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ORIGINAL RESEARCH COMMUNICATION

# Cockayne Syndrome B Protects Against Methamphetamine-Enhanced Oxidative DNA Damage in Murine Fetal Brain and Postnatal Neurodevelopmental Deficits

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## Abstract

Methamphetamine (METH) increases the oxidative DNA lesion 8-oxoguanine (8-oxoG) in fetal mouse brain, and causes postnatal motor coordination deficits after *in utero* exposure. Like oxoguanine glycosylase 1 (OGG1), the Cockayne syndrome B (CSB) protein is involved in the repair of oxidatively damaged DNA, although its function is unclear. Here we used CSB-deficient $Csb^{m/m}$ knockout mice to investigate the developmental role of DNA oxidation and CSB in METH-initiated neurodevelopmental deficits. METH (40 mg/kg intraperitoneally) administration to pregnant *Csb* females on gestational day 17 increased 8-oxoG levels in $Csb^{m/m}$ fetal brains ($p < 0.05$). CSB modulated 8-oxoG levels independent of OGG1 activity, as 8-oxoG incision activity in fetal nuclear extracts was identical in $Csb^{m/m}$ and $Csb^{+/+}$mice. This CSB effect was evident despite 7.1-fold higher OGG1 activity in $Csb^{+/+}$ mice compared to outbred CD-1 mice. Female $Csb^{m/m}$ offspring exposed *in utero* to METH exhibited motor coordination deficits postnatally ($p < 0.05$). *In utero* METH exposure did not cause dopaminergic nerve terminal degeneration, in contrast to adult exposures. This is the first evidence that CSB protects the fetus from xenobiotic-enhanced DNA oxidation and postnatal functional deficits, suggesting that oxidatively damaged DNA is developmentally pathogenic, and that fetal CSB activity may modulate the risk of reactive oxygen species-mediated adverse developmental outcomes. *Antioxid. Redox Signal*. 14, 747–756.

## Introduction

Methamphetamine (METH, "speed," "ice") is an addictive psychomotor stimulant that is neurotoxic in humans and adult animal models [reviewed in (7)]. Despite the widespread use of METH in women of childbearing age, little is known about the long-term effects of gestational exposure to this drug. In humans, *in utero* METH exposure of the fetus is associated with structural anomalies as well as postnatal functional deficits (10, 38).

It has been proposed that METH-initiated neurotoxicity occurs through the generation of reactive oxygen species (ROS) (6, 21) that can cause damage to cellular macromolecules, including DNA. Oxidative DNA damage, in particular, the lesion 8-oxoguanine (8-oxoG), may mispair with adenine, resulting in transversions mutations, or alternatively, alter the DNA binding of nuclear transcriptions factors and/or block RNA polymerase, resulting in altered or delayed transcription of proteins (17, 35, 40).

Previously, we have shown that CD-1 embryos and fetuses exposed to METH *in utero* have increased oxidative DNA damage in brain and liver, and exhibit postnatal motor coordination deficits (22). Unlike adult mice treated with METH, striatal dopaminergic nerve terminal degeneration is not detected in mice exposed to the drug *in utero*. Also, oxoguanine glycosylase 1 (*Ogg1*) knockout mice, which are deficient in base excision repair (BER) of 8-oxoG, are more susceptible to METH-enhanced oxidative DNA damage and postnatal neurodevelopmental deficits, indicating that oxidative DNA damage plays a role in the pathogenic mechanism of METH (52).

In addition to OGG1, repair of 8-oxoG may involve the Cockayne syndrome B (CSB) protein, which is deficient in 80% of Cockayne syndrome (CS) patients [reviewed in (28)]. CSB is involved in the transcription-coupled repair (TCR)

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subpathway of nucleotide excision repair, which removes UV-induced and other bulky helix distorting lesions from actively transcribed genes (48). Additional roles of CSB in DNA metabolism have been speculated to account for the greater severity of symptoms in CS patients compared to patients with xeroderma pigmentosum, the latter of whom often have combined defects in the TCR and global genome repair subpathways of nucleotide excision repair. Numerous studies in cell lines and mice deficient in CSB protein have observed an enhanced sensitivity to treatments that initiate oxidative DNA damage (13, 14, 42, 45). The precise role of CSB in the repair of oxidatively damaged DNA has not been elucidated, but may include its involvement in TCR. Spivak and Hanawalt concluded that little repair of 8-oxoG can occur within transcription complexes in plasmid sequences in CSB-deficient fibroblasts transfected with reporter plasmids containing randomly induced oxidative lesions. A role for CSB in the TCR of 8-oxo-G is also supported by the observation of a 40%–50% reduction in luciferase activity in $Csb^{m/m}$ knockout mouse embryo fibroblasts (MEFs) compared to wild-type MEFs when transfected with a luciferase reporter gene containing a single 8-oxo-G residue in the transcribed strand (35). CSB may also participate in the BER of 8-oxoG. Human cells expressing CSB mutations have a decreased capacity to repair 8-oxoG presumably due in part to a downregulation of expression of OGG1 mRNA (15, 44), and *CSB*-deficient cells accumulate more 8-oxoG than wild-type cells after exposure to gamma irradiation (45, 46).

We hypothesized that 8-oxoG is a pathogenic molecular lesion that causes neurodevelopmental deficits, and knockout mice deficient in the *Csb* gene are more susceptible to enhanced fetal oxidative DNA damage and neurodevelopmental deficits resulting from *in utero* exposure to xenobiotics like METH that enhance the fetal formation of ROS. Our results provide the first evidence that, in addition to OGG1, CSB protects the fetus from xenobiotic-enhanced DNA oxidation and postnatal functional deficits, suggesting that oxidatively damaged DNA is developmentally pathogenic, and that individual variations in fetal CSB activity may contribute to the risk of ROS-mediated adverse developmental outcomes.

## Materials and Methods

### Chemicals

8-oxo-2′-deoxyguanosine (8-oxo-dG) was obtained from Cayman Chemical Co., nuclease P1 and *Escherichia coli* alkaline phosphatase from Sigma-Aldrich, chloroform:isoamyl alcohol:phenol (24:1:25) from Life Technologies, Inc. and proteinase K from Roche Diagnostics. All other reagents used were of analytical or high-performance liquid chromatography (HPLC) grade.

