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In vivo evidence for free radical involvement in the degeneration of rat brain 5-HT following administration of MDMA ('ecstasy') and *p*-chloroamphetamine but not the degeneration following fenfluramine

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1 Administration of 3,4-methylenedioxymethamphetamine (MDMA or 'ecstasy') to several species results in a long lasting neurotoxic degeneration of 5-hydroxytryptaminergic neurones in several regions of the brain. We have now investigated whether this degeneration is likely to be the result of free radical-induced damage.

2 Free radical formation can be assessed by measuring the formation of 2,3- and 2,5-dihydroxybenzoic acid (2,3-DHBA and 2,5-DHBA) from salicylic acid. An existing method involving implantation of a probe into the hippocampus and *in vivo* microdialysis was modified and validated.

3 Administration of MDMA (15 mg kg^{-1}, i.p.) to Dark Agouti (DA) rats increased the formation of 2,3-DHBA (but not 2,5-DHBA) for at least 6 h. Seven days after this dose of MDMA, the concentration of 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) was reduced by over 50% in hippocampus, cortex and striatum, reflecting neurotoxic damage. There was no change in the concentration of dopamine or 3,4-dihydroxyphenylacetic acid (DOPAC) in the striatum.

4 *p*-Chloroamphetamine (PCA), another compound which produces a neurotoxic loss of cerebral 5-HT content, when given at a dose of 5 mg kg^{-1} also significantly increased the formation of 2,3-DHBA (but not 2,5-DHBA) in the dialysate for over 4.5 h. *post*-injection starting 2 h after treatment.

5 In contrast, fenfluramine administration (15 mg kg^{-1}, i.p.) failed to increase the 2,3-DHBA or 2,5-DHBA concentration in the dialysate. A single fenfluramine injection nevertheless also markedly decreased the concentration of 5-HT and 5-HIAA in the hippocampus, cortex and striatum seven days later.

6 When rats pretreated with fenfluramine (15 mg kg^{-1}, i.p.) seven days earlier were given MDMA (15 mg kg^{-1}, i.p.) no increase in 2,3-DHBA was seen in the dialysate from the hippocampal probe. This indicates that the increase in free radical formation following MDMA is occurring in 5-HT neurones which have been damaged by the prior fenfluramine injection.

7 Administration of the free radical scavenging agent α-phenyl-N-tert-butyl nitrone (PBN; 120 mg kg^{-1}, i.p.) 10 min before and 120 min after an MDMA (15 mg kg^{-1}, i.p.) injection prevented the acute rise in the 2,3-DHBA concentration in the dialysate and attenuated by 30% the long term damage to hippocampal 5-HT neurones (as indicated by a smaller MDMA-induced decrease in both the concentration of 5-HT and 5-HIAA and also the binding of [^{3}H]-paroxetine).

8 These data indicate that a major mechanism by which MDMA and PCA induce damage to 5-hydroxytryptaminergic neurones in rat brain is by increasing the formation of free radicals. These probably result from the degradation of catechol and quinone metabolites of these substituted amphetamines. In contrast, fenfluramine induces damage by another mechanism not involving free radicals; a proposal supported by some of our earlier indirect studies.

9 We suggest that these different modes of action render untenable the recent suggestion that MDMA will not be neurotoxic in humans because fenfluramine appears safe at clinical doses.

Keywords: 3,4-Methylenedioxymethamphetamine; ecstasy; neurodegeneration; 5-hydroxytryptamine; free radicals; fenfluramine; hyperthermia; *p*-chloroamphetamine; 2,3-dihydroxybenzoic acid; α-phenyl-N-tert-butyl nitrone

Introduction

The recreationally used drug 3,4-methylenedioxymethamphetamine (MDMA or 'ecstasy') has two distinct actions in the brains of experimental animals. The first effect is that of producing a rapid and major release of 5-hydroxytryptamine (5-HT) from neuronal stores, resulting in substantial depletion of this monoamine (see for example Schmidt *et al*., 1986; Stone *et al*., 1986; Colado & Green, 1994). Many of the acute functional effects of MDMA, including behavioural excitation and hyperthermia (for review see Green *et al*., 1995), can be associated with the 5-HT release and it is probable that the release is induced by the action of the parent compound (see Green *et al*., 1995). It is also noteworthy that hyperthermia and also behavioural changes similar to those induced in rats can both occur in those people using the drug recreationally (see Steele *et al*., 1994; Green *et al*., 1995).

The second action of MDMA in the brain is that of producing long term neurotoxic damage to 5-HT axon terminals in several areas of the brain and this is reflected in a significant loss in the 5-HT content in several brain regions. This damage has been shown both histologically (O'Hearn *et al*., 1988;

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Molliver *et al*., 1990) and biochemically – for example, loss of [^{3}H]-paroxetine binding to the presynaptic 5-HT transporter in the cerebral cortex and hippocampus (Battaglia *et al*., 1987; Sharkey *et al*., 1991; Hewitt & Green, 1994). This neurodegeneration has been shown to occur not only in rodent brain, but also in several species of primate (Ricaurte *et al*., 1988a,b; Insel *et al*., 1989; Wilson *et al*., 1989). In squirrel monkeys several brain regions showed no recovery even after a year and where recovery of 5-HT content was observed, the innervation was found to be highly abnormal (Fischer *et al*, 1995).

Whether long term brain damage occurs in people who misuse the drug is unknown. However, damage does occur in at least one strain of rats following a single dose of MDMA which produced plasma concentrations of the drug similar to those that have been measured in the plasma of recreational users of the drug (Colado *et al*., 1995). Furthermore, studies in humans misusing the drug, in which both behavioural and biochemical indices of cerebral 5-HT function were used, suggest that damage may occur (Green *et al*., 1995; Green & Goodwin, 1996).

Neurodegeneration following MDMA does not appear to be either the result of the acute release of 5-HT which occurs (Hekmatpanah *et al*., 1989; Colado & Green, 1994) or the action of the parent compound (Molliver *et al*., 1986; Paris & Cunningham, 1992). It seems reasonable therefore to propose that damage might be caused by metabolites of MDMA. There is evidence that MDMA is metabolized to catechol and quinone metabolites (Hiramatsu *et al*., 1990; Lim & Foltz, 1991; Lim *et al*., 1992; Tucker *et al*., 1994) and we have proposed that further metabolism of such compounds would result in the formation of free radicals which induce oxidative stress and membrane damage (Colado *et al*., 1995). Indeed there is evidence to support this proposal since Sprague & Nichols (1995) have shown that L-deprenil administration protected against both the MDMA-induced increase in lipid peroxidation and also the long term loss of 5-HT. Also, Colado & Green (1995) showed that administration of a free radical scavenger, the spin trap reagent α-phenyl-N-tert-butyl nitrone (PBN), prevented MDMA-induced degeneration of 5-hydroxytryptaminergic neurones in the rat cortex and hippocampus.

Other substituted amphetamines are also known to induce neurotoxic damage to 5-HT nerve terminals in rat brain, including *p*-chloroamphetamine (PCA; Sanders-Bush *et al*., 1972; Fuller *et al*., 1975; Colado *et al*., 1993; Murray *et al*., 1996) and the clinically used anorectic agent fenfluramine (Harvey & McMaster, 1975; Neckers *et al*., 1976; Colado *et al*., 1993; Murray *et al*., 1996). We have recently shown that PCA-induced damage was attenuated by PBN, while this free radical scavenger was without effect on fenfluramine-induced damage (Murray *et al*., 1996). These data suggest therefore that PCA also causes damage through a free radical mediated mechanism while the damage which follows fenfluramine administration does not.

We have now undertaken a study to examine whether MDMA, PCA and fenfluramine administration results in the formation of free radical species in the brain. This has been done by using *in vivo* microdialysis and measuring the formation of 2,3- and 2,5-dihydroxybenzoic acid formation from perfused salicylic acid as an index of free radical formation (see for example Chiueh *et al*., 1992).

Our results suggest strongly that free radical production is involved in MDMA-induced neurotoxic damage, the free radicals presumably resulting from the further degradation of MDMA metabolites. A preliminary account of some of this work has been presented at a meeting of the British Pharmacological Society (Colado *et al*., 1997c).

Methods

Animals

Adult male Dark Agouti (DA) rats (Harlan Olac, Bicester, Oxon) weighing 150 – 170 g were used. They were housed in groups of 5, in conditions of constant temperature (21°C ± 2°C) and a 12 hour light/dark cycle (lights on: 07 h 00 min) and given free access to food and water.

