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TABLE OF CONTENTS

FRONT COVER	1
FORM 298	2
TABLE OF CONTENTS.....	3
INTRODUCTION.....	4
BODY OF THE REPORT	4
Statement of work for Year 2 of funding	4
Cytotoxicity of HN2 and MAM in C57BL/6 neuronal and astrocyte cultures	5
DNA damage in HN2 and MAM in C57BL/6 neuronal and astrocyte cultures	7
Cytotoxicity of HN2 and MAM in <i>Aag</i> -deficient neuronal and astrocyte cultures.....	7
Cytotoxicity of HN2 and MAM in <i>xpa</i> -deficient neuronal and astrocyte cultures	7
Cytotoxicity of HN2 and MAM in <i>Mgmt</i> -deficient neuronal and astrocyte cultures.....	8
Dose-range finding studies of HN2 and MAM in <i>Xpa</i> -deficient mice.....	9
KEY RESEARCH ACCOMPLISHMENTS	9
REPORTABLE OUTCOMES	9
CONCLUSIONS.....	10
REFERENCES.....	10
APPENDICES.....	12

INTRODUCTION

Experiments are proposed to examine the molecular mechanism by which mustard chemical warfare agents induce neuronal cell death. DNA damage is the proposed underlying mechanism of mustard-induced neuronal cell death. We propose a novel research strategy to test this hypothesis by using mice with perturbed DNA repair to explore the relationship between mustard-induced DNA damage and neuronal cell death. Initial *in vitro* studies (Years 1, 2 & 3) are proposed to examine the cytotoxic and DNA damaging properties of the sulfur mustard analogue mechlorethamine (nitrogen mustard or HN2) and the neurotoxic DNA-damaging agent methylazoxymethanol (MAM) using neuronal and astrocyte cell cultures from different brain regions of mice with perturbed DNA repair. Findings from these studies will be used to examine the *in vivo* neurotoxic effects of HN2 and MAM (Years 3 & 4) in mice with perturbed DNA repair.

BODY OF THE REPORT

STATEMENT OF WORK FOR YEAR 2 of FUNDING

The overall goal of studies proposed in Year 2 are to: (i) examine the cytotoxicity of HN2 and MAM in neuronal and astrocyte cell cultures of wild type mice, (ii) examine the cytotoxicity of HN2 and MAM in neuronal and astrocyte cell cultures of DNA repair-mutant mice and (iii) use results from these *in vitro* studies to begin dose-range finding studies of HN2 and MAM in DNA repair-deficient mice. Specific objectives proposed in Year 2 of the Statement of Work are as follows:

1. Measure cytotoxicity of HN2 and MAM in C57BL/6 neuronal and astrocyte cell cultures.
2. Measure DNA damage of HN2 and MAM in C57BL/6 neuronal and astrocyte cell cultures.
3. Examine cytotoxicity of HN2 and MAM in *Aag*-deficient neuronal and astrocyte cell cultures.
4. Examine cytotoxicity of HN2 and MAM in *Xpa*-deficient neuronal and astrocyte cell cultures.
5. Examine cytotoxicity of HN2 and MAM in *Mgmt*-deficient neuronal and astrocyte cell cultures.
6. Commence dose-range finding studies of HN2 and MAM in *Xpa*-deficient mice.

For the ongoing studies, we are currently breeding three strains of mice with deficiencies for DNA repair pathways. These are XPA, MGMT, and AAG. Over the last year we encountered significant difficulties in maintaining these strains as a result of the tragic death of one of our technicians, Mr. Greg Komma, who had sole responsibility for the mouse room. Although several technicians helped to maintain our wild-type stocks, they were not trained to take over this responsibility on a moment's notice and we recognized eventually that animals had been identified incorrectly as bearing MGMT and AAG deficiencies were used for additional breedings. Therefore, rather than to try to recover these strains in house, we eliminated these strains and re-derived them again from breeder pairs sent to us in July from Dr. Leona Samson (Harvard University, Boston, MA). We are now expanding both colonies of *Mgmt*- and *Aag*-deficient mice. Despite these problems, we were successful in keeping the XPA strain and have started working with cultures from homozygous deficient animals (see Objective 4 below). The only problem encountered with this strain is reduced fertility for null X null breedings. We are currently increasing the number of XPA null mice to ensure an ample supply of these animals for the proposed *in vitro* and *in vivo* experiments. Finally, to prevent further disruptions to our animal colony, we have trained two technicians to be fully proficient in all aspects of animal breeding and genotyping.

Because of a lack of some DNA repair-deficient mice, only certain research objectives were achieved during the 2nd year of funding (i.e., Objectives 1, 4). We were successful in examining Objectives 1 & 4 of the Statement of Work in Year 2 by focusing on: (i) the establishment of neuronal and astrocyte cell cultures from different brain regions (i.e., cerebellum, cortex and midbrain) of wild-type mice, (ii) characterizing the DNA repair level and activity of the CNS of wild type and DNA repair-overexpressing mice, and (iii) examining the sensitivity of neurons from XPA mice to MAM & HN2. A detailed description of the research accomplishments for each objective of Year 2 follows.

1. Measure cytotoxicity of HN2 and MAM in C57BL/6 neuronal and astrocyte cell cultures.

The colony of wild-type mice that was established during the 1st year of study was used primarily during the 2nd year to: (i) re-derive and expand our colonies of *Aag*-, *Mgmt*-, and *Xpa*-mutant mice, (ii) train technical staff in the timing of pregnant mice and (iii) develop cultures of cortex, midbrain, and cerebellar neurons and astrocytes.

Cerebellar Cultures

Cerebellar neuronal and astrocyte cell cultures were successfully developed from litters of wild type and *Mgmt*⁺ mice and examined for sensitivity to HN2 and MAM. Initial studies compared the relative toxicity of the sulfur mustard analogue mechlorethamine (HN2, nitrogen mustard) and the neurotoxin methylazoxymethanol (MAM) among cerebellar neuronal and astrocyte cell cultures prepared from mice that overexpress human *O*⁶-methylguanine-DNA alkyltransferase (MGMT⁺). Human MGMT (~22-23 kDA, *black arrow*) was detected by western blotting studies in the cerebellum, hippocampus, and midbrain of MGMT⁺ mice, but not the brain of wild-type mice (**Figure 1, top; Appendix**). As expected, the endogenous mouse protein (mMGMT, *white arrow*) was detected in both wild type and MGMT⁺ mice. Consistent with these observations, MGMT activity was higher in the cerebellum (~50-fold) and other brain regions (12 to 80-fold) of MGMT⁺ mice when compared with similar brain regions of wild-type mice (**Figure 1, bottom**). Interestingly, brain levels and activity of MGMT were observed to be higher than previously reported in other organs (e.g., thymus, spleen, muscle) [1] suggesting that the DNA repair protein may have other functions in the CNS. Therefore, cerebellar neurons and astrocytes of MGMT⁺ mice have elevated levels and activity of MGMT.

The relative toxicity of HN2 and MAM was examined in wild type and MGMT⁺ cerebellar neurons and astrocytes using the vital fluorochromes calcein-AM (*green fluorescence*) and propidium iodide (PI, *red fluorescence*) (**Figure 2, Appendix**). Calcein-AM is a cell permeant dye that is taken up by active mitochondria while a loss in membrane integrity leads to uptake of PI and nuclear staining of dying cells. Cell loss was extensive in wild-type mouse cerebellar neurons treated with 100 μ M MAM (>70%) and 1.0 μ M HN2 (>90%) (**Figure 2, left panels**). In contrast, MGMT⁺ cerebellar neurons were protected from 100 μ M MAM, but not from 1.0 μ M HN2-induced cytotoxicity. Additional studies using lower concentrations of HN2 are underway to confirm these findings. MGMT⁺ cerebellar neurons were also protected at higher concentrations of MAM as indicated by the significantly lower LDH activity measured in cell culture media (**Figure 3, right**) or the higher mitochondrial redox activity (**Figure 3,**

bottom) than comparably treated wild-type cells. The increased resistance of *Mgmt*⁺ neurons to high concentrations of MAM may be explained by the elevated repair of *O*⁶-methylguanine DNA adducts, a lesion that may be responsible for MAM-induced neuronal cell death. *Mgmt*⁺ neurons were not protected from HN2-induced cytotoxicity because this genotoxin produces predominantly *N*⁷-alkyl DNA adducts and x-links, adducts not repaired by MGMT.

In comparison to cerebellar neurons, wild type or MGMT⁺ astrocytes were relatively insensitive to 100 μ M MAM or 1.0 μ M HN2-induced cytotoxicity (**Figure 2, right panels**). In fact, concentrations greater than 10 μ M HN2 or 500 μ M MAM were required to induce significant cell loss (>50%) in astrocyte cultures (*data not shown*). Similar results have also been reported in studies that compared the relative toxicity of HN2 in rat cerebellar neuronal and astrocyte cell cultures [2], an effect probably related to the high basal levels of this DNA repair protein in glial cells [3,4].

Cortical & Midbrain Cell Cultures

Because of problems in generating a sufficient number of *Aag*-, *Mgmt*-, or *Xpa*-deficient mice, cortical and midbrain neuronal cultures were developed from wild-type mice and used to examine their sensitivity to MAM and HN2. For these studies, cortical and midbrain neuronal cultures were grown in antioxidant-enriched culture media (NeurobasalTM/B27) and the viability (mitochondrial redox activity) assessed by incubating the cells with Alamar BlueTM. Using the same litter of animals, we were able to develop separate viable (80-90%) neuronal cell cultures from the cortex and midbrain of mouse embryos (GD14-15). Cerebral cortical neurons were sensitive to low concentrations (< 500 μ M) of MAM when compared with similarly treated midbrain (**Figure 4, Appendix**) or wild-type cerebellar (**Figure 6, Appendix**) neurons. In contrast, cortical neurons and cerebellar neurons were equally insensitive to low concentrations of MAM or HN2. Additional studies using higher concentrations of MAM or HN2 will be conducted to confirm these findings and determine if there are regional differences in the sensitivity of neurons to MAM or HN2.