### Drugs

Pure racemic (*d*/*l*)-METH was provided by the Healthy Environments and Consumer Safety Branch of Health Canada. The identity and absolute purity of (*d*/*l*)-METH was determined by diode array detection using a Bio-Rad REMEDi™ HS system and confirmed by liquid chromatography-mass spectrometry-mass spectrometry.

### Mice

The generation of a CSB-deficient knockout mouse model has been previously described (47). Heterozygous (+/*m*) females were housed overnight three to a cage with a +/*m* male breeder. The presence of a vaginal plug the next morning was designated as gestational day (GD) 1. Pregnant females were isolated and housed in plastic cages with ground corn-cob bedding (Beta Chip; Northeastern Products) and maintained in temperature-controlled rooms with a 12-h light-dark cycle. Food (Laboratory Rodent Chow 5001; Ralston Purina) and tap water were provided *ad libitum*. All animal studies were approved by the University of Toronto Animal Care Committee in accordance with the standards of the Canadian Council on Animal Care.

### Animal treatment

Drugs were dissolved with sterilized 0.9% saline, and the drug or its vehicle was injected intraperitoneally in a fixed volume of 0.1 ml/10 g body weight. Mice were administered a single dose of METH (40 mg/kg) or 0.9% saline (vehicle) on GD 17 at 10:00 h. For analysis of DNA oxidation and 8-oxoG repair activity, pregnant dams were sacrificed 4 h after drug treatment, the time of maximal oxidative DNA damage in fetal brain (22), and the fetal brain and liver were isolated. Isolated organs were subsequently rinsed in ice-cold 1.15% KCl solution, snap-frozen in liquid nitrogen, and stored at −80°C until sampling. Fetal tails were frozen for genotyping. For behavioral studies, dams were allowed to deliver spontaneously, and tail snips were obtained from the offspring for genotyping. For immunohistochemistry, brains were isolated and fixed in 10% formalin until further processing.

### Genotyping

DNA was isolated from fetal or adult tail snips using a standard DNA extraction kit (Sigma-Aldrich). Purified DNA (100–300 ng) was added to a PCR master mix containing 50 m*M* KCl, 10 m*M* Tris-HCl, pH 8.3, 2 m*M* $MgCl_2$, primers, 0.8 m*M* deoxynucleoside triphosphates, 0.02 U/l Taq polymerase and $ddH_2O$. The primers (0.43 $\mu M$) used to amplify the bands (195 bp for the wild-type allele [CSB6-CSB4] and 490 bp for the targeted allele [CSB6-CSB5]) were CSB4 (5′-GCTGCTTATAATAATCCTCATCTCC-3′), CSB5 (5′-ATCTG CGTGTTCGAATTCGCCAATG-3′), and CSB6 (5′-GTCTTCT GATGACGTTAGCTATGAG-3′). The final reaction volume was 45 $\mu$l. Samples were placed in a thermal cycler (Eppendorf Master Cycler® Gradient, Eppendorf Scientific Inc., Westbury, NY) and run under the following conditions: 94°C, 1 min; followed by 30 cycles of 94°C, 1 min; 58°C, 1.5 min; 72°C, 2 min, and a final extension at 94°C for 10 min. PCR samples were combined with a 10×gel loading buffer (0.24% bromophenol blue; 0.25% xylene cyanol and 15% Ficoll type 400 in $ddH_2O$) and loaded onto a 1.5% agarose gel prepared with 1×TRIS–acetic acid–ethylenediaminetetraacetic acid (EDTA) running buffer and ethidium bromide. The agarose gel was run at a constant 100 V for 1 h, viewed under UV light, and photographed.

### Analysis of METH-initiated DNA oxidation in CSB-deficient mice

Brain and liver were homogenized in 500 $\mu$l DNA digestion buffer (100 m*M* Tris-HCl, pH 8.0; 5 m*M* EDTA, pH 8.0; 0.2% sodium dodecyl sulfate; and 200 m*M* NaCl) and allowed to digest overnight with proteinase K (50 $\mu$g/ml) at 55°C. DNA

was extracted as described previously (29). Isolated DNA (RNA free) was digested to nucleotides by incubation with nuclease P1 and incubated with *E. coli* alkaline phosphatase, and the resulting deoxynucleoside mixture was filtered and analyzed by HPLC with electrochemical (EC) detection.

### *Detection of 8-oxo-dG*

Oxidation of 2′-deoxyguanosine to 8-oxo-dG was quantified using an isocratic Series 200 HPLC system (Perkin-Elmer Instruments LLC) equipped with a 5-$\mu$m Exsil 80A-ODS C-18 column (5 cm $\times$ 4.6 mm; Jones Chromatography, Ltd.), an EC detector (Coulochem® II), a guard cell (model 5020), an analytical cell (model 5010) (Coulochem, ESA, Inc.), and an integrator (Perkin-Elmer NCI 900 Interface). Samples were filtered (0.22 $\mu$m), injected into the HPLC-EC system, and eluted using a mobile phase consisting of 50 m*M* $KH_2PO_4$ buffer (pH 5.5)-methanol (95:5, v/v) at a flow rate of 0.8 ml/min with a detector oxidation potential of +0.4 V (50, 51). Chromatographs were analyzed using the TotalChrom chromatography software version 6.2.0 (Perkin-Elmer Instruments LLC).

### *Immunohistochemistry*

Brain sections (5 $\mu$m) were deparaffinized in xylene and ethanol, followed by a high temperature unmasking in 0.01 *M* sodium citrate buffer (pH 6.0) for 5 min. Tissue sections were blocked for 3 h with 3% bovine serum albumin, 20 m*M* $MgCl_2$, 0.3% Tween 20, and 5% goat serum in phosphate-buffered saline followed by an overnight incubation with the goat anti-rabbit tyrosine hydroxylase (TH) primary antibody (1:600; Chemicon International, Inc.). Sections were incubated with biotinylated goat anti-rabbit IgG reagent (1:200; Vector Laboratories) for 30 min at room temperature. To quench endogenous peroxidase activity, slides were incubated in 3% $H_2O_2$ in methanol for 30 min at room temperature. Detection was performed using the Vectastain Elite ABC Reagent kit and 3,3′-diaminobenzidine kit (Vector Laboratories).

