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The acute and long-term neurotoxic effects of MDMA on marble burying behaviour in mice

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Abstract

When mice are exposed to harmless objects such as marbles in their cage they bury them, a behaviour sometimes known as defensive burying. We investigated the effect of an acute dose of MDMA ('ecstasy') and other psychoactive drugs on marble burying and also examined the effect of a prior neurotoxic dose of MDMA or p-chloroamphetamine (PCA) on burying. Acute administration of MDMA produced dose-dependent inhibition of marble burying (EC_{50}: 7.6 μmol/kg). Other drugs that enhance monoamine function also produced dose-dependent inhibition: methamphetamine > PCA > paroxetine > MDMA > GBR 12909 > methylphenidate. None of these drugs altered locomotor activity at a dose that inhibited burying. A prior neurotoxic dose of MDMA, which decreased striatal dopamine content by 60%, but left striatal 5-HT content unaltered, did not alter spontaneous marble burying 18 or 40 days later. However, a neurotoxic dose of PCA which decreased striatal dopamine by 60% and striatal 5-HT by 70% attenuated marble burying 28 days later. Overall, these data suggest that MDMA, primarily by acutely increasing 5-HT function, acts like several anxiolytic drugs in this behavioural model. Long-term loss of cerebral 5-HT content also produced a similar effect. Since this change was observed only after 28 days, it is probably due to an adaptive response in the brain.

Keywords

5-HT, amphetamines, anxiety, defensive burying, dopamine, ecstasy, marbles, MDMA, p-chloroamphetamine

Introduction

Administration of 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) to rodents results in an acute release of both 5-hydroxytryptamine (5-HT) and dopamine from nerve endings in the forebrain (Nash *et al.*, 1988; Gordon *et al.*, 1991; Colado *et al.*, 1999; O'Shea *et al.*, 2001). High or repeated doses of this popular recreational drug also result in long-term selective neurotoxic damage to 5-HT nerve endings in rat brain and, in contrast, selective loss of dopamine nerve endings in mouse brain (see Green *et al.*, 2003).

Studies on the effect of an acute dose of MDMA on anxiety-like behaviour have produced conflicting data in both rats and mice. Morley and McGregor (2000) reported an anxiogenic-like effect of MDMA in rats using the plus maze test, but an anxiolytic-like effect using the social interaction test, while Bhattacharya *et al.* (1998) observed an anxiogenic effect of the drug in both tests. In mice, MDMA had an anxiogenic effect at low dose and an anxiolytic effect at high dose when animals were tested on the plus maze (Lin *et al.*, 1999). An anxiogenic effect of the drug was also reported to occur in mice examined in the social interaction test (Maldonado and Navarro, 2001).

Experiments on the effect of a prior MDMA-induced neurotoxic lesion on the response of rats in various behavioural tests of anxiety-like behaviour have also produced results that are conflicting. Morley *et al.* (2001), Gurtman *et al.* (2002) and Fone *et al.* (2002) all reported that the lesion resulted in a long-term anxiogenic-like effect while Mechan *et al.* (2002a) observed an anxiolytic-like effect. Re-evaluation of the data suggested that the results obtained might have been dependent on the rat strain used and the basal (control) level of anxiety-like behaviour in the plus maze (Green and McGregor, 2002). Dark Agouti rats injected with saline spent little time on the open arm and this time was increased following the MDMA-induced lesion (Mechan *et al.*, 2002a). In contrast, both Sprague Dawley (Mechan *et al.*, 2002b) and Wistar rats (Morley *et al.*, 2001) injected with saline spent a considerably

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greater period of time on the open arms and a MDMA-induced lesion decreased this time (Morley *et al.*, 2001). Another possibility for the contradictory results is that the plus maze was evaluating, at least in part, impulsivity or risk-taking behaviour and this was altered by MDMA-induced loss of cerebral 5-HT (Mechan *et al.*, 2002a). No results appear to have been published on the long-term consequences of a MDMA-induced neurotoxic lesion on anxiety-like behaviour in mice.

Overall, therefore, the majority of studies in both rats and mice appear to suggest that an acute dose of MDMA produces an anxiogenic effect, and also suggest that a similar effect is seen in rats when a neurotoxic dose of the drug has been given some weeks prior to testing.

