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In vivo evidence against clomethiazole being neuroprotective against MDMA ('ecstasy')-induced degeneration of rat brain 5-HT nerve terminals by a free radical scavenging mechanism

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Abstract

Clomethiazole is an effective neuroprotective agent against the degeneration of 5-HT neurones that follows administration of 3,4-methylenedioxymethamphetamine (MDMA or 'ecstasy'). Since there is good evidence that free radical formation resulting from auto-oxidation of MDMA metabolites is responsible for the degeneration we have examined whether clomethiazole is a free radical scavenger. MDMA (15 mg/kg i.p.) increased the formation of 2,3- and 2,5-dihydroxybenzoic acids (2,3-DHBA and 2,5-DHBA) from salicylic acid perfused through a microdialysis tube implanted in the hippocampus, indicating increased free radical formation. Clomethiazole (50 mg/kg i.p.) administered 5 min prior and 55 min post MDMA prevented both the acute MDMA-induced hyperthermia and the rise in 2,3- and 2,5-DHBA. However, when the temperature of the MDMA + clomethiazole treated rats was kept elevated to that of the MDMA treated rats with a homeothermic blanket there was no inhibition of the MDMA-induced increase in 2,3-DHBA or 2,5-DHBA. These data suggest firstly that free radical formation is inhibited when the acute MDMA-induced hyperthermia is prevented. Secondly the data further indicate that clomethiazole has no free radical scavenging activity since the drug produces substantial neuroprotection when MDMA + clomethiazole treated rats are kept hyperthermic. This conclusion was strengthened by our observation that clomethiazole is a weak inhibitor ($IC_{50} > 1$ mM) of lipid peroxidation in synaptosomes when it had been induced by addition of $FeCl_2$ + ascorbic acid.

Keywords: Clomethiazole; 3,4-Methylenedioxymethamphetamine; Ecstasy; MDMA; Free radicals; 5-Hydroxytryptamine; Neuroprotection; Lipid peroxidation; Neurodegeneration; Hyperthermia

1. Introduction

It is well established that administration of 3,4-methylenedioxymethamphetamine (MDMA or 'ecstasy') to a variety of animal species results in long term neurotoxic degeneration of 5-hydroxytryptamine (5-HT) nerve terminals in several areas of the brain (see Steele et al., 1994; Green et al., 1995; White et al., 1996). This damage has been shown both histologically (O'Hearn et al., 1988; 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 and Green, 1994).

Most of the studies in rats have reported that several high doses of MDMA (typically 4×20 mg/kg) are required to produce neurodegeneration. However, in the last few years we have shown that the Dark Agouti strain of rat is particularly susceptible to the long term effects of the neurotoxic amphetamine derivatives (Colado and Green, 1995; Colado et al., 1995; Murray et al., 1996; Colado et al., 1997b, 1998a) and a recent investigation in this strain emphasised that a single dose of 10 mg/kg produced substantial loss of 5-HT nerve endings in several areas of the brain 7 days later (O'Shea et al., 1998).

Various compounds when given concurrently with

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MDMA have been reported to prevent the neurodegeneration (see Green et al., 1995). However, recent studies have indicated that a significant number of such compounds, including several with clear efficacy as neuroprotective agents in animal models of acute ischaemic stroke, only prevent MDMA-induced damage because they either induce hypothermia (Farfel and Seiden, 1995a,b; Malberg et al., 1996; Colado et al., 1998a) or prevent the acute hyperthermia which follows MDMA administration (Colado et al., 1998a). In contrast, clomethiazole, a compound which has excellent neuroprotective efficacy in a wide range of experimental models of stroke (see Green, 1998), protects against MDMA-induced damage by a mechanism which does not involve an effect on body temperature (Colado et al., 1998a).

There is now substantial evidence that increased free radical formation, probably resulting from the auto-oxidation of metabolites of MDMA (see Colado et al., 1995), is responsible for the neurotoxic damage. Such evidence includes the observation that MDMA administration increases 2,3-DHBA formation from salicylate (Colado et al., 1997b), the fact that the free radical scavenger α-phenyl-*N*-tert-butyl nitrone (PBN) is neuroprotective (Colado and Green, 1995; Yeh, 1996; Colado et al., 1997b) and also abolishes the MDMA-induced rise in 2,3-DHBA (Colado et al., 1997b), the observation that MDMA increases lipid peroxidation in the brain (Sprague and Nichols, 1995; Colado et al., 1997a) and the report that transgenic mice overexpressing CuZn superoxide dismutase (SOD) are resistant to the neurotoxic actions of the drug (Cadet et al., 1994). It thus seemed possible that at least part of the mechanism of the neuroprotective action of clomethiazole involved a free radical scavenging action. This has now been investigated by using in vivo microdialysis and measuring the formation of 2,3- and 2,5-dihydroxybenzoic acid from perfused salicylic acid as an index of free radical formation (see for example Chiueh et al., 1992; Colado et al., 1997b).