Measurement of monoamines and their metabolites

Rats were killed by cervical dislocation and decapitation, the brains rapidly removed and cortex, hippocampus and striatum dissected out on ice. Tissue was homogenized and 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) measured by high performance liquid chromatography (h.p.l.c.). Briefly, the mobile phase for 5-HT, 5-HIAA, dopamine and DOPAC analysis consisted of KH_2PO_4 (0.05 M), octanesulphonic acid (0.4 mM), EDTA (0.1 mM) and methanol (14%), and was adjusted to pH 3 with phosphoric acid, filtered and degassed. The flow rate was 1 ml min^{-1} and the working electrode potential was set at 0.85 V.

The h.p.l.c. system consisted of a pump (Waters 510) linked to an automatic sample injector (Loop 200 μl, Waters 712 WISP), a stainless steel reversed-phase column (Spherisorb ODS2, 5 μm, 150 × 3.9 mm) with a precolumn and an amperometric detector (Waters M460). The current produced was monitored by using an integrator (Waters M745).

[^{3}H]-paroxetine binding in tissue homogenates

[^{3}H]-paroxetine binding was measured by the method described in detail in Hewitt & Green (1994). Briefly, the animals were killed, the brain rapidly removed and dissected on ice within 2 min. Tissue from individual animals was homogenized in ice-cold Tris-HCl (50 mM; pH 7.4) containing NaCl (120 mM) and KCl (5 mM) by use of an Ultra-Turrax. The homogenate was centrifuged at 30 000 *g* for 10 min at 4°C. The supernatant was discarded and the wash procedure repeated twice more. The pellet finally resuspended in the Tris buffer at a concentration of 10 mg tissue ml^{-1}. The assay solution (1 ml) contained [^{3}H]-paroxetine (1 nM) and 800 μl tissue preparation with the addition of 5-HT (100 μM) for determination of non specific binding. Incubation was for 60 min at room temperature. Assays were terminated by rapid filtration and counting of the radioactivity by scintillation spectrometry. Protein concentrations were measured by the method of Lowry *et al*. (1951).

Measurement of rectal temperature

Temperature was measured by insertion of a thermocouple probe (connected to a digital readout) inserted 2.5 cm into the rectum, the rat being lightly restrained by holding in the hand. A steady readout was obtained within 10 s of probe insertion.

Implantation of microdialysis probe in the hippocampus

Rats were anaesthetised with sodium pentobarbitone ('Sagatal', 40 mg kg^{-1}, i.p.) and secured in a Kopf stereotaxic frame with the tooth bar at −3.3 mm below the interaural zero. The dialysis probe (3.5 mm × 200 μm: Cuprophan) was implanted in the hippocampus +2.2 cm from the interaural line, −4.3 mm lateral and −8 mm below the surface of the brain (König & Klippel 1963). Probes were secured to the skull as described by Baldwin *et al*. (1994).

Twenty-four hours after implantation, probes were perfused with artificial cerebrospinal fluid (KCl 2.5 mM, NaCl 125 mM, $MgCl_2.6H_2O$ 1.18 mM, $CaCl_2.2H_2O$ 1.26 mM) at a rate of 1 μl min^{-1} and samples collected from the freely moving animals at 30 min intervals. The first 60 min sample was discarded and the next three 30 min baseline samples collected.

Measurement of free radical formation in vivo *by microdialysis*

Hydroxyl free radicals react with salicylic acid and generate 2,3- and 2,5-dihydroxybenzoic acid (2,3-DHBA and 2,5-

DHBA). These compounds can be measured electrochemically by use of h.p.l.c. (Radzik *et al*., 1983; Floyd *et al*., 1984). Systemic administration of salicylate has been used. However, for studies on free radical formation in the brain, this technique requires measurement of salicylic acid in the brain and 2,5-DHBA can be formed by liver cytochrome P450 and microsomal enzymes (Halliwell *et al*., 1991). Use of microdialysis probe containing salicylic acid in the perfusate obviates these problems and allows measurement of free radical formation in a selected brain region (Chiueh *et al*., 1992).

During initial experiments establishing the techniques as described by others (Chiueh *et al*., 1992; Ciovanni *et al*., 1995; Globus *et al*., 1995; Maruyama *et al*., 1995) some difficulties were encountered. Modifications were therefore made as described below to develop a reliable technique in our hands.

The recovery 2,3-DHBA and 2,5-DHBA across the microdialysis membrane was similar for both Hospal membrane and Cuprophan membrane when examined *in vitro* and Cuprophan was therefore used routinely (recovery of a 160 pg μl^{-1} sample: 2,5-DHBA: $25.7 \pm 3.1\%$; 2,3-DHBA: $16.2 \pm 2.3\%$). When salicylic acid was present in the perfusate at a concentration of 5 mM, as used by others (Chiueh *et al*., 1992; Wu *et al*., 1993; Globus *et al*., 1995) a decrease in the basal concentration of both 2,3-DHBA and 2,5-DHBA was observed over time (Figure 1). The pH of the artificial CSF containing salicylic acid (5.0 mM) was found to be very acid (pH 2.7). We therefore examined the effect of perfusing a much lower concentration of salicylic acid (0.5 mM) as recently used by others (e.g. Obata & Yamanaka, 1995). This produced a slightly less acidic medium (pH 3.5) and a stable baseline which was easily measured despite the much lower concentration of 2,3-DHBA and 2,5-DHBA (Figure 1). This lower concentration of salicylic acid was therefore used in all our subsequent studies.

Measurement of 2,3-DHBA and 2,5-DHBA

The two compounds 2,3-DHBA and 2,5-DHBA were measured by h.p.l.c. and electrochemical detection. The mobile phase consisted of KH_2PO_4 (0.025 M), acetonitrile (20%) and methanol (10%) and was adjusted to pH 3.7 with phosphoric acid, filtered and degassed. The flow rate was 1 ml min^{-1}.

The h.p.l.c. system consisted of a pump (Waters 510) linked to manual sample injector (Loop 20 μl Rheodyne), a stainless steel reversed-phase column (250×4.6 mm, 5 μm C8 Ultracarb, Phenomenex) with a precolumn (30×4.60 mm, 5 μm C8 Ultracarb, Phenomex) and an coulometric detector (Coulochem 5100A) with 5011 analytical cell. The working electrode potential was set at 400 mV with 1 μA gain. The current produced was monitored by using a computer data handling system (AXXIOM 747).

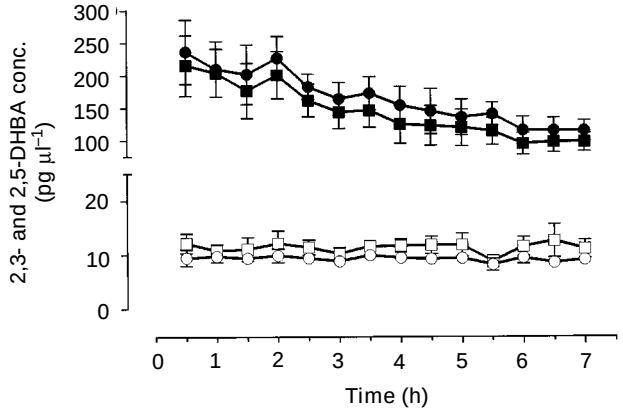

Figure 1 Measurement of 2,3-DHBA (■, □) and 2,5-DHBA (●, ○) concentrations in dialysate from rats perfused with artificial CSF containing salicylic acid 0.5 mM (□,○; $n=11$) or 5 mM (■, ●; $n=7$). Sample collection began after a resting period of 1 h. Results shown as mean and vertical lines indicate s.e.mean.

Drugs

(±)-Methylenedioxymethamphetamine HCl was obtained from the Ministry of Health (Spain). Fenfluramine HCl, *p*-chloroamphetamine HCl (PCA) and α-phenyl-N-tert-butyl nitrone (PBN) were obtained from Sigma Chemical Co. All drugs were dissolved in 0.9% w/v NaCl (saline) and injected i.p. Doses are quoted in terms of the base. Control animals were injected with saline.

Statistics

Statistical analyses of the microdialysis experiments were performed by use of the statistical computer package BMDP/386 Dynamic (BMDP Statistical Solutions, Cork, Eire). Data were analysed by analysis of variance (ANOVA) with repeated measures (program 2V) or, where missing values occurred, an unbalanced repeated measure model (program 5V) was used. Both used treatment as the between subjects factor and time as the repeated measure. ANOVA was performed on both pretreatment and *post*-treatment data. Temperature data were also analysed by ANOVA with repeated measures. Data from experiments in which monoamines and [^{3}H]-paroxetine binding were measured were analysed by Newman-Keuls test.

