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Role of hyperthermia in the protective action of clomethiazole against MDMA ('ecstasy')-induced neurodegeneration, comparison with the novel NMDA channel blocker AR-R15896AR

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1 The immediate effect of administration of 3,4-methylenedioxymethamphetamine (MDMA or 'ecstasy') on rectal temperature and the effect of putative neuroprotective agents on this change has been examined in rats. The influence of the temperature changes on the long term MDMA-induced neurodegeneration of cerebral 5-hydroxytryptamine (5-HT) nerve terminals was also examined.

2 The novel low affinity N-methyl-D-aspartate (NMDA) receptor channel blocker AR-R15896AR (20 mg kg^{-1}, i.p.) given 5 min before and 55 min after MDMA (15 mg kg^{-1}, i.p.) did not prevent the MDMA-induced hyperthermia and did not alter either the MDMA-induced neurodegenerative loss of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) in cortex, striatum and hippocampus or loss of [^{3}H]-paroxetine binding in cortex 7 days later.

3 The neuroprotective agent clomethiazole (50 mg kg^{-1}, i.p.) given 5 min before and 55 min after MDMA (15 mg kg^{-1}) abolished the MDMA-induced hyperthermic response and markedly attenuated the loss of 5-HT, 5-HIAA and [^{3}H]-paroxetine binding in the brain regions examined 7 days later.

4 When rats treated with MDMA plus clomethiazole were kept at high ambient temperature for 5 h post-MDMA, thereby keeping their body temperature elevated to near that seen in rats given MDMA alone, the MDMA-induced loss of 5-HT, 5-HIAA and [^{3}H]-paroxetine was still attenuated. However, the protection (39%) afforded by the clomethiazole administration was less than seen in rats kept at normal ambient temperature (75%).

5 These data support the proposals of others that NMDA receptor antagonists are neuroprotective against MDMA-induced degeneration only if they induce hypothermia and further suggest that increased glutamate activity may not be involved in the neurotoxic action of MDMA.

6 These data further demonstrate that a proportion of the neuroprotective action of clomethiazole is due to an effect on body temperature but that, in addition, the compound protects against MDMA-induced damage by an unrelated mechanism.

Keywords: 3,4-Methylenedioxymethamphetamine; ecstasy; clomethiazole; AR-R15896AR; NMDA antagonists; 5-hydroxytryptamine; neuroprotection; hypothermia; neurodegeneration

Introduction

3,4-Methylenedioxymethamphetamine (MDMA or 'ecstasy') administration to a variety of animal species results in a long-term neurotoxic degeneration of 5-hydroxytryptamine (5-HT) nerve terminals in several regions of the brain (Steele *et al*., 1994; Green *et al*., 1995). The degeneration has been demonstrated histologically and is reflected in the marked loss in the concentration of 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) and by the loss of [^{3}H]-paroxetine binding to the presynaptic transporter (see review of Green *et al*., 1995). There have been a variety of compounds which, when given concurrently with the MDMA, have been shown to protect against the neurodegenerative loss of 5-HT (see Green *et al*., 1995). It is also well established that hypothermia will attenuate or prevent neurodegeneration in a variety of animal models of acute ischaemic stroke (Busto *et al*., 1987; Buchan & Pulsinelli, 1990; Corbett *et al*., 1990; Nurse & Corbett, 1996). However much of this evidence became available either before or during the time that many of the neuroprotective studies were being conducted on MDMA. Consequently, until recently few studies on MDMA-induced neurodegeneration examined the body temperature of the animals under investigation.

In the last few years Seiden and colleagues have presented compelling data to support their proposal that several compounds previously shown to be neuroprotective against MDMA-induced damage (including certain NMDA antagonists) only had this property because, when combined with MDMA, they produced a significant hypothermia (Malberg *et al*., 1996; Farfel & Seiden, 1995a;b). However, there is a further complication in that MDMA administration alone produces a significant hyperthermia (Nash *et al*., 1988; Gordon *et al*., 1991; Colado *et al*., 1993; Dafters, 1994) and Broening *et al*. (1995) have shown that hyperthermia plays a significant role in the expression of 5-hydroxytryptaminergic neurotoxicity following MDMA.