A preliminary account of some of this work has been presented at a meeting of the British Pharmacological Society (Colado et al., 1998b)

2. Methods

2.1. Animals, drugs and reagents

Adult male Dark Agouti (DA) rats (Interfauna, Barcelona, Spain) weighing 150–170 g were used. They were housed in groups of five, 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.

(±)-Methylenedioxymethamphetamine HCl was obtained from the Ministry of Health (Spain). Clomethiazole edisylate was obtained from Astra Arcus, Södertälje, Sweden. Both compounds 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.

Butylated hydroxytoluene (BHT) and thiobarbituric acid were purchased from Sigma and ferrous chloride tetrahydrate and trichloroacetic acid from Aldrich.

2.2. 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.

2.3. Elevation of body temperature with a homeothermic blanket

In one experiment rats which were injected with MDMA + clomethiazole had their rectal temperature kept elevated to near that seen in rats given MDMA alone (see Fig. 3). This was achieved by placing the rats in a cage containing a Harvard Homeothermic Blanket system (Model 50-7087).

2.4. Implantation of microdialysis probe in the hippocampus

Rats were anaesthetised with sodium pentobarbitone ('Euta-Lender', 40 mg/kg i.p.) and secured in a Kopf stereotaxic frame with the tooth bar at -3.3 mm below the interaural 0. The dialysis probe (3.5 mm × 200 μm: Cuprophan) was implanted in the hippocampus $+2.2$ mm from the interaural line, -4.3 mm lateral and -8 mm below the surface of the brain (König and Klippel, 1963). Probes were secured to the skull as described by Baldwin et al. (1994).

2.5. Measurement of free radical formation in vivo using microdialysis

Free radical formation in the brain in vivo was measured by the method recently described in detail by Colado et al. (1997b). The method relies on the fact that hydroxyl free radicals react with salicylic acid to generate 2,3- and 2,5-dihydroxybenzoic acids (2,3-DHBA and 2,5-DHBA) and this reaction is utilised by measuring the formation of 2,3- and 2,5-DHBA in the dialysate of a microdialysis probe implanted in the hippocampus (see above) and which is being perfused with salicylic acid (see Chiueh et al., 1992; Giovanni et al. 1995). 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) containing salicylic acid (0.5

mM) at a rate of 1 μl/min 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.

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.

The HPLC 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 a 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).

2.6. $FeCl_2$ + ascorbic acid induced lipid peroxidation

Rat cortical synaptosomes were prepared as described by Lynch and Voss (1990) and resuspended in 0.9% NaCl, pH 7.4 to produce a protein concentration of approx. 400 μg/ml. The suspension was aliquoted and frozen at −30°C. Membranes (400 μl) were added to 50μl of $FeCl_2$ (30 μM) and 50 μl of ascorbic acid (1 mM) and incubated at 37°C for 30 min. Malondialdehyde, a product of lipid peroxidation was assayed by the thiobarbituric acid reaction (Wilbur et al., 1949). Briefly, 0.5 ml HCl (0.8M) containing trichloroacetic acid (12.5%) was added to the incubation followed by thiobarbituric acid (1.23%). Samples were boiled for 15 min, cooled and centrifuged at 1500 × *g* at 4°C for 15 min. The pink colour developed by the reaction was measured by recording the optical density at 523 nm. All assays were performed in triplicate.

2.7. Statistics

Statistical analyses of the microdialysis experiments were performed using 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 pre-treatment and post-treatment data. Temperature data were also analysed by ANOVA with repeated measures. Differences among groups were considered significant at $P < 0.05$.

3. Results

3.1. The effect of clomethiazole on rectal temperature of MDMA-treated rats kept at normal ambient temperature

Administration of MDMA (15 mg/kg i.p.) produced a clear and sustained hyperthermia (Fig. 1). In contrast, clomethiazole (50 mg/kg i.p.) injected 5 min before a saline injection produced hypothermia which was sustained and enhanced following the second dose 60 min later (Fig. 1). Administration of clomethiazole 5 min prior to the MDMA (15 mg/kg) injection resulted not only in the abolition of the MDMA-induced hyperthermic response but a clear hypothermia which was sustained by the second dose (Fig. 1).

3.2. The effect of clomethiazole on 2,3-DHBA and 2,5-DHBA in the dialysate of MDMA-treated rats kept at normal ambient temperature

MDMA (15 mg/kg i.p.) was administered to rats in which a microdialysis probe had been implanted in the right hippocampus and through which salicylic acid