Results

Effect of MDMA on brain monamine concentrations 7 days later

A single injection of MDMA (10 or 15 mg kg^{-1}, i.p.) produced a substantial decrease in the concentration of 5-HT and its metabolite 5-HIAA in cortex, hippocampus and striatum 7 days later, with the higher dose resulting in a more marked effect (Figure 2). In contrast, MDMA administration produced no change in the concentration of dopamine or its metabolite DOPAC in the striatum (Table 1).

Acute effect of MDMA on the formation of 2,3-DHBA and 2,5-DHBA in the dialysate

MDMA (10 mg kg^{-1}, i.p.) was administered to rats in which a microdialysis probe had been implanted in the right hippocampus and through which salicylic acid was being perfused (see Methods). No significant change in the formation of 2,3-DHBA from salicylic acid was observed in the drug-treated animals compared with saline injected control rats (Figure 3a). However, MDMA 15 mg kg^{-1} produced a sustained rise of approximately 50% in the concentration of 2,3-DHBA in the perfusate (Figure 3a). No change in the concentration of 2,5-DHBA in the perfusate was observed following either dose of MDMA (Figure 3b).

Rectal temperature of the rats following MDMA

The rectal temperature of the rats injected with MDMA was measured over the time of the dialysis experiment. Injection of MDMA (10 mg kg^{-1}) produced a modest but statistically significant elevation in rectal temperature (Figure 4). A marked hyperthermic response was seen in the rats injected with MDMA (15 mg kg^{-1}) (Figure 4).

Effect of p*-chloroamphetamine on the formation of 2,3-DHBA and 2,5-DHBA in the dialysate*

A single dose of PCA (5 mg kg^{-1}, i.p.) produced a sustained and statistically significant increase in the 2,3-DHBA concentration in the dialysate from 2 h post-injection which lasted for

at least another 4.5 h (Figure 5a). The mean 2,5-DHBA concentration also increased over the same period (Figure 5b), but this change was not significantly different.

Effect of p-*chloroamphetamine on rectal temperature*

Rectal temperature increased rapidly after PCA administration, peaking after 1 h and declining thereafter (Figure 6a).

Effect of fenfluramine on brain monoamine concentrations 7 days later

Seven days after administration of fenfluramine (15 mg kg^{-1}, i.p.) there was a significant decrease in the concentration of 5-HT and 5-HIAA in the hippocampus, cortex and striatum (Figure 7). No change in striatal content of dopamine or DOPAC was observed (Table 1).

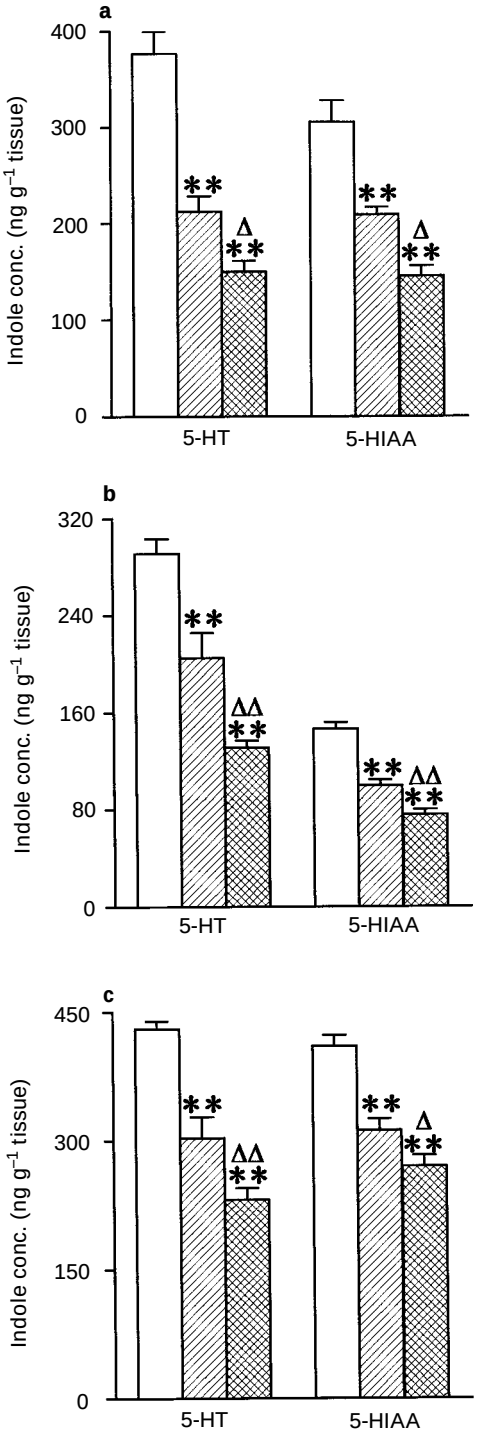

Figure 2 Effect of MDMA (10 and 15 mg kg^{-1}, i.p.) on indole concentration in (a) hippocampus, (b) cortex and (c) striatum 7 days after administration. Results shown as mean ± s.e.mean of values of rats given saline (open columns; $n=9$), MDMA (10 mg kg^{-1}, hatched columns; $n=8$) or MDMA (15 mg kg^{-1}, cross-hatched columns; $n=10$). Different from saline, $^{**}P<0.01$. Different from MDMA (10 mg kg^{-1}), $\Delta P<0.05$; $\Delta\Delta P<0.01$.

Table 1 Effect of administration of MDMA and fenfluramine on the concentration of dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) in the striatum 7 days later

Injected	n	*Dopamine*	*DOPAC*
Saline	9	9104 ± 230	1089 ± 48
MDMA (10 mg kg^{-1})	9	8817 ± 375	1043 ± 54
MDMA (15 mg kg^{-1})	10	8754 ± 256	984 ± 19
Fenfluramine (15 mg kg^{-1})	5	8388 ± 341	1300 ± 85

Results shown as mean ± s.e mean in ng g^{-1} tissue (wet weight).

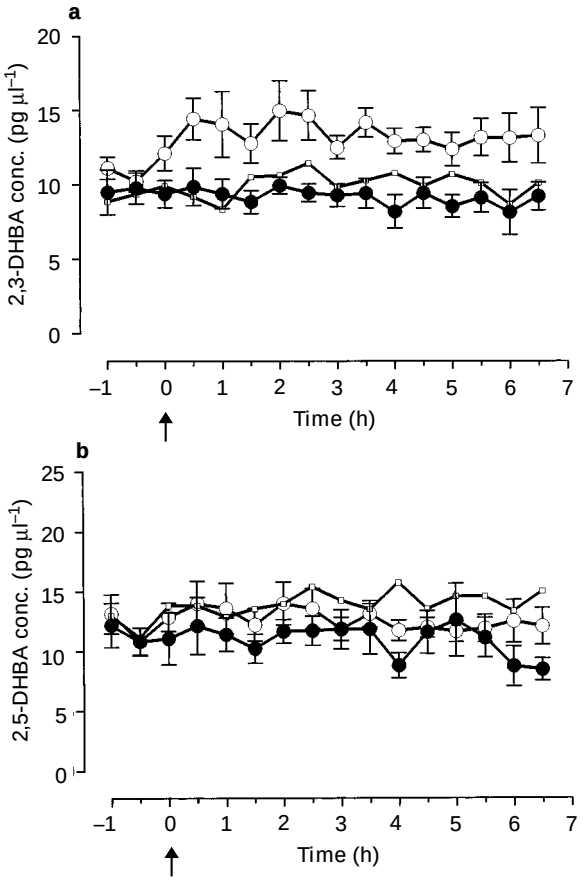

Figure 3 Measurement of 2,3- and 2,5-dihydroxybenzoic acid (DHBA) concentration in dialysate from rats given saline (●; $n=11$), MDMA 10 mg kg^{-1} (□; $n=6$) or MDMA 15 mg kg^{-1} (○; $n=10$). The arrows show the time of drug administration. (a) 2,3-DHBA concentration (pg μl^{-1}) in dialysate. Before drug treatment there was no difference in the levels of 2,3-DHBA. Following drug treatment there was a significant effect of treatment ($F(2,24)=19.48$, $P<0.001$). Further analysis revealed that only the 15 mg kg^{-1} dose of MDMA produced a significant increase in the level of 2,3-DHBA ($F(1,19)=31.3$, $P<0.001$), whereas the group given 10 mg kg^{-1} was no different from the saline-treated group. The symbol size of the 10 mg kg^{-1} group has been decreased to help emphasize the difference between the saline and 15 mg kg^{-1} group. (b) 2,5-DHBA concentration (pg μl^{-1}) in dialysate. Results shown as mean and vertical lines indicate s.e.mean.

