Levels of 4-Hydroxynonenal and Malondialdehyde Are Increased in Brain of Human Chronic Users of Methamphetamine¹

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Running title: 4-Hydroxynonenal/Malondialdehyde in Methamphetamine Users

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Text pages: 15
Tables: 3
Figures: 1
References: 40

Number of words
Abstract: 222
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Abbreviations: BSTFA, N,O-bis(trimethylsilyl)trifluoroacetamide; GC-MS, gas chromatography-mass spectrometry; HNE, 4-hydroxynonenal; MA, methamphetamine; MDA, malondialdehyde; NICI, negative ion chemical ionization; O-PFB, O-(2,3,4,5,6-pentafluorobenzyl; PMI, postmortem interval; TBARS, thiobarbituric acid reactive substances; TMCS, trimethylchlorosilane, TMS, trimethylsilyl

Recommended section assignment: Toxicology
Abstract

Animal studies suggest that the widely used psychostimulant drug methamphetamine (MA) can harm brain dopamine neurones, possibly by causing oxidative damage. However, evidence of oxidative damage in brain of human MA users is lacking. We tested the hypothesis that levels of two “gold standard” products generated from lipid peroxidation, 4-hydroxynonenal (one of the most reactive lipid peroxidation aldehyde products) and malondialdehyde, would be elevated in postmortem brain of 16 dopamine-deficient chronic MA users as compared with those in 21 matched control subjects. Derivatised aldehyde concentrations were determined by gas chromatography-mass spectrometry. In the MA group we found significantly increased levels of 4-hydroxynonenal and malondialdehyde in the dopamine-rich caudate nucleus (by 67% and 75%, respectively) and to a lesser extent in frontal cortex (48% and 36%, respectively) but not in the cerebellar cortex. Approximately one-half of the MA users had levels of 4-hydroxynonenal falling above the upper limit of the control range in caudate and frontal cortex. A subgroup of MA users with high brain drug levels had higher concentrations of the aldehydes. Our data suggest that MA exposure in the human causes, as in experimental animals, above-normal formation of potentially toxic lipid peroxidation products in brain. This provides evidence for involvement of oxygen-based free radicals in the action of MA in both dopamine-rich (caudate) and poor (cerebral cortex) areas of human brain.
Introduction

The amphetamines, amphetamine and methamphetamine (MA), are psychostimulant drugs that can be abused for their euphoric effects, whereas at low doses amphetamine is clinically useful for the management of conditions such as attention-deficit hyperactivity disorder (see Ricaurte et al., 2005). The behavioural actions of amphetamines are related to some extent to the ability of the drugs to cause release in brain of the neurotransmitter dopamine (Laruelle et al., 1995). This feature probably explains in large part our postmortem brain observation of markedly decreased dopamine levels in striatum (caudate, putamen) of chronic MA users who all tested positive for the drug at the time of death (Wilson et al., 1996; Moszczynska et al., 2004).

Because of evidence from experimental animal studies that high doses of MA can harm brain dopamine nerve endings (see McCann and Ricaurte, 2004 for a review), there is public health concern that the drug could damage dopamine neurones in humans, even when administered under medical supervision for the treatment of attention-deficit hyperactivity disorder (Ricaurte et al., 2005). However, the absence of Parkinsonism reported as a characteristic of chronic MA exposure in human drug users indicates that such damage to nigrostriatal dopamine neurones, if present, could not be severe (see Moszczynska et al., 2004 and Discussion). There also exists in the imaging literature reports of a variety of different structural abnormalities in the brain of some self-reported human MA users (cf. Chang et al., 2005). Although a consistent pattern of structural damage has yet to emerge from the somewhat contradictory data, findings in some drug users of changes in brain that do not receive intense dopaminergic innervation (e.g., cerebral cortex, hippocampus, Thompson et al., 2004) allow for the possibility that some aspects of MA toxicity might not be dopamine-related (Gluck et al., 2001; see also Yuan et al., 2001).
One approach to the question of MA neurotoxicity in human brain is the measurement of indices of oxidative stress and damage. Animal data suggest that MA-induced damage to dopamine nerve endings could be explained by excessive oxidative stress possibly related to the formation of dopamine-derived oxidation products (see Davidson et al., 2001 for review). As short-lived reactive oxygen species cannot yet be directly measured in human brain, the question could be addressed by measurement of levels of antioxidants (which might be expected to show compensatory changes) or concentrations of oxidatively damaged proteins, lipids, or DNA or products thereof. Previously, we showed that in postmortem brain of human MA users levels of key antioxidant defenses were relatively preserved, but with some markers showing modest changes suggestive of a compensatory response to oxidative stress (Mirecki et al., 2004).

