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DNA Repair Modulates The Vulnerability of The Developing Brain to Alkylating Agents

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Abstract

Neurons of the developing brain are especially vulnerable to environmental agents that damage DNA (i.e., genotoxins), but the mechanism is poorly understood. The focus of the present study is to demonstrate that DNA damage plays a key role in disrupting neurodevelopment. To examine this hypothesis, we compared the cytotoxic and DNA damaging properties of the methylating agents methylazoxymethanol (MAM) and dimethyl sulfate (DMS) and the mono- and bifunctional alkylating agents chloroethylamine (CEA) and nitrogen mustard (HN2), in granule cell neurons derived from the cerebellum of neonatal wild type mice and three transgenic DNA repair strains. Wild type cerebellar neurons were significantly more sensitive to the alkylating agents DMS and HN2 than neuronal cultures treated with MAM or the half-mustard CEA. Parallel studies with neuronal cultures from mice deficient in alkylguanine DNA glycosylase (Aag-/-) or O6-methylguanine methyltransferase (Mgmt-/-), revealed significant differences in the sensitivity of neurons to all four genotoxicants. Mgmt-/- neurons were more sensitive to MAM and HN2 than the other genotoxicants and wild type neurons treated with either alkylating agent. In contrast, Aag-/- neurons were for the most part significantly less sensitive than wild type or Mgmt-/- neurons to MAM and HN2. Aag-/- neurons were also significantly less sensitive than wild type neurons treated with either DMS or CEA. Granule cell development and motor function were also more severely disturbed by MAM and HN2 in Mgmt-/- mice than in comparably treated wild type mice. In contrast, cerebellar development and motor function were well preserved in MAM treated Aag-/- or MGMT overexpressing (MgmtTg+) mice, even as compared with wild type mice suggesting that AAG protein increases MAM toxicity, whereas MGMT protein decreases toxicity. Surprisingly, neuronal development and motor function were severely disturbed in MgmtTg+ mice treated with HN2. Collectively, these in vitro and in vivo studies demonstrate that the type of DNA lesion and the efficiency of DNA repair are two important factors that determine the vulnerability of the developing brain to long-term injury by a genotoxicant.

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Introduction

DNA damage occurs in children and newborns after exposure to environmental pollutants [1] or chemotherapeutic agents [2,3]. A consistent finding is frequent association between the level of DNA damage, impaired growth during the prenatal or postnatal period [4,5], and disruption of neuronal and cognitive development [6-8]. Since most neurodevelopmental disorders are categorized as migrational disorders [9], environmental agents that preferentially target DNA of migrating post-mitotic neurons would be expected to interfere with key steps involved in laying down the final cytoarchitecture of the mature brain. Consistent with this hypothesis, DNA damage and perturbation of developmentally regulated genes occur well before neurodevelopmental changes induced by the genotoxicant methylazoxymethanol (MAM) in neonatal animals [10]. Moreover, the vulnerability of immature neurons to individual genotoxicants correlates with the accumulation of specific DNA lesions and distinct alterations in gene expression [11]. While DNA damage is a characteristic feature of certain neurodevelopmental disorders [12] and neurological diseases [13-15], our understanding of how genotoxicants contribute to these conditions is poorly understood. Since the brain is especially inefficient at repairing DNA damage [16-18] the preferential accumulation and persistence of DNA lesions in immature neurons may be key factors to explain the increased vulnerability of the developing brain to genotoxicants.

Alkylating agents are an important class of genotoxicants because they occur in the environment (e.g., tobacco specific nitrosamines) and are used as cytotoxic drugs in cancer chemotherapy; DNA lesions produced by this class of genotoxicants play an important role in their mutagenic, carcinogenic, and cytotoxic properties [20]. Exposure of mammalian cells to alkylating agents activates important defensive pathways by inducing multiple proteins involved in DNA repair, cell cycle checkpoint control and potentially apoptosis [21]. These cellular processes are important for recognizing and repairing the DNA damage to maintain genomic stability and prevent cell death or mutations. While there is ample evidence that the DNA lesions formed by alkylating agents are processed by distinct repair pathways in somatic cells or tissues [21,22], relatively little information exists showing how proliferating or post-mitotic neurons in the developing brain respond to these DNA lesions [23]. Since a majority of the cells in the developing brain are post-mitotic, the persistence of DNA lesions in these cells could disrupt key developmental programs that are required to achieve the mature adult brain. However, little evidence exists to show that DNA lesions are important initiators of neuronal injury or that they disrupt brain function. Therefore, major questions that have yet to be answered include: 1) are certain DNA lesions more toxic to neurons than others, 2) how do neurons process different types of DNA lesions, and 3) do neurons require one or multiple pathways to repair DNA lesions? Answers to these questions are likely to advance our understanding not only about how the developing brain responds to genotoxicant insult, but whether DNA repair plays a critical role in protecting the immature brain from long-term brain injury (e.g., neurodevelopmental disorders).

