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MDMA Induces EPSP–Spike Potentiation in Rat Ventral Hippocampus *In Vitro* Via Serotonin and Noradrenaline Release and Coactivation of 5-HT_4 and β_1 Receptors

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It is well documented that *N*-methyl-3,4-methylenedioxyamphetamine (MDMA, ecstasy) releases brain serotonin (5-HT; 5-hydroxytryptamine), noradrenaline (NE; norepinephrine), and dopamine, but the consequent effect on brain functioning remains elusive. In this study, we characterized the effects of MDMA on electrically evoked responses in the ventral CA1 region of a rat hippocampal slice preparation. Superfusion with MDMA (10 μM, 30 min) increased the population spike amplitude (PSA) by 48.9 ± 31.2% and decreased population spike latency (PSL) by 103 ± 139 μs (both: mean ± SD, $n = 123$; $p < 0.0001$, Wilcoxon test), without affecting field excitatory postsynaptic potential (fEPSP). This effect persisted for at least 1 h after MDMA washout; we have called this EPSP–spike potentiation (ESP) by MDMA, ESP_{MDMA}. Antagonism of GABAergic transmission did not prevent ESP_{MDMA}, suggesting that an increase in excitability of pyramidal cells underlies this MDMA action. Block of serotonin transporter (SERT) with citalopram or 5-HT depletion with (±)-p-chlorophenylalanine pretreatment partially inhibited the ESP_{MDMA}. Block of both SERT and NE transporter prevented ESP_{MDMA}, indicating its dependence on release of both 5-HT and NE. ESP_{MDMA} is produced by simultaneous activation of 5-HT_4 and β_1 receptors, with a predominant role of 5-HT_4 receptors. Block of both 5-HT_4 and β_1 receptors revealed an inhibitory component of the MDMA action mediated by 5-HT_{1A} receptor. The concentration range of MDMA which produced ESP_{MDMA} (1–30 μM) corresponds to that commonly reached in human plasma following the ingestion of psychoactive MDMA doses, suggesting that release of both 5-HT and NE, and consequent ESP_{MDMA} may underlie some of the psychoactive effects of MDMA in humans.
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INTRODUCTION

N-methyl-3,4-methylenedioxyamphetamine (MDMA) has unique psychoactive properties that differ from psychostimulants or hallucinogens (Nichols, 1986; Nichols *et al*, 1986). In humans, acute MDMA produces positive mood changes, enhances empathy toward others, and increases social interaction (Downing, 1986; Peroutka *et al*, 1988; Dumont and Verkes, 2006). In spite of its well-documented potential to cause neurotoxic damage to serotonergic axons in the forebrain (Battaglia *et al*, 1987; O'Hearn *et al*, 1988), MDMA has gained wide popularity as a recreational drug (Parrott, 2001) and has been promoted as a psychotherapeutic tool (Grinspoon and Bakalar, 1986; Doblin, 2002; Check, 2004).

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At present, mechanisms by which acute MDMA affects brain processes underlying emotional and affective behavior are largely unknown. For example, neither receptors mediating psychotropic effects of MDMA, nor the brain regions involved have been identified. Acute effects of MDMA in humans and in experimental animals have generally been attributed to release of monoamine neuromodulators and in particular to serotonin transporter (SERT)-dependent brain serotonin (5-HT, 5-hydroxytryptamine) release (Bengel *et al*, 1998; Parrott, 2001; Green *et al*, 2003; Morton, 2005). In humans, chronic treatment with selective serotonin reuptake inhibitors (SSRI) prevented the MDMA-induced euphoria (Stein and Rink, 1999), while pretreatment with a single dose of the SSRI citalopram markedly attenuated most of the psychological effects of MDMA (Liechti *et al*, 2000). *In vitro* studies have demonstrated that MDMA binds to the plasma membrane monoamine transporters with relative selectivity for SERT (Steele *et al*, 1987; Battaglia *et al*, 1988; Rudnick and Wall, 1992). Although most studies suggest that MDMA mainly targets brain serotonergic system, some of them indicate the

possible involvement of the noradrenergic system. Namely, MDMA is essentially equipotent at 5-HT and noradrenaline (NE, norepinephrine) release from both rat-brain slices (Fitzgerald and Reid, 1993) and synaptosomal preparations (Rothman *et al*, 2001).

The ventral hippocampus (anterior hippocampus in primates) is one of the brain structures which is potentially important to generation of acute psychoactive effects of MDMA. It is densely innervated by serotonergic and noradrenergic axons (Oleskevich *et al*, 1989; Oleskevich and Descarries, 1990; Schroeter *et al*, 2000), projects to other limbic regions, eg prefrontal cortex, amygdala, hypothalamus, and nucleus accumbens (Kelley and Domesick, 1982; Van Groen and Wyss, 1990; Verwer *et al*, 1997) and is involved in mood- and emotion-related behaviors (Bannerman *et al*, 2004; Dolcos *et al*, 2004; Richardson *et al*, 2004; Herman and Mueller, 2006). Acute release of 5-HT and NE by MDMA has been demonstrated *in vitro* in rat hippocampal slices (Johnson *et al*, 1986; Fitzgerald and Reid, 1990, 1993). In the CA1 hippocampal region, 5-HT and NE exert multiple effects on excitability of both pyramidal cells and interneurons (Madison and Nicoll, 1986; Colino and Halliwell, 1987; Andrade, 1998; Hoffman and Johnston, 1999) as well as on synaptic transmission (Segal, 1990; Mlinar *et al*, 2001; Otmakhova *et al*, 2005). Furthermore, in the CA1 hippocampal region, 5-HT and NE affect synaptic plasticity (Corradetti *et al*, 1992; Villani and Johnston, 1993; Thomas *et al*, 1996; Katsuki *et al*, 1997; Gelinas and Nguyen, 2005) and are involved in the action of antidepressant drugs (Mongeau *et al*, 1997). In this study, we sought to characterize electrophysiological effects of acute MDMA application in the CA1 region of the ventral hippocampus *in vitro*. In addition, we attempted to identify monoamine neuromodulators and their receptors that mediate the MDMA effects.