Cortical and midbrain neuronal cultures were also examined for the density of neurons or different neurotransmitter cell types (**Figure 5, Appendix**). Cortical neuronal cultures were immunoprobed with an antibody to microtubule associated protein (MAP2) to label dendrites and axons of neurons (**Figure 5, top**). MAP2 localization is specific for neurons and neural precursor cells, but not for glial cells. A majority (>90%) of the cells in cortical cultures were neurons. Midbrain neuronal cultures were immunoprobed with antibodies to glutamate decarboxylase (GAD) and tyrosine hydroxylase (TH) to determine the density of GABAergic and dopaminergic neurons (**Figure 5, Appendix**). A majority of the neurons (>90%) were determined to be GABAergic (*white arrow*), while only a small fraction (<1%) were dopaminergic (*black arrow*). The low yield of dopaminergic (DA) neurons was likely due to the age of the wild-type mice (GD15-16), since higher densities of DA neurons (~5-10%) have been reported for mesencephalic neurons that were prepared from the brains of younger (GD13) mice [5]. In the future, younger timed-pregnant mice (GD13-14) will be used to prepare midbrain cell cultures. An

advantage to this technique is the simultaneous culturing of neurons from two different brain regions (cortex and midbrain) from the same litter of animals. This method will reduce the time required for examining the relative toxicity of HN2 and MAM in separate cortical and midbrain neuronal and astrocyte cell cultures. Moreover, the neurotransmitter characterization of these neuronal cultures will provide a method to assess the relative sensitivity of different CNS cell types to HN2 or MAM.

2. Measure DNA damage of HN2 and MAM in C57BL/6 neuronal and astrocyte cell cultures.

Breeder pairs of C57BL/6 mice were mated and colonies established for wild-type mice. A majority of the animals from this colony were used to expand the three DNA repair-deficient mutant mice (i.e., *XPA*, *MGMT*, and *Aag*). Consequently, a sufficient number of animals were not available to develop high density neuronal cell cultures ($1-2 \times 10^7$ cells/flask or dish) that are required for the DNA damage studies. By the end of this year, we will have expanded the 3 colonies of DNA repair-deficient mice and be able to commence the DNA damage studies with wild type mice.

3. Examine cytotoxicity of HN2 and MAM in *Aag*-deficient neuronal and astrocyte cell cultures.

Heterozygous crosses of mice with *Aag* knockout mutations (obtained from L. Samson, Harvard University, Boston MA) were set up to obtain mice with both heterozygous and homozygous deficiencies. PCR conditions were established and primers specific for the *Aag* knockout mutation were successfully optimized in Year 2. These findings allowed us to create litters with specific genotypes, i.e. wild type, heterozygous-deficient, and homozygous-deficient. The ability to generate litters of a specific genotype is especially important for the successful development of neuronal and astrocyte cell cultures from embryonic nervous tissue (E13-14) of DNA repair-deficient mice. We are currently expanding the number of *Aag*-deficient mice so that we will have a sufficient number of animals to complete studies that compare the relative sensitivity of *Aag*-deficient cerebellar, cortical and midbrain neuronal and astrocyte cell cultures to MAM and HN2. More importantly, an aggressive expansion of this homozygous mutant mouse colony will be essential to provide a sufficient number of animals for the preparation of neurons from both postnatal (i.e., cerebellum) and fetal (i.e., cortex, midbrain) brain tissue and to complete *in vivo* studies.

4. Examine cytotoxicity of HN2 and MAM in *XPA*-deficient neuronal and astrocyte cell cultures.

The *Xpa* gene codes for a NER protein that is responsible for recognizing bulky adducts and x-links [6,7]. Mice homozygous for *Xpa* were observed to produce small litters (average 3-5 pups) and a high frequency of pseudopregnancies. To overcome these problems, the colony of *XPA*^{-/-} mice was extensively expanded in Year 2 to generate a sufficient number of animals for both neuronal and astrocyte cell cultures. Comparable expansion of *Aag* and *Mgmt* homozygous mutant mice was also conducted in parallel. The cytotoxic properties of HN2 and MAM were examined in cerebellar granule cell cultures prepared from wild type (C57BL/6) mice and crosses of *XPA*^{-/-} mice. Wild-type neuronal

cultures were treated for 24 h with control culture media or culture media containing various concentrations of HN2 (0.1 μM -10 μM) or methylazoxymethanol (MAM; 50 μM – 1000 μM) (**Figure 6, Appendix**). For comparison, neuronal cultures were prepared from $XPA^{-/-}$ mice and treated in a similar manner with HN2 and MAM. Viability was determined in HN2 and MAM treated neuronal cell cultures by adding Alamar Blue™, a non-toxic metabolic indicator that is widely used to measure mitochondrial function in different cell systems (including neurons) [8,9]. These concentrations of HN2 or MAM have been shown to produce cell death of rat cerebellar [2] or cortical neurons [10], respectively.

The viability of untreated wild type and $XPA^{-/-}$ cerebellar neurons was ~100%. Neuronal survival after exposure to 50 μM – 500 μM MAM for 24 h was similar for wild type and $XPA^{-/-}$ cells. At higher concentrations of MAM (1000 μM), wild type cells appeared very sensitive to the alkylating agent. The lack of sufficient $XPA^{-/-}$ cells prevented us from determining if $XPA^{-/-}$ cells exhibit a similar or different sensitivity as wild-type cells to high concentrations of MAM. An interesting observation is that cerebellar granule cells prepared from the C57BL/6 strain of mice used in the XPA studies were more resistant to MAM than comparably treated cells prepared from wild-type littermates of $MGMT^{+}$ heterozygous mice (C57Bl/6 x SJL) (**see Figures 3 & 6, Appendix**). This differential sensitivity may be due to differences in the response of some strains to alkylation-induced DNA damage, as previously reported for the MAM precursor azoxymethane [11] or other neurotoxins [12]. For example, the level of O^6 -methylguanine and N^7 -methylguanine DNA adducts in tissues of SWJ/J and AKR/J mice treated with azoxymethane can differ by a factor of up to 5-fold. Therefore, all current colonies of DNA repair deficient mice are being backcrossed into C57BL/6 (i.e., *Xpa*) or have been extensively backcrossed into C57BL/6 (~80-90%, *Aag*, *Mgmt*).

Like MAM, neuronal survival after treatment of wild type and $XPA^{-/-}$ cerebellar neurons with 0.1 μM – 1.0 μM HN2 was similar. However, $XPA^{-/-}$ cerebellar neurons were significantly more sensitive to higher concentrations of HN2 (5.0 μM and 10 μM) than wild-type cells. Additional studies using lower concentrations of HN2 (1.0 μM – 5.0 μM) after various exposure times will be conducted to determine if this effect is both time- and dose-dependent. These findings and the increased sensitivity of $XPA^{-/-}$ cerebellar neurons to UV-irradiation determined from Year 1 studies strongly suggest that HN2-induced neurotoxicity is due to cross-link formation. Additional studies are underway to confirm these findings by examining similarly treated wild type and $XPA^{-/-}$ cerebellar granule cell cultures for the level of cross-links. Parallel studies conducted in HN2 treated cerebellar neurons from *Mgmt*^{-/-} and *Aag*^{-/-} mice will provide additional support for DNA damage as an important mechanism of HN2-induced neurotoxicity.

5. Examine cytotoxicity of HN2 and MAM in *MGMT*-deficient neuronal and astrocyte cell cultures.

Heterozygous crosses of mice with *Mgmt* knockout mutations (obtained from L. Samson, Harvard University, Boston MA) were set up to obtain mice with both heterozygous and homozygous

deficiencies. PCR conditions were established and primers specific for the *Mgmt* knockout mutation were successfully optimized in Year 2. We are currently expanding the number of *Mgmt*-deficient mice so that we will have a sufficient number of animals to complete the proposed *in vitro* and *in vivo* studies.

6. Commence dose-range finding studies of HN2 and MAM in XPA-deficient mice.

Because of the difficulties experienced with the generation of DNA repair-deficient mice for the preparation of neuronal and astrocyte cell cultures, dose-range finding studies proposed in Year 2 are postponed for Year 3. We anticipate that we will have a sufficient number of animals from the DNA repair-deficient colonies to commence *in vivo* studies with HN2 and MAM in Year 3. Evidence from studies with the *MGMT*⁺ and *XPA*^{-/-} mice conducted in Years 1 and 2 suggest that we will begin the dose-range finding studies with these DNA repair mutant mice. Plans are already underway (in collaboration with Dr. S. Gerson, Case Western University) to obtain adult *MGMT*⁺ for dose-range findings studies with MAM and HN2.

KEY RESEARCH ACCOMPLISHMENTS

- Successfully optimized the genotyping (i.e., wild, heterozygous, homozygous).of DNA repair mutant mice.
- Examined the regional distribution of DNA repair proteins in the brains of wild type and DNA repair mutant (i.e., *MGMT*⁺) mice.
- Demonstrated that neurons are more sensitive to HN2 and MAM than comparably treated astrocytes.
- Demonstrated that neurons overexpressing *Mgmt* are protected from MAM-induced toxicity.
- Developed viable cultures of two different neurons (i.e., midbrain, and cortex) from the same litter of mice.
- Demonstrated that neurons from nucleotide excision repair (NER)-deficient mice (i.e., *Xpa*) are more sensitive to HN2 than MAM.

REPORTABLE OUTCOMES

1. Kisby, G.E., Gilchrist, J., Zelenka, J., Vemana, S., Komma, G., Wong, V., Qin, X., Gerson, S.L., Turker, M.S. Differential sensitivity of mutant DNA repair neurons to mustard-induced cytotoxicity. Bioscience Review (*In press*). Findings from the second year of studies were presented at the USARMC Bioscience Review Meeting on June 6-9, 2000 in Baltimore, MD.

