Protection against 3,4-methylenedioxymethamphetamine-induced neurodegeneration produced by glutathione depletion in rats is mediated by attenuation of hyperthermia

E. O’Shea,* N. Easton,* J. R. Fry,* A. R. Green† and C. A. Marsden*

*School of Biomedical Sciences, Queen’s Medical Centre, Nottingham, UK
†Pharmacology Research Group, School of Pharmacy, De Montfort University, Leicester, UK

Abstract

3,4-Methylenedioxymethamphetamine (MDMA) administration produces neurotoxic degeneration of serotonin terminals in rat brain. These effects occur only after systemic administration and not after central injection, suggesting that peripheral metabolism, possibly hepatic, is required for toxicity. Glutathione is one of the principal cellular defence mechanisms, but conjugation with glutathione can, on some occasions, increase the reactivity of certain molecules. Previous studies have shown that central administration of glutathione adducts of a MDMA metabolite produces a neurotoxicity profile similar to that of systemic MDMA. In the present study, depletion of peripheral (hepatic) glutathione by 43% with DL-buthionine-(S,R)-sulfoximine (an inhibitor of glutathione synthesis) did not attenuate MDMA-induced neurotoxicity as indicated by the 34% loss of [3H]paroxetine binding to the serotonin uptake sites in Dark Agouti rats treated with the inhibitor. However, a more profound depletion (92%) of glutathione by diethylmaleate (direct conjugation) administration significantly reduced the serotonergic neurotoxicity produced by MDMA. This depletion protocol also attenuated the hyperthermic response to MDMA. A combination protocol utilising both buthionine-(S,R)-sulfoximine and diethylmaleate that did not alter the hyperthermic response of the rats given MDMA also failed to attenuate the neurotoxicity. These findings indicate that glutathione depletion does not offer specific protection against MDMA-induced serotonin neurotoxicity in Dark Agouti rats.

Keywords: glutathione, hyperthermia, metabolite, 3,4-methylenedioxymethamphetamine, neuroprotection, neurotoxicity.


3,4-Methylenedioxymethamphetamine (MDMA or ‘ecstasy’) is a commonly used drug of abuse which, in recent years, has led to a number of deaths among young people due to the acute effects of the drug including hyperthermia, acute renal failure, rhabdomyolysis, arrhythmias, intravascular disseminated coagulation and cardiovascular collapse, as well as thrombosis and/or haemorrhage (Brown and Osterloh 1987; Dowling et al. 1987; Suarez and Riemersma 1988; Henry 1992). There is also evidence that MDMA causes long-term neurotoxic effects in various species of animals (Green et al. 1995), including humans (McCann et al. 1998, 1999). This damage has been observed up to 7 years after treatment in some species of non-human primates (Hatzidimitriou et al. 1999) and manifests itself as a loss of indole content and terminals of the fine serotonergic projections from the dorsal raphe nucleus (Battaglia et al. 1987; O’Hearn et al. 1988; Molliver et al. 1990; Hewitt and Green 1994; Colado and Green 1995).

This toxicity to the serotonergic neurones occurs after systemic (Colado et al. 1997a, 1997b; Finnegan et al. 1988; Slikker et al. 1988; O’Shea et al. 1998) but not central administration of the drug (McCann and Ricaurte 1991; Paris and Cunningham 1992; Esteban et al. 2001; Nixdorf et al. 2001), and has led to the proposal that peripheral metabolism of MDMA, possibly hepatic, is required before the neurotoxic profile can develop (Esteban et al. 2001). Various studies have

Received October 2, 2001; revised manuscript received January 16, 2002; accepted January 21, 2002.