METHODS

All animal manipulations were carried out according to the European Community guidelines for animal care (DL 116/92, application of the European Communities Council Directive 86/609/EEC) and approved by the Italian Ministry of Health and the Committee for Animal Care and Experimental Use of the University of Florence.

Electrophysiological Recordings

Experiments were done on transversal slices of ventral hippocampus. Slices were taken from the part of hippocampus approximately 20–40% distant from the ventral pole of the hippocampus. Slices were prepared from 6- to 9-week-old male Wistar rats (Harlan-Nossan, Milan, Italy). Rats were anesthetized with halothane and decapitated with a guillotine. The hippocampi were rapidly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF), which contained the following: NaCl, 126 mM; KCl, 2 mM; KH_2PO_4, 1.25 mM; $NaHCO_3$, 26 mM; $MgSO_4$, 1.5 mM; $CaCl_2$, 2 mM; and D-glucose 10 mM. The solution was bubbled with a 95% O_2/5% CO_2 gas mixture (pH 7.4). Transversal hippocampal slices of 400 μM nominal thickness were cut with a McIlwain tissue chopper (Gomshall, UK) and kept for at least 1.5 h at room temperature until recording. Before transferring to the recording chamber, a single slice was temporarily transferred to a Petri dish, where the CA1 region was disconnected from the CA3 region by a surgical cut. The slice was then placed on a nylon mesh, completely submerged in a recording chamber and superfused on both sides with oxygenated ACSF. The flow rate of 1.8–2.2 ml min^{-1} was kept constant during the experiment. Experiments were carried out at 31–32°C. Slices were incubated for 15 min in the recording chamber before initiating electrical stimulation that was continuous throughout the experiment. All drugs were applied via bath perfusion. Synaptic responses of CA1 pyramidal neurons were elicited by stimulation of the Schaffer collateral/commissural pathway. Stimulation pulses (80 μs duration; 15 s interpulse interval), triggered by a PC controlled by either LTP230D (Anderson and Collingridge, 2001) or by pClamp software (Molecular Devices, Foster City, CA) were delivered by a stimulus isolation unit (DS2, Digitimer, Welwyn Garden City, UK) through a twisted bipolar nichrome electrode. In a minority of experiments, stimulation was done by a constant current stimulation unit (NL800, Digitimer) through a monopolar tungsten electrode. Field potentials were recorded with glass electrodes filled with 150 mMNaCl (2–10 MΩ resistance) placed in the distal third of the stratum radiatum to record fEPSP and/or in the stratum pyramidale to record population spikes (PS). The distance between recording electrodes and stimulating electrode was 300–500 μM. In some experiments, two recording electrodes were placed in the stratum pyramidale on opposite sides of the stimulation electrode, enabling recording of two separate PS in each slice. Alternatively, PS were recorded simultaneously from two slices with one stimulating and one recording electrode per slice. Recorded potentials were amplified with Neurolog NL 104 amplifiers (Digitimer), digitized with TL-1 interface (Molecular Devices) with the sampling rate of 10–50 kHz and stored in a PC for off-line analysis. The stimulus intensity of test pulses was set to evoke a PS that in control had an amplitude greater than 1 mV and was equal to 20–30% of the maximum. At least 10 min of stable responses were used to generate the baseline values. Stimulus intensity was held constant throughout the experiment. At the beginning and the end of each recording, the maximal population spike amplitude (PSA) was assessed and those experiments in which the maximal PSA changed by more than 10%, indicating probable artificial change in recording conditions, were discarded. The PSA was measured as the length of the vertical line from the minimum of the PS to the line that joined the two positive peaks of the field response recorded in the stratum pyramidale. fEPSP was determined as the slope of the initial falling phase of the response recorded in the stratum radiatum.