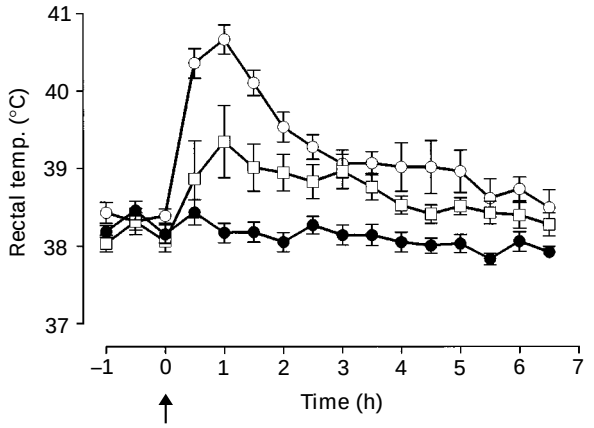

Figure 4 Rectal temperature of dialysed rats given saline (●; $n=11$), MDMA 10 mg kg^{-1} (□; $n=6$) or MDMA 15 mg kg^{-1} (○; $n=10$). The arrow shows the time of drug administration. Results shown as mean and vertical lines indicate s.e.mean. There was no difference in basal temperature of the groups. Following drug treatment there was a significant increase in body temperature ($F(2,24)=27.8$, $P<0.001$). Further analysis revealed that both doses of MDMA significantly increased temperature compared with that of saline-treated rats (for the dose of 10 mg kg^{-1}: $F(1,15)=11.7$, $P<0.01$; for the dose of 15 mg kg^{-1}: $F(1,19=66.1$, $P<0.001$). However, the 15 mg kg^{-1} dose produced a significantly greater increase than the lower dose ($F(1,14)=7.24$, $P<0.025$).

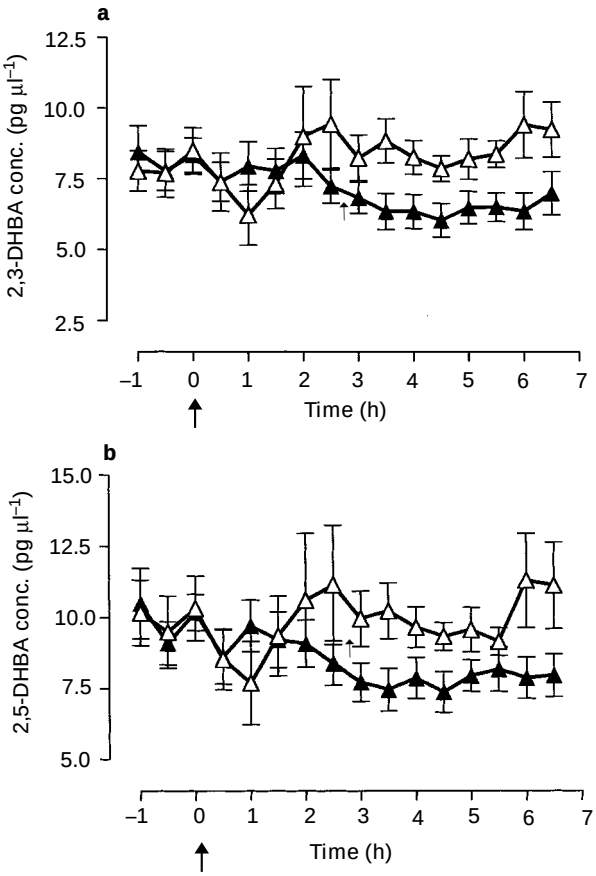

Figure 5 Measurement of 2,3-DHBA and 2,5-DHBA concentration in the dialysate of rats given saline (▲; $n=9$) or PCA 5 mg kg^{-1} (△; $n=9$). The arrows show the time of drug administration. (a) 2,3-DHBA concentration (pg µl^{-1}) in dialysate, (b) 2,5-DHBA concentration (pg µl^{-1}) in dialysate. Before treatment there was no difference in the levels of 2,3- or 2.5-DHBA. After treatment there was significant effect ($F(1,16)=4.6$, $P<0.05$) in the case of the 2,3-DHBA concentration, but not in the case of the 2,5-DHBA concentration ($F(1,16)=2.46$, NS).

Acute effect of fenfluramine on the formation of 2,3-DHBA and 2,5-DHBA in the dialysate

Rats with a microdialysis probe in the hippocampus were injected with fenfluramine and 2,3-DHBA and 2,5-DHBA in the dialysate measured over the next 6.5 h. Fenfluramine failed to alter the concentration of 2,3-DHBA in the dialysate, compared to saline injected controls (Figure 8a). The concentration of 2,5-DHBA was modestly decreased in the fenfluramine treated rats when compared to control values (Figure 8b).

Rectal temperature of the rats following fenfluramine

The rectal temperature of the rats given fenfluramine (15 mg kg^{-1}) was similar to that of the saline injected rats over the 6.5 h following drug administration (Figure 6b).

The effect of MDMA administration on 2,3-DHBA and 2,5-DHBA in the dialysate in rats pretreated with fenfluramine

The results above suggested that MDMA and PCA increased free radical formation in the hippocampus but that fenfluramine did not have this effect. However, all 3 compounds damage central 5-HT nerve terminals (Figures 2 and 7 and see Discussion). To examine whether the increase in free radical formation following MDMA resulted from changes occurring in 5-hydroxytryptaminergic neurones we examined whether MDMA administration would increase free radical formation in rats which had been subjected to a neurotoxic lesion of 5-HT nerve terminals by prior fenfluramine administration.

Rats were injected with fenfluramine (15 mg kg^{-1}) or saline. Seven days later both groups were implanted with microdialysis probes in the hippocampus and then injected with MDMA (15 mg kg^{-1}) on day 8. MDMA administration failed to increase significantly the dialysate concentration of 2,3-DHBA and 2,5-DHBA in rats pretreated with fenfluramine (Figure 9).

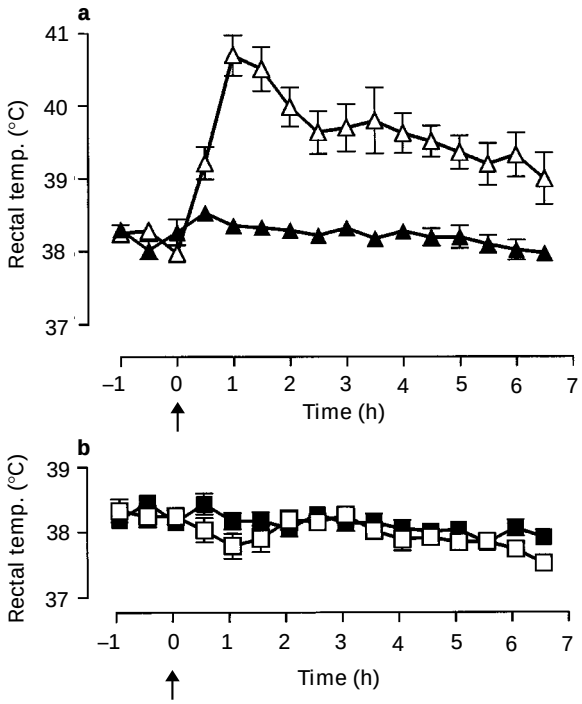

Figure 6 The effect of PCA or fenfluramine on rectal temperature. (a) Effect of saline (▲; $n=9$) or PCA (5 mg kg^{-1}; △; $n=8$) on rectal temperature. Temperature of PCA treated rats was different from saline treated rats ($P<0.05$ or better) at all times from +0.5 h to 6.5 h. (b) Effect of saline (■; $n=9$) or fenfluramine (15 mg kg^{-1}; □; $n=12$) on rectal temperature. Arrows show time of drug administration.

The effect of MDMA on rectal temperature in rats pretreated with fenfluramine

The rise in rectal temperature following MDMA (15 mg kg^{-1}) was markedly attenuated in rats pretreated with fenfluramine (15 mg kg^{-1}) 8 days earlier (Figure 10).