2. 33rd Annual Winter Conference on Brain Research (WCBR) at Breckinridge, CO (January 22-29, 2000). A panel of speakers (S. LeDoux, G. Kisby, P. Brooks, T. Nospikel) were invited by Dr. P. Hanawalt (Stanford University) to speak on DNA Repair in the Nervous System .

CONCLUSIONS

The primary objective of the 2nd year of study was to generate a sufficient number of postnatal and fetal DNA repair-deficient mice to assess the cytotoxicity of HN2 and MAM in cerebellar or cortical and midbrain neuronal cell cultures (respectively). While we experienced difficulty in generating *Aag*- and *Mgmt*-deficient mice, we were successful in determining the relative sensitivity of HN2 and MAM using *MGMT*⁺ and *XPA*^{-/-} mice. The *MGMT*⁺ studies examined whether the neurotoxicity of HN2 and MAM could be prevented in cerebellar neurons that overexpress *MGMT*. Since MAM produces *O*⁶-methylguanine DNA adducts, *MGMT*⁺ cerebellar neurons should exhibit resistance to MAM, but not to HN2. As proposed, neuronal survival, membrane integrity and mitochondrial function were essentially maintained in cerebellar *MGMT*⁺ neurons treated with MAM, but not after HN2 treatment. These findings suggest that *MGMT* protects neurons from MAM-induced cytotoxicity, but not from HN2-induced cytotoxicity. The increased resistance of *MGMT*⁺ neurons to high concentrations of MAM may be explained by the elevated repair of *O*⁶-methylguanine DNA adducts, a lesion that may be responsible for MAM-induced neuronal cell death. In support of this notion, astrocytes were relatively insensitive to MAM and HN2 because this CNS cell type is reported to contain high levels of *MGMT* [3,4] and possibly other DNA repair proteins (e.g., *NER*).

In separate experiments, cerebellar neurons from mice defective in the nucleotide excision repair (*NER*) protein *XPA*, a key protein that repairs x-links and UV DNA damage [6], were examined for their sensitivity to HN2 and MAM. The increased sensitivity of *NER*-deficient neurons to HN2 and UV-irradiation (Year 1 studies) and not to MAM suggests that the inefficient removal of x-links play an important role in mustard-induced neurotoxicity. Additional studies are required with neuronal and astrocyte cell cultures of other DNA repair-deficient mice to determine if mustard-induced neurotoxicity is dependent upon the inefficient removal of a specific mustard-induced DNA adduct (i.e., *N*⁷-alkylguanine vs. x-links). These findings also suggest that the survival of CNS neurons depend upon the efficient repair of alkylation-induced DNA damage.

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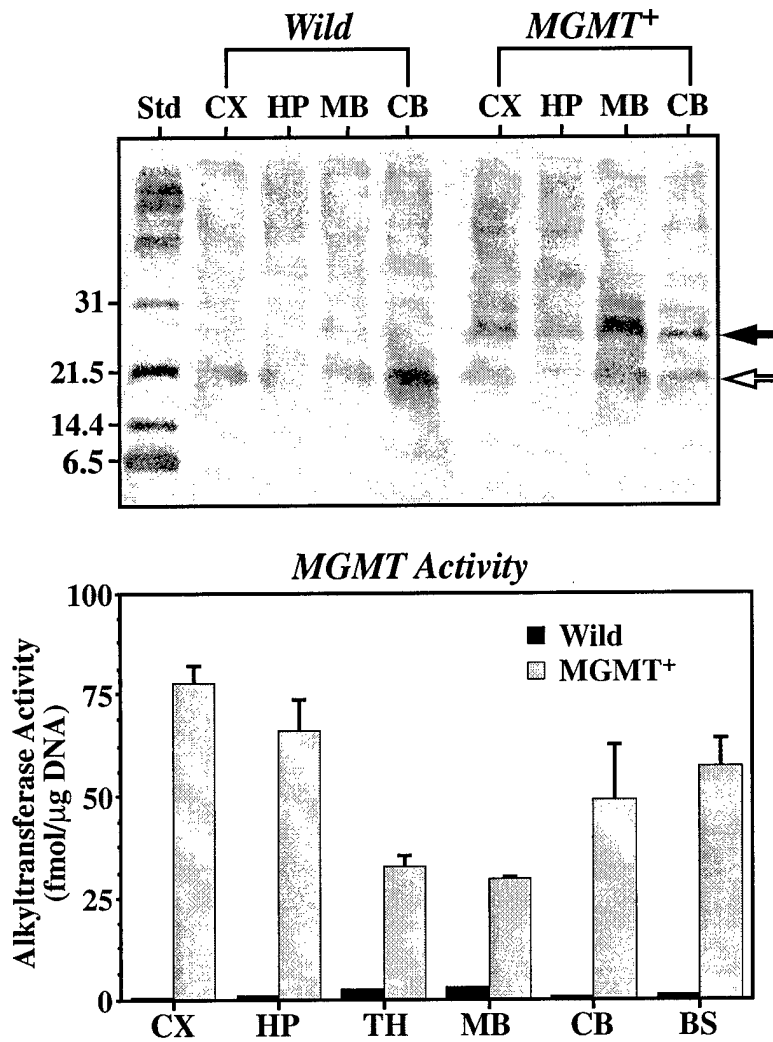


Figure 1. *O*⁶-Methylguanine methyltransferase (MGMT) levels and activity in different brain regions of wild type (Wild) and MGMT-overexpressing (MGMT⁺) mice. Postnatal (6-8 day old) brain tissue of Wild and MGMT⁺ mice were homogenized in lysis buffer, centrifuged, and the tissue extracts analyzed for MGMT⁺ levels (*top*) or MGMT activity (*bottom*). Protein extracts (25 μg) were electrophoresed on a SDS-PAGE, transferred to a nitrocellulose membrane, and the membrane blocked and immunoprobed with a monoclonal antibody to MGMT (MT 22.1; gift from Dr. D. Bigner, Duke University). Specific binding of MGMT was visualized using a HRP-conjugated goat anti-mouse antibody (1:1000) and enhanced chemiluminescence. For MGMT activity, an aliquot of protein extract (100 μg or 250 μg) was incubated with [³H] methyl DNA and the amount of [³H] *O*⁶-methylguanine determined by HPLC with liquid scintillation counting. Values are the mean ± SEM (*n* = 3-4). Std=biotinylated markers. Human MGMT (hMGMT, *black arrow*) and mouse MGMT (mMGMT, *white arrow*). CX=cortex; HP=hippocampus; TH=thalamus; MB= midbrain; CB=cerebellum; BS=brainstem.

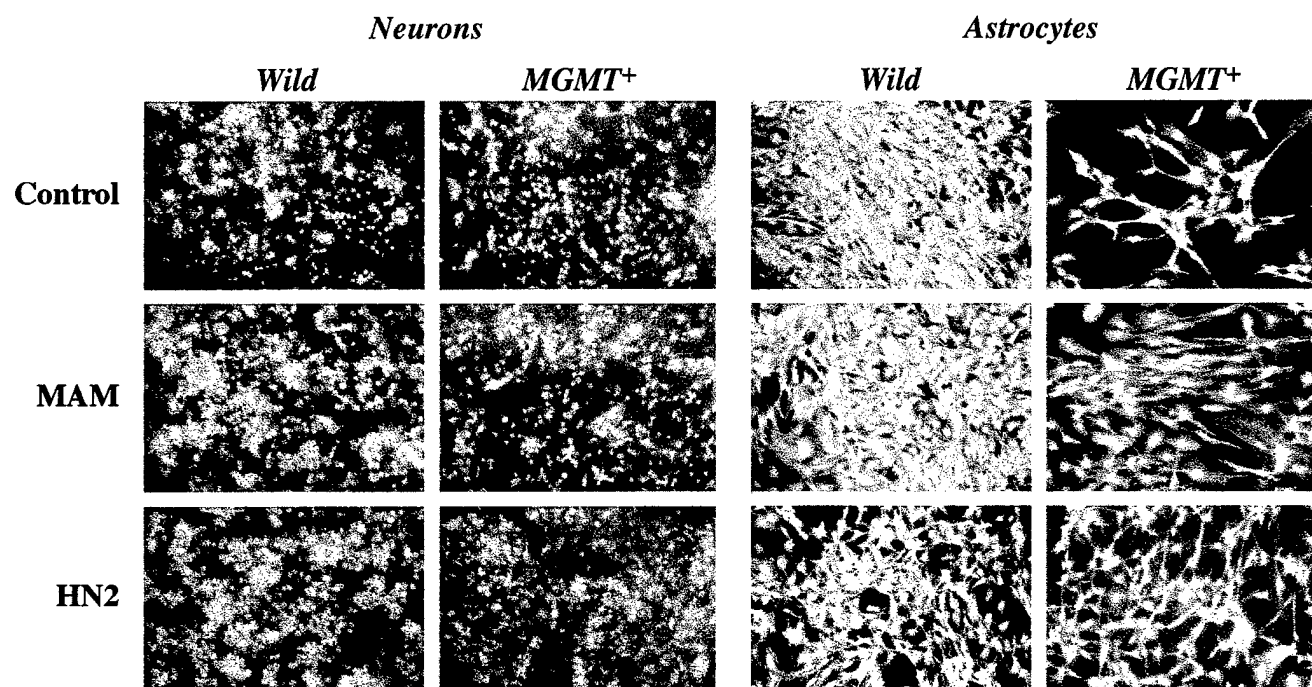


Figure 2. Photomicrographs of representative fields from wild type and $MGMT^+$ neuronal and astrocyte cell cultures treated with MAM or HN2. The relative toxicity of HN2 and MAM was examined in cerebellar neurons and astrocytes using the vital fluorochromes calcein-AM (*green fluorescence*) and propidium iodide (PI, *red fluorescence*). Cerebellar granule and astrocyte cell cultures from wild-type and $MGMT^+$ littermates were treated with 100 μ M MAM or 1.0 μ M HN2 for 24h, the culture media removed and the cultures incubated for 30 min with fluorochrome (calcein-AM and propidium iodide) containing culture media.

