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ORIGINAL INVESTIGATION

Thomas J. R. Beveridge · Annis O. Mechan · Marie Sprakes · Qi Pei · Tyra S. C. Zetterstrom · A. Richard Green · J. Martin Elliott

Effect of 5-HT depletion by MDMA on hyperthermia and Arc mRNA induction in rat brain

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Abstract *Rationale:* 3,4-Methylenedioxymethamphetamine (MDMA) administration to rats produces an acute hyperthermic response and induces localised neuronal activation, which can be visualised via expression of immediate-early genes. The pharmacological and anatomical basis of these effects are unclear. At high doses, MDMA also causes selective neurotoxicity at serotonergic nerve terminals. *Objective:* We investigated the effect of 5-hydroxytryptamine (5-HT) depletion on the acute hyperthermic response to MDMA and the pattern of neuronal excitation indicated by Arc (activity-regulated cytoskeleton associated gene) in naive rats and following administration of MDMA at a neurotoxic dose. *Methods:* Expression of Arc mRNA was investigated by in situ hybridisation histochemistry using ^{35}S-labelled oligonucleotide probe. *Results:* MDMA induced a significant hyperthermia together with increased Arc mRNA expression in cortical regions, caudate-putamen and CA1 hippocampus but not hypothalamus. At 21 days after a neurotoxic dose of MDMA, brain 5-HT and 5-HIAA levels were significantly reduced by 21–32%. In these animals, both the hyperthermic response and the pattern and extent of Arc mRNA expression induced by a subsequent dose of MDMA were unaltered. However, basal Arc expression was significantly increased in cortical regions and CA1 hippocampus. *Conclusion:* We conclude that the acute hyperthermic response induced by MDMA is not attenuated by moderate depletion of 5-HT, further questioning mediation via a serotonergic mechanism. Arc mRNA induction by MDMA exhibits highly localised expression, which is not altered following 5-HT depletion. However, following a neurotoxic dose of MDMA, basal expression of Arc is increased, particularly in cortex and CA1, suggesting that mechanisms underlying synaptic plasticity might also be modified.

Keywords MDMA · Arc · Immediate-early genes · Synaptic plasticity · 5-HT depletion · Hyperthermia · Rat brain · Neurotoxicity

T. J. R. Beveridge · A. O. Mechan · T. S. C. Zetterstrom · A. R. Green · J. M. Elliott (✉)
School of Pharmacy,
De Montfort University,
Leicester, LE1 9BH, UK
e-mail: jme@dmu.ac.uk
Tel.: +44-116-2577126
Fax: +44-116-2577287

M. Sprakes · Q. Pei
Department of Pharmacology,
University of Oxford,
Oxford, OX1 3QT, UK

T. J. R. Beveridge
Department of Physiology and Pharmacology,
Wake Forest University School of Medicine,
Medical Center Boulevard,
Winston-Salem, NC 27157-1083, USA

A. O. Mechan
Department of Neurology,
Johns Hopkins University School of Medicine,
5501 Hopkins Bayview Circle, Baltimore, MD 21224, USA

Introduction

Administration of 3,4-methylenedioxymethamphetamine (MDMA, 'ecstasy') causes the rapid release of 5-hydroxytryptamine (5-HT) and dopamine in rodent and primate brains (Gough et al. 1991; Brodkin et al. 1993). This coincides with the appearance of an acute hyperthermic response (Colado et al. 1995; Malpass et al. 1999) and the characteristic behavioural effects associated with the serotonin syndrome, including hyperactivity, head-weaving and low body posture (Slikker et al. 1989). To localise those brain areas specifically activated by MDMA, the pattern of expression of immediate-early genes (IEGs), including c-fos and zif 268, has been studied (Stephenson et al. 1999; Shirayama et al. 2000), and results indicate activation of many cortical, limbic and striatal brain regions. Unlike c-fos and zif 268, which code for nuclear transcription factors, Arc (activity-regulated cytoskeleton associated gene) is an IEG which is translated to a cytosolic protein implicated in the

mechanism of synaptic plasticity associated with long-term potentiation (LTP) and learning (Guzowski et al. 2000). Expression of Arc mRNA and/or protein is increased by neuronal excitation induced by electrical stimulation (Lyford et al. 1995) or by activation of glutamatergic, dopaminergic or serotonergic receptors (Kodama et al. 1998; Pei et al. 2000; Steward and Worley 2001b). Both mRNA and protein expression are concentrated within specific dendritic domains associated with increased synaptic activity (Steward and Worley 2001a).

