



The relationship between the degree of neurodegeneration of rat brain 5-HT nerve terminals and the dose and frequency of administration of MDMA ('ecstasy')

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Abstract

The effect of varying the dose and frequency of administration of 3,4-methylenedioxymethamphetamine (MDMA or 'ecstasy') on both the acute hyperthermic response and the long term neurodegeneration of 5-hydroxytryptamine (5-HT) nerve terminals in the brain has been studied in Dark Agouti rats. A single injection (4–15 mg/kg i.p.) of MDMA produced immediate dose-related hyperthermia and a dose-related decrease in 5-HT, 5-hydroxyindoleacetic acid (5-HIAA) and [³H]paroxetine binding in regions of the brain 7 days later, with a dose of 4 mg/kg having no degenerative effect. This dose was also without effect when given once daily for 4 days, but produced a marked loss of [³H]paroxetine binding and indole concentration ($\approx 55\%$) when given twice daily for 4 days. When a dose of 4 mg/kg was given twice weekly for 8 weeks it had no effect on these serotonergic markers, despite a clear anorectic effect of the drug being seen. These data demonstrate that MDMA-induced neurodegeneration is related to both the dose and frequency of administration and indicate that damage to 5-HT neurones can occur in the absence of a hyperthermic response to the drug. We suggest that damage occurs when endogenous free radical scavenging mechanisms become overwhelmed or exhausted. © 1998 Elsevier Science Ltd. All rights reserved.

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1. Introduction

The recreationally used drug 3,4-methylenedioxymethamphetamine (MDMA or 'ecstasy') has been shown in many studies to produce long term neurotoxic damage to 5-hydroxytryptamine (5-HT) axon terminals in several areas of the brain (Steele et al., 1994; Green et al., 1995; Seiden and Sabol, 1996). The damage has been shown both histologically and biochemically (reviewed in Green et al., 1995) and there have been many studies on the mechanisms involved in the neurodegenerative change (Colado et al., 1997a and reviewed in McKenna and Peroutka, 1990; Steele et al., 1994; Green et al., 1995; Seiden and Sabol, 1996; White et al., 1996).

There is a view among some recreational users that the doses required to induce damage in experimental

animals are so high that they have little relevance to those taken by humans and that the drug is therefore 'safe'. This view is weakened by the observation that a single dose of MDMA of 10 mg/kg produced drug levels similar to those admitted with acute adverse reactions and that this dose caused considerable neurotoxic damage to 5-HT nerve terminals (Colado et al., 1995). McCann et al. (1996) recently pointed out the similarity in the dose taken by recreational users and that producing damage in the primate brain and Green and Goodwin (1996) emphasised the possible long term neurological hazards of 'ecstasy' use.

However, an additional complication is that clinical data have indicated that there may not be a clear relationship between the dose ingested and the adverse reactions that occur (Henry et al., 1992). Furthermore, even in rodents there have been few studies on the dosing schedules or minimum doses required to induce damage. It is acknowledged that neurodegeneration occurs after either one large single dose (20 mg/kg or

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more) or several lower doses given over one or two days (Battaglia et al., 1988; Ricaurte et al., 1988; Colado et al., 1993) but there appears to have been no systematic examination of dosing schedules in a single strain of rat, even though dose response studies have been conducted in primates (Battaglia et al., 1988; Ali et al., 1993). We have therefore now investigated both the dose of MDMA required both to induce the acute hyperthermic response (Nash et al., 1988; Schmidt et al., 1990; Gordon et al., 1991; Colado et al., 1993; Dafters, 1994) and also the dose required to induce long term neurotoxic damage to 5-HT nerve terminals. The latter was examined by measuring the loss of both the cerebral content of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) (Stone et al., 1986; Battaglia et al., 1987; Schmidt et al., 1987; Colado et al., 1993) and the binding of [3 H]paroxetine to presynaptic 5-HT nerve endings (Battaglia et al., 1987; Sharkey et al., 1991; Hewitt and Green, 1994; Colado et al., 1997a). We also examined whether toxicity occurred when a dose of MDMA that does not cause overt damage when given once will do so when given repeatedly over several days or weeks. A preliminary account of some of this work was given at a meeting of the British Pharmacological Society (Colado et al., 1997d).

