Suppression of Uracil-DNA Glycosylase Induces Neuronal Apoptosis*

Inna I. Kruman‡§, Elena Schwartz§, Yuri Kruma, Roy G. Cutler¶, Xiaoxiang Zhu†, Nigel H. Greig, and Mark P. Mattson**

From the ‡Sun Health Research Institute, Sun City, Arizona 85351, §Laboratory of Molecular Growth Regulation, NICHD, National Institutes of Health, Bethesda, Maryland 20892, the ¶Laboratory of Neurosciences, National Institute on Aging Gerontology Research Center, Baltimore, Maryland 21224, and the **Department of Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

A chronic imbalance in DNA precursors, caused by one-carbon metabolism impairment, can result in a deficiency of DNA repair and increased DNA damage. Although indirect evidence suggests that DNA damage plays a role in neuronal apoptosis and in the pathogenesis of neurodegenerative disorders, the underlying mechanisms are poorly understood. In particular, very little is known about the role of base excision repair of misincorporated uracil in neuronal survival. To test the hypothesis that repair of DNA damage associated with uracil misincorporation is critical for neuronal survival, we employed an antisense (AS) oligonucleotide directed against uracil-DNA glycosylase encoded by the UNG gene to deplete UNG in cultured rat hippocampal neurons. AS, but not a scrambled control oligonucleotide, induced apoptosis, which was associated with DNA damage analyzed by comet assay and up-regulation of p53. UNG mRNA and protein levels were decreased within 30 min and were undetectable within 6–9 h of exposure to the UNG AS oligonucleotide. Whereas UNG expression is significantly higher in proliferating as compared with nonproliferating cells, such as neurons, the levels of UNG mRNA were increased in brains of cystathionine β-synthase knockout mice, a model for hyperhomocysteinemia, suggesting that one-carbon metabolism impairment and uracil misincorporation can induce the up-regulation of UNG expression.

It is well established that damage to DNA can lead to mutations, chromosomal rearrangement, oncogenic transformation, and cell death (1). Genomic stability in the brain is of particular interest because terminally differentiated neurons cannot divide and must stay active for the lifetime of the animal. In addition, neurons have a high level of metabolic activity and accordingly produce high amounts of reactive oxygen species (2). The importance of DNA repair to the nervous system is illustrated by the neurological abnormalities observed in patients with hereditary diseases associated with defects in DNA repair, such as xeroderma pigmentosum and ataxia telangiectasia (3). Recently, DNA-damaging agents have been shown to activate death programs in terminally differentiated postmitotic neurons (4). Thus, neuroblastoma cells become extremely UV-sensitive after terminal differentiation (5), and neurons are more vulnerable than astrocytes to DNA-damaging conditions such as ionizing radiation (6) and camptothecin (7), possibly because functionally mature neurons are generally deficient in the repair of their DNA (8) and therefore more sensitive to DNA damage. A number of terminally differentiated cell systems, including neurons, have been demonstrated to have attenuated DNA repair at the global genome level (9). Since neurons have an elevated rate of metabolism, particularly oxidative metabolism, which may result in accumulation of numerous lesions in the genome and compromise transcription (10), it would be logical to suggest that a mechanism exists to maintain the integrity of those genes needed for viable cell function. Primary neurons can repair strand breaks, but the process is slower than in astrocytes, and neurons are more prone to apoptosis (6). Upon terminal differentiation of neurons, there is a sharp decrease in global genomic repair. However, repair of active genes has been found to be maintained or even enhanced by differentiation (11). Differentiated cells have been shown to repair transcribed genes using the specialized repair pathway termed transcription-coupled repair (TCR), which targets repair systems to transcribed genes (9). The mechanisms of this type of DNA repair are not yet fully understood. Several DNA repair systems, including base excision repair (BER) may be involved in TCR (12).