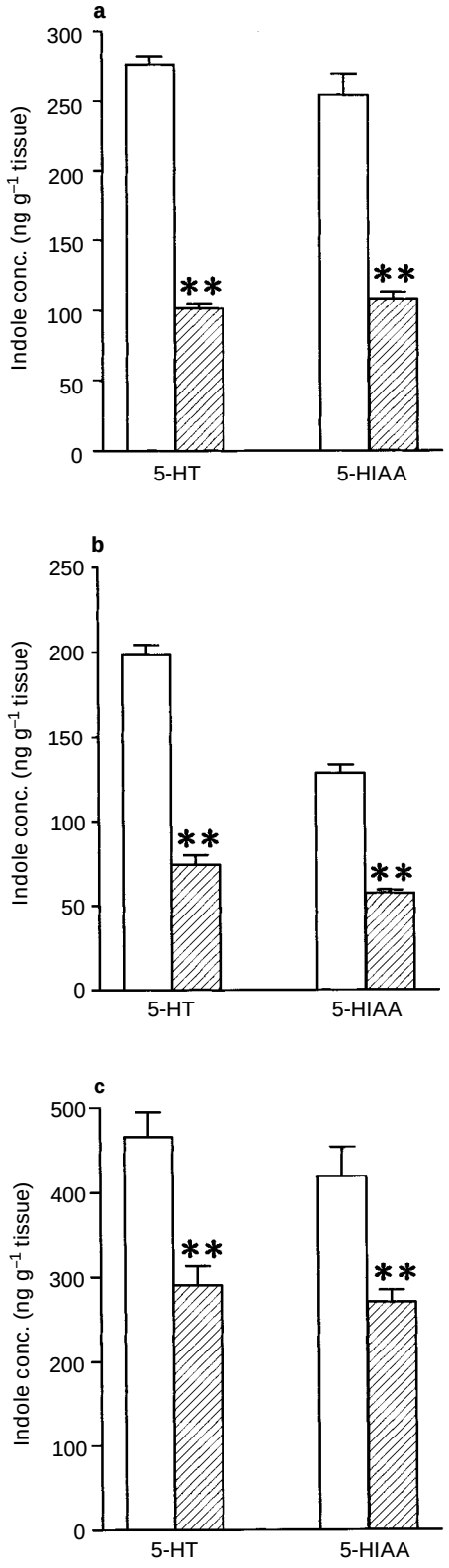

Figure 7 Effect of fenfluramine (15 mg kg^{-1}, i.p.) on indole concentration in (a) hippocampus, (b) cortex and (c) striatum 7 days after administration. Results shown as mean ± s.e.mean of rats injected with saline (open columns, $n=4$) or fenfluramine (hatched columns, $n=5$). Different from saline: $^{**}P<0.01$.

Effect of PBN on the formation of 2,3-DHBA and 2,5-DHBA following administration of MDMA

In an earlier study we showed that injection of the free radical scavenger PBN attenuated the neurodegeneration of 5-HT pathways produced by MDMA (Colado & Green, 1995). We therefore next examined whether injection of PBN decreased the rise in 2,3-DHBA concentration in the dialysate which occurs after MDMA injection.

Two injections of PBN (120 mg kg^{-1}) 130 min apart failed to alter the dialysate concentration of 2,3-DHBA compared to saline injected controls (data not shown). However, when PBN (120 mg kg^{-1}) was given 10 min before and 120 min after MDMA (15 mg kg^{-1}) it prevented the MDMA-induced rise in the concentration of 2,3-DHBA (Figure 11). No changes were seen in the concentration of 2,5-DHBA in any of these experiments (data not shown).

Effect of PBN administration to MDMA-treated rats on brain indole concentration and [^{3}H]-paroxetine binding 7 days later

Rats were treated with PBN and MDMA exactly as detailed in the previous section and killed 7 days later for measurement of 5-HT and 5-HIAA concentrations in the cortex, hippocampus and striatum. Administration of PBN alone did not affect the concentration of either 5-HT or 5-HIAA, compared to saline treated controls (Figure 12). MDMA

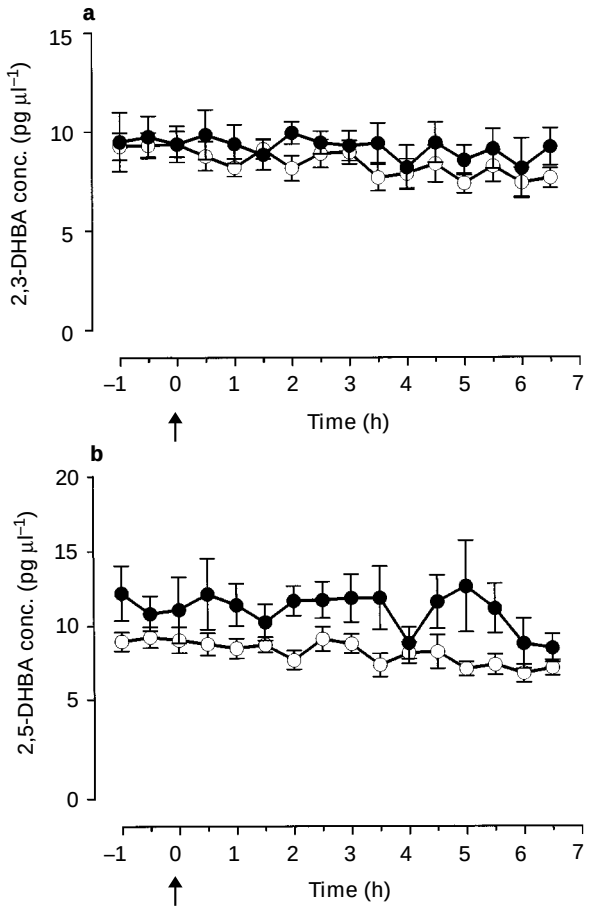

Figure 8 Measurement of 2,3- and 2,5-dihydroxybenzoic acid (DHBA) concentration in dialysate from rats receiving either saline (●; $n=11$) or fenfluramine 15 mg kg^{-1} (○; $n=9$). The arrows show the time of drug administration. (a) 2,3-DHBA concentration (pg µl^{-1}) in dialysate, (b) 2,5-DHBA concentration (pg µg^{-1}) in dialysate. Before drug treatment there was no difference in the levels of 2,5-DHBA. Fenfluramine significantly lowered the level of 2,5-DHBA ($F(1,18)=13.37$, $P<0.01$). Results expressed as mean and vertical lines indicate s.e.mean.

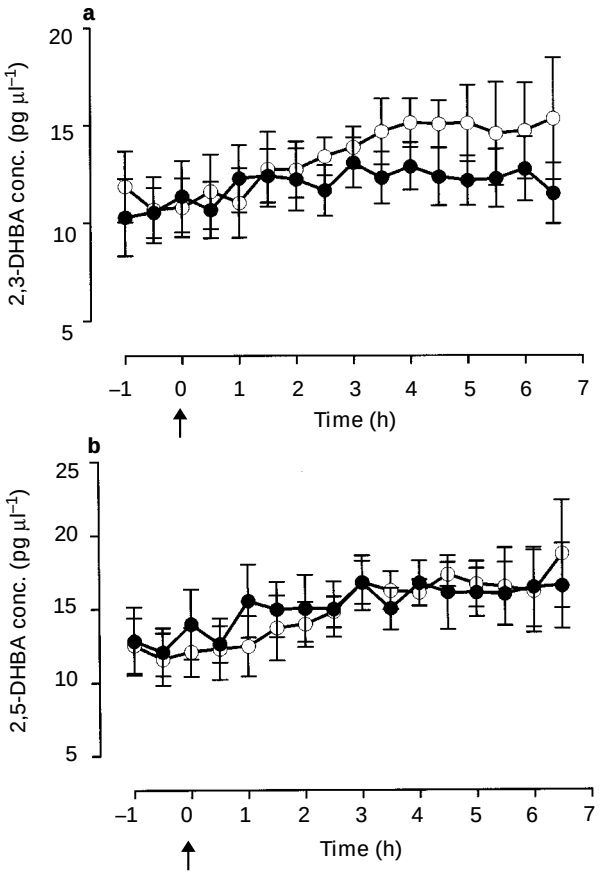

Figure 9 Measurement of 2,3- and 2,5-dihydroxybenzoic acid (DHBA) concentration in dialysate from rats pretreated with fenfluramine (15 mg kg^{-1}, i.p.) 8 days before receiving either saline (●; $n=7$) or MDMA 15 mg kg^{-1} (○; $n=6$). The arrows show the time of drug administration. (a) 2,3-DHBA, (b) 2,5-DHBA concentration (pg μl^{-1}) in dialysate. Results expressed as mean and vertical lines indicate s.e.mean.