At high doses, MDMA causes a selective neurotoxic effect on serotonergic nerve terminal arborisation, as revealed by depletion of 5-HT and its principal metabolite 5-hydroxyindoleacetic acid (5-HIAA) (Battaglia et al. 1987; O'Shea et al. 1998), reduction in the density of the serotonin transporter (Battaglia et al. 1987; Hewitt and Green 1994) and signs of axonal degeneration (O'Hearn et al. 1988). These effects are evident in rats and non-human primates within 7 days of drug administration and persist for many months in the rat and even longer in primates (Sabol et al. 1996). In man, brain imaging studies quantifying the serotonin transporter site report significantly lower density (20–30%) in the brains of persistent MDMA users relative to drug-free control subjects (McCann et al. 1998; Semple et al. 1999; Reneman et al. 2001), suggesting that similar neurotoxic changes may result from illicit drug use. The loss in forebrain 5-HT concentration following a neurotoxic dose of MDMA does not however appear to necessarily alter 5-HT release from cerebral tissue. In the rat, reduction of the tissue 5-HT concentration following a neurotoxic dose of MDMA did not alter basal 5-HT release (Series et al. 1994; Gartside et al. 1996), while 5-HT release induced by electrical stimulation of raphé nuclei showed regional variability, with attenuation in hippocampus but not frontal cortex (Gartside et al. 1996). Release of cerebral 5-HT in rats pre-treated with a neurotoxic dose of MDMA by administration of a subsequent dose of MDMA or fenfluramine was however attenuated as was the MDMA-induced characteristic serotonin-meditated behavioural syndrome (Series et al. 1995; Shankaran and Gudelsky 1999). MDMA-induced neuroendocrine changes (prolactin and ACTH release) were unaltered however (Series et al. 1995).

The effects of a prior neurotoxic dose of MDMA on the subsequent MDMA-induced hyperthermic response has produced conflicting data (Dafters 1995; Shankaran and Gudelsky 1999). However, recent data has indicated that MDMA-induced hyperthermia is probably the result of dopamine release rather than 5-HT release (Mechan et al. 2002a), so loss of 5-HT function might not be expected to change the temperature response following a subsequent MDMA dose.

Since both biochemical and functional studies have indicated that following administration of MDMA there is a complex pattern of effects that may be regionally discrete, we have investigated the effect of acute MDMA administration on Arc mRNA expression in naive rats and in animals previously treated with a neurotoxic dose of MDMA to determine whether depletion of brain 5-HT levels might reduce the serotonergic component of the Arc response. We have also examined the acute hyperthermic response to MDMA in these animals to further characterise the pharmacological basis of this effect.

Materials and methods

Treatment protocol

Male Dark Agouti rats (250–300 g) housed at an ambient room temperature of 20°C initially received either saline vehicle (1 ml/kg) or MDMA (12.5 mg/kg IP), and body temperature was measured at intervals immediately before and up to 4 h after drug administration using a rectal thermocouple probe. They were then returned to their home cages and allowed food and water ad libitum for 21 days. On day 22, each rat again received either saline or MDMA (12.5 mg/kg IP) and body temperature was measured as before. In a second group of animals, MDMA or saline was administered on day 1 and day 22 according to the same protocol, and the rats were killed by cervical dislocation 2 h after drug administration on day 22. In the third group of animals, MDMA or saline was administered on day 1, and the rats were killed on day 22 without any further injections. In the latter two cases, the brains were rapidly removed after killing the animals and frozen by immersion in cooled isopentane (−30°C). The brains were then stored at −80°C until processing. All animal studies were carried out under Home Office licence and with the approval of De Montfort University ethics review board.