The development of ‘knock-out’ and transgenic mice for DNA repair [24] provides a novel approach for assessing the influence of different types of DNA lesions on brain development. This approach was used in the present study to examine the influence of DNA lesions on brain development by comparing the sensitivity of neurons from the cerebellum of neonatal mice that are deficient in base-excision repair (BER) (i.e., alkyladenine alkyltransferase, Aag^−/−) and O6-methylguanine methyltransferase (Mgmt^−/−), or that overexpress MGMT (Mgmt^Tg^+/+), to the developmental neurotoxins MAM and HN2. The complex responses of neurons from these mice to alkylating agents demonstrates that the vulnerability of the developing brain to a particular genotoxicant depends not only upon the type of DNA lesion formed, but also the inherent DNA repair capacity.
Materials and Methods

Animals

Alkyladenine DNA glycosylase knock-out (Aag\(^{-/-}\)) mice were generated by inserting a neo expression cassette into exon 2 of the mouse Aag gene using ES cell techniques and the intercrossing of heterozygous (Aag\(^{+/+}\)) mice [25]. Aag\(^{-/-}\) mice were generated from intercrosses of Aag heterozygous mice (\(+/\)) and litters from the intercrossing of Aag\(^{-/-}\) mice used to prepare neuronal cell cultures. Mgmt knock-out (Mgmt\(^{-/-}\)) mice were generated by inserting a pSL-PGH-neo cassette into pBS containing exon 5 of the mouse Mgmt gene (pMT42-ΔE5-NEO-TK) using ES cell techniques and the intercrossing of heterozygous (Mgmt\(^{-/-}\)) mice [26]. Both Aag\(^{-/-}\) and Mgmt\(^{-/-}\) mice were backcrossed for >12 generations onto the C57Bl/6J background. Mgmt transgenic (Mgmt\(^{Tg}\)) mice were generated in C57BL/6J × SJL mice using a chimeric gene consisting of the chicken \(\gamma\)-actin promoter, the human Mgmt cDNA, the poly A region from bovine growth hormone and the locus control region from the human CD2 gene [27]. All neonates from homozygous intercrosses were genotyped after each cell culture experiment.

Neuronal Cell Cultures

Primary neuronal cultures were prepared from the cerebral cortex and midbrain of gestational day 14 C57BL/6J mice or the cerebella of 6-8-day old neonatal C57BL/6J (wild type) mice, Aag\(^{-/-}\), Mgmt\(^{-/-}\) or Mgmt\(^{Tg}\) mice by placing the tissue in ice-cold Hibernate/B27 cell culture media (GibcoBRL) and dissociating the tissue in BSS with 0.1% trypsin as previously described by Brewer et al. [28], Kisby et al.[29] and Meira et al. [30]. Cortical, midbrain or cerebellar granule cell cultures were prepared by diluting the cell pellet with plating media (Neurobasal™ media with 0.5 mM glutamine, 10% FCS, 10% HS, 2% B27 supplement) containing 5 mM (low) or 25 mM (high) potassium and seeded at a density of 0.65-0.75 × 10\(^5\) cells/well of a 48-well plate coated with poly-D-lysine (BioCoat™, BD Biosciences). Neurons were allowed to attach for 2h before replacing the plating media with low or high potassium containing maintenance media (Neurobasal™ media with 0.5 mM glutamine, 2% B27 supplement). Neuronal cell cultures were fed weekly by adding fresh culture media to the wells and the cells maintained for 7 days before treatment with 10 \(\mu\)M to 2000 \(\mu\)M methylazoxymethanol (MAM) or dimethyl sulfate (DMS) and 0.1-20 \(\mu\)M mechlorethamine (nitrogen mustard or HN2) or 2-chloroethylamine (CEA).

Cell Viability

Redox Function—Alamar blue™ (Trek Diagnostic Systems, Inc.) is a non-toxic metabolic indicator of viable cells that becomes fluorescent upon mitochondrial reduction and has been widely used to measure mitochondrial function in different cell culture systems [31,32]. Mitochondrial function was determined in genotoxicant treated neuronal cultures by adding Alamar Blue™ to a final concentration of 10% and the cells incubated at 37°C in a humidified 5%CO\(_2\)/O\(_2\) incubator for 4h. Viability was measured when the medium in control wells turned blue to pink, typically at ~4h. Alamar blue™ fluorescence was measured in a Gemini-XS™ (Molecular Devices) automated plate-reading fluorometer, with excitation at 530 nm and emission at 590 nm. Values are reported as % redox activity of controls.

Live/Dead Assay—Murine neuronal cultures treated with control media or media supplemented with various concentrations of genotoxicants were examined by fluorescence microscopy for cell viability using the fluorochromes propidium iodide (PI) and calcein-AM as previously described by Kisby et al. [29,33] and Meira et al. [30]. Briefly, the media over control, genotoxicant-treated cultures was removed, replaced with control media containing 3.0 \(\mu\)M PI (a marker of cell damage) and 0.26 \(\mu\)M calcein-AM (a marker of cell viability), and the cultures treated for 10 min in a humidified 5%CO\(_2\)/O\(_2\) incubator. The fluorochrome containing media was aspirated, the cultures washed once with control media and cell survival...
examined on a fluorescence microplate reader (GeminiXS™, Molecular Devices) with well scanning capabilities. Values were expressed as the mean % surviving cells of controls ± SEM (n= 6/treatment group × 3-5 separate experiments).

**TUNEL labeling**

Primary cerebellar neuronal cultures treated for 24h with MAM (10 μM, 100 μM, 1000 μM) or HN2 (1.0 μM, 5.0 μM, 10 μM) were examined for DNA damage using the NeuroTacs™ kit (Trevigen, Inc.). After toxin treatment, cells (on 8-well chamber slides) were fixed with 4% buffered paraformaldehyde and the incorporation of biotinylated nucleotides determined by incubating the cells with NovaRed™ (Vector Labs, Inc.) according to the manufacturer’s protocols. The cells were washed, lightly counterstained with methyl green (Vector Labs, Inc.), mounted and the cells examined by light microscopy on a Zeiss Axioskop 2 microscope with digital imaging software (i.e., AxioVision 3.0) [33]. For quantitative studies, 5 random fields (~200-500 cells/field of cells with prominent nuclei) were counted and the values expressed as the mean ± SEM of immunopositive cells (TUNEL+).