### *Behavioral studies*

Mice were conditioned and trained on a constant-speed rotarod before performing the motor coordination test. Briefly, mice were required to perch on the stationary rod for 30 s to accustom themselves before being allowed to run with the rod rotating at a constant speed of 5 rpm for 90 s. Once conditioned, mice were tested at a constant speed of 20 rpm. The performance time and speed at which the mice fell from the rod were recorded at 6, 8, 10, and 12 weeks postnatally, and an average performance was calculated for each mouse.

### *8-oxoG repair activity*

8-oxoG repair activity was assayed by an oligonucleotide incision assay using as the substrate a double-stranded oligonucleotide containing a single 8-oxoG residue. Briefly, 100 nmol of a 49-mer oligonucleotide containing a single, internal 8-oxoG residue (5′-TAGACATTGCCATTCTCGA TA-8-oxoG-GATCCGGTCAAACCTAGACGAATTCCG-3′; [sequence from (24)] (Sigma-Genosys) was labeled at the 3′-terminus with biotin (Pierce Biotechnology, Inc.). The labeled oligonucleotide was annealed to a twofold molar excess of its complementary sequence (5′-CGAATTCGTCTAGGTTT GACCGGATCCTATCGAGAATGGCAATGTCTA-3′) with C opposite 8-oxoG, using an annealing buffer (0.1 *M* NaCl, 10 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA), by heating to 95°C for 5 min, and cooling to room temperature.

Nuclear extracts were prepared from adult or fetal (GD 17) brains and livers according to the small-scale procedure described by Lee *et al*. (27) with modifications. Briefly, 100 mg tissues samples were homogenized in 1 ml of buffer A (10 m*M* HEPES, pH 7.9, 1.5 m*M* $MgCl_2$, 10 m*M* KCl, and 0.5 m*M* DTT) and centrifuged at 1000 *g* for 10 min at 4°C. The pellet was resuspended in 140 $\mu$l of buffer C (20 m*M* HEPES, pH 7.9, 25% glycerol, 0.42 *M* NaCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* EDTA, 10 m*M* KCl, 0.5 m*M* DTT, and 0.5 m*M* PMSF) and mixed gently for 30 min at 4°C. The nuclear debris was pelletted by spinning for 10 min at 14,000 *g* and the supernatant dialyzed against buffer D (20 m*M* HEPES, pH 7.9, 20% (v/v) glycerol, 0.1 *M* KCl, 0.2 m*M* EDTA, 0.2 m*M* PMSF, and 0.5 m*M* DTT) for 4 h using Slide-A-lyzer® Mini Dialysis units (Pierce Biotechnology, Inc.). The protein concentration was determined by the bicinchoninic acid method (Pierce Biotechnology, Inc.), and the nuclear extracts were stored at −80°C before use.

Incision reactions (20 $\mu$l) contained 25 m*M* Tris-HCl (pH 7.6), 5 m*M* EDTA, 50 m*M* KCl, 500 fmol of biotin labeled oligonucleotide duplex, and 2.5–20 $\mu$g of nuclear protein (or purified formamidopyrimidine glycosylase enzyme [2 units; Trevigen]) as a positive control. The reactions were incubated for 1 h at 37°C and were terminated by the addition of 0.8 $\mu$l of both 5 mg/ml Proteinase K and 10% sodium dodecyl sulfate followed by incubation at 55°C for 15 min. DNA was ethanol-precipitated by the addition of 20 $\mu$g of glycogen and 5.9 $\mu$l of 7.5 *M* ammonium acetate and 60 $\mu$l of ethanol. The dried DNA pellet was resuspended in loading buffer (80% formamide, 50 m*M* Tris–borate, 1 m*M* EDTA, pH 8.0, 0.1% cyanol [w/v], and 0.1% bromophenol blue [w/v]) and resolved by denaturing electrophoresis in a 15% polyacrylamide (19:1) gel containing 8.3 *M* urea. After electrophoresis samples were transferred (80 V; 1 h) in 50 m*M* Tris-borate, pH 8.3, 1 m*M* EDTA buffer to a Biodyne B nylon membrane (Pall Life Sciences) using a Mini Trans-Blot® electrophoretic transfer cell (Bio-Rad). DNA was cross-linked to the membrane using a GS Gene Linker® UV chamber (Bio-Rad) set at program mode C-L (125 mJ). Biotin-labeled DNA was detected using a Phototope®-Star detection kit (New England Biolabs, Ltd.) according to the manufacturer's instructions.

### *Quantification of 8-oxoG incision activity data*

Gel images were captured under linear exposure conditions using an Alpha Innotech Fluorochem™ gel documentation system (Cell Biosciences). Band intensities were calculated using the 1D-multi lane densitometry tool in the AlphaEase® FC software package (version 6.0). The percentage of cleaved product was calculated by dividing the mean integrated density of the cleaved product band by the sum of the mean integrated densities of the cleaved product and substrate bands.

### *Statistical analysis*

Statistical significance of differences between paired data was determined by the two-tailed Student's *t*-test, whereas multiple comparisons among groups were analyzed by one-way analysis of variance with a subsequent Tukey's test

(GraphPad Prism® 3.02; GraphPad Software, Inc.). The level of significance was determined to be at $p < 0.05$.

## Results

### *Oxidative DNA damage is increased in METH-exposed GD 17 fetal brain in CSB-deficient mice*

In fetal brain, there were no significant differences in oxidative DNA damage among saline-exposed wild-type (+/+) CSB-normal, or +/*m* or homozygous (*m*/*m*) CSB-deficient fetuses (Fig. 1). However, with METH exposure, a 58% increase in 8-oxoG levels in *m*/*m versus* +/+ fetal brains was observed ($p < 0.05$). Oxidative DNA damage was also elevated in fetal brain in METH-exposed *m*/*m* fetuses compared to saline-exposed controls of the same genotype ($p = 0.0002$), and in +/m fetuses exposed to METH compared to saline-exposed fetuses of the same genotype ($p < 0.02$).

