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BEHAVIOURAL, HYPERTHERMIC AND NEUROTOXIC EFFECTS OF 3,4-METHYLENEDIOXYMETHAMPHETAMINE ANALOGUES IN THE WISTAR RAT

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Abstract

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1. The ability of N-ethyl (MDEA) and N-butyl (MDBA) analogues of 3,4-methylenedioxymethamphetamine (MDMA, 'Ecstasy') to induce acute behavioural changes and increases in body temperature, and to cause serotonergic neurotoxicity, was assessed in young adult male Wistar rats. The *in vitro* ability of MDMA analogues to evoke presynaptic monoamine release from crude rat forebrain synaptosomal preparations pre-labelled with [^{3}H]5-HT or [^{3}H]DA was also measured.
2. In behavioural experiments, acute MDMA and MDEA (20 mg/kg, i.p.) significantly increased rat open-field locomotion scores, decreased open-field rearing, and induced stereotypy, Straub tail and head weaving. MDBA did not produce any of these behaviours.
3. After repeated dosing (8 x 20 mg/kg, i.p., twice daily for 4 days), MDMA > MDEA >> MDBA ≥ saline at decreasing forebrain [^{3}H]paroxetine binding levels and concentrations of 5-HT and 5-HIAA at 14 days post-treatment. None of the analogues caused any long-term changes in dopamine or noradrenaline concentrations in the forebrain.
4. Acute MDMA and MDEA (20 mg/kg, i.p.) produced significant acute increases in rat aural temperature compared with saline-treated animals, while 20 mg/kg MDBA caused no significant effects.
5. MDA, MDMA and MDEA were equipotent at inducing [^{3}H]5-HT release from frontal cortex/hippocampal synaptosomes, while MDBA only evoked a significant release at 100 μM concentrations. The potency order for inducing [^{3}H]DA release from striatal synaptosomes was MDA > MDMA > MDEA = MDBA.
6. This study shows that large N-alkyl substitution decreases the ability of MDMA analogues to evoke presynaptic 5-HT and DA release, induce acute hyperthermia, hyperlocomotion and behavioural changes, and cause long-term serotonergic neurotoxicity.
7. The structure-activity relationship data presented here indicate that the neurotoxic damage caused by substituted amphetamines requires a combination of acute hyperthermia and increased

neurotransmitter release. Induction of one of these effects in isolation is not sufficient to cause serotonergic nerve terminal degradation.

Keywords: behaviour, dopamine, hyperthermia, [^{3}H]monoamine release, MDA, MDBA, MDEA, MDMA, neurotoxicity, [^{3}H]paroxetine, serotonin.

Abbreviations: dopamine (DA), high performance liquid chromatography (HPLC), 5-hydroxyindoleacetic acid (5-HIAA), monoamine oxidase (MAO), 3,4-methylenedioxy-amphetamine (MDA), 3,4-methylenedioxybutylamphetamine (MDBA), 3,4-methylene-dioxyethylamphetamine (MDEA), 3,4-methylenedioxymethamphetamine (MDMA), open-field (OF), serotonin (5-HT).

Introduction

3,4-methylenedioxymethamphetamine (MDMA or 'Ecstasy') is a ring-substituted amphetamine derivative, which is a popular recreationally used drug in Europe and North America (Cregg and Tracey, 1993; Cuomo et al., 1994; Green et al., 1995). MDMA and its N-desmethyl and N-ethyl analogues, MDA and MDEA, have been proposed as a novel class of drugs, labelled enactogens, which produce euphoria and empathy and are distinct from classical hallucinogens or psychomotor stimulants (Hermle et al., 1993; Nichols and Oberlender, 1990). However there is growing concern in the medical community about the long-term effects of repeated methylenedioxyamphetamines on human users, as MDMA has been shown to be neurotoxic in animals at doses similar to those used recreationally by humans (Colado et al., 1997; O'Shea et al., 1998).

MDMA and analogues induce acute behavioural changes in laboratory rats including locomotor hyperactivity, olfactory exploration, head-weaving and other components of the 'serotonin syndrome' (Green et al., 1995; Hegadoren et al., 1995). Moreover, these agents have been shown to induce serotonergic neurotoxicity when administered acutely or repeatedly to laboratory rats or primates. Serotonergic changes observed have included long-term reductions in forebrain tissue levels of serotonin (5-hydroxytryptamine, 5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA), as well as reductions in 5-HT uptake sites and tryptophan hydroxylase, the synthetic enzyme for 5-HT (Battaglia et al., 1987; Ricaurte et al., 1992; Schmidt et al., 1987; Schmidt and Taylor, 1987). These neurochemical changes have been correlated with histochemical and immunocytochemical evidence that MDA and MDMA selectively destroy the fine 5-HT axon terminals that originate in the dorsal raphe nucleus (Commins et al., 1987; Molliver, 1987; O'Hearn et al., 1988; Ricaurte et al., 1985).

The mechanisms underlying the neurotoxic effects of MDMA are not clear at present, but production of a hyperthermic response has been implicated as a necessary factor for MDMA-induced loss of serotonergic axons and nerve terminals (Huether et al., 1997; Malberg et al., 1996). MDMA produces an acute dose-dependent hyperthermic response in rats which is related to ambient temperature. At normal (20°C) and high ambient temperatures MDMA produces a hyperthermic response, but evokes a hypothermic response at lower ambient temperatures (Dafters, 1994; Gordon et al., 1991; Malberg and Seiden, 1998; Schmidt et al., 1990). The presence of this hyperthermia is thought to be critical for MDMA-induced neurotoxicity, and neurodegeneration can be attenuated if body temperature is kept low using low ambient temperatures or drugs that produce hypothermia in combination with MDMA (Hewitt and Green, 1994; Malberg et al., 1996;

Malberg and Seiden, 1998). However, there is also a mechanism of protection against MDMA-induced neurotoxicity that is independent of prevention of the hyperthermic response, as evidenced by drugs that block neurodegeneration without attenuating MDMA-induced hyperthermia (Malberg et al., 1996; Schmidt et al., 1990).

