

Brain antioxidant systems in human methamphetamine users

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Abstract

Animal data suggest that the widely abused psychostimulant methamphetamine can damage brain dopamine neurones by causing dopamine-dependent oxidative stress; however, the relevance to human methamphetamine users is unclear. We measured levels of key antioxidant defences [reduced (GSH) and oxidized (GSSG) glutathione, six major GSH system enzymes, copper–zinc superoxide dismutase (CuZnSOD), uric acid] that are often altered after exposure to oxidative stress, in autopsied brain of human methamphetamine users and matched controls. Changes in the total ($n = 20$) methamphetamine group were limited to the dopamine-rich caudate (the striatal subdivision with the most severe dopamine loss) in which only activity of CuZnSOD (+ 14%) and GSSG levels (+ 58%) were changed. In the six methamphetamine

users with severe (– 72 to – 97%) caudate dopamine loss, caudate CuZnSOD activity (+ 20%) and uric acid levels (+ 63%) were increased with a trend for decreased (– 35%) GSH concentration. Our data suggest that brain levels of many antioxidant systems are preserved in methamphetamine users and that GSH depletion, commonly observed during severe oxidative stress, might occur only with severe dopamine loss. Increased CuZnSOD and uric acid might reflect compensatory responses to oxidative stress. Future studies are necessary to establish whether these changes are associated with oxidative brain damage in human methamphetamine users.

Keywords: dopamine, glutathione, human brain, methamphetamine, oxidative brain damage, oxidative stress.

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Methamphetamine (MA) is a widely abused drug that causes the release in human brain of the neurotransmitter dopamine (Laruelle *et al.* 1995; Martinez *et al.* 2003), an

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Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; CuZnSOD, copper–zinc superoxide dismutase; DAT, dopamine transporter; γ -GCS, γ -glutamylcysteine synthetase; G6PD, glucose-6-phosphate dehydrogenase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione S-transferase; γ -GTP, γ -glutamyltranspeptidase; INT, 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride; MA, methamphetamine; MnSOD, manganese superoxide dismutase; VMAT2, vesicular monoamine transporter.

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action considered to explain, at least in part, the euphoric effects of the psychostimulant (Wise 1996).

In experimental animals, high doses of MA can lead to a persistent, possibly reversible reduction of dopamine and other biochemical markers of dopamine neuronal integrity in the striatal (caudate, putamen) nerve terminal (Fibiger and McGeer 1971; Seiden *et al.* 1975/1976; Frey *et al.* 1997; Harvey *et al.* 2000a,b). However, unlike the pathology of idiopathic Parkinson's disease, the brain morphological changes following MA exposure do not include obvious loss of pigmented dopamine cell bodies in substantia nigra of either experimental animals (Woolverton *et al.* 1989; Linder *et al.* 1995; but see Harvey *et al.* 2000b; Sonsalla *et al.* 1996) or of humans (Wilson *et al.* 1996; Moszczynska *et al.* 2004). The animal findings of long-term reduction of brain dopaminergic markers, together with histological signs suggestive of nerve terminal injury following acute MA exposure (Linder *et al.* 1995), have led to the conclusion that MA damages brain dopamine nerve endings.

In human forensically confirmed MA users who had recently used the drug, we originally reported a post-mortem striatal loss of three dopamine nerve terminal markers [dopamine, dopamine transporter (DAT), tyrosine hydroxylase (EC 1.14.16.2)], but normal levels of two other markers [dopa decarboxylase (EC 4.1.1.28), vesicular monoamine transporter (VMAT2)]. The latter appears, at least in experimental animals, to be a relatively stable marker of striatal dopamine nerve terminal integrity (Wilson *et al.* 1996 and references therein). Subsequent brain imaging studies (by positron emission tomography) in self-reported (i.e. not forensically confirmed) MA users withdrawn from the drug confirmed the DAT reduction (McCann *et al.* 1998; Sekine *et al.* 2001; Volkow *et al.* 2001a,b) and demonstrated, in a preliminary investigation, the finding that striatal VMAT2 levels are, at most, only slightly decreased (C. Schuster, C-E. Johanson and K. Frey, personal communication). However, because of the possibility that striatal levels of the dopaminergic markers might change independently of changes in number of dopamine nerve terminals (Harvey *et al.* 2000b), the question of whether MA causes actual physical loss of dopamine nerve endings in human brain remains unanswered.

Although the mechanism by which MA might damage brain dopamine neurones is not understood, much animal data suggest that an early event is oxidative stress, possibly related to the excessive release or intraneuronal mobilization of dopamine and formation of toxic dopamine-derived oxyradicals (see Cadet and Brannock 1998 for review). In this regard, our finding that striatal dopamine can be decreased by up to 97% in post-mortem brain of some MA users who had recently used the drug (Wilson *et al.* 1996; Moszczynska *et al.* 2004) implies a massive release and metabolism of striatal dopamine, and the likelihood of some dopamine-related oxidative stress.

The major objective of our post-mortem brain investigation was therefore to establish whether levels of indices sensitive to oxidative stress are selectively altered in striatum of MA users, and whether the extent of changes might be related to the dopamine reduction. It is not yet possible to measure oxidative stress directly in either living or post-mortem human brain. However, measurement of brain levels of components of antioxidant systems, such as glutathione and its metabolizing enzymes (Sen 1997), superoxide dismutase (Yoo *et al.* 1999) and uric acid (Desole *et al.* 1995a,b), which are often altered in a characteristic direction after exposure to oxidative stress, can provide information suggesting that oxidative stress had occurred. As changes in antioxidant system concentrations have also been reported in brain of MA-exposed animals (glutathione system: Jayanthi *et al.* 1998; Moszczynska *et al.* 1998; Kim *et al.* 1999; Harold *et al.* 2000; superoxide dismutase: Acikgoz *et al.* 1998, 2000; Kim *et al.* 1999; uric acid: Yamamoto and Zhu 1998), we measured levels of reduced (GSH) and oxidized (GSSG) glutathione, six enzymes involved in GSH metabolism [glutathione peroxidase (EC 1.11.1.9) (GPx), glutathione reductase (EC 1.8.1.7) (GR), γ -glutamyltranspeptidase (EC 2.3.2.2) (γ -GTP), glutathione S-transferase (EC 2.5.1.18) (GST), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (G6PD) and γ -glutamylcysteine synthetase (EC 6.3.2.2) (γ -GCS)], copper-zinc superoxide dismutase (EC 1.15.1.1) (CuZnSOD) and uric acid in autopsied brain of chronic MA users. As oxidative stress caused by MA is likely to occur during or shortly after MA exposure (Cubells *et al.* 1994; Yokoyama *et al.* 1997; Yamamoto and Zhu 1998), subjects were restricted to those who tested positive at autopsy for MA in blood and brain. Given the experimental finding that GSH levels can be decreased following severe oxidative stress (Moszczynska *et al.* 1998; Kim *et al.* 1999), our major hypothesis was that striatal GSH levels would be decreased in brain of the human MA users.

Materials and methods

Subjects and brain material

Post-mortem brain material from MA users ($n = 20$) and control subjects ($n = 16$) was obtained from medical examiner offices in the US and Canada. The control and MA groups were matched with respect to age (mean \pm SEM, controls 35.1 ± 2.7 years; MA users 31.4 ± 1.9 years), post-mortem interval between death and autopsy (mean \pm SEM, controls 13.7 ± 1.7 h; MA users 14.6 ± 1.5 h) ($p > 0.1$, Student's two-tailed t -test) and sex (controls 14M : 2F; MA users 15M : 5F) ($p > 0.1$, χ^2 test with Yates' correction).

All MA users had evidence, from the medical examiner investigation and/or structured interview with the next of kin, of use of MA as the primary drug of abuse for at least 1 year; presence of MA [assessed by GC-MS (Wilson *et al.* 1996)] in blood, brain (occipital cortex) and, in the 14 of 20 MA users from whom hair was available, in sequential scalp hair samples; and absence, at autopsy,

of brain pathology unrelated to MA use. Sixteen MA users tested negative for other drugs of abuse, including alcohol in blood, hair and brain; and for other psychostimulants, opiates, PCP (phencyclidine). Heroin or cocaine and/or their metabolites were also detected in blood and brain of three subjects (559, 726, 478) and in hair of four subjects (559, 726, 478, 678). The suspected or known causes of death of MA users were acute aortic dissection ($n = 1$), gunshot wounds to chest ($n = 2$), severe coronary artery atherosclerosis with MA toxicity as a possible contributing factor ($n = 2$), multiple drug toxicity ($n = 3$) and MA intoxication ($n = 12$). Examination of the body at autopsy (mean 15 h after death) revealed no evidence of hyperthermia, with the exception of one subject who had a raised temperature. All control subjects were neurologically normal, had no history of drug use and tested negative for any drug of abuse in blood, autopsied brain and, in the 14 subjects for whom scalp hair was available, sequential scalp hair samples. The causes of death of control subjects were electrocution ($n = 1$), cut throat ($n = 1$) morbid obesity ($n = 1$), trauma ($n = 3$), pulmonary embolism ($n = 2$), cardiomyopathy ($n = 2$) and atherosclerotic cardiovascular disease ($n = 6$).