2. Materials and methods

2.1. Animals and drug administration

Adult male Dark Agouti (DA) rats (Interfauna, Barcelona, Spain) 150–180 g were used. They were housed in groups of 5 in conditions of constant temperature (21–22°C) and a 12 hour light/dark cycle (lights on 07:00) with free access to food and water. MDMA (obtained from the Ministry of Health, Spain) was dissolved in saline (0.9% NaCl) and given i.p. Doses are reported in terms of the base.

2.2. Measurement of 5-HT and 5-HIAA

The rats were killed by cervical dislocation and decapitation, the brains rapidly removed and the cortex, hippocampus and striatum dissected out on ice. The tissue was homogenised and 5-HT and 5-HIAA measured by high performance liquid chromatography (HPLC) as described by Colado et al. (1997a). 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 and the working electrode potential was set at 0.85 V. The HPLC 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 mm $\times$ 3.9 mm) with a precolumn and an amperometric detector (Waters M460). The current produced was monitored by using an integrator (Waters M745).

2.3. [3 H]paroxetine binding in tissue homogenates

[3 H]paroxetine binding was measured by the method described in detail by Hewitt and 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) using an Ultra-Turrax. The homogenate was centrifuged at $30000 \times g$ for 10 min at 4°C. The supernatant was discarded and the wash procedure repeated twice more. The pellet was finally resuspended in the Tris buffer at a concentration of 10 mg tissue/ml. 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. The assays were terminated by rapid filtration and counting of the radioactivity by scintillation spectrometry. The protein concentrations were measured by the method of Lowry et al. (1951).

2.4. Measurement of rectal temperature

The temperature was measured by insertion of a thermocouple probe (with a digital readout) inserted 2.5 cm into the rectum, the rat being lightly restrained by hand.

2.5. Statistics

All neurochemical data were analysed by one way ANOVA followed the 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, Ireland).

3. Results

3.1. Effect of an acute dose of MDMA on rectal temperature

Following MDMA (4, 10 and 15 mg/kg, i.p.) administration there was a dose related increase in the rectal temperature lasting several hours (Fig. 1).

3.2. Effect of an acute dose of MDMA on 5-HT and 5-HIAA concentrations and [3 H]paroxetine binding in regions of the rat brain

A single 15 mg/kg dose of MDMA produced a greater than 50% loss in 5-HT and 5-HIAA in the cortex, hippocampus and striatum (Fig. 2) seven days later. This loss reflected neurodegenerative changes as was indicated by the approximately parallel loss of [3 H]paroxetine binding (Fig. 2). A major neurotoxic loss was also observed after a dose of 10 mg/kg, with no significant effect after 4 mg/kg (Fig. 2).

3.3. Effect of a dose of 4 mg/kg MDMA given once or twice daily for 4 days on rectal temperature

Immediately following the first dose of 4 mg/kg MDMA the rectal temperature of the animals was increased for several hours compared to the control (saline injected) rats, whose temperature decreased over the period of observation (Fig. 3a–c). Measurements made on the second, third and fourth days in the morning (reflecting the 2nd, 3rd, and 4th doses of the 'once daily' group and the 3rd, 5th, and 7th doses of the 'twice daily' group) failed to detect any hyperthermic response to the MDMA (Fig. 3 shows the results