Address correspondence and reprint requests to E. O’Shea, Dpto Farmacologia, F. Medicina, Universidad Complutense de Madrid, Avda Complutense s/n, 28040 Madrid, Spain, E-mail: estheros@farm.ucm.es

Abbreviations used: BSO, DL-buthionine-[S,R]-sulfoximine; DEM, diethylmaleate; GSH, reduced glutathione; 5-GSH-α-MeDA, 5-(glutathion-S-yl)-α-methylodopamine; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine (serotonin); MDA, 3,4-methylenedioxyamphetamine; MDMA, 3,4-methylenedioxymethamphetamine.
been carried out to identify the metabolic products of MDMA (Lim and Foltz 1988, 1991a, 1991b) but, to date, none has been found with a toxicity profile matching that of MDMA (Elayan et al. 1992; Johnson et al. 1992). More recently, studies have indicated that reduced glutathione (GSH) adducts of putative MDMA metabolites have some properties similar to systematically administered MDMA. MDMA is metabolized in the liver by demethylation to 3,4-methylenedioxyamphetamine (MDA), and both this metabolite and MDMA can be demethylated, yielding 3,4-dihydroxyamphetamine (α-methylamphetamine) and 3,4-dihydroxyamphetamine, respectively (Cho et al. 1990; Lin et al. 1992). These metabolites have catechol structures and can be converted to their corresponding quinones, compounds that do not easily pass the blood–brain barrier. They may, however, conjugate with GSH to form adducts such as 5-(glutathion-α-y1)-α-methylamphetamine (5-GSH-α-MeDA) and 5-(glutathion-S-yl)-N-methyl-α-methylamphetamine (Hiramatsu et al. 1990; Miller et al. 1995) that may cross the blood–brain barrier using a GSH transporter. Indeed, the brain uptake index of the compound is reduced by GSH co-administration, indicating a competitive uptake mechanism (Miller et al. 1996). 5-GSH-α-MeDA produces, after repeated central administration, selective 5-HT terminal degeneration (Bai et al. 1999).

Once in the brain, 5-GSH-α-MeDA is metabolized via a cysteine derivative to 5-(N-acety1-L-cystein-S-yl)-α-methylamphetamine (Miller et al. 1995). This latter compound is cleared at a much slower rate than its precursor and is more toxic, possibly due to the conservation of a quinone structure that allows further redox cycling (Miller et al. 1997; Bai et al. 1999).

5-GSH-α-MeDA may also undergo a further addition of a GSH molecule yielding the di-conjugate 2,5-bis-(glutathion-S-yl)-α-methylamphetamine. This compound has been shown to be neurotoxic following central administration and could also be further metabolized to a more toxic derivative (Miller et al. 1997; Bai et al. 1999). It seems reasonable to propose that a reduction in the rate and extent of peripheral conjugation with GSH would reduce the formation of the conjugated metabolites of MDMA and so possibly attenuate its toxic effects.

Therefore, our aim in this study was to investigate the role of peripheral GSH in the neurotoxicity of MDMA. Modulation of peripheral GSH was achieved by inhibition of GSH synthesis (using BSO, an inhibitor of γ-glutamylcysteine synthetase, the rate-limiting step in GSH synthesis) and/or by depletion of preformed GSH (using DEM, which conjugates directly with GSH).

**Materials and methods**

**Animals**

Adult male Dark Agouti rats (Harlan, UK) weighing 170–210 g were used. They were housed in groups of 4–6, in conditions of constant temperature (21 ± 2°C) and a 12-h light/dark cycle (lights on 07:00 h) and given free access to food and water. All animal procedures were carried out with the approval of the Home Office (UK) under Project Licence 40/1955.

**Drugs and administration protocol**

The following drugs were used: (+/−) 3,4-methylenedioxymethamphetamine HCl (MDMA, synthesized by Department Chemistry, University College Dublin, Ireland), dl-buthionine-[(S,R)-sulfoximine (BSO) and liquid diethylmaleate (DEM) from Sigma (Dorset, UK). All drug injections were intraperitoneal (i.p.). MDMA (dose quoted in terms of the base) was dissolved in 0.9% w/v NaCl (saline) and injected in a volume of 1 mL/kg. BSO was suspended in saline containing 10% ethanol and 10% Tween-80 (vehicle) and given in a volume of 5 mL/kg. DEM was diluted in peanut oil (vehicle) and injected in a volume of 1 mL/kg. Control animals for each treatment were injected with saline or vehicle at the corresponding times.

**Study I**

Animals were treated with BSO (890 mg/kg) 4 h before administration of MDMA (12.5 mg/kg) and maintained on BSO in drinking water (4.446 g/L) for 24 h after MDMA injection.