What is perhaps confusing about these data is that they do not fully accord with clinical experience on the acute effects of MDMA in recreational users. Nichols (1986) classed MDMA as an entactogen, producing feelings of 'closeness' while Davison and Parrott (1997) reported that human subjects generally experience elation, increased energy, happiness, calmness and relaxation having ingested the drug. Similarly, Farré *et al.* (2004) also cited stimulation, euphoria, liking and well-being as reported feelings of subjects ingesting the drug in laboratory conditions. Harris *et al.* (2002) also found some evidence for euphoria. 'Low mood' can, however, occur during the subsequent withdrawal period (Curran and Travill, 1997; Parrott and Lasky, 1998). In contrast, long-term effects in chronic users can include increased anxiety (Gamma *et al.*, 2000; Parrott *et al.*, 2000; de Win *et al.*, 2004; McCardle *et al.*, 2004; Roiser and Sahakian, 2004), although separating the effect of long-term use of MDMA from the concomitant use by the subjects of other psychoactive drugs has proved impossible (Parrott *et al.*, 2001) and some investigators have found evidence for increased impulsivity rather than anxiety (Morgan, 1998).

In the current study we have used another behavioural model to examine the acute effects of MDMA in rodents, namely marble burying behaviour in mice. Basically, when mice are presented with harmless objects such as marbles in their cage, they bury them in the bedding (see review of De Boer and Koolhaas, 2003). While it has been argued that this behaviour does not reflect an anxiety-like response it is nevertheless modified by several anxiolytic drugs and it has, therefore, been proposed to be a useful model to examine the putative anxiolytic action of drugs (Broekkamp *et al.*, 1986; Njung'e and Handley 1991a, 1991b). However, other evidence does indicate significant shortcomings in the model for predicting anxiolytic activity (De Boer and Koolhaas, 2003) and some other investigators have proposed that a drug-induced attenuation of the burying response might indicate that the compound has anti-impulsive/compulsive activity (Martin *et al.*, 1998; Millan *et al.*, 2001, 2002). Again, this proposal also lacks robust validation.

We have also examined the effect of methamphetamine, p–chloroamphetamine, methylphenidate, paroxetine and GBR 12909 on marble burying behaviour since all these compounds are known to acutely alter monoamine function in the brain.

Methods and materials

Animals and drug administration

Male NIH-Swiss mice (Harlan Olac, Bicester, Oxfordshire, UK) weighing 25–30 g at the start of experiments were used in all experiments. They were housed in groups of eight at an ambient temperature of 20 ± 2 °C and a 12 h light/dark cycle (lights on: 0730 h). Both food and water were freely provided.

All procedures were carried out following approval by the De Montfort University Experimental Ethics Committee and in accordance with United Kingdom Home Office regulations which ensure humane and proper care of research animals.

(±)-MDMA was obtained from Ultrafine Chemicals Ltd, Manchester, UK and dissolved in 0.9 % w/v saline and injected intraperitoneally (i.p.). The dose of MDMA administered to produce neurotoxic damage was 25 mg/kg i.p., administered three times at 3 h intervals, a dosing regime previously demonstrated to induce neurotoxic damage (O'Shea *et al.*, 2001). Acute doses of MDMA were as detailed in the results. Other drugs used in the study (supplier in brackets) were: paroxetine (GlaxoSmithKline, Harlow, UK); methylphenidate HCl, p-chloroamphetamine HCl and methamphetamine HCl (all from Sigma, Poole, UK); GBR 12909.2HCl (Tocris, Avonmouth, UK). Doses of all compounds are reported in terms of the salt, but all comparisons on potency use molar concentrations. All compounds were dissolved in saline and injected i.p. as described in results.

Marble burying behaviour

The methodology used was essentially as described by Njung'e and Handley (1991a, 1991b). Mice were placed individually in a novel polypropylene cage ($42 \times 25 \times 12$ cm) containing 14 glass marbles evenly spaced about 10 cm from the walls and resting on small wood-chip bedding 10 cm deep. The number of marbles at least two-thirds buried was counted 30 min later and results are expressed as the % of total marbles buried or the % change in the response following drug administration compared to that seen in the control (saline-injected) group.