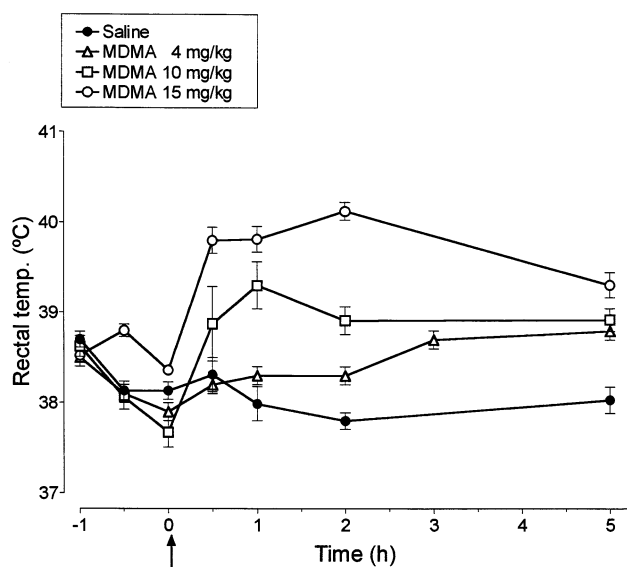


Fig. 1. Rectal temperature of rats injected with a single dose of MDMA (4, 10 and 15 mg/kg, i.p.) or saline. The results are shown as mean and the vertical lines indicate S.E.M., ($n=6-9$). MDMA produced a significant increase in temperature at doses of 4 ($F(1,13)=17.0$, $P<0.01$), 10 ($F(1,15)=46.35$, $P<0.001$) and 15 mg/kg ($F(1,15)=189.35$, $P<0.001$) vs. saline. The hyperthermic response induced by 15 mg/kg MDMA was significantly larger than that induced by 10 mg/kg MDMA ($F(1,14)=19.47$, $P<0.001$) and 4 mg/kg MDMA ($F(1,12)=117.45$, $P<0.001$). Similarly, 10 mg/kg MDMA vs. 4 mg/kg MDMA was significant ($F(1,12)=12.77$, $P<0.01$).

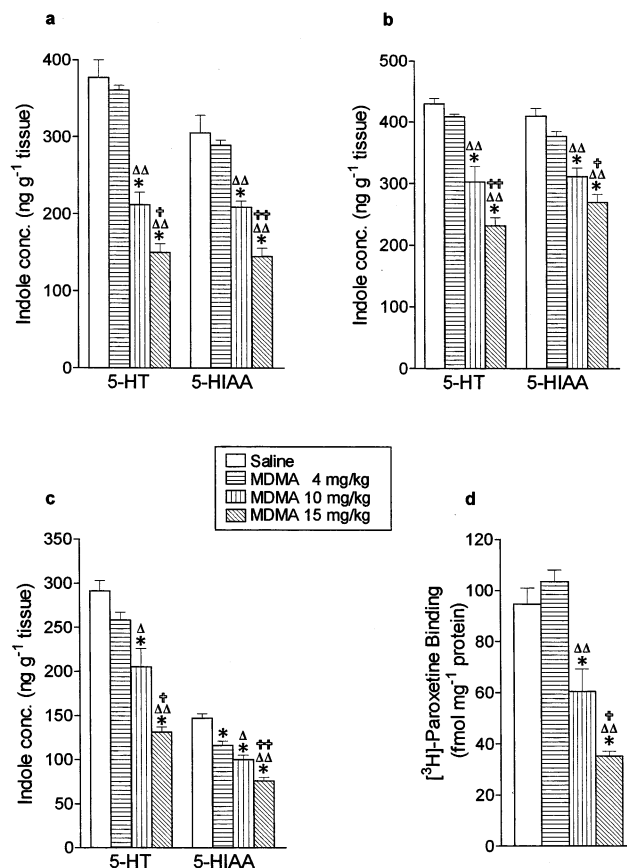


Fig. 2. Effect of a single administration of MDMA (4, 10 and 15 mg/kg) on the hippocampal (a), striatal (b), and cortical (c), indole concentration and on the cortical [3 H]paroxetine binding (d) 7 days after injection. The results are shown as mean $\pm$ S.E.M. ($n=6-9$). Different from saline: * $P<0.01$. Different from 4 mg/kg MDMA: $\Delta P<0.05$, $\Delta\Delta P<0.01$. Different from 10 mg/kg MDMA: $\dagger P<0.05$, $\dagger\dagger P<0.01$, (Newman–Keuls test).

for values obtained on: (a) day 1, (b) day 2 and (c) day 4).

3.4. Effect of a dose of 4 mg/kg MDMA given once or twice daily on 5-HT and 5-HIAA concentrations and [3 H]paroxetine binding in regions of the rat brain

A 4 mg/kg dose of MDMA given once daily for 4 days produced a small, and generally non-statistically significant effect in 5-HT, 5-HIAA and [3 H]paroxetine binding 7 days after the final dose (Table 1). However, when the 4 mg/kg dose of MDMA was given twice daily for 4 days substantial damage was detected (Table 1).