**In vivo studies**

Neonatal C57BL/6J or DNA repair mutant (i.e., Aag−/−, Mgmt−/−, MgmtTg+−) mice (postnatal day 3, PND3; n=3/timepoint) were administered a single mid-scapular subcutaneous injection of MAM (325 μmol/kg), HN2 (13 μmol/kg), DMS (325 μmol/kg) or CEA (13 μmol/kg). When the animals reached PND22, they were euthanized and each animal perfused for histological examination of the brain. A separate set of mice were injected with either saline (control) or DMSO.

**Histology**

Saline or genotoxicant treated C57BL/6J or DNA repair mutant mice were perfused with 4% buffered paraformaldehyde, the brain cryoprotected in sucrose (10-30%) and the tissue rapidly frozen in Tissue-Tek™ as previously described by Kisby et al. [10]. Sagittal brain tissue sections (10 μm) were made with a cryostat through one half of the cerebellum of saline or genotoxicant treated mice (n=3/treatment) and the corresponding sections of each treatment placed on a glass slide. The sections were air-dried overnight and the next day stained with cresyl violet. Every tenth section was examined for morphology (cresyl violet staining) by light microscopy.

**Statistical Analysis**

Data are expressed as the mean ± S.E.M. All data obtained were evaluated for statistical significance by one-way analysis of variance (ANOVA) and Scheffe’s method of comparison (StatView™). A probability value of p < 0.05 was considered significant unless otherwise noted.

**Results**

**Viability of Genotoxicant Treated Neurons**

The objective of the present study was to determine if alkylating agents induce their long-term effects on the developing brain by selectively targeting neurons through a DNA damage mechanism. To test this hypothesis, we compared the sensitivity of neuronal cultures prepared from the cerebellum of wild type neonatal mice with mice deficient in the repair of O6-methylguanine DNA lesions (Mgmt−/−) or N7-methyl- or N7-alkylguanine DNA lesions (Aag−/−) after treatment with methylating agents (i.e., MAM, DMS) and monofunctional and bifunctional chloroethylating agents (CEA, HN2) (Figure 1). MAM methylates DNA to produce the DNA lesions N7-methylguanine (N7-mG) and O6-methylguanine (O6-mG) [34],

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whereas HN2 rapidly and irreversibly alkylates guanine and adenine (e.g., N7-alkylG and 3-alkylA, respectively) of DNA to produce monoadducts, intrastrand crosslinks and interstrand cross-links [35]. Dimethyl sulfate (DMS) is a methylating agent that produces primarily N7-mG and 3-methyladenine (3-mA) DNA lesions while chloroethylamine (CEA), the monofunctional analogue of HN2, produces predominantly N7-alkylG DNA lesions, but not cross-links [36]. Since the greatest number of neurons (i.e., granule cells) are produced in the cerebellum during neonatal development and neurons from this brain region in wild type mice showed the greatest sensitivity to MAM or HN2 (see Supplement Figure 1), granule cell cultures were used as the *in vitro* model to test cytotoxicity in the different DNA repair backgrounds. The increased sensitivity of wild type granule cells to MAM or HN2 is likely due to the accumulation of unrepaired DNA lesions (i.e., O6-mG, N7-mG, N7-alkylG, 3-alkylA or cross-links).

To clarify the relationship between the DNA lesions formed by the genotoxicants in Fig. 1 and the sensitivity of immature cerebellar neurons to alkylating agents, we compared the sensitivity of cerebellar neuronal cultures derived from wild type, *Aag*-/-, and *Mgmt*-/- mice to MAM, DMS, HN2 and CEA (Figure 2). DMS was more toxic than MAM to wild type cerebellar neurons, whereas HN2 was more toxic to cerebellar neurons than CEA (Fig. 2). The greater sensitivity of cerebellar neurons to DMS and HN2 is likely due to the formation of DNA lesions not produced by MAM or CEA.

Neuronal cultures from DNA-repair mutant neurons were then used to examine the relationship between DNA damage and DNA repair. Neuronal cultures from the cerebellum of *Mgmt*-/- and *Aag*-/- mice were treated with the same alkylating agents and the results compared with that of similarly treated wild type cells (Fig. 2). *Aag*-/- neurons were equally sensitive to MAM and DMS, but significantly less sensitive than wild type neurons to both agents. In contrast, *Mgmt*-/- neurons were more sensitive to MAM than DMS and MAM treated wild type neurons. These results suggest that O6-mG DNA lesions play an important role in MAM-induced neurotoxicity. Differences were also noted among the DNA repair mutant neurons treated with HN2 or CEA. *Aag*-/- neurons were more sensitive to CEA than HN2, but only at the highest concentration tested (20 μM). The relative insensitivity of *Mgmt* or *Aag* null neurons to DMS suggests that N7-mG DNA lesions are less toxic to immature post-mitotic neurons that O6-mG DNA lesions. At the highest dose tested (20 μM), *Aag*-/- neurons were more resistant to HN2 than wild type neurons. Unexpectedly, *Mgmt*-/- neurons were more sensitive to HN2 than CEA suggesting that HN2 produces DNA lesions that are repaired by MGMT. Table I shows the LD50 of neurons isolated from both wild type and DNA repair mutants after treatment with the individual alkylating agents. A similar pattern of sensitivity was observed in parallel cultures of fibroblasts and epithelial cells from the skin and kidney of wild type, *Aag*-/-, and *Mgmt*-/- mice that were treated for 24h with low concentrations of MAM or HN2 and then examined for cloning efficiency (Supplement Figure 2).

