Alcohol-induced One-carbon Metabolism Impairment Promotes Dysfunction of DNA Base Excision Repair in Adult Brain*

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Background: DNA repair dysfunction leads to genomic instability and neuron death.
Results: Long term alcohol exposure results in reduced DNA repair, increased DNA damage, and neuron death in adult brain.
Conclusion: Long term alcohol exposure in adult brain promotes genomic instability mediated by impairment in one-carbon metabolism.
Significance: This is the first demonstration of alcohol-induced genomic instability in brain.

The brain is one of the major targets of chronic alcohol abuse. Yet the fundamental mechanisms underlying alcohol-mediated brain damage remain unclear. The products of alcohol metabolism cause DNA damage, which in conditions of DNA repair dysfunction leads to genomic instability and neural death. We propose that one-carbon metabolism (OCM) impairment associated with long term chronic ethanol intake is a key factor in ethanol-induced neurotoxicity, because OCM provides cells with DNA precursors for DNA repair and methyl groups for DNA methylation, both critical for genomic stability. Using histological (immunohistochemistry and stereological counting) and biochemical assays, we show that 3-week chronic exposure of adult mice to 5% ethanol (Lieber-Decarli diet) results in increased DNA damage, reduced DNA repair, and neuronal death in the brain. These were concomitant with compromised OCM, as evidenced by elevated homocysteine, a marker of OCM dysfunction. We conclude that OCM dysfunction plays a causal role in alcohol-induced genomic instability in the brain because OCM status determines the alcohol effect on DNA damage/repair and genomic stability. Short ethanol exposure, which did not disturb OCM, also did not affect the response to DNA damage, whereas additional OCM disturbance induced by deficiency in a key OCM enzyme, methylenetetrahydrofolate reductase (MTHFR) in Mthfr+/− mice, exaggerated the ethanol effect on DNA repair. Thus, the impact of long term ethanol exposure on DNA repair and genomic stability in the brain results from OCM dysfunction, and MTHFR mutations such as Mthfr 677C→T, common in human population, may exaggerate the adverse effects of ethanol on the brain.

Nearly 100 million people worldwide have alcohol use disorders. Brain damage is a common and potentially severe consequence of alcohol abuse (1). It is estimated that 50–75% of long term alcoholics exhibit cognitive impairment and structural damage to the brain. It is also known that chronic alcohol abuse is associated with cerebral cortical atrophy (1). Neuropathological analyses have provided evidence for loss of neurons in certain brain regions such as frontal lobes, which are highly affected in the alcoholic brain (1, 2). This region is essential for executive functions, whose loss may lead to a progressive loss of these functions, as seen in people addicted to alcohol as well as in individuals with frontal cortical damage (2, 3). These structural and cognitive changes have been suggested as the primary consequence of alcohol toxicity (4).

Alcohol consumption is associated with generation of genotoxic metabolites producing reactive oxygen species (ROS) and acetaldehyde-derived DNA lesions. The most abundant oxidative DNA lesion is 7,8-dihydro-8-oxo-2′-deoxyguanosine (oxo8dG) (5, 6). The primary acetaldehyde-derived DNA adduct is N2-ethyl-2-deoxyguanosine (N2-ethyl-dG) (7), which has been found in the DNA of ethanol-treated mice (8) and human alcoholics (9).