Locomotor activity

Mice were placed individually in a novel polypropylene cage ($42 \times 25 \times 12$ cm) which was divided equally on the base by one longitudinal line and two cross lines. Thirty min after drug administration the total number of line crossings was counted during a 30 min period. This was done by videotaping the response for later counting. This also enabled the experimenter to be blind to treatment during the measurement. Animals used in this study were drug naive and had not been examined in any of the marble burying studies.

Effect of a neurotoxic dose regime of MDMA or PCA on striatal monoamine concentration: dose protocol and measurement of monoamines

Prior to the study on the neurotoxic effect of MDMA and PCA a study was performed to examine the effect of each of these two compounds on the concentration of dopamine and 5-HT in the striatum.

MDMA (25 mg/kg) was given to mice at 3-hourly intervals for a total of three doses. Two doses of PCA (15 mg/kg) were given 6 h apart. To prevent excessive hyperthermia and possible mortality the PCA-treated mice were kept in pairs for the first 24 h post treatment, then grouped eight to a cage as normal. Seven days following treatment with either of the neurotoxic drugs animals were killed by cervical dislocation and decapitation, the brains rapidly removed and the striatum dissected out on ice. Tissue was homogenized and 5-HT and dopamine were measured by high performance liquid chromatography (HPLC) with electrochemical detection. The method used was based on that published in detail by Colado *et al.* (1997). Briefly, the mobile phase consisted of KH_2PO_4 (1.0 M), octanesulfonic acid (0.25 mM), EDTA (0.1 mM) and methanol (10%), and was adjusted to pH 3 with phosphoric acid, filtered and degassed. The flow rate was 1.2 ml/min and the working electrode potential was set at +0.8 V.

The HPLC system consisted of a pump (Pharmacia LKB 2150) linked to a stainless steel reversed-phase column (C18 Phenomenex Lichrosher Select B, 5 μm, 150 × 4.6 mm) with a precolumn and a BAS LC-4C amperometric detector (Bioanalytical Systems Inc, Congleton, Cheshire, UK). The current produced was monitored by using integration software (SP4400 Chromjet with Nelson Chromatography Software).

Statistics

All marble burying data are presented as the number of the marbles buried as a percentage of the number buried by a control (saline-injected) group. Throughout the study the control group consistently buried seven to ten of the 14 marbles presented. Data on the acute effect of drug administration were analysed by one-way analysis of variance (ANOVA) and *post hoc* analysis performed with Dunnett's test, examining the effect of each dose administered. Comparison of marble burying behaviour at various times following a neurotoxic dose of MDMA or PCA was performed with a two-way ANOVA with day-of-test × treatment as factors followed by a *post hoc* test where appropriate. ED_{50} values were calculated by non-linear regression (Graph Pad Prism).

The effect of a neurotoxic dose regime of MDMA and PCA on striatal monoamine concentration was analysed by Student's t test.

Results

Time course of marble burying behaviour

While earlier studies on burying generally appear to have used a 30 min measurement period, the time course of the response does not appear to have been reported. We therefore performed an initial experiment to examine whether this was an optimum measurement period. A group of mice were injected with saline and 30 min later placed in individual cages with the marbles and the number buried counted after 10, 20 and 30 min exposure. Results suggest that the majority of the response had occurred by 30 min (Fig. 1), so this time was adopted for all subsequent experiments.

Effect of MDMA on marble burying behaviour

When an approximate ED_{50} dose (11 μmol/kg; 2.5 mg/kg) was given to a group of mice, the maximum inhibitory response was seen 30 min post-injection (Fig. 2). At this time, MDMA produced a dose-dependent inhibition of the marble burying response (Fig. 3a).

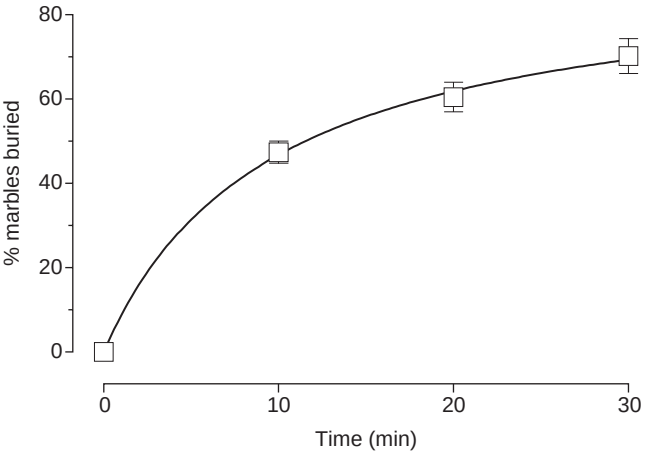

Figure 1 The percentage of the marbles buried by a group of saline-injected mice over a 30 min exposure period. Results are expressed as mean ± SEM ($n = 8$) per test group