At autopsy, one half of the brain was fixed in formalin fixative whereas the other half was immediately frozen until dissection. Brain neuropathological analysis of the MA users and of the control subjects showed no significant abnormalities in any brain area, with

the exception of prominent gliosis in the putamen in subject 879 and mild gliosis in the substantia nigra in subject 478. However, detailed quantitative assessment of gliosis was not performed, allowing for the possibility that some above-normal gliosis in brain of MA users might have occurred that could not be detected by routine histological analysis. For the biochemical analyses brain areas were excised using Brodmann classification and the atlas of Riley (1960). For some assays, a sample of white matter taken dorsal to the anterior head of the caudate was excised.

Drug history, drug concentrations in post-mortem tissue, brain neuropathological findings, and striatal dopamine levels of the 20 MA users have been reported previously (Wilson *et al.* 1996; Moszczynska *et al.* 2004). In the group of 20 MA users, we reported that mean striatal dopamine levels were reduced by 50% in putamen and by 61% in caudate compared with control levels (Moszczynska *et al.* 2004). Some demographic information on the MA users, together with dopamine levels in caudate nucleus expressed as a percentage of control values, are provided in Table 1.

In order to establish whether levels of the biochemical outcome measures were similar in living and autopsied brain, biopsied temporal cortex was obtained from three patients immediately following operations for the treatment of intractable hippocampal focal epilepsy (mean \pm SEM 31.0 \pm 6.1 years of age; 2M : 1F). The biopsied tissue was taken from non-epileptic lateral temporal

Table 1 Subject information and drug histories of 20 chronic MA users

Case ^a	Sex	Age (years)	Years of MA use	Amount/pattern of recent MA use	Route of administration	Suspected/known cause of death	Dopamine in caudate (%) ^c
374	F	34	10	\$10 per day, daily	nasal	acute MA toxicity	- 77
375	M	36	> 10	~ once per month	nasal and i.v.	acute MA toxicity	- 44
407	M	22	8	daily, if money available	i.v.	acute MA toxicity	- 13
422	M	42	> 20	3-4 times per week	oral and nasal	acute MA toxicity	- 51
424	M	20	1	unknown	oral	acute MA toxicity	- 53
442	M	28	16	binges for 2-3 days, 7-10 days apart	i.v. and smoking	gunshot wound to chest	- 68
448	M	39	23	daily, 4-5 hits per day	i.v.	gunshot wound to chest	- 95
510	M	28	10	every 2 weeks	i.v.	acute MA toxicity	- 57
523	F	44	10	1-2 lines per day	nasal	coronary artery atherosclerosis	- 57
590	M	33	18	daily, limited only by funds	smoking, oral and nasal	coronary artery atherosclerosis	- 45
524	M	20	3 - 4	chronic user, probably daily	oral and nasal	acute MA toxicity	- 51
447	F	44	15	line every 2weeks	nasal	acute MA toxicity	- 82
677	M	29	> 8	daily, 1/4 g per day	nasal	acute aortic dissection	- 67
767	M	35	> 1	unknown	unknown	acute MA toxicity	- 85
867	F	39	15	every few days	oral and i.v.	acute MA toxicity	- 55
879	M	26	> 1	unknown	unknown	acute MA toxicity	- 42
559 ^b	M	26	10	binges for 3-4 days, few weeks apart;	i.v.	acute multiple drug toxicity	- 73
				2-3 days MA, 1 day heroin			
678 ^b	M	30	> 1	MA: unknown, cocaine: unknown	i.v.	acute MA toxicity	- 97
726 ^b	F	15	> 1	MA: unknown, heroin: unknown	i.v.	acute multiple drug toxicity	- 51
478 ^b	M	37	8	MA: unknown, cocaine: 2-3 times/week	oral and nasal	acute multiple drug toxicity	- 54

For cases 523, 590, and 677 MA toxicity was considered to be a possible contributing factor to the cause of death. ^a The 20 MA users were originally described in Wilson *et al.* (1996) and Moszczynska *et al.* (2004). ^b MA users who tested positive for other drugs (cocaine and/or heroin) in addition to MA in blood, brain and/or hair. ^c Dopamine levels in MA users are expressed as percentage change of the control mean and were calculated from values provided in Wilson *et al.* (1996) and Moszczynska *et al.* (2004), and are reproduced by permission of Oxford University Press.

regions that, by anatomical necessity, were excised (and frozen within 15 min) during an anterior temporal lobectomy (Kish *et al.* 1988).

Biochemical assays

Tissue homogenization

For the HPLC assay of GSH and metabolites, uric acid and methionine, brain tissue (30–40 mg) was homogenized on ice, in 300 μ L NaH₂PO₄ (10 mM) containing octane sulfonate (0.05 mM) using a Bronwill Biosonic Sonicator (Bronwill Scientific, Rochester, NY, USA). Proteins were precipitated by addition of an equivalent volume of ice-cold methanol and incubated at 0°C for 15 min. The homogenates were then centrifuged at 17 000 *g* for 15 min, the supernatant removed and diluted with five volumes NaH₂PO₄ and filtered (0.25 μ m, syringe filter) before injection. For the enzyme spectrophotometric assays and western blotting analysis, adjacent tissue samples (40 mg) from each brain area were homogenized by sonication at 0°C in 10 volumes (w/v) of ice-cold distilled water. Each homogenate was divided into three portions: one portion was taken for γ -GCS immunolabelling; to the two other portions was added one volume of phosphate-based (for GPx, GR, GST and CuZnSOD assay) [0.04 M K₂HPO₄, pH 7.0, 0.2 M KCl, 2 mM EDTA, 1% (v/v) Triton X-100] or Tris-based (for G6PD and γ -GTP assay) [0.2 M Tris-HCl, pH 8.0, 1% (v/v) Triton X-100] homogenization buffer. The tissue sample homogenates were sonicated again, divided (for each assay and determination of protein content), and stored at –80°C until assayed, with the exception of G6PD which was assayed on the same day.

Glutathione and its metabolites, uric acid, and methionine

The levels of GSH, GSSG, GSH–cysteine mixed disulfide (a metabolite of GSH) and uric acid, as well as methionine, an amino

acid used to provide a general index of post-mortem protein degradation (Perry *et al.* 1981), were measured by HPLC with electrochemical detection (minor modifications of Harvey *et al.* 1989; Fitzmaurice *et al.* 2003). The eluent consisted of 10 mM NaH₂PO₄ and 0.05 mM octane sulfonate adjusted to pH 2.7 using 12 M phosphoric acid and 6.9% methanol, at a flow rate of 1 mL/min and a reverse-phase ODS spherosorb column (250 \times 4.7 mm, 5 μ m) (Hichrom, Theale, Berkshire, UK).

Glutathione metabolizing and related enzymes

Because the enzymes in our investigation had not been fully characterized in autopsied human brain, we first determined Michaelis constants (K_m) for the substrates and maximal velocity (V_{max}) values, and established that enzyme activity was linear with respect to time and protein homogenate concentration, and was inhibited by known inhibitors of the enzyme (Table 2).

GPx activity was measured by a modified spectrophotometric procedure (Paglia and Valentine 1967) with the reaction mixture containing 0.1 M K₂HPO₄ (pH 7.0), 4 mM EDTA, 1.2 mM NaN₃, 10 mM GSH, 1 unit GR, 0.15 mM NADPH, 0.1 mM H₂O₂ and brain tissue homogenate (3 mg wet tissue) in a final volume of 1 mL. The reaction was initiated by addition of H₂O₂ and the decrease in absorbance at 340 nm was monitored at 37°C. The results were corrected for non-enzymatic oxidation of GSH and NADPH by H₂O₂. GR activity was measured by a modified spectrophotometric method (Smith *et al.* 1988) in a reaction mixture containing 0.1 M K₂HPO₄ (pH 7.0), 1 mM EDTA, 0.06 mM 5,5'-dithiobis(2-nitrobenzoic acid), 2 mM GSSG, 0.15 mM NADPH and brain tissue homogenate (3 mg wet tissue) in a final volume of 1 mL. The reaction was initiated by addition of NADPH and the increase in absorbance at 412 nm was monitored at 37°C. To correct for non-specific oxidation of NADPH, a no-GSSG blank was used as a baseline. For measurement of G6PD activity (modified spectropho-

Table 2 Kinetic parameters and inhibition characteristics of five glutathione system enzymes in control human cerebral cortex