In our earlier studies on clomethiazole (INN: clomethiazole; BAN: chlormethiazole) and dizocilpine we noted that at the doses used neither compound when given with MDMA appeared to produce frank hypothermia (Colado *et al*., 1993; Hewitt & Green, 1994). Nevertheless both compounds were neuroprotective. However, both compounds did abolish the MDMA-induced hyperthermia (Hewitt & Green, 1994). We have now therefore re-examined the effect of clomethiazole to determine whether its neuroprotective effect against MDMA-

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induced damage is due to its effect on body temperature or whether this compound had an additional neuroprotective action in this neurodegenerative model as it has in animal models of acute ischaemic stroke (Green & Cross, 1994; Green, 1998). In addition we examined the effect of the novel low affinity NMDA receptor channel blocker AR-R15896AR (Greene *et al*., 1996; Cregan *et al*., 1997; Palmer *et al*., 1997) to see whether the proposal of Farfel & Seiden (1995a) that NMDA antagonists may not be neuroprotective against MDMA induced damage in the absence of hypothermia could be confirmed and extended. Some of these results were given in preliminary form to a meeting of the British Pharmacological Society (Colado *et al*., 1997a).

Methods

Animals

Adult male Dark Agouti rats (Interfauna, Barcelona) 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 h light dark cycle (lights on: 07 h 00 min) and given free access to food and water.

Elevation of body temperature with a homeothermic blanket

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

Measurement of 5-HT and 5-HIAA

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) and 5-hydroxyindoleacetic acid (5-HIAA) measured by high performance liquid chromatography (h.p.l c.) as described by Colado *et al*. (1997c). Briefly, the mobile phase for 5-HT and 5-HIAA 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 by Hewitt & Green (1994). 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 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 use of a digital readout thermocouple (Type K thermometer, Portec, U.K.) with a resolution of ±0.1°C and accuracy of ±0.2°C attached to a CAC-005 Rodent Sensor which was 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.

Drugs

(±)-Methylenedioxymethamphetamine HCl was obtained from the Ministry of Health (Spain). Clomethiazole edisylate was obtained from Astra Arcus (S-151 85 Södertälje, Sweden) and AR-R15896AR (S-(+)-alpha-phenyl-2-pyridine ethanamine dihydrochloride) from Astra Charnwood (Bakewell Road, Loughborough, LE11 5RH). All drugs were dissolved in 0.9% w/v NaCl (saline) and injected i.p. Control animals were injected with saline. Doses are quoted in terms of the base.

Statistics

All neurochemical data were analysed by one way ANOVA followed by Newman-Keuls test (Pharmacological Calculations, Tallarida). Analysis of the temperature data was by use of the statistical computer package BMDP/386 Dynamic (BDMP Statistical Solutions, Cork, Eire).

Results

The effect of AR-R15896AR on rectal temperature in MDMA treated rats

MDMA (15 mg kg^{-1} i.p.) produced a rapid and clear hyperthermic response lasting more than 5 h (Figure 1). Two doses of AR-R15896AR (20 mg kg^{-1}) given 60 min apart produced a modest rise in rectal temperature towards the end of the observation period (Figure 1). AR-R15896AR administration did not significantly alter the hyperthermic response of the rats given MDMA (Figure 1).

The effect of AR-R15896AR on the neurodegenerative effect of MDMA

AR-R15896AR had no effect on the MDMA-induced loss of cerebral 5-HT and 5-HIAA in hippocampus, striatum and cortex or on the loss of [^{3}H]-paroxetine binding in the cortex 7 days later (Table 1).

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

Clomethiazole (50 mg kg^{-1}) produced hypothermia after the first dose which was sustained and enhanced following the second dose (Figure 2a). Administration of clomethiazole 5 min before MDMA (15 mg kg^{-1}) injection resulted in the

abolition of the MDMA-induced hyperthermic response for the first 60 min and a brief and modest hypothermia following the second dose (Figure 2a).

The effect of clomethiazole on the neurodegenerative effect of MDMA 7 days later

Administration of a single dose of MDMA (15 mg kg^{-1}) resulted in a substantial loss of 5-HT and 5-HIAA in cortex, hippocampus and striatum 7 days later (Figure 3). There was a similar loss in [^{3}H]-paroxetine binding in the cortex (Figure 3). Clomethiazole markedly attenuated the MDMA-induced loss of these parameters (Figures 3 and 5b).