**Study II**

Animals were administered DEM (3 mmol/kg) 30 min before and 2 h after MDMA.

**Study III**

Animals received BSO (890 mg/kg) 18 h before MDMA and maintained on BSO (4.446 g/L) in drinking water until 24 h after the MDMA treatment. In addition, 30 min before MDMA treatment animals were administered DEM (1.5 mmol/kg).

In all neurotoxicity experiments, animals were killed 7 days after the administration of MDMA.

[3H]Paroxetine (specific activity = 25 Ci mmol) was obtained from NEN (UK). All other chemicals were obtained from Sigma and BDH (Poole, Dorset, UK).

**Measurement of rectal temperature**

Temperature was measured by use of a digital readout thermocouple (Type K thermometer, Portec, Wrestlingworth, UK) with a resolution of ±0.1°C and accuracy of ±0.2°C attached to a CAC-005 Rodent Sensor which was inserted into the rectum of the rat, the animal being lightly restrained by holding in the hand. A steady readout was obtained within 10s of probe insertion.

**Tissue extraction**

Rats were killed by cervical dislocation and decapitation, the liver and brain rapidly removed and cortex, hippocampus and striatum dissected out on a cold plate. Tissue for GSH and monoamine measurements was immediately frozen in liquid nitrogen and kept at −80°C until processed. Cerebral cortices for [3H]paroxetine binding studies were placed on ice.

**Measurement of reduced GSH levels in liver and brain**

GSH determinations were carried out by the method described by Sassame and Boyd (1978) with minor modifications. Briefly, tissue
was homogenized in 4% sulfosalicylic acid and centrifuged at 18 000 g for 20 min at 4°C. Aliquots of the supernatants and GSH standards were reacted with 0.1 mM 5,5'-dithio-bis(2-nitrobenzoic acid) for 5 min and the absorbance determined at 405 nm.

**Measurement of monoamines and their metabolites in cerebral tissue**
Quantification of monoamines and their metabolites was carried out following the method previously described (Colado et al. 1997a) with minor modifications. Tissue was sonicated in 0.2 M perchloric acid containing 0.1% cysteine, 0.1% sodium metabisulfite and 0.01% EDTA and centrifuged at 18000 g for 20 min at 4°C. Levels of 5-HT and 5-HIAA in the supernatant were determined by high-performance liquid chromatography (HPLC) with electrochemical detection. The mobile phase consisted of KH2PO4 (50 mM), octanesulfonic acid (sodium salt, 0.16 mM), EDTA (0.1 mM) and methanol (16%), adjusted to pH 3 with o-phosphoric acid, filtered and degassed.

The HPLC system consisted of a pump (HPLC technology RR/066 L solvent pump) linked to a manual injector (Loop 20 μL, Rheodyne 7125 Manual Injector), a stainless steel reverse-phase column (Sphericlon ODS2, 5 μm, 150 x 4.6 mm, Phenomenex, Macclesfield, UK) fitted with a pre-column (ODS, 4 x 3 mm, Phenomenex) and an amperometric detector (Antec EC Controller CU-04AZ). The flow rate was 0.6 mL/min and the glassy carbon working electrode potential was set at +0.75 V with reference to a saturated KCl-filled Ag/AgCl reference electrode. The current produced was monitored by means of an integrator (Spectra-Physics Chromojet integrator, Spectra-Physics, Darmstadt, Germany).

Quantification of [3H]paroxetine binding in the cerebral cortex
[3H]Paroxetine binding was measured in fresh cortical tissue by the method described in detail by Hewitt and Green (1994). Briefly, tissue was homogenized in ice-cold Tris–HCl (50 mM; pH 7.4) containing NaCl (120 mM) and KCl (5 mM) using an Ultra-Turrax. The homogenate was centrifuged at 30000 g for 10 min at 4°C. The supernatant was discarded and the wash procedure repeated twice more. The pellet was finally resuspended in the Tris buffer at a concentration of 10 mg tissue/mL. Aliquots of tissue (800 μL) were incubated with [3H]paroxetine (1 nM) for 60 min at room temperature in the absence and presence of 5-HT (100 μM) for determination of total and nonspecific binding, respectively. Assays were terminated by rapid filtration through GF/B filters and radioactivity determined by scintillation spectrometry. Protein was determined by the method of Lowry et al. (1951).

