptor in mitochondrial electron transport. Folate deprivation has previously been shown to produce a reduction in cytochrome *c* activity in rat liver (Chang et al., 2007). Here, cytochrome *c* oxidase activity in brain was also significantly reduced as an effect of folate-deficient feeding (Fig. 4B; $F_{1,28} = 4.9$, $p = 0.04$; post-hoc analysis for factor experimental diet within wildtype animals: $p = 0.04$). Interestingly, this effect appeared to be blunted in *Ung*^{-/-} mice.

Finally, we assessed LDH activity as a measure of the brain's glycolytic capacity. Insufficiency of ATP production via oxidative phosphorylation can be compensated for by substrate level phosphorylation with a corresponding increase in glucose fermentation to lactate (Rohas et al., 2007). We hypothesized that LDH activity may be elevated in *Ung*^{-/-} mice, particularly in the context of folate deficiency. However, independent of *Ung* genotype, folate deficiency resulted in a strong reduction in LDH activity (two-way ANOVA for factor feeding: $p < 0.001$; Fig. 4C).

Discussion

Strong epidemiological and experimental evidence links folate deficiency to neurodegenerative and neuropsychiatric disease (Kronenberg et al., 2009). Using a 2×2 design, we here assessed the effects of the absence of efficient uracil excision repair and of folate

deprivation on mitochondrial DNA in aged brain. The close proximity to the electron transport chain and the relative lack of repair enzymes render mtDNA particularly susceptible to being a target for reactive oxygen species (ROS). Accumulation of mtDNA mutations may contribute to brain dysfunction and, ultimately, to neuronal loss.

The current study yielded the following major findings: 1. Folate deprivation increased mtDNA content in brain of *Ung*^{-/-} mice, but not of wild-type controls. 2. Independent of folate status, the frequency of the D-1 deletion in mtDNA was significantly increased in *Ung*^{-/-} mice. 3. Mitochondrial biogenesis as assessed by PGC-1 α and Tfam mRNA expression levels was not affected by either *Ung*^{-/-} genotype or short-term folate deficiency at six weeks of folate-deficient feeding. Furthermore, CS activity in brain did not differ across experimental groups. 4. Finally, independent of efficient uracil excision repair, low folate status resulted in a significant reduction of LDH activity in brain.

Mitochondria are semiautonomous organelles responsible for generating ATP through the coupling of oxidative phosphorylation to respiration. They exert central functions in energy production, cellular metabolism and ion homeostasis. Mitochondria therefore play an important role in regulating cell survival and differentiation as well as apoptosis (Chan, 2006). Several mechanisms including lack of protective histones, proximity to the ROS-producing electron transport chain and paucity of DNA repair enzymes render mtDNA particularly susceptible to oxidative damage (Mandavilli et al., 2002). In mice, the so-called mitochondrial mutator phenotype is associated with reduced lifespan and premature onset of aging-related phenotypes such as weight loss, osteoporosis, sarcopenia, cardiomyopathy and reduced fertility (Kujoth et al., 2005; Trifunovic et al., 2004).

It has previously been shown that four weeks of a folate deficient diet promotes mitochondrial dysfunction and reduces cytochrome *c* oxidase activity in rat liver (Chang et al., 2007), and our previous work suggests this could occur also in brain (Kronenberg et al., 2008). Our measurements of cytochrome *c* oxidase activity are in good agreement with this, as folate deficiency reduced cytochrome *c* oxidase activity by 43% in brain tissue. However, our hypothesis that *Ung* deficiency would exasperate this effect was not borne out by the data, as the effect was partially rescued by *Ung* deficiency.