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

The experiment above was repeated but with the MDMA plus clomethiazole rats being kept at high ambient temperature

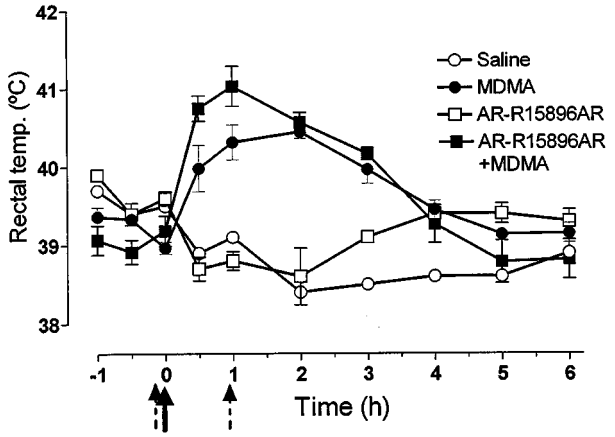

Figure 1 Rectal temperature of rats following injection of AR-R15896AR (20 mg kg^{-1}, i.p.) and MDMA (15 mg kg^{-1}, i.p.). Saline or MDMA was given at time zero (at large arrow). AR-R15896AR or saline was given 5 min before and 55 min after saline or MDMA (at dotted arrows). MDMA significantly increased temperature compared to saline treatment ($F(1,9)=52.03$, $P<0.001$). Administration of AR-R15896AR to MDMA-treated rats did not modify the hyperthermic response induced by MDMA ($F(1,10)=0.71$). AR-R15896AR given to saline treated rats produced a rise in body temperature ($F(1,8)=5.84$, $P<0.05$). All results shown as mean ± s.e.mean of $n=5-6$.

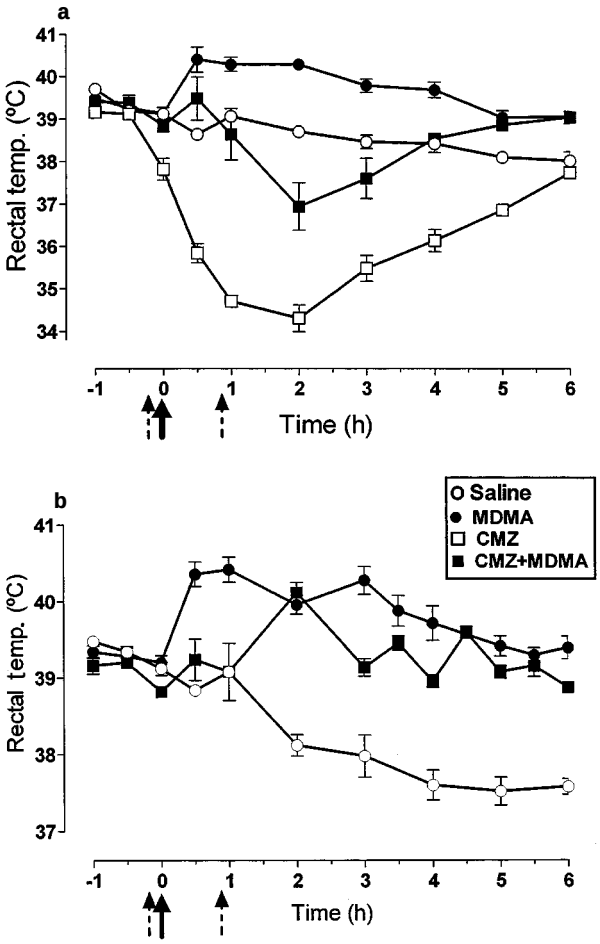

Figure 2 Effect of clomethiazole on MDMA-induced hyperthermia. (a) Rectal temperature of rats injected with saline or MDMA (15 mg kg^{-1}, i.p.) at time zero (at large arrow) and of rats injected with clomethiazole (50 mg kg^{-1}, i.p.) at −5 min and +55 min (at dotted arrows) and treated with either saline or MDMA at time zero. There was no difference in the basal temperature of the groups. MDMA produced a significant rise in body temperature ($F(1,9)=87.68$, $P<0.001$), compared with the saline injected group. Administration of clomethiazole (CMZ) to MDMA treated rats prevented the MDMA-induced hyperthermia ($F(1,9)=25.25$, $P<0.001$), the rats temperature not being significantly different from controls ($F(1,8)=0.07$). (b) Effect of clomethiazole on MDMA-induced hyperthermia when the group injected with clomethiazole + MDMA were kept at high ambient temperature to prevent the hypothermia resulting from this drug combination. All results shown as mean ± s.e.mean of $n=5-6$ rats.