**Data analyses**
Results are expressed as mean ± SEM. Data from monoamine, binding and GSH studies were analysed either by one-way ANOVA followed by the Tukey multiple comparison test when a significant F-value was obtained or by an unpaired t-test (GraphPad PrismSoftware, Version 3.00 for Windows, GraphPad, San Diego, CA, USA). Statistical analyses of the temperature measurements were performed using repeated measures ANOVA with treatment as the between-subjects factor and time as the repeated measure, followed by the Tukey Compromise test when a significant F-value was obtained. ANOVA was performed on both pre-MDMA (basal temperature) and post-MDMA data (SuperANOVA, Abacus Concepts, Inc., Berkeley, CA, USA).

**Results**

**Study I**

**Effect of BSO on hepatic and central GSH levels**
Administration of BSO (one injection of 890 mg/kg and 4.446 g/L in drinking water until the animals were killed) reduced hepatic GSH levels by 43% 4 h later without affecting hippocampal, cortical or striatal levels (Fig. 1). This depletion of hepatic GSH was increased slightly at 24 h (67%) but with little effect on central GSH levels (Fig. 1).

**Effect of BSO on MDMA-induced neurodegeneration**
Seven days after administration, MDMA (12.5 mg/kg) produced a significant reduction in the number of [3H]paroxetine-labelled cortical 5-HT uptake sites (Fig. 2a) which was unaffected by pre-treatment with BSO (Fig. 2a).

Administration of MDMA also produced a significant reduction in the levels of 5-HT and its metabolite, 5-HIAA, in the hippocampus, striatum and cortex (Table 1).
Pre-treatment with BSO did not modify this reduction nor did it affect the indole levels in saline-treated rats (Table 1).

**Effect of BSO on MDMA-induced hyperthermia**

Administration of MDMA produced an increase in the rectal temperature that peaked at 30 min and persisted for at least 4 h. Pre-treatment with BSO did not alter the hyperthermia produced by MDMA nor the temperature of saline-treated animals (Fig. 3a).

**Study II**

**Effect of DEM on hepatic and central GSH levels**

Administration of DEM (3 mmol/kg) induced a 92% depletion of hepatic GSH levels 60 min after treatment. This depletion was reduced to 37% at 180 min (Fig. 4). DEM treatment also significantly reduced levels of GSH in various brain regions 60 min after administration (Fig. 4).

**Effect of DEM on MDMA-induced neurodegeneration**

DEM administered 30 min before and 120 min after MDMA attenuated the reduction in [³H]paroxetine-labelled cortical 5-HT uptake sites induced by MDMA 7 days later (Fig. 2b). However, MDMA-induced reductions in 5-HT and 5-HIAA levels in the hippocampus, cortex and striatum were unaltered by DEM treatment (Table 2).

**Effect of DEM on MDMA-induced hyperthermia**

Administration of DEM to MDMA-treated animals significantly reduced, but did not abolish, MDMA-induced hyperthermia (Fig. 3b). However, DEM did not modify the magnitude of peak increase in temperature of MDMA-treated animals (vehicle/MDMA/vehicle: 1.64 ± 0.17°C; DEM/MDMA/DEM: 1.63 ± 0.30°C). Administration of DEM to saline-treated animals produced an initial slight hypothermia that was increased by the second DEM dose and persisted for at least 4 h after the second DEM dose (Fig. 3b).

**Study III**

**Effect of BSO and DEM on hepatic and central GSH levels**

Administration of BSO (890 mg/kg i.p. and 4.446 g/L in drinking water) 18 h before and DEM (1.5 mmol/kg) 30 min before MDMA treatment, respectively, i.p., 18 h and 30 min before MDMA treatment, respectively) failed to modify the reduction. Results shown as mean ± SEM, n = 3–7. Different from corresponding controls: *p < 0.05 and 0.001, respectively; different from MDMA-treated: *p < 0.05 (one-way ANOVA followed by Tukey’s test).