Enzyme	Substrate	Co-substrate	K_m	V_{max}	IC_{50}
GPx	GSH	0.1 mM H ₂ O ₂	12.0 \pm 1.1 mM	256 \pm 6	64.4 \pm 7.2 μ M
	H ₂ O ₂	10 mM GSH	40.0 \pm 2.1 μ M	184 \pm 25	
GR	GSSG	0.15 mM NADPH	61.8 \pm 7.5 μ M	60.6 \pm 2.3	4.87 \pm 1.23 mM
	NADPH	2 mM GSSG	9.01 \pm 0.94 μ M	62.5 \pm 1.5	
G6PD	G6P	1 mM NADP ⁺	69.7 \pm 9.3 μ M	35.9 \pm 1.9	31.1 \pm 8.0 μ M
	NADP ⁺	1 mM G6P	14.7 \pm 1.5 μ M	30.1 \pm 0.2	
GST	GSH	1 mM CDNB	0.22 \pm 0.02 mM	166 \pm 31	2.11 \pm 0.36 μ M ^a
		5 mM GSH	0.71 \pm 0.08 mM	297 \pm 17	8.65 \pm 1.35 μ M ^b
					22.8 \pm 4.38 μ M ^c
γ -GTP	GlyGly	3.8 mM L-GpNA	20.2 \pm 1.6 mM	10.5 \pm 0.3	1.38 \pm 0.18 mM
		100 mM GlyGly	1.02 \pm 0.06 mM	9.85 \pm 0.38	

Values are mean \pm SEM of three autopsied human cortices (frontal: GPx, GR, G6PD; occipital: γ -GTP; parietal: GST) analyzed in independent experiments. The K_m and V_{max} (nmol per min per mg) values were calculated using 8–13 substrate concentrations, and were derived by non-linear regression analysis using GraphPad Prism computer software program (GraphPad, San Diego, CA, USA). The influence of inhibitors on activity was examined by comparing reaction rates in the presence and absence of inhibitors: dehydroepiandrosterone (G6PD), β -mercaptosuccinate (GPx), 1,3-bis (2-chloroethyl)-1-nitrosourea (GR), acivicin (γ -GTP), and ^a cibacron blue (characteristic for class μ) ^b hematin (characteristic for class α) and ^c sulfasalazine (α , μ , and π class inhibitor) for GST. G6P, glucose-6-phosphate; GlyGly, glycylglycine; L-GpNA, L- γ -glutamyl-*p*-nitroanilide.

tometric method; Lee 1982) the reaction mixture contained 0.1 M Tris-HCl (pH 8.0), 1 mM glucose-6-phosphate, 1 mM NADP⁺, 10 mM 2,3-diphosphoglycerate, 10 mM MgCl₂ and brain tissue homogenate (2 mg wet tissue) in a final volume of 1 mL. The reaction was initiated by addition of NADP⁺ and the increase in absorbance at 340 nm was monitored at 37°C. A blank employing no homogenate was used as a baseline. GST activity was measured using a modified spectrophotometric method (Spearman and Leibman 1984) with the reaction mixture containing 0.1 M K₂HPO₄ (pH 6.5), 5 mM GSH, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) and brain tissue homogenate (0.8 mg wet tissue) in a final volume of 1 mL. The reaction was initiated by addition of CDNB and the increase in absorbance at 340 nm was monitored at 25°C. A blank employing no homogenate was used as a baseline. For measurement of γ -GTP activity (modified spectrophotometric method; Tate and Meister 1985) the reaction mixture contained 0.1 M Tris-HCl (pH 8.0), 3.8 mM L- γ -glutamyl-*p*-nitroanilide, 100 mM glycylglycine and brain tissue homogenate (5 mg wet tissue) in a final volume of 1 mL. The reaction was initiated by addition of homogenate and the increase in absorbance at 401 nm was monitored at 37°C. A blank employing no homogenate was used as a baseline.

Although γ -GCS activity has been reported previously in autopsied human brain (Sian *et al.* 1994b), we found that its activity was too low to be measured reliably when employing procedures that followed formation of ADP (Seelig and Meister 1985), GSH (Fernandez-Checa and Kaplowitz 1990) or inorganic phosphorus (Fiske and SubbaRow 1925). However, we were successful in developing an assay for measuring protein levels of the catalytic heavy subunit of γ -GCS by quantitative immunolabeling using a polyclonal rabbit anti-rat antibody against the heavy subunit of γ -GCS protein (provided by Dr Henry Jay Forman, Division of Natural Sciences, Founding Faculty, University of California, Merced, CA, USA). Brain tissue homogenates were diluted in sample buffer to a concentration of 1.2 μ g protein/ μ L and samples containing 30 μ g protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 7.5% gels. The resolved proteins were electrophoretically transferred to a polyvinylidene difluoride membrane using a wet transfer tank (32 V overnight). The blot was incubated with 5% non-fat dry milk in TTBS buffer (Tris-buffered saline with 0.1% Tween-20) for 1 h at room temperature (RT, 22°C), rinsed three times in TTBS, incubated with anti- γ -GCS antibody in 5% milk-TTBS (1 : 5000) for 1 h at RT, rinsed several times in TTBS, incubated with secondary antibody (horseradish peroxidase-conjugated Protein A, 1 : 5000; Bio-Rad, Hercules, CA, USA) for 1 h at RT, and rinsed again several times in TTBS. Immunoreactivity was visualized by exposure to autoradiographic film after incubation with chemiluminescence reagents (WB Chemiluminescence reagent plus; New England Nuclear, Boston, MA, USA) and quantified using a computer-based imaging device (MCID; Imaging Research Inc., St Catharines, Ontario, Canada). Levels of γ -GCS in samples were calculated from a five-point standard curve of tissue standard (10–50 μ g protein) and expressed as microgram protein of tissue standard per microgram protein. Purified γ -GCS from human blood and all brain samples, with the exception of human cerebellar cortex, showed only one band at approximately 74 kDa, which corresponded to the molecular mass of the γ -GCS heavy subunit reported in

mouse brain (70 kDa; Thompson *et al.* 1999) and other human and rodent tissues (73–74 kDa; Yan and Meister 1990; Gipp *et al.* 1992; Reid *et al.* 1997a). Human brain cerebellar cortex showed a second band at approximately 30 kDa, which corresponded to the molecular mass of the γ -GCS light subunit (mouse brain: 27 kDa, other sources: 27–31 kDa; Huang *et al.* 1993; Reid *et al.* 1997b; Thompson *et al.* 1999). In other brain regions the second band could be also seen upon prolonged exposure.

CuZnSOD

CuZnSOD activity was measured by adaptation of an erythrocyte SOD spectrophotometric assay kit (Ransod Kit; Randox Laboratories Ltd, Crumlin, Co. Antrim, UK). The assay is based on 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) reduction by superoxide radical followed at 505 nm at 37°C. A xanthine-xanthine oxidase system is used to generate a superoxide radical flux. The initial assay mixture contained 850 μ L mixed substrate (0.05 mM xanthine plus 0.025 mM INT) in 40 mM cyclohexylaminopropane buffer (pH 10.2) containing 0.94 mM EDTA and 25 μ L of SOD standard, brain tissue homogenate (0.1 mg wet tissue) or blank employing no homogenate. The reaction was initiated by addition of 125 μ L xanthine oxidase. The reaction of the superoxide free radical with INT provides a spectrophotometrically measurable formazan product. The activity of SOD (total SOD and MnSOD) was measured by the degree of inhibition of the formazan product reaction (via superoxide free radical consumption) and calculated from the standard curve (% inhibition vs. log₁₀[standard]). For measurement of MnSOD activity, the samples were incubated for 1 h at 37°C with 5 mM NaCN before the assay. The assay was optimized for measurement of CuZnSOD and the enzyme activity was calculated by subtracting the cyanide-resistant MnSOD from the total SOD activity. SOD activity was expressed as units per milligram of protein, a unit being defined as the amount of the enzyme that inhibits the reaction of INT reduction by 50%.

Protein concentration

Protein concentration was determined using the Bio-Rad protein assay kit with bovine serum albumin as a standard.

Statistical analysis

Differences between the control and the total MA groups were analyzed by the Student's two-tailed unpaired *t*-test whereas differences between controls and subgroups of MA users in the caudate and putamen were analyzed by ANOVA followed by the Fisher's least significant difference (LSD) test. Correlations between biochemical outcome measures and age or post-mortem time were determined using the Pearson correlation test, with the exception of correlations between neurochemical outcome measures and brain MA levels in which the Spearman rank correlation test was employed. The criterion for statistical significance for all comparisons was *p* < 0.05. Based on animal findings (see below), some of which were obtained in our laboratory (Moszczynska *et al.* 1998), our hypotheses were that levels of GSH would be reduced and those of CuZnSOD and uric acid increased in the (dopamine-rich) caudate and putamen only of the MA users. For comparisons involving the outcome measures in brain areas outside the striatum, statistical differences were assessed using standard *t*-tests as well as *t*-tests

with a conservative adjustment for Type I errors (Bonferroni adjustment).