According to the generally accepted mechanism, endogenous DNA damage is constantly being produced in normal conditions but is also repaired, resulting in a low steady-state level of damage compatible with normal cellular function. However, under conditions of DNA repair deficiency, endogenous damage is not repaired and therefore accumulates over time causing unscheduled alterations within the genome or genomic instability, which can involve simple DNA base changes that occur due to defects in the DNA repair (10, 11), or may be character-
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alyzed by grossly abnormal karyotypes, featuring structural and numerical chromosome abnormalities (12, 13). The latter is the major form of genomic instability in cancers and is predominantly accepted as one of the most important factors of carcinogenesis (10, 12, 13). Another major clinical feature of DNA repair deficiency is neurological disease, and accordingly, DNA repair deficiencies are implicated in various diseases that feature progressive neurodegeneration, such as Werner syndrome, ataxia telangiectasia, and ataxia-oculomotor apraxia-1 (11). An emerging mechanism for how aberrant DNA repair and persistent DNA damage may affect cell viability of postmitotic neurons is via impairment in transcription (14). Therefore, ethanol-induced DNA damage, if not repaired, may also lead to genomic instability and its consequences, carcinogenesis and neurodegeneration. Indeed, chronic alcoholism is associated with both carcinogenesis and brain damage (1, 6). What can compromise DNA repair in conditions of chronic long term ethanol exposure? The impairment of OCM can play such a role. It is known that long term alcohol exposure is associated with OCM dysfunction and the suppression of OCM reactions. Elevated Hcy, an indication of OCM dysfunction (15, 16), is often observed in patients with chronic alcoholism (6, 17). OCM is critical for maintaining genomic stability (16), because OCM reactions are involved in the biosynthesis of DNA nucleotides (dNTPs), precursors for DNA repair, and producing S-adenosylmethionine involved in DNA methylation (15, 16). Chronic alcohol consumption has long been associated with an increased risk of a variety of cancers, including hepatocellular carcinoma (18, 19). Recent evidence demonstrates that alcohol cancer initiation is caused by genomic instability (19), which may be a result of OCM impairment (16, 17). However, it is not known whether these factors play a role in alcohol-induced neurodegeneration. Here, we report that long term ethanol intake increased DNA damage, compromised DNA repair, and induced neuron death in the cerebral cortex of adult mice. These were concomitant with compromised OCM, as evidenced by elevated Hcy and global DNA hypomethylation. We show a causal role of OCM impairment in long term ethanol-induced DNA repair dysfunction and ensuing genomic instability by demonstrating how modulation of OCM status affects DNA repair in the neurons of cerebral cortex in alcohol-exposed mice. A short ethanol exposure, which did not disturb OCM, also induced a normal reaction to DNA damage and activation of DNA damage repair. These mice were characterized by lower levels of DNA damage and neuronal death in the brain, compared with those after long term ethanol exposure. In contrast, additional OCM impairment caused by deficiency in essential the OCM enzyme, MTHFR in Mthfr<sup>+/−</sup> mice, exaggerated the effect of long term ethanol exposure on DNA repair. Our findings are the first demonstration of alcohol-induced genomic instability mediated by OCM impairment in the brain.

EXPERIMENTAL PROCEDURES

Mice and Ethanol Exposure—All experiments were approved by the IACUC at the Texas Tech University Health Sciences Center. 10–12-Week-old C57BL/6 male mice (The Jackson Laboratory) were fed ad libitum a nutritionally adequate Lieber-DeCarli liquid diet containing 5% (v/v) ethanol or a control diet in which ethanol was substituted iso- calorically with dextrin maltose (BioServ). MTHFR-deficient mice on C57BL/6 background with heterozygous (+/−) disruption of the gene (a breeding pair of these mice were kindly provided by Dr. Richard Finnell, UT at Austin) were PCR-genotyped as described (20).

Homocysteine Analysis—The experiments were carried out on a Prominence HPLC system (Shimadzu Scientific Instruments), using Hcy, acetonitrile, formic acid (Sigma), and dL-[<sup>3</sup>H]<sub>4</sub>Hcy (Cambridge Isotopes Laboratories) as standards (21). [<sup>3</sup>H]<sub>4</sub>Hcy stock solution was prepared as described (21), and serial dilutions of the solution were used to prepare the samples for the calibration curves. The quantification of Hcy was accomplished using area ratios calculated using [<sup>3</sup>H]<sub>4</sub>Hcy as the internal standard, where the concentration of the internal standard was set at 2000 ng/ml. For the plasma samples, 5 µl of 20 µg/ml of [<sup>3</sup>H]<sub>4</sub>Hcy and 15 µl of water was added to 30 µl of plasma samples, and Hcy was separated using an Eclipse XDB-C18 guard column (4.6 × 12.5 mm) and an analytical column (150 × 4.6 mm inner diameter, 5 µm, Zorbax C18 Agilent). The mobile phase consisted of water with 0.5% formic acid (A) and methanol (B). An isocratic gradient was run at 60% B at a flow rate of 0.5 ml/min (10 min/sample). Mass spectrometry (MS/MS) assay was performed using a triple quadrupole mass spectrometer (API 4000 system) equipped with Turbo Ion Spray® (Applied Biosystems). The data were acquired and analyzed using Analyst Version 1.4.2 (Applied Biosystems). Positive electrospray ionization data were acquired using multiple reactions monitoring. The Turbo Ion Spray® instrumental source settings for temperature (curtain gas, ion source gas 1, ion source gas 2, and ion spray voltage) were 200 °C, 10, 60, and 60 p.s.i., and 5500 V. The Turbo Ion Spray® compound parameter settings for declustering potential, entrance potential, and collision cell exit potential were 30, 10, and 12 V, respectively. The collision energy setting was 20 V. Hcy and [<sup>3</sup>H]<sub>4</sub>Hcy were characterized using the multiple reactions monitoring ion transitions 136.1–90 and 140.0–94, respectively. Hcy was extracted as follows: 1 ml of solid phase extraction cartridges (Oasis MCX, Waters Corp.) were preconditioned with 1 ml of methanol, followed by 1 ml of water. The plasma samples were added and washed with 1 ml of 1% formic acid and 1 ml of methanol, and the compounds were eluted with 1 ml of 5% ammonium hydroxide. The eluate was stream-dried under nitrogen at 60 °C, reconstituted in 100 µl of methanol, and then transferred to the autosampler vial for analysis.