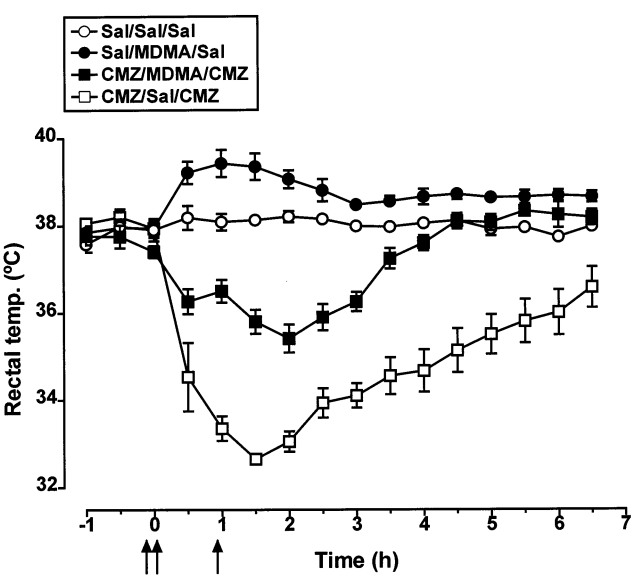

Fig. 1. Rectal temperature in rats injected with clomethiazole (CMZ) and MDMA. CMZ (50 mg/kg, i.p.) or saline was administered 5 min before and 55 min after MDMA (15 mg/kg, i.p.) or saline. Results shown as mean and vertical lines indicate SEM, $n = 5–9$. There was no difference in the basal temperature of the groups. MDMA produced a significant rise in body temperature ($F(1,12) = 40.90$, $P < 0.001$) compared with the saline-treated group. CMZ prevented the MDMA-induced hyperthermia ($F(1,15) = 144.94$, $P < 0.001$), the rats showing marked hypothermia ($F(1,11) = 39.21$, $P < 0.001$) compared with the saline group. CMZ administered to saline-treated rats also produced a significant hypothermia ($F(1,9) = 64.56$, $P < 0.001$).

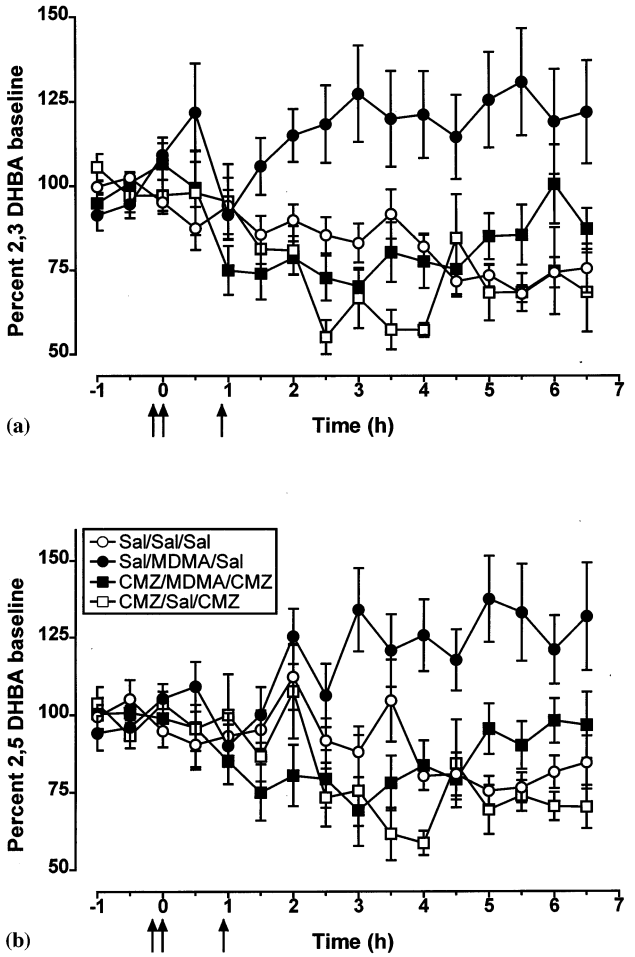

Fig. 2. Changes on the concentration of (a) 2,3-DHBA dihydroxybenzoic acid (DHBA) and (b) 2,5-DHBA in the hippocampal dialysate of rats injected with clomethiazole (CMZ) and MDMA. CMZ (50 mg/kg, i.p.) or saline was administered 5 min before and 55 min after MDMA (15 mg/kg, i.p.) or saline. Values are expressed as a percentage of the mean of three measurements before drug administration. Results shown as mean and vertical lines indicate SEM, $n = 6$–9. There was no difference in the basal levels of 2,3-DHBA and 2,5-DHBA. MDMA produced a significant increase in the levels of 2,3-DHBA ($F(1,15) = 20.03$, $P < 0.001$) and 2,5-DHBA ($F(1,15) = 18.62$, $P < 0.001$). CMZ abolished the MDMA-induced rise in the concentration of 2,3-DHBA ($F(1,15) = 19.59$, $P < 0.001$) and 2,5-DHBA ($F(1,15) = 19.43$, $P < 0.001$). Statistical analysis among groups was performed over the 6.5 h following MDMA administration. The basal concentrations in saline-treated rats ($n = 8$) were: 2,3-DHBA (9.9 ± 0.9 pg μl^{-1}) and 2,5-DHBA (9.0 ± 0.9 pg μl^{-1}).

was being perfused (Section 2). This produced a sustained rise of approximately 50% in the concentration of 2,3-DHBA and 2,5-DHBA in the perfusate (Fig. 2). Clomethiazole (50 mg/kg × 2) alone did not alter the formation of either 2,3- or 2,5-DHBA in the perfusate (Fig. 2) but did abolish the MDMA-induced rise in these compounds (Fig. 2).