Dopamine (DA) has been shown to be clearly involved in MDMA-induced neurotoxicity, and there is a direct correlation between the acute release of DA and the long-term depletion of 5-HT (Nash and Nichols, 1991). MDMA directly releases DA via the DA transporter (Johnson et al., 1986; Johnson et al., 1991; Schmidt et al., 1987) and indirectly through 5-HT release-induced activation of 5-HT_{2A} receptors which stimulate acute DA synthesis and release (Nash, 1990). The neurodegeneration of 5-HT nerve terminals caused by MDMA can be blocked using anti-dopaminergic agents (α-methyl-*p*-tyrosine, haloperidol, reserpine, 8-OH DA) as well as anti-serotonergic agents (ketanserin, MDL 11,939, fluoxetine), or potentiated by pretreatment with the DA precursor L-DOPA (Colado et al., 1999a; Green et al., 1995; Schmidt et al., 1991a; Schmidt et al., 1991b). For these reasons, the ability of substituted amphetamines to cause a large acute release of both 5-HT and DA is considered to be intrinsic to their neurotoxic potential. However, the relative importance of hyperthermia, DA release and 5-HT release for induction of serotonergic nerve damage is not clear.

The aim of this study was to examine structure-activity relationships within a series of methylenedioxy analogues: MDMA, its N-ethyl analogue MDEA and its N-butyl analogue MDBA. MDEA is known to be less potent than either MDA or MDMA at decreasing neuronal markers of serotonergic function (Ricaurte et al., 1987; Stone et al., 1987). This is the first report on the actions of MDBA. Our expectation was that comparison of the acute and long-term effects of the analogues would permit relationships between induction of serotonergic neurotoxicity and occurrence of other events to be more closely established. Thus, we have compared for the relative potency of MDMA analogues at producing acute behavioural changes and long-term decreases in forebrain markers of serotonergic function in young adult male Wistar rats. Mechanisms underlying these actions, and their relative importance to MDMA-induced neurotoxicity, were investigated by measuring the ability of analogues to cause an acute hyperthermic response and *in vitro* release of 5-HT and DA.

Methods

Drug Synthesis and Purification

Ring-subsituted amphetamines were synthesised following the methods of Braun et al. (1980). Briefly, isosafrole was oxidised to 3,4-methylenedioxyphenylacetone, which was then reacted with ammonium acetate, methylamine, ethylamine or butylamine. This was followed by reduction with sodium cyanoborohydride to yield the corresponding MDA, MDMA, MDEA or MDBA derivative. The products were prepared as their (±)-HCl salts and their structures were confirmed by nuclear magnetic resonance spectroscopy, microanalysis and mass spectroscopy.

Animals and Drug administration

Young adult male Wistar rats (200-300g, Biomedical Facility, U.C.D.) were used. They were singly housed in conditions of constant temperature (20-22°C) and a 12 hour light/dark cycle with free access to food and water. Substituted amphetamines (HCl base) were dissolved in 0.9% NaCl (saline) and administered i.p. at a volume of 2 ml/kg. Rats given eight injections were dosed twice daily for four consecutive days. Rats were sacrificed by cervical dislocation at 14 days post-treatment and brains were removed on ice. Frontal cortical, striatal, amygdala and hippocampal regions were dissected and stored whole, in Tris buffer for [^{3}H]paroxetine binding experiments or without buffer for determination of biogenic amine concentrations, at -70°C.

Behavioural Measurements

The effects of the substituted amphetamine analogues on acute rat locomotor behaviour was measured in a square open field (OF) box (60 x 60 x 14 cm), in the morning of treatment days under conditions of red light. Rats were placed in the OF box for 5 min on the two days preceding amphetamine treatment and at +30 min post-first injection on each of the treatment days (0 – 3 inclusive). Rats were also tested one day and seven days after cessation of treatment. Treatments were blinded and rat ambulation and rearing behaviour over 5 min was measured by visual tracking, and changes in movement, activity and posture were observed. Presence of Straub tail, head weaving and stereotypy (non-directed movements and continuous turning) were given a score of 0 – 3, where 0 = absent, 1 = rare or trace, 2 = moderate, 3 = severe (Hegadoren et al., 1995; Pranzatelli and Pluchino, 1991).

Aural Temperature Measurements

Rat aural temperature measurements (average of 3 readings) were taken from –1 to +5 hr post-injection, using a Braun Thermoscan infrared aural thermometer with digital readout, with the probe being placed just inside the rat's ear canal and the rat being lightly restrained by hand.

[^{3}H]paroxetine Binding Studies

Rat brain regions were thawed and homogenised in 50 mM Tris HCl (pH 7.4 @ 25°C) buffer containing 120 mM NaCl and 5 mM KCl. The homogenate was washed by centrifuging at 80,000xg in a Beckman L7-55 Ultracentrifuge for 10 min at 4°C. This process was repeated twice with the final pellet resuspended in Tris buffer (10 mg tissue wet wt/ml buffer).