Among the factors that promote genetic instability and carcinogenesis, the impairment of homocysteine/folate (one-carbon metabolism) is considered to be an important risk factor associated with an unbalanced diet or genetic defects. A growing body of evidence suggests that the disturbance of this metabolism is also involved in neuronal abnormalities. The disturbance of one-carbon metabolism is thought to cause a chronic DNA precursor imbalance by blocking synthesis of purines and thymidine (13–15). Thymidylate synthase converts dUMP to TMP. However, when this reaction is blocked, it depresses thymidylate synthesis and results in the accumulation of excess dUTP and in increased dUMP misincorporation into DNA during replication or repair. Hydroxylative deamination of cytosine residues in DNA also generates aberrant uracil (1). If unrepaired, the resulting premutagenic U:G or U:A mispairings lead to transition mutations. To protect the cell from these consequences, all organisms studied to date have uracil-DNA

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¶ To whom correspondence should be addressed: Sun Health Research Institute, 10515 Santa Fe Dr., Sun City, AZ 85351. Tel.: 623-876-5328; Fax: 623-876-5695; E-mail: inna.kruman@sunhealth.org.

1 The abbreviations used are: TCR, transcription-coupled repair; Z, N-benzoyloxycarbonyl; BER, base excision repair; OGG1, oxoguanine-DNA glycosylase 1; AS, antisense oligonucleotide; S, scrambled oligonucleotide; CBS, cystathionine β-synthase; 3AB, 3-aminobenzamide; PARP, poly(ADP-ribose) polymerase; WT, wild type; RT, reverse transcriptase.

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Glycosylase activity, responsible for the excision of uracil residues and initiation of the BER pathway (16). The BER pathway repairs lesions involving modifications to the DNA bases. The specificity of BER is provided by DNA glycosylases with precise substrate specificities. The apurinic site generated as a consequence of one-carbon metabolism impairment is thought to be linked with proliferating cells and carcinogenesis, the mechanism of neuronal cell death caused by this disturbance remains to be clarified.

In the present study, we demonstrate that UNG is essential for the survival of embryonic rat hippocampal neurons and that depletion of UNG results in a DNA damage response that triggers p53-dependent apoptosis. In addition, we show that UNG expression is increased in the brains of cystathionine β-synthase knockout mice, a well-known mouse model of hyperhomocysteinemia (21) associated with one-carbon metabolism disturbance, suggesting that one-carbon metabolism impairment and uracil misincorporation can induce a compensatory up-regulation of UNG expression in the brain (22, 23).

EXPERIMENTAL PROCEDURES

Oligonucleotide Synthesis—18-Mer oligonucleotides were used in this study. The antisense (AS) sequence 5′-ACA AGG TGT TTA CCC GCA-3′ is complementary to bases 484–501 of human UNG mRNA (GenBank™ accession number X79909). Scrambled (S) oligonucleotide 5′-CAC ACT GCG TGT GTA AAC-3′ with bases identical to that of the antisense, but with a scrambled sequence, was used as a control. The oligonucleotides were synthesized by Integrated DNA Technologies, Inc.

Cell Cultures, Experimental Treatments, and Assessment of Cell Survival—Primary hippocampal cell cultures were established from embryonic day 18 Sprague-Dawley rats or from embryonic day 16 p53+/− (heterozygous) female mice, +/−p53 −/− and +/−p53 −/− were obtained from mouse embryos resulting from heterozygous matings (C57BL/6 background, provided by Jackson laboratories). Genotyping was performed on each individual embryo by PCR. The primers used were as described previously (26). For experimental analyses, cells were grown on poly-L-lysine-coated culture dishes. Immediately prior to experimental treatments, the growth medium was replaced with Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (Invitrogen) and 5% horse serum (Invitrogen) in a humidified atmosphere of 5% CO2, 95% air at 37°C, as described previously (28). For flow cytometry, cells were prepared as described earlier (29). Cells were harvested by incubation with 0.25% trypsin for 3 min. The cell suspension was fixed in 70% ethanol. Prior to propidium iodide staining (50 μg/ml in the dark for 30 min), suspended cells were treated for 1 h at 37°C with RNase (100 μg/ml). DNA content and ploidy were assessed with a BD Biosciences FACScan flow cytometer, and cell cycle distributions were analyzed using Multicycle (Phoenix Flow Systems).