### *No increase in fetal liver DNA oxidation levels in METH-exposed CSB-deficient mice*

In fetal liver, *in utero* METH exposure did not cause an increase in 8-oxoG levels compared to saline-exposed controls (Fig. 2). Additionally, no significant differences in DNA oxidation were observed among saline-exposed +/+, +/*m*, or *m*/*m*- littermates, nor among METH-exposed fetuses of different *Csb* genotypes (Fig. 2).

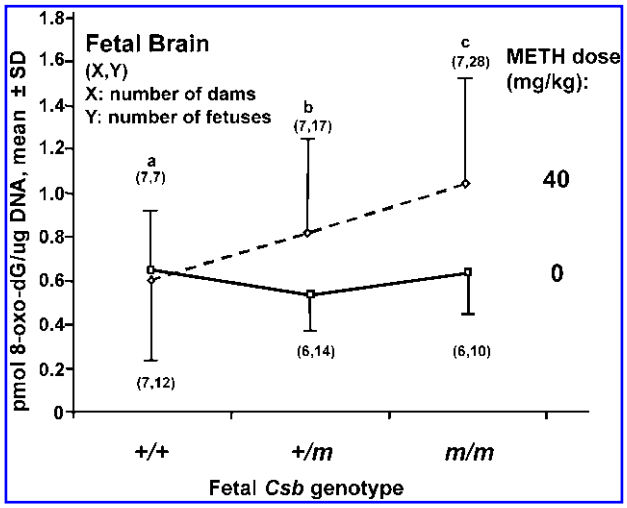


**FIG. 1. Increased DNA oxidation in Cockayne syndrome B (CSB)-deficient fetal brain after *in utero* methamphetamine (METH) exposure.** METH was dissolved in 0.9% saline and administered in a single dose (40 mg/kg intraperitoneally) to gestational day (GD) 17 pregnant $Csb^{+/m}$ dams mated with $Csb^{+/m}$ males. Control dams were treated with the saline vehicle. The dams were sacrificed 4 h after injection. Tissue was isolated from fetal brain and analyzed for oxidative DNA damage reflected by 8-oxoguanine (8-oxoG) formation. $^{a}p < 0.05$ indicates a difference between +/+ and *m*/*m* fetal brains exposed to METH. $^{b}p < 0.02$ indicates a difference between 40 mg/kg and the corresponding saline control in +/*m* fetal brains. $^{c}p = 0.0002$ indicates a difference between METH and the corresponding saline control in *m*/*m* fetal brains. (*X*,*Y*) indicates the number of dams (*X*) and fetuses (*Y*) analyzed.

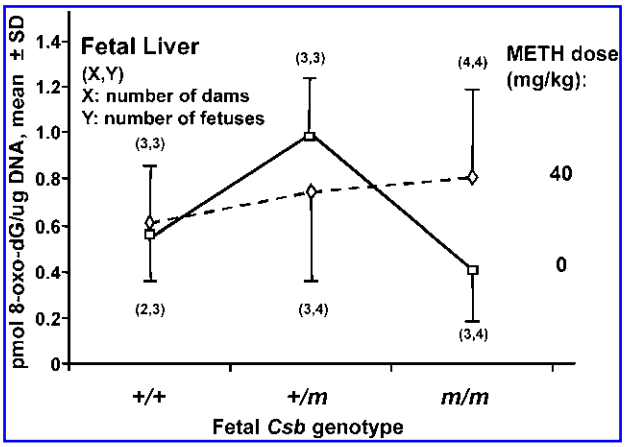


**FIG. 2. No increase in fetal liver oxidative DNA damage after *in utero* METH exposure.** Pregnant $Csb^{+/m}$ dams were treated on GD 17 as described in fig. 1, and 8-oxoG levels were analyzed in fetal liver 4 h later. No significant differences were observed in oxidative DNA damage among saline-exposed +/+, +/*m*, or *m*/*m* littermates, nor among METH-exposed fetuses of different *CSB* genotypes.

### *Enhanced motor coordination deficits in female CSB-deficient mice exposed in utero to METH*

Motor coordination deficits were observed in METH-exposed *m*/*m* female offspring compared to saline controls ($p < 0.05$). No significant differences were observed in METH-exposed +/*m* and +/+ female offspring compared to saline controls (Fig. 3). A similar trend for decreased rotarod performance was observed in CSB-deficient males exposed to METH, but the differences were not statistically significant (Fig. 3).

### *Absence of postnatal striatal dopaminergic nerve terminal degeneration in CSB-deficient offspring exposed in utero to METH*

Brain sections from 12-week-old mice that were exposed *in utero* to METH on GD 17 were stained for TH indicative of dopaminergic nerve terminals. Unlike in adult mice treated with METH (21), there was no evidence of postnatal degeneration of nerve terminals after METH exposure in CSB-deficient mice compared to wild-type littermates, nor compared to saline controls (Fig. 4).

### *8-oxoG incision activity*

Due to potential product inhibition at high rates of incision (53), we employed a substrate concentration that yielded 5%–10% product conversion to allow an adequate signal-to-noise ratio to accurately quantify the rate of 8-oxoG incision activity (36). We found that a 25 n*M* substrate concentration produced a linear increase in incision rate between nuclear extract amounts of 2.5 and 10 $\mu$g from fetal brain and liver in both $Csb^{+/+}$ and $Csb^{m/m}$ mice with saturation beginning at 20 $\mu$g of nuclear extract (Fig. 5A; data not shown). The same level of 8-oxoG incision activity was detected in 5 $\mu$g of nuclear extract from $Csb^{+/+}$ GD 17 fetal brain and liver with an incision rate of 7.9% (Figs. 5B and 6). No statistically significant difference in incision activity was