**Delayed Cytotoxicity of Alkylating Agents in DNA Repair-Deficient Neurons**

Additional studies were conducted with wild type and DNA repair mutant mice to determine if the differential sensitivity of *Mgmt*-/- and *Aag*-/- neurons to MAM or HN2 also occurs after sub-chronic treatment (*up to 7 days*) with lower concentrations of alkylating agents (Figure 3). As in the acute studies (Fig. 2), *Mgmt*-/- neurons were significantly more sensitive to sub-chronic treatment with low concentrations of MAM (<500 μM) or HN2 (<1.0 μM) than similarly treated wild type or *Aag*-/- cells, but differences were now observed at lower concentrations of these genotoxicants. However, the protective effect for AAG on both MAM and HN2 that we observed in the short-term studies (see Fig. 2) disappeared after *Aag* null neurons were treated for prolonged periods (> 3 days) with low concentrations of MAM (500 μM) or HN2 (>2.5 μM). These studies demonstrate that low concentrations of MAM or
mustards induce delayed neurotoxicity in Mgmr<sup>−/−</sup> neurons and the resistance of Aag null neurons to both genotoxicants disappears after prolonged exposure to low concentrations.

**DNA Fragmentation in DNA Repair Mutant Neurons Treated with Alkylation Agents**

Previous studies suggest that the DNA damage produced by MAM and HN2 is an important mechanism of cell death in non-nervous tissue [37,38]. The basis for the increased sensitivity of cells to MAM and HN2 is likely to be an increase in unrepaired DNA lesions, which would lead to strand breaks and ultimately cell death. To clarify further the role of specific DNA lesions in MAM or HN2 induced neuronal cell death, we compared the extent of DNA fragmentation (i.e., TUNEL labeling) induced by MAM and HN2 in wild type and DNA repair mutant neurons. As in Fig. 2, cerebellar neurons from wild type, Aag<sup>−/−</sup>, and Mgmt<sup>−/−</sup> mutant mice were treated for 24h with 10 μM to 1000 μM MAM or 1.0 μM to 10 μM HN2 and the cells examined for DNA strand breaks by the TUNEL technique (Figure 4). Since O<sup>6</sup>-mG DNA lesions may be an important mechanism by which MAM induces its neurotoxic effects, we also examined neurons from MGMT-overexpressing mice for DNA strand breaks after treatment with MAM or HN2. In general, significant changes in the number of TUNEL labeled cells were only observed in neuronal cultures treated with >100 μM MAM or > 1 μM HN2. The extent of DNA fragmentation in Mgmr<sup>−/−</sup> and Aag<sup>−/−</sup> neurons correlated with the sensitivity of the DNA repair mutant neurons to MAM or HN2 in Fig. 2. DNA fragmentation was more extensive in Mgmr<sup>−/−</sup> neurons than corresponding wild type neurons treated with MAM. Both the Mgmt<sup>Tg</sup> and Aag<sup>−/−</sup> genotypes were protective. The Aag<sup>−/−</sup> genotype was also protective after treatment with HN2. Much to our surprise, DNA fragmentation in Mgmt<sup>Tg</sup> neurons was more extensive than corresponding wild type neurons treated with HN2. The reduced DNA fragmentation in MAM or HN2 treated Aag<sup>−/−</sup> neurons is consistent with the relative insensitivity of Aag null neurons to either genotoxicant (Fig. 2).

**Effect of Alkylation Agents on Growth and Cerebellar Development in Wild Type Mice**

The above in vitro studies demonstrate that immature cerebellar granule cells are especially sensitive to alkylation agents and this sensitivity appears to depend upon the formation of specific DNA lesions (e.g., O<sup>6</sup>-methyl, N3-methyl and N7-methyl DNA lesions, cross-links). An important question is whether alkylation agents disrupt the in vivo development of cerebellar granule cells by a similar mechanism to induce persistent neuropathological changes and associated motor dysfunction (i.e., ataxia). To test this hypothesis, we examined the influence of all four genotoxicants on granule cell development in the cerebellum of wild type and DNA repair deficient mice. Wild type and DNA repair mutant mice were administered a single subcutaneous injection of saline, DMSO (vehicle for DMS) or equimolar doses of a methylating agent (325 μmol of MAM or DMS) or bifunctional and monofunctional agents (13 μmol HN2 or CEA) at postnatal day 3 (PND3, the peak period of granule cell development) [39]. The cerebellum was examined for neuropathology at different times (i.e., PND4, PND8, PND15, PND22) after dosing mice with MAM or HN2 and at PND22 for mice treated with DMS or CEA.