Assessments of DNA Damage and Apoptosis—DNA damage was assessed using the alkaline single-cell gel electrophoresis (“comet assay” methodology) as described previously (30). The comet assay has been shown to be a sensitive and reliable measure of DNA strand breaks associated with incomplete excision repair sites and alkali-labile sites. Following experimental treatments, neutrons were scraped, and cell suspensions were embedded in 0.5% low melting agarose on slides (Trevigen). After treatment with lysis buffer (1% Triton X-100, 10% Me2SO, 2.5 mM NaCl, 100 mM EDTA, 10 mM Tris, pH 10), the slides were transferred to a horizontal electrophoresis unit containing buffer (1 mM EDTA and 0.3 mM NaOH, pH 12.4), and electrophoresis was performed at 25 V and 300 mA for 30 min. The slides were then stained with ethidium bromide and analyzed using an epifluorescence microscope and the comet assay image analysis software (Komet 4.0 Kinetic Imaging Ltd.). Nuclei with damaged DNA were scored as comets with a bright head and a tail, whereas nuclei with undamaged DNA appear round, with no tail. Olive tail moment is one of the parameters that is commonly measured with the comet assay. It represents the product of the amount of DNA in the tail (expressed as a percentage of the total DNA) and the distance between the centers of mass of the head and tail regions as the measure of DNA damage. For each experiment regime, 100 nucleoids or comets were assessed in each of 3–4 samples. Apoptosis was quantified in cultures after exposure to experimental treatments. Cells were fixed and stained with fluorescent DNA-binding dye Hoechst 33342, and the percentage of cells with apoptotic nuclei was calculated as described previously (31).

Acetylcholinesterase and butyrylcholinesterase—The cultures were immunostained with an antibody specific for α3 nicotinic receptor on serine 15 (S15 polyclonal antibody; 1:100; Cell Signaling Technology, Inc.) or microtubule-associated protein 2 (monoclonal antibody; 1:2000; Sigma) followed by exposure to goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (1:200 dilution; Jackson ImmunoResearch) for p53 detection. A fluorescein isothiocyanate (FITC)-conjugated mouse IgG second antibody or mouse IgG second antibody (Tebu-bio, Eugene, OR) was used to detect microtubule-associated protein 2 expression. Cells were then imaged using a Zeiss CLSM 510 confocal microscope with a ×40 water immersion objective. Relative levels of immunoreactivity (average pixel intensity/neuron) were quantified as described previously (31).

Neuronal Survival Assay—Experiments were performed in CBS knockout mice (21), which were purchased from Jackson Laboratories. To minimize the potential influence of differences in genetic background, heterozygous CBS-deficient (CBS+/−) mice were bred, and comparisons were performed between CBSI/− mice and wild-type, heterozygous CBS+/− mice.
reverse-transcribed cDNA was carried out to generate cDNA fragments corresponding to the coding regions of UNG. PCR amplification of this cDNA was performed from cultured hippocampal neurons exposed to saline (Con), 20 μM AS, or 20 μM of a control oligonucleotide with a scrambled sequence (S), and neuronal survival was quantified at the indicated time points. The values are the mean ± S.D. (n = 6), * p < 0.01; ** p < 0.001. C, representative RT-PCR analysis of mRNA for UNG and ribosomal 16 S protein (internal control) in hippocampal cultures treated either with saline (Con) or 20 μM AS for increasing time periods. HI, heat-inactivated reverse transcriptase (negative control); M, marker. Similar results were obtained in two separate experiments. Note the clear time-dependent effect of UNG AS on the UNG expression.