We have now extended our biochemical analyses to include measurement, in postmortem brain of MA users, of levels of 4-hydroxynonenal (HNE) and malondialdehyde (MDA), two aldehydic products of oxidatively damaged lipids that have historically served as markers for lipoperoxidation (Esterbauer et al., 1991). We hypothesized that, as in most of the experimental animal studies in which MDA or MDA-like thiobarbituric acid reactive substances (TBARS) were reported to be increased in brain of MA-treated rodents (Yamamoto and Zhu, 1998; Jayanthi et al., 1998; Kim et al., 1999; Acikgoz et al., 1998; 2000; Kita et al., 2000; Wan et al., 2000; Gluck et al., 2001; Flora et al., 2002; Iwashita et al., 2004), levels of both oxidative markers would be above-normal in the dopamine-rich caudate nucleus of human MA users, but less markedly increased (if at all) in brain areas having very low (frontal and cerebellar cortices) concentrations of dopamine. Our human postmortem data provide additional support to the notion based on animal models that MA can cause oxidative damage in human brain.
Materials and methods

Brain Materials. Postmortem brain from a total of 21 controls (19 males and 2 females) and 16 chronic users of methamphetamine (11 males and 5 females) was obtained from medical examiner offices in the US using a standardized protocol. Drug histories and patient information are summarized in Table 1, with the information having been previously reported (Wilson et al., 1996; Tong et al., 2003; Moszczynska et al., 2004). The regional distribution of MA and its metabolite amphetamine in autopsied brain has been reported for 12 of the 16 MA cases (Kalasinsky et al., 2001). Dopamine levels in the caudate have been reported for all of the MA cases (see Table 1 and Moszczynska et al., 2004). This study was approved by Institutional Review Board of the Centre for Addiction and Mental Health at Toronto. There were no statistical differences in age (control, 33.6 ± 2.2 years; MA, 32.4 ± 2.0 years; mean ± SEM) or postmortem intervals (PMI, interval between death and freezing of the brain; control, 13.1 ± 1.3 hours; MA, 15.3 ± 1.7 hours) between the control and MA users (Student’s two-tailed t-tests).

At autopsy, one half-brain was fixed in formalin fixative for neuropathological analysis, whereas the other half was immediately frozen until dissection for neurochemical analysis. Blood samples were obtained from all of the MA users and the control subjects for drug screening. Scalp hair samples for drug analyses could be obtained from 18 of the 21 controls and 11 of the 16 MA users. Levels of drugs of abuse in blood and other bodily fluids were measured by the local medical examiner whereas drug analyses in brain and hair samples were conducted at the Armed Forces Institute of Pathology (KK, Washington, DC, USA). All MA users met the following selection criteria: (a) presence of MA on toxicology screens in blood, autopsied brain, and, where available, sequential scalp hair samples; (b) absence of any other drugs of abuse in these tissues (and absence of blood ethanol); (c) evidence from the case records and structured
interviews with medical examiner investigators, next of kin, and informants, of use of MA for at least 1 year before death; and (d) absence of neurological illness or brain pathology unrelated to use of the drug. The finding of MA (and metabolite amphetamine) in sequential hair samples in the absence of other psychostimulant and opiate drugs provides the strongest evidence that the subjects did not use these non-MA drugs of abuse in the recent (months) past. Examination of the case information disclosed no report of neurological illness (i.e., Parkinsonism) in any of MA users, although formal neurological testing had not been conducted.

All control subjects were neurologically normal and had no evidence of brain pathology on neuropathological examination. All had no history of drug use and tested negative for drugs of abuse in blood, autopsied brain, and, in the 18 subjects in which scalp hair was available, sequential scalp hair samples. The cause of death for the controls were cardiovascular disease (n = 12), trauma (n = 7), drowning (n = 1), and leukemia (n = 1).