Pre-treatment with the combination of BSO and DEM did not produce any change in the MDMA-induced reduction in the number of [³H]paroxetine-labelled cortical 5-HT uptake sites, 7 days after treatment (Fig. 2c). Similarly, it did not modify the
reduction in indole levels induced by MDMA in the hippocampus, cortex or striatum, 7 days after treatment (Table 3).

Effect of BSO and DEM on MDMA-induced hyperthermia
Administration of MDMA produced a sustained hyperthermia that peaked at 30 min. Pre-treatment with BSO and DEM did not modify MDMA-induced hyperthermia (Fig. 3c) nor did it modify the saline response.

Discussion
Administration of a single dose of MDMA produced neurodegeneration of the 5-HT terminals in several brain regions of Dark Agouti rats and this effect appeared to be unaffected by hepatic glutathione depletion.

Initially, studies were carried out using a glutathione depleting protocol involving BSO, an irreversible inhibitor of glutathione peroxidase synthesis. The effects of BSO on MDMA-induced hyperthermia were observed in the first 24 h after treatment (Fig. 3a). BSO treatment was started 4 h before MDMA injection and continued for 24 h. The results show that BSO treatment reduced the basal temperature of the animals compared with control animals (p < 0.01). However, the effect of BSO on MDMA-induced hyperthermia was not statistically significant (p > 0.05). In contrast, DEM treatment, which is a potent activator of glutathione peroxidase, produced a marked hypothermia in animals not treated with MDMA (p < 0.01). BSO/DEM treatment did not modify this response nor did it modify the temperature of animals not treated with MDMA.

Table 1: Effect of buthionine-[S,R]-sulfoximine (BSO) on MDMA-induced reductions in indole levels in the brain

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hippocampus</th>
<th>Cortex</th>
<th>Striatum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5-HT</td>
<td>5-HIAA</td>
<td>5-HT</td>
</tr>
<tr>
<td>Vehicle/saline</td>
<td>1.77 ± 0.07</td>
<td>1.81 ± 0.06</td>
<td>1.90 ± 0.07</td>
</tr>
<tr>
<td>Vehicle/MDMA</td>
<td>0.83 ± 0.07*</td>
<td>1.04 ± 0.05*</td>
<td>1.09 ± 0.10*</td>
</tr>
<tr>
<td>BSO/MDMA</td>
<td>0.91 ± 0.07*</td>
<td>1.19 ± 0.07*</td>
<td>1.15 ± 0.07*</td>
</tr>
<tr>
<td>BSO/saline</td>
<td>1.58 ± 0.16</td>
<td>1.85 ± 0.11</td>
<td>2.01 ± 0.09</td>
</tr>
</tbody>
</table>

Animals were treated with BSO (890 mg/kg, i.p. 4 h before and 4.446 g/L in drinking water for 24 h after MDMA treatment) or vehicle before MDMA (12.5 mg/kg, i.p.) or saline and killed 7 days later. Data expressed as pmol/mg tissue. Results shown as mean ± SEM, n = 5–12. Different from vehicle/saline-treated animals: *p < 0.001; different from vehicle/MDMA: ^p < 0.05 (one-way ANOVA followed by Tukey’s test).