N<sup>2</sup>-Ethyl-dG Analysis—DNA hydrolysis, sample enrichment, and purification were carried out as reported (22). Briefly, DNA was dissolved in 400 µl of 10 mM Tris, 5 mM MgCl<sub>2</sub> buffer containing N<sup>8</sup>-[<sup>15</sup>N<sub>5</sub>]ethyl-dG (50 fmol) and NaBH<sub>4</sub>CN (30 mg) to convert N<sup>2</sup>-ethyldene-dG to N<sup>2</sup>-ethyl-dG (7). The DNA was digested overnight at room temperature with 1300 units of DNase I, and then 1300 additional units of DNase I, 0.07 units of phosphodiesterase I (type II, from Crotalus adamanteus venom), and 750 units of alkaline phosphatase were added to the resulting mixture and incubated at 37 °C for 70 min and then overnight at room temperature. Enzymes were removed by using an MPS device (30 kDa, Amicon). After removal of an aliquot for dG analysis, the hydrolysate was desalted and puri-
fied using a solid-phase extraction (Strata-X 33 μm; 30 mg/1 ml, Phenomenex). The 70% CH$_3$OH fraction was collected, evaporated, dissolved in 300 μl of H$_2$O, and purified by anion exchange reversed phase extraction (Oasis MAX, 30 mg/cartridge, Waters). Adducts were eluted with 500 μl/ml of 70% CH$_3$OH. After evaporation, the sample was dissolved in 20 μl of H$_2$O, and aliquots were analyzed by LC-ESI-MS/MS. Samples from each dose were processed together as a set (three sets/subject). Buffer blanks containing internal standard were analyzed for MS instrument base line and contamination. Calf thymus DNA (0.1 mg) with internal standard was used as a positive control. dG was quantified with an Agilent 1100 capillary flow HPLC with a diode array UV detector (Agilent Technologies, 254 nm). A 4.6-mm × 25-cm Luna 5-μm C18 column (Phenomenex) was used with a gradient from 5 to 40% CH$_3$OH in H$_2$O (35 min, 10 μl/min). LC-ESI-MS/MS analysis was performed with an Agilent 1100 capillary flow HPLC (Agilent Technologies) with a 250-mm × 0.5-mm 5-μm particle size Polar RP column (Phenomenex) and a Vantage (ThermoElectron) triple quadrupole mass spectrometer. The solvent elution program was a 10 μl/min gradient from 5 to 40% CH$_3$OH in 35 min at 30 °C. The ESI source was set in the positive ion mode as follows: voltage, 3.7 kV; current, 3 μA; and heated ion transfer tube, 275 °C. The collision energy was 12 eV, and the argon collision gas pressure was 1.0 millitorr. Adducts were quantified by MS/MS with selected reaction monitoring at m/z 296 → m/z 180 ([M + H]$^+$→[BH$^+$]) for N$^2$-ethyl-dG and at the corresponding transition m/z 301→m/z 185 for N$^2$-[15N$_3$]ethyl-dG. A calibration curve was used for each analysis (standard solution: a mixture 0.1, 0.5, 4, 10, or 100 fmol of N$^2$-ethyl-dG and 5 fmol of [15N$_3$]N$^2$-ethyl-dG).