[^{3}H]paroxetine binding experiments in rat forebrain regions were performed using the methods of Habert et al. (1985). Briefly, 0.4 ml of membrane preparation was incubated with [^{3}H]paroxetine (NEN, S.A. = 17.10 Ci/mmol) in concentrations ranging from 0.03 to 2.5 nM, at 22°C for 120 min in a final volume of 2.0 ml (duplicate experiments). Specific binding was measured by performing the incubations in the presence and absence of 10 µM fluoxetine. At the end of the incubation, the reactions were terminated by rapid

filtration through Whatman GF/C filters, pre-soaked in 0.3% polyethyleneinine, using a Brandel cell harvester. The filters were washed three times with 4 ml ice-cold buffer, and then placed in 5 ml of Ecoscint and counted the following day. Membrane protein concentrations were determined by the method of Lowry et al. (1951), and [^{3}H]paroxetine binding was expressed in fmol/mg protein.

Determination of Brain Biogenic Amine Concentrations

Rat forebrain levels of biogenic amines were determined using the methods of McNamara et al. (1995). Concentrations of noradrenaline, DA, 5-HT and 5-HIAA were measured by high performance liquid chromatography (HPLC) with electrochemical detection (Seyfried et al. 1986) using an automated Shimadzu HPLC system. Rat forebrain regions were thawed and homogenised by sonication in 1.0 ml elution buffer (pH 2.8), containing 0.1 M citric acid, 0.1 M sodium dihydrogen phosphate, 1.4 mM octane-1-sulphonic acid and 0.1 mM EDTA. The elution buffer differed from the mobile phase in that it was "spiked" with 20 ng/50 µl *N*-methyl dopamine as an internal standard. Homogenates were centrifuged at 15,000xg in a Hettich Mikro/K refrigerated centrifuge for 15 min. A 20 µl sample of the supernatant was injected directly into a reverse phase column (RP18, 25 cm X 4 mm internal diameter, particle size 5 µm) for separation of indoles and catecholamines (flow rate 1 ml/min). The neurotransmitters were quantified using a Merck-Hitachi D-2000 integrator, and expressed as ng/g tissue wet wt.

[^{3}H]monoamine Release Studies

The ability of substituted amphetamines to evoke presynaptic monomamine neurotransmitter release was assessed *in vitro* using a crude synaptosomal preparation of rat forebrain tissue (Clements, 1997). Rat frontal cortex and hippocampal tissues were pooled for [^{3}H]5-HT release studies and striatal tissue was used for [^{3}H]DA release studies. Rat brain tissue was homogenised in Krebs-Hepes buffer (pH 7.4 @ 25°C, containing 130 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 20 mM Hepes, 1.2 mM Na_2HPO_4, 10 µM pargyline, 10 mM glucose) containing 0.32 M sucrose, and centrifuged at 1000xg for 5 min at 4°C. The supernatant was then diluted 1:1 with Krebs-Hepes buffer and centrifuged at 15,000xg for 15 min at 4°C. The pellet was resuspended in oxygenated Krebs-Hepes buffer and prelabelled for 30 min with 50 nM [^{3}H]5-HT (Amersham, S.A. = 14.3 Ci/mmol) or 10 nM [7,8-^{3}H]DA (Amersham, S.A. = 47.0 Ci/mmol) at 37°C in a shaking water bath. After this time, the crude synaptosomal preparation was washed 3 times by centrifugation at 1500xg for 3 min and resuspension of in 25 volumes of fresh Krebs-Hepes buffer. The final pellet was resuspended in 20 volumes (frontal cortex/hippocampus) or 150 volumes (striatum) Krebs-Hepes.

0.5 ml of crude synaptosomes was added to 1.5 ml plastic eppendorf tubes containing oxygenated Krebs-Hepes buffer and drugs in a final assay volume of 1.0 ml (triplicate experiments). The tubes were capped and vortexed, and then incubated for 30 min in a 37°C water bath. Incubations were terminated by centrifugation at 110xg for 10 min at 4°C, then 0.5 ml of supernatant was removed and added to scintillation vials containing 10 ml Ecoscint A. 0.5 ml 2% Triton-X was added to the eppendorf tubes and the pelleted

synaptosomes were resuspended by vortexing the tubes. The tubes were left for 60 min to allow lysis of synaptosomes, vortexed, and then 0.5 ml was removed to scintillation vials. The release of [^{3}H]monoamines from crude rat brain synaptosomes was calculated as (radioactivity in supernatant / total radioactivity in supernatant and pellet) x 100%. The effect of drugs on [^{3}H]monoamine release was expressed as % Basal Release.

Data Analysis

Data are presented as mean ± S.E.M. of n independent experiments. Binding isotherms and dose-response curves were fitted using the non-linear curve fitting software Graphpad Prism. Statistical significance of differences between treatment groups was determined by ANOVA, or repeated measures ANOVA, followed by a Dunnett's or Bonferroni's post-ANOVA test, as indicated in the text.

Results

Behavioural Effects of MDMA Analogues

Male Wistar rats were randomly assigned to 4 treatment groups, and were acclimatised to the open-field apparatus for two days prior to commencement of treatment (days -2, -1). The effects of repeated doses of MDMA, MDEA and MDBA (8 x 20 mg/kg, i.p., twice daily for four days) on rat locomotion were quantified at +30 min post–first injection (Fig. 1A).