It has been postulated that an increase in mtDNA or mitochondrial abundance generally may compensate for mitochondrial respiratory dysfunction resulting from DNA damage or mutation (Lee & Wei, 2005). The increase in mtDNA observed in aging tissues also suggests a compensatory response to the accumulated effects of ROS or mtDNA mutation load (e.g. Gadaleta et al., 1992; Barrientos et al., 1997). Under pharmacological stress conditions such as treatment with taxol or etoposide, cultured mammalian cells also upregulate the abundance of mtDNA (Karbowski et al., 2001; Reipert et al., 1995). In the aged animals investigated here, we did not detect an effect on the amount of mtDNA of either short-term folate deficiency or of the *Ung* null genotype alone. However, there was a marked increase in the amount of mtDNA specifically in *Ung*^{-/-} mice that had been fed the folate-deficient diet for six weeks. Similarly, the amount of the D-1 mtDNA deletion was significantly increased in brain of folate-deprived *Ung*^{-/-} mice. Conceptually, these findings fit rather well with an earlier study from our laboratory demonstrating a distinct interaction between the *Ung* null genotype and of low folate status resulting in neurodegeneration along with significant alterations on the molecular, neurochemical, and behavioral levels (Kronenberg et al., 2008). It should be noted that a decrease in the amount of mtDNA has previously been reported in folate-deficient tissues of rats (Chou et al., 2007). However, that study was conducted in weaning rats whereas we used aged mice for this investigation. Clearly, the effects of folate deficiency will differ markedly between a growing organism with high rates of cell proliferation and an old organism with considerably less proliferative activity. It should also be noted that

although overall mtDNA content was significantly increased in folate-deprived *Ung*^{−/−} mice, the rate of ongoing mitochondrial biogenesis as reflected by Tfam or PGC-1 α mRNA levels did not differ between groups at six weeks of folate-deficient feeding. Similarly, activity of the mitochondrial marker protein CS did not differ significantly between experimental groups at six weeks. Taken together, these results are consistent with the notion that the increase in mtDNA observed in *Ung*^{−/−} mice following a brief episode of folate deprivation constitutes a compensatory response.

The results reported here support our previous hypothesis that in folate deficiency, the detrimental effects of impaired uracil base excision repair on the CNS are due in large part to mitochondrial dysfunction (Kronenberg et al., 2008 and 2009). There is currently an intense debate about the precise molecular mechanisms (e.g. point mutations, large deletions, linear mtDNA molecules caused by replication stalling) underlying progeroid phenotypes associated with mtDNA variations (Edgar & Trifunovic, 2009). In this study, we only measured the D-1 deletion of mtDNA which we used as a bona fide index of overall mtDNA damage. It has yet to be clarified in more detail how deletions in mtDNA are produced. The direct link between uracil misincorporation and a large deletion event is therefore a significant aspect of our data. We note that the frequency of the D-1 deletion as such may be too low to have a direct functional impact. However, since the burden of point mutations was not assessed, our study does not lend itself to the debate as to whether mtDNA deletions versus mtDNA point mutations are more detrimental.

LDH is a key metabolic enzyme catalyzing the interconversion of pyruvate and lactate in brain tissue, which is a net producer of lactate under basal conditions (reviewed in van Hall, 2010). LDH activity is found in both neuronal and glial fractions of cerebral cortex (Hrachovina & Mourek, 1990). Importantly, mitochondrial LDH has recently been demonstrated both in neurons (Hashimoto et al., 2008) and in a human astrocytic cell line (Lemire et al., 2008). We had originally hypothesized that LDH activity might be elevated in brain tissue of *Ung*^{−/−} and/or folate deficient mice to compensate for ATP insufficiency associated with mitochondrial dysfunction. However, instead, LDH activities were significantly reduced by folate deficiency. The unexpected decrease in LDH activity may suggest a disruption of redox balance elicited by folate deficiency. This, however, remains to be more fully elucidated. At any rate, reduced LDH activity is expected to further depress ATP production in brain tissue, exacerbating the dysregulation of energy metabolism and placing neurons and glial cells at risk of apoptotic or necrotic death. In hypoxemia, for example, the glycolytic capacity of the brain may be the deciding factor in determining neuronal survival. Reduced LDH activity may therefore contribute to the increased susceptibility of the brain to neurodegeneration under low-folate conditions (e.g. Endres et al., 2005).

In conclusion, our study provides new insights into how folate deficiency compromises brain function. Whereas folate deficiency reduced LDH activity in brain independent of *Ung* genotype, the effects of folate deficiency on mtDNA were aggravated in animals lacking efficient uracil excision repair.

Acknowledgments

The authors wish to thank Bettina Herrmann for excellent technical assistance. This study was supported by grants from the Volkswagen Foundation (Lichtenberg program), BMBF (Center for Stroke Research Berlin), Deutsche Forschungsgemeinschaft, and the Hermann and Lilly Schilling Foundation.

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