Histological Assays—Brains were collected, fixed in Bouin’s fixative, embedded in gelatin blocks, and serially sectioned in coronal planes at 25 μm by Neuroscience Associates, Inc. Before incubation with primary antibody, sections were subjected to antigen retrieval (ProHisto, 15 min) Endogenous peroxidase activity was blocked with 0.3% H$_2$O$_2$ in 100% methanol. The sections were then incubated with the primary antibody (overnight at 4 °C). Antibodies used were as follows: AIF (1:500; Abcam ab32516); active caspase 3 (1:500; R&D Systems AF835); oxo8dG (1:100; Abcam ab64548), PAR (1:100; Enzo Life Sciences; BML-SA216). The sections were then incubated with the primary antibody (overnight, 4 °C). Antibodies used were as follows: AIF (1:500; Abcam ab32516); active caspase 3 (1:500; R&D Systems AF835); and PAR (1:100; Enzo Life Sciences; BML-SA216). The sections were then stained for TUNEL using the In Situ Cell Death Detection kit (Roche Applied Science). A positive control was performed by incubating with DNase I (Invitrogen; 18047-019) prior to labeling; a negative control was performed by incubating the slides with the TUNEL label in the absence of the enzyme. Labeling was performed by incubating sections with a 1:10 ratio of enzyme to label solution (1 h, 37 °C). The signal was converted to a peroxidase using a POD kit (Roche Applied Science; 11426346910, 30 min, 37 °C), and then sections were developed as described above. Both sets of slides were blocked again with 10% horse serum (20 min), and MAP2 (1:500; Millipore; AB5622) was applied either for 2 h (oxo8dG subset) or overnight at 4 °C (TUNEL subset). Sections were then developed (see above) and counterstained with Methyl Green (Vector Laboratories; H3402) for oxo8dG subset or Mayer’s hematoxylin (Electron Microscopy Services, 26043) for TUNEL subset.

Stereological counting was performed using the CASTGRID software (Olympus DK). An Olympus BX51 microscope with a motorized stage (x, y), attached microcator (z axis, Heidenhain), and color camera (Hitachi HV-C20) was used. Both the optical dissector and fractionator methods were employed to compare the two estimates of total cell number and relative efficiency. Cavalieri’s principle was used to determine cerebral cortex volume (V$_{rep}$ 23). The density ($N_v$) of total labeled neurons, positive for oxo8dG or TUNEL, was established using the optical dissector method: $N_v = \frac{\Sigma Q - \frac{1}{V_{dis}} \Sigma P_v}{V_{rep}}$, whereby $\Sigma Q$ is the total number of cells counted; $V_{dis}$ is the counting frame area multiplied by the height of the optical dissector, and $\Sigma P_v$ is the total number of stops within the reference volume counted. For each cortex, a random systematic sampling of every 12th section was analyzed (20 and 22 sections/animal). All counting was done with an oil immersion x60 objective.

For confocal microscopy analysis, brains were collected, rapidly fresh frozen, and serially cryosectioned in the coronal plane at 10 μm. Sections from prefrontal cerebral cortex were analyzed in fluorescence experiments. Before incubation with primary antibody, sections were briefly fixed with 4% paraformaldehyde for 10 min and then subjected to antigen retrieval (ProHisto, 15 min). Endogenous peroxidase activity was blocked with 0.3% H$_2$O$_2$ in 100% methanol. The sections were then incubated with the primary antibody (overnight, 4 °C). Antibodies used were as follows: AIF (1:500; Abcam ab32516); active caspase 3 (1:500; R&D Systems AF835); and PAR (1:100; Enzo Life Sciences; BML-SA216). The sections were then stained for TUNEL using the In Situ Cell Death Detection kit (Roche Applied Science). A positive control was performed by incubating with DNase I (Invitrogen; 18047-019) prior to labeling; a negative control was performed by incubating the slides with the TUNEL label in the absence of the enzyme. Labeling was performed by incubating sections with a 1:10 ratio of enzyme to label solution (1 h, 37 °C). The signal was converted to a peroxidase using a POD kit (Roche Applied Science; 11426346910, 30 min, 37 °C), and then sections were developed as described above. Both sets of slides were blocked again with 10% horse serum (20 min), and MAP2 (1:500; Millipore; AB5622) was applied either for 2 h (oxo8dG subset) or overnight at 4 °C (TUNEL subset). Sections were then developed (see above) and counterstained with Methyl Green (Vector Laboratories; H3402) for oxo8dG subset or Mayer’s hematoxylin (Electron Microscopy Services, 26043) for TUNEL subset.
Neither the number of MAP-2-positive cells (neurons) nor cerebral cortex volume was affected by chronic ethanol exposure (data not shown).