3.3. The effect of clomethiazole on rectal temperature of MDMA-treated rats kept at high ambient temperature

The experiment above was repeated but with the rats which had been injected with MDMA + clomethiazole being kept at high ambient temperature (Section 2) in order to prevent the decrease in rectal temperature that occurred in this group when kept at normal ambient temperature, an aim which was achieved (Fig. 3).

3.4. The effect of clomethiazole on 2,3-DHBA and 2,5-DHBA in the dialysate of MDMA-treated rats kept at high ambient temperature

When the MDMA plus clomethiazole treated rats were kept at high ambient temperature the concentration of both 2,3- and 2,5-DHBA in the dialysate from the hippocampal microdialysis probe was similar to that seen in the rats given MDMA alone (Fig. 4). Both treatments resulted in significantly higher levels of 2,3- and 2,5-DHBA when compared with those in saline treated rats which showed a slight decrease in these two compounds with time.

3.5. Effect of clomethiazole on $FeCl_2$ + ascorbic acid-induced lipid peroxidation in vitro

Incubation of synaptosomes with $FeCl_2$/ascorbate resulted in an approximate 80-fold increase in malondi-

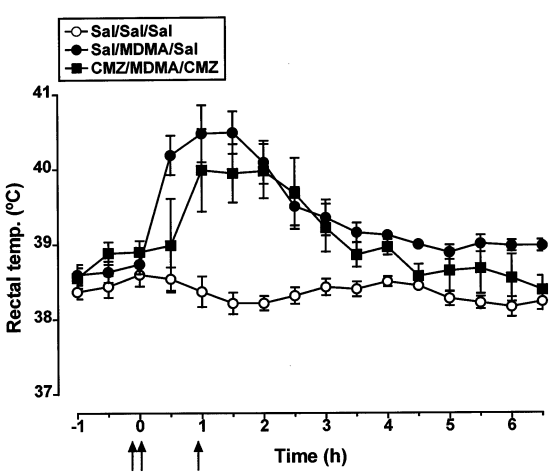

Fig. 3. Rectal temperature in rats injected with clomethiazole (CMZ) and MDMA and kept at high ambient temperature. CMZ (50 mg/kg, i.p.) or saline was administered 5 min and 55 min after MDMA (15 mg/kg, i.p.) or saline. Results shown as mean and vertical lines indicate SEM, $n = 7$–8. There was no difference in the basal temperature of the groups. MDMA produced a significant rise in body temperature ($F(1,13) = 49.95$, $P < 0.001$) compared with the saline-treated group. Using the homeothermic blanket, the rectal temperature of the CMZ + MDMA treated rats was similar to that observed when MDMA is given alone ($F(1,13) = 1.70$, n.s.), and higher than that of the saline group ($F(1,12) = 9.10$, $P < 0.01$).