In situ hybridisation histochemistry

In situ hybridisation histochemistry (ISHH) was performed as previously described (Pei et al. 2000). Briefly, coronal sections (12 μm) were cut from the frozen brains at levels corresponding to plates 7–8 (cingulate and orbital cortex), 14–15 (caudate-putamen), 24–25 (hypothalamus) and 32–33 (parietal cortex and hippocampus) of the stereotaxic atlas (Paxinos and Watson 1986) and thaw mounted onto gelatinised slides. Following pretreatment, ISHH was carried out using an oligonucleotide complementary to bases 789–833 of the rat Arc gene (Lyford et al. 1995) (59-CTTGGTTG-CCCATCCTCACCTGGCACCCAAGACTGGTATT-GCTGA-39) labelled with [^{35}S]dATP (NEN DuPont, 1250 Ci/mmol) by terminal transferase (Promega) to a specific activity of approximately 1×10^9 d.p.m./mg oligonucleotide, in hybridisation buffer. After incubation at 36°C for 14–16 h, slides were rinsed to high stringency, air-dried and apposed to autoradiographic film (Biomax, Amersham) for 48 h at room temperature.

Brain indoleamine analysis

Rats received either saline or MDMA (12.5 mg/kg IP) and were killed 21 days later. The brain was rapidly dissected on ice and 5-HT, 5-HIAA and dopamine was measured by means of high-performance liquid chromatography (HPLC) with electrochemical detection as described previously (Colado et al. 1995).

Data analysis

The relative abundance of mRNAs in selected brain areas was determined by densitometric quantification of autoradiograms using NIH Image v1.61 (http://rsb.info.nih.gov/nih-image/index.html) on a Macintosh G3 computer. Optical density values were calibrated to ^{35}S-tissue equivalents using ^{14}C microscales

(Amersham, UK). Mean densitometric values were measured from three sections in each rat brain and expressed as nCi/g tissue.

Data are presented as mean±SEM with generally n=5–7 per group. Statistical analysis of Arc mRNA expression and body temperature was performed by two-way or one-way ANOVA as appropriate and post-hoc comparisons between individual groups by Newman-Keuls test (GraphPad Prism, San Diego, CA, USA). Statistical analysis of tissue indoleamine levels was performed by Student's unpaired t-test.

Results

MDMA-induced hyperthermia

Administration of MDMA (12.5 mg/kg) to naive rats produced a significant rise in body temperature relative to rats that received saline (Fig. 1). Administration of MDMA to rats that had previously received a neurotoxic dose of MDMA 21 days earlier induced a similar rise in temperature which was significantly greater than saline controls but not significantly different from the effect of the initial administration of MDMA (Fig. 1).

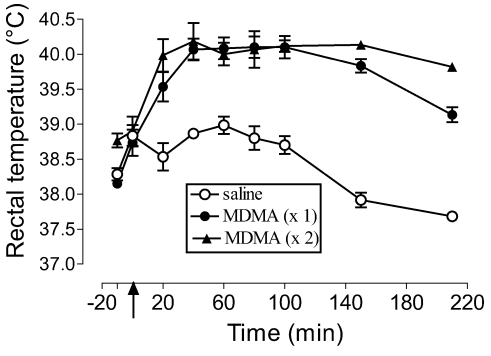

Fig. 1 Effect of MDMA on body temperature in naive rats relative to a previous administration of a neurotoxic dose of MDMA. On day 1, MDMA (12.5 mg/kg IP) or saline (1 ml/kg) was administered to rats and rectal body temperature measured over the subsequent 3.5 h as described in Methods. On day 22, rats that had previously received MDMA were again challenged with MDMA (12.5 mg/kg IP) and rectal temperature measured as before. Data shown represent Mean ±SEM (n=6). MDMA induced a significant hyperthermic response in naive rats relative to saline (P<0.001, Newman-Keuls test) when measured as area-under-the-curve for the period 0–210 min. The second challenge with MDMA also induced a significant hyperthermic effect relative to saline controls (P<0.001), but this response was not significantly different from that initially induced by MDMA in naive rats

Brain indoleamine concentrations

Administration of MDMA caused a significant depletion of both 5-HT and 5-HIAA levels of 21–32% in frontal cortex, striatum and hippocampus when measured 21 days later (Fig. 2). There was no significant change in the striatal dopamine concentration (data not shown).