3.5. The effect of 4 mg/kg MDMA given twice weekly for 8 weeks on body weight

The administration of 4 mg/kg MDMA twice weekly for 8 weeks produced a slowing in the rate of weight gain, compared to control animals (Fig. 4).

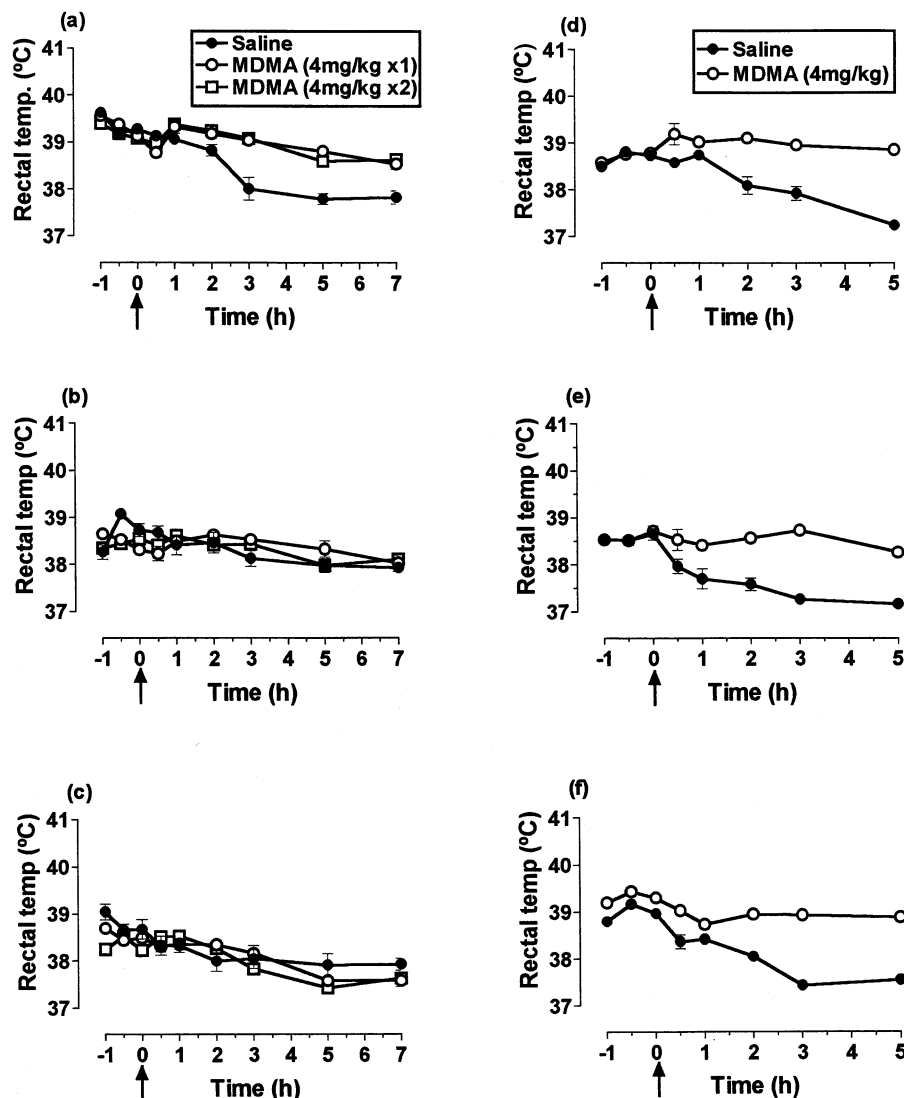


Fig. 3. Left hand panels. Rectal temperature of rats injected with 4 mg/kg MDMA once or twice daily (09:00 and 17:00) for 4 days. Results show changes in temperature in the 7 h following the first injection on: (a) day 1, (b) day 2 and (c) day 4. Results shown as mean and vertical lines indicate S.E.M., ($n = 6$). MDMA produced significant changes in rectal temperature only after the first injection ($F(1,10) = 27.71$, $P < 0.001$). Right hand panels. Rectal temperature of rats injected with 4 mg/kg MDMA twice weekly (monday and friday) for 8 weeks. The graphs show the response to MDMA following: (d) the 1st injection (week 1), (e) the 2nd injection (week 1) and (f) the 16th injection (week 8). The results are shown as mean and the vertical lines indicate S.E.M. ($n = 6$). There was an overall effect of treatment ($F(1,30) = 298.4$, $P < 0.001$), MDMA producing a significant increase in temperature. The interaction of week $\times$ group was not significant ($F(3,30) = 0.08$), indicating that the effect of MDMA did not differ for the 2 groups across weeks. There was a significant interaction week $\times$ time ($F(8,120) = 2.94$, $P < 0.01$), indicating that the temperature response differed across weeks. Finally, the interaction week $\times$ time $\times$ group was significant ($F(8,120) = 2.43$, $P < 0.05$), indicating that the effect produced by treatment on temperature differed slightly between weeks.

3.6. The effect of 4 mg/kg MDMA given twice weekly for 8 weeks on rectal temperature

Control (saline injected) rats consistently showed a slow decrease in rectal temperature in the 5 h following the injection every time they were tested over the 8 week study (Fig. 3d–f). In contrast, rats injected with 4 mg/kg MDMA remained stable, thus showing a hyperthermic response compared to the control group (Fig. 3 shows the data obtained following: (d) the 1st dose