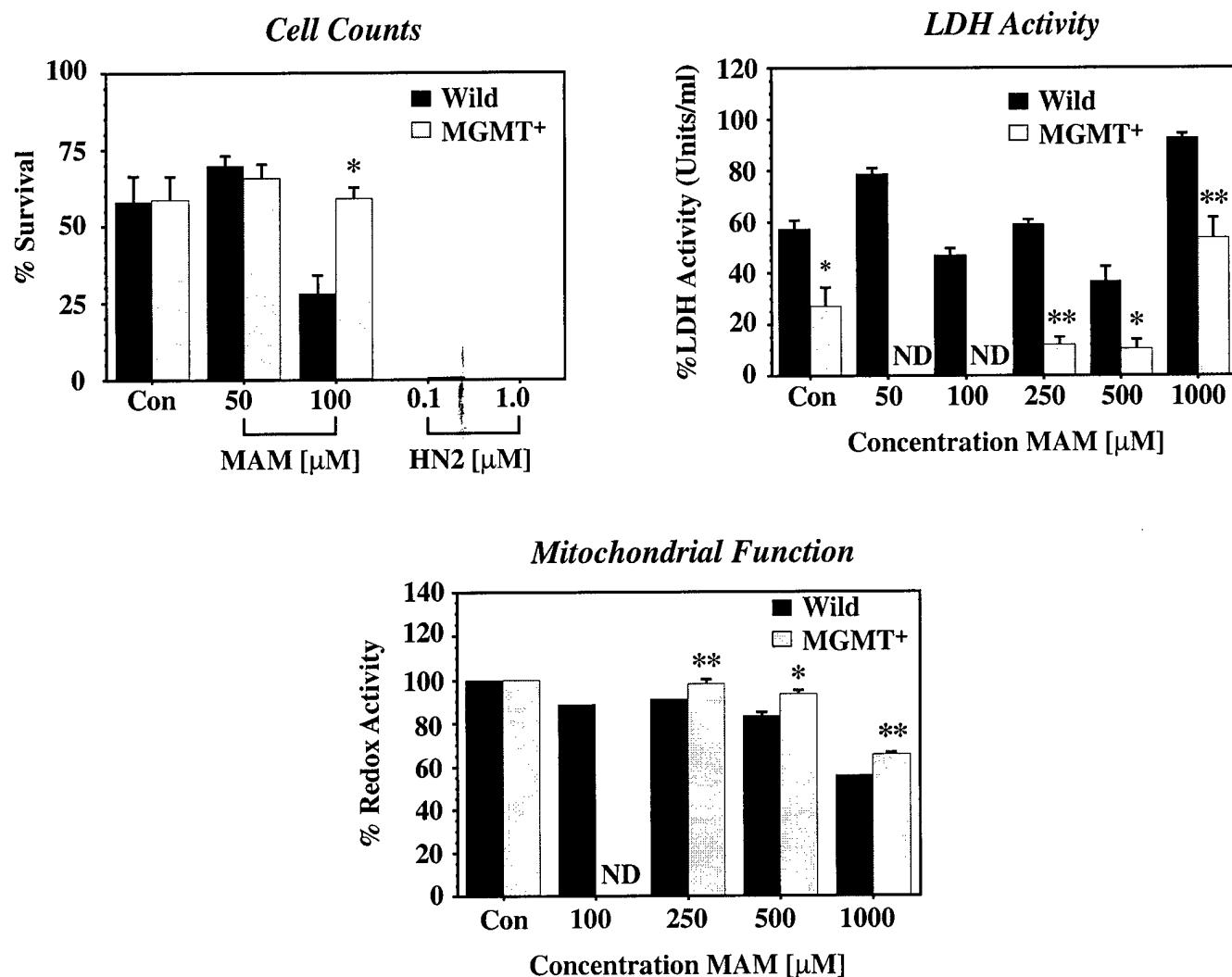
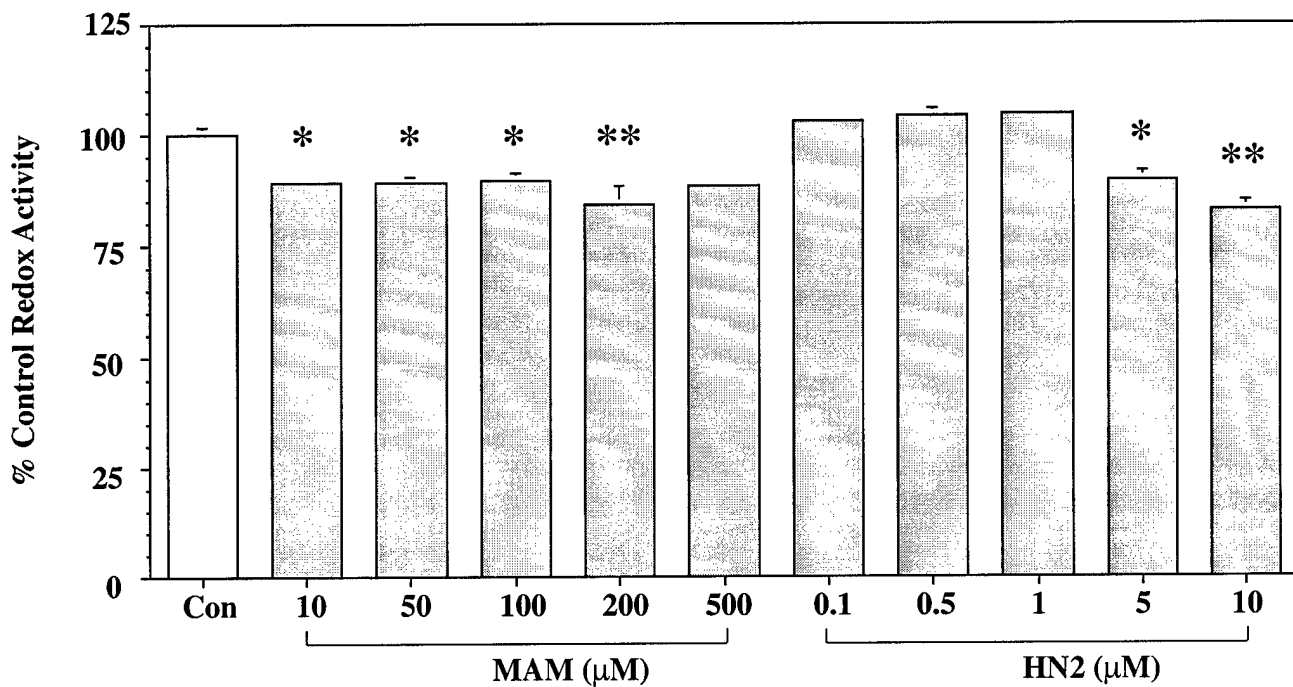


Figure 3. Viability of HN2 and MAM treated cerebellar neurons from wild type (Wild) and *Mgmt*-overexpressing (MGMT⁺) mice. Mouse cerebellar granule cell cultures were treated with various concentrations of HN2 (0.1 μ M, 1.0 μ M) or MAM (50 μ M - 1000 μ M) for 24h, an aliquot of the culture media removed and analyzed for LDH activity, the cultures incubated with Alamar Blue™ for 4h and examined for fluorescence. After 4h, the cultures were incubated with fluorochrome containing culture media (0.26 μ M calcein-AM and 3.0 μ M propidium iodide). Cell viability was assessed by counting the total number of live (*green*) and dead (*red*) cells of fluorescent photomicrographs taken from 3 random fields (~500-1000 cells/field) of each well, as previously described by Kisby *et al.* [2]. Values represent the mean \pm SEM ($n=4$). Significantly different from MAM treated wild-type cells (* $p < 0.05$, ** $p < 0.01$, ANOVA).

Cerebral Cortex Neuronal Cultures



Midbrain Neuronal Cultures

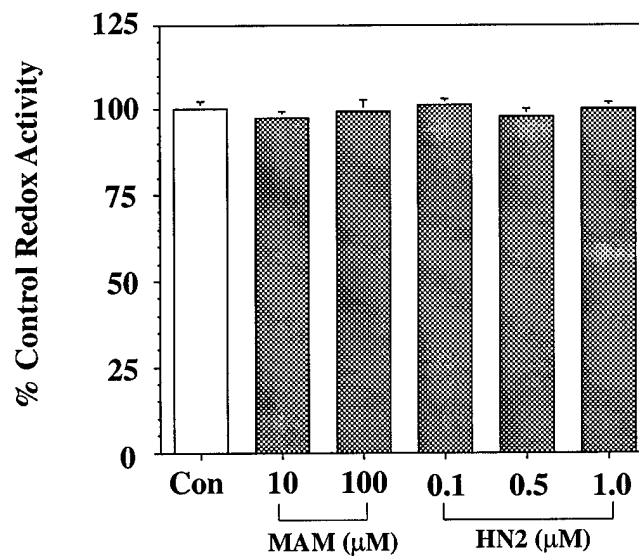


Figure 4. Viability of HN2 and MAM treated cerebral cortical and midbrain neurons from wild-type (C57BL/6) mice. Mouse cortical and midbrain neuronal cultures were treated with various concentrations of HN2 (0.1 μ M - 10 μ M) or MAM (10 μ M - 500 μ M) for 24h, the cultures incubated with Alamar Blue™ for 4h and examined for fluorescence. Values represent the mean \pm SEM ($n=4$) (except for 500 μ M MAM, $n=1$). Significantly different from controls (* $p < 0.05$, ** $p < 0.001$, ANOVA).