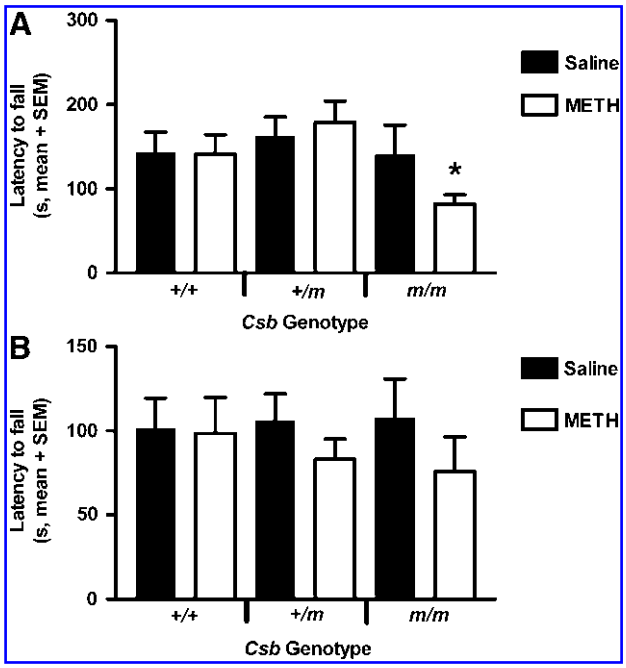


**FIG. 3. Motor coordination deficits in CSB-deficient offspring exposed *in utero* to METH.** Pregnant $Csb^{+/m}$ dams were treated on GD 17 as described in Figure 1, and motor coordination impairment was assessed in the offspring using the rotarod test at 6, 8, 10, and 12 weeks after birth. The average latency or time at which the mice fell from the rotating rod was calculated. $(x,y)$ indicates the number of dams $(x)$ and fetuses $(y)$ analyzed. **(A)** Females. $^*p < 0.05$ indicates a difference between METH exposed $m/m$ compared to the corresponding saline control. $+/+$[saline (14,16), METH (11,11)] $+/m$ [saline (15,20), METH (11,13)]; $m/m$ [saline (9,10), METH (10,10). **(B)** Males. $+/+$ [saline (11,13), METH (14,14)]; $+/m$ [saline (16,18), METH (12,21)]; $m/m$ [saline (10,10), METH (9,11).

observed in 5 $\mu$g of nuclear extract from $Csb^{m/m}$ GD 17 fetal brain and liver compared to $Csb^{+/+}$ extracts with relative incision activities of 7.3% and 7.0%, respectively (Figs. 5B and 6). We obtained similar results using 2.5 $\mu$g of nuclear extract. Incision rates for $Csb^{+/+}$ GD 17 fetal brain and liver were 4.2% and 4.1%, respectively, with incision rates of 3.9% and 3.7% being observed for GD 17 fetal brain and liver in $Csb^{m/m}$ mice.

Comparison of incision rates in GD 17 fetal brain nuclear extracts from $Csb^{+/+}$ mice and outbred CD-1 mice revealed 7.1-fold higher repair activity in $Csb^{+/+}$ mice (Fig. 7).

## Discussion

The results from this study provide the first evidence for the increased susceptibility of CSB-deficient knockout fetuses to oxidative DNA damage and postnatal neurodevelopmental deficits after *in utero* exposure to METH, indicating the importance of CSB-mediated repair in protecting the fetus from ROS-initiated oxidative DNA damage during development. These observations in CSB-deficient mice corroborate results from OGG1-deficient mice (52), indicating that oxidatively damaged DNA is developmentally pathogenic, and contributes to the mechanism of METH teratogenicity.

The increase in oxidative DNA damage, as well as susceptibility to postnatal motor coordination deficits, in CSB-deficient offspring exposed to METH compared to wild-type littermates was not as dramatic as in CD-1 outbred mice and *Ogg1* knockout mice (22, 52). To investigate the mechanism responsible for the relative resistance of the $Csb^{+/+}$ mice to METH-initiated 8-oxoG formation and subsequent postnatal motor coordination deficits, we compared 8-oxoG repair capacity in the fetal brain of the $Csb^{+/+}$ mice to that in the more sensitive CD-1 strain. This comparison revealed a dramatic 7.1-fold higher OGG1-dependent 8-oxoG incision activity in the $Csb^{+/+}$ mice. A previous report supported an OGG1-independent accessory role for CSB in 8-oxoG repair: $Ogg1^{-/-}$ knockout MEFs repaired 8-oxoG faster than $Csb^{m/m}/Ogg1^{-/-}$ double knockout cells, but cells from $Csb^{m/m}$ mice did not accumulate 8-oxoG at significantly higher rates than wild types with age, in contrast to cells from $Ogg1^{-/-}$ mice (34). Thus, the less dramatic increases in 8-oxoG observed in this study of $Csb^{m/m}$ mice, compared with our previous studies in CD-1 and $Ogg1^{-/-}$ mice (22, 52), may be accounted for by the substantially higher intrinsic OGG1-dependent 8-oxoG repair activity in the *Csb* strain, together with the secondary independent role that CSB plays in 8-oxoG repair. The enhanced susceptibility to oxidative DNA damage in METH-exposed fetal brains from $Csb^{m/m}$ mice was not observed in fetal liver, and may reflect tissue-specific differences in CSB function, ROS formation and/or activity of antioxidative enzymes. In regard to ROS formation, a contributing factor could be greater ROS formation in the brain secondary to monoamine metabolism. In addition, we have shown that prostaglandin H synthase, which is constitutively expressed in brain but not liver, can bioactivate METH to free radical intermediates capable of producing ROS-initiated formation of 8-oxoG in adult brain (21).

The importance of functional CSB in the developing brain is reflected by the characteristics of CS patients, which include mental growth retardation, microcephaly, progressive neurological and retinal degeneration, and delayed psychomotor development (32). Further, neuropathological examinations have revealed various changes in the CNS indicative of neurodegeneration (5, 18, 20, 25). Enhanced oxidative stress caused by xenobiotic exposure combined with CSB deficiency may result in more severe or additional developmental consequences in embryos or fetuses. However, there was no evidence of increased *in utero* death of $+/m$ or $m/m$ fetuses with or without METH exposure.