**RESULTS**

**3-Week Ethanol Consumption Induces Neuronal Death in the Cerebral Cortex of Mice**—We first set out to identify whether a 3-week exposure to the Lieber-Decarli liquid diet containing 5% ethanol causes neuron death in the brains of the C57BL/6 mice. The C57BL/6 mouse is one of few inbred strains that consume sufficient amounts of alcohol to allow biologically relevant blood alcohol levels to be achieved (25). The Lieber-Decarli liquid diet (LD82, Bioserv) contains standard (corresponding to standard rodent diet) composition of ingredients, including vitamins. The use of a liquid diet is based on need for higher blood alcohol levels and lesser stress to the animal compared with forced-feeding regimens (aerosolized inhalation and intravenous or gavage administration) (26). The mice were also exposed to a control diet in which ethanol was substituted isocalorically with dextrin maltose (27). The consumption of control and ethanol diets did not differ significantly (data not shown), suggesting that the diet paradigm utilized in these experiments does not affect ethanol consumption. Blood ethanol levels in all groups of animals exposed to ethanol were 55.2 ± 7.2 mm. Given that chronic ethanol consumption leads, among other lesions, to cortical cerebral atrophy (1, 2), we quantified the density of neurons (MAP-2 staining) with terminal transferase-mediated dUTP-biotin nick end-labeling (TUNEL) throughout the entire cortex using histological techniques and stereological counting. This detects DNA fragmentation resulting from apoptotic signaling and may also label cells undergoing other types of cell death, as well as active DNA repair and gene transcription (28, 29). However, TUNEL labeling is too crude and very limited in sensitivity for detecting repairable DNA damage (30, 31). 3-Week ethanol exposure significantly increased the density of TUNEL-labeled neurons in the mouse cerebral cortex (Fig. 1, A and B), suggesting that chronic ethanol exposure results in neuronal damage but did not affect the number of neurons (MAP-2-positive cells) in the cerebral cortex (data not shown), indicating that 3-week exposure is sufficient for activation of neuronal death but not sufficient for neuronal loss.

**Ethanol Induces ROS- and Acetaldehyde-derived DNA Lesions in the Cerebral Cortex**—Not much is known about genotoxic effects of ethanol in brain. We examined DNA damage in brain tissue of mice exposed to ethanol for 3 weeks and demonstrated the formation of both ROS-derived oxo8dG and acetaldehyde-derived N²-ethyl-dG DNA lesions. Histological techniques and stereological counting (23) allowed us to quantify the density of neurons (MAP-2 staining) labeled with anti-oxo8dG antibody in the cerebral cortex of mice. Fig. 2, A and B, shows a significant increase in density of neurons containing oxo8dG following long term ethanol exposure, compared with mice exposed to control diet. Using liquid chromatography-tandem mass spectrometry (LC/MS/MS), we determined the
content of acetaldehyde-derived DNA adduct $N^2$-ethyl-$d$G in brain. Fig. 2C shows a significant increase in levels of this adduct in brains of ethanol-exposed mice, compared with mice on the control diet. These data indicate that ethanol exposure induces formation of both ROS- and acetaldehyde-derived DNA lesions in the brain.

Ethanol-induced DNA Damage Response and Neuronal Death in the Brain—We determined the mode(s) of cell death produced in response to ethanol-induced DNA damage. Fig. 3 exhibits fluorescently stained sections representing the co-expression of TUNEL with other markers of cell death in neurons of prefrontal cortex in mice exposed to control and ethanol-containing diets for short (4 days) and long (3 weeks) periods of time (Fig. 3, A and B). Although caspases, and specifically caspase-3, have been implicated in a nonapoptotic role in the regulation of the cell cycle, cell proliferation, and cell differentiation (32, 33), active caspase-3 is intimately linked to apoptotic events and is a key downstream effector in the execution of apoptosis. Neuron apoptosis is predominantly associated with high levels of caspase-3 activation following insult, although low levels of caspase-3 activation were found in astrocytes in the absence of cell death (34). Fig. 3A demonstrates significant levels of active caspase-3 expression in the nucleus (35) of neurons (NeuN staining), typical of apoptosis. Fig. 3A shows that neurons positive for locations in the nucleus-active (cleaved) caspase-3 and TUNEL are more prevalent in the prefrontal cortex of mice exposed to long term ethanol than those in the control group or after short ethanol exposure. Relatively high levels of TUNEL-positive neurons in untreated mice and mice following short ethanol exposure may be explained by the fact that tissue sectioning also creates TUNEL reactivity (31). The coincidence of active caspase-3 with TUNEL reveals that these neurons undergo caspase-dependent apoptosis (29).