Fig. 3: Effect of glutathione depletion on MDMA-induced hyperthermia. Pre-treatment with BSO (a) slightly increased the basal temperature of the animals compared with pre-treatment with vehicle (p < 0.01), whereas pre-treatment with DEM (b) reduced the basal temperature of the animals compared with pre-treatment with vehicle (p < 0.05). Pre-treatment with BSO and DEM (c) had no effect on basal temperatures of the animals. MDMA (12.5 mg/kg, i.p., indicated by the solid arrow) produced a significant increase in the rectal temperature of the rats (p < 0.01). BSO pre-treatment (a, 890 mg/kg, i.p. 4 h before MDMA and 4.446 g/L in drinking water for 24 h after MDMA treatment) did not modify this response nor modify the temperature of animals not treated with MDMA, whereas DEM (b, 3 mmol/kg, i.p., indicated by the broken arrows) attenuated the MDMA-induced response (p < 0.01) and produced a marked hypothermia in animals not treated with MDMA (p < 0.01). BSO/DEM pre-treatment (c, 890 mg/kg and 1.5 mmol/kg i.p., 18 h and 30 min indicated by the broken arrow, before MDMA treatment, respectively) did not modify this response nor did it modify the temperature of animals not treated with MDMA. Results shown as mean ± SEM, n = 4–8 (repeated measures ANOVA followed by Tukey Compromise test). For time points –0.5 h and 0 h, open circles correspond to all animals pre-treated with vehicle and closed squares to all animals pre-treated with (a) BSO (b) DEM or (c) BSO/DEM. Thereafter the symbols are as explained in the figure.
γ-glutamylcysteine synthetase (Griffith and Meister 1979a). This enzyme is the rate-limiting step in the synthesis of glutathione and therefore inhibition of the enzyme function leads to a progressive depletion in GSH levels as the existing GSH is mobilized from different stores and utilized (Griffith and Meister 1985). The reduction is long-lasting since synthesis of new enzyme is required to overcome the block and it can be maintained by administration of BSO in drinking water after an initial i.p. administration; no rebound increase in GSH content appears to occur after cessation of treatment (Robertson et al. 1998). The compound is relatively non-toxic and has been tested in humans for the treatment of cancer (O’Dwyer et al. 1992). In our initial studies (study I), the depletion in hepatic GSH produced was approximately 43% and although levels in other peripheral tissues were not determined, other studies have shown that this dosing schedule results in significant reductions in the GSH levels in heart, kidney and blood (Griffith and Meister 1979b, 1985; Robertson et al. 1998). It seems reasonable therefore to assume that levels in these tissues were also lowered. This treatment, however, produced only a slight depletion in brain tissue. This relative selectivity of GSH depletion was of benefit in our experimental design as it avoided any non-specific neurodegeneration which may occur in a GSH-depleted brain, especially one subjected to MDMA-induced changes, for example the substantial increases in neurotransmitter release, hyperthermia and increases in free radical formation (Colado et al. 1997a, 1997b, 1999a; Esteban et al. 2001).

Peripheral depletion of GSH by 43% failed to modify the neurodegeneration induced by MDMA. Previous studies, however, have shown that a high degree of GSH depletion is required to have any effect on the rate of substrate conjugation (Mulder and Ouwerkerk-Mahadevan 1997). Indeed, according to Polhuijs et al. (1992) hepatic levels of GSH must be reduced to below 1 μmol/g tissue before significantly affecting the conjugation rate. This corresponds to a depletion of about 90%, substantially more than that produced by this initial protocol. DEM conjugates directly with GSH and requires no preliminary biotransformation so the effect, which is catalysed by cytosolic glutathione S-alkenetransferase and consists of the addition of GSH across an α,β-unsaturated bond (Boyland and Chasseaud 1967), is rapid, reaching a maximum hepatic depletion by 30 min which lasts for 2 h (Ecobichon 1984). However, a disadvantage of this compound is that the induced depletion is short-lived, with recovery reaching 50% by 8 h followed by a rebound increase in GSH (Ecobichon 1984). Thus, it is essential to give further administrations of the drug to maintain depletion. In the present study, DEM produced decreased central as well as peripheral GSH thus exposing the brain to potential non-specific neurodegenerative effects.

### Table 2 Effect of diethylmaleate (DEM) on MDMA-induced reductions in indole levels in the brain

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hippocampus</th>
<th>Cortex</th>
<th>Striatum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5-HT</td>
<td>5-HIAA</td>
<td>5-HT</td>
</tr>
<tr>
<td>Vehicle/saline/vehicle</td>
<td>2.91 ± 0.19</td>
<td>2.45 ± 0.17</td>
<td>2.07 ± 0.03</td>
</tr>
<tr>
<td>Vehicle/MDMA/vehicle</td>
<td>1.23 ± 0.14**</td>
<td>1.40 ± 0.20**</td>
<td>1.47 ± 0.20**</td>
</tr>
<tr>
<td>DEM/MDMA/DEM</td>
<td>1.35 ± 0.09**</td>
<td>1.52 ± 0.11**</td>
<td>2.25 ± 0.51</td>
</tr>
<tr>
<td>DEM/saline/DEM</td>
<td>2.85 ± 0.16</td>
<td>2.57 ± 0.11</td>
<td>2.00 ± 0.39</td>
</tr>
</tbody>
</table>