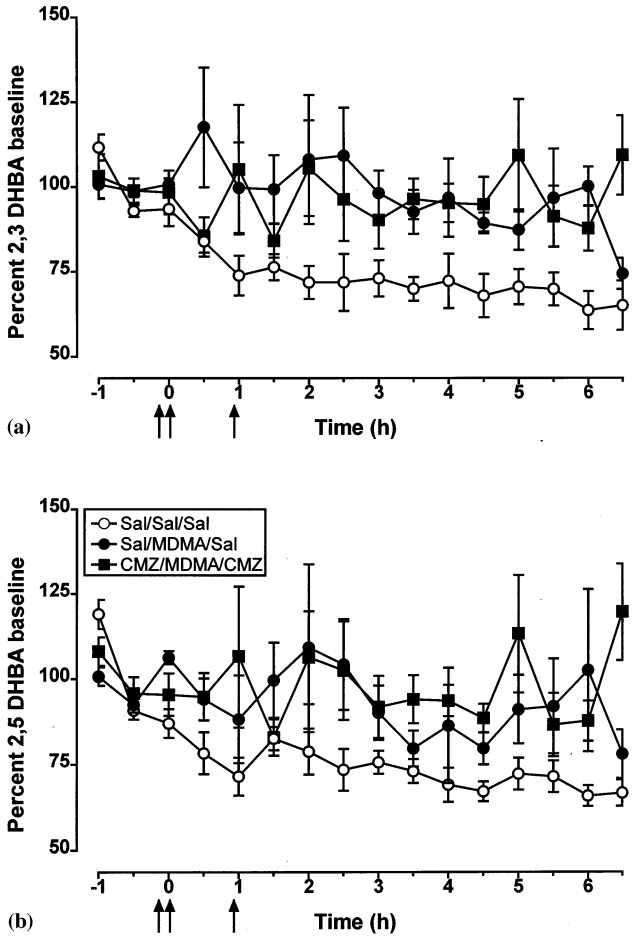

Fig. 4. Changes on the concentration of (a) 2,3-DHBA dihydroxybenzoic acid (DHBA) and (b) 2,5-DHBA in the hippocampal dialysate of rats injected with clomethiazole (CMZ) and MDMA and kept at high ambient temperature. CMZ (50 mg/kg, i.p.) or saline was administered 5 min before and 55 min after MDMA (15 mg/kg, i.p.) or saline. Values are expressed as a percentage of the mean of three measurements before drug administration. Results shown as mean and vertical lines indicate SEM, $n = 7–8$. There was no difference in the basal levels of 2,3-DHBA and 2,5-DHBA. MDMA produced a significant increase in the levels of 2,3-DHBA ($F(1,13) = 16.00$, $P < 0.001$) and 2,5-DHBA ($F(1,13) = 10.82$, $P < 0.001$). Using the homeothermic blanket, the levels of 2,3-DHBA ($F(1,13) = 0.017$, n.s.) and 2,5-DHBA ($F(1,13) = 0.66$, n.s.) in the dialysate of CMZ + MDMA treated rats were similar to that seen when MDMA is given alone. Statistical analysis among groups was performed over the 6.5 h following MDMA administration. The basal concentrations in saline-treated rats ($n = 7$) were: 2,3-DHBA (6.0 ± 0.6 pg μl^{-1}) and 2,5-DHBA (6.7 ± 0.6 pg μl^{-1}).

aldehyde formation (Data not shown). The free radical scavenger BHT inhibited the peroxidation -induced increase in a concentration dependent way (Fig. 5) with an IC_{50} of below micromolar while clomethiazole produced only modest inhibition at a millimolar concentration (Fig. 5).

4. 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 (Radzik et al., 1983; Floyd et al., 1984), although the concentration of 2,3-DHBA is considered a more reliable index of free radical formation because 2,5-DHBA can also be formed enzymatically from salicylate (Halliwell et al., 1991). However the use of a selectively implanted microdialysis probe allows measurement of free radical formation in the hippocampus, an area known to be damaged by MDMA and removes the problem both of assessing the cerebral concentration of salicylic acid and also its possible peripheral metabolism to 2,5-DHBA.

In our earlier study (Colado et al., 1997b) we observed a significant increase of approximately 50% in the concentration of 2,3-DHBA in the dialysate following MDMA (15 mg/kg) and this finding was confirmed in the present investigation. The 2,5-DHBA concentration however did not increase significantly after MDMA in the study of Colado et al. (1997b). In contrast, the current study did detect a statistically significant rise in the 2,5-DHBA concentration after MDMA (15 mg/kg). We cannot explain why the results differ in the two studies other than point out that while some studies on free radical producing compounds have found that both salicylate metabolites changed in a similar fashion (e.g. Chiueh et al., 1993), others have reported a statistically significant rise in 2,3-DHBA but not 2,5-DHBA (e.g. Giovanni et al., 1995). Since 2,3-DHBA is the metabolite that cannot be formed enzymatically (Halliwell et al., 1991), this compound is probably a more reliable indicator of increased free radical production.

There is now evidence that increased free radical formation is responsible for MDMA-induced neurotoxicity (see Section 1 for details). We therefore investigated whether the neuroprotective action of clomethiazole involved a free radical scavenging action since it is

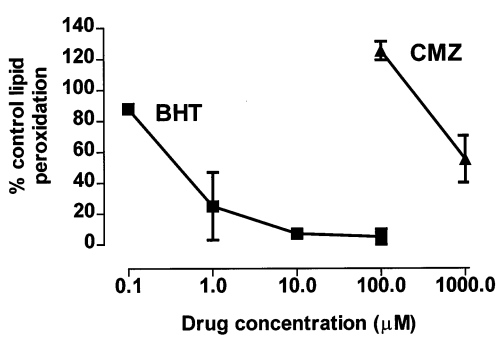

Fig. 5. The effect of increasing concentrations of BHT and clomethiazole on $FeCl_2$ (3 μM) plus ascorbic acid (0.1 M) on lipid peroxidation in rat brain synaptosomes. Data is shown as mean ± SEM of $n = 3–6$.