PCPA Pretreatment and Measurement of 5-HT and NE Content

To deplete brain 5-HT, rats were pretreated with the tryptophan hydroxylase inhibitor p-chlorophenylalanine (PCPA). PCPA, at the dose of 400 mg kg^{-1} day^{-1}, was injected intraperitoneally for 3 consecutive days, the last application being 1 day before the experiment. PCPA was

dissolved at 20 mg ml^{-1} in 0.5% solution of carboxy-methylcellulose immediately before injection. The control group (sham) was pretreated the same way but with the omission of PCPA. Hippocampal 5-HT and NE content was determined as described previously (Mlinar *et al*, 2005). The slices were sonicated with a Labsonic dismembranator (1510, B. Braun Melzungen AG, Melzungen, Germany) for 15 s in 200 μl of an ice-cold solution containing 0.05% EDTA and 0.05% $Na_2S_2O_5$ in 0.1 M perchloric acid. The sample was centrifuged, the supernatant was neutralized with K_2HPO_4/KH_2PO_4 buffer, and loaded in a refrigerated autosampler (SIL-10ADVP, Shimatzu, Kyoto, Japan) connected to a computer-controlled high-performance liquid chromatography system (ESA 5006, ESA, Chelmsford, MA) equipped with a Nucleosil column (100-5 C18 AB 125/3, Macherey-Nagel GmbH, Düren, Germany) and an electrochemical detector (ESA 6210). The mobile phase consisted of 75 mM NaH_2PO_4, 3 mM octyl sodium sulfate, 1.2 mM EDTA, and 8% CH_3CN (pH 3.4).

Pharmacology

All drugs used in electrophysiological experiments were prepared as stock solutions (1000 times concentrated) in distilled water, aliquoted and stored at −20°C until use. All antagonists were perfused for at least 30 min before as well as during MDMA application. Antagonists applied individually or in a mixture, by themselves did not provoke significant effects. Citalopram was used as SSRI and nisoxetine was used as selective noradrenaline reuptake inhibitor (NRI). Citalopram (IC_{50} values for SERT and NET of 1.8 nM and 8.8 μM, respectively; Hyttel and Larsen, 1985) was used at 1 μM concentration while nisoxetine (IC_{50} values for NET and SERT of 3.4 and 610 nM, respectively; Hyttel and Larsen, 1985) was used at 200 nM concentration. These concentrations of reuptake inhibitors ensured full block of the respective transporter with neglectable effects on other monoamine transporters and receptors (Sanchez *et al*, 2003). *N*-(2-(-4(2-Methoxyphenyl)-1-piperazinyl)ethyl)-*N*-(2-pyridinyl) cyclohexane carboxamide (Way-100635, 50 or 100 nM) was used as the selective 5-HT_{1A} receptor antagonist. 3-(Piperdine-1-yl)-propyl-4-amino-5-chloro-2-methoxy benzoate hydrochloride (RS-23597-190, 10 μM) and 1-piperidinylethyl 1H-indole-3-carboxylate hydrochloride (SB-203186, 500 nM or 1 μM) were used as selective 5-HT_4 receptor antagonists. WAY-100635, SB-203186 and RS-23597-190 were used at concentrations which, in our experimental conditions, completely inhibited 5-HT_{1A} and 5-HT_4 receptor-mediated responses evoked by corresponding receptor agonists, as well as responses evoked by up to 30 μM 5-HT (Pugliese *et al*, 1998; Mlinar *et al*, 2001, 2006). Other 5-HT and NE antagonists used were applied at concentrations $\geqslant$100 folds their reported K_i (or K_d when available) values. The selective 5-HT_6 antagonist 4-Iodo-*N*-[methoxy-3-(4-methyl-1-piperazinyl)phenyl]benzenesulfonamide hydrochloride (SB-258585; pK_d = 8.56; Hirst *et al*, 2000) was applied at 100 nM. The selective 5-HT_7 receptor antagonist [R]-3-[2-(2-[4-methylpiperidin-1-yl]ethyl)pyrrolidine-1-sulfonyl]phenol hydrochloride (SB-269970; pK_i = 8.3 Hagan *et al*, 2000) was used at 1 μM concentration. 1-[2-((3-carbamoyl-4-hydroxy)phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl) phenoxy]-2-propanol methanesulfonate (CGP-20712) was used as the selective β_1-adrenergic antagonist at 200 or 500 nM (K_i = 4.5 nM; Hoffmann *et al*, 2004). The $GABA_A$ receptor antagonist bicuculline (10 μM) was used to block inhibitory neurotransmission.

Materials

N-methyl-3,4-methylenedioxyamphetamine was purchased from Ultrafine (UFC Ltd, Manchester, UK). CGP-20712, SB-203186, SB-258585, SB-269970, and SR-95531 were purchased from Tocris Cookson (Bristol, UK), and all other drugs were from Sigma-Aldrich (Steinheim, Germany).

Statistical Analysis

Data were analyzed using LTP230D, Clampfit (Molecular Devices) and Prism 4 software (GraphPad Software, San Diego, CA, USA). All data are expressed as the mean ± SD. Two-tailed nonparametric tests were used for statistical analysis. The significance of change caused by a drug superfusion compared to the baseline values was assessed by Wilcoxon matched-pair test. Differences between experimental groups were estimated by the Mann–Whitney test and Kruskal–Wallis test with Dunn's multiple comparison *post hoc* test. A value of $p < 0.05$ was considered significant.