MDMA caused a pronounced increase in locomotor counts over the 4 treatment days ($p < 0.001$ Vs. saline treated animals, repeated measures ANOVA, followed by Bonferroni's test). MDEA caused a smaller ($p < 0.01$) but also significant increase in OF locomotion for the duration of the treatment. MDBA did not alter animal locomotor scores on any of the treatment days.

Rats were weighed each morning during this treatment period. Repeated administration of MDMA significantly reduced rat body weight ($p < 0.001$, repeated measures ANOVA, followed by Bonferroni's test) compared with saline-, MDEA- and MDBA-treated animals. There were no significant differences between the other treatment groups (Fig. 1B).

The acute effects of the MDMA analogues (20 mg/kg, i.p.) on rat behaviour was examined in more detail by scoring individual elements in the rats behavioural repertoire at +30 min post-first injection. Both MDMA and MDEA significantly decreased ($p < 0.05$) the amount of rearing in the open field, whilst MDBA had no effect (Fig. 2A). MDMA and MDEA both caused significant occurrence of stereotypy, Straub tail and head weaving ($p < 0.001$). MDBA failed to evoke these behaviours (Fig. 2B).

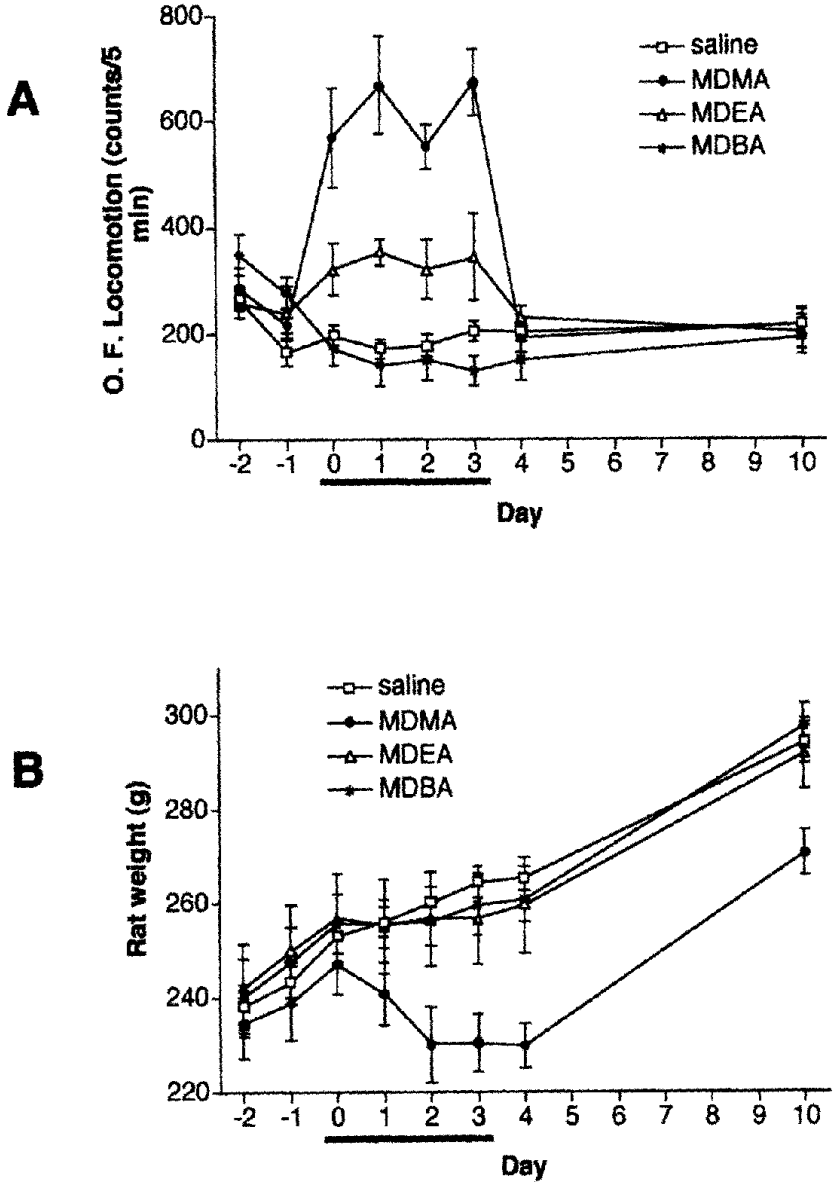


Fig. 1: Effect of repeated administration of MDMA analogues (8 x 20 mg/kg, i.p., twice daily for four days) on rat open field locomotion (A) at +30 min post-first injection on each of the treatment days (days 0 - 3, indicated by black bar), and on rat body weight (B). Data are means ± S.E.M. of 6-7 independent experiments. MDMA ($p<0.001$) and MDEA ($p<0.01$) significantly increased rat locomotion. MDMA also decreased rat body weight ($p<0.001$). (Repeated measures ANOVA followed by Bonferroni's test.)