A potential gender difference was evident. Although male METH-exposed CSB-deficient offspring exhibited a trend for decreased rotarod performance, the differences were not significant, unlike with the METH-exposed CSB-deficient female offspring. This may reflect a reduced sensitivity of this measure in male mice as their basal performance is approximately two-thirds that of female mice. The enhanced susceptibility of female mice maybe due to disruption of hormonally dependent pathways. Recent evidence suggests that transcription of estrogen responsive genes is regulated by a controlled DNA damage and repair response (37), and it is possible that female mice are more susceptible due to 8-oxoG dysregulation of this hormone-dependent signaling pathway. We have observed a similar gender difference for decreased rotarod performance in offspring exposed *in utero* to METH in some (52), but not other (22), strains of mice.

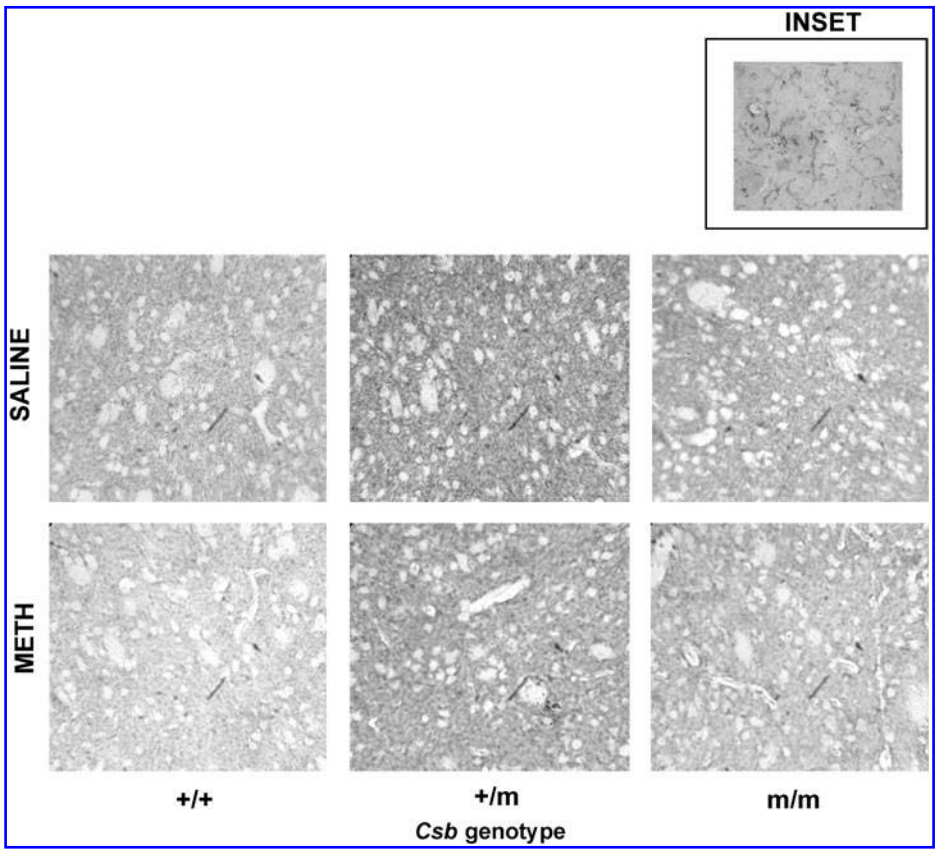


**FIG. 4. Prenatal METH exposure does not cause postnatal degeneration of dopaminergic nerve terminals in the striatum of offspring.** Pregnant $Csb^{+/m}$ dams were treated on GD 17 as described in Figure 1, and the progeny were sacrificed at 12 weeks of age. Brain sections were stained for tyrosine hydroxylase (TH) indicative of dopaminergic nerve terminals. Immunohistochemical staining is representative of $n = 4$/treatment group at 400× magnification. (*Inset*) Positive control: reduced TH staining of adult CD-1 mice treated 1 week previously with 40 mg/kg of METH.

In BER, CSB may regulate OGG1 expression, as a decreased ability to repair 8-oxoG in CS-B cells is associated with a downregulation of human *OGG1* mRNA and protein levels (15, 44). Transfection of the cells with the wild-type *CSB* gene increases *OGG1* mRNA levels by 50% (15), suggesting that CSB stimulates the transcription of the *OGG1* gene. Comparable rates of 8-oxoG incision activity were observed in GD 17 fetal brain and liver nuclear extracts from $Csb^{m/m}$ and $Csb^{+/+}$ mice, suggesting that the transcriptional regulation of *OGG1* observed in CSB-deficient human fibroblasts transfected with wild-type *CSB* (15, 44) does not occur in murine fetal brain and liver. These observations are consistent with the findings in whole cell extracts from $Csb^{m/m}$ and $Csb^{+/+}$ MEFs where no differences in 8-oxoG incision activity and Ogg1 expression were observed (34).

There is evidence for a novel role of the CSB protein in the repair of 8-oxoG that is independent of both Ogg1-dependent BER and transcription of genes (34). Cells from tissues of $Ogg1^{-/-}/Csb^{m/m}$ mice display higher steady-state levels and accumulation with age of oxidative DNA lesions than *Ogg1* knockout mice. Further, 60% of oxidative lesions induced by a photosensitizer are repaired after 16 h in OGG1-deficient MEFs, whereas only 20% are repaired in $Ogg1^{-/-}/Csb^{m/m}$ MEFs, indicating that the predominant role of CSB in the repair of 8-oxoG is independent of OGG1, and that CSB plays an important role in the repair of 8-oxoG in the absence of OGG1. The mechanism how CSB participates in this repair pathway has not been elucidated. Within the nucleus, CSB may cooperate with poly(ADP-ribose) polymerase-1 (PARP-1) to stimulate the later steps of BER, as has recently been suggested (43). Alternatively, CSB may remodel chromatin to allow access of DNA repair proteins to the site of the damage (12, 33). The findings of comparable 8-oxoG incision activity in fetal brain and liver nuclear extracts from $Csb^{+/+}$and $Csb^{m/m}$ mice, combined with the observations of METH-dependent increases in 8-oxoG in $Csb^{m/m}$ mice, suggest that CSB is not involved in the Ogg1-dependent incision step of 8-oxoG repair during fetal development and instead may act in an OGG1- and transcription-independent repair pathway.