The covalent modification of proteins by PARylation is an immediate and dramatic biochemical response to DNA damage, leading to a vivid increase (by ~500 times) in levels of PARs (39). Although PARylation is also involved in other processes such as differentiation, it is primarily attributed to the DNA damage response, critical for DNA repair, and elimination of cells with irreparable DNA damage by executing programmed cell death (40). Neurons of the cerebral cortex in mice exposed to ethanol exhibit higher expression of PARs, compared with control mice. This is consistent with our results (Fig. 2) that ethanol produces DNA damage and activates DNA repair (Fig. 4) in the brain and also with previous in vitro findings (40).
number of neurons coexpressing a marker of the DNA damage response, PAR and TUNEL, in the cerebral cortex of mice exposed to ethanol for 3 weeks (Fig. 3C) is notably higher than in mice exposed to ethanol for a short time and in control mice. This suggests that long term ethanol-induced neuron death is initiated by DNA damage.

Long Term Ethanol Exposure Compromises DNA Base Excision Repair in the Brain—The classic model for neurodegeneration due to dysfunctional DNA repair represents the idea that DNA damage accumulates in the absence of repair, resulting in the death of neurons (10, 11). This prompted us to determine whether long term ethanol exposure is associated with dysfunctional DNA repair. ROS-induced DNA damage in mammalian cells is subject to repair primarily by BER (41), whereas a pathway for repair of the most abundant acetaldehyde-derived DNA lesion, N\(^2\)-ethyl-dG, has not been identified. We therefore focused on BER and determined how long term ethanol exposure impacts BER activity in mouse brain using real time PCR (qPCR). This method detects BER activity analyzing integrity of the template DNA containing a single nucleotide lesion after its exposure to whole cell extract where the DNA repair activity is measured. The assay is based on the ability of DNA lesions to block or slow down the progression of DNA polymerase, resulting in decreased amplification of the target DNA (24). This is expected to result in reduced amplification (24). A template containing DNA lesion and control template (Table 1) was incubated with brain extracts. BER activity was calculated by comparing the \(\Delta Ct\) values (\(Ct\) is the number of cycles required for the fluorescent signal to cross the threshold) of the repaired and control templates.

It is known that all cells respond to DNA damage by activating DNA repair and DNA damage signaling pathways (42). DNA repair is generally attenuated by differentiation in most types of cells, and DNA repair activity in postmitotic cells is low.

**FIGURE 3. Ethanol exposure induces different modes of cell death and DNA damage response (PARylation).** The brain sections (prefrontal cerebral cortex) of mice chronically exposed for 3 weeks (long term) or 4 days (short term) to the Lieber-Decarli liquid diet with or without ethanol (5%) were triple-labeled with neuronal marker NeuN (purple), TUNEL (green), and cleaved caspase-3 (A), AIF (B), or antibody to PAR (C), all red. Hoescht (blue) was used to identify all cell nuclei. Fluorescence was visualized by confocal microscopy. Note increased number of cells in which TUNEL is colocalized with NeuN and active caspase-3, AIF, or PAR (yellow double arrows) in the prefrontal cerebral cortex of mice exposed to long term ethanol compared with control mice and mice exposed to short term ethanol. Of particular interest is the translocation of active caspase-3 into the nucleus (A) and the movement of both AIF (B) and PAR (C) to the periphery of the nucleus after ethanol, especially after long term exposure. Single arrowhead, non-neuronal cell; double arrowhead, neuronal cell (NeuN positive); white, normal; green, TUNEL-positive; red, active caspase-3/AIF/PAR-positive concentrated around nucleus; yellow, TUNEL and active capase-3/AIF/PAR concentrated around nucleus; blue, TUNEL and active caspase-3 nuclear location. Scale bar, 20 \(\mu m\).
Accordingly, ethanol-induced DNA damage should activate DNA repair in neurons. Fig. 4A shows that short term ethanol exposure activated DNA repair, making the level of DNA repair activity higher than in control mice. Long term ethanol exposure significantly reduced the response, and the level of DNA repair activity was lower, compared with those after short term exposure. B, quantitative analysis of acetaldehyde-derived DNA lesions, $N^2$-ethyl-dG in DNA from brain tissue of mice chronically or acutely exposed to the Lieber-Decarli liquid diet with or without ethanol (5%), using LC/MS/MS. Note that $N^2$-ethyl-dG levels are significantly increased in DNA from mice after long term ethanol exposure, compared with mice after short term exposure and control (Con) mice. Values are means ± S.E.; *$p < 0.05$; **$p < 0.001$.