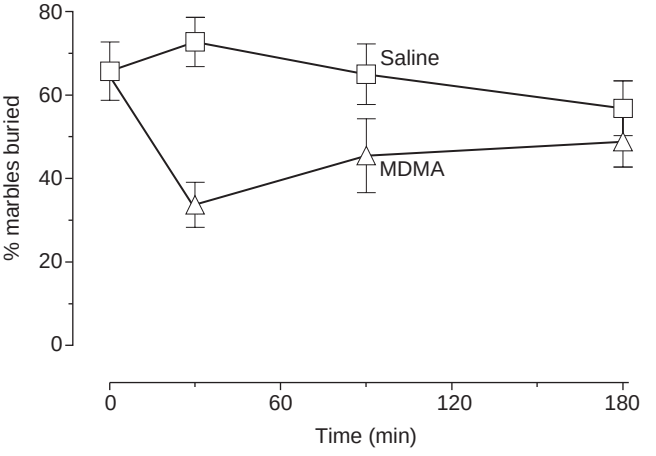

Figure 2 The percentage of the marbles buried by mice when exposed to the marbles at a specific time following the injection with saline or MDMA (11 μmol/kg (2.5 mg/kg) i.p.). Results are expressed as mean ± SEM ($n = 8$) for each treatment group. $^{**}p < 0.01$ compared to saline-injected mice at the same time point, unpaired t-test