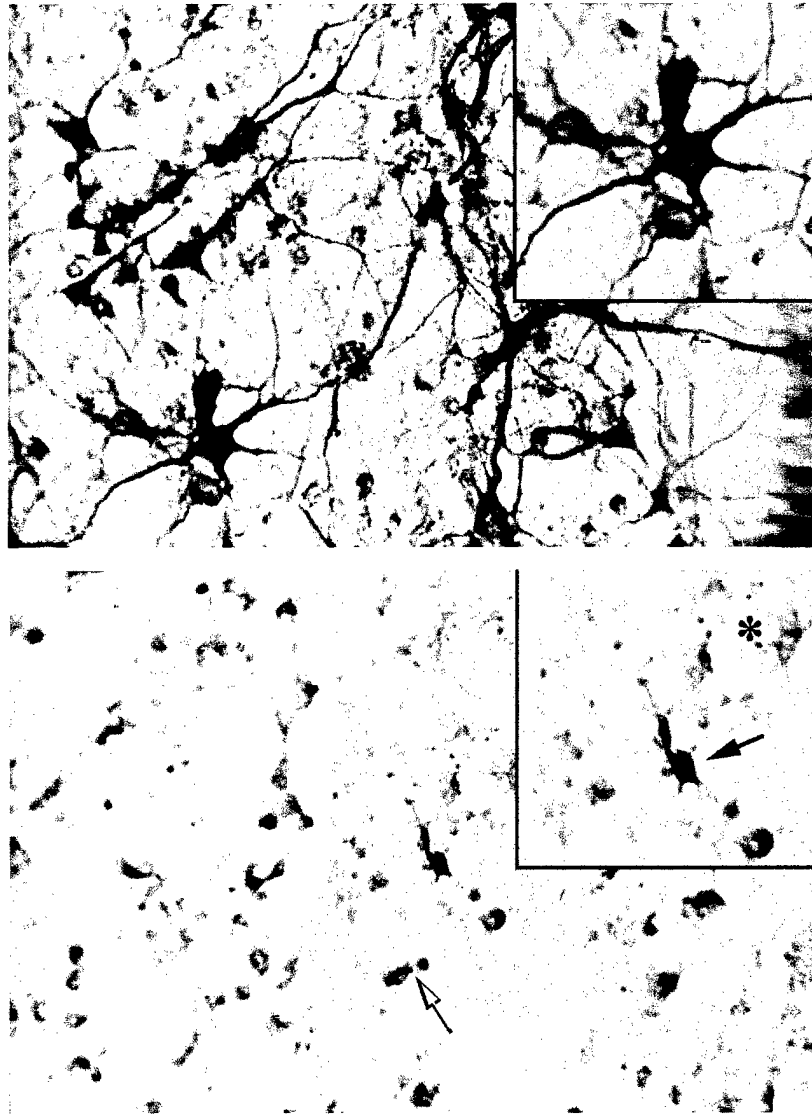


Figure 5. Photomicrographs of representative fields from wild-type cerebral cortical (*top*) and midbrain (*bottom*) neuronal cell cultures immunoprobed for markers of neuronal (microtubule associated protein, MAP2), dopaminergic (tyrosine hydroxylase, TH) or GABAergic (glutamic acid decarboxylase, GAD) cell types. Mature cortical and midbrain neuronal cultures (6-7 days *in vitro*) were fixed with 4% buffered paraformaldehyde in sucrose and the coverslips microwaved 3x for 5 min in 0.2 M Tris buffer (pH 10). Microwaved coverslips were rinsed with PBS (pH 7.4), incubated with blocking solution and immunoprobed overnight at 4°C with a monoclonal antibody to TH (1:1,000; INCSTAR). After removal of the primary antibody, the coverslips were incubated with a biotinylated secondary antibody (goat anti-mouse or anti-rabbit IgG) and processed using the avidin biotin-peroxidase method for immunohistochemical staining (Vectastain M.O.M.TM Peroxidase kit, Vector Laboratories), according to the manufacturer's instructions. Cortical neuronal cultures were incubated with Vector Nova-REDTM kit (*brownish-red*) to detect MAP2 immunostaining of neurons. Midbrain neuronal cultures were incubated with Vector Nova-REDTM kit to detect TH immunostaining and subsequently immunoprobed with anti-GAD and TrueBlueTM (KPL, *blue*) to detect GABAergic neurons. *Insets:* Higher magnification of

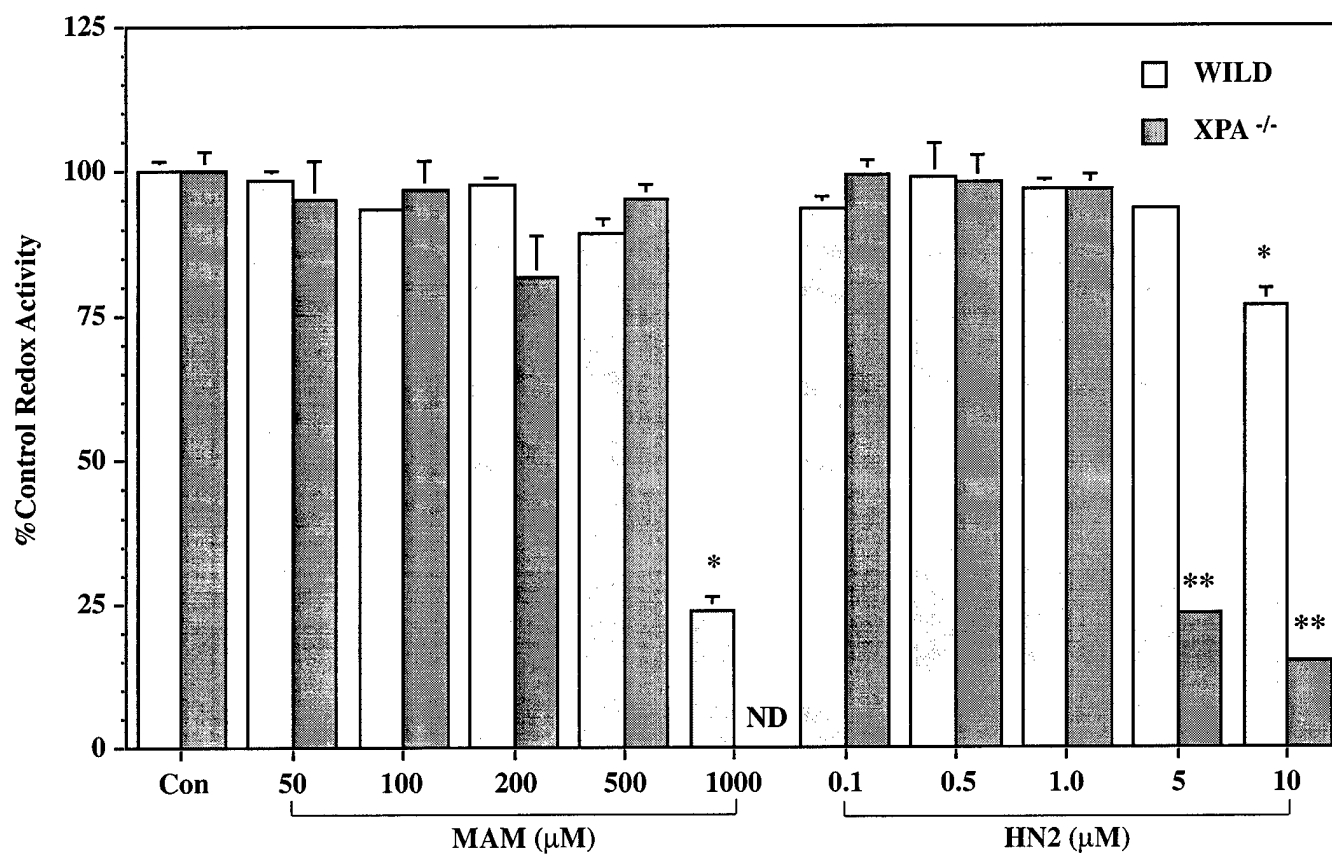


Figure 6. Viability of HN2 and MAM treated cerebellar neurons from wild type (WILD;C57BL/6) and XPA^{-/-} mice. Mouse cerebellar granule cell cultures were treated with various concentrations of HN2 (0.1 μM - 10 μM) or MAM (50 μM - 1000 μM) for 24h, the cultures incubated with Alamar Blue™ for 4h and examined for fluorescence. Values represent the mean ± SEM ($n = 4-12$). Significantly different from controls ($*p < 0.001$, ANOVA) or control and HN2 treated wild-type cells ($**p < 0.001$, ANOVA). ND = not determined.

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DIFFERENTIAL SENSITIVITY OF MUTANT DNA REPAIR NEURONS TO MUSTARD-INDUCED CYTOTOXICITY

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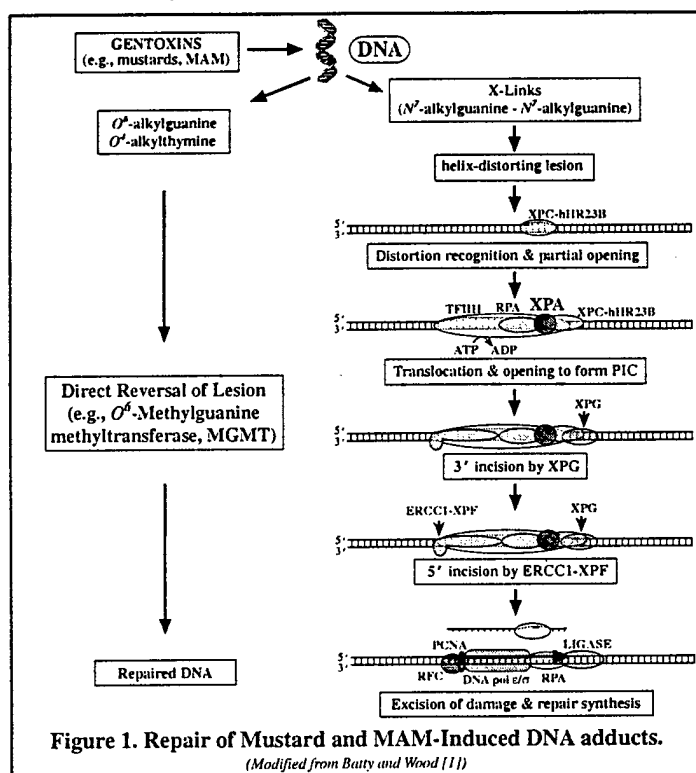
Abstract