Wild type mice that had been treated with MAM or HN2 were about one half to 2/3 the size of animals treated with saline (Figure 5A), whereas growth of neonatal mice treated with DMS or CEA was similar to that of saline treated animals. The effect of MAM on growth was dose-dependent (see Supplement Figure 3). We examined the PND22 cerebella of treated mice for morphological changes (Figure 5B). At the light microscopic level, smaller cerebellar folia, a thinner cerebellar cortex and a large loss of neurons in the granule cell layer (i.e., Nissl staining) were observed in both MAM and HN2 treated mice, but not in DMS or CEA treated mice. The cerebella from MAM treated wild type mice were also examined at PND4 and PND22 for both N7-mG and O<sup>6</sup>-mG DNA lesions by HPLC with electrochemical detection [40] or LC/MS [41] to determine if the cytoarchitectural changes induced by this alkylating agent are...
associated with elevated levels of DNA lesions (Figure 5C). N7-mG and O\textsuperscript{6}-mG levels were both significantly higher in the cerebellum of PND4 and PND22 mice treated with MAM than saline treated mice. The decline of these DNA lesions over time was greater for O\textsuperscript{6}-mG than N7-mG DNA lesions indicating that the immature cerebellum is more efficient at repairing O\textsuperscript{6}-mG than N7-mG DNA lesions. Collectively, these studies indicate that only certain alkylating agents influence the growth and neuronal development of the neonate and these effects are correlated with the accumulation and persistence of specific DNA lesions.

The cerebella of younger animals were then examined for morphological changes (i.e., folia and granule cell development) at different time-points after treatment of PND3 mice with MAM or HN2 to determine when neurodevelopmental changes first appear (Figure 6). No gross morphological changes in the cerebella were noted 24h (i.e., PND4) after the administration of 325 μmol MAM or at other doses of MAM (see Supplement Figure 3). However, morphological changes were detected in the cerebella of PND8 mice after treatment with MAM or HN2 and these pathological changes became increasingly more evident with age (Fig. 6, middle and right panels). Differences were also noted for the influence of MAM and HN2 on cerebellar development. Development of the cerebellum was initially slower after MAM treatment than HN2 (compare PND8 vs. PND4) whereas HN2 had a greater effect at later timepoints (i.e., PND15 and PND22). Similar cytoarchitectural changes were reported in the cerebella of neonatal C57BL/6 mice injected with a 2-fold higher concentration of methylazoxymethanol (MAM) [10] or after injection with increasing concentrations of MAM (see Supplement Figure 3), indicating that the effects of these alkylating agents on cerebellar development are delayed and dose dependent. In addition to the cerebellum, other brain regions (e.g., hippocampus, olfactory bulbs) were also targeted by either MAM or HN2, but the effects on neurodevelopment were subtle (data not shown).

Effect of Alkylating Agents on Cerebellar Development in DNA Repair Altered Mice

The studies in Fig. 6 indicate that granule cell development is severely disrupted after brief exposure of neonatal wild type mice to MAM or HN2 and based upon the results from the above studies in Fig. 5C and our previous work [10], this was probably due to the accumulation and persistence of specific DNA lesions. This hypothesis was investigated further by comparing the effects of MAM or HN2 on granule cell development in DNA repair-deficient (i.e., Aag\textsuperscript{-/-}, Mgmt\textsuperscript{-/-}) or DNA repair-overexpressing (i.e., Mgmt\textsuperscript{Tg}++) mice. As in Figs. 5 and 6, neonatal wild type and DNA repair mutant mice were administered a single equimolar injection of MAM or HN2 and the cerebellum examined at PND22 for morphological changes (Figures 7A and 7B). As in Fig. 5B, MAM and HN2 induced pronounced changes in the morphology of the developing cerebellum (Fig. 7A and 7B) and these changes were associated with a disruption in both exploratory activity and motor function (Figure. 7C). The most striking observation was the difference in the morphology of the cerebellum of neonatal wild type and DNA repair mutant mice after treatment with either MAM or HN2 (Fig. 7A). In wild type mice, MAM and HN2 reduced the density of neurons in the granule cell layer (Fig. 7B, stars) and disrupted the organization of the Purkinje cell layer (Fig. 7B, arrows). The disorganization of Purkinje cells was more pronounced in specific folia (i.e., 2-8) of HN2 treated mice than that of MAM treated mice (compare Figs. 7A and 7B). In comparison, development of folia and the organization of the Purkinje cell layer were well preserved and granule cell loss was minimal in the cerebellum of Aag\textsuperscript{-/-} mice treated with MAM or HN2. In marked contrast, the changes in granule cell and folia development were significantly greater in Mgmt\textsuperscript{Tg}++ mice treated with MAM than wild type mice or Mgmt\textsuperscript{-/-} mice treated with HN2. Also note the severe cerebellar atrophy (Fig. 7A) and disorganization of the granule cell and Purkinje cell layers in Mgmt\textsuperscript{Tg}++ mice treated with HN2 in comparison to Mgmt\textsuperscript{Tg}++ mice treated with MAM (Fig. 7B, lower panels). These studies further suggest that the DNA lesions produced by HN2 are more
toxic to MGMT-overexpressing neurons than the DNA lesions produced by MAM as seen in Figure 4.