**Aldehyde derivatisation and extraction.** HNE and MDA were measured by gas chromatography negative ion chemical ionization mass spectrometry (GC-NICI-MS) using minor modifications of the method of Luo et al. (1995). All samples were analyzed in replicate by an unbiased observer (M.Y.) without knowledge of the source of the brain tissues. Standard solutions of 4-hydroxy-2-trans-nonenal (Calbiochem, La Jolla, CA, USA), malondialdehyde (Sigma, Louis, USA), trans-4-hydroxy-nonenal-5,5,6,7,7,8,8,9,9,9-d_{11} HNE(d_{11}) (CDN Isotopes, Quebec, Canada; synthesised by the acid hydrolysis of the diethyl and dimethyl acetylated parent compound) and deuterated benzaldehyde-2,3,4,5,6-d_{5} (CDN Isotopes, Quebec, Canada) were used as deuterated internal and non-deuterated external calibration standards.

To 50-70 mg of frozen brain tissue (800 pmol) benzaldehyde-2,3,4,5,6-d_{5} and HNE(d_{11}) internal standards were added and homogenised by sonication (Bronwill Biosonik, USA) on ice
in 800 µl deionised H$_2$O containing EDTA (400 µM) (Sigma, Louis, USA), butylated hydroxy
toluene (20 µM) (Sigma, Louis, USA) and desferal (20 µM) (Novartis, Dorval, Quebec,
Canada). Aldehydes were derivatised by the addition of O-(2,3,4,5,6-pentafluoro-benzyl)(O-
PFB)hydroxylamine hydrochloride (Sigma, Louis, USA) (0.05 M, 200 µl) and incubating for 60
min at room temperature. Tissue proteins were precipitated with 1 ml of ethanol and O-PFB-
oxime aldehyde derivatives were hexane-extracted by the addition of 2 ml hexane, vortex-mixing
for 1 min and centrifugation for 5 min at 3000 rpm. The top hexane layer was transferred into
clean borosilicate tubes and the hexane extraction was repeated. The two hexane phases were
combined and residual moisture was removed with 0.5 g of sodium sulphate. The hexane solvent
was evaporated under a stream of nitrogen gas and the residue was incubated with 50 µL of N,O-
bis(trimethylsilyl)trifluoroacetamide (BSTFA) in 1% trimethylchlorosilane (TMCS) (Pierce
Rockford, USA) for 15 min at 80°C forming the trimethylsilyl (TMS) ether derivatives of HNE.
50 µL of hexane was added to each sample, vortex-mixed and transferred into GC auto sampler
vials and 1 µL was analysed by GC-NICIMS.

**Chromatography.** Derivatised aldehydes were analysed using an Agilent Technologies Inc.
5973N mass selective detector (MSD) GC-MS equipped with an HP-5ms capillary column
(0.25mm internal diameter, 0.25 µm film thickness, 30m length). Sample introduction was
conducted using pulsed-splitless mode with helium carrier gas set at a constant flow rate of 1
ml/min. Mass spectrometry was performed in the negative-ion chemical ionization mode with
methane (1.2 ml/min) as the reagent gas. An initial oven temperature of 50°C was maintained for
1 min followed by an increase to 240°C at 10°C/min and to 300°C at 20°C/min and maintained
at 300°C for 1 min. The injector temperature was maintained at 250°C, the GC to MS transfer
line was maintained at 280°C and the source temperature was set at 150°C.
Derivatised aldehyde chromatographs were acquired in single ion monitor (SIM) mode by recording m/z 333.1 and 283.3 for HNE-PFB-TMS derivative, m/z 204.1 for MDA-PFB derivative, 344.1 and 294.3 for HNE(d11)-PFB-TMS derivative and m/z 286.1 for benzaldehyde-2,3,4,5,6-d5–PFB derivative.

HNE and MDA levels in tissues were quantitated by dividing the HNE or MDA chromatographic peak area by the internal standard peak area (i.e. HNE/HNE(d11) and MDA/benzaldehyde-2,3,4,5,6-d5) and interpolating the result via comparison with a standard curve constructed from ratios of known amounts of HNE/HNE(d11) and MDA/benzaldehyde-2,3,4,5,6-d5. The matrix used to construct this 7 point curve (linearity curve) was homogenised brain tissue. To estimate recovery a standard mixtures of HNE, MDA (including internal standards HNE (d11) and benzaldehyde-2,3,4,5,6-d5) was added to brain tissue homogenate. Following the procedures described above the levels of HNE and MDA were quantified in both tissue and spiked tissue samples and % recovery calculated.