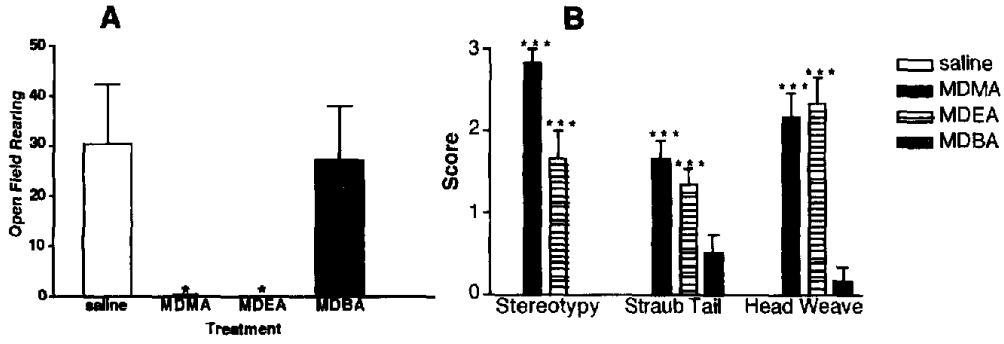


Fig. 2: Acute effect of MDMA analogues (20 mg/kg, i.p.) on rat open field rearing (A) and behaviours (B) at +30 min post-injection. Data are means ± S.E.M. of 6-7 independent experiments. * $p < 0.05$, *** $p < 0.001$ Vs. saline-treated animals, ANOVA followed by Bonferroni's test.

Neurochemical Effects of MDMA Analogues

Preliminary studies established that [^{3}H]paroxetine bound to a single site in membranes prepared from rat cortex, striatum, hippocampus and amygdala, with a K_D value of 0.3 – 0.8 nM. Specific binding was displaced by nM concentrations of the selective-serotonin-reuptake-inhibitors citalopram and fluoxetine, indicating that [^{3}H]paroxetine was binding to the serotonin transporter. The effects of repeated administration of MDMA analogues (8 x 20 mg/kg, i.p.) on [^{3}H]paroxetine binding levels at 14 days post-treatment are shown in Fig. 3A. MDMA caused severe reductions (≈ 70-80%) in [^{3}H]paroxetine binding in all brain regions examined. MDEA only caused significant decreases in the frontal cortex and the striatum, but not in the hippocampus or amygdala. MDBA failed to cause a decrease in binding levels in any of the forebrain regions examined.

The long-term effects of repeated administration of MDMA, MDEA and MDBA on concentrations of 5-HT and its major metabolite, 5-HIAA, were also investigated. 5-HT concentrations were significantly decreased by repeated MDMA treatment in all of the forebrain regions studied. MDEA only decreased 5-HT levels in the frontal cortex, and MDBA did not have a long-term effect on 5-HT levels in the forebrain (Fig. 3B). 5-HIAA concentrations were significantly decreased by repeated MDMA treatment in all of the forebrain regions examined. MDEA and MDBA significantly decreased long-term 5-HIAA levels in the frontal cortex, but not in any other brain region (Fig. 3C). None of the analogues caused any long-term changes in DA or noradrenaline concentrations in the rat forebrain (data not shown).