Arc mRNA expression

Acute administration of MDMA to rats that had received saline 21 days earlier induced discrete expression of Arc mRNA in the cerebral cortex, caudate-putamen and hippocampus but not the hypothalamus, relative to saline/saline controls (Table 1). Within the hippocampus, significant Arc induction was visible in the CA1 region, but not CA3 or dentate gyrus (Fig. 3A, B). An acute challenge with MDMA to rats administered a neurotoxic dose of MDMA 21 days earlier again induced an increase in Arc mRNA expression in cortical areas, caudate-putamen and CA1 region of the hippocampus (Fig. 3C), which was not significantly different from that observed in rats that had received MDMA following saline pretreatment (Table 1). Analysis of the Arc mRNA data by two-way ANOVA for first versus second treatments indicated no significant interaction effects between the two treatment groups in any of the individual brain areas.

Rats that had initially received MDMA and received saline 21 days later displayed an increase in Arc mRNA expression when compared against saline/saline controls in the cortical regions and CA1 hippocampus (33–82% increase), but post-hoc analysis of individual brain areas demonstrated statistical significance only in the case of

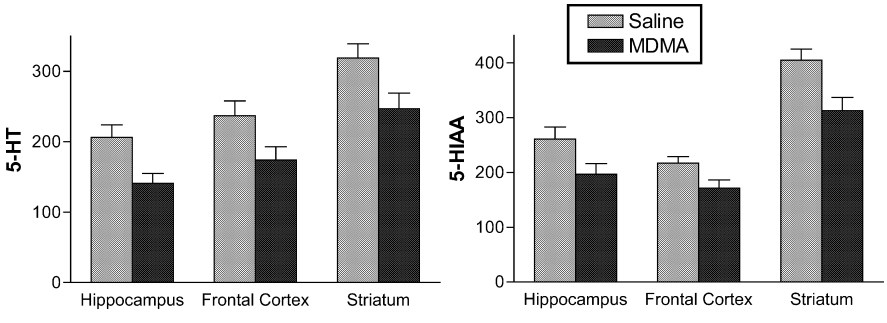

Fig. 2 Effect of MDMA on brain indoleamine content. Rats received MDMA (12.5 mg/kg IP) or saline (1 ml/kg) and were killed 21 days later. Brain regions were rapidly dissected on ice and indoleamine content estimated by high-performance liquid chromatography with electrochemical detection as described in Methods. Values for 5-HT and 5-HIAA content are expressed in ng/g tissue wet weight and represent Mean ±SEM for 5–7 samples per group. MDMA significantly reduced both 5-HT and 5-HIAA levels in each of the brain regions studied (P<0.05, Student's unpaired t-test)