TABLE 1
Templates for qPCR-based BER activity assay

| (A) | Primer 1 | Probe 1 target |
|-----|----------|----------------|
| 5'-ATGGCGGGCCTCAGAAACATCATGAGTCAACTTCGCCGGAGCTCT-3' | 3'- (ddC) GAGGTCCAGGTCTGGAAGGCTGTGGGCAAGGTCATATA-5' |
| Forward S CGGGCCTCAGAAACAT | Probe 1: 5'- FAM -CAATTCCCGGACGTCTAAACCAAACCACTTTC-TAMRA -3' |
| Reverse AS ATGACCTTGCCCACAGCCT | |

Template A for BER activity assay

| (B) | Primer 2 | Probe 2 target |
|-----|----------|----------------|
| 5'-ATGGCGGGCCTCAGAAACATCATGAGTCAACTTCGCCGGAGCTCT-3' | 3'- (ddC) GAGGTCCAGGTCTGGAAGGCTGTGGGCAAGGTCATATA-5' |
| Forward S CGGGCCTCAGAAACAT | Probe 2: 5'- TET-CCGGGACCTGACTGACTTGTGAGGTCCAGGTCAAGGTCATATA-3' |
| Reverse AS ATGACCTTGCCCACAGCCT | |

Template B for the endogenous control

(43, 44). Accordingly, ethanol-induced DNA damage should activate DNA repair in neurons. Fig. 4A shows that short term ethanol exposure activated DNA repair, making the level of DNA repair activity higher than in control mice. Long term ethanol exposure significantly reduced the response, and the level of DNA repair activity was lower, compared with short term exposure. This is consistent with high levels of DNA damage in brains of mice after long term ethanol exposure (Figs. 2, A and B, and 4B). These data suggest that long term ethanol exposure disturbs DNA repair in the brain.

**Long Term Ethanol Consumption Affects OCM**—It is known that ethanol interferes with OCM (17, 45). We therefore determined OCM status in mice exposed to ethanol for short (4 days) and long (3 weeks) periods of time. We measured blood Hcy levels, a functional OCM marker using LC/MS/MS. Fig. 5 demonstrates that long term ethanol exposure induced elevation of blood Hcy levels. In contrast, short term ethanol exposure did not raise Hcy, suggesting that long term but not short term ethanol exposure affects OCM. Although OCM is a major source of methyl groups for cellular methylation reactions (15–17), global hypomethylation of DNA isolated from mouse cerebral cortex following long term alcohol exposure represents alcohol-induced OCM impairment in brain (Fig. 5B). Thus, long term but not short term ethanol exposure leads to OCM impairment seen from increased blood Hcy levels and a significant decrease in global DNA methylation in brain.

**OCM Impairment Mediates Alcohol-induced DNA Repair Dysfunction**—What can cause the reduction of DNA repair capacity and ensuing genomic instability in the conditions of long term ethanol exposure? Ethanol affects OCM (17, 45), which is critical for maintaining genomic stability (15–17). To define a role for OCM impairment of the effect of ethanol on
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DISCUSSION

Although ethanol is known to generate genotoxic metabolites, and the DNA damaging effect of ethanol was well studied in the context of carcinogenesis (6, 18, 19), it is unclear whether genotoxicity of ethanol can play a role in ethanol-induced brain damage. Our results show that ethanol generates both ROS- and acetaldehyde-derived DNA damage in the adult mouse brain. The role of DNA damage in alcohol effects in brain is supported by PARylation seen in brains of mice exposed to ethanol. PARylation is widely documented to play an essential role in DNA damage response, a fundamental genome surveillance mechanism that integrates DNA damage detection, DNA repair, and death of cells with irreparable DNA damage (40). Different degrees of PARylation ascertain a mode of cell death (37, 39). We demonstrated at least two types of neuron death associated with long term ethanol exposure, caspase-dependent apoptosis, and caspase-independent parthanatos. Parthanatos is PAR-dependent (36, 49). In addition, we found that in neurons of the cerebral cortex, PARylation coincided with TUNEL. Together, these findings suggest that ethanol-induced neuron death in adult brain is initiated by DNA damage, consistent with the role of DNA damage in neuron death and neurodegeneration in other circumstances (50). Neuron death has been related to unrepaired DNA damage and is associated with DNA repair deficiencies implicated in various diseases that feature progressive neurodegeneration (11, 51). Thus, DNA repair is critically important for neuron survival. Although endogenous DNA damage is constantly being produced in normal conditions, DNA repair dysfunction leads to accumulation of DNA damage. Induction of DNA damage and repression of repair of damaged DNA are considered to play the most important roles in genomic instability (52). Genomic instability encompasses alterations at the nucleotide level mainly due to faulty DNA repair and at the chromosome level. Thus, genomic instability usually results from an aberrant cellular response to DNA damage when the rate of DNA damage exceeds the DNA repair capacity of the cell. In the framework of alcohol abuse, genomic instability has been studied mainly in the context of cancer (6, 17, 19). Our results demonstrate that long term ethanol intake results in DNA repair dysfunction and neuron death in adult brain. This is consistent with previously