Results

Possible influence of post-mortem time on levels of oxidative stress indices

To establish whether there might be a substantial difference between brain levels of the oxidative stress outcome measures in living and post-mortem brain, we compared concentrations in autopsied temporal cortex (mean time after death 15 h) with those of electrographically normal biopsied temporal cortex from three patients with intractable epilepsy. As shown in Table 3, levels of all neurochemicals in biopsied and autopsied brains were generally similar, with the expected exception of those of GSH, which were markedly lower (−48%) in autopsied brain and of methionine (+1291%), a constituent of proteins that markedly increases after death (Perry *et al.* 1981), and uric acid (+660%) that were much higher in autopsied brain.

Possible influence of time after death was also assessed by examination of correlations (Pearson correlation test) in control subjects between post-mortem time and brain levels of the neurochemicals. Statistically significant correlations that were observed across multiple brain areas were limited to GST in all seven brain regions examined (range $r = -0.39$ to -0.70 ; statistically significant in five of the seven brain areas). The other statistically significant corre-

lations were limited to GSH in cerebellar cortex ($r = -0.50$), uric acid ($r = 0.53$) and methionine ($r = 0.54$) in temporal cortex, and γ -GCS ($r = -0.52$) and CuZnSOD ($r = -0.54$) in caudate nucleus ($p < 0.05$).

No statistically significant differences (data not shown) were observed between brain methionine levels (which increase after death; see above) in 11 cerebral cortical and subcortical brain areas of the control and MA groups.

Oxidative stress indices in MA users

Statistically significant changes in levels of the primary outcome measures (GSH, CuZnSOD, uric acid) in the MA group as a whole were limited to modestly increased activity of CuZnSOD (+14%) in the caudate nucleus (Tables 4 and 5). When the four subjects who had used other drugs of abuse in addition to MA were excluded, CuZnSOD activity was similarly increased (+14%; $p < 0.05$). No significant group differences were observed for any of the other outcome measures (GSSG, GSH–cysteine mixed disulfide, GPx, GR, γ -GTP, GST, G6PD, γ -GCS) in any brain region examined ($p > 0.05$), with the exception of increased GSSG (+58%) in the caudate nucleus (Tables 4 and 5).

Statistically significant correlations (Spearman rank correlation test) between brain levels of oxidative stress indices and brain levels of MA plus its metabolite amphetamine (measured in occipital cortex) were limited to positive correlations between uric acid and total MA levels in caudate, putamen, frontal, temporal and insular cortex ($r_s = 0.49$ – 0.71), and negative correlations between total MA levels and G6PD in putamen, cerebellum, frontal, occipital and insular cortex ($r_s = -0.46$ to -0.78), GST in putamen and temporal cortex ($r_s = -0.59$ and -0.51), γ -GCS, MnSOD in temporal cortex ($r_s = -0.48$), and γ -GTP in putamen ($r_s = -0.56$) and temporal cortex ($r_s = -0.47$).

To establish whether there might be any relationship between the extent of MA exposure, as inferred from the extent of striatal dopamine depletion, the levels of oxidative stress indices in control subjects were compared with those in the six MA users who had severe (>70%) dopamine reduction in caudate nucleus (73–97% loss compared with control mean) and those in the 14 MA users who had normal to moderately decreased levels (13–68% dopamine loss) (Table 6). Statistically significant differences between the MA user subgroups and the control subjects were limited to caudate nucleus levels of GSSG in the group with moderate dopamine loss (+85%), and caudate nucleus CuZnSOD (+20%) and uric acid (+63%) in those with severe dopamine loss (Table 6), and also putamen activity of GST (−19%) in the latter group (data not shown). A non-significant trend was observed for decreased caudate nucleus GSH (−35%) and putamen GSH (−31%) in the group with severe dopamine loss and increased caudate nucleus GSH (+26%) in those with moderate dopamine loss compared

Table 3 Comparison of levels of oxidative stress indices and methionine in temporal cortex in control biopsied ($n = 3$) and autopsied ($n = 16$) human brain

Index	Biopsied temporal cortex	Autopsied temporal cortex	% change
GSH	9517 ± 1142	4926 ± 832*	−48
GSSG	607 ± 119	178 ± 37*	−71
GSH-Cys	136 ± 12	180 ± 28	28
GPx	162 ± 5	141 ± 5	−13
GR	61.9 ± 2.7	44.8 ± 2.0*	−28
G6PD	42.7 ± 2.0	34.8 ± 1.3*	−18
γ -GTP	7.34 ± 0.44	7.25 ± 0.32	−1
GST	142 ± 17	136 ± 8	−5
CuZnSOD	93.6 ± 15.5	119 ± 8	27
MnSOD	16.5 ± 0.5	17.2 ± 2.4	4
Uric acid	75 ± 18	570 ± 143	660
Methionine	66 ± 18	918 ± 158*	1291

Values are mean ± SEM. Activities of GPx, GR, G6PD, GST and γ -GTP are expressed as nmoles per minute per milligram protein, whereas activity of CuZnSOD is expressed as units per milligram protein. Levels of GSH, GSSG, GSH-Cys, uric acid and methionine are expressed as nanograms per milligram protein. * $p < 0.05$ versus biopsied (Student's two-tailed *t*-test).

Region		GSH	GSSG	GSH-Cys	Uric acid
Caudate	Controls	7895 ± 1336	435 ± 58	700 ± 72	461 ± 65
	MA	8494 ± 996	688 ± 100*	788 ± 57	538 ± 63
Putamen	Controls	10 365 ± 1390	463 ± 102	460 ± 45	482 ± 44
	MA	9816 ± 1397	449 ± 107	507 ± 32	558 ± 47
Cerebellar cortex	Controls	9895 ± 1176	195 ± 27	497 ± 46	683 ± 61
	MA	10 409 ± 1184	198 ± 40	586 ± 52	706 ± 70
Temporal cortex	Controls	4926 ± 832	178 ± 37	180 ± 28	570 ± 143
	MA	5448 ± 526	178 ± 25	171 ± 15	593 ± 85
Occipital cortex	Controls	7534 ± 1722	224 ± 50	406 ± 54	831 ± 127
	MA	6738 ± 1716	183 ± 41	479 ± 97	635 ± 100
Insular cortex	Controls	6399 ± 1324	359 ± 90	399 ± 59	786 ± 132
	MA	6165 ± 789	255 ± 58	345 ± 43	816 ± 155
Frontal cortex	Controls	8125 ± 820	204 ± 48	520 ± 62	473 ± 90
	MA	7856 ± 814	215 ± 47	581 ± 71	376 ± 59
Parietal cortex	Controls	6155 ± 543	195 ± 38	282 ± 39	400 ± 57
	MA	6868 ± 515	185 ± 33	255 ± 30	493 ± 49
White matter	Controls	10 862 ± 1105	485 ± 118	1717 ± 297	708 ± 315
	MA	12 273 ± 1581	297 ± 66	1353 ± 308	343 ± 78
Hippocampus	Controls	7027 ± 187	51 ± 14	1467 ± 187	498 ± 105
	MA	10 524 ± 2085	72 ± 22	1435 ± 232	539 ± 104
Thalamus– medial pulvinar	Controls	4269 ± 791	269 ± 76	1570 ± 201	725 ± 128
	MA	6107 ± 1050	164 ± 44	1761 ± 396	868 ± 121

Table 4 Levels of reduced glutathione, oxidized glutathione, glutathione–cysteine mixed disulfide and uric acid in brain of the control subjects ($n = 16$) and MA users ($n = 20$)

Values are mean ± SEM and are expressed as nanograms per milligram protein. * $p < 0.05$ versus controls in caudate or putamen (Student's two-tailed t -test). In extrastriatal brain areas no statistically significant changes were observed by t -test either without ($p > 0.05$) or with ($p > 0.0007$) Bonferroni adjustments.

Table 5 Glutathione system enzymes and CuZnSOD in brain of control subjects ($n = 16$) and MA users ($n = 20$)

Region		GPx	GR	G6PD	GST	γ -GTP	γ -GCS	CuZnSOD
Caudate	Controls	121 ± 3	52.0 ± 2.5	42.0 ± 1.1	170 ± 10	10.6 ± 0.4	1.38 ± 0.08	148 ± 6
	MA	123 ± 5	49.7 ± 1.2	41.2 ± 1.0	158 ± 9	10.5 ± 0.3	1.30 ± 0.07	168 ± 7*
Putamen	Controls	113 ± 5	44.2 ± 2.6	38.6 ± 1.0	148 ± 7	11.2 ± 0.4	1.28 ± 0.05	177 ± 7
	MA	117 ± 5	40.2 ± 1.4	38.7 ± 1.0	133 ± 6	11.0 ± 0.3	1.18 ± 0.05	198 ± 9
Cerebellar cortex	Controls	96.9 ± 4.2	61.4 ± 3.0	35.8 ± 1.3	210 ± 15	8.65 ± 0.26	0.85 ± 0.04 ^{HS} 1.41 ± 0.14 ^{LS}	120 ± 6
	MA	95.9 ± 3.9	57.1 ± 1.5	37.1 ± 0.7	199 ± 11	8.25 ± 0.32	0.79 ± 0.07 ^{HS} 1.48 ± 0.19 ^{LS}	111 ± 4
Temporal cortex	Controls	141 ± 5	44.8 ± 2.0	34.8 ± 1.3	136 ± 8	7.25 ± 0.32	1.03 ± 0.06	119 ± 9
	MA	136 ± 6	41.5 ± 1.3	33.3 ± 1.0	132 ± 6	7.08 ± 0.16	1.00 ± 0.03	106 ± 6
Occipital cortex	Controls	122 ± 5	38.4 ± 1.9	42.3 ± 2.2	121 ± 6	10.6 ± 0.5	ND	93.5 ± 4.5
	MA	123 ± 5	37.3 ± 1.4	41.2 ± 1.0	118 ± 6	11.1 ± 0.5		98.5 ± 4.2
Insular cortex	Controls	160 ± 6	40.1 ± 1.9	45.1 ± 1.3	181 ± 8	9.42 ± 0.44	ND	113 ± 5
	MA	163 ± 8	37.4 ± 1.4	45.7 ± 1.4	167 ± 9	8.40 ± 0.27		113 ± 4
Frontal cortex	Controls	166 ± 6	54.0 ± 1.8	39.7 ± 1.0	165 ± 10	7.85 ± 0.22	ND	116 ± 4
	MA	165 ± 6	51.7 ± 1.8	39.4 ± 1.0	160 ± 8	7.96 ± 0.19		121 ± 4