CSB may also function in the repair of oxidatively damaged DNA in actively transcribed genes, as evidenced by the observations of a 40%–50% reduction in luciferase activity in $Csb^{m/m}$ MEFs compared to $Csb^{+/+}$ MEFs when transfected with a luciferase reporter gene containing a single 8-oxo-G residue in the transcribed strand (35). Although the contribution of a TCR pathway to the overall repair of oxidatively damaged DNA is estimated to be low since it only repairs DNA on the transcribed sequence, which constitutes <5% of the genome, the consequences of unrepaired oxidatively damaged DNA in the transcribed genome could alter expression of key developmental proteins through alterations in gene expression. Unrepaired 8-oxoG could potentially block transcription by RNA polymerase II (49), or lead to transcriptional mutagenesis by 8-oxoG bypass (11). Experimental discrepancies in the literature regarding the ability of 8-oxoG to block transcription may be explained by recent observations that the repair level and/or the transcriptional arrest caused by a unique 8-oxoG lesion varies according to the promoter strength and nucleotidic sequence surrounding the lesion (35). CSB also may protect in part through TCR repair of other unique ROS-initiated DNA lesions, including lipid peroxidation products adducted to DNA (30), and

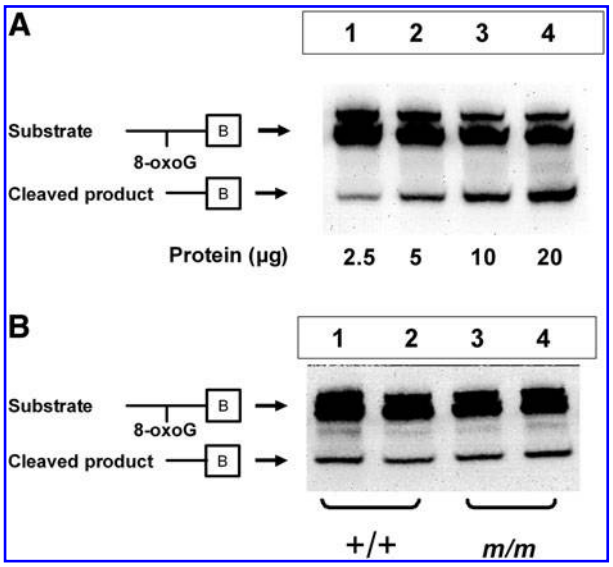


**FIG. 5. Endogenous 8-oxoG repair activity in fetal brain nuclear extracts from CSB mice. (A)** About 2.5–20 $\mu$g of pooled fetal brain nuclear extract (lanes 1–4) from three $Csb^{+/+}$ mice was incubated with 25 n*M* of a biotin-labeled oligonucleotide containing 8-oxoG that was annealed to its complementary strand containing C opposite 8-oxoG. Reaction products were resolved by 15% denaturing polyacrylamide gel electrophoresis. **(B)** Lanes 1 and 2: 25 n*M* oligonucleotide incubated with 5 $\mu$g of nuclear extract from GD 17 brain from $Csb^{+/+}$ fetuses from different litters. Lanes 3 and 4: 25 n*M* oligonucleotide incubated with 5 $\mu$g of nuclear extract from GD 17 brain from $Csb^{m/m}$ fetuses from different litters.

oxidized DNA lesions other than 8-oxoG, including 8,5′-cyclopurine-2′-deoxynucleosides (23) and ring-opened formamidopyrimidine lesions (19).

After oxidative stress, CS-B cells display a general deficiency in transcription, suggesting that CSB influences the

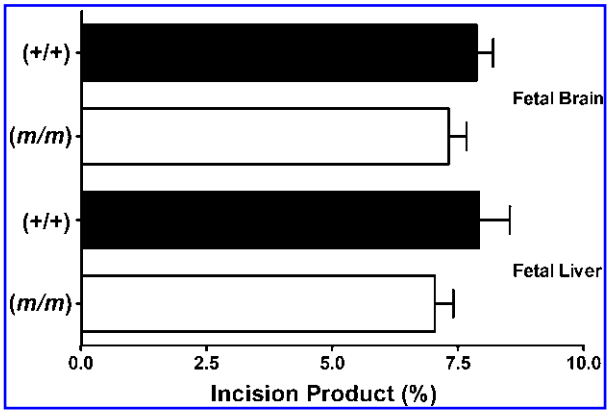


**FIG. 6. Densitometric analysis of endogenous fetal brain and liver 8-oxoG repair activity in nuclear extracts from $Csb^{+/+}$ and $Csb^{m/m}$ mice.** Extracts were incubated with a biotin-labeled oligonucleotide containing 8-oxoG that was annealed to its complementary strand containing C opposite 8-oxoG. 25 n*M* oligonucleotide incubated with 5 $\mu$g of nuclear extract from GD 17 brain or liver ($n = 8$; mean ± SD). Fetuses were selected randomly and evenly from six different litters.