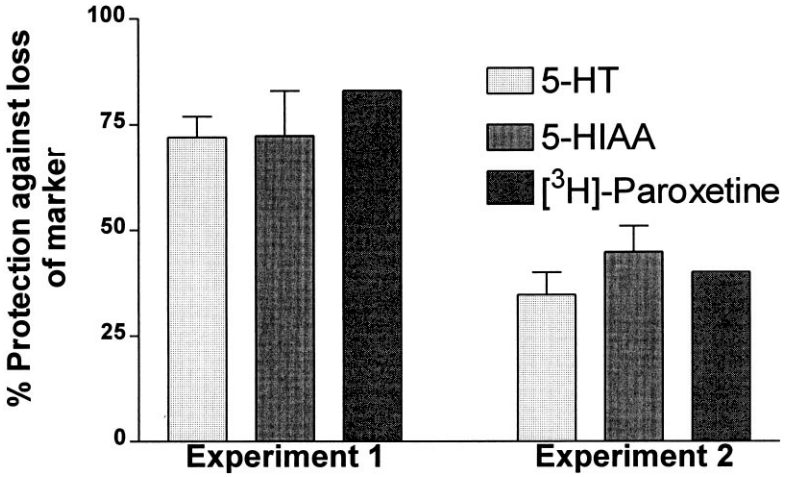

Fig. 6. The mean ± SEM of the percentage protection against the MDMA-induced loss of 5-HT and 5-HIAA in cortex, hippocampus and striatum and mean percentage loss of cortical [^{3}H]paroxetine binding in a MDMA + clomethiazole treated group of rats kept at normal ambient temperature (Experiment 1) and a group given MDMA + clomethiazole and kept at high ambient temperature (Experiment 2). Data taken and figure redrawn from results presented in Colado et al. (1998a).

an effective neuroprotective agent not only against MDMA-induced damage (Colado et al., 1993; Hewitt and Green, 1994; Colado et al., 1998a), but also in various models of acute ischemic stroke (see Green, 1998), a property it shares with the free radical scavenger PBN (Carney and Floyd, 1991; Zini et al., 1992; Sens and Phillis, 1993; Zhao et al., 1994; Colado and Green, 1995; Yeh, 1996; Colado et al., 1997b).

Clomethiazole provides substantial neuroprotection against MDMA-induced damage in rats (Colado et al., 1993) and does so even in rats kept at high ambient temperature, thereby maintaining their hyperthermic response to MDMA (see Fig. 6, Colado et al., 1998a). Therefore, the fact that it failed to inhibit the MDMA-induced rise in 2,3- or 2,5-DHBA in the dialysate of hyperthermic animals suggests strongly that its protective effect does not involve free radical scavenging.

When clomethiazole was given to MDMA treated rats kept at normal ambient temperature, thereby resulting in the animals becoming hypothermic, no elevation of the 2,3- and 2,5-DHBA concentrations in the dialysate was detected. This observation is consistant with the fact that hyperthermia markedly enhances free radical formation (Globus et al., 1995; Kil et al., 1996) and supports the contention that free radical production is favoured by the acute hyperthermia normally produced by MDMA administration (Colado et al., 1998a). It also explains why almost total neuroprotection can be achieved when clomethiazole is allowed to induce normothermia or hypothermia since the drug is producing protection through two mechanisms, a reduction in the hyperthermic response and another unrelated mechanism which may relate to the ability of clomethiazole to enhance the function of GABA (Colado et al., 1998a).

While the long term neurodegeneration which follows administration of MDMA has been shown to be related to the acute hyperthermia produced by the drug (Broening et al., 1995) neurodegeneration does occur in normothermic animals (Broening et al., 1995; Farfel and Seiden, 1995a). However any increase in free radical production occurring in the normothermic animals studied in the current investigation is below the limits of detection of the in vivo method used here, as we have discussed elsewhere (Colado et al., 1997b).

Finally, in support of our contention that clomethiazole is not a free radical scavenger, our in vitro study also failed to detect any scavenging activity, other than at a concentration at least 100-fold greater than a concentration previously shown in vivo to provide neuroprotection (Cross et al., 1995).

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References

Baldwin, H.A., Williams, J.L., Snares, M., Ferreira, T., Cross, A.J., Green, A.R., 1994. Attenuation by chlormethiazole administration of the rise in extracellular amino acids following focal ischaemia in the cerebral cortex of the rat. British Journal of Pharmacology 112, 188–194.

Battaglia, G., Yeh, S.Y., O'Hearn, E., Molliver, M.E., Kuhar, K.J., De Souza, E.B., 1987. 3,4-Methylenedioxymethamphetamine and 3,4-methylenedioxyamphetamine destroy serotonin terminals in rat brain: quantification of neurodegeneration by measurement of

[^{3}H]paroxetine-labelled serotonin uptake sites. Journal of Pharmacology and Experimental Therapeutics 242, 911–916.

Broening, H.W., Bowyer, J.F., Slikker, W. Jr., 1995. Age dependent sensitivity of rats to the long term effects of the serotonergic neurotoxicant (±)-3,4-methylenedioxymethamphetamine (MDMA) correlates with the magnitude of the MDMA-induced thermal response. Journal of Pharmacology and Experimental Therapeutics 275, 325–333.

Cadet, J.L., Ladenheim, B., Baum, I., Carlson, E., Epstein, C., 1994. CuZn-superoxide dismutase (CuZn SOD) transgenic mice show resistance to the lethal effects of methylenedioxyamphetamine (MDA) and methylenedioxymethamphetamine (MDMA). Brain Research 655, 259–262.

Carney, J.M., Floyd, R.A., 1991. Protection against oxidative damage to CNS by α-phenyl-*tert*-butyl-nitrone (PBN) and other spin-trapping agents. A novel series of nonlipid free radical scavengers. Journal of Molecular Neuroscience 3, 47–57.