Table 1 Changes in the concentration of 5-HT (ng g^{-1} tissue wet wt.), 5-HIAA (ng g^{-1} tissue wet wt.) and [^{3}H]-paroxetine binding (fmol mg^{-1} protein) in rat brain following MDMA and the lack of effect of AR-R15896AR on these changes

	Saline	*MDMA*	*MDMA + AR-R15896AR*	*AR-R15896AR*
Hippocampus				
5-HT	286 ± 2 (4)	146 ± 9 (5)**	161 ± 18 (6)**	282 ± 10 (5)
5-HIAA	320 ± 8 (5)	176 ± 12 (5)**	184 ± 22 (6)**	329 ± 8 (5)
Striatum				
5-HT	394 ± 15 (5)	326 ± 25 (6)*	281 ± 19 (6)**	397 ± 23 (5)
5-HIAA	422 ± 18 (5)	352 ± 22 (6)*	332 ± 15 (6)*	434 ± 25 (5)
Cortex				
5-HT	259 ± 6 (5)	166 ± 8 (6)**	148 ± 9 (6)**	252 ± 16 (5)
5-HIAA	158 ± 4 (5)	103 ± 5 (6)**	95 ± 8 (6)**	160 ± 5 (5)
[^{3}H]-paroxetine	58 ± 4 (6)	29 ± 3 (6)**	26 ± 6 (6)**	60 ± 8 (5)

AR-R15896AR (20 mg kg^{-1}, i.p.) was injected 5 min before and 55 min after MDMA (15 mg kg^{-1}, i.p.) or saline, the rats being killed 7 days later. Results reported as mean ± s.e.mean (n). Different from saline injected: *$P<0.05$, **$P<0.01$. Values of the MDMA and AR-R15896AR + MDMA groups not statistically different from each other.

(see methods), in order to try and maintain their rectal temperature elevated to a similar degree to that occurring in the rats given MDMA plus saline. This aim was achieved (Figure 2b).

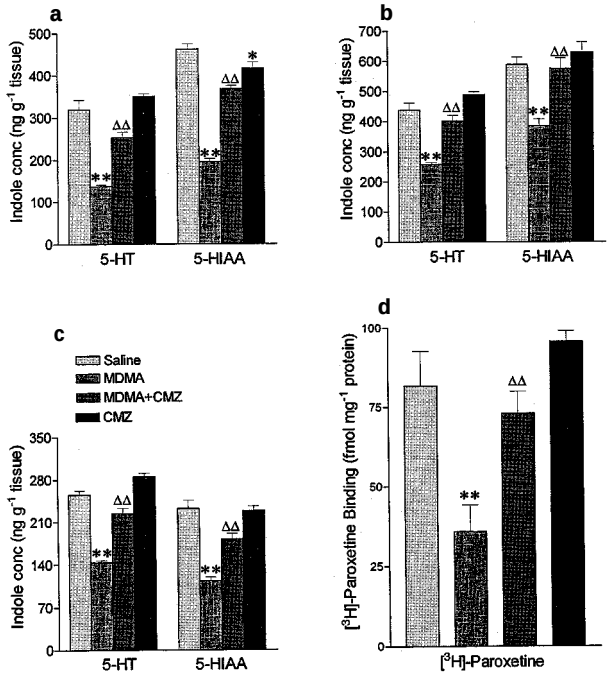

Figure 3 The indole concentrations in (a) hippocampus, (b) striatum, (c) cortex and (d) [^{3}H]-paroxetine binding values in cortex 7 days following saline or clomethiazole (CMZ, 50 mg kg^{-1}) 5 min before and 55 min after saline or MDMA (15 mg kg^{-1}). Results shown as mean ± s.e.mean, $n=5$. Different from saline-treated: $^*P<0.05$, $^{**}P<0.01$; different from MDMA-treated: $\Delta\Delta P<0.01$.