Genomic stability in the brain, we utilized MTHFR-deficient mice with heterozygous (+/−) disruption of the gene that represents a mild MTHFR deficiency in humans with 677C→T mutation in the gene encoding MTHFR and is common in human populations (46, 47). The mice were phenotypically normal but more sensitive to conditions affecting OCM (e.g., folate deficiency), exaggerating the effect of these conditions (48). If OCM is involved in chronic ethanol-induced genomic instability and affected DNA repair, MTHFR deficiency should modulate the effect of ethanol on DNA repair. Fig. 6A demonstrates that MTHFR deficiency, which exaggerates the effect of ethanol on OCM, as evidenced by a significantly higher growth of blood Hcy levels in response to ethanol exposure, also augmented the effect of ethanol on DNA repair activity in the brain (Fig. 6B). These data are the first to demonstrate that long term ethanol consumption can impact DNA repair (and genomic stability) in brain via OCM impairment. More evidence of OCM involvement in alcohol-induced DNA repair dysfunction is that in contrast to long term exposure, short term ethanol exposure, which does not raise Hcy levels (Fig. 5), facilitates more active response to DNA damage (higher activity of DNA repair) in the brain, which results in lower levels of DNA damage and neuron death (Figs. 3 and 4).

FIGURE 5. Chronic ethanol exposure leads to OCM impairment. A, blood Hcy levels in mice exposed to the Lieber-Decarli liquid diet during 3 weeks or 4 days were determined using LC/MS/MS. Note significant increase in blood Hcy levels in mice exposed to ethanol for 3 weeks but not for 4 days. B, global DNA methylation was determined by methylation quantification assay (Epigenetek kit) in DNA isolated from mouse brains after long term exposure to the Lieber-Decarli liquid diet. Note a significant reduction in DNA methylation in brains of ethanol-exposed mice. Values are means ± S.E. *p < 0.01, Con, control.

FIGURE 6. OCM impairment mediates alcohol-induced DNA repair dysfunction. A, blood Hcy levels in MTHFR and WT mice chronically exposed to the Lieber-Decarli liquid diet with or without ethanol (5%) were determined using LC/MS/MS. Note the effect of ethanol on Hcy levels in MTHFR, compared with WT mice. B, additional OCM impairment in MTHFR mice exaggerates the effect of ethanol on DNA repair activity. Note a significant decrease in DNA repair activity in MTHFR mice after long term ethanol exposure, compared with WT mice. *p < 0.001, Con, control.
shown relevance of DNA damage/DNA repair and genomic instability to the mechanisms of neurodegeneration (10, 50, 53). It is well known that long term alcohol exposure is associated with OCM dysfunction (17, 45). The phenotype of OCM impairment includes reduced tolerance to DNA-damaging agents and genomic instability (54, 55). This can be explained by the critical importance of OCM for biosynthesis of DNA precursors, essential for DNA repair, and as the major source of methyl groups for DNA methylation. Thus, ethanol-induced OCM dysfunction can result in genomic instability caused by DNA repair dysfunction due to a shortage of DNA precursors or by aberrant DNA methylation (15–17, 55). Genomic instability leads to a hypermutable cell, a central step for the induction of the cancer phenotype (10, 12, 13). Indeed, chronic alcohol abuse is associated with an increased risk of a variety of cancers (6, 19). *Mthfr* 677C→T mutation, common in the human population and associated with increased OCM impairment, is known to increase the risk of alcohol-induced cancer (46, 47). Thus, OCM impairment as a part of the mechanism involved in alcohol genotoxicity was mainly studied in the context of carcinogenesis (6, 17–19). However, the involvement of OCM impairment in the context of neurodegeneration due to alcohol exposure has not previously been shown. Our results demonstrate that short term ethanol exposure not associated with OCM impairment does not affect DNA repair. In addition, long term exposure results in neuronal DNA damage, DNA repair deficiency, initiation of neuronal cell death, and OCM impairment, although MTHFR deficiency, which exaggerates alcohol-induced OCM impairment, also impedes DNA repair. Therefore, this study is the first to indicate that OCM impairment plays an important role in alcohol-induced brain damage. Our results also suggest that under conditions of long term ethanol intake, *Mthfr* 677C→T mutation, common in the human population (46, 47), not only increases risk for cancer but also for neurodegeneration. Future work to study these effects will require characterization of particular pathways by which ethanol-induced OCM dysfunction impacts genomic instability.

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