Animals were treated with DEM (3 mmol/kg, i.p. 30 min before and 2 h after MDMA) or vehicle before and after MDMA (12.5 mg/kg, i.p) or saline and killed 7 days later. Data expressed pmol/mg tissue. Results shown as mean ± SEM, n = 3–7. Different from vehicle/saline/vehicle-treated animals: **p < 0.01, 0.001, respectively (one-way ANOVA followed by Tukey’s test).
In order to exclude a non-specific component to the neurodegeneration observed after MDMA, we determined the levels of dopamine and its main metabolites (3,4-dihydroxyphenylacetic acid and homovanillic acid) and also quantified the uptake of \([3H]\)noradrenaline by cortical synaptosomes since both the dopaminergic and noradrenergic systems are spared by MDMA (Stone et al. 1986; Battaglia et al. 1987, 1988; Commins et al. 1987; Schmidt and Kehne 1990). No changes in these parameters were observed (data not shown). However, DEM administration did attenuate the 5-HT neurodegeneration induced by MDMA.

Measurement of the rectal temperature of the animals during the 6 h after MDMA treatment revealed that DEM pre- and post-treatment also attenuated the hyperthermia induced by MDMA. The mechanism of DEM-induced hypothermia is not fully understood but it is thought that its effect on temperature is independent of its GSH depleting effect (Costa and Murphy 1986) and could possibly be related to its impairment of glycogen synthesis and metabolism leading to an impairment of energy production (Bignall et al. 1977; Krack et al. 1980; Steffen and Musacchia 1985). Prevention, or even attenuation, of the hyperthermic response to MDMA has been widely shown to be neuroprotective against the degeneration induced by MDMA (Farfel and Seiden 1995a; Malberg et al. 1996; Colado et al. 1998, 1999a, 1999b) and other substituted amphetamines (Farfel and Seiden 1995b), leading to the question of whether or not the attenuation in hyperthermic response to MDMA induced by DEM treatment might have produced non-specific protection.

In order to answer this, we designed a protocol that depleted peripheral GSH to a similar extent to that observed in study II, but that did not alter the hyperthermic response to MDMA and evaluated the capacity it had to protect against MDMA-induced neurodegeneration. The combination of inhibition of GSH synthesis afforded by BSO and conjugation of existing GSH by DEM produced no attenuation in the hyperthermic response to MDMA but also failed to protect against the reduction in \([3H]\)paroxetine-labelled 5-HT sites produced by MDMA, 7 days after administration. This finding would seem to suggest that the protection observed in the earlier study when DEM alone was used (study II) might have been due solely to its attenuation of MDMA-induced hyperthermia.

Although the results obtained indicate that the neuroprotection observed in GSH-depleted animals (study II) was due

In order to exclude a non-specific component to the neurodegeneration observed after MDMA, we determined the levels of dopamine and its main metabolites (3,4-dihydroxyphenylacetic acid and homovanillic acid) and also quantified the uptake of \([3H]\)noradrenaline by cortical synaptosomes since both the dopaminergic and noradrenergic systems are spared by MDMA (Stone et al. 1986; Battaglia et al. 1987, 1988; Commins et al. 1987; Schmidt and Kehne 1990). No changes in these parameters were observed (data not shown). However, DEM administration did attenuate the 5-HT neurodegeneration induced by MDMA.

Measurement of the rectal temperature of the animals during the 6 h after MDMA treatment revealed that DEM pre- and post-treatment also attenuated the hyperthermia induced by MDMA. The mechanism of DEM-induced hypothermia is not fully understood but it is thought that its effect on temperature is independent of its GSH depleting effect (Costa and Murphy 1986) and could possibly be related to its impairment of glycogen synthesis and metabolism leading to an impairment of energy production (Bignall et al. 1977; Krack et al. 1980; Steffen and Musacchia 1985). Prevention, or even attenuation, of the hyperthermic response to MDMA has been widely shown to be neuroprotective against the degeneration induced by MDMA (Farfel and Seiden 1995a; Malberg et al. 1996; Colado et al. 1998, 1999a, 1999b) and other substituted amphetamines (Farfel and Seiden 1995b), leading to the question of whether or not the attenuation in hyperthermic response to MDMA induced by DEM treatment might have produced non-specific protection.