Statistical analyses. Statistical analyses were performed by using the StatSoft STATISTICA 7.1 (Tulsa, Oklahoma, USA). Differences in levels of the aldehydes between control and MA cases and between the brain regions were analyzed by one-way or two-way ANCOVA, with PMI as the covariate, followed by post hoc Bonferroni tests. Differences in percentage changes of the aldehydes between the brain regions were analyzed by paired Student’s t-tests. Correlations were examined by Pearson product moment correlation or Spearman ranking order correlation as indicated in the text.
Results

Validation of the GC-MS assay of HNE and MDA. Standard curves were linear (r>0.99) from 50 pmol to 1600 pmol HNE and MDA. The coefficient of variation of replicate samples (within run) was 3.4% (HNE) and 4.2% (MDA). Mean recovery was 87% for HNE and 93% for MDA. Signals at the retention times of HNE and MDA were always present in the ion chromatograms obtained on analyzing brain tissue samples (10-100mg tissue weight, data not shown). In order to confirm that these signals originated from endogenous aldehydes, spiked and basal brain tissue samples were analysed focusing on three ions for each aldehyde, m/z 152, 283, and 333 for HNE, and m/z 204, 281, and 442 for MDA respectively and the ion ratios compared. Comparison of the ratios of the signal intensities for both HNE and MDA ions indicated that m/z 333 (for HNE) and m/z 204 (for MDA) were the most suitable ions for quantification within tissues.

Possible influence of age and PMI on brain levels of HNE and MDA. No statistically significant correlations (Pearson correlation coefficient r) were found between the age of the subjects, either controls or MA users, and levels of either HNE or MDA. Significant correlations between levels of HNE or MDA and PMI were limited to the caudate nucleus of the control subjects (HNE: r = 0.58, p = 0.01; MDA: r = 0.54, p = 0.01) and the frontal cortex of the MA users (HNE: r = 0.51, p = 0.04).

Increased levels of HNE and MDA in brain of human chronic MA users. Regional levels of HNE and MDA in the MA users were significantly correlated (Pearson) with each other (caudate: r = 0.60, p = 0.01; frontal cortex: r = 0.90, p < 0.001; cerebellar cortex: r = 0.84, p < 0.001) whereas in the controls, the correlation was significant for caudate (r = 0.77, p < 0.001) and cerebellar cortex (r = 0.92, p < 0.001) but not for frontal cortex (r = 0.17, p = 0.47). As
shown in Table 2 and Figure, HNE and MDA levels were significantly elevated in the caudate nucleus (+67% and +75%, respectively) and, to a lesser extent, in the frontal cortex (+48% and +36%, respectively) of the MA users as compared with those in the control subjects. The changes were also statistically significant using ANCOVA with postmortem time and/or age as covariates and when using ANOVA. The slight (+14-15%) increases in HNE and MDA concentrations in cerebellar cortex of the MA users did not achieve statistical significance. Examination of the individual subject data revealed that the caudate striatal and frontal cortical values for the MA users were somewhat more scattered than the control subjects (see Figure).

**Aldehyde correlations with MA and dopamine in the MA users.** Previously we showed that levels of MA plus its metabolite amphetamine (“total MA levels”) are homogenously distributed throughout the postmortem brain of chronic MA users (Kalasinsky et al., 2001). To establish whether aldehyde concentrations might have been influenced by the amount of MA recently taken, correlations (Spearman) were made between levels of aldehydes in the three brain regions examined and MA plus amphetamine concentrations in the occipital cortex. The correlations between brain levels of the aldehydes and drug levels were positive but only modest and not statistically significant ($r = 0.37$ to $0.47$, $n = 16$), with that for MDA in the caudate nucleus just missing statistical significance ($r = 0.47$, $p = 0.06$). However, by sub-grouping the MA users according to the brain drug level (Table 3), those with high brain drug levels (> 20 nmol/g tissue) had significantly higher (by 41 and 53% in the caudate and by 69 and 73% in the frontal cortex) concentrations of the aldehydes and higher percentage increases (vs. control levels) of the aldehydes in both the caudate and frontal cortex than those with lower drug levels (< 16 nmol/g tissue). As shown in Figure, the high drug level group had most (HNE and MDA
in caudate and MDA in frontal cortex) or all (HNE in frontal cortex) of the individual subject aldehyde values above the upper limit of the control range.