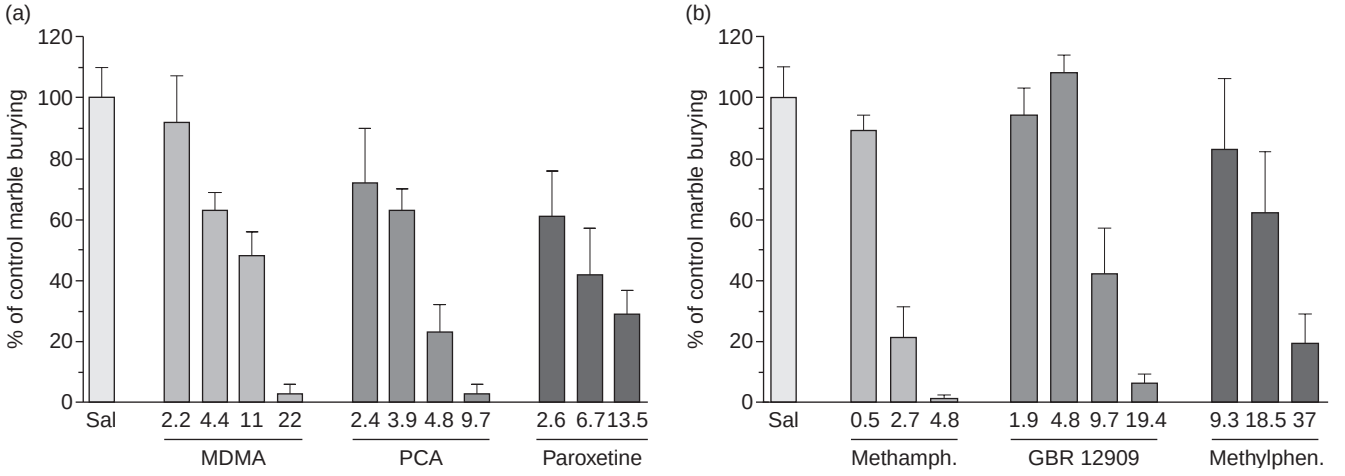

Figure 3 The percentage of marbles buried by mice administered various doses of the drugs shown when tested 30 min later relative to their corresponding saline control. There was an overall effect of treatment with each of the drugs investigated as follows: MDMA, $F(4,30) = 16.82$, $p < 0.0001$; PCA, $F(4,20) = 13.01$, $p < 0.0001$; paroxetine $F(3,19) = 6.19$, $p = 0.004$; methamphetamine (Methamph.), $F(3,28) = 25.65$, $p < 0.0001$; GBR 12909, $F(4,20) = 19.16$, $p < 0.0001$; Methylphenidate (Methylphen.), $F(3,28) = 5.017$, $p = 0.007$. All doses of drugs are expressed in μmol/kg. *Post hoc* analysis (Dunnett's) shown on graph as follows: different from the appropriate saline-injected control group for the experimental set examined: $*p < 0.05$; $**p < 0.01$

Effect of other compounds that increase the cerebral extracellular monoamine concentration on marble burying

The next experiments examined other compounds known to alter 5-HT or dopamine function or both. The 5-HT and dopamine releasing compound p-chloroamphetamine (PCA) and the 5-HT uptake inhibitor paroxetine inhibited marble burying in a dose dependent way and both were modestly more potent than MDMA (Fig. 3a; Table 1).

Methamphetamine, a drug that is also both a 5-HT and dopamine releasing agent, potently inhibited marble burying, while methylphenidate was also effective but was less potent than any other compound examined (Fig. 3a; Table 1).

Administration of the dopamine uptake inhibitor GBR 12909 also resulted in an inhibition of marble burying (Fig. 3b; Table 1).

Locomotor activity

To determine whether inhibition of marble burying was due to an alteration of locomotor activity, individual mice were injected with approximate ED_{50} doses for inhibition of burying behaviour and their locomotor activity measured individually 30 min later. No effect on locomotor activity was seen over the same time period (30 min) as that used for marble burying behaviour for any of the drugs tested (Table 2).

Effect of a neurotoxic dose of MDMA and PCA on cerebral 5-HT and dopamine content

One week following a neurotoxic dose regime of MDMA (25 mg/kg given at 3 h intervals to a total of three doses) there was a significant loss of dopamine in the striatum of treated mice, compared to saline injected control animals (Table 3). There was no statistically significant loss of 5-HT in the striatum of the same animals (Table 3).

One week following a neurotoxic dose regime of PCA (15 mg/kg given twice 6 h apart) there was a significant loss of both dopamine and 5-HT in the striatum of treated mice, compared to saline injected control animals (Table 3).

Table 1 The ED_{50} for inhibition of marble burying behaviour of the compounds investigated

	ED_{50} value	
Drug	μmol/kg (95% confidence intervals)	mg/kg (base) (mean value)
MDMA	7.60 [4.0–14.2]	1.35
Methamphetamine	1.44 [1.43–1.46]	0.21
PCA	3.66 [2.62–5.12]	0.62
Paroxetine	4.59 [4.13–5.09]	1.51
GBR 12909	Approx 9.5	4.27
Methylphenidate	21.31 [16.36–27.76]	4.96

The ED_{50} value for each compound calculated by non-linear regression of the data presented in Fig. 3. Results obtained with GBR 12909 differed substantially from a Hill plot of near unity and did not allow confidence limits to be calculated.

Table 2 Effect of MDMA and other drugs altering monoamine function on locomotor activity

Injected	Dose (μmol/kg)	Locomotion (total crossings/30 min)
Saline	–	306 ± 16
MDMA	7.6	306 ± 31
Methamphetamine	1.35	261 ± 41
PCA	3.9	239 ± 55
Paroxetine	3.3	348 ± 71
GBR 12909	11.1	285 ± 30
Methylphenidate	19.5	256 ± 64

All drugs were given 30 min before locomotor activity was examined over a 30 min period. Results shown as mean ± SEM ($n = 4$) of total crossings in the 30 min period.

Table 3 The concentration of 5-HT and dopamine in mouse striatum 7 days after administration of a neurotoxic dose regime of MDMA or PCA

Treatment	*n*	5-HT	Dopamine
Saline	6	437 ± 43	6606 ± 760
MDMA	5	351 ± 58	2657 ± 719†
PCA	5	122 ± 23**	2571 ± 684**

The concentration of the monoamines reported in ng/g tissue wet weight and shown as mean ± SEM together with number of observations (*n*). Different from saline-injected control group: †$p < 0.02$; **$p < 0.01$.