Chiueh, C.C., Krishna, G., Tulsi, P., Obata, T., Lang, K., Huang, S.J., Murphy, D.L., 1992. Intracranial microdialysis of salicylic acid to detect hydroxyl radical generation through dopamine autooxidation in the caudate nucleus: effect of MPP^+. Free Radicals in Biology and Medicine 13, 581–583.

Chiueh, C.C., Miyake, H., Peng, M.-T., 1993. Role of dopamine autoxidation, hydroxyl radical generation and calcium overload in underlying mechanisms involved in MPTP- induced Parkinsonism. Advances in Neurology 60, 251–258.

Colado, M.I., Green, A.R., 1995. The spin trap reagent α-phenyl-*N*-tert-butyl nitrone prevents neurodegeneration following administration of 'ecstasy'. European Journal of Pharmacology 280, 343–346.

Colado, M.I., Murray, T.K., Green, A.R., 1993. 5-HT loss in rat brain following 3,4-methylenedioxymethamphetamine (MDMA), *p*-chloroamphetamine and fenfluramine administration and effects of chlormethiazole and dizocilpine. British Journal of Pharmacology 108, 583–589.

Colado, M.I., O'Shea, E., Granados, R., Misra, A., Murray, T.K., Green, A.R., 1997a. A study of the neurotoxic effect of MDMA ('ecstasy') on 5-HT neurones in the brains of mothers and neonates following administration of the drug during pregnancy. British Journal of Pharmacology 121, 827–833.

Colado, M.I., O'Shea, E., Granados, R., Murray, T.K., Williams, J.L., Green, A.R., 1997b. 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. British Journal of Pharmacology 121, 889–900.

Colado, M.I., Granados, R., O'Shea, E., Esteban, B., Green, A.R., 1998a. Role of hyperthermia in the protective action of clomethiazole against MDMA ('ecstasy')-induced neurodegeneration, comparison with the novel NMDA channel blocker AR-R15896AR. British Journal of Pharmacology 124, 479–484.

Colado, M.I., O'Shea, E., Esteban, B., Granados, R., Green, A.R., 1998b. Evidence against clomethiazole preventing MDMA-induced neurodegeneration by free radical scavenging. British Journal of Pharmacology 124, 43P.

Colado, M.I., Williams, J.L., Green, A.R., 1995. The hyperthermic and neurotoxic effects of 'ecstasy' (MDMA) and 3,4-methylenedioxyamphetamine (MDA) in the Dark Agouti (DA) rat, a model of the CYP2D6 poor metaboliser phenotype. British Journal of Pharmacology 115, 1281–1289.

Cross, A.J., Jones, J.A., Snares, M., Jostell, K.-G., Bredberg, U., Green, A.R., 1995. The protective action of chlormethiazole against ischaemia-induced neurodegeneration in gerbils when infused at doses having little sedative or anticonvulsant activity. British Journal of Pharmacology 114, 1625–1630.

Farfel, C.M., Seiden, L.S., 1995a. Role of hypothermia in the mechanism of protection against serotonergic toxicity. I. Experiments with 3,4-methylenedioxymethamphetamine, dizocilpine, CGS 19755 and NBQX. Journal of Pharmacology and Experimental Therapeutics 272, 860–867.

Farfel, C.M., Seiden, L.S., 1995b. Role of hypothermia in the mechanism of protection against serotonergic toxicity. II. Experiments with methamphetamine, *p*-chloroamphetamine, fenfluramine, dizocilpine and dextromethorphan. Journal of Pharmacology and Experimental Therapeutics 272, 868–875.

Floyd, R.A., Watson, J.J., Wong, P.K., 1984. Sensitive assay of hydroxyl free radical formation utilizing high pressure liquid chromatography with electrochemical detection of phenol and salicylate hydroxylation products. Journal of Biochemical and Biophysical Methods 10, 221–235.

Giovanni, A., Liang, L.P., Hastings, T.G., Zigmond, M.J., 1995. Estimating hydroxyl radical content in rat brain using systemic and intraventricular salicylate: impact of methamphetamine. Journal of Neurochemistry 64, 1819–1825.

Globus, M.Y.-T., Busto, R., Lin, B., Schnippering, H., Ginsberg, M.D., 1995. Detection of free radical formation during transient global ischemia and recirculation: effects of intraischemic brain temperature modulation. Journal of Neurochemistry 65, 1250–1256.

Green, A.R., 1998. Clomethiazole (Zendra®) in acute ischemic stroke: basic pharmacology and biochemistry and clinical efficacy. Pharmacology and Therapeutics (in press).

Green, A.R., Cross, A.J., Goodwin, G.M., 1995. Review of the pharmacology and clinical pharmacology of 3,4-methylenedioxymethamphetamine (MDMA or 'ecstasy'). Psychopharmacology 119, 247–260.

Halliwell, B., Kaur, H., Ingelman-Sundberg, M., 1991. Hydroxylation of salicylate as an assay for hydroxyl radicals: a cautionary note. Free Radicals in Biology and Medicine 10, 439–441.