Sulfur and nitrogen mustards induce neurobehavioral and neuropathological changes in animals. One potential mechanism for these changes is unrepaired DNA damage. To determine the importance of DNA repair for neuronal survival, we compared the cytotoxic properties of nitrogen mustard (HN2) in neuronal cultures from wild-type mice and mice that overexpress or are deficient for DNA repair enzymes. Cultures of cerebellar granule cell neurons from mice that overexpress *O*⁶-methylguanine methyltransferase (MGMT⁺) were treated for 24h with various concentrations of HN2 (0.1 μ M, 1.0 μ M) or methylazoxymethanol (MAM; 50 μ M-1000 μ M) and examined for viability by determining the number of surviving cells (calcein-AM and propidium iodide), membrane integrity (LDH activity) and mitochondrial function (Alamar BlueTM). Cerebellar neuronal cultures prepared from mice that are heterozygous (+/-) and homozygous (-/-) for the nucleotide excision DNA repair gene xeroderma pigmentosum group A (*Xpa*) were also treated for 24h with HN2 (0.1 μ M-5.0 μ M) or UV irradiation (2 J/m²- 10 J/m²) and examined for LDH activity. MGMT⁺ cerebellar neurons were protected from exposure to high concentrations of MAM (>100 μ M), but not protected from HN2-induced cell death. In contrast, XPA^{-/-} deficient cerebellar neurons were more sensitive to HN2 and UV irradiation exposures than comparably treated XPA^{+/+} deficient neurons. A similar sensitivity of XPA^{-/-} deficient cerebellar neurons to HN2 and UV irradiation suggests that cross-links play an important role in HN2-induced neuronal cell death while *O*⁶-methylguanine DNA adducts are more important in MAM-induced neuronal cell death. These findings are consistent with HN2 and MAM selectively targeting neurons *in vivo* possibly through a mechanism involving DNA damage.

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Introduction

Sulfur mustard has been used as a chemical warfare agent because it is extremely effective at incapacitating opposing forces by acutely injuring the eyes, respiratory tract, and skin tissue. Because sulfur mustard is lipophilic, it is rapidly absorbed through the skin or by inhalation to induce additional systemic injury to gastrointestinal, cardiac, and nervous tissue. CNS symptoms are the second most frequently reported delayed effects in persons exposed to mustard gas (WHO Reports 1984 and 1986). These reports are supported by recent findings of psychological disturbances in WWII veterans exposed to mustard gas [31]. Following intoxication by sulfur mustard, nerve cells are reportedly damaged in the cerebral cortex, basal



ganglia, hippocampus and cerebellum of humans and animals [28,32], probably because the agent concentrates in brain tissue [5,8,13,35]. Animals exposed to either mustard gas or nitrogen mustard also develop acute and delayed neurobehavioral changes consisting of ataxia, tremors, memory deficits, and motor dysfunction [13]. However, the molecular mechanism by which mustards induce nervous tissue injury is unknown. In non-nervous tissue, mustards interfere with glycolysis, antioxidant enzyme levels and glutathione levels [8,15], but these biochemical changes are considered secondary to its direct damage to DNA [3,20]. Since the cytotoxic and long-term effects of mustards in non-nervous tissue have been ascribed to its DNA-damaging properties, a similar mechanism may also explain its neurotoxic effects.

The major alkylation site of nucleic acids by mustards (nitrogen or sulfur) in mammals is the N^7 -position of guanine to produce N^7 -alkylguanine DNA adducts. Cross-links can form between adjacent (intrastrand-) or opposing (interstrand-) N^7 -alkylguanines, which may increase further the cytotoxic action of mustard-induced DNA damage. The N^7 -alkylguanine DNA adduct is repaired by the base-excision DNA repair protein 3-methylpurine DNA glycosylase (MPG, also termed alkylguanine DNA glycosylase, AAG) [24,25] while cross-links are repaired predominantly by nucleotide excision repair (NER) [23]. There are 5-10 proteins involved in base-excision DNA repair and over 20 different proteins involved in NER of DNA adducts in mammalian cells (see Figure 1). Xeroderma group A (XPA) was the first human NER protein demonstrated to have a preference for damaged DNA, and in its absence cells have absolutely no capacity for NER and no incisions are made. Therefore, neurons that lack XPA should be especially sensitive to HN2-induced cross-links while comparable cells that lack MPG should be especially sensitive to mustard-induced alkylguanine DNA adducts.

The present studies examine the relationship between mustard-induced cross-links and neuronal cell death by examining the sensitivity of neuronal cultures from XPA-deficient mice to the sulfur mustard analogue mechlorethamine (nitrogen mustard or HN2). For comparison, HN2

and methylazoxymethanol (MAM), a related DNA damaging agent that produces both *N*⁷-alkylguanine and *O*⁶-alkylguanine (repaired by methylguanine methyltransferase, MGMT) DNA adducts, and is strongly linked to a prototypical neurodegenerative disorder [10,17], were also examined for their neurotoxic properties in neuronal cultures from MGMT-overexpressing (MGMT⁺) mice. MGMT⁺ mice were used as a negative control for HN2-induced toxicity because HN2 does not produce *O*⁶-alkylguanine DNA adducts [20,29], while MAM does [7,10].

Methods

Mutant Mice

MGMT transgenic (MGMT⁺) mice were generated in C57Bl/6 x SJL mice using a chimeric gene consisting of the chicken β -actin promoter, the human MGMT cDNA, the poly A region from bovine growth hormone and the locus control region from the human CD2 gene [6]. XPA knock-out (XPA^{-/-}) mice were generated in C57Bl/6 mice by inserting a pMC1 neo/poly (A)-cassette into exon 4 of the XPA gene using ES cell techniques and the intercrossing of heterozygous (XPA^{+/-}) mice [9]. XPA^{-/-} mice were generated from intercrosses of XPA heterozygous mice (+/-) that were obtained from Dr. K. Tanaka (Osaka University Medical School, Osaka, Japan). The brains of MGMT⁺ and wild-type littermates were placed in Hibernate/B27 cell culture media (GibcoBRL) and sent overnight on ice to our laboratory (GK) for the preparation of cerebellar and astrocyte cell cultures. The brains of XPA^{-/-} mice were obtained by intercrossing XPA^{+/-} mice.

Cerebellar Neuronal and Astrocyte Cell Cultures

Primary mouse granule or astrocyte cell cultures were prepared from the cerebella of 6-8-day old neonatal C57Bl/6 (wild type), MGMT⁺, XPA^{+/-}, or XPA^{-/-} mice by dissociating the tissue in BSS with 0.1% trypsin as previously described [16]. Cell cultures were prepared by diluting the cells with high potassium (25 mM) containing plating media (DMEM with 10% FCS, 10% HS, 600 mg/ml glucose and 30 μ g/ml insulin) and seeded at a density of 0.1-0.2 x 10⁶ cells/well of a 24-well plate pre-treated for 1h with (*neurons*) or without (*astrocytes*) poly-D-lysine (100 μ g/ml). Cultures were fed twice weekly and maintained for 7 days before treatment with nitrogen mustard (HN2) or methylazoxymethanol acetate (MAM) for 24h.

Cell Counts

Cell cultures treated with control media or media supplemented with various concentrations of HN2 were examined by light and fluorescence microscopy for cell viability using the fluorochromes propidium iodide (PI) and calcein-AM as previously described by Kisby *et al.* [19]. Briefly, the media over control, HN2, or MAM treated cultures was removed, replaced with control media containing 3 μ M PI (a marker of cell damage) and 0.26 μ M calcein-AM (a marker of cell viability), and the cultures treated for 30 min in a humidified 5%CO₂/O₂ incubator. The fluorochrome containing media was aspirated, the cultures washed once with control media, photomicrographs taken of the cell monolayer by fluorescence microscopy, and 35 mm slides from each treatment group scanned and the numbers of calcein-AM and PI cells counted manually from each image. A second observer replicated all manual counts to ensure count accuracy and minimal interobserver variability (< 1%).

LDH Activity

Neuronal cell injury was determined 24h after treatment with HN2 or MAM by measuring LDH released from damaged cells by a standard kinetic assay for pyruvate. Briefly, media over control and treated cultures was removed, placed in a microcentrifuge tube, and stored at -90°C until analysis for LDH activity. An aliquot (100 μ l) of thawed cell culture media from control, HN2 or MAM treated neuronal cultures or plating media (blank) was incubated with 200 μ l NADH (25 mg/ml), 200 μ l sodium pyruvate (1 mg/ml) and 2.5 ml of 0.1 M phosphate buffer (pH 7.4) by measuring the decrease in absorbance of NADH at 340 nm over a 3-min time period

at 30-s intervals as previously described by Kisby *et al.* [16]. LDH activity was reported as Units/ml for samples and the values corrected by subtracting the blank value.

Mitochondrial 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 systems (including neurons) [34,36]. Mitochondrial function was determined in HN2 and MAM treated neuronal cell cultures by adding Alamar Blue™ to a final concentration of 10% and the cells incubated at 37°C in a humidified 5%CO₂/O₂ incubator for 4h. Viability was measured when the medium in control wells turned blue to pink, typically at ~4h for granule cell neurons. Alamar blue™ fluorescence was measured in a FLUOstar™ (BMG LabTechnologies) automated plate-reading fluorometer, with excitation at 530 nm and emission at 590 nm. Values are reported as % redox activity of controls.