**Effect of Alkylating Agents on Behavior in DNA Repair Altered Mice**

The behaviors of wild type and DNA repair mutant mice treated with MAM or HN2 were observed using a video camcorder to determine if the genotoxins exert an effect on exploratory activity (e.g., rearing, sniffing) and motor function (Figure 7C)(see video clip in Supplement 4). The neuropathological changes induced by MAM and HN2 in Figs. 7A and 7B and the increased sensitivity of \( \text{Mgmt}^{+/-} \) granule cell cultures to MAM or HN2 in Fig. 2. are consistent with the more severe gait disturbances (i.e., hindlimb splay, ‘goose-stepping’, ataxia) and reduced exploratory activity observed in MAM and HN2 treated \( \text{Mgmt}^{-/-} \) mice (Fig. 7C). The preservation of exploratory activity and motor function in \( \text{Mgmt}^{Tg+} \) mice treated with MAM and \( \text{Aag}^{-/-} \) mice treated with MAM or HN2 is consistent with the relative lack of neuropathological changes in these mouse mutants after either MAM or HN2 treatment (Figs. 7A and 7B) and the insensitivity of neuronal cultures treated with these genotoxins (Fig. 2). Moreover, the severe neurobehavioral deficits observed in \( \text{Mgmt}^{Tg+} \) mice after HN2 treatment (Fig 7C, lower panels), but not after MAM treatment, are consistent with the increased vulnerability of \( \text{Mgmt}^{Tg+} \) cerebellar neurons to apoptosis (Fig. 3) and dysgenesis (Fig. 7B, lower panels) after HN2 treatment. The locomotor deficits (see video clip in Supplement 4) in this mouse mutant after HN2 treatment were also more severe than any other genotype treated with either HN2 or MAM. These *in vivo* studies are strong evidence that DNA damage is an important mechanism by which MAM and mustards disrupt neurodevelopment and induce neurobehavioral changes.

**Discussion**

The striking observation from our study was the difference in the vulnerability of cerebellar neurons from the three DNA repair (i.e., \( \text{Aag}^{-/-} \), \( \text{Mgmt}^{-/-} \), and \( \text{Mgmt}^{Tg+} \)) mutant mice to genotoxins that induce either methyl (i.e., MAM, DMS) or alkyl (i.e., CEA, HN2) DNA lesions. In addition, the effect of these classes of genotoxins on growth and cerebellar development was significantly different among the three DNA repair mutant or wild type mice. These findings suggest that the specific combination of DNA lesion and DNA repair capacity within a neuron are key factors that determine whether the immature brain is vulnerable to a particular genotoxicant. Such factors are expected to be particularly important for understanding how environmental genotoxins or chemotherapeutic agents induce their long-term effects on the developing brain.

**DNA Repair Capacity, Methylated DNA Lesions and Neurodevelopment**

An important finding from our studies with methylating agents is that DNA repair proteins can either increase or reduce the vulnerability of neurons to genotoxins. \( \text{Mgmt}^{-/-} \) neuronal cultures were more sensitive to MAM than cultures derived from wild type mice and this sensitivity occurred through an apoptotic mechanism. Neurodevelopment and motor function were also more severely affected by MAM in \( \text{Mgmt}^{-/-} \) mice than wild type mice indicating that the direct reversal DNA repair pathway plays an important role in protecting neurons of the immature brain from DNA damage induced by methylating agents. The reduced number of \( \text{Mgmt}^{Tg+} \) neurons undergoing apoptosis after MAM treatment and the preservation of cerebellar morphology and motor function after \( \text{Mgmt}^{Tg+} \) mice were administered MAM is additional evidence that MGMT protects immature neurons from genotoxicant induced injury. Collectively, these findings indicate that one pathway by which methylating agents induce their neurotoxic effects is through the production of \( O^6\)-mG DNA lesions.
Conversely, Aag−/− neuronal cultures were relatively insensitive to MAM and this correlated with the preservation of cerebellar development and motor function in Aag null treated mice. Thus, AAG and MGMT protein have opposite effects on the vulnerability of the developing brain to methylating agents. The BER enzyme AAG converts the non-lethal N7-mG DNA lesions produced by methylating agents into more lethal DNA lesions (i.e., abasic sites) [42-44] causing increased neurotoxicity whereas neurons that lack AAG are relatively insensitive to methylating agents because they are unable to convert N7-mG DNA lesions into abasic sites. In support of this hypothesis, embryonic stem cells from Aag−/− mice are relatively insensitive to a variety of methylating and alkylating chemotherapeutic agents [45] while cells that overexpress AAG are hypersensitive to alkylating agents [43,46]. Although previous studies consistently reported that the N7-mG DNA lesions formed by MAM are responsible for its neurodevelopmental effects [47], our studies demonstrate that O6-mG DNA lesions also influence neurodevelopment. Therefore, O6-mG and N7-mG DNA lesions both have an important role in MAM induced neurotoxicity. These studies also demonstrate that multiple DNA repair pathways are required to protect the developing brain from long-term injury by methylating agents. Since methylating agents that are used in cancer chemotherapy impair learning and memory [3,48], this information may explain how these agents induce their neurotoxic effects. Our model emphasized the impact of genotoxicants on neurodevelopment while the clinical observational studies take place in children at various stages of development. Nonetheless, the cumulative data suggests a significant impact of genotoxicants on neurons, neural precursors and presumably neural stem cells with a protective effect of MGMT, and as discussed below, a tolerance induced by the loss of AAG.