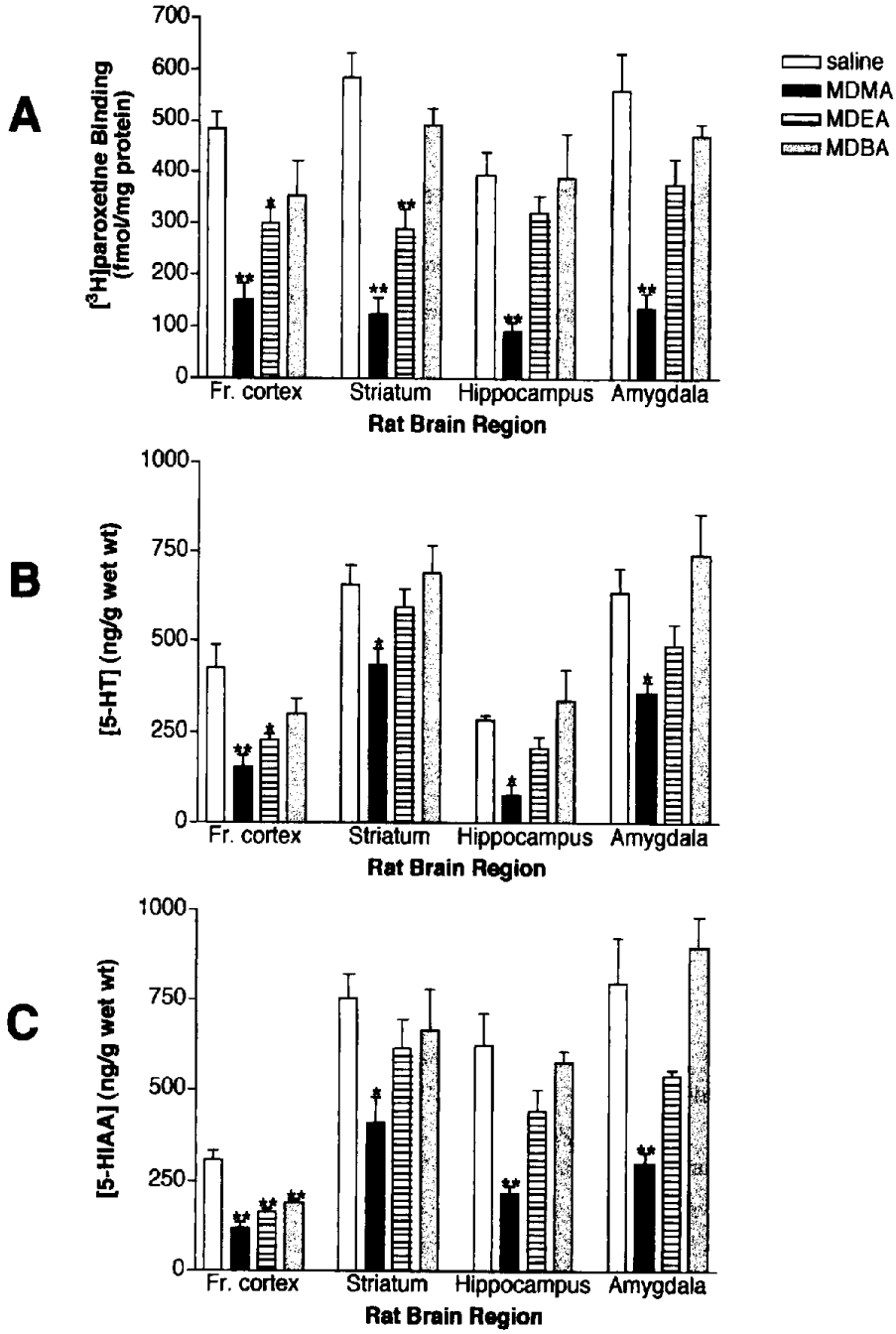


Fig. 3: The effect of repeated dose of MDMA analogues (8 x 20 mg/kg, i.p., twice daily for four days) on [^{3}H]paroxetine binding levels (A), and 5-HT (B) and 5-HIAA (C) concentrations in rat forebrain regions at 14 days post-treatment. Data are means ± S.E.M. of 6-9 independent experiments. * $p < 0.05$, ** $p < 0.01$ Vs. corresponding saline group, ANOVA followed by Dunnett's test.

Acute Effects of MDMA Analogues on Rat Aural Temperature

MDMA (5 – 20 mg/kg, i.p.) caused a dose-dependent increase in rat aural temperature (data not shown). The acute effects of 20 mg/kg MDMA, MDEA and MDBA on rat aural temperature are shown in Fig. 4. MDMA ($p < 0.001$) and MDEA ($p < 0.01$, repeated measures ANOVA, followed by Bonferroni's test) caused a significant increase in rat aural temperature compared with saline-treated animals, while MDBA caused no significant effects. There was no significant difference between MDMA- and MDEA-induced hyperthermia. (Note: results were identical when the data were analysed as area under the curve rather than by repeated measures ANOVA). 10 mg/kg MDA caused a significantly greater ($p < 0.05$) increase in aural temperature than did 10 mg/kg MDMA (data not shown).

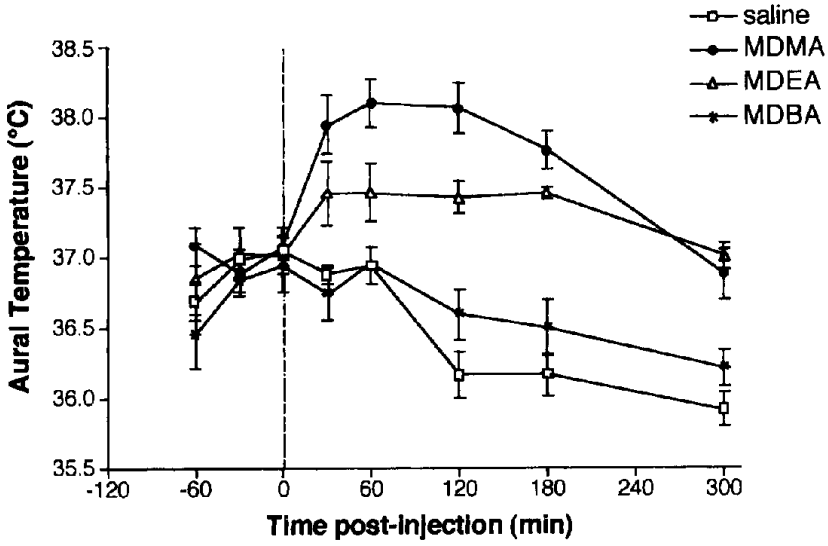


Fig. 4: Acute effect of MDMA analogues (20 mg/kg, i.p.) on rat aural temperature. Points are means ± S.E.M. of 6-7 independent experiments. See text for results of statistical comparison.

Effects of MDMA Analogues on [^{3}H]monoamine Release

Pooled rat frontal cortex/hippocampal synaptosomes were pre-labelled with [^{3}H]5-HT, and striatal synaptosomes were pre-labelled with [^{3}H]DA. Basal release of [^{3}H]5-HT from synaptosomes was 44.1 ± 2.0 % of total radioactivity in the samples. The [^{3}H]5-HT release evoked by the direct application of MDMA analogues from crude frontal cortex/hippocampal synaptosomes was calcium-independent and temperature-dependent (data not shown) as reported by others (McKenna et al., 1991). Maximum responses were achieved by 10^{-5} M concentrations of MDMA analogue; thereafter responses declined. Hence, responses generated by 10^{-4} M concentrations were not included in the analysis. For comparative purposes, MDA was

included in this study. MDA, MDMA and MDEA were equipotent (EC_{50} = 0.3-0.4 μM) at inducing [^{3}H]5-HT release (Fig. 5A), and had similar maximum responses (≈ 150 – 170% basal release). MDBA only evoked a significant increase in [^{3}H]5-HT release at 100 μM concentrations.