MGMT Levels

Wild type and MGMT⁺ mice (6-8 days old) were killed by CO₂ inhalation, the brains dissected, and immediately snap frozen in liquid N₂. Frozen brain tissue was homogenized in lysis buffer [50 mM Tris (pH 7.5), 2 mM EDTA, 0.1 M NaCl, 1 mM dithiothreitol, and 200 μM phenylmethylsulfonyl fluoride], sonicated for 90 s on ice, centrifuged at 3,000 x g for 10 min at 4°C, and the supernatant analyzed for protein concentration (Bradford). Protein extracts (25 μg) were applied to a 12% SDS-PAGE gel, the gel electrophoresed, the samples transferred onto nitrocellulose membranes, and the membrane immunoprobed with a monoclonal antibody to MGMT (MT 22.1, a kind gift from Dr. T. Brent, St. Jude's Hospital, Memphis, TN). Bands were detected by incubating the membrane with an HRP-conjugated goat anti-mouse antibody, enhanced chemiluminescence (ECL™, Amersham), and phosphor image analysis of the blot as previously described by Kisby *et al.* [18]. Sets of biotinylated and kaleidoscope markers were also run along with the samples.

MGMT Activity

The activity of O⁶-methylguanine-DNA methyltransferase (MGMT) in brain tissue extracts was measured as the removal of the [³H]-methyl adduct from the O⁶-position of guanine in [³H]methyl DNA alkylated with [³H]N-methylnitrosourea ([³H]MNU) as previously described [11]. An aliquot of wild-type (250 μg) or MGMT⁺ (100 μg) brain tissue extract was incubated with [³H]methyl containing DNA in assay buffer for 60 min at 37°C [12]. The reaction was stopped by the addition of 50% TCA and samples incubated at 4°C for 30 min. The precipitate was collected by centrifugation, washed with 80% ethanol and the pellet hydrolyzed with 0.1 N HCl at 80°C for 60 min. The reaction was stopped by the addition of 0.01 M Tris, the hydrolyzed purines present in the supernatant separated by HPLC and the radioactivity quantified using a liquid scintillation counter.

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 and the Scheffe comparison method. A probability value of $p < 0.05$ was considered significant unless otherwise noted.

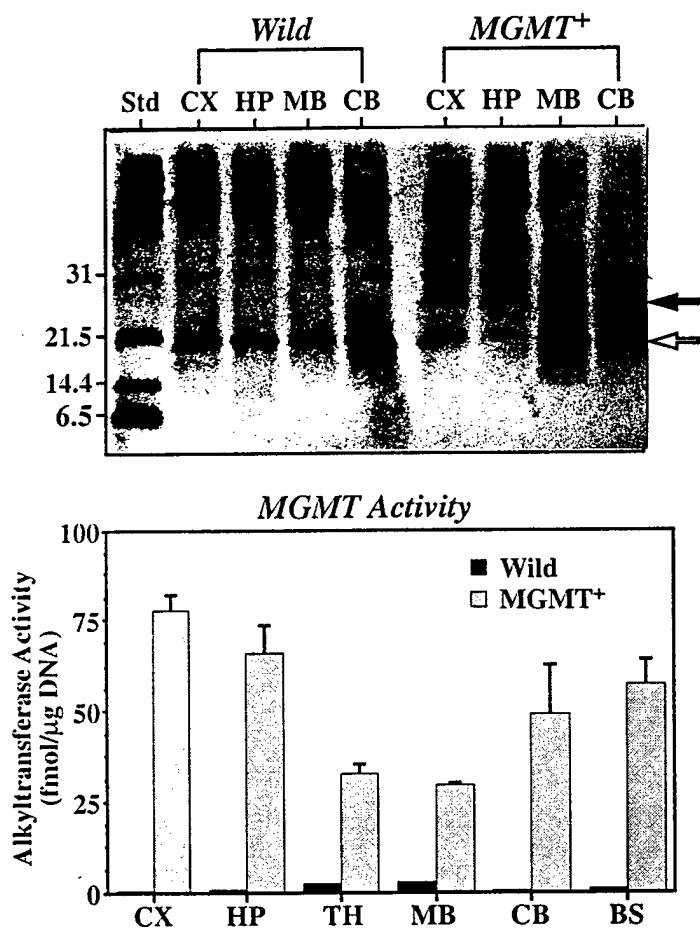
Results

MGMT-Overexpressing Neuronal and Astrocyte Cultures

Initial studies compared the relative toxicity of the sulfur mustard analogue mechlorethamine (HN2, nitrogen mustard) and the neurotoxin methylazoxymethanol (MAM) among cerebellar neuronal and astrocyte cell cultures prepared from mice that overexpress human O⁶-

methylguanine-DNA alkyltransferase (MGMT⁺). hMGMT (~22-23 kDA) was detected (*black arrow*) by western blotting studies in the cerebellum, hippocampus, and midbrain of MGMT⁺ mice, but not in brain tissue of wild-type mice (Figure 2, *top*). As expected, the endogenous mouse protein (mMGMT, *white arrow*) was detected in both wild type and MGMT⁺ mice. Consistent with these observations, MGMT activity was higher in the cerebellum (~50-fold) and other brain regions (12 to 80-fold) of MGMT⁺ mice when compared with similar brain regions of wild-type mice (Figure 2, *bottom*). Therefore, cerebellar neurons and astrocytes of MGMT⁺ mice have elevated levels and activity of MGMT.

Figure 2. *O*⁶-Methylguanine methyltransferase (MGMT) levels and activity in different brain regions of wild type (Wild) and MGMT overexpressing (MGMT⁺) mice. Postnatal (6-8 day old) brain tissue of wild and MGMT⁺ mice were homogenized in lysis buffer, centrifuged, and the tissue extracts analyzed for MGMT⁺ levels or MGMT activity. Protein extracts (25 μ g) were



electrophoresed on a SDS-PAGE, transferred to a nitrocellulose membrane, and the membrane blocked and immunoprobed with a monoclonal antibody to MGMT (MT 22.1). Specific binding of MGMT was visualized using a HRP-conjugated goat anti-mouse antibody (1:1000) and enhanced chemiluminescence. For MGMT activity, an aliquot of protein extract (100 μ g or 250 μ g) was incubated with [³H]methyl DNA and the amount of [³H] *O*⁶-methylguanine determined by HPLC with liquid scintillation counting. Values are the mean \pm SEM. Std= biotinylated markers. hMGMT (*black arrow*) and mMGMT (*white arrow*). CX=cortex; HP=hippocampus; TH=thalamus; MB= midbrain; BS=brainstem.

The relative toxicity of HN2 and the genotoxin MAM were examined in cerebellar neurons and astrocytes using the vital fluorochromes calcein-AM (*green fluorescence*) and propidium iodide (PI, *red fluorescence*) (Figures 3 and 4, *next page*). Calcein-AM is a cell permeant

dye that is taken up by active mitochondria while a loss in membrane integrity leads to uptake of PI and nuclear staining of dying cells. Cell loss was extensive in wild-type mouse cerebellar neurons treated with 100 μ M MAM (>70%) and 1.0 μ M HN2 (>90%). In contrast, MGMT⁺ cerebellar neurons were protected from 100 μ M MAM, but not from 1.0 μ M HN2-induced cytotoxicity. Additional studies using lower concentrations of HN2 will be necessary to confirm these findings. MGMT⁺ cerebellar neurons were also protected at higher concentrations of MAM as indicated by the significantly lower LDH activity measured in cell culture media (Figure 4, *right*) or the higher mitochondrial redox activity (Figure 4, *bottom*) than comparably treated wild-type cells.

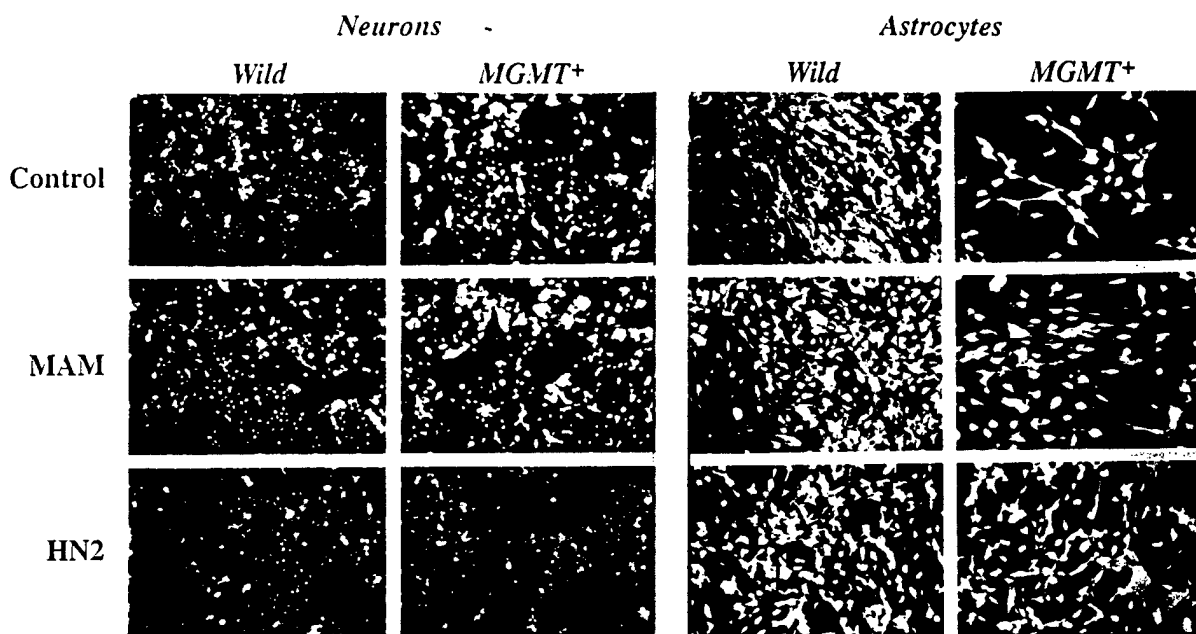


Figure 3. Photomicrographs of representative fields from wild type and MGMT⁺ neuronal and astrocyte cell cultures treated with MAM or HN2. Cerebellar granule and astrocyte cell cultures from wild type and MGMT⁺ littermates were treated with 100 μ M MAM or 1.0 μ M HN2 for 24h, the culture media removed and the cultures incubated for 30 min with fluorochrome (calcein-AM and propidium iodide) containing culture media.