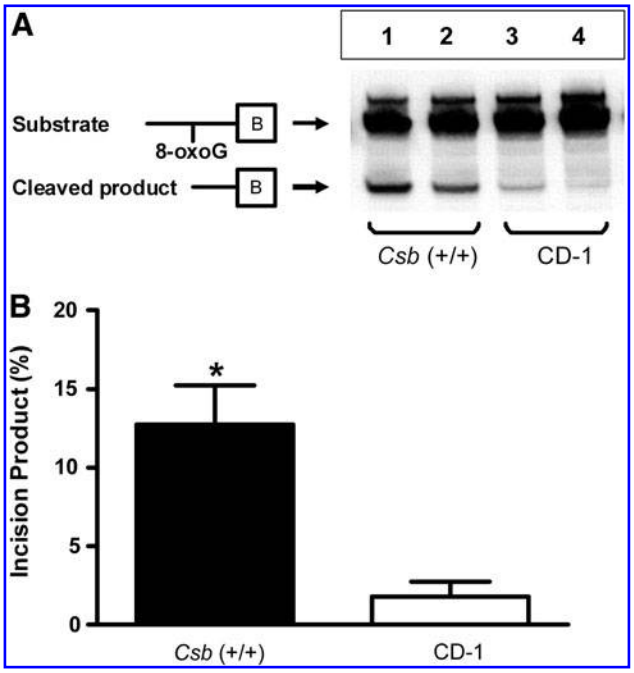


**FIG. 7. Endogenous 8-oxoG repair activity in fetal brain nuclear extracts from $Csb^{+/+}$ and CD-1 mice. (A)** A 10 $\mu$g aliquot of pooled fetal brain nuclear extract from $Csb^{+/+}$ mice (lanes 1 and 2) and CD-1 mice (lanes 3 and 4) was incubated with 25 n*M* of a biotin-labeled oligonucleotide substrate containing 8-oxoG that was annealed to its complementary strand containing C opposite 8-oxoG. Reaction products were resolved by 15% denaturing polyacrylamide gel electrophoresis. **(B)** Densitometric analysis of endogenous fetal brain 8-oxoG repair activity in nuclear extracts from $Csb^{+/+}$ and CD-1 mice. The oligonucleotide substrate (25 n*M*) was incubated with 10 $\mu$g of nuclear extract from GD 17 brain ($n = 4$; mean ± SD). *A difference from CD-1 mice ($p < 0.05$).

regulation of transcription of certain genes, including those involved in stress response, DNA repair, cell cycle arrest, signal transduction, and ribosomal functions (26). Similarly, defective transcription in $Csb^{m/m}$ mice after oxidative stress caused by *in utero* METH exposure may alter expression of key proteins required for normal brain development.

Previously, we have shown that mouse embryos and fetuses exposed to METH *in utero* have increased oxidative DNA damage in brain, and exhibit postnatal motor coordination deficits (22, 52). Unlike adult mice treated with METH (7, 21), striatal dopaminergic nerve terminal degeneration and METH-dependent apoptotic cell death is not detected in mice exposed to the drug *in utero* (22, 52). DNA repair genes, including members of the BER pathway, are upregulated in adult mice after METH administration, suggesting that increased repair activity may counteract METH-initiated oxidative DNA damage (8). In addition, the observation that more DNA repair genes are upregulated in wild-type mice than in c-fos-deficient adult mice, which are more susceptible to the toxic effects of METH, suggests that the inability to trigger DNA repair responses contributes to the increased sensitivity of these mice to the drug. Enhanced 8-oxoG BER activity in fetal tissues compared to those of adults (16, 52) may contribute to the absence of METH-initiated apoptotic changes or dopaminergic nerve terminal degeneration in fetal

brain. Other contributing factors to the differential fetal *versus* adult response may include differences in neurotransmitter levels and thermoregulatory responses. Mice are born with around 20% of the dopamine that is observed in adult brain (1). Early studies indicated that developing mice are more resistant to lethal doses of amphetamine than adults (2). More recent work in rats has shown that weanling rats are resistant to losses of TH-immunoreactivity and dopamine depletion in the caudate-putamen after METH (9, 39) or amphetamine (3) treatment. This observed resistance maybe due in part to a blunted hyperthermic response in young rats as the severity of METH-induced neurotoxicity correlates with the degree of hyperthermia induced by treatment in adult rats, with sustained elevations in core temperature between 39.5°C and 41°C required to observe dopaminergic nerve terminal degeneration in the striatum (4). The studies of Cappon *et al*. (9) point out a developmental distinction between METH response and hyperthermia between postnatal day (PND) 20 and 40 rats. Both PND 20 and 40 rats are resistant to METH-induced hyperthermia, neostriatal dopamine depletion, and elevations in glial fibrillary acidic protein content when dosed at an ambient temperature of 22°C. However, raising the ambient temperature to 30°C restores an adult pattern of response in PND 40 rats, but not PND 20 rats despite a comparable hyperthermic response in PND 20 rats. It is possible that pregnancy itself affords some protection to the developing fetus against METH-neurotoxicity through altered thermoregulatory responses, given reports of neuroimmune hyporesponsiveness in late gestation (31, 41). The postnatal neurodevelopmental deficits observed herein in CSB-deficient females after *in utero* exposure to METH, in the absence of dopaminergic nerve terminal damage observed after adult exposure (21), is consistent with our results in other mouse strains (22, 52). These results suggest that the neurodevelopmental deficits after *in utero* exposure may result from altered formation and/or integration of neural structures, rather than the degenerative process observed after adult exposures, although contributions from nondopaminergic degenerative processes cannot be excluded.

In summary, the results from this study are the first to demonstrate that CSB contributes to the protection of fetus against METH-initiated toxicity. The magnitude of CSB-mediated developmental protection appears to be considerably less than that provided by fetal OGG1 (52). Interindividual variability in the various components of repair of oxidative DNA damage may contribute to the risk of neurodevelopmental deficits and other anomalies after exposure to METH and possibly other ROS-initiating teratogens. These findings also support our studies with OGG1-deficient mice in providing the most direct evidence to date that oxidatively damaged DNA is pathogenic and contributes to the mechanism of METH-initiated neurodevelopmental deficits, and perhaps other embryopathic effects of ROS-initiating teratogens.

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## Author Disclosure Statement

No competing financial interests exist.

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**Abbreviations Used**

8-oxo-dG = 8-oxo-2′-deoxyguanosine
8-oxoG = 8-oxoguanine
BER = base excision repair
CS = Cockayne syndrome
CSB = Cockayne syndrome B
EC = electrochemical
EDTA = ethylenediaminetetraacetic acid
GD = gestational day
MEFs = mouse embryo fibroblasts
METH = methamphetamine
OGG1 = oxoguanine glycosylase 1
PND = postnatal day
ROS = reactive oxygen species
TCR = transcription-coupled repair
TH = tyrosine hydroxylase

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