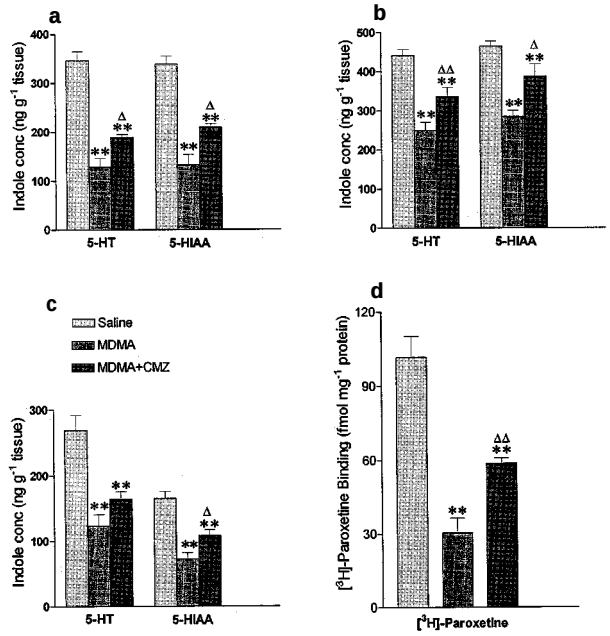

Figure 4 The indole concentrations in (a) hippocampus, (b) striatum, (c) cortex and (d) [^{3}H]-paroxetine binding values in cortex 7 days following saline or clomethiazole (CMZ, 50 mg kg^{-1}) 5 min before and 55 min after saline or MDMA (15 mg kg^{-1}). The MDMA + clomethiazole rats were kept at high ambient temperature during the 5 h after the second CMZ injection. Results shown as mean ± s.e.mean, $n=4-5$. Different from saline-treated: $^{**}P<0.01$. Different from MDMA-treated: $\Delta P<0.05$, $\Delta\Delta P<0.01$.

The effect of clomethiazole on the neurodegenerative effect of MDMA in rats housed at high ambient temperature

The degree of damage to 5-HT nerve terminals (as judged by loss of 5-HT, 5-HIAA and [^{3}H]-paroxetine binding) induced by MDMA was similar in this study to the previous experiment (Figures 4 and 5a). However, the degree of protection provided by clomethiazole in rats kept at high ambient temperature was significantly less than that seen when the animals were housed at normal ambient temperature (Figures 4 and 5b).

Discussion

There have been several studies, including those conducted in our laboratory, which have demonstrated a protective effect of various NMDA antagonists against MDMA-induced neurotoxicity (Finnegan *et al*., 1990; Farfel *et al*., 1992; Colado *et al*., 1993; Hewitt & Green, 1994). However, recently Farfel & Seiden (1995a) observed that the combination of dizocilpine or CGS 19755 and MDMA produced frank hypothermia and suggested that it was the hypothermia that produced the neuroprotection. They strengthened this proposal by showing that dizocilpine was no longer neuroprotective when rats were kept at high ambient temperature, thereby preventing a decrease in body temperature in the dizocilpine/MDMA

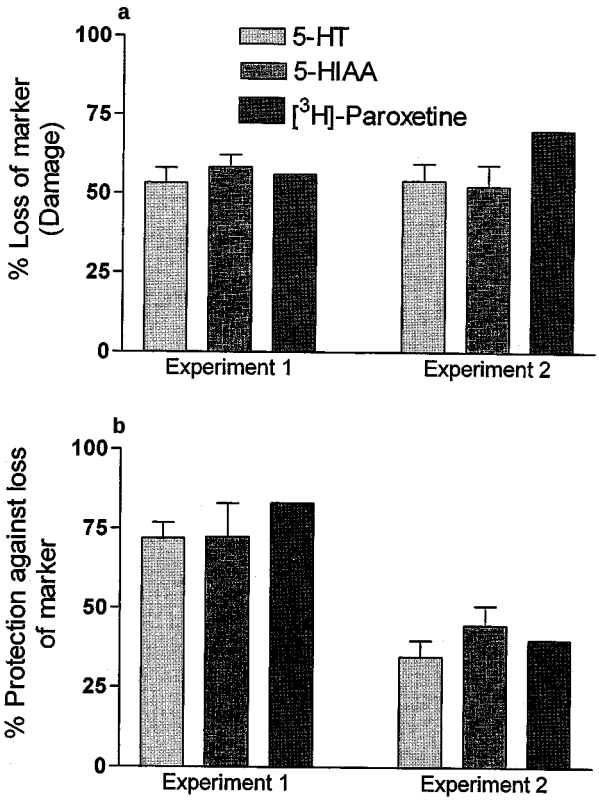

Figure 5 (a) The mean % loss of 5-HT and 5-HIAA in the 3 areas examined (± s.e.mean) and mean % loss of [^{3}H]-paroxetine binding in the MDMA-treated group in the experiment presented in Figure 3 (Experiment 1) and the experiment shown in Figure 4 (Experiment 2). (b) The mean % protection against MDMA-induced loss of 5-HT and 5-HIAA and mean % loss of [^{3}H]-paroxetine binding in the MDMA + clomethiazole treated group kept at normal ambient temperature and shown in Figure 3 (Experiment 1) and the group given MDMA + clomethiazole kept at high ambient temperature in the experiment illustrated in Figure 4 (Experiment 2).