Although the high drug level group had a non-significant trend for lower dopamine levels in the caudate (Table 3), no significant correlation (Pearson) was observed between aldehyde and dopamine levels in the caudate (HNE: $r = -0.18$, $p = 0.51$; MDA: $r = -0.15$, $p = 0.58$). There was also no significant correlation (Pearson, $r = 0.07$ to $-0.32$, $p > 0.33$) between levels of the aldehydes and the duration of drug use in those cases ($n = 11$) for which some estimate of duration information was available.

**Correlations with brain levels of the antioxidants in the MA users.** Possible correlations (Pearson) in the MA users between levels of HNE or MDA and our previous reported (Mirecki et al., 2004) concentrations of several indices of brain antioxidant systems, namely reduced glutathione, Cu-Zn superoxide dismutase, and uric acid, were explored. Statistically significant correlations were limited to a positive correlation between levels of uric acid and HNE ($r = 0.57$, $p < 0.05$) and MDA ($r = 0.51$, $p < 0.05$) in the frontal cortex. A trend was also observed for a positive correlation between levels of uric acid and HNE ($r = 0.46$, $p = 0.07$) and MDA ($r = 0.49$, $p = 0.05$) in the caudate.
Discussion

We found above normal levels of two markers of oxidative damage to lipids in brain of human MA users, an observation which suggests that animal data indicating that MA can induce brain oxidative stress and damage may be relevant to the human.

Limitations of the postmortem brain study. We attempted to address, as much as possible, a variety of confounding variables (e.g., age, postmortem time, uncertainty of drugs used) that are generic to autopsied human brain investigations of psychostimulant drug users. In this regard, the control and MA groups were matched with respect to age and postmortem time. Like the MA users, most of the control subjects died a “sudden” death. The limited or absence of significant correlations between the postmortem time and tissue levels of the aldehydes was consistent with earlier findings on MDA-like TBARS levels in a prospective animal study (up to 48 hours postmortem; Dexter et al., 1989) and in a retrospective human brain study (10-70 hours; Lyras et al., 1997). To ensure, as much as possible that any differences in the MA group could be attributed to MA, forensic drug analyses were conducted in blood, brain, and, where available, hair of the drug users and controls to confirm use of MA and lack of use of other drugs that could be detected by these methods. However, we cannot exclude the possibility that the MA users might have taken other CNS active drugs or had other characteristics which could have influenced levels of the outcome measures.

HNE and MDA in normal human brain. A variety of procedures to measure levels of HNE and MDA in biological samples have been developed (for review see Esterbauer and Zollner, 1989). The GC-MS method employed in the present study has the advantages of mass specificity, high sensitivity allowing for detection of HNE at the level of 50 fmol (Luo et al., 1995), mild derivatization conditions to minimize the artefactual production of lipid peroxidation...
aldehydic products during the experimental processes (see also below), and simultaneous
detection and quantification of both HNE and MDA in one sample. The use of stable, deuterated
internal standards has addressed early concerns that the recovery of HNE might not be
quantitative (Luo et al., 1995).

The concentration of MDA we observed in postmortem brain of normal subjects is similar to
that in reports employing the improved TBARS assay coupled to HPLC separation (Hayn et al.,
1996; Lyras et al., 1997; Odetti et al., 2000) and in studies using the colorimetric assay based on
reactions of the aldehyde to N-methyl-2-phenyl-indole (Yoshida et al., 2000; Schuessel et al.,
2004). The brain levels of HNE we obtained (~1 pmol/mg wet tissue) are within the
physiological concentration range of HNE in biological tissues or fluids (~1 µM, Esterbauer et
al., 1991) and also agree with those reported using direct HPLC/UV detection of HNE (Odetti et
al., 2000). However, postmortem human brain HNE concentrations reported by one group using
an LC-MS method (Williams et al., 2005b) and earlier by HPLC-fluorescence detection
(Markesbery and Lovell, 1998) are about 10 fold higher than those we obtained. This
discrepancy could be explained by differences in the derivatization procedures, as the above
studies employed incubation at 60°C for 1 hour in the absence of chelating reagents and
antioxidants (Williams et al., 2005a; vs. room temperature for 1 hour in the presence of butylated
hydroxy toluene and desferal in our study), which might promote in vitro lipid decomposition
and production of aldehyde products including HNE.