**Influence of Cross-Links on Neurodevelopment**

HN2 is a bifunctional alkylating agent that induces both cross-links and monoadducts [38], whereas the half-mustard CEA only produces monoadducts [49]. The monoadducts (i.e., N7-alkylguanine) formed by HN2 and CEA are primarily repaired by the BER pathway [37,50,51] whereas the cross-links produced by HN2 are primarily repaired by the nucleotide-excision repair pathway [37,50,51]. Estimates indicate that only 1-5% of the DNA lesions produced by HN2 are interstrand cross-links while the majority are N7-alkylguanine (70%) or N3-alkyladenine (17%) monoadducts [38,47]. Our initial goal was to determine the relative contributions of the monoadducts (i.e., N7-alkylG) and cross-links to the neurotoxicity of bifunctional alkylating agents by comparing the in vitro and in vivo response of cerebellar neurons from DNA repair-proficient and DNA repair-mutant mice to both monofunctional (CEA) and bifunctional (HN2) mustards. The remarkable difference in the sensitivity of DNA repair-proficient immature neurons (whether in culture or in vivo) to bifunctional vs. monofunctional mustards is strong evidence that cross-links are more lethal to neurons in the developing brain than monoadducts. The greater sensitivity of kidney epithelial cells, other animal cells or bacteria to bifunctional as opposed to monofunctional nitrogen mustards [51] suggests that cross-links are the key lesions that activate cell death mechanisms in both neurons and non-neural cells exposed to mustards.

**DNA Repair Capacity Influences Mustard-Induced Neurotoxicity**

Aag null neurons were more tolerant of HN2-induced DNA damage in neural tissues than neurons from either wild type or Mgmt null mice. Since wild type and DNA repair mutant neurons are relatively insensitive to monofunctional mustards, these studies suggest that immature neurons use AAG to process cross-links. A manuscript in press also supports AAG acting on DNA interstrand crosslinks [52]. Support for this hypothesis comes from studies that compared the sensitivity of AlkA-deficient bacteria (the homologue of AAG) or AAG null mouse embryo fibroblasts (MEFs) to sulfur mustard and its monofunctional analogue chloroethyl ethyl sulfide (CEES) [46]. Sulfur mustard and HN2 are bifunctional alkylating agents that produce cross-links whereas the corresponding monofunctional analogues CEES.
and CEA produce N7-alkylguanine DNA lesions. AlkA null bacteria or AAG null mouse embryo fibroblasts (MEFs) were significantly less sensitive to sulfur mustard and CEES than similarly treated wild type cells. Moreover, wild type bacteria and MEFs were ∼10-20-fold more sensitive to sulfur mustard than CEES. These findings are consistent with the differential vulnerability of wild type or Aag null neurons to HN2 or CEA. Therefore, neurons and non-neuronal cells appear to use BER enzymes to repair the cross-links produced by bifunctional mustards. While most cell types from AAG null mice show an increased sensitivity to alkylating agents, previous studies showed that some cell types (e.g., bone marrow) are relatively resistant [42]. Consequently, the processing of mustard-induced DNA lesions by AAG may be incomplete in neurons resulting in reduced levels of toxic intermediates as previously noted in bone marrow cells [42]. Taken together, the neuronal BER pathway appears to play an important role in repairing mustard induced monoadducts, but in contrast to MGMT, the processing of specific DNA lesions by BER can either reduce (e.g., cross-links) or increase (e.g., N7-mG) the neurotoxic effects of mustards.

Neurons of Mgmt−/− and MgmtTg+ mice were more sensitive to HN2 than neurons of the other genotypes. The increased sensitivity of Mgmt−/− neurons to HN2 indicates that the direct reversal pathway plays an important, but undefined role in protecting neurons from both the acute and delayed toxic effects of bifunctional mustards. There are at least two possible explanations for these unexpected results. First, HN2 may produce O6-alkyl lesions that are especially lethal to developing neurons. Second, the targeting of Mgmt via a knockout or transgenic strategy may increase the vulnerability of neurons to HN2 either by influencing cellular pathways that repair cross-links (e.g., recombination, translesion synthesis) [53-55] or by activating cell death mechanisms (e.g., apoptosis) [21,56]. Like sulfur mustard [57], HN2 may react with the O6-position of guanine to generate the unstable haloalkylguanine lesion, which under physiological conditions would generate the more stable hydroxyalkylguanine DNA lesion (S. Ludeman, personal communication). Although formation of an O6-alkylguanine DNA lesion by HN2 has never been reported, the identification of O6-alkylguanine in sulfur mustard treated DNA [57] and the increased sensitivity of MGMT-depleted CHO cells to nitrogen mustards [58] suggests that this DNA lesion may be formed in HN2 treated neurons. The ability of MAM and HN2 to produce similar neuropathological and neurobehavioral features in Mgmt−/− mice is additional evidence that O6-alkylguanine DNA lesions may be formed in neurons by mustards. However, such a mechanism does not explain the more severe neuropathological and neurobehavioral features that were observed in MgmtTg+ mice treated with HN2. One strong possibility is that HN2 reacted with neuronal proteins to produce a more highly reactive intermediate that covalently binds to DNA (e.g., protein-DNA adducts). Previous studies indicate that bis alkylating agents like HN2 are capable of reacting with either glutathione (GSH) [59] or MGMT [60] to form chemical cross-links with DNA. Loeber and colleagues [61] recently demonstrated that HN2 forms a cross-link between the N7 position of guanine in DNA and the two cysteine residues within the active site of MGMT. The MGMT-DNA conjugate induced by HN2 was isolated from protein extracts of MGMT-overexpressing CHO cells, but not control cells demonstrating that the increased sensitivity of MGMT-overexpressing cells to mustards occurs through the production of DNA-protein adducts. These protein cross-links are especially toxic to cells [62,63] and their formation may occur more readily in neurons with high intracellular levels of both GSH and MGMT.