In comparison to mouse cerebellar neurons, wild type or MGMT⁺ astrocytes were relatively insensitive to 100 μ M MAM or 1.0 μ M HN2-induced cytotoxicity (Figure 3, right). In fact,

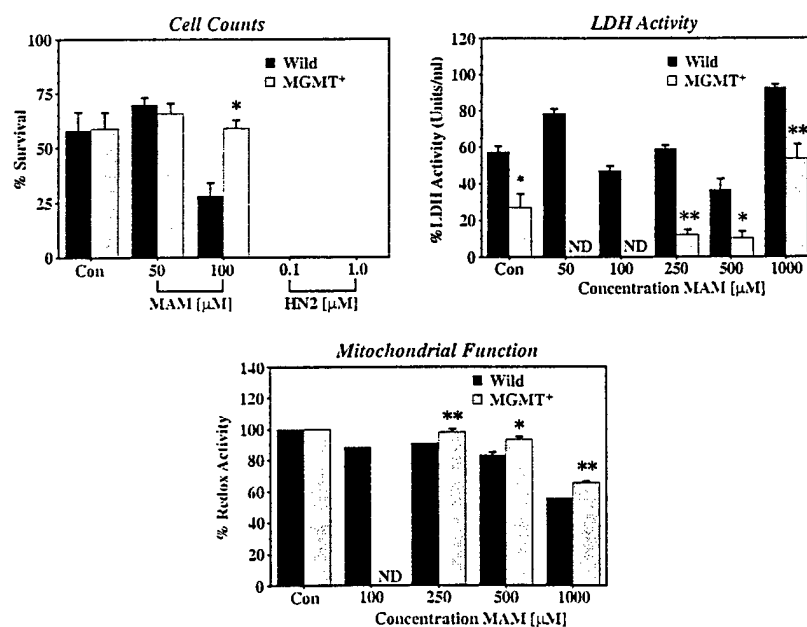


Figure 4. Viability of HN2 and MAM treated cerebellar neurons from wild type (Wild) and *Mgmt*-overexpressing (MGMT⁺) mice. Mouse cerebellar granule cell cultures were treated with various concentrations of HN2 (0.1 μ M, 1.0 μ M) or MAM (50 μ M - 1000 μ M) for 24h, an aliquot of the culture media removed and analyzed for LDH activity, the cultures incubated with Alamar BlueTM for 4h and examined for fluorescence. After 4h, the cultures were incubated with fluorochrome containing culture media

(0.26 μ M calcein-AM and 3.0 μ M propidium iodide). Cell viability was assessed by counting the total number of live (*green*) and dead (*red*) cells of fluorescent photomicrographs taken from 3 random fields (~500-1000 cells/field) of each well, as previously described by Kisby *et al.*

[16]. Values represent the mean \pm SEM ($n = 4$). Significantly different from MAM treated wild-type cells (* $p < 0.05$, ** $p < 0.01$, ANOVA).

concentrations greater than 10 μM HN2 or 500 μM MAM were required to induce significant cell loss (>50%) in astrocyte cultures (*data not shown*). Similar results have also been reported for studies that compared the relative toxicity of HN2 in rat cerebellar neuronal and astrocyte cell cultures [19], an effect probably related to the high basal levels of DNA repair [21].

XPA Deficient Neuronal Cultures

The XPA gene codes for a nucleotide excision DNA repair (NER) protein that is responsible for recognizing bulky adducts and x-links [2,30](*see also* Figure 1). Since x-links are considered a primary mechanism of sulfur and nitrogen mustard-induced cell death, we compared the sensitivity of XPA deficient ($XPA^{-/-}$) with partially defective XPA ($XPA^{+/-}$) cerebellar neurons after exposure to HN2 (Figure 5).

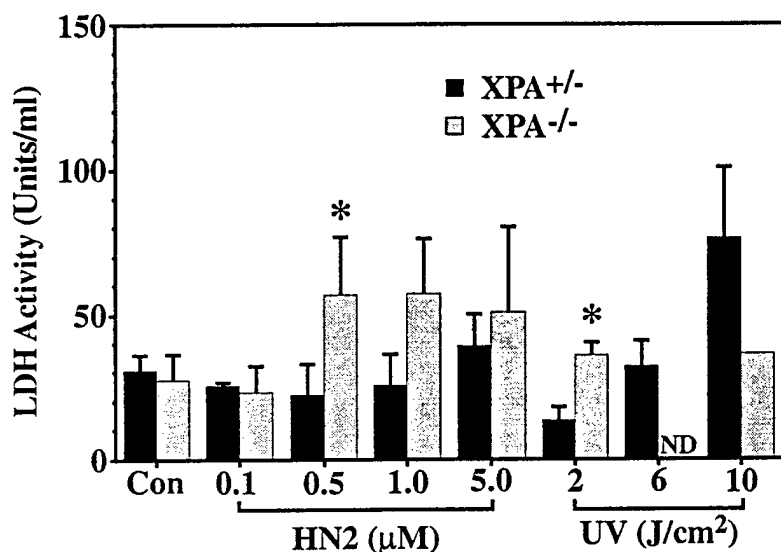


Figure 5. Lactate dehydrogenase (LDH) release from HN2 and MAM treated cerebellar neurons of mice heterozygous ($XPA^{+/-}$) or homozygous ($XPA^{-/-}$) for XPA. Mouse cerebellar granule cell cultures were treated with various concentrations of HN2 (0.1 μM , 0.5 μM , 1.0 μM , 5.0 μM) for 24h or UV irradiation (2 J/cm^2 , 6 J/cm^2 , 10 J/cm^2), the cell culture media removed and

assayed for LDH activity as previously described by Kisby *et al.* (14). Values represent the mean \pm SEM ($n = 4$). ND = not determined. * Significantly different from 0.5 μM HN2-treated or UV-irradiated $XPA^{+/-}$ cells ($p < 0.05$, ANOVA).

LDH activity (an indicator of loss of membrane integrity) was 1.5 to 3.0-fold higher in HN2-treated $XPA^{-/-}$ cerebellar neurons than comparably treated $XPA^{+/-}$ neuronal cultures. LDH activity was also ~3.0-fold higher in low-dose UV-irradiated $XPA^{-/-}$ deficient cerebellar neurons than comparably treated $XPA^{+/-}$ cerebellar neurons. Cerebellar neurons of $XPA^{-/-}$ deficient mice have been reported to respond in a similar manner to UV irradiation [9].

Discussion

Depletion of glutathione, generation of reactive oxygen species, and the formation of stable DNA adducts are considered key molecular mechanisms by which mustard chemical warfare agents induce tissue injury [33]. Recent *in vitro* studies demonstrate that DNA damage and changes in DNA repair occur soon (~ 1-2h) after exposure to sulfur mustard [4,14,27] suggesting that DNA damage precedes mustard-induced tissue injury. We propose that nervous tissue injury is induced in a similar manner by mustards. The purpose of the present studies was to test this hypothesis by comparing the cytotoxicity of the sulfur mustard analogue nitrogen mustard (HN2) in neuronal cultures from mice with perturbed DNA repair. Methylazoxymethanol

(MAM), a related alkylating agent, was also included to specifically examine the relationship between the type of HN2-induced DNA damage and neurotoxicity.

Initial studies examined whether the neurotoxicity of HN2 and MAM differs in cerebellar neurons that overexpress MGMT. Since MAM produces *O*⁶-methylguanine DNA adducts, MGMT⁺ cerebellar neurons should exhibit resistance to MAM, but not to HN2. As proposed, neuronal survival, membrane integrity and mitochondrial function were essentially maintained in cerebellar MGMT⁺ neurons treated with MAM but not after HN2 treatment. These findings suggest that MGMT protects neurons from MAM-induced cytotoxicity, but not from HN2-induced cytotoxicity. The increased resistance of MGMT⁺ neurons to high concentrations of MAM may be explained by the elevated repair of *O*⁶-methylguanine DNA adducts, a lesion that may be responsible for MAM-induced neuronal cell death. In support of this notion, astrocytes were relatively insensitive to MAM and HN2 because this CNS cell type is reported to contain high levels of MGMT [17,21] and possibly other DNA repair proteins (e.g., NER). In contrast, overexpressing MGMT failed to protect cerebellar neurons from HN2-induced cytotoxicity probably because this genotoxin produces predominantly *N*⁷-alkyl DNA adducts and x-links, DNA adducts that are not repaired by MGMT.

While sulfur and nitrogen mustards [22,26] react with DNA to generate x-links and *N*⁷-alkylguanine DNA adducts, recent studies suggest a strong correlation between the formation of mustard-induced x-links and cytotoxicity [22,26]. Consequently, cerebellar neurons that are defective in the repair of x-links should be especially sensitive to mustards. Cerebellar neurons from mice defective in the nucleotide excision repair (NER) protein XPA, a key protein that repairs x-links and UV DNA damage [1], were examined for their sensitivity to HN2. The increased sensitivity of NER-deficient neurons to HN2 and UV-irradiation suggests that the inefficient removal of x-links play an important role in mustard-induced neurotoxicity. Additional studies are required with neuronal and astrocyte cell cultures of XPA^{-/-} and other DNA repair-deficient mice to determine if mustard-induced neurotoxicity is dependent upon the inefficient removal of a specific mustard-induced DNA adduct (i.e., *N*⁷-alkylguanine vs. x-links).

In summary, the findings of the present work suggest that perturbing neuronal DNA repair (i.e., NER or MGMT) can influence the cytotoxicity of mustards and related environmental genotoxin agents. More importantly, these preliminary studies suggest for the first time that DNA damage is likely to play a key role in mustard-induced neurotoxicity. Additional studies are underway to confirm these findings and to explore the potential role of other DNA repair pathways (i.e., base-excision) in protecting neurons from mustard-induced toxicity. Results from these studies will provide a better understanding of the therapeutic measures that may be required to counteract the neurotoxic effects of mustards.

Acknowledgements

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