Hewitt, K.E., Green, A.R., 1994. Chlormethiazole, dizocilpine and haloperidol prevent the degeneration of serotonergic nerve terminals induced by administration of MDMA ('ecstasy'). Neuropharmacology 33, 1589–1595.

Kil, H.Y., Zahng, J., Piantadosi, L.A., 1996. Brain temperature alters hydroxyl radical production during cerebral ischemia/reperfusion in rats. Journal of Cerebral Blood Flow and Metabolism 16, 100–106.

König, J.F.R., Klippel, R.A., 1963. The Rat Brain. A Stereotaxic Atlas of the Forebrain and Lower Parts of the Brain Stem. Robert E. Krieger, New York.

Lynch, M.A., Voss, K.L., 1990. Arachidonic acid increases inositol phospholipid metabolism and glutamate release in synaptosomes prepared from hippocampal tissue. Journal of Neurochemistry 55, 215–221.

Malberg, J.E., Sabol, K.E., Seiden, L.S., 1996. Co-administration of MDMA with drugs that protect against MDMA neurotoxicity produces different effects on body temperature in the rat. Journal of Pharmacology and Experimental Therapeutics 278, 258–267.

Molliver, M.E., Berger, U.V., Manounas, L.A., Molliver, D.L., O'Hearn, E., Wilson, M.A., 1990. Neurotoxicity of MDMA and related compounds: anatomic studies. Annals of the New York Academy of Sciences 600, 640–661.

Murray, T.K., Misra, A., Williams, J.L., Colado, M.I., Green, A.R., 1996. The spin trap reagent PBN attenuates degeneration of 5-HT neurones in rat brain induced by *p*-chloroamphetamine but not fenfluramine. Neuropharmacology 35, 1615–1620.

O'Hearn, E., Battaglia, G., De Souza, E.B., Kuhar, M.J., Molliver, M.E., 1988. Methylenedioxyamphetamine (MDA) and methylenedioxymethamphetamine (MDMA) cause ablation of serotonergic nerve terminals in forebrain: immunocytochemical evidence. Journal of Neuroscience 8, 2788–2803.

O'Shea, E., Granados, R., Esteban, B., Colado, M.I., Green, A.R., 1998. The relationship between the degree of neurodegeneration of rat brain 5-HT nerve terminals and the dose and frequency of administration of MDMA ('ecstasy'). Neuropharmacology 37, 919–926.

Radzik, D.M., Roston, D.A., Kissenger, P.T., 1983. Determination of hydroxylated aromatic compounds produced in a superoxide-dependent formation of hydroxyl radicals by liquid chromatography/electrochemistry. Analytical Biochemistry 131, 458–464.

Sens, S., Phillis, J.W., 1993. α-Phenyl-*tert*-butyl nitrone (PBN) attenuates hydroxyl radical production during ischaemia-reperfusion injury of rat brain: an EPR study. Free Radical Research Communications 19, 255–265.

Sharkey, J., McBean, D.E., Kelly, P.A.T., 1991. Alterations in hippocampal function following repeated exposure to the amphetamine derivative methylenedioxymethamphetamine ('Ecstasy'). Psychopharmacology 105, 113–118.

Sprague, J.E., Nichols, D.E., 1995. The monoamine oxidase-B inhibitor L-deprenyl protects against 3,4-methylenedioxymethamphetamine-induced lipid peroxidation and long-term serotonergic deficits. Journal of Pharmacology and Experimental Therapeutics 273, 667–673.

Steele, T.D., McCann, U.D., Ricaurte, G.A, 1994. 3,4-Methylenedioxymethamphetamine (MDMA, 'ecstasy'); pharmacology and toxicology in animals and humans. Addiction 89, 539–551.

White, S.R., Obradovic, T., Imel, K.M., Wheaton, M.J., 1996. The effects of methylenedioxymethamphetamine (MDMA, 'ecstasy') on monoaminergic neurotransmission in the central nervous system. Progress in Neurobiology 49, 455–479.

Wilbur, K.M., Bernheim, F., Shapiro, O.W., 1949. The thiobarbituric acid reagent as a test for the oxidation of unsaturated fatty acid by various agents. Archives of Biochemistry and Biophysics 24, 305–313.

Yeh, S.Y., 1996. Attenuation of 3,4-methylenedioxymethamphetamine (MDMA)-induced neurotoxicity by phenyl-*t*-butyl nitrone (PBN) in rats. American Society of Neuroscience, Abst. 749.2.

Zhao, Q., Pahlmark, K., Smith, M.L., Siesjö, B.K., 1994. Delayed treatment with the spin trap α-phenyl-*N*-*tert*-butyl nitrone (PBN) reduces infant size following transient middle cerebral artery occlusion in rats. Acta Physiologica Scandinavica 152, 349–350.

Zini, I., Tomasi, A., Grimaldi, R., Vanninin, V., Agnati, L.F., 1992. Detection of free radicals during brain ischaemia and reperfusion by spin-trapping and microdialysis. Neuroscience Letters 138, 279–282.