**RESULTS**

To test the hypothesis that uracil-DNA glycosylase, as a part of the BER mechanism responsible for DNA repair associated with uracil misincorporation, is critical for neuronal survival, we employed an AS oligonucleotide directed against the mRNA of human uracil-DNA glycosylase encoded by the *UNG* gene.
UNG is highly conserved and is characterized by high homology between the rodent and human enzymes (34). The selection of target sites for antisense oligonucleotides was based on functional and structural considerations. We chose the transcriptional start site as one of the potentially most vulnerable positions (35). A control scrambled oligonucleotide (S) had a base composition identical to that of AS but with a scrambled sequence. The cultures contained 90–95% of the neuron-specific microtubule-associated protein 2-positive cells (data not shown). A 24-h treatment of hippocampal neurons with 20 μM AS, but not with 20 μM S, significantly reduced neuronal survival (Fig. 1, A and B). To determine the effect of the AS on UNG mRNA levels, we performed RT-PCR analysis using primers corresponding to the coding regions of UNG and the ribosomal 16 S protein as an internal control, PCR amplification of this reverse-transcribed cDNA was carried out to generate cDNA fragments for each probe. The 298-bp UNG PCR fragment was obtained using primers 5’-CCG CTT TTG CTG GGA CCT G-3’ and 5’-AAC ATC ATG GCT TTC TTT TAC C-3’. The 198-bp ribosomal 16 S PCR fragment was obtained using primers 5’-CAC TGG AAA CGG GGA AAT GG-3’ and 5’-TGA GAT GGA ATG TCG TCA CAC C-3’ and used as the internal control (33). Untreated rat emryonal hippocampal neurons expressed UNG (Fig. 1C). The level of UNG mRNA, measured by RT-PCR, was found to be decreased already within 0.5 h and remained at undetectable levels through 3 h of treatment with the UNG AS and then reappeared after 12–18 h of AS exposure. mRNA levels of UNG were not different from the mRNA levels in untreated rat hippocampal neurons. As a positive control, we used UNG expression in the rat thymus, since UNG expression is known to be high in proliferating tissues (23) (Fig. 1C). These differences were significant because the reaction conditions were developed in the dynamic range of the assay to reflect quantitative differences in the samples. To determine the effect of the AS on UNG protein, we performed immunoblot analyses with a UNG antibody on whole-cell extracts of rat hippocampal neurons treated with AS.

Untreated rat emryonal hippocampal neurons expressed UNG (Fig. 1D). The protein level of UNG was found to be decreased already within 0.5 h and remained at undetectable levels through 3 h of treatment with the UNG AS and then reappeared as weak bands at 6 and 9 h of AS exposure compared with untreated control. As positive and negative controls, we used UNG expression in the rat thymus and brain, since UNG expression is typically significantly higher in proliferating (thymus) as compared with nonproliferating (brain) tissues (23). Thus, results of both RT-PCR and immunoblot analyses demonstrate the depletion of mRNA and protein levels of UNG with UNG AS treatment.

To determine whether or not neurons that died in response to depletion of UNG with AS treatment underwent apoptosis, we evaluated nuclear chromatin morphology in the cells. The dy-
ing neurons exhibited chromatin condensation as assessed by Hoechst staining, consistent with an apoptotic mode of cell death (Fig. 2A). To confirm that UNG depletion triggers neuronal apoptosis, we co-treated cultures with the pancaspase inhibitor Z-VAD-fluoromethyl ketone (10 μM) and UNG AS. Z-VAD-fluoromethyl ketone prevented the death of neurons exposed to UNG AS, demonstrating a requirement for caspase activation in the cell death process (Fig. 2B).