RESULTS

Acute Effects of MDMA in the CA1 Region of Ventral Hippocampal Slices

The effects of 10 μM MDMA on population responses, evoked by electrical stimulation of the Schaffer collaterals/commissural fibers in the stratum radiatum, are summarized in Figure 1. Bath superfusion with 10 μM MDMA augmented PS recorded from the somatic region of pyramidal cells without changing fEPSP recorded from the dendritic region (Figure 1a–c). In individual experiments, effects of 30-min MDMA application were measured such that control values corresponded to those of the mean response obtained by averaging the last 20 data traces before MDMA application, and values in MDMA corresponded to the mean response obtained by averaging five data traces recorded during the 30th min of MDMA application. Analysis of pooled data from all experiments in which 10 μM MDMA was applied (Figure 1d and e) shows that 30-min MDMA application did not significantly affect fEPSP slope (2.8 ± 9.2%, $n = 23$, slices obtained from 14 animals; $p = 0.15$, Wilcoxon test) while it significantly increased the PSA (48.9 ± 31.2%, $n = 123$ from 68 animals; $p < 0.0001$, Wilcoxon test) and decreased population spike latency (PSL) (−103 ± 139 μs, $n = 123$ from 68 animals; $p < 0.0001$, Wilcoxon test). These results suggest that 10 μM MDMA facilitates fEPSP–PS coupling in the CA1 area of the ventral hippocampus.

Concentration dependence of the MDMA effect on PSA was assessed for concentrations ranging from 1 to 30 μM (Figure 2). Measured at 30 min of superfusion, 1 μM MDMA increased the PSA submaximally (22.3 ± 13.8%, $n = 8$ from six animals; significant, $p < 0.01$, Wilcoxon test), while at 3 μM concentration, it induced a nearly maximal response

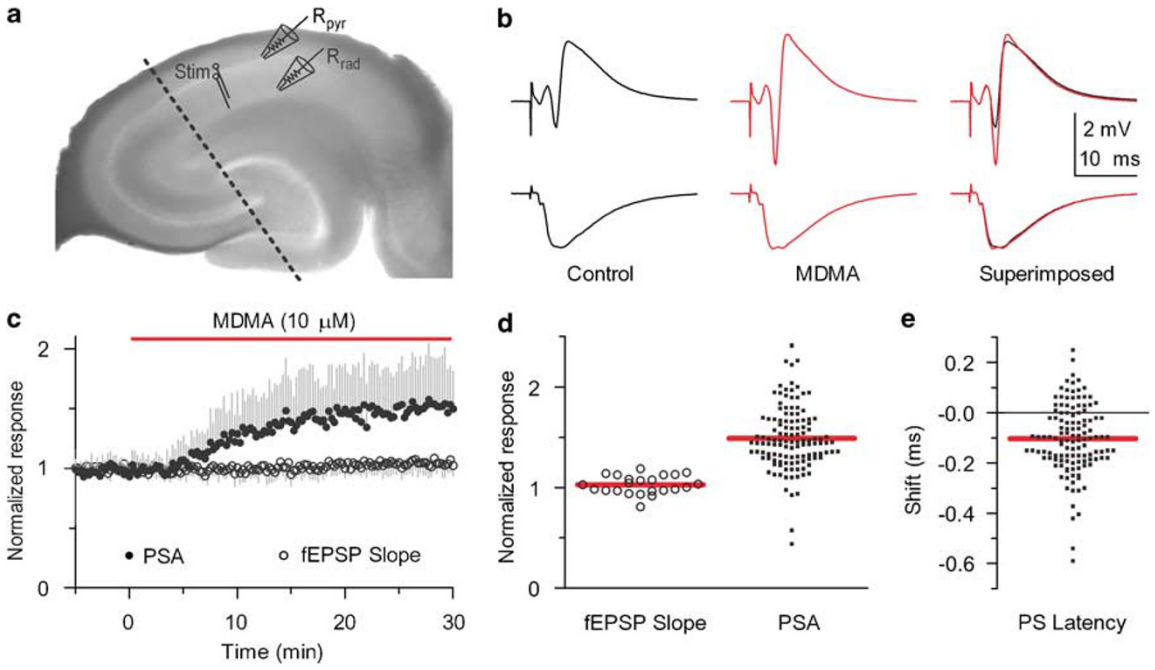

Figure 1 Effects of *N*-methyl-3,4-methylenedioxyamphetamine (MDMA) on population responses. (a) Photomicrograph scheme shows the arrangement of the recording (R_{rad}, R_{pyr}) and stimulus (S) electrodes in the CA1 region of hippocampal slices. Dotted lines indicate position of a cut made to separate CA1 and CA3 regions. (b) Data traces recorded with electrodes positioned in the stratum pyramidale (R_{pyr}, PS (population spikes); above) and in the distal part of stratum radiatum (R_{rad}, fEPSP (field excitatory postsynaptic potential); below). Traces were averaged over 20 sweeps recorded immediately before (black lines) and 25–30 min after the beginning of perfusion with 10 μM MDMA (red lines). (c) Summary time–course plot (mean ± SD; $n = 9$ from six animals) of MDMA (10 μM) effects on PSA (filled circles) and the fEPSP slope (open circles) recorded as shown above. (d) Scatter plot of effects of MDMA (10 μM, 30 min) on PSA and fEPSP slope for all 123 experiments. (e) Scatter plot shows the shift of PSL induced by MDMA (10 μM, 30 min) for all experiments.