treated animals. We have now found that the novel low affinity NMDA channel blocker AR-R15896AR did not attenuate the MDMA-induced hyperthermia and also had no neuroprotective effect at a dose known to be neuroprotective in animal models of ischaemic stroke (Palmer *et al*., 1997; Cregan *et al*., 1997). All these data therefore suggest that the neurodegeneration which follows MDMA may not involve an excitotoxic cascade involving glutamate, as has been suggested to occur in ischaemia-induced neurodegeneration (see Small & Buchan, 1997).

These data raised the possibility that other drugs previously shown to be neuroprotective might also have induced protection by lowering body temperature, rather than through a specific neuroprotective mechanism. We therefore examined clomethiazole, another compound we had previously investigated, and which has been found to be neuroprotective in various animal models of acute ischaemic stroke (see Green & Cross, 1994; Green, 1998).

In our previous studies clomethiazole did not produce hypothermia in the MDMA-treated Lister Hooded rats, but nevertheless clearly abolished the hyperthermia induced by MDMA and was an effective neuroprotective drug (Colado *et al*., 1993; Hewitt & Green, 1994). In this study, using Dark Agouti rats this finding was confirmed.

When the temperature of the rats given clomethiazole plus MDMA was kept elevated to approximately that of those given only MDMA, there was a reduction in the degree of neuroprotection. Nevertheless, there was still considerable protection, in contrast to the studies with dizocilpine (Farfel & Seiden, 1995a) where it was abolished. This observation also indirectly supports our evidence that the neuroprotective action of clomethiazole does not involve an action of the drug at NMDA receptors (Cross *et al*., 1993; Green *et al*., 1997).

Damage following MDMA occurs in normothermic animals (Broening *et al*., 1995; Farfel & Seiden, 1995a) but hyperthermia worsens the damage (Broening *et al*., 1995). Our data are consistent with this indicating that the protection afforded by clomethiazole at normal ambient temperatures comprises two additive mechanisms. One is a reduction of the rat temperature to normothermia and the second is an unrelated neuroprotective mechanism.

There is now a substantial body of evidence that increased free radical formation is responsible for MDMA-induced neurotoxicity (Cadet *et al*., 1994; Colado & Green, 1995; Sprague & Nichols, 1995; Murray *et al*., 1996; Colado *et al*., 1997b,c). This, in turn, would explain why hyperthermia exacerbates the damage, since it has been shown to enhance free radical production in the brain (Globus *et al*., 1995; Kil *et al*., 1996). The MDMA-induced increase in free radical formation only becomes apparent 1 h after administration and continues for several hours thereafter (Colado *et al*., 1997c). It seems unlikely therefore that the lower body temperature of the clomethiazole/MDMA treated rats compared to the MDMA treated rats in the first hour explains the substantial neuroprotection seen.

What remains uncertain is the mechanism(s) by which clomethiazole affords protection against MDMA-induced damage. Although there is good evidence that the damage results from increased free radical formation in the brain (see above), recent studies have indicated that clomethiazole is not a free radical scavenger (Colado *et al*., unpublished observations). Clomethiazole is known to potentiate the action of γ-aminobutyric acid (GABA) in the brain and does so in a way that differs from benzodiazepines and barbiturates (see Cross *et al*., 1989; Green *et al*., 1996), compounds that are not neuroprotective against ischaemic stroke (Green, 1998). There are reasonable grounds for believing that it is this GABA potentiating action that is responsible for the drug being neuroprotective in animal models of acute ischaemic stroke (see Green, 1998 for review). However, whether this property is involved in the neuroprotective action of the drug against MDMA-induced damage requires further investigation.

Finally, the current data and that of others (Farfel & Seiden 1995a,b; Malberg *et al*., 1996) suggest that re-evaluation is required of some of the earlier reports on the neuroprotective properties of various compounds against MDMA-induced neurodegeneration, if the studies were conducted in the absence of good measures of body temperature.

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