We have shown previously that neuronal apoptosis associated with one-carbon metabolism impairment is accompanied by p53 up-regulation (27, 31). Exposure of cultured hippocampal neurons to UNG AS, but not with S, resulted in significant increase in the levels of activated (phosphorylated on serine 15) p53 that occurred within 5–7 h after exposure to AS (Fig. 3, A–C), a time course that clearly preceded morphological signs of apoptosis such as nuclear chromatin condensation (Fig. 2B) or fragmentation of neurites (Fig. 1A). The neuronal death was completely prevented when cultures where pretreated with the p53 inhibitor PFTα (200 nM) (Fig. 4A), demonstrating that p53 activation is involved in neuronal death induced by UNG AS. To confirm the involvement of p53 in neuronal death initiated by UNG suppression, we quantified neuronal apoptosis in hippocampal neurons derived from mice with targeted deletion in the p53 gene (p53-/- mice) and from wild-type (p53+/+) mice, as assessed by cell counts of Hoechst-stained cultures. Embryos obtained from parental p53+/+ mice were dissected and processed independently. From each embryo, tissue samples were used for PCR-based genotyping. AS-induced apoptosis was significantly reduced in p53 knockout cell cultures compared with wild-type cultures (Fig. 4B), lending additional support to the notion that p53 is essential for neuronal apoptosis initiated by the impairment of uracil-BER mediated by UNG suppression.

To address the question of whether neuronal cell death induced by depletion of UNG is initiated by DNA damage, we used the single-cell gel electrophoresis “comet assay” method (27, 30). The assay has been shown to be a direct, sensitive, and reliable measure of DNA strand breaks associated with incomplete excision repair sites and alkali-labile sites (27). A 5-h incubation with the UNG AS but not S resulted in significant DNA damage (Fig. 5, A and B), indicating that the suppression of UNG by AS produces a DNA-damaging effect, leading to apoptosis.

Co-treatment of neurons with PARP inhibitor 3AB attenuated AS-induced cell death (Fig. 5C). Since PARP is a DNA repair enzyme selectively activated by DNA strand breaks (36), its blockage by 3AB associated with the attenuation of neuronal toxicity supports our results indicating that AS-induced neuronal apoptosis is mediated by DNA damage.

In order to demonstrate how one-carbon metabolism impairment may affect UNG expression in vivo, we determined mRNA levels of UNG in tissues of CBS knockout mice, a model of hyperhomocysteinemia associated with an increase in dUMP misincorporation into DNA via depression of the TTP pool. Since uracil-DNA glycosylase activity is typically significantly higher in proliferating as compared with nonproliferating tissues (22) and expression of mRNA for UNG has been shown to be lower in brain than in thymus (23), we determined UNG mRNA levels in brain and thymus of CBS+/+ (WT) and CBS-/- mice. Homozygous mutants completely lacking CBS have been shown to have highly increased plasma homocysteine levels, suffer from severe growth retardation, and die within 5–8 weeks after birth (21). In our experiments, CBS knockout mice exhibited very high blood homocysteine levels compared with WT mice as determined by an IMX® immunoassay analyzer (Fig. 6A). As expected, WT mice had relatively low levels of UNG mRNA expressed in the brain and high levels of UNG mRNA in thymus (Fig. 6B). In homozygous CBS knockout mice, the UNG expression profile was reversed. The mRNA levels were undetectable in thymus but
relatively high in brain samples (Fig. 6B). The latter results suggest that under conditions of increased dUMP incorporation into DNA, UNG is up-regulated in the brain and down-regulated in the thymus. Given that folic acid deficiency (and elevated homocysteine levels) is a known risk factor for neural tube defects (37) and one-carbon metabolism is essential for the synthesis of DNA precursors and thereby DNA synthesis and cell proliferation (38, 39), we tested the effect of impaired one-carbon metabolism on the proliferation of neural progenitor cells in vitro. Methotrexate, a widely used anti-cancer agent, is known to block homocysteine/folate metabolism (40). A 24-h treatment of C17.2 neural progenitor cells with 20 μM methotrexate resulted in a significant decrease in cell proliferation but did not affect viability of the C17.2 cells (3.4 ± 0.5% of apoptotic cells versus 2.4 ± 0.7% in control) (Fig. 6C). Flow cytometric analyses of control and methotrexate-treated C17.2 cells showed that methotrexate induced S phase arrest (Fig. 6D). These effects of methotrexate can be explained by its impact on one-carbon metabolism and DNA precursor balance and furthermore demonstrate a link between one-carbon metabolism impairment and the inhibition of proliferating activity of cells in vitro.