HNE and MDA in MA users. The simplest explanation for the HNE and MDA increases in
the human MA users, is that the psychostimulant induces oxidative stress in human brain, as in
the case in animal models.
We predicted that aldehyde levels might be related to the recent “extent” of MA used by the drug users as indicated by brain drug levels and by the extent of (caudate) dopamine depletion likely caused by MA, and also by regional differences in dopamine levels in normal brain.

Brain levels of MA can provide some general index of recent drug use; however, interpretation of correlations of outcome measures with this estimate will be confounded to some extent by individual differences in drug metabolism, interval (usually uncertain) between last use of the drug and death, and in post-mortem redistribution of MA between brain and other organs. Although only a non-significant trend for a positive association between brain levels of total MA and concentrations of the aldehydes could be observed, the subgroup of MA users having high brain drug levels had significantly higher concentrations of the aldehydes. To the extent that brain levels of MA are representative of amount of recent drug use, this suggests that part of the aldehyde increase could be explained by an acute (e.g., hours-days) exposure to MA as is the case in animal models (Yamamoto and Zhu, 1998; Jayanthi et al., 1998; Kim et al., 1999; Acikgoz et al., 1998; 2000; Kita et al., 2000; Wan et al., 2000; Gluck et al., 2001; Flora et al., 2002; Iwashita et al., 2004). Nevertheless, the possibility also has to be considered that chronic drug exposure (e.g., involving tolerance, sensitization) might also have modulated the effects of an acute exposure, as suggested by some animal data (Acikgoz et al., 1998, 2000).

Our working model, based on animal findings, was that the aldehyde increase would be inversely related to the extent of tissue dopamine depletion, which we suspect is an index of the severity of a pharmacological action of MA, and would be more marked in dopamine-rich vs. poor regions. Although we could not find an association in the MA users between caudate dopamine levels and HNE/MDA concentrations, we did find, as did those animal investigations in which multiple regions were examined (Kim et al., 1999; Gluck et al., 2001), that the extent of
increase in the lipoperoxidation markers was most marked in the dopamine-rich (~5000 ng/g tissue, Wilson et al., 1996) caudate and least (+14-15%, not significant) in the dopamine-poor (~3 ng/g tissue; Kish, unpublished observations) cerebellar cortex. Given that MA is homogeneously distributed throughout the post-mortem brain (including cerebellar cortex) of the MA users (Kalasinsky et al., 2001), the animal and human postmortem data showing partial dopamine selectivity of changes in lipoperoxidation markers point towards the involvement of dopamine as a factor involved in promoting lipoperoxidation (e.g., MA-induced formation of dopamine-derived oxyradicals; Davidson et al., 2001). Although dopamine levels are very low and similar in frontal and cerebellar cortices (Scatton et al., 1983; Kish, unpublished observations), our finding that aldehyde levels were substantially elevated in frontal cortex but not in the cerebellar cortex could be explained by a more intense dopaminergic innervation of cerebral cortex (Melchitzky and Lewis, 2000; Swanson et al., 2006) although the question of cerebellar cortical innervation appears not to have been resolved in the human (Olsson et al., 1999; Melchitzky and Lewis, 2000). However, a case can also be made that part of the increase in HNE/MDA was not restricted to dopamine neurones. Indeed, it has been recently suggested that MA neurotoxicity might not be dopamine dependent (Gluck et al., 2001; Yuan et al., 2001).

**HNE, MDA and brain damage in MA users?** Although this topic is debated, findings in abstinent MA users suggest that MA might cause some structural damage to dopamine neurones, but also that the damage is probably modest (see Moszczynska et al., 2004 and Johanson et al., 2006 for reviews). In this regard, although formal neurological testing had not been conducted, examination of the MA case information did not disclose any report of obvious neurological illness. As MDA and especially HNE have been used historically as gold standard markers of lipoperoxidation (Esterbauer et al., 1991), our data strongly suggest that oxidative damage to
lipids in striatum and cerebral cortex of human MA users is, on average, increased over normal basal levels. The finding that one half of the MA users, in particular those with high brain drug levels, had values for HNE (considered to be one of the most reactive aldehydes) falling above the upper limit of the control range in caudate and frontal cortex suggests that brain lipoperoxidative changes in some MA users can be “robust”. The extent of increase in the lipoperoxidation markers we observed was also similar to that reported in experimental animal toxicity studies where MA was administered at doses that can cause persistent loss of dopamine nerve terminal markers, morphological signs of structural damage (cf. McCann and Ricaurte, 2004), and gliosis (O’Callaghan and Miller, 1994). Although the finding of increased levels of oxidatively damaged lipid products does not constitute proof of neurotoxic damage, it does suggest that the risk of toxicity due to oxidative stress is probably increased with recreational use of MA.