Values are mean ± SEM. Activities of GPx, GR, GST, γ -GTP, and G6PD are expressed as nmoles per minute per milligram protein, activity of CuZnSOD as units per milligram protein, and levels of γ -GCS are microgram protein tissue standard per microgram protein. HS, heavy subunit; LS, light subunit; ND, not determined. * $p < 0.05$ versus controls in caudate or putamen (Student's two-tailed t -test). In extrastriatal brain areas no statistically significant changes were observed by t -test without ($p > 0.05$) or with ($p > 0.0007$) Bonferroni adjustments.

Table 6 Levels of dopamine in the striatum of the control subjects ($n = 14$ – 16) and in methamphetamine users ($n = 20$) subgrouped according to the levels of dopamine in the caudate nucleus

	DA	GSH	GSSG	CuZnSOD	Uric acid
Caudate					
Controls	7.03 ± 0.50	7895 ± 1336	435 ± 57	148 ± 6	461 ± 65
Severe DA loss ($n = 6$)	1.08 ± 0.28* (– 85%)	5135 ± 1536 (– 35%)	414 ± 61 (– 5%)	178 ± 6* (+ 20%)	750 ± 103* (+ 63%)
Moderate DA loss ($n = 14$)	3.48 ± 0.35* (– 50%)	9933 ± 1073 (+ 26%)	805 ± 130* (+ 85%)	164 ± 9 (+ 11%)	448 ± 66 (– 3%)
Putamen					
Controls	7.61 ± 0.61	10 365 ± 1390	463 ± 102	177 ± 7	482 ± 44
Severe DA loss ($n = 6$)	1.70 ± 0.30* (– 78%)	7160 ± 1446 (– 31%)	163 ± 48 (– 65%)	194 ± 16 (+10 %)	649 ± 101 (+ 35%)
Moderate DA loss ($n = 14$)	4.68 ± 0.50* (– 39%)	10 955 ± 1844 (+ 6%)	571 ± 140 (+ 23%)	200 ± 11 (+13%)	518 ± 51 (+7%)

Data are the mean ± SEM. Levels of dopamine (DA) are expressed as nanograms per milligram tissue, levels of GSH, GSSG, and uric acid as nanograms per milligram protein, and activity of CuZnSOD as units per milligram protein. Values in parentheses are percentage change with respect to control. Striatal dopamine levels of the 20 MA users have been published previously (Wilson *et al.* 1996; Moszczynska *et al.* 2004) and are reproduced by permission of Oxford University Press. * $p < 0.05$ versus control (one-way ANOVA followed by Fisher's LSD test).

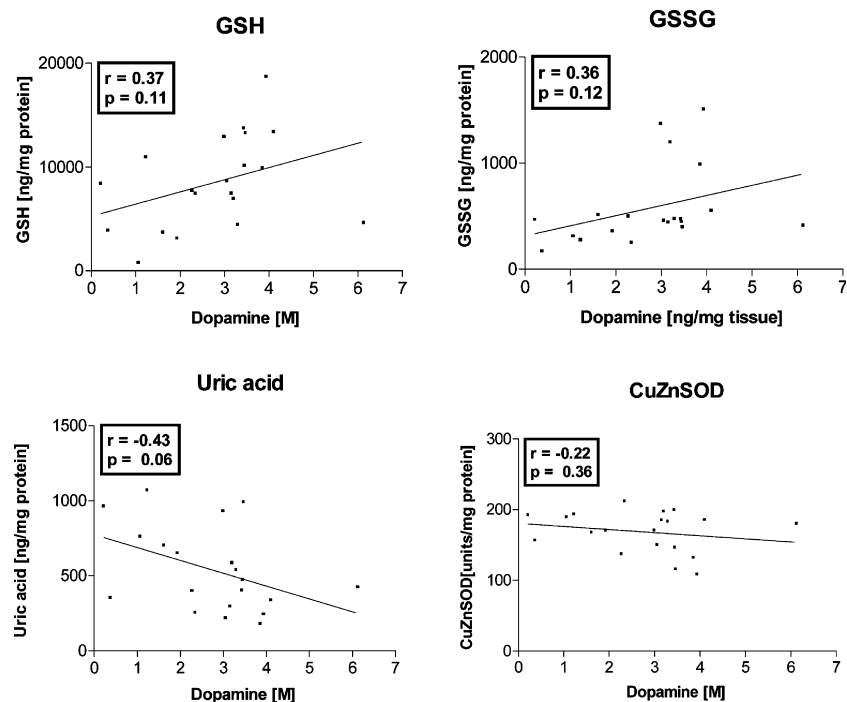


Fig. 1 Regression analyses showed a lack of statistically significant correlations between dopamine levels and GSH, GSSG, uric acid and CuZnSOD in the caudate nucleus of 20 MA users, although a trend was observed for decreased GSH and GSSG and increased uric acid with declining dopamine in the MA users. Striatal dopamine levels in the 20 MA users have been published previously (Wilson *et al.* 1996; Moszczynska *et al.* 2004) and are reproduced by permission of Oxford University Press.

with control levels (Table 6). In caudate nucleus of the 20 MA users, regression analyses revealed no statistically significant correlations between dopamine levels and GSH, GSSG, CuZnSOD and uric acid, although a trend was observed for decreased GSH and GSSG and increased uric acid with declining dopamine concentration (see Fig. 1).

Discussion

We found that in the MA group as a whole, brain levels of most antioxidant systems were generally preserved. Changes were limited to the dopamine-rich caudate and were more marked in those MA users with a very low striatal dopamine level.

Limitations of study

We attempted to address, as much as possible, some of the potential confounding factors inherent in studies of post-mortem human brain of drug users. In order to prove that the MA users had, in fact, used MA both acutely and chronically, levels of the drug were determined in post-mortem blood, brain and, in 14 of the 20 subjects for which autopsied hair was available, hair samples. Although the results of the forensic drug analyses, medical examiner investigation and structured interview with the next of kin suggest that most of the MA users (with the exception of four subjects who tested positive for other drugs of abuse) had used only MA, it is possible that all of the MA users in this study had previously

used other drugs that might somehow have influenced the levels of our biochemical outcome measures. Similarly, accurate information could not be obtained for most of the subjects regarding amount or daily pattern of drug use or interval between last use of drug and death. Notwithstanding these difficulties, the presence of MA in blood and brain of all of the drug users provides absolute confidence that they had all recently used the drug and could therefore be employed to investigate the influence of MA on oxidative stress.

The possible influence of age, agonal status and post-mortem time on brain levels of oxidative stress indices was addressed first by matching the control and MA user groups as closely as possible to these variables. Our finding that levels of methionine, an amino acid that markedly increases after death (Perry *et al.* 1981) were similar in all examined brain areas of the control and MA user groups also suggests that the two groups were reasonably matched with respect to post-mortem time. We also assessed group differences in light of comparison of levels of the biochemical outcome measures in autopsied (mean postmortem time 15 h) and living (biopsied) brain. Concentrations of most of the markers were similar in biopsied and autopsied brain, suggesting relative stability of these markers during the first 15 h after death, but with the expected finding of lower levels of GSH (Perry *et al.* 1981; Perry 1982) and higher concentrations of uric acid in autopsied compared with biopsied brain (Iriyama *et al.* 1986). Although this suggests that post-mortem glutathione and uric acid measurements cannot predict absolute concentrations in living brain, it is not unreasonable to expect that qualitative differences observed in post-mortem brain might also occur *in vivo*.

Oxidative stress indices

Our goal was to establish whether levels of components involved in antioxidant reactions are altered in brain of human MA users. We selected indices that are often altered in a variety of organ systems after exposure to experimental conditions associated with oxidative stress or damage, with a special focus on outcome measures that have been shown to be altered in brain of rodents exposed to MA.