(week 1), (e) the 2nd dose (week 1) and (f) the 16th dose (week 8)).

3.7. The effect of 4 mg/kg MDMA given twice weekly for 8 weeks on 5-HT and 5-HIAA concentrations and [3 H]paroxetine binding in regions of the rat brain

When 4 mg/kg MDMA was given twice weekly (monday and friday) over an 8 week period no loss of 5-HT, 5-HIAA or [3 H]paroxetine binding was observed 7 days after the last dose (Table 2).

Table 1

Effect of 4 mg/kg MDMA (i.p.) given once or twice daily (09:00 and 17:00) for 4 days on the indole concentrations in cortex, hippocampus and striatum and [^3H]paroxetine binding in the cortex 7 days after the last injection

	Saline	MDMA (4 mg/kg)	
		Once daily	Twice daily
Cortex			
5-HT	283 $\pm$ 2 (5)	269 $\pm$ 9 (6)	172 $\pm$ 6 (6) ^a
5-HIAA	186 $\pm$ 5 (6)	165 $\pm$ 4 (6)	111 $\pm$ 3 (6) ^a
[^3H]paroxetine	82 $\pm$ 5 (6)	69 $\pm$ 4 (5)	37 $\pm$ 4 (6) ^a
Hippocampus			
5-HT	323 $\pm$ 5 (6)	279 $\pm$ 9 (6)	185 $\pm$ 11 (5) ^a
5-HIAA	360 $\pm$ 7 (6)	314 $\pm$ 8 (6)	202 $\pm$ 13 (5) ^a
Striatum			
5-HT	466 $\pm$ 6 (6)	432 $\pm$ 7 (6)	365 $\pm$ 16 (6) ^a
5-HIAA	514 $\pm$ 16 (6)	474 $\pm$ 11 (6)	390 $\pm$ 14 (6) ^a

Results shown as mean $\pm$ S.E. mean, $n = 5-6$.

^a Different from saline $P < 0.01$. Indole concentrations reported in ng/g tissue and [^3H]paroxetine binding in fmol/mg protein.

4. Discussion

There is now substantial evidence implicating free radical formation as being responsible for the neurotoxic degeneration of 5-HT nerve terminals which occurs after MDMA administration. Briefly, this evidence includes increased formation of free radicals in the brain as indicated by an increased conversion of salicylic acid to 2,3-DHBA in microdialysis probes implanted in the hippocampus of rats injected with

Table 2

Effect of 4 mg/kg MDMA (i.p.) given twice weekly (monday and friday) for 8 weeks on indole concentrations in the cortex, hippocampus and striatum and [^3H]paroxetine binding in the cortex 7 days after the last injection