In conclusion, the present studies demonstrate that the response of developing neurons to DNA damaging agents is complex. For Mgmt null neurons, the absence of DNA repair leads to increased sensitivity to DNA damaging agents, consistent with a straightforward cause and effect relationship in which persistence of DNA damage is toxic to neurons. However, Aag null neurons are resistant to some DNA damaging agents suggesting that the repair process can be toxic to neurons under certain circumstances. More work will be required to better...
understand DNA repair in neurons and why some pathways are toxic when repairing specific types of lesions.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Alkylating agents used to examine the influence of DNA damage on neurodevelopment

- Methylazoxymethanol (MAM)
- Mechlorethamine (HN2)
- Dimethyl sulfate (DMS)
- 2-Chloroethylamine (CEA)
Figure 2. Comparative sensitivity of DNA repair mutant neurons to different alkylating agents
Primary granule cell cultures from the cerebellum of C57BL/6 (wild type) or DNA repair-deficient (i.e., Aag<sup>-/-</sup>, Mgmt<sup>-/-</sup>) neonatal mice were treated for 24h with various concentrations of MAM, DMS, HN2 or CEA, incubated with calcein-AM and the cells examined for fluorescence. Values represent the mean % survival of 2-3 separate experiments (n=6/tx group). Significantly different from toxin treated cells (* p < 0.01, ** p < 0.001, ANOVA), wild type cells (a p < 0.05, b p < 0.001, ANOVA) or DNA repair mutant cells (c p < 0.05, d p < 0.001, ANOVA).
Cerebellar granule cell cultures from C57BL/6 (wild type), Aag-/- or Mgmt-/- mice were treated continuously with MAM (10 µM-500 µM) or HN2 (0.1 µM-5.0 µM) and at various time periods (1, 3, 5, 7 days), the cell cultures incubated with calcein-AM and examined for fluorescence. Values represent the mean % survival of controls ± SEM (n= 6tx group, 2-3 separate experiments). Significantly different from controls (* p < 0.05, ** p <0.01, ANOVA).

Figure 3. Long-term viability of DNA repair-deficient neurons treated with MAM or HN2

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Figure 4. DNA fragmentation in DNA repair mutant neurons treated with MAM or HN2
Primary cerebellar neurons were treated for 24h with various concentrations of MAM (10 μM, 100 μM, 1000 μM) or HN2 (1.0 μM, 5.0 μM, 10 μM) and the cells examined for DNA strand breaks by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) technique (NeuroTacs™ kit, Trevigen, Inc). Significantly different from controls (* p < 0.01) or wild type cells (** p < 0.01, † p < 0.001, ANOVA).
Figure 5. Comparative sensitivity of C57BL/6 mice to various alkylating agents

A. Growth of neonatal (postnatal day 22) mice after treatment on postnatal day 3 with a single subcutaneous injection of 325 µmol of MAM and DMS or 13 µmol HN2 and CEA. B. Light micrographs of representative areas from cresyl violet stained parasagittal sections (10 µm) of the cerebellum from PND22 mice treated on PND3 with saline, DMSO (vehicles) or the genotoxicants MAM, DMS, CEA, or HN2. Note reduced staining of the granule cell layer and atrophy of the cerebellar folia in PND22 mice treated with MAM or HN2. C. DNA isolated from the cerebellum of PND 4 or PND 22 mice (n=3) treated on PND 3 with either saline or 325 µmol MAM was analyzed by HPLC/EC [10] or LC/MS/MS [41] for the DNA lesions N7-
methylguanine (N7-mG) and $O^6$-methyldeoxyguanosine ($O^6$-mG), respectively. Significantly different from saline treated mice (* $p < 0.05$, ANOVA). ND= not detectable.
Figure 6. Effect of MAM and HN2 on the cytoarchitecture of the developing cerebellum of C57BL/6 mice
Light micrographs of representative areas from cresyl violet stained parasagittal sections (10 μm) of the cerebellum from postnatal day 4, 8, 15 and 22 day-old pups treated at postnatal day 3 (PND3) with saline (left panels), MAM (middle panels) or HN2 (right panels). Magnification × 3.85.
Figure 7. Cytoarchitecture of the cerebellum of wild type and DNA repair-mutant mice treated with MAM or HN2
A. Light micrographs of representative areas from cresyl violet stained parasagittal sections (10 μm) of the PND22 cerebellum from C57BL/6J (wild), Aag<sup>-/-</sup>, Mgmt<sup>-/-</sup> or Mgmt<sup>+</sup> mice treated on PND3 with a single injection of saline (left panels), MAM (325 μmol, s.c., middle panels) or HN2 (13 μmol, s.c., right panels). Mag ×3.85. B. Higher magnification (×77) of the cerebellum in tissue sections from wild type or DNA repair mutant mice treated with MAM (left panels) or HN2 (right panels). f= folia, ml= molecular layer, pc= Purkinje cell layer, gc= granule cell layer. C. Photomicrographs of the gait of PND22 wild type and DNA repair mutant mice after treatment on PND3 with either 325 μmol MAM or 13 μmol HN2.
Table I
Relative Sensitivity (LD50) of DNA Repair-Deficient Neurons to Alkylating Agents

| Genotype | MAM     | DMS     | CEA     | HN2     |
|----------|---------|---------|---------|---------|
| Wild     | 500-1000 μM | 200-500 μM | >20 μM  | 10 μM   |
| Aag<sup>-/-</sup> | 1000-2000 μM | 1000-2000 μM | >20 μM  | 10 μM   |
| Mgmt<sup>-/-</sup> | 500 μM   | 1000-2000 μM | >20 μM  | 1.0 μM  |