**DISCUSSION**

Data obtained in the present study provide evidence that the uracil-DNA glycosylase encoded by the UNG gene, known as a major carrier of uracil-DNA glycosylase activity, is necessary for the survival of cultured rat embryonic hippocampal neurons. Using AS oligonucleotide directed against UNG mRNA, we showed that UNG depletion induces DNA damage-mediated apoptosis of cultured rat hippocampal neurons. The effect of AS was time-dependent. The depletion of UNG mRNA and protein occurred within 30 min of exposure to UNG AS oligonucleotide and was totally undetectable at 3 h but returned to the control level at 12 h after exposure to the AS. We next addressed the question of whether the observed phenomenon of induced neuronal cell death was relevant to DNA damage. AS induced p53 activation, typical for apoptosis initiated by DNA damage (41). Neuronal apoptosis was attenuated by suppression of the function of p53. The involvement of DNA damage in apoptosis of hippocampal neurons induced by AS was suggested by experiments in which neurons were protected against AS-initiated toxicity when pretreated with either p53 inhibitor PFTα or as a result of p53 deficiency in hippocampal neurons derived from p53 knockout mice. These results were supported by the notion
that an inhibitor of another DNA damage response-related factor, PARP (36), also produces significant protective effect against AS-induced apoptosis. Rapid p53 activation; attenuation of AS-induced apoptosis by p53 inhibitor, by p53 deficiency, and by the PARP blockage; and direct evidence of DNA strand breaks observed within 5–6 h following exposure to AS suggest that UNG depletion mediates DNA damage-initiated apoptosis of primary hippocampal neurons. However, the mechanism by which UNG suppression leads to apoptosis in cultured hippocampal neurons remains a mystery. The greatly increased frequency of mutations in Saccharomyces cerevisiae ung– mutants deficient in UNG demonstrates the importance of this DNA uracil glycosylase (43). Surprisingly, UNG-deficient mice lack the mutator genotype characteristic of bacterial and yeast ung–/– mutations (42), suggesting the existence of complementary uracil-DNA glycosylase activity. Similarly, deficiency in oxoguanine-DNA glycosylase (OGG1), which recognizes and removes 8-oxoguanine, is also not exhibited through an overt phenotype (44).

Since TCR is operative in terminally differentiated cells (9), we can analyze our results in the context of this pathway. TCR is a discrete pathway for initiating the rapid removal of lesions that block transcription. It has been proposed that TCR has two functions: the recruitment of appropriate repair enzymes to lesions blocking transcription and the removal of the stalled RNA polymerase II to allow repair to occur (9). Unrepaired uracil may be a block to transcription as it has previously been shown for 8-oxoguanine (12). One possible explanation for our results is that UNG deficiency may lead to defective TCR and may result in the arrest of RNA polymerase II translocation. The latter has been found to constitute a signal for p53 activation and apoptosis, especially if transcription-blocking DNA damage occurs in genes that encode essential proteins (45).

Our findings suggest the importance of UNG for neuronal survival. However, further studies will be required to elucidate the mechanisms of uracil-BER in neurons. Such studies may have important implications for neuronal toxicity associated with hyperhomocysteinemia and other clinical abnormalities associated with perturbed one-carbon metabolism. Hyperhomocysteinemia is associated with uracil misincorporation into DNA and chromosomal breakage in proliferative cells (46, 47), which is probably the result of a chronic DNA precursor imbalance (48). We previously reported that elevated homocysteine levels induce uracil misincorporation and apoptosis of cultured hippocampal neurons (27), suggesting that hyperhomocysteinemia-mediated neuronal apoptosis is linked to uracil-BER.
Methotrexate has been shown to induce DNA damage and to be toxic for neurons in vitro (49). One of the major clinical manifestations of homozygous individuals with the most common type of hereditary homocystinuria, caused by deficiency in cystathionine β-synthase, is mental retardation (50). This demonstrates the association between elevated homocysteine levels and neurological abnormalities. To elucidate if hyperhomocysteinemia is linked to uracil-BER in vivo, we used a genetic model of hyperhomocysteinemia in mice in which hyperhomocysteinemia results from a targeted disruption of the CBS gene. As follows from the results presented in this study and those previously published (21), homozygous CBS mice have highly increased blood homocysteine levels and severe growth retardation.