Glutathione

The glutathione system is recognized as a major antioxidant defense against the toxic effects of reactive oxygen species (Ceballos-Picot 1997; Griffith and Mulcahy 1999). The reaction of the tripeptide GSH with reactive oxygen species leads to the conversion of GSH to its oxidized form (GSSG), the latter then being reconverted to GSH via GR, which utilizes NADPH as a reducing agent. In contrast, GSH reaction with most electrophiles leads to formation of a stable thiol adduct in which loss of GSH can be reversed only by *de novo* synthesis involving the sequential action of rate-limiting γ -GCS and glutathione synthetase. Oxidative

stress can cause either decreased or increased GSH depending on the severity of the stress, experimental system and time at which GSH levels are measured, whereas GSSG levels are often, but not always, increased (Halliwell and Cross 1994; Benzi and Moretti 1995). Thus, mild oxidative stress can often result in an increase in GSSG followed by adaptational increase in GSH levels, whereas severe oxidative stress typically causes GSH depletion (Seyfried *et al.* 2000).

Employing the same HPLC–electrochemical detection procedure for GSH measurement as that used in the present study, we first showed that we could detect the expected (Perry *et al.* 1982; Riederer *et al.* 1989; Sian *et al.* 1994a), but slight (30%) reduction of GSH concentration in autopsied substantia nigra of patients with Parkinson's disease (Fitzmaurice *et al.* 2003), a 'gold standard' of GSH deficiency in a neurodegenerative disorder, indicating that this procedure could successfully be used to detect modest group differences in GSH concentration in a post-mortem human brain study.

Experimental animal data suggest that a high binge dose of MA can cause a modest reduction in striatal GSH (Mosczyńska *et al.* 1998) whereas striatal GSH concentration can be raised following a low dose of the drug (Harold *et al.* 2000) (but see Jayanthi *et al.* 1998; Kim *et al.* 1999). We found that in the entire group of 20 MA users mean GSH levels were normal in all examined brain areas. This suggests that any MA-related oxidative stress was not sufficiently severe, in the group of MA users as a whole, to cause depletion of tissue stores of GSH. However, the lack of any overall change in GSH might be explained by the possible inclusion of subjects who died before or after a time-dependent, short-lasting change in GSH levels, or who had developed a partial tolerance to the effects of MA on oxidative stress. Nevertheless, we did observe a non-significant trend in the subgroup of MA users with a severely decreased dopamine level for a reduction in GSH (–35%) in caudate nucleus, the brain area with the most marked dopamine loss, and in the putamen (–31%). This suggests that in brain of MA users who take a dose of the drug sufficient to cause marked dopamine depletion, GSH might be overutilized in antioxidant reactions related to excessive release of dopamine, a scenario supported by animal data showing decreased glutathione concentration following intrastriatal injection of dopamine (Rabinovic *et al.* 2000). In principle, any dopamine-related oxidative stress might be related to changes within dopamine nerve terminals or postsynaptic elements.

Levels of GSSG were significantly increased in caudate nucleus of MA users, with the increase limited to the subgroup of MA users with only moderate dopamine loss (see Table 6). Although increased levels of GSSG limited to a dopamine-rich area suggests the possibility of dopamine-related oxidative stress, these changes cannot be considered

to be robust as the intersubject variability, as expected (Adams *et al.* 1991; Sian *et al.* 1994a; Gotz *et al.* 2001), of GSSG in normal human brain is high and the overall GSSG change was represented by very high GSSG levels in only four of the 20 subjects.

GSH-related enzymes

Surprisingly, no changes were observed in levels of any of the enzymes involved in glutathione metabolism (GPx, GR, G6PD, GST, γ -GTP, γ -GCS) either in the MA group as a whole or in the subgroup with marked dopamine reduction, with the exception of a modestly (-19%) decreased GST level in the putamen of those with severe dopamine loss. This suggests that no substantial damage (e.g. by oxidative stress) to the enzymes or compensatory increase in protein levels occurs in humans chronically exposed to MA, as can occur in brain of experimental animals exposed to MA (decreased GPx activity: Jayanthi *et al.* 1998; Kim *et al.* 1999) and in other models of oxidative stress (decreased activity of glutathione metabolic enzymes: Aspberg and Tottmar 1994; Thiffault *et al.* 1995; Binienda *et al.* 1998; increased activity: Rahman *et al.* 1998; Shimizu *et al.* 1998; Singhal *et al.* 1999).

CuZn SOD

Activity of the enzyme CuZnSOD (SOD1), responsible for conversion of superoxide radical to hydrogen peroxide and molecular oxygen, can be induced in a variety of chronic and acute experimental animal conditions associated with oxidative stress (Ohtsuki *et al.* 1993; Thiffault *et al.* 1995; Binienda *et al.* 1998; Hung and Lee 1998; Shimizu *et al.* 1998) and in GSH-depleted neuroblastoma cells (Ceballos-Picot 1997). CuZnSOD or total SOD activity is also increased in brain of rodents exposed to MA in some (Acikgoz *et al.* 1998, 2000; Kim *et al.* 1999) but not all investigations (D'Almeida *et al.* 1995; Jayanthi *et al.* 1998). Inhibition of CuZnSOD exacerbates MA toxicity to brain dopaminergic neurones (De Vito and Wagner 1989), whereas overexpression is neuroprotective (Cadet *et al.* 1994; Hirata *et al.* 1995).

As in many animal models of oxidative stress, CuZnSOD activity was modestly increased in brain of human MA users. This suggests that in MA-exposed human brain, the activity of an enzyme involved in protection from the toxic effects of superoxide radicals is increased as a compensatory phenomenon caused by oxidative stress. As the increase in activity of CuZnSOD was restricted to a dopamine-rich area (caudate) and was greater in the subgroup of MA users with markedly reduced dopamine ($+20\%$; $p < 0.05$) than in those with moderate dopamine depletion ($+11\%$; $p = 0.10$), oxidative stress may have been related to MA-induced release and subsequent oxidation of dopamine to form superoxide radical and hydrogen peroxide, the latter being an activator of *SOD1* gene expression (Yoo *et al.* 1999).

Uric acid

Uric acid is an end product of metabolism of purines (adenine, guanine, xanthine, and hypoxanthine) and nucleotides that is formed from xanthine or hypoxanthine by xanthine oxidase or xanthine dehydrogenase (Parks and Granger 1986). Uric acid has also been suggested to act as an antioxidant *in vivo* (Ames *et al.* 1981), with levels increased in some experimental conditions considered to involve oxidative stress (Desole *et al.* 1995a,b) including exposure to MA (Yamamoto and Zhu 1998).

Although mean uric acid levels were not altered in brain of the entire group of MA users, concentrations of the purine metabolite were markedly and significantly increased ($+63\%$) in the caudate nucleus and displayed a trend for an increase in the putamen ($+35\%$; $p = 0.08$) of the MA users who had severely decreased striatal dopamine. These findings are consistent with the results of recent experimental studies reporting increased uric acid in striatal tissue (Yamamoto and Zhu 1998) and extracellular fluid (Mueller 1990; Saponjic *et al.* 1994) of rodents exposed to MA or amphetamine. Although the function of uric acid in human brain is not yet defined, animal data showing that uric acid can protect against a variety of insults associated with oxidative stress suggest that uric acid levels might have been increased, perhaps by activation of xanthine oxidase, as a possible compensatory response to acute and/or chronic oxidative stress.

Conclusions

We found that levels of key antioxidant systems were generally preserved in post-mortem brain of human MA users. However, in the subgroup of MA users with very low brain dopamine levels changes were observed in caudate levels of several antioxidant systems that are altered in MA animal models, suggesting that MA might cause dopamine-related oxidative stress in human brain. Further studies are required to establish whether any oxidative stress is sufficient to cause actual oxidative brain damage and whether antioxidant therapy might be neuroprotective for the high-dose MA user.