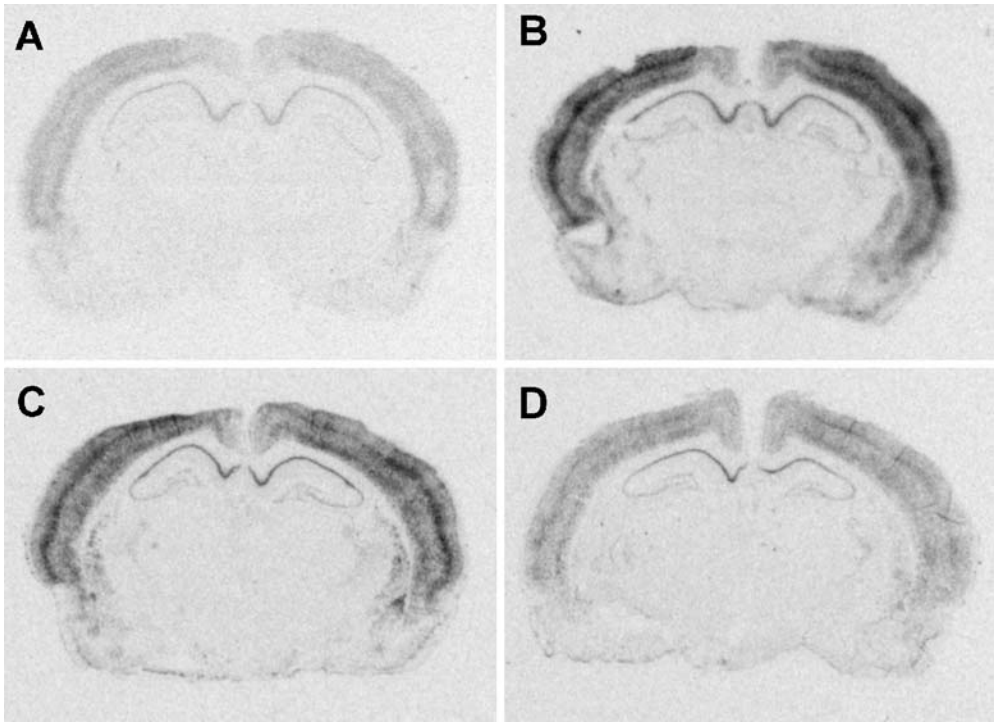

Fig. 3 Effect of sequential administration of saline/MDMA on Arc mRNA expression in rat parietal cortex and hippocampus. Rats were treated with saline (1 ml/kg) or MDMA (12.5 mg/kg IP) on day 1 and again on day 22 then killed 2 h after the second treatment. Brains were isolated and Arc mRNA expression visualised by in-situ hybridisation histochemistry as described in Methods. **A** saline/saline, **B** saline/MDMA, **C** MDMA/MDMA, **D** MDMA/saline

Table 1 Effect of sequential administration of saline and/or 3,4-methylenedioxymethamphetamine (MDMA) on Arc (activity-regulated cytoskeleton associated gene) mRNA expression in rat brain. Saline (1 ml/kg) or MDMA (12.5 mg/kg IP) was administered on day 1 and again on day 22, and rats were killed 2 h after the second dose. Values for Arc mRNA were quantified from in situ hybridisation histochemistry (ISHH) studies as described in Methods and are expressed in nCi/g tissue weight as mean ±SEM (n=6)

	Saline/saline	Saline/MDMA	MDMA/MDMA	MDMA/saline
Orbital cortex	319±54	639±81**	799±80***	425±41
Cingulate cortex	242±63	576±71**	747±66***	343±22
Parietal cortex	353±57	1427±103***	1352±73***	605±105
Caudate putamen	111±33	719±72***	774±81***	98±29
Hypothalamus	111±19	105±16	111±29	103±18
CA1	320±30	554±60*	509±46*	487±63*
CA3	201±25	254±40	211±32	194±16
Dentate gyrus	166±27	177±27	144±27	134±5

*P<0.05; **P<0.01; ***P<0.001 compared with corresponding saline/saline group (Newman-Keuls post-hoc test following 1-way ANOVA)

Table 2 Effect of a single administration of 3,4-methylenedioxymethamphetamine (MDMA) or saline on Arc (activity-regulated cytoskeleton associated gene) mRNA expression in rat brain. Saline (1 ml/kg) or MDMA (12.5 mg/kg IP) was administered on day 1 and rats were killed on day 22. Values for Arc mRNA are expressed in nCi/g tissue weight as mean ±SEM (n=3)

Treatment	Orbital cortex	Cingulate cortex	Parietal cortex	CA1
Saline	253±107	227±70	279±61	412±40
MDMA	738±110*	531±60	701±61*	771±76*

*P<0.05 compared with saline group (Newman-Keuls post-hoc test following 1-way ANOVA)

the hippocampal CA1 region (Fig. 3D). To determine whether the stress associated with the saline injection on day 22 might have been pertinent to the effect of the neurotoxic regimen of MDMA on day 1, Arc expression was examined in a group of rats injected on day 1 with MDMA (12.5 mg/kg) or saline and then killed on day 22 without further drug administration. As before, animals receiving MDMA exhibited increased Arc mRNA expression relative to the saline controls (Table 2) which was statistically significant in orbital and parietal cortex as well as CA1 hippocampus.