	Saline	MDMA
Cortex		
5-HT	340 $\pm$ 7	326 $\pm$ 13
5-HIAA	294 $\pm$ 19	264 $\pm$ 6
[^3H]paroxetine	64.7 $\pm$ 4.2	62.1 $\pm$ 4.3
Hippocampus		
5-HT	282 $\pm$ 10	286 $\pm$ 6
5-HIAA	489 $\pm$ 14	438 $\pm$ 8
Striatum		
5-HT	466 $\pm$ 15	421 $\pm$ 18
5-HIAA	706 $\pm$ 20	659 $\pm$ 23

Results shown as mean $\pm$ S.E. mean, $n = 6$. Indole concentrations expressed in ng/g tissue and [^3H]paroxetine binding in fmol/mg protein.

MDMA (Colado et al., 1997a); attenuation of MDMA-induced damage by administration of the free radical scavenger PBN (Colado and Green, 1995; Yeh, 1996; Colado et al., 1997a) together with evidence that PBN attenuates the rise in free radical formation using the microdialysis probe technique (Colado et al., 1997a) and evidence of increased lipid peroxidation in cerebral tissue after MDMA (Sprague and Nichols, 1995; Colado et al., 1997b). Finally it has also been reported that transgenic mice carrying the sequence of the human CuZn superoxide dismutase enzyme are resistant to MDMA-induced damage (Cadet et al., 1994).

If the proposal that MDMA-induced damage is caused by increased free radical production is correct then there is an obvious corollary, which is that MDMA will only produce damage when endogenous free radical scavenging systems are either exhausted or overwhelmed, which in turn suggests that the damage will be related to the dose or the frequency of drug ingestion. The current results support this view. Thus there is dose-related damage to 5-HT neurones following a single dose of MDMA. Furthermore, while a single dose of 4 mg/kg produced no overt damage when given as either a single dose, or even daily for 4 days, this dose produced marked damage when given twice daily for 4 days. However, when this dose was given only twice weekly even over a period of 2 months, damage was not detected, presumably because endogenous free radical scavenging mechanism recovered between each administration and did not become exhausted.

Both Broening et al. (1995) and Farfel and Seiden (1995) have demonstrated that neurodegeneration can occur after MDMA administration even when the drug does not produce a hyperthermic response. This sug-

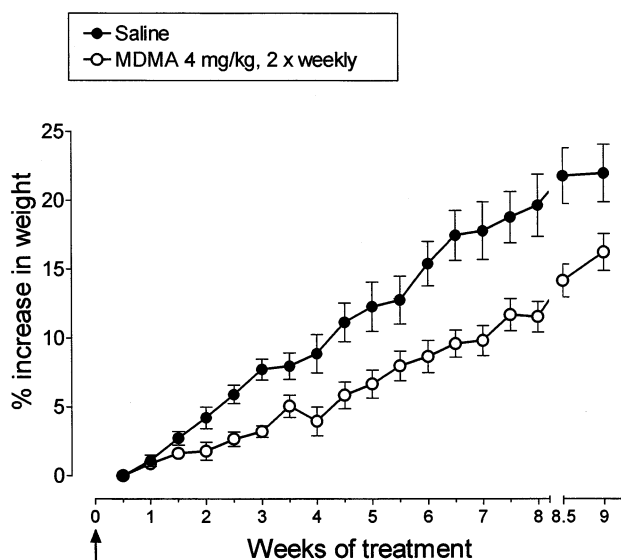


Fig. 4. Effect of 4 mg/kg MDMA or saline given twice weekly for 8 weeks on the weight gain of the rats. The results show the percentage increase in weight per day during the treatment period. Breaks show where the treatment stopped. Symbols show mean and vertical lines indicate S.E.M., ($n = 6$). MDMA (vs. saline) significantly reduced the weight gain ($F(1,10) = 10.35$, $P < 0.01$).