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References

- Acikgoz O., Gonenc S., Kayatekin B. M., Uysal N., Pekcetin C. and Semin I. (1998) Methamphetamine causes lipid peroxidation and an increase in superoxide dismutase activity in the rat striatum. *Brain Res.* **813**, 200–202.
- Acikgoz O., Gonenc S., Kayatekin B. M., Pekcetin C., Uysal N., Dayi A., Semin I. and Gure A. (2000) The effects of single dose of meth-

- amphetamine on lipid peroxidation levels in the rat striatum and prefrontal cortex. *Eur. Neuropsychopharmacol.* **10**, 415–418.
- Adams J. D. Jr, Klaidman L. K., Odunze I. N., Shen H. C. and Miller C. A. (1991) Alzheimer's and Parkinson's disease. Brain levels of glutathione, glutathione disulfide, and vitamin E. *Mol. Chem. Neuropathol.* **14**, 213–226.
- Ames B. N., Cathcart R., Schwiers E. and Hochstein P. (1981) Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer: a hypothesis. *Proc. Natl Acad. Sci. USA* **78**, 6858–6862.
- Aspberg A. and Totmar O. (1994) Oxidative stress decreases antioxidant enzyme activities in reaggregation cultures of rat brain cells. *Free Radic. Biol. Med.* **17**, 511–516.
- Benzi G. and Moretti A. (1995) Age- and peroxidative stress-related modifications of the cerebral enzymatic activities linked to mitochondria and the glutathione system. *Free Radic. Biol. Med.* **19**, 77–101.
- Bieniada Z., Simmons C., Hussain S., Slikker W. Jr and Ali S. F. (1998) Effect of acute exposure to 3-nitropropionic acid on activities of endogenous antioxidants in the rat brain. *Neurosci. Lett.* **251**, 173–176.
- Cadet J. L. and Brannock C. (1998) Free radicals and the pathobiology of brain dopamine systems. *Neurochem. Int.* **32**, 117–131.
- Cadet J. L., Sheng P., Ali S., Rothman R., Carlson E. and Epstein C. (1994) Attenuation of methamphetamine-induced neurotoxicity in copper/zinc superoxide dismutase transgenic mice. *J. Neurochem.* **62**, 380–383.
- Ceballos-Picot I. (1997) *The Role of Oxidative Stress in Neuronal Death*. Landes Bioscience, Austin.
- Cubells J. F., Rayport S., Rajendran G. and Sulzer D. (1994) Methamphetamine neurotoxicity involves vacuolation of endocytic organelles and dopamine-dependent intracellular oxidative stress. *J. Neurosci.* **14**, 2260–2271.
- D'Almeida V., Camarini R., Azzalis L. A., Mattei R., Junqueira V. B. and Carlini E. A. (1995) Antioxidant defense in rat brain after chronic treatment with anorectic drugs. *Toxicol. Lett.* **81**, 101–110.
- De Vito M. J. and Wagner G. C. (1989) Methamphetamine-induced neuronal damage: a possible role for free radicals. *Neuropharmacology* **28**, 1145–1150.
- Desole M. S., Esposito G., Migheli R., Fresu L., Sircana S., Zangani D., Miele M. and Miele E. (1995a) Cellular defence mechanisms in the striatum of young and aged rats subchronically exposed to managane. *Neuropharmacology* **34**, 289–295.
- Desole M. S., Miele M., Esposito G., Fresu L. G., Migheli R., Zangani D., Sircana S., Grella G. and Miele E. (1995b) Neuronal antioxidant system and MPTP-induced oxidative stress in the striatum and brain stem of the rat. *Pharmacol. Biochem. Behav.* **51**, 581–592.
- Fernandez-Checa J. C. and Kaplowitz N. (1990) The use of monochlorobimane to determine hepatic GSH levels and synthesis. *Anal. Biochem.* **190**, 212–219.
- Fibiger H. C. and McGeer E. G. (1971) Effect of acute and chronic methamphetamine treatment on tyrosine hydroxylase activity in brain and adrenal medulla. *Eur. J. Pharmacol.* **16**, 176–180.
- Fiske C. H. and SubbaRow Y. (1925) The colorimetric determination of phosphorus. *J. Biol. Chem.* **66**, 375.
- Fitzmaurice P. S., Ang L., Guttman M., Rajput A. H., Furukawa Y. and Kish S. J. (2003) Nigral glutathione deficiency is not specific for idiopathic Parkinson's disease. *Mov. Disord.* **18**, 969–976.
- Frey K., Kilbourn M. and Robinson T. (1997) Reduced striatal vesicular monoamine transporters after neurotoxic but not after behaviorally-sensitizing doses of methamphetamine. *Eur. J. Pharmacol.* **334**, 273–279.
- Gipp J. J., Chang C. and Mulcahy R. T. (1992) Cloning and nucleotide sequence of a full-length cDNA for human liver gamma-glutamylcysteine synthetase. *Biochem. Biophys. Res. Commun.* **185**, 29–35.
- Gotz M. E., Janetzky B., Pohli S. *et al.* (2001) Chronic alcohol consumption and cerebral indices of oxidative stress: is there a link? *Alcohol. Clin. Exp. Res.* **25**, 717–725.
- Griffith O. W. and Mulcahy R. T. (1999) The enzymes of glutathione synthesis: gamma-glutamylcysteine synthetase. *Adv. Enzymol. Relat. Areas Mol. Biol.* **73**, 209–267.
- Halliwell B. and Cross C. E. (1994) Oxygen-derived species: their relation to human disease and environmental stress. *Environ. Health. Perspect.* **102**, 5–12.
- Harold C., Wallace T., Friedman R., Gudelsky G. and Yamamoto B. (2000) Methamphetamine selectively alters brain glutathione. *Eur. J. Pharmacol.* **400**, 99–102.
- Harvey P. R., Ilson R. G. and Strasberg S. M. (1989) The simultaneous determination of oxidized and reduced glutathiones in liver tissue by ion pairing reverse phase high performance liquid chromatography with a coulometric electrochemical detector. *Clin. Chim. Acta* **180**, 203–212.
- Harvey D. C., Lacan G. and Melega W. P. (2000a) Regional heterogeneity of dopaminergic deficits in vervet monkey striatum and substantia nigra after methamphetamine exposure. *Exp. Brain Res.* **133**, 349–358.
- Harvey D. C., Lacan G., Tanious S. P. and Melega W. P. (2000b) Recovery from methamphetamine reduced long-term nigrostriatal dopaminergic deficits without substantia nigra cell loss. *Brain Res.* **871**, 259–270.
- Hirata H., Ladenheim B., Rothman R. B., Epstein C. and Cadet J. L. (1995) Methamphetamine-induced serotonin neurotoxicity is mediated by superoxide radicals. *Brain Res.* **677**, 345–347.
- Huang C. S., Anderson M. E. and Meister A. (1993) Amino acid sequence and function of the light subunit of rat kidney gamma-glutamylcysteine synthetase. *J. Biol. Chem.* **268**, 20578–20583.
- Hung H. C. and Lee E. H. (1998) MPTP produces differential oxidative stress and antioxidative responses in the nigrostriatal and mesolimbic dopaminergic pathways. *Free Radic. Biol. Med.* **24**, 76–84.
- Iriyama K., Iwamoto T., Yoshiura M. and Aoki T. (1986) Postmortem changes in uric acid and ascorbic acid in human cerebral cortex tissues excised after cardiac death. *Biochem. Med. Metab. Biol.* **36**, 186–193.
- Jayanthi S., Ladenheim B. and Cadet J. L. (1998) Methamphetamine-induced changes in antioxidant enzymes and lipid peroxidation in copper/zinc-superoxide dismutase transgenic mice. *Ann. N. Y. Acad. Sci.* **844**, 92–102.
- Kim H. C., Jhoo W. K., Choi D. Y., Im D. H., Shin E. J., Suh J. H., Floyd R. A. and Bing G. (1999) Protection of methamphetamine nigrostriatal toxicity by dietary selenium. *Brain Res.* **851**, 76–86.
- Kish S. J., Olivier A., Dubeau F., Robitaille Y. and Sherwin A. L. (1988) Increased activity of cholineacetyltransferase and acetylcholinesterase in actively epileptic human cerebral cortex. *Epilepsy Res.* **2**, 227–231.
- Laruelle M., Abi-Dargham A., van Dyck C. H., Rosenblatt W., Zea-Ponce Y., Zoghbi S. S., Baldwin R. M., Charney D. S., Hoffer P. B. and Kung H. F. (1995) SPECT imaging of striatal dopamine release after amphetamine challenge. *J. Nucl. Med.* **36**, 1182–1190.
- Lee C.-Y. (1982) Glucose-6-phosphate dehydrogenase from mouse. *Meth. Enzymol.* **89**, 252–257.
- Linder J. C., Young S. J. and Groves P. M. (1995) Electron microscopic evidence for neurotoxicity in the basal ganglia. *Neurochem. Int.* **26**, 195–202.