Discussion

Administration of MDMA (12.5 mg/kg) produced an acute hyperthermic response and subsequent neurotoxic loss of 5-HT in several brain regions in agreement with previous studies (Green et al. 2003b). Interestingly, a second dose of MDMA (12.5 mg/kg) evoked a similar hyperthermic response to that seen following the first. Previous studies on the effect of prior treatment with MDMA on temperature response to a subsequent dose of the drug have produced conflicting data with both attenuation and enhanced sensitivity being reported (Dafters 1995; Shankaran and Gudelsky 1999). However, the current data support the contention (Mechan et al. 2002a) that MDMA-induced acute hyperthermia results primarily from dopamine release since a 20–30% loss of 5-HT did not appear to alter the response. Although tissue 5-HT loss in the hypothalamus, the central thermoregulatory centre, was not measured here previous studies suggest that this region is less affected by the neurotoxic action of MDMA than cortical regions (Green et al. 2003b). Furthermore, it cannot be argued that this degree of 5-HT loss is insufficient to produce a functional change since we have previously shown that it can alter both behavioural and thermoregulatory responses (Mechan et al. 2001, 2002b; Green et al. 2003a). It is also similar to the degree of loss in 5-HT markers reported to occur in the brains of heavy recreational users of MDMA (McCann et al. 1998).

With regard to the main part of the investigation on Arc, our results demonstrate that acute administration of MDMA induces substantial, but highly localised, increases in the expression of Arc mRNA in rat brain. Expression is evident throughout the cerebral cortex with discrete banding at inner and outer layers, similar to the pattern of expression induced by *p*-chloroamphetamine and the selective 5-HT_2 receptor agonist DOI (Pei et al. 2000) and by combined administration of paroxetine and WAY100635 (Castro et al. 2003). In the latter study, Arc expression was accredited to the three-fold increase in extracellular 5-HT concentration resulting from combined administration of the selective 5-HT uptake inhibitor and 5-HT_{1A} receptor antagonist and was abolished by prior inhibition of 5-HT synthesis using *p*-chlorophenylalanine. It seems likely, therefore, that 5-HT is the principal mediator in the induction of Arc mRNA which follows MDMA administration, but further studies using selective antagonists are needed to confirm this. Significant expression of Arc mRNA was also induced by MDMA in the caudate putamen. A similar effect has previously been reported in response to cocaine (Fosnaugh et al. 1995) and methamphetamine (Kodama et al. 1998) and this was blocked by the dopamine D1 receptor antagonist SCH23390. Since MDMA induces both 5-HT and dopamine release, it is possible that either or both amines may mediate this effect of MDMA, so again specific antagonist studies will be required to clarify the pharmacology of the response. The pattern of Arc expression induced by MDMA in the cortex and caudate putamen is similar to that of other IEGs such as c-fos (Stephenson et al. 1999) and zif 268 (Shirayama et al. 2000), but induction of Arc mRNA in the CA1 region of the hippocampus is quite distinct from these other IEGs. Furthermore, Arc induction was not observed in CA1 following acute administration of *p*-chloroamphetamine and was significantly decreased following extracellular 5-HT release induced by combined administration of the precursor amino-acid L-tryptophan and the monoamineoxidase (MAO) inhibitor tranylcypromine (Pei et al. 2000). Since Arc is involved in the mechanism underlying synaptic plasticity associated with learning (Guzowski et al. 2000) and the hippocampus is critically concerned with the anatomical pathways for learning and memory (Temple et al. 2003) this response within CA1 region may represent a unique effect of MDMA which is not shared by other amphetamines or by simple enhancement of 5-HT release and could have particular implications for the effects of MDMA on cognitive function, as discussed below. No localised induction of Arc mRNA by MDMA was apparent within the lateral, anterior and paraventricular areas of the hypothalamus, although several of these nuclei are innervated by serotonergic neurones (Milton 1977); hence, the interpretation of Arc expression is likely to be of limited value in resolving the anatomical basis of the hyperthermic response to MDMA.