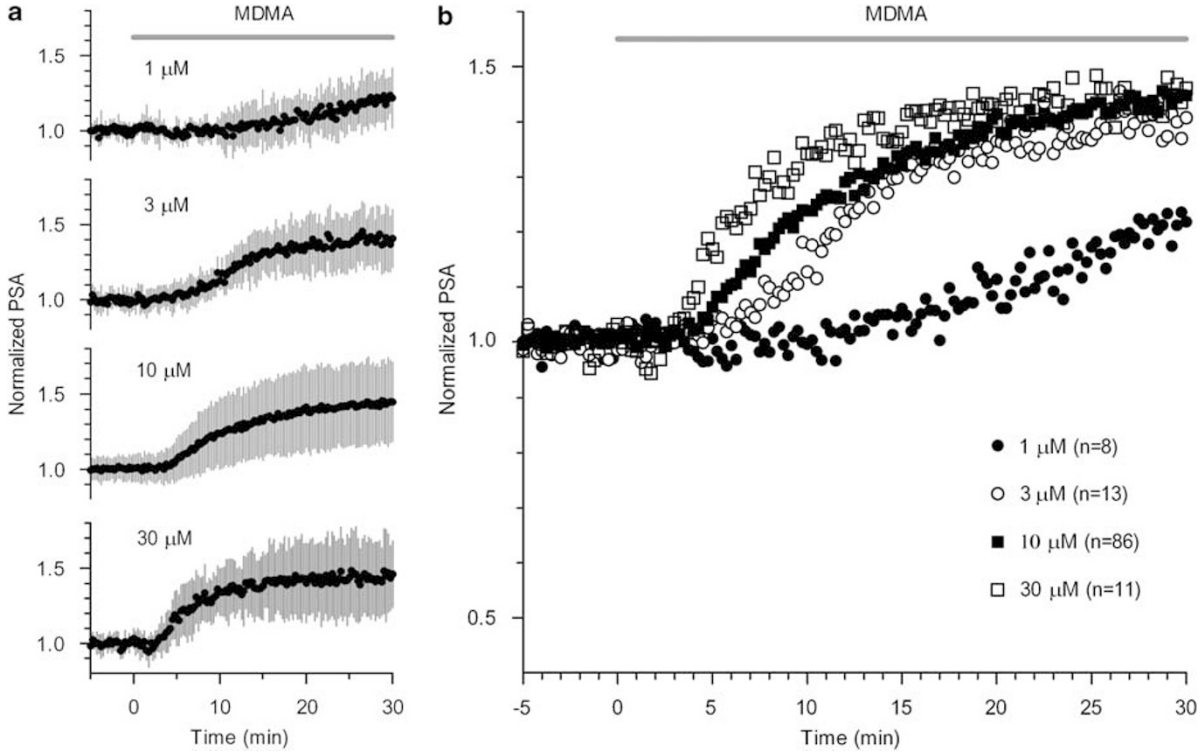

Figure 2 Concentration-dependence of the increase in population spike amplitude (PSA) induced by *N*-methyl-3,4-methylenedioxyamphetamine (MDMA). (a) Summary time–courses of effects of indicated MDMA concentrations on PSA (mean ± SD; $n = 8$, 13, 86, and 11, slices obtained from 6, 7, 47, and 7 animals, respectively). (b) Superimposed mean time–course of the indicated concentrations of MDMA showing faster PSA increase in response to the application of higher MDMA concentrations.

(39.3 ± 19.4%, $n = 13$ from seven animals; significant $p = 0.002$, Wilcoxon test). An increase in MDMA concentration to 10 and 30 μM did not appreciably increase the steady-state effect (mean values of 44.4 and 45.8%, respectively), but reduced the lag before onset of the effect and accelerated its time–course (Figure 2b). The lag before the onset of the effect was approximately 6.5, 5.0, and 3.75 min for MDMA concentrations of 3, 10, and 30 μM, respectively.

We next studied the reversibility and repeatability of the MDMA effect (Figure 3). The PSA increase induced by MDMA persisted after the washout of MDMA and recovered to near-control values only after 2 h of washout. The second MDMA application, after the reversal, induced an increase in PSA similar to the first application (Figure 3a and b). In fourteen experiments, including the above six, 1-h washout of MDMA did not reverse the induced increase in PSA (−12.9 ± 32.9%, $n = 14$ from eight animals; nonsignificant, $p = 0.15$, Wilcoxon test, Figure 3c and d). We have termed EPSP–spike potentiation induced by MDMA (ESP_{MDMA}). Two-hour washout of MDMA reversed the increase in PSA by 80.6 ± 23.9% ($n = 6$ from four animals; significant, $p < 0.05$, Wilcoxon test).

To test for the involvement of interneurons in the action of MDMA, we carried out a set of experiments in the presence of the $GABA_A$ receptor antagonist, bicuculline (Figure 4). In the presence of 10 μM bicuculline, stimulation of the stratum radiatum afferents evoked multiple PS in somatic recordings. Application of MDMA (10 μM, 30 min) in these disinhibited slices significantly increased the amplitude of the first PS in the burst (22.4 ± 26.7%, $n = 22$ from 13 animals; $p < 0.01$, Wilcoxon test) and significantly reduced its latency (−0.560 ± 0.212, $n = 22$ from 13 animals; $p < 0.0001$, Wilcoxon test), suggesting a lack of involvement of GABAergic interneurons in the MDMA action.