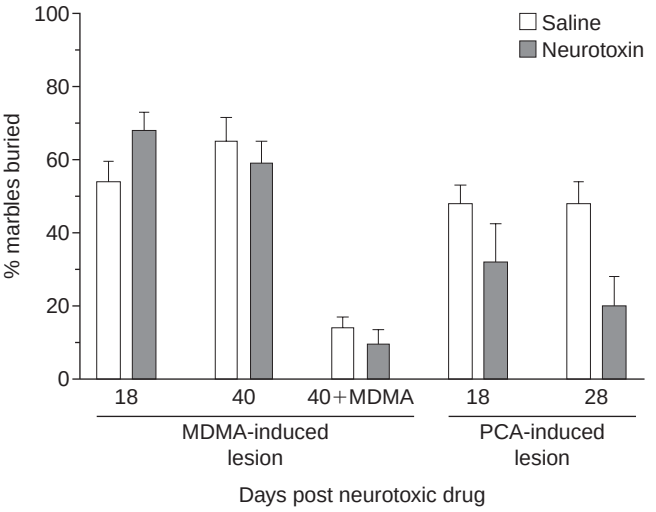

Figure 4 The percentage of marbles buried by mice at various times following administration of either saline or a neurotoxic drug (MDMA or PCA). Graph also shows the response of saline or MDMA-lesioned rats to a further acute dose of MDMA (2.5 mg/kg) on day 40. There was no significant difference between the saline-injected control group and the MDMA-lesioned group on either day 18 or day 40, or in the response of the two groups to a challenge dose of MDMA (2.5 mg/kg) on day 40. In the PCA study there was a clear effect of drug on the response: $F(1,21) = 8.58$, $p = 0.05$ and a *post hoc* test demonstrated that the lesioned group buried fewer marbles at 28 days ($p < 0.05$)

Effect of a neurotoxic dose of MDMA and PCA on marble burying behaviour

One day after the neurotoxic dose regime of MDMA there was no difference in spontaneous marble burying behaviour of the mice compared to saline injected control animals. No difference was also seen between the groups either 18 or 40 days later. When both groups were then challenged with MDMA (2.5 mg/kg) at day 40 a similar inhibition of the behaviour was seen (Fig. 4).

When PCA-lesioned mice were examined a different pattern of response was seen. There was no statistically significant decrease in marbles buried at 18 days compared to the control group, but a significant decrease in marble burying at day 28 (Fig. 4). The experiment was terminated at this point on ethical grounds as the PCA-treated mice had become very aggressive with each other when housed together.

Discussion

Administration of MDMA to mice produced a dose-dependent inhibition of marble burying with the maximum inhibition occurring 30 min after drug administration and the time course of the effect appears to match well the increase in MDMA-induced monoamine release *in vivo* (Camarero *et al.*, 2002). The response of the control group in this part of the study also confirmed the report of Njung'e and Handley (1991b) that repeat testing does not induce habituation in this response.

Previous data have indicated that an increase in 5-HT functional activity is a major determinant in the inhibition of marble burying by mice since the selective uptake inhibitors fluvoxamine, indalpine, zimeldine and citalopram all attenuated the behaviour (Njung'e and Handley, 1991a, 1991b; De Boer and Koolhaas, 2003). Our observation that paroxetine administration also inhibited the burying response is consistent with these data.

The results on MDMA-induced inhibition of marble burying could also be interpreted in the light of it being a 5-HT-mediated effect since this compound markedly enhances 5-HT release (Green *et al.*, 2003). However, MDMA also enhances dopamine release in the brain of both rats and mice (Colado *et al.*, 2004). PCA and methamphetamine also release both 5-HT and dopamine in the brain (Sharp *et al.*, 1986; Baldwin *et al.*, 1993; Iyer *et al.*, 1994; Sugita *et al.*, 1994) and both compounds were also very effective in producing a dose dependent inhibition of marble burying behaviour. Consequently a role for dopamine in modulating this behaviour cannot be ruled out on the basis of these studies. Methylphenidate, a compound that elevates the synaptic concentration of both dopamine and noradrenaline (Kuczenski and Segal, 1997; Leonard *et al.*, 2004), had an ED_{50} for inhibiting marble burying approximately three- to 14-fold weaker than the

three amphetamine derivatives. Since methylphenidate does not have any effect on cerebral 5-HT release, this result suggests that catecholamine release may play a minor role in inhibiting marble burying behaviour. This interpretation is supported by the observation that the dopamine uptake inhibitor GBR 12909 was also active in inhibiting marble burying behaviour. However, this compound produced a very 'steep' inhibition curve which differed substantially from a Hill plot of near unity and this therefore prevented accurate assessment of the non-linear regression curve and calculation of an ED_{50} value.