Whereas the physiological function of the other classes of uracil-DNA glycosylases is unclear, the function and mechanism of the specific UNG is well documented (51). The UNG gene encodes nuclear and mitochondrial UNG isoforms generated via transcription from different promoters and alternative RNA splicing (18). The expression of UNG has been demonstrated to be under cell cycle regulation (52, 53). It has been proposed to be mainly regulated at the transcriptional level (19) and has a higher turnover rate ($k_{cat} = 4.6$ s$^{-1}$) than other DNA glycosylases consistent with a role in DNA replication (54). The UNG activity is in general significantly higher in proliferating as compared with nonproliferating tissues (22), and expression of UNG mRNA correlates with its activity in different tissues of an adult organism. UNG mRNA levels have been shown to be much higher in proliferating tissues like testis and thymus than in brain (23). Based on these findings, we compared expression of UNG mRNA in brain and thymus of wild-type and CBS knockout mice. UNG mRNA levels were undetectable in brains of wild-type mice, in contrast to mRNA levels in thymus consistent with previously published notions. Surprisingly, we found that UNG mRNA was undetectable in thymus of CBS knockout mice, in contrast to brain tissue of these animals. One possible explanation for these observations may be that severe hyperhomocysteinemia and one-carbon metabolism impairment block the synthesis of DNA precursors, which results in the inhibition of proliferating activity in tissues of CBS knockout mice, including the thymus. Since the expression of UNG is under cell cycle control and is associated with the DNA replication machinery (52, 54), the suppression of this machinery may down-regulate UNG expression. Perturbed one-carbon metabolism has been suggested to play a role in the etiology of neural tube defects and anemia, both

![Fig. 6. Up-regulation of UNG mRNA levels in brains of CBS knockout mice. A, levels of homocysteine in serum samples from CBS+/+(WT) and CBS−/−(CBS) mice. Values are the mean and S.D. (n = 4). *, p < 0.0001. B, representative RT-PCR analysis of mRNA for UNG and ribosomal 16 S protein (internal control) in thymus (T) or brain (B) of WT or CBS knockout mice. HI, heat-inactivated reverse transcriptase (negative control); M, marker. Similar results were obtained in two separate experiments. Note the higher expression of UNG mRNA in thymus compared to brain in WT mice in contrast to mRNA levels in brain and thymus of CBS knockout mice. C, C17.2 cells were exposed for 24 h to saline (Con) or 20 µM methotrexate (Meth) in Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum. Cell densities were quantified and expressed as percentage of control. Values are the mean and S.D. (n = 6). *, p < 0.001. D, C17.2 cells were exposed for 24 h to saline (Con) or 20 µM methotrexate (Meth), and cell cycle distributions were analyzed using flow cytometry. *, p < 0.001. Note the significantly elevated percentage of S cells in the population treated with methotrexate.](image-url)
associated with diminished cell division (37, 55). In addition, CBS knockout mice have highly reduced body weights as compared with wild-type mice, a factor that may be linked to decreased proliferating rate in the tissues of these animals. Our results demonstrating the retardation of the proliferating activity of C17.2 cells as well as their arrest at the S phase of the cell cycle upon one-carbon metabolism impairment by methotrexate are consistent with previously published data suggesting the impact of one-carbon metabolism disturbance on DNA synthesis (55). The other surprising finding in our experiments was the up-regulation of UNG mRNA in brains of CBS knockout mice. One possible explanation may be that this up-regulation may serve as a compensatory mechanism in which cells attempt to activate uracil-BER when uracil misincorporation is elevated.