gests that the neurotoxicity and hyperthermia are separable and Broening et al. (1995) concluded that neurotoxicity was exacerbated but not induced by hyperthermia, a conclusion also reached by Colado et al. (1997c) following studies on certain neuroprotective compounds. This conclusion is also consistent with the idea of the damage being produced by free radicals because their formation in the brain is known to be enhanced by hyperthermia (Globus et al., 1995; Kil et al., 1996). The current data also demonstrate that hyperthermia and neurodegeneration are separable. For example, MDMA administration produced a hyperthermic response after every dose when the drug was given twice weekly but no neurodegeneration was detected. In contrast, twice daily MDMA had no effect on rectal temperature after the first day but nevertheless produced substantial neurotoxic damage. When a single dose of 4 mg/kg MDMA was given on day 1 no hyperthermic response following the second dose on day 2 (Fig. 3b) was seen. In contrast, when the second dose was given 4 days later on day 5 a hyperthermic response was observed (Fig. 3e). The attenuation of the temperature response when 4 mg/kg MDMA was given daily presumably reflects the depletion of 5-HT that is occurring during the treatment period (Colado and Green, 1994) since repeated doses of MDMA or lesioning of the 5-HT nerve endings have both previously been shown to abolish the MDMA-induced hyperthermic response (Colado et al., 1997a,b). When the drug was only given twice weekly it seems reasonable to assume that some restoration of the amine level occurred thereby resulting in a hyperthermic response again being detected after the MDMA injection.

What remains difficult is the extrapolation of these data to human recreational use and the danger therefrom. It does appear clear that the pharmacokinetics of the drug in rats and humans may be similar. For example a dose of 1.5 mg/kg MDMA to humans resulted in a peak plasma concentration of 331 ng/ml (Helmlin et al., 1996). Extrapolation from data obtained with a dose of 10 mg/kg to female DA rats (Colado et al., 1995) suggests that administration of a dose of 1.5 mg/kg would produce a very similar plasma concentration. Furthermore neurotoxic brain damage occurs in rats at plasma levels of MDMA that have been recorded in human recreational users (Colado et al., 1995). We previously suggested that neurotoxic damage occurred in the rat brain because MDMA was metabolised to certain catechol and quinone metabolites which produced free radicals on further metabolism (Colado and Green, 1995; Colado et al., 1995, 1997a). Since there is evidence that these metabolites are formed not only in the rat (Hiramatsu et al., 1990; Lim and Foltz, 1991; Lin et al., 1992; Kumagai et al., 1994; Chu et al., 1996), but probably also in the human (Tucker et al., 1994), there is little reason to

suppose that neurodegeneration will not occur in the brains of heavy users of this popular recreational drug.

The current study also suggests that even though a clear relationship between dose and acute toxicity was not apparent in an earlier clinical report (Henry et al., 1992), such a relationship probably exists. What complicates matters is that toxicity also depends on other factors. These include the endogenous free radical scavenger status of the brain of the subject (which is influenced by ingestion of other drugs which compromise free radical scavenging activity, for example methamphetamine (Giovanni et al., 1995; Cappon et al., 1996)) and whether the subject is an extensive or poor metaboliser of debrisoquine since the CYP2D6 enzyme is involved in the metabolism of MDMA to the free radical producing metabolites (Tucker et al., 1994; Colado et al., 1995; Chu et al., 1996). A further factor is the phenomenon of 'aggregation toxicity'. Many years ago Gunn and Gurd (1940) reported that aggregation (or grouping) of mice, as opposed to single housing resulted in the behavioural and toxic effects of amphetamine being markedly enhanced. This work was confirmed and extended by Chance (Chance, 1946, 1947) who demonstrated that it was the number housed together that was important, not the total floor area available for each animal. He also showed that elevated temperature, external sound and poor hydration worsened the toxicity. The relevance of these findings to the conditions existing at dance parties or 'raves' is striking and suggests that the social environment in which the user is present could be crucial to the toxic response to the drug.

However, even when all the complications reviewed above are taken into account, the fact remains that the current study indicates that high doses or frequent doses are probably the major determinants of damage. Furthermore the absence of an acute hyperthermic response appears to offer no guarantee that long term brain damage is being avoided.

Finally it should be realised from the foregoing discussion that there is no way that the current data can be interpreted in terms of what might or might not be 'safe' recreational doses of MDMA, other than to suggest that frequent doses or high doses must carry significantly greater risk of brain damage, evidence for which now appears to be accumulating (McCann et al., 1996).

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