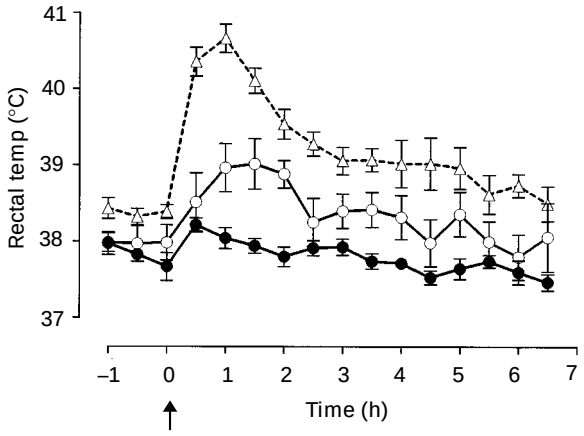

Figure 10 Rectal temperature of dialysed rats pretreated with fenfluramine (15 mg kg^{-1}, i.p.) 8 days before receiving either saline (●; $n=7$) or MDMA 15 mg kg^{-1} (○; $n=6$). The arrows show the time of drug administration. Results shown as mean and vertical lines indicate s.e.mean. The body temperature of rats treated with MDMA 8 days after administration of fenfluramine was found to be significantly higher than that of rats treated with saline ($F(1,13)=23.8$, $P<0.001$). (△) The rectal temperature of rats not pretreated with fenfluramine but receiving MDMA; results taken from an earlier experiment (Figure 4).

administration produced the expected decrease in indole concentration and this decrease was modestly but significantly attenuated by PBN (Figure 12).

To confirm that the attenuation of the 5-HT loss seen when PBN was given to MDMA-treated rats was due to a neuroprotective action of the free radical scavenger we next repeated the experiment but measured [^{3}H]-paroxetine binding in both hippocampus and cortex. Results obtained with the measurement of binding of [^{3}H]-paroxetine to the presynaptic 5-HT transporter were very similar to those obtained when measuring tissue 5-HT content. That is there was an approximately 65% loss of binding in cerebral tissue from MDMA-treated rats, whereas the decrease was only 45% in tissue from animals also given PBN (Figure 13). PBN thus produced a 30% neuroprotection.

Rectal temperature in MDMA-injected rats pretreated with PBN

Administration of two doses of PBN to MDMA-injected rats (see above) failed to alter the hyperthermic response which follows MDMA injection (Figure 14). PBN administration alone resulted in a modest hypothermia (Figure 14).

Discussion

The technique of examining the formation of 2,3-DHBA and 2,5-DHBA from salicylic acid as an index of ·OH radical formation is well established having been first developed over 10 years ago (Radzik *et al*., 1983; Floyd *et al*., 1984). It is considered that 2,3-DHBA is a more reliable index of free radical formation because, 2,5-DHBA can also be formed enzymatically from salicylate (Halliwell *et al*., 1991). The use of a selectively implanted microdialysis probe as described in the methods allowed measurement of free radical formation in a selective brain region known to be susceptible to MDMA-induced neurodegeneration (the hippocampus) and circumvented the problem of assessing the concentration of salicylic acid in the brain and also its possible peripheral metabolism to 2,5-DHBA. We undertook initial studies to try and optimize the experimental conditions and found that both the membranes routinely used in the laboratory (Cuprophan and Hospal) had similar recovery characteristics. However, using a salicylic acid concentration (5 mM) used by others (e.g. Chiueh *et al*., 1992; 1993; Busto *et al*., 1995) resulted in a baseline that decreased with time, which it was felt might be due to damage caused by the high acidity of the dialysis medium. A ten fold decrease in salicylate concentration resulted in a somewhat less acidic solution and a stable baseline (Figure 1).

Initial studies confirmed the ability of MDMA to produce neurotoxic damage (as reflected in the long term loss in 5-HT content) when given as a single low dose to DA rats (see also Colado *et al*., 1995; Colado & Green, 1995). No change in dopamine or DOPAC concentration was seen in the striatum, in agreement with data from other studies on the relative selectivity of this neurotoxic drug for 5-hydroxytryptaminergic neurones (Schmidt & Kehne, 1990; Colado *et al*., 1997a).

There was a small but non-significant increase in the concentration of 2,3-DHBA (but not 2,5-DHBA) in the dialysate following MDMA (10 mg kg^{-1}). However, a dose of MDMA of 15 mg kg^{-1} produced a significant and sustained rise of approximately 50% in the dialysate concentration of 2,3-DHBA. Again the 2,5-DHBA concentration did not alter. Why the 2,5-DHBA concentration did not change is not known. Studies on the neurotoxin MPP^+ and some other free radical producing compounds have found that both salicylate metabolites changed in a similar fashion (e.g. Chiueh *et al*., 1993). However, the recent study on the effect of neurotoxic doses of methamphetamine also noted a statistically significant rise in the dialysate concentration of 2,3-DHBA but not 2,5-DHBA (Giovanni *et al*., 1995). It was reassuring that it was 2,3-DHBA, the metabolite that cannot be formed enzymati-

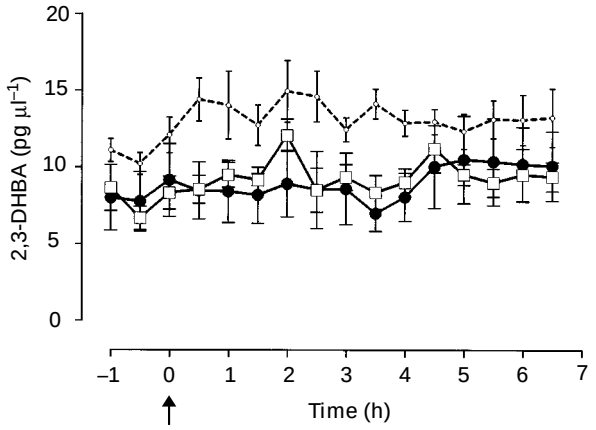

Figure 11 Measurement of 2,3-dihydrobenzoic acid (DHBA) concentration in dialysate from rats given PBN (120 mg kg^{-1}, i.p., $n=10$) 10 min before and 120 min after MDMA (15 mg kg^{-1}, i.p.) at time 0 h (□). (●) The 2,3-DHBA concentration in dialysate from saline-only treated rats. As the response of PBN-only treated rats was the same as saline treated animals the line has been omitted for clarity. (○) The response of MDMA-treated rats from the earlier experiment (Figure 3). Results shown as mean and vertical lines indicate s.e.mean.

cally (Halliwell *et al*., 1991), that changed since this increased confidence in the view that free radical formation had increased.

Administration of PCA is also known to produce neurotoxic degeneration of cerebral 5-HT (Sanders-Bush *et al*., 1972; Fuller *et al*., 1975; Colado *et al*., 1993; Murray *et al*., 1996). Our recent study suggested that this compound might also produce damage by increasing free radical formation (Murray *et al*., 1996 and see later) and this view was strengthened by the current study, since administration of the compound also increased 2,3-DHBA formation. Unlike MDMA, where an increase was seen to occur rapidly, no increase was seen until 2 h after the PCA injection. This may reflect differences between the two compounds in rates of degradation to free radical producing metabolites. While the 2,5-DHBA formation also appeared to increase after PCA, the change was not statistically significant.

Injection of fenfluramine also produced a long term neurotoxic loss of 5-HT in several brain regions, a change first demonstrated many years ago (Harvey & McMaster, 1975; Neckers *et al*., 1976; Colado *et al*., 1993) and recently also shown in the DA strain of rat (Murray *et al*., 1996). The values of 2,3-DHBA and 2,5-DHBA following fenfluramine did not change and thus provided no evidence to suggest that free radicals are formed following injection of this compound. These data, suggesting that fenfluramine does not produce neurotoxic damage through free radical formation, are consistent with our recent findings that the free radical scavenger PBN failed to prevent damage when given before fenfluramine (Murray *et al*., 1996).