The study on locomotor activity suggested that none of the compounds inhibited marble burying due to an alteration in locomotor activity as none of them had an effect on locomotor activity at a dose that produced an approximate 50% reduction in marble burying.

The behavioural data on mice given a prior neurotoxic dose of MDMA also support the contention that the inhibition of marble burying is primarily via a 5-HT-mediated mechanism. In agreement with earlier studies (Colado *et al.*, 2001; O'Shea *et al.*, 2001) we found that a neurotoxic dose regime of MDMA in mice produced a major (60%) loss in striatal dopamine content, but no significant loss in striatal 5-HT concentration (Logan *et al.*, 1988; Camarero *et al.*, 2002). This major loss in cerebral dopamine concentration nevertheless failed to influence marble burying behaviour during the following 40 days or the inhibition of marble burying produced by a 'challenge' dose of MDMA at the end of the study period. This 'challenge' result again suggests that it may be 5-HT rather than dopamine release which plays the primary role in the attenuation of marble burying produced by an acute dose of MDMA. However, we acknowledge the fact that the striatum may not be the neuroanatomical locus of the behaviour being investigated so a firm conclusion is unwarranted.

When a neurotoxic dose of PCA was given it produced a similar loss in striatal dopamine content, but also a substantial (70%) loss in 5-HT. This allowed an examination of the consequences of a long-term decrease in cerebral 5-HT function which has relevance to the possible effects of MDMA in the human since long-term MDMA-induced neurotoxic damage is restricted to 5-HT nerve endings in rats and non-human primates, but crucially also possibly in human brain (see Green *et al.*, 2003). Although the defensive burying model has been described in rats, it has only been produced with aversive stimuli such as electrified probes rather than marbles (De Boer and Koolhaas, 2003; Green *et al.*, unpublished observations) and is therefore not distinct from other conditioned anxiety models. Therefore mice must be used to study unconditioned defensive burying.

Eighteen days after treatment the PCA-lesioned group displayed a non-significant decrease in marble burying behaviour. At 28 days a clear statistically significant attenuation of the burying was seen. This effect is presumably consequent on the decrease in cerebral 5-HT concentration since the similar, but selective, dopamine loss generated by MDMA administration was without effect. While it would have been interesting to continue this part of the study, the fact the PCA-treated mice had become aggressive towards each other meant that we felt that ethically it was unreasonable to continue. It has been known for many years that a lesion of 5-HT pathways in mice with PCA can produce aggressive behaviour (Gianutsos and Lal, 1975), and our current observation provided further evidence for the effectiveness of the lesion on 5-HT pathways. No aggression had been seen in the MDMA-treated animals. The apparent anxiolytic-like effect of the 5-HT lesion is consistent with other data. Decreased 5-HT function has long been reported to result in behaviour consistent with an 'anxiolytic' effect (Tye *et al.*, 1977, 1979; Iversen, 1984; Briley *et al.*, 1990), although the role of 5-HT in anxiety remains controversial (see Green and McGregor 2002). Furthermore, since substantial 5-HT loss would have occurred in the brain in the first few days following administration of PCA these data suggest that the change in behavioural response at 28 days may be a long-term adaptive response to 5-HT loss. Indeed adaptive changes in the sensitivity of 5-HT receptors have been reported following administration of MDMA in both rats and man (Reneman *et al.*, 2002; McGregor *et al.*, 2003).

In conclusion, the current data suggest that low doses of MDMA and other amphetamines produce an effect in this behavioural model that is qualitatively similar to that seen following an acute dose of several antidepressant or anxiolytic drugs and this effect is probably due primarily to the 5-HT releasing properties of MDMA and related compounds. A long-term lesion of 5-HT pathways in the mouse produced a similar effect and the timing of this change suggests that it is an adaptive response.

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