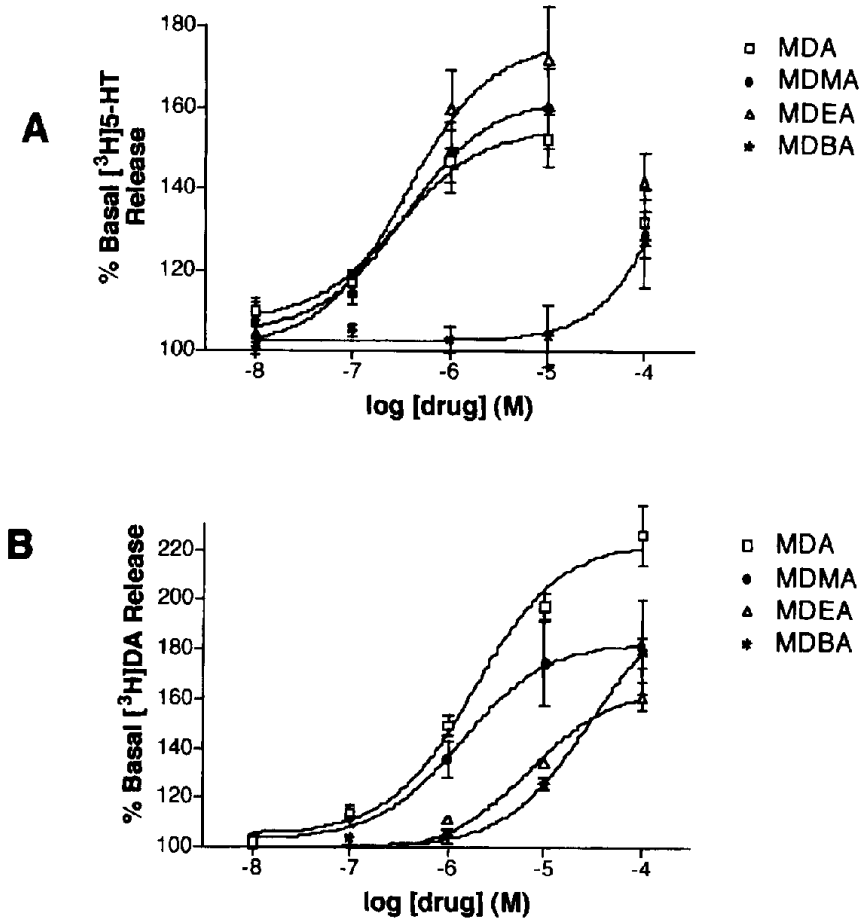


Fig. 5: The in vitro monoamine releasing effects of MDMA and analogues from rat brain crude synaptosomes. [^{3}H]5-HT release experiments (A) were performed in pooled frontal cortex/hippocampal synaptosomes, and [^{3}H]DA release (B) was assessed in striatal synaptosomes. Points are means ± S.E.M. of 4-7 independent experiments.

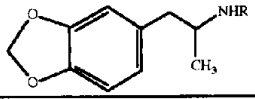
Basal release of [^{3}H]DA from rat striatal synaptosomes was 37.0 ± 1.3 % of total radioactivity in the samples. [^{3}H]DA release was temperature-dependent, and calcium-independent release accounted for 85% of MDMA analogue-induced release (data not shown). MDA and MDMA had similar potencies in evoking [^{3}H]DA release (≈ 1.6 μM), but the E_{max} of MDA-induced release (223.4 ± 10.7%) was higher than that of MDMA (183.4 ± 19.5%). MDEA and MDBA were less potent (6.91 and 29.12 μM, respectively) at

inducing [^{3}H]DA release and their E_{max} values were similar (≈ 165%) but less than that produced by MDMA (Fig. 5B).

A summary of all these results is presented in Table 1.

Table 1

Comparative Effects of Ring-Substituted Amphetamine Analogues

Methylenedioxy pharmacophore 	**MDMA** R = CH_3	**MDEA** R = C_2H_5	**MDBA** R = C_4H_9
Acute effects			
Hyperlocomotion	**	*	-
↓ Rearing	**	**	-
Behavioural changes	**	**	-
Hyperthermia	**	*	-
Weight loss	**	-	-
In vitro effects			
5-HT release	***	***	-
DA release	**	*	*
Long-term Neurotoxicity			
↓ [^{3}H]paroxetine binding	***	**	-
↓ 5-HT	***	**	-
↓ 5-HIAA	***	**	*

Discussion

The aim of the present study was to assess the relative abilities of the ring-substituted amphetamine derivatives MDMA, MDEA and MDBA to produce acute behavioural changes and long-term neurodegeneration of serotonergic nerve terminals. The relationship between the the length of N-alkyl substitution on the methylenedioxyamphetamine molecule and induction of these behavioural and neurochemical effects was also examined, by measuring the relative potency of the analogues at inducing hyperthermia and *in vitro* release of 5-HT and DA.

Acute Effects on Rat Behaviour and Body Temperature

MDMA and MDEA (20 mg/kg, i.p.) both produced hyperlocomotion, decreased rearing behaviour and behavioural changes associated with the 'serotonin syndrome', including head weaving and stereotypy, in rats in the OF environment at +30 min post-injection. MDMA and MDEA both also induced significant hyperthermia in the animals, but as with the increase in locomotor scores, MDMA caused a greater response than did MDEA. These results are largely in agreement with the results of others which indicate that MDMA is more potent than MDEA at inducing behavioural and locomotor changes (Gold et al., 1988; Paulus and Geyer, 1992). The largest N-alkyl derivative of MDMA, MDBA, which to our knowledge has not been examined before, failed to evoke acute behavioural changes or a significant increase in rat body temperature compared with saline-treated animals.