In order to clarify whether the MDMA-induced increase in free radical formation in the hippocampus was associated with changes occurring in 5-hydroxytryptaminergic neurones, we next performed an experiment where we first injected fenfluramine to produce neurodegeneration of 5-HT nerve terminals in the hippocampus (see Figure 7 and Murray *et al*., 1996). We postulated that following MDMA little or no increase in 2,3-DHBA would be seen in the lesioned animals. While a modest rise in 2,3-DHBA did appear to occur (Figure 8), the response of the lesioned animals was not found to be significantly different from the control animals. A small rise would not have been unexpected given the fact that the fenfluramine only produces an approximate 70% loss of 5-HT terminals (Figure 7), a magnitude of loss also indirectly indicated by the fact that the MDMA-induced hyperthermic response was attenuated

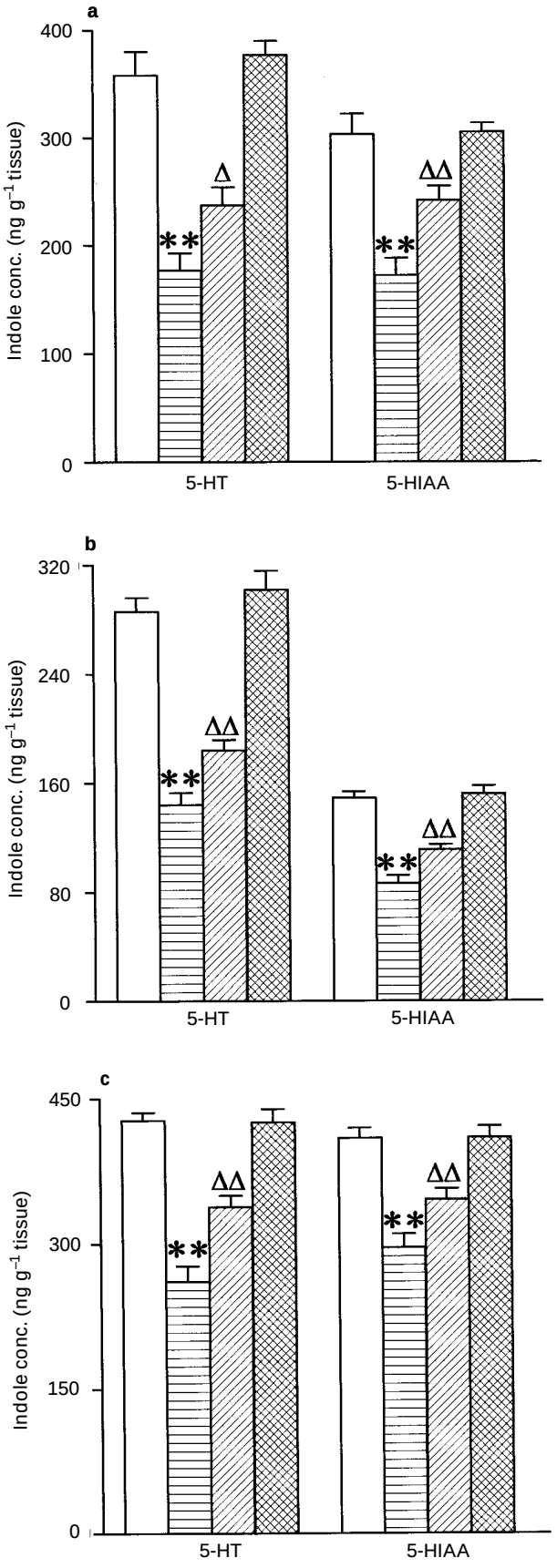

Figure 12 Effect of PBN (120 mg kg^{-1}, i.p.) given 10 min before (diagonally-hatched columns) and 120 min after MDMA (15 mg kg^{-1}, i.p.) (horizontally-hatched columns) on (a) hippocampal, (b) cortical and (c) striatal indole concentration 7 days after administration. Results shown as mean ± s.e.mean ($n=11-16$). Different from saline (open columns): $^{**}P<0.01$. Different from MDMA (horizontally-hatched columns): $\Delta P<0.05$, $\Delta\Delta P<0.01$. Cross-hatched columns show effects of PBN alone.

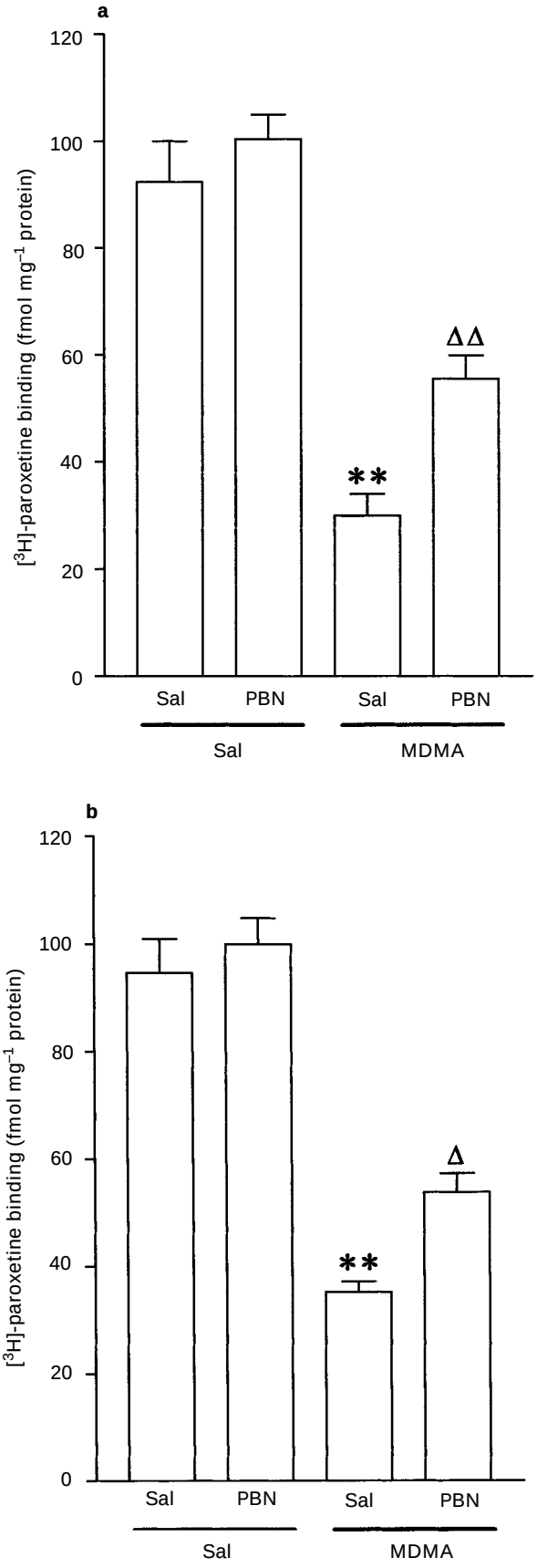

Figure 13 Effect of PBN (120 mg kg^{-1}, i.p.) given 10 min before and 120 min after MDMA (15 mg kg^{-1}, i.p.) on (a) hippocampal and (b) cortical [^{3}H]-paroxetine binding 7 days after administration. Results shown as mean ± s.e.mean ($n = 8-14$). Different from saline: $^{**}P < 0.01$; different from MDMA: $\Delta P < 0.05$, $\Delta\Delta P < 0.01$.

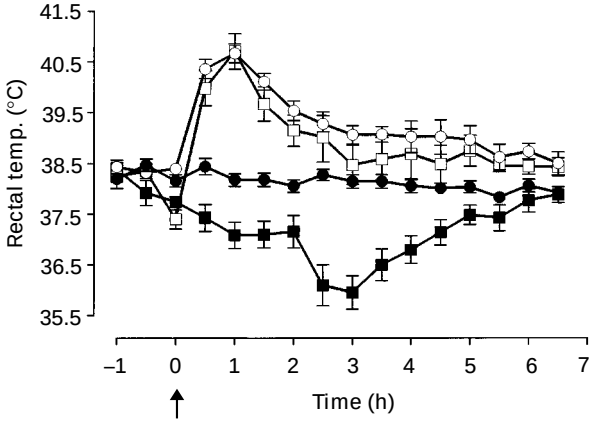

Figure 14 Rectal temperature of rats receiving PBN (120 mg kg^{-1}, i.p.) 10 min before and 120 min after saline (■; $n = 7$) or MDMA (15 mg kg^{-1}, □; $n = 10$). Also shown are the rectal temperature of animals injected with saline instead of PBN and administered either saline (●; $n = 11$) or MDMA (○; $n = 10$). Results shown as mean and vertical lines indicate s.e.mean. The basal temperatures of the group did not differ. Following drug treatment there was a significant effect on body temperature ($F(3,29) = 28.82$; $P < 0.001$). Further analysis revealed that PBN significantly decreased temperature compared to that of saline treated rats ($F(1,11) = 36.3$; $P < 0.001$) whereas MDMA produced a significant rise in body temperature ($F(1,14) = 30.9$; $P < 0.001$) compared to the saline-treated group. In addition the group administered both PBN and MDMA also had a rectal temperature significantly different from the control group ($F(1,14) = 5.57$; $P < 0.05$).

but not abolished by the fenfluramine pretreatment. These data suggest strongly that the increase in free radical formation must follow uptake of MDMA into 5-hydroxytryptaminergic neurones and that the free radicals are being formed in 5-hydroxytryptaminergic nerve endings.