Acute administration of MDMA to rats which had received a prior neurotoxic dose of MDMA produced a similar pattern and extent of Arc mRNA expression to that seen in saline pre-treated rats. Depletion of tissue 5-HT content by approximately 30% did not, therefore, attenuate the induction of Arc by MDMA in any brain region, even though this loss produces functional changes (see earlier). It could be concluded that Arc induction by MDMA is not mediated by 5-HT, but this seems unlikely in view of the similarity in the pattern of expression observed here compared to other direct or indirect serotonergic agonists described above and, indeed, preliminary studies indicate that Arc expression following MDMA is attenuated by pre-treatment with *p*-chlorophenylalanine (Aston and Elliott, unpublished observation). It seems more likely, therefore, that the induction of Arc is less sensitive to reduction in tissue 5-HT levels than the other responses reported above. Similar discrepancies have been noted in the responses to fenfluramine following 5-HT depletion by MDMA, whereby 5-HT release and several behavioural responses were attenuated whilst the neuroendocrine responses of prolactin and ACTH were unchanged (Series et al. 1994, 1995).

Following administration of a neurotoxic regimen of MDMA, Arc mRNA levels were significantly increased in cortical brain areas and specifically in the CA1 hippocampal region after an acute saline injection relative to saline/saline controls. This sustained increase in Arc expression may be associated with the neurotoxic consequences of MDMA since a similar effect has been reported within the hippocampus following lesion of the afferent pathway from the entorhinal cortex (Temple et al.

2003). In the latter case, however, the localised expression of Arc induced by exposure to a novel environment initially diminished 4–8 days following the lesion, but then recovered after 16 days coincident with the time course of re-innervation. In the present study, Arc expression was investigated 21 days after induction of the serotonergic lesion by MDMA, but the rats were kept in their home cage throughout the treatment period and were not exposed to any novel situation. It is possible that the saline injection given 2 h prior to the animals being killed could have initiated Arc expression if the animals had been sensitised by the initial MDMA administration (Kodama et al. 1998). We therefore examined Arc expression in rats that received either saline or MDMA (12.5 mg/kg IP) on day 1 and were then killed on day 22 without any additional saline injection. The effect of MDMA on the pattern of Arc expression was retained; in fact, levels were significantly increased in two of the cortical regions as well as CA1 following the neurotoxic dose of MDMA relative to saline. In the absence of any defined instigator of Arc expression, the interpretation of this effect is unclear. Whilst some re-innervation of neuronal pathways generated by the serotonergic lesion may be ongoing 21 days following MDMA administration, increased expression of Arc is evident throughout the cortex as well as CA1 hippocampus. The selective reinforcement or elimination of synapses forms the principal basis of our current understanding of synaptic plasticity associated with learning and memory and the expression and localisation of Arc to precise areas of neuronal activation indicate a principal role for Arc in this process (Steward and Worley 2001a). To be effective in this role, therefore, Arc expression must be tightly regulated. The generalised expression of Arc resulting from MDMA-induced neurotoxicity may therefore mask the discrete expression required to develop the synaptic reorganisation associated with specific learning paradigms and so could disrupt neuronal plasticity and hence lead to cognitive dysfunction. Such an outcome has been reported in the rat following administration of a neurotoxic regimen of MDMA (Marston et al. 1999; Broening et al. 2001), and cognitive impairment has been cited in several studies of human subjects with a history of frequent MDMA use (Krystal et al. 1992; Bhattachary and Powell 2001; Parrott 2001). Further studies are needed to determine whether such effects occur only at neurotoxic doses of MDMA or are also apparent at lower doses, particularly those in the range found in people who regularly self-administer MDMA for recreational purposes.

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