Although it is widely accepted that postmitotic cells and tissues have decreased DNA repair capacity (9), several findings indicate that postmitotic cells such as muscle cells and neurons express DNA glycosylases (56) and may up-regulate such expression in certain conditions. Thus, OGG1 activity in rat heart increases significantly with age (57). In brain, up-regulation of BER activity was detected after ischemia-reperfusion injury (58). This may indicate that BER in general is essential for maintaining genomic integrity in the brain. Oxidative stress and oxidative DNA damage induce up-regulation of OGG1 activity associated with this type of DNA damage. Our results indicate that, like the up-regulation of OGG1 in response to oxidative DNA damage, uracil misincorporation mediated by hyperhomocysteinemia induces up-regulation of UNG mRNA as a compensatory mechanism for uracil removal.

In conclusion, our findings demonstrate that the suppression of UNG expression induces neuronal apoptosis in vitro, suggesting the important role of DNA-uracil glycosylase activity and particularly UNG activity in neuronal survival. We establish a link between hyperhomocysteinemia and DNA repair events in the brain, demonstrating the up-regulation of UNG mRNA in brains of CBS knockout mice, a model of hyperhomocysteinemia. This association of one-carbon metabolism impairment (itself associated with uracil misincorporation) with cysteinemia is associated with diminished cell division (37, 55). In addition, CBS knockout mice have highly reduced body weights as compared with wild-type mice, a factor that may be linked to decreased proliferating rate in the tissues of these animals. Our results demonstrating the retardation of the proliferating activity of C17.2 cells as well as their arrest at the S phase of the cell cycle upon one-carbon metabolism impairment induced by methotrexate are consistent with previously published data suggesting the impact of one-carbon metabolism disturbance on DNA synthesis (55). The other surprising finding in our experiments was the up-regulation of UNG mRNA in brains of CBS knockout mice. One possible explanation may be that this up-regulation may serve as a compensatory mechanism in which cells attempt to activate uracil-BER when uracil misincorporation is elevated.

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REFERENCES
1. Lindahl, T. (1993) Nature 362, 709–715
2. Kandel, E. R. (1998) Principles of Neural Science, pp. 19–32, Elsevier, New York
3. Rolig, R. L., and McKinnon, P. J. (2000) Trends Neurosci. 23, 417–424
4. Park, D. S., Morris, E. J., Stefanis, L., Troy, C. M., Shalanski, M. L., Geller, H. M., and Green, L. A. (1998) J. Neurosci. 18, 830–840
5. McCombe, P., Lavin, M., and Kisdon, C. (1976) Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med. 29, 523–531
6. Gobbel, G. T., Bellinsonan, M., Vogt, A. R., Gupta, N., Fike, J. R., and Chan, P. H. (1998) J. Neurosci. 18, 147–155
7. Morris, E. J., and Geller, H. M. (1996) J. Cell Biol. 134, 757–770
8. Hanawalt, P. C., Lee, P. H., Hirozawa, K., and Kopecky, K. E. (1992) Ann. N. Y. Acad. Sci. 663, 17–25
9. Nouspikel, T., and Hanawalt, P. C. (2002) DNA Repair (Amst.) 1, 59–75
10. Brooks, P. J. (2000) Neurochem. Int. 37, 403–412
11. Hirozawa, K., and Hanawalt, P. C. (1991) Mutat. Res. 255, 123–141
12. Le Page, F., Randrianarison V., Marot, D., Cabannes, J., Perricaudet, M., Feunteun, J., and Sarasin, A. (2000) Cancer Res. 60, 5548–5552
13. Scott, J. M. (1999) Proc. Natl. Acad. Sci. 96, 441–448