In conclusion, our post-mortem data suggest that MA can increase pro-oxidant processes in both dopamine rich and poor areas of human brain and, in this respect, provide support for the relevance to the human of animal models of MA exposure in which similar findings have been reported.
References


Footnotes

This work was supported by the US NIDA/NIH DA07182 and the New Zealand Institute of Environmental Science and Research, Ltd.

\footnote{The assertions and opinions contained herein are the private views of the authors and are not to be construed as official or as reflecting views of the United States Department of Army or Department of Defense.}
Legends for Figures

**Figure.** Increased levels of 4-hydroxynonenal (HNE) and malondialdehyde (MDA) in brain of human chronic users of methamphetamine (MA) as compared to the control subjects. Shown are individual values and the group mean. The MA cases are sub-grouped according to the brain drug levels (MA plus the metabolite amphetamine) into a MA-low (< 16 nmol/g tissue) and a MA-high (> 20 nmol/g tissue) group. * $p < 0.05$, *** $p < 0.001$, MA vs. control (two-way ANCOVA with postmortem interval as the covariate, followed by post hoc Bonferroni tests).
TABLE 1: Characteristics and drug use histories of the 16 methamphetamine users.

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (yrs), PMI (h) Duration of recent drug use pattern</th>
<th>Route of drug administration</th>
<th>Suspected/known cause of death</th>
<th>Toxicology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>34,F 14 10 Daily, $10</td>
<td>Nasal</td>
<td>MA intoxication</td>
<td>Brain drug Caudate HAIR level &lt;sup&gt;b&lt;/sup&gt; DA level &lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>36,M 5 &gt;10 Once per month</td>
<td>Nasal; intravenous</td>
<td>MA intoxication</td>
<td>---</td>
</tr>
<tr>
<td>3</td>
<td>22,M 16 8 Daily, limited only by funds</td>
<td>Intravenous</td>
<td>MA intoxication</td>
<td>---</td>
</tr>
<tr>
<td>4</td>
<td>42,M 10 &gt;20 3-4 times per week</td>
<td>Nasal; oral</td>
<td>MA intoxication</td>
<td>---</td>
</tr>
<tr>
<td>5</td>
<td>20,M 21 1 Unknown</td>
<td>Oral</td>
<td>MA intoxication</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>28,M 14 16 Daily and 2-3-day binges</td>
<td>Smoked; intravenous</td>
<td>Gunshot wound to chest</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>44,F 24 15 Every two weeks</td>
<td>Nasal</td>
<td>MA intoxication</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>39,M 19 23 Daily 4-5 hits</td>
<td>Intravenous</td>
<td>Gunshot wound to chest</td>
<td>+</td>
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<tr>
<td>9</td>
<td>28,M 4 10 Every two weeks</td>
<td>Intravenous</td>
<td>MA intoxication</td>
<td>+</td>
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<td>10</td>
<td>44,F 23 10 1-2 lines per day</td>
<td>Nasal</td>
<td>Coronary artery atherosclerosis</td>
<td>+</td>
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<td>11</td>
<td>20,M 21 3-4 Daily</td>
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<td>Nasal; oral; smoked</td>
<td>Coronary artery atherosclerosis</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>29,M 11 &gt;8 Daily 0.25 g</td>
<td>Nasal</td>
<td>Acute aortic dissection</td>
<td>---</td>
</tr>
<tr>
<td>14</td>
<td>35,M 22 &gt;1 Unknown</td>
<td>Unknown</td>
<td>MA intoxication</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>39,F 22.5 15 Every few days</td>
<td>Oral; intravenous</td>
<td>MA intoxication</td>
<td>+</td>
</tr>
<tr>
<td>16</td>
<td>26,F 12 &gt;1 Unknown</td>
<td>Unknown</td>
<td>MA intoxication</td>
<td>+</td>
</tr>
</tbody>
</table>