This indirect evidence that the free radicals produced by an MDMA injection result from changes occurring selectively within 5-HT nerve terminals in turn explains why the rise in 2,3-DHBA is relatively modest. Following a dose of MDMA of 15 mg kg^{-1} there was an approximate 50% rise in 2,3-DHBA concentration which is almost exactly the same increase as that found to occur after the administration of a neurotoxic dose of methamphetamine (Giovanni *et al*., 1995). Even global ischaemia, which presumably induces damage in most cerebral tissue, was only found to produce an increase in the concentration of 2,3-DHBA of 200% – 300% (e.g. Globus *et al*., 1995). One is clearly limited in the detection of change with this method. We were unable to study higher doses of MDMA as these tended to be fatal.

An earlier study (Colado & Green, 1995) demonstrated that the neurodegenerative effect of MDMA could be attenuated by co-administration of the spin trap reagent PBN, a free radical scavenger. This observation has recently been confirmed (Yeh, 1996). The current study has shown that PBN does prevent the MDMA-induced increase in free radical formation which occurs *in vivo*. We were also able to show, by use of the same dose schedule, that there was a significant attenuation of the MDMA-induced damage following PBN administration. This result strengthens the earlier proposal that the neuroprotective effect of PBN is the result of its free radical scavenging activity. While the protection (a 30% decrease in the damage induced by the MDMA injection) was less than that obtained in our earlier study (Colado & Green 1995), this is almost certainly because the dose of MDMA was higher and the dose of the neuroprotective agent (PBN) lower than in the previous study.

We recently demonstrated that PBN administration also prevented damage to 5-hydroxytryptaminergic neurones when induced by *p*-chloroamphetamine, but not when induced by fenfluramine (Murray *et al*, 1996). These data, together with

the earlier findings that PBN protects against MDMA-induced damage (Colado & Green, 1995; Yeh, 1996), indicate strongly that MDMA and PCA induce damage by mechanisms involving free radical formation, whilst fenfluramine induces damage through another mechanism not involving free radical formation. The current findings support this proposition since both MDMA and PCA increased 2,3-DHBA formation in the dialysis probe while fenfluramine did not.

Particular attention was paid in this study to changes in body temperature since it is well established that hypothermia attenuates the neurodegeneration produced not only by cerebral ischaemia (Busto *et al*., 1987) but also the degeneration produced by administration of substituted amphetamines (Farfel & Seiden, 1995; Broening *et al*., 1995). It is also possible that damage is exacerbated by hyperthermia (Broening *et al*., 1995).

The rectal temperature of the DA rats increased following MDMA, as has previously been shown (Colado *et al*., 1995; Colado & Green, 1995). This is an established response to MDMA administration (see review by Green *et al*., 1995) and has been proposed to be due to an action of 5-HT following its rapid release from nerve endings induced by the drug (see Green *et al*., 1995). This proposal is supported by the current results, since the animals with depleted 5-HT stores following fenfluramine pretreatment showed a markedly reduced hyperthermic response following MDMA. This is somewhat analogous to our recent observation that repeated doses of MDMA result in an increasingly attenuated hyperthermic response (Colado *et al*., 1997a,b). PCA also induced hyperthermia, in agreement with our earlier findings (Colado *et al*., 1993), while fenfluramine injection did not alter rectal temperature, again in agreement with earlier observations (Colado *et al*., 1993). Nevertheless it seems unlikely that the temperature change can be associated directly with either the neurodegeneration or the increase in free radical formation. In the first place, a lower dose of PCA (2.5 mg kg^{-1}) was previously found to have no significant effect on rectal temperature but nevertheless induce damage to cerebral 5-HT (Colado *et al*., 1993; Murray *et al*., 1996). Secondly, in the current study, there appears to be no obvious relationship, either in magnitude or temporally, between the temperature change and the 2,3-DHBA concentration change. We do nevertheless acknowledge that free radical formation can be markedly enhanced in damaged tissue when the animal is hyperthermic, as has been demonstrated by studies in hypoxic ischaemia (e.g. Globus *et al*., 1995) and it seems probable that following MDMA free radical production is favoured by the hyperthermia that the drug induces (Kil *et al*, 1996).

What can also be stated with some degree of confidence is that PBN is not being neuroprotective because it either prevents hyperthermia or induces hypothermia. PBN alone in a high dose can induce hypothermia (Colado & Green, 1995). However at the lower doses used in the current study its effects were small and the temperature responses of the MDMA-treated rats given PBN were identical to those not given PBN, and yet the latter had significantly greater neurodegeneration.

The major question that arises from the study is whether the rise in free radical production that follows MDMA administration can be associated with the neurodegeneration that occurs and whether the neuroprotective action of PBN is due to the free radical scavenging action of that compound. In the case of hypoxic-ischaemic damage these links appear to be well established. The results obtained with experimental models of stroke have resulted in a substantial body of evidence which implicates oxyradicals in the tissue damage (Oliver *et al*., 1990; Carney & Floyd, 1991; Hensley *et al*., 1997). The major property of PBN is to scavenge oxyradicals (see Hensley *et al*., 1997). The compound has good brain penetration (Cheng *et al*., 1993), decreases free radical production in the brains of ischaemic rats (Zini *et al*., 1990; Sen & Phillis, 1993) and is an effective neuroprotective compound in animal models of stroke (Carney & Floyd, 1991; Zini *et al*., 1992; Sen & Phillis, 1993; Zhao *et al*., 1994). The general view therefore is that free radicals are a major determinant of ischaemia-induced damage in the brain and that PBN is neuroprotective because of its free radical scavenging action (see Hensley *et al*., 1997).

The foregoing gives weight to our proposition that MDMA, PCA and probably methamphetamine, produce damage to 5-HT nerve terminals because of the involvement of free radicals in the process. In support of the proposal it can be cited that methamphetamine (Giovanni *et al*., 1995), MDMA (this paper) and PCA (this paper) have all been shown to increase free radical formation in the brain by the use of microdialysis probes containing salicylic acid. The neurotoxic damage produced by administration of MDMA and PCA can be prevented or attenuated by injection of the free radical scavenger PBN (Colado & Green, 1995; Murray *et al*., 1996; Yeh, 1996; this paper). PBN also prevented the MDMA induced increase in free radical formation measured *in vivo* (this paper). Another free radical scavenging compound, L-cysteine has also been shown to attenuate PCA-induced damage (Steranka & Rehind, 1987). Elevated levels of free radicals in tissue leads to increased lipid peroxidation and it has been shown that following MDMA there is an increase in lipid peroxidation in the brain (Sprague & Nichols, 1995; Colado *et al*., 1997a). Administration of L-deprenil prevents both the MDMA-induced increase in lipid peroxidation and the neurotoxic damage (Sprague & Nichols, 1995). We also recently found that administration of MDMA to neonates neither increased lipid peroxidation in the brain nor induced neurotoxic loss of 5-HT (Colado *et al*., 1997a), possibly because of the high free radical scavenging activity that is present in the tissues of young animals (Floyd, 1990). Furthermore, transgenic mice carrying the sequence of human CuZn superoxide dismutase show increased resistance to the toxic effects of MDMA and its metabolite 3,4-methylenedioxyamphetamine (MDA) compared to mice not carrying the gene (Cadet *et al*., 1994). It is also noteworthy that prior destruction of the 5-HT nerve terminals normally damaged by MDMA administration results in the compound no longer producing an increase in free radical production (Figure 8). Finally, as we have pointed out elsewhere (Colado *et al*., 1995; Murray *et al*., 1996), there is good evidence that both MDMA and PCA (but not fenfluramine) are metabolized to compounds (catechols and quinones), that have long been known on further degradation to form free radicals which induce associated tissue damage (Graham *et al*., 1978).

These observations have another important implication. The fact that fenfluramine has been used clinically with apparent safety for many years was used recently as an argument to support the proposition that MDMA will not produce neurotoxic damage in man when used recreationally (Saunders, 1996). However, leaving aside the fact that in man the recreational doses of ecstasy are sometimes 10 fold greater than the clinical doses of fenfluramine (while the dose of these two compounds required to produce toxicity in rodents is similar); the fact that the mechanism of neurotoxic damage probably differs between the drugs makes extrapolation from one drug to another impossible and the claim by Saunders (1996) that ecstasy is safe, on the basis that fenfluramine has a product licence, a possibly very dangerous fallacy.

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