ESP_{MDMA} is SERT and NET Dependent

The possible role of serotonergic and noradrenergic systems in the effect of MDMA observed in this study was assessed with citalopram, a high-affinity SSRI and with nisoxetine, a high-affinity NRI (Figure 5). PSA was not affected by a 60 min application of 1 μM citalopram (−1.0 ± 3.3%, $n = 8$ from seven animals), 30-min application of 200 nM nisoxetine (−1.5 ± 4.7%, $n = 10$ from five animals) or by their co-application (30 min; 2.3 ± 5.4%, $n = 8$ from five

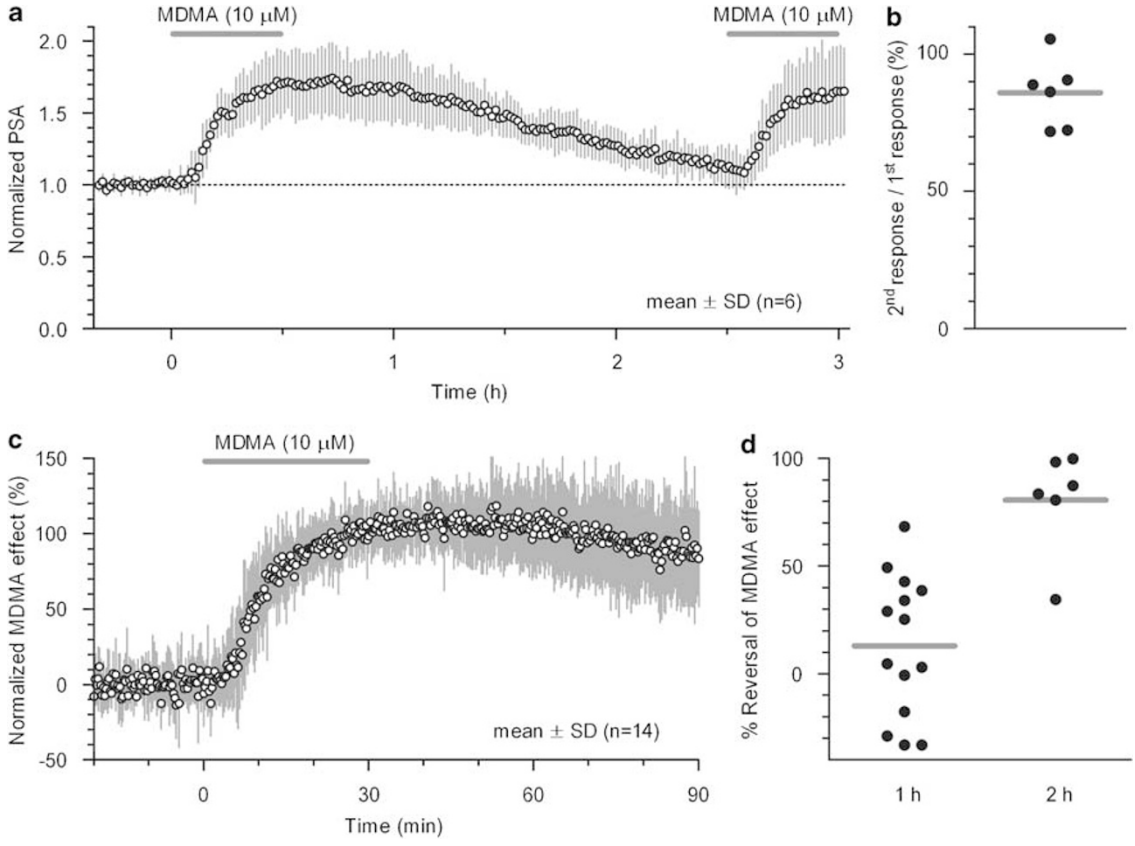

Figure 3 Reversibility and repeatability of the *N*-methyl-3,4-methylenedioxyamphetamine (MDMA) effect. (a) Summary time–course plot of effects on population spike amplitude (PSA) for experiments in which MDMA application was repeated following 2-h washout. Data points are rebinned over 1-min intervals for clarity. (b) Scatter plot shows responses to the second MDMA application relative to responses to the first application for single experiments. (c) Summary time–course plot of effects of MDMA application and the subsequent 1-h washout on the PSA. Data are expressed as the percent of the MDMA effect, normalized such that 0 and 100% correspond to mean PSA recorded over the last 20 min before MDMA application and over the last min of MDMA application. (d) Scatter plot shows reversal of ESP_{MDMA} following the 1-h and 2-h washout in individual experiments. Data were normalized so that the reversal of 0 and 100% corresponds to average PSA values obtained during the last 1 min of MDMA application and during the last 20 min before the MDMA application, respectively.

animals; all three groups: nonsignificant, Wilcoxon test; data not shown), indicating that inhibition of SERT and/or NET does not change PSA in our experimental conditions.