- Martinez D., Slifstein M., Broft A. *et al.* (2003) Imaging human meso-limbic dopamine transmission with positron emission tomography. Part II: amphetamine-induced dopamine release in the functional subdivisions of the striatum. *J. Cereb. Blood Flow Metab.* **23**, 285–300.
- McCann U. D., Wong D. F., Yokoi F., Villemagne V., Dannals R. F. and Ricaurte G. A. (1998) Reduced striatal dopamine transporter density in abstinent methamphetamine and methcathinone users: evidence from positron emission tomography studies with [¹¹C]WIN-35,428. *J. Neurosci.* **18**, 8417–8422.
- Moszczynska A., Turenne S. and Kish S. J. (1998) Rat striatal levels of the antioxidant glutathione are decreased following binge administration of methamphetamine. *Neurosci. Lett.* **255**, 49–52.
- Moszczynska A., Fitzmaurice P., Ang L., Kalasinsky K. S., Schmunk G. A., Peretti F. J., Aiken S. S., Wickham D. J. and Kish S. J. (2004) Why is Parkinsonism not a feature of human methamphetamine users? *Brain* **127**, 363–370.
- Mueller K. (1990) The effects of haloperidol and amphetamine on ascorbic acid and uric acid in caudate and nucleus accumbens of rats as measured by voltammetry *in vivo*. *Life Sci.* **47**, 735–742.
- Ohtsuki T., Matsumoto M., Suzuki K., Taniguchi N. and Kamada T. (1993) Effect of transient forebrain ischemia on superoxide dismutases in gerbil hippocampus. *Brain Res.* **620**, 305–309.
- Paglia D. E. and Valentine W. N. (1967) Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J. Lab. Clin. Med.* **70**, 158–169.
- Parks D. A. and Granger D. N. (1986) Xanthine oxidase: biochemistry, distribution and physiology. *Acta Physiol. Scand. Suppl.* **548**, 87–99.
- Perry T. L. (1982) Cerebral amino acid pools, in *Handbook of Neurochemistry* (Lajtha A., ed.), pp. 151–180. Plenum Publishing Corporation, New York, NY USA.
- Perry T. L., Hansen S. and Gandham S. S. (1981) Postmortem changes of amino compounds in human and rat brain. *J. Neurochem.* **36**, 406–410.
- Perry T. L., Godin D. V. and Hansen S. (1982) Parkinson's disease: a disorder due to nigral glutathione deficiency? *Neurosci. Lett.* **33**, 305–310.
- Rabinovic A. D., Lewis D. A. and Hastings T. G. (2000) Role of oxidative changes in the degeneration of dopamine terminals after injection of neurotoxic levels of dopamine. *Neuroscience* **101**, 67–76.
- Rahman I., Bel A., Mulier B., Donaldson K. and MacNee W. (1998) Differential regulation of glutathione by oxidants and dexamethasone in alveolar epithelial cells. *Am. J. Physiol.* **275**, L80–L86.
- Reid L. L., Botta D., Lu Y., Gallagher E. P. and Kavanagh T. J. (1997a) Molecular cloning and sequencing of the cDNA encoding the catalytic subunit of mouse glutamate-cysteine ligase. *Biochim. Biophys. Acta* **1352**, 233–237.
- Reid L. L., Botta D., Shao J., Hudson F. N. and Kavanagh T. J. (1997b) Molecular cloning and sequencing of the cDNA encoding mouse glutamate-cysteine ligase regulatory subunit. *Biochim. Biophys. Acta* **1353**, 107–110.
- Riederer P., Sofic E., Rausch W. D., Schmidt B., Reynolds G. P., Jellinger K. and Youdim M. B. (1989) Transition metals, ferritin, glutathione, and ascorbic acid in parkinsonian brains. *J. Neurochem.* **52**, 515–520.
- Riley H. A. (1960) *An Atlas of the Basal Ganglia, Brainstem and Spinal Cord*. Hafner, New York.
- Saponjic R. M., Mueller K., Krug D. and Kunko P. M. (1994) The effects of haloperidol, scopolamine, and MK-801 on amphetamine-induced increases in ascorbic and uric acid as determined by voltammetry *in vivo*. *Pharmacol. Biochem. Behav.* **48**, 161–168.
- Seelig G. F. and Meister A. (1985) Glutathione biosynthesis; gamma-glutamylcysteine synthetase from rat kidney. *Meth. Enzymol.* **113**, 379–390.
- Seiden L. S., Fischman M. W. and Schuster C. R. (1975/1976) Long-term methamphetamine induced changes in brain catecholamines in tolerant rhesus monkeys. *Drug Alcohol Depend.* **1**, 215–219.
- Sekine Y., Iyo M., Ouchi Y., Matsunaga T., Tsukada H., Okada H., Yoshikawa E., Futatsubashi M., Takei N. and Mori N. (2001) Methamphetamine-related psychiatric symptoms and reduced brain dopamine transporters studied with PET. *Am. J. Psychiatry* **158**, 1206–1214.
- Sen C. K. (1997) Nutritional biochemistry of cellular glutathione. *Nutr. Biochem.* **8**, 660–672.
- Seyfried J., Soldner F., Kunz W. S., Schulz J. B., Klockgether T., Kovar K. A. and Wullner U. (2000) Effect of 1-methyl-4-phenylpyridinium on glutathione in rat pheochromocytoma PC 12 cells. *Neurochem. Int.* **36**, 489–497.
- Shimizu T., Iwanaga M., Yasunaga A., Urata Y., Goto S., Shibata S. and Kondo T. (1998) Protective role of glutathione synthesis on radiation-induced DNA damage in rabbit brain. *Cell. Mol. Neurobiol.* **18**, 299–310.
- Sian J., Dexter D. T., Lees A. J., Daniel S., Agid Y., Javoy-Agid F., Jenner P. and Marsden C. D. (1994a) Alterations in glutathione levels in Parkinson's disease and other neurodegenerative disorders affecting basal ganglia. *Ann. Neurol.* **36**, 348–355.
- Sian J., Dexter D. T., Lees A. J., Daniel S., Jenner P. and Marsden C. D. (1994b) Glutathione-related enzymes in brain in Parkinson's disease. *Ann. Neurol.* **36**, 356–361.
- Singhal S. S., Godley B. F., Chandra A., Pandya U., Jin G. F., Saini M. K., Awasthi S. and Awasthi Y. C. (1999) Induction of glutathione S-transferase hGST 5.8 is an early response to oxidative stress in RPE cells. *Invest. Ophthalmol. Vis. Sci.* **40**, 2652–2659.
- Smith I. K., Vierheller T. L. and Thorne C. A. (1988) Assay of glutathione reductase in crude tissue homogenates using 5,5'-dithiobis (2-nitrobenzoic acid). *Anal. Biochem.* **175**, 408–413.
- Sonsalla P. K., Jochnowitz N. D., Zeevalk G. D., Oostveen J. A. and Hall E. D. (1996) Treatment of mice with methamphetamine produces cell loss in the substantia nigra. *Brain Res.* **738**, 172–175.
- Spearman M. E. and Leibman K. C. (1984) Effects of aging on hepatic and pulmonary glutathione S-transferase activities in male and female Fischer 344 rats. *Biochem. Pharmacol.* **33**, 1309–1313.
- Tate S. S. and Meister A. (1985) gamma-Glutamyl transpeptidase from kidney. *Meth. Enzymol.* **113**, 400–419.
- Thiffault C., Aumont N., Quirion R. and Poirier J. (1995) Effect of MPTP and 1-deprenyl on antioxidant enzymes and lipid peroxidation levels in mouse brain. *J. Neurochem.* **65**, 2725–2733.
- Thompson S. A., White C. C., Krejsa C. M., Diaz D., Woods J. S., Eaton D. L. and Kavanagh T. J. (1999) Induction of glutamate-cysteine ligase (gamma-glutamylcysteine synthetase) in the brains of adult female mice subchronically exposed to methylmercury. *Toxicol. Lett.* **110**, 1–9.
- Volkow N. D., Chang L., Wang G. J. *et al.* (2001a) Loss of dopamine transporters in methamphetamine abusers recovers with protracted abstinence. *J. Neurosci.* **21**, 9414–9418.
- Volkow N. D., Chang L., Wang G. J. *et al.* (2001b) Association of dopamine transporter reduction with psychomotor impairment in methamphetamine abusers. *Am. J. Psychiatry* **158**, 377–382.
- Wilson J. M., Kalasinsky K. S., Levey A. I., Bergeron C., Reiber G., Anthony R. M., Schmunk G. A., Shannak K., Haycock J. W. and Kish S. J. (1996) Striatal dopamine nerve terminal markers in human, chronic methamphetamine use. *Nat. Med.* **2**, 699–703.
- Wise R. A. (1996) Neurobiology of addiction. *Curr. Opin. Neurobiol.* **6**, 243–251.

- Woolverton W. L., Ricaurte G. A., Forno L. S. and Seiden L. S. (1989) Long-term effects of chronic methamphetamine administration in rhesus monkeys. *Brain Res.* **486**, 73–78.
- Yamamoto B. K. and Zhu W. (1998) The effects of methamphetamine on the production of free radicals and oxidative stress. *J. Pharmacol. Exp. Ther.* **287**, 107–114.
- Yan N. and Meister A. (1990) Amino acid sequence of rat kidney gamma-glutamylcysteine synthetase. *J. Biol. Chem.* **265**, 1588–1593.
- Yokoyama H., Tsuchihashi N., Kasai N., Matsue T., Uchida I., Mori N., Ohya-Nishiguchi H. and Kamada H. (1997) Hydrogen peroxide augmentation in a rat striatum after methamphetamine injection as monitored *in vivo* by a Part-disk microelectrode. *Biosens. Bioelectron.* **12**, 1037–1041.
- Yoo H. Y., Chang M. S. and Rho H. M. (1999) The activation of the rat copper/zinc superoxide dismutase gene by hydrogen peroxide through the hydrogen peroxide-responsive element and by paraquat and heat shock through the same heat shock element. *J. Biol. Chem.* **274**, 23887–23892.