In order to answer this, we designed a protocol that depleted peripheral GSH to a similar extent to that observed in study II, but that did not alter the hyperthermic response to MDMA and evaluated the capacity it had to protect against MDMA-induced neurodegeneration. The combination of inhibition of GSH synthesis afforded by BSO and conjugation of existing GSH by DEM produced no attenuation in the hyperthermic response to MDMA but also failed to protect against the reduction in \([3H]\)paroxetine-labelled 5-HT sites produced by MDMA, 7 days after administration. This finding would seem to suggest that the protection observed in the earlier study when DEM alone was used (study II) might have been due solely to its attenuation of MDMA-induced hyperthermia.

Although the results obtained indicate that the neuroprotection observed in GSH-depleted animals (study II) was due

---

**Table 3** Effect of the combination of buthionine-[S,R]-sulfoximine (BSO) and diethylmaleate (DEM) on MDMA-induced reductions in indole levels in the brain

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hippocampus</th>
<th>Cortex</th>
<th>Striatum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5-HT</td>
<td>5-HIAA</td>
<td>5-HT</td>
</tr>
<tr>
<td>Vehicle/vehicle/saline</td>
<td>1.54 ± 0.06</td>
<td>1.94 ± 0.10</td>
<td>1.49 ± 0.04</td>
</tr>
<tr>
<td>Vehicle/vehicle/MDMA</td>
<td>1.00 ± 0.10**</td>
<td>1.20 ± 0.09**</td>
<td>1.05 ± 0.08*</td>
</tr>
<tr>
<td>BSO/DEM/MDMA</td>
<td>0.80 ± 0.09**</td>
<td>1.31 ± 0.12**</td>
<td>1.07 ± 0.11*</td>
</tr>
<tr>
<td>BSO/DEM/saline</td>
<td>1.58 ± 0.06</td>
<td>2.06 ± 0.10</td>
<td>1.52 ± 0.07</td>
</tr>
</tbody>
</table>

Animals were treated with BSO (890 mg/kg, i.p. 18 h before and 4.446 g/L in drinking water for 24 h after MDMA treatment) and DEM (1.5 mmol/kg, i.p. 30 min before MDMA) or vehicle before MDMA (12.5 mg/kg, i.p.) or saline and killed 7 days later. Data expressed as pmol/mg tissue. Results shown as mean ± SEM, n = 4–6. Different from vehicle/vehicle/saline-treated animals: *, ** p < 0.01, 0.001, respectively (one-way ANOVA followed by Tukey’s test).
to the attenuation of the MDMA-induced increase in rectal temperature they do not, however, exclude a role for GSH in the neurodegeneration induced by MDMA. The GSH adducts of MDA and MDMA and GSH itself appear to share a common saturable carrier across the blood–brain barrier (Patel et al. 1993), as demonstrated by a decrease in brain adduct uptake in the presence of GSH (Miller et al. 1996). This interpretation is also supported by the finding that the majority of α-MeDA-thioethers are transported into the brain in the form of intact GSH conjugates, as indicated by the fact that acivicin (an inhibitor of γ-glutamyl transpeptidase) pre-treatment potentiates the neurotoxicity of MDA and MDMA (Bai et al. 2001). If this were the case, then perhaps a substantial decrease in the concentration of peripheral GSH could lead to an increase in the fraction of 5-GSH-α-MeDA taken up into the brain. Thus, any decrease in the formation of the conjugate would be offset by increased uptake of a GSH-conjugated metabolite of MDMA.

In conclusion, extensive depletion of the peripheral GSH pool by DEM attenuated the MDMA-mediated neurotoxicity in the Dark Agouti rats, and this action appears to be related to the attenuation of the MDMA-mediated hyperthermic response, rather than to the GSH depletion per se. These findings, in themselves, do not exclude a pivotal role of quinone metabolite-GSH adducts in the neurotoxicity of MDMA.

Acknowledgements

The research was supported by The Wellcome Trust (UK). EOS would like to thank the Ministerio de Ciencia y Tecnologia (Spain) for financial support. The authors would like to thanks Mrs Clare Roe for technical assistance.

References