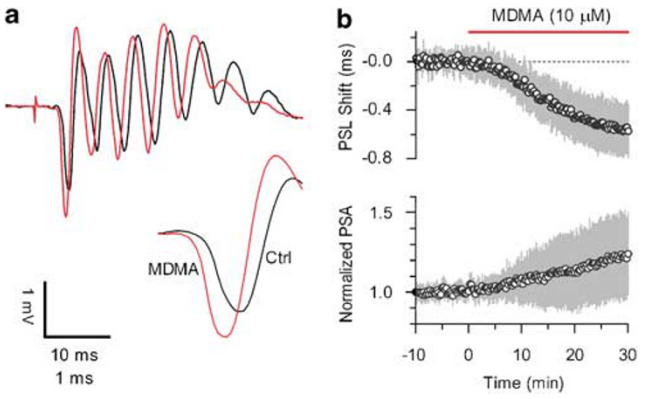

Figure 4 *N*-methyl-3,4-methylenedioxyamphetamine (MDMA) increases population spike amplitude (PSA) by action on pyramidal neurons. (a) Superimposed data traces recorded in the stratum pyramidale showing the effect of MDMA application in continuous presence of the $GABA_A$ antagonist, bicuculline (10 μM). Traces were averaged over 20 sweeps recorded immediately before (black line) and 25–30 min after the beginning of perfusion with 10 μM MDMA (red line). Initial parts of the traces including the first PS of the burst are shown on the expanded scale in the inset. (b) Summary time–courses of effects of application of 10 μM MDMA on the population spike latency (PSL) (above) and PSA (below) in the continuous presence of 10 μM bicuculline (mean ± SD; $n = 22$).

In the presence of 1 μM citalopram, the MDMA-induced PSA increase (10 μM, 30 min; 21.6 ± 15.9%, $n = 22$ from 10 animals) was significantly reduced compared to controls in the absence of reuptake inhibitors (52.3 ± 24.8%, $n = 24$ from 10 animals; $p < 0.001$, Kruskal–Wallis test with Dunn's *post hoc* analysis), indicating partial SERT-dependence of the observed MDMA effect. In the presence of nisoxetine, the PSA increase induced by MDMA (10 μM, 30 min; 44.0 ± 25.8%, $n = 11$ from five animals) was not significantly different from the control, suggesting lack of involvement of the noradrenergic system. On the other hand, in the presence of both 1 μM citalopram and 200 nM nisoxetine, a 30-min application of MDMA did not significantly change the PSA (5.8 ± 9.4%, $n = 10$ from five animals; $p = 0.11$, Wilcoxon test), suggesting an essential role of both SERT and NET in the observed MDMA action.

Effect of 5-HT Depletion on ESP_{MDMA}

Since MDMA is generally considered to be a 'serotonergic' drug, we also wanted to test whether the drug-induced PSA increase in the presence of citalopram was due to residual MDMA action on the serotonergic system. We therefore carried out a set of experiments in slices prepared from animals depleted of 5-HT. Pretreatment of animals for 3 consecutive days with the tryptophan hydroxylase inhibitor PCPA, at the dose of 400 mg kg^{-1} day^{-1} (i.p.), reduced 5-HT content in slices prepared 1 day after the last PCPA