M = male; F = female; PMI = postmortem interval; MA = methamphetamine; DA = dopamine. + Drug hair analyses confirmed use of MA. For cases 10, 12 and 13, MA toxicity was considered to be a possible contributing factor to the cause of death. a Information on the 16 cases including brain drug MA levels has been published previously in Wilson et al. (1996), Kalasinsky et al. (2001), Tong et al. (2003) and Moszczynska et al. (2004). b Measured in nmol (MA plus metabolite amphetamine)/g tissue (occipital cortex). c Measured as percentage decrease of the control mean and the data were calculated from values provided in Wilson et al. (1996) and Moszczynska et al. (2004).
TABLE 2. Levels of 4-Hydroxynonenal (HNE) and malondialdehyde (MDA) in brain of human chronic methamphetamine users and control subjects

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 21)</th>
<th>MA users (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HNE</td>
<td>MDA</td>
</tr>
<tr>
<td>Caudate</td>
<td>12.0 ± 0.9</td>
<td>418 ± 43</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>15.6 ± 0.7</td>
<td>912 ± 56c</td>
</tr>
<tr>
<td>Cerebellar cortex</td>
<td>13.9 ± 1.1</td>
<td>623 ± 49</td>
</tr>
</tbody>
</table>

Data are in mean ± SEM. Frontal cortex was taken from Brodmann area 9. Levels of HNE and MDA are expressed as pmol/mg protein. Values in parentheses are percentage change with respect to the mean control value.

* a p < 0.05, b p < 0.001, MA vs. control; c p < 0.05, frontal cortex vs. the other two regions (two-way ANCOVA with postmortem interval as the covariate, followed by post hoc Bonferroni tests).

* d p < 0.05, caudate vs. frontal cortex percentage change from controls (paired Student’s t-tests).
TABLE 3. Levels of 4-Hydroxynonenal (HNE) and malondialdehyde (MDA) in brain of human chronic methamphetamine users subgrouped according to the brain drug level (methamphetamine plus metabolite amphetamine)

<table>
<thead>
<tr>
<th></th>
<th>Dopamine</th>
<th>HNE</th>
<th>MDA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(±104%)</td>
<td>(+124%)</td>
</tr>
<tr>
<td>High brain drug level</td>
<td>n = 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caudate</td>
<td>2.3 ± 0.4 (-67%)</td>
<td>24.5 ± 2.0&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>935 ± 108&lt;sup&gt;b,d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>NE</td>
<td>31.1 ± 3.5&lt;sup&gt;b,e&lt;/sup&gt;</td>
<td>1689 ± 228&lt;sup&gt;b,e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cerebellar cortex</td>
<td>NE</td>
<td>18.1 ± 1.6</td>
<td>857 ± 109</td>
</tr>
</tbody>
</table>

| Low brain drug level | n = 10  |          |          |
| Caudate              | 3.3 ± 0.5 (-53%) | 17.4 ± 2.0<sup>a</sup> | 611 ± 34<sup>a</sup> |
| Frontal cortex       | NE       | 18.4 ± 2.2 | 976 ± 96  |
| Cerebellar cortex    | NE       | 14.6 ± 1.1 | 635 ± 46  |

Data are in mean ± SEM. NE = not examined. Brain (occipital cortex) drug levels for the high and low groups are 124 ± 47 (range 20-319, median 90) and 10.0 ± 1.3 (range 2.8-15.6, median 10) nmol/g tissue, respectively ($p < 0.01$, Mann-Whitney U tests). Dopamine levels (previously reported in Wilson et al. [1996] and Moszczynska et al. [2004]) are expressed as ng/mg wet tissue, levels of HNE and MDA as pmol/mg protein. Values in parentheses are percentage change with respect to the mean control value.

<sup>a</sup> $p < 0.05$, <sup>b</sup> $p < 0.001$, MA vs. control; <sup>c</sup> $p < 0.05$, <sup>d</sup> $p < 0.01$, <sup>e</sup> $p < 0.001$, high vs. low brain MA cases (one-way ANCOVA with PMI as the covariate, followed by post hoc Bonferroni tests).