Long-term Effects on Brain 5-HT Function

The effects of repeated administration of MDMA analogues (8 x 20 mg/kg, i.p., twice daily for 4 days) on markers of serotonergic nerve terminal integrity in the rat forebrain was examined at 14 days post-treatment. This dose regimen is well documented at producing large reductions in forebrain markers of 5-HT nerve terminal integrity (Battaglia et al., 1987; O'Hearn et al., 1988; Ricaurte et al., 1987), and was used to achieve substantial decreases in 5-HT, 5-HIAA and [^{3}H]paroxetine binding levels (~75%) so that the relative toxicity of the less toxic analogues could be assessed with comparison to MDMA. MDMA was more potent than MDEA at reducing forebrain concentrations of 5-HT and 5-HIAA and [^{3}H]paroxetine binding levels. MDEA displayed a regionally selective neurotoxic effect as it only reduced these markers in the frontal cortex and striatum, but did not affect levels in the hippocampus or amygdala. This regionally selective toxicity of MDEA is in contrast with other studies which have demonstrated MDEA-induced neurotoxic loss of 5-HT in the hippocampus but not in the striatum (Colado et al., 1999c; Ricaurte et al., 1987) or the cortex (Stone et al., 1987). MDBA failed to produce significant reductions in [^{3}H]paroxetine binding levels or 5-HT concentrations in any of the regions studied, and only decreased 5-HIAA in the frontal cortex. As with the acute effects of the analogues, increasing the N-alkyl substitution decreases the potency of the compound at producing serotonergic neurotoxicity after repeated administration.

In Vitro [^{3}H]monoamine Releasing Effects

In vitro [^{3}H]monoamine release studies showed that MDA, MDMA and MDEA were equipotent at evoking [^{3}H]5-HT release from rat frontal cortex/hippocampal synaptosomes, but MDBA only induced a significant release of 5-HT at 100 μM concentrations. MDA was more potent than MDMA which was more potent than MDEA at inducing [^{3}H]DA release from striatal synaptosomes. MDA, MDMA, and MDEA have similar efficacies in the micromolar concentration range in releasing [^{3}H]5-HT from striatal slices, but MDA is more potent than MDMA which is more potent than MDEA at causing DA release *in vitro* and *in vivo* (Johnson et al., 1986; Schmidt, 1987; Nash and Nichols, 1991). This potency order at induction of DA release is also the rank order of potency of these analogues at producing long-term serotonergic deficits. MDBA had no effect on 5-HT release but was equipotent to MDEA at inducing DA release.

Neurotoxicity of MDMA Analogues

Several theories have been proposed to explain the toxicity of MDMA. Recent evidence suggests that MDMA produces a DA-dependent increase in hydroxy radicals in the forebrain (Colado et al., 1997; Shankaran et al., 1999) which may contribute to the mechanism of toxicity. Sprague et al. (1998) have proposed an integrated hypothesis which accounts for the accumulation of DA in 5-HT nerve terminals and its subsequent deamination by monoamine oxidase B (MAO-B), resulting in free radical products. 5-HT, GABA and DA have all been implicated in this process. The role of core body temperature in the process is unclear as many, but not all, agents that protect against MDMA-induced neurotoxicity also attenuate the acute hyperthermic effects of MDMA (Hewitt and Green, 1994; Malberg et al, 1996). Lower body temperature has long been known to protect against many types of brain damage by slowing chemical reactions that could lead to neurotoxic events. Blockade of MDMA-induced hyperthermia or induction of hypothermia may afford protection through decreasing the rate of production of oxidising species (Colado et al., 1999b)

Relationship Between Analogue Structure and Acute and Long-term Effects

The ability of the methylenedioxyamphetamine derivatives to cause acute behavioural changes and long-term decreases in forebrain 5-HT nerve terminals seems to be dependent on the induction of an acute release of both 5-HT and DA. MDBA, which produces the same level of *in vitro* DA release as MDEA, but does not produce 5-HT release, failed to induce noticeable behavioural changes or hyperthermia, and had only a minimal effect on measures of serotonergic nerve terminal integrity. The present data support the view that the activation of post-synaptic 5-HT receptors is a prerequisite for MDMA-induced behavioural changes and hyperthermia (Schmidt et al., 1990; Green et al., 1995) since MDBA did not alter behaviour or induce hyperthermia. MDEA, which is equipotent to MDA and MDMA at evoking 5-HT release but less potent at inducing DA release, is also less neurotoxic than MDMA emphasising the role of DA release in the neurodegeneration of 5-HT nerve terminals by substituted amphetamines. The ability to induce an hyperthermic response also seems to be involved in the neurotoxic potential of the derivatives, as there is a direct correlation between the magnitude of the hyperthermic response and the severity of the long-term serotonergic neurotoxicity.

Our study has shown that the acute release of 5-HT and DA coupled with a hyperthermic response are necessary for long-term serotonergic neurotoxicity. The structure-activity relationships for MDMA and its N-ethyl and N-butyl derivatives suggest that bulky N-alkyl groups reduce monoamine transporter-dependent release of 5-HT and to a lesser extent DA, resulting in less apparent acute effects and decreased ability to cause neurodegeneration of 5-HT nerve terminals.

Conclusion

Methylenedioxy analogues of amphetamine produce acute behavioural changes and hyperthermia through an interaction with monoamine transporters to release 5-HT and DA. Repeated administration of MDMA causes long-term neurodegeneration of rat forebrain 5-HT axons and nerve terminals. Increasing the size of the N-alkyl substitution on the amphetamine derivative decreases the ability of the compound to cause *in vitro* monoamine release, and to produce acute behavioural changes, hyperthermia and long-term serotonergic neurotoxicity in the Wistar rat.

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