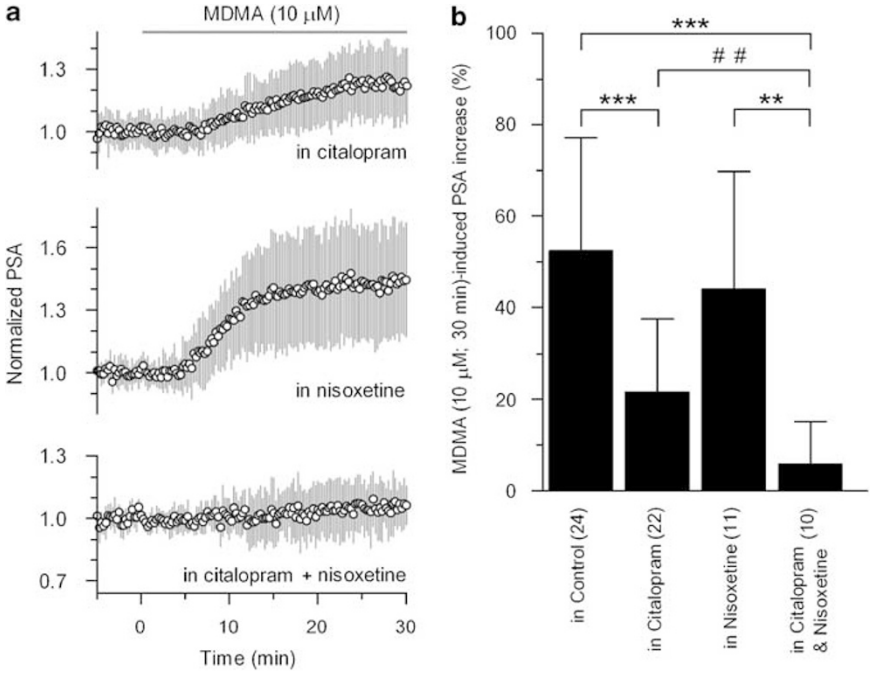

Figure 5 Antagonism of the *N*-methyl-3,4-methylenedioxyamphetamine (MDMA) effect by citalopram and nisoxetine. (a) Summary time–courses (mean ± SD) of the effect of MDMA application on population spike amplitude (PSA) in the presence of 1 μM citalopram ($n = 22$, above), 200 nM nisoxetine ($n = 11$, middle), and both 1 μM citalopram and 200 nM nisoxetine ($n = 10$, bottom). (b) Bar graph shows the effect of 30-min application of 10 μM MDMA on the PSA in the control, in the presence of 1 μM citalopram, 200 nM nisoxetine, and both 1 μM citalopram and 200 nM nisoxetine. The error bars correspond to SD. *n* values are indicated. MDMA-induced PSA increase reached significance in control, in the presence of citalopram and nisoxetine (all three groups: $p \leqslant 0.001$, Wilcoxon test), but not in the presence of both citalopram and nisoxetine ($p = 0.11$, Wilcoxon test). The groups are significantly different ($p < 0.0001$, Kruskal–Wallis test). Dunn's multiple comparison *post hoc* analysis revealed significantly different effects of MDMA between citalopram and control groups (***$p < 0.001$), between citalopram + nisoxetine and control groups (***$p < 0.001$) and between nisoxetine and citalopram + nisoxetine groups (**$p < 0.01$). Although Dunn's analysis revealed no significant difference between citalopram and citalopram + nisoxetine groups, the Mann–Whitney test showed a significant difference ($^{\#\#}p < 0.01$).

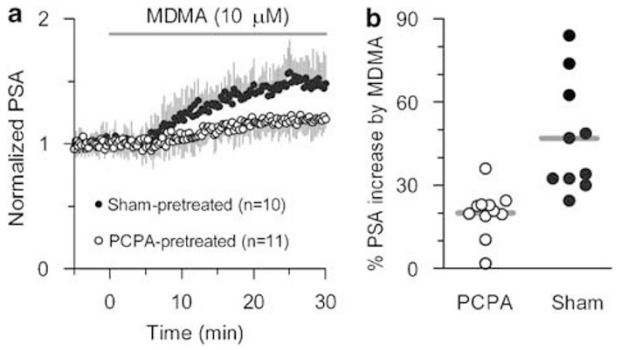

Figure 6 p-Chlorophenylalanine (PCPA)-pretreatment reduces, but does not abolish the increase in population spike amplitude (PSA) induced by *N*-methyl-3,4-methylenedioxyamphetamine (MDMA). (a) Superimposed are summary time–courses (mean ± SD) of the effect of MDMA on PSA in slices obtained from PCPA-pretreated (open circles) and sham-operated (filled circles) rats. (b) Scatter plot shows the effect of PCPA and sham pretreatment on the increase in PSA induced by a 30-min application of 10 μM MDMA. MDMA significantly increased PSA in both groups (PCPA = 20.0 ± 8.5%, $n = 11$ from five animals; Sham = 47.0 ± 20.3%, $n = 10$ from five animals; Wilcoxon signed rank test, PCPA: $p < 0.001$; Sham: $p = 0.002$). The effect of MDMA is significantly different between the groups (Mann–Whitney test, $p < 0.001$).

injection by 98% compared to 5-HT content in slices from sham-pretreated animals (5-HT: 0.58 ± 0.27 pmol slice^{-1}, $n = 3$ *vs* 29.7 ± 3.2 pmol slice^{-1}, $n = 3$). On the other hand, pretreatment with PCPA did not markedly affect the NE content when compared to sham-pretreated controls (NE: 11.7 ± 7.2 pmol slice^{-1}, $n = 3$ *vs* 14.0 ± 2.8 pmol slice^{-1}, $n = 3$). In slices obtained from PCPA-pretreated animals, a 30-min perfusion of 10 μM MDMA increased the PSA by 20.0 ± 8.5% (Figure 6, $n = 11$ from five animals; significant, $p < 0.001$, Wilcoxon test). These results suggest that MDMA-induced PSA increase is, in part, mediated by a non-serotonergic mechanism.

Receptor Pharmacology of MDMA Effects

To identify receptors mediating MDMA-induced PSA increase, we examined the effects of application of single receptor antagonists on MDMA-induced response. The response to MDMA application was excitatory and long lasting, resembling that observed after activation of 5-HT$_4$ (Mlinar *et al*, 2006) and β-adrenergic (Heginbotham and Dunwiddie, 1991) receptors. Since 5-HT$_6$ and 5-HT$_7$ receptors also produce excitatory effects in CA1 pyramidal cells through activation of adenylate cyclase, selective antagonists for these receptors, as well as for the inhibitory 5-HT$_{1A}$ receptor, were included in our study. The possible contribution of other 5-HT receptors was not assessed because their activation does not produce direct, electrophysiologically detectable effects on CA1 pyramidal cells (Andrade, 1998).

As shown in Figure 7a, the MDMA-induced PSA increase was not significantly affected by the presence of 5-HT$_{1A}$ receptor antagonist, Way-100635 (50 nM; $n = 21$ from 10 animals), 5-HT$_6$ receptor antagonist, SB-258585 (200–500 nM; $n = 10$ from seven animals) or 5-HT$_7$ receptor antagonists, SB-269970 (500 nM; $n = 8$ from five animals). On the contrary, in the presence of 5-HT$_4$ receptor antagonist, RS-23597-190 (10 μM), MDMA did not change the PSA (0.0 ± 7.4%, $n = 8$ from five animals; nonsignificant, Wilcoxon test), a result significantly different from the control (45.2 ± 24.9%, $n = 31$ from 16 animals; $^{***}p < 0.001$, Dunn's test). Although the MDMA-induced PSA increase reached significance (13.3 ± 14.7%, $n = 12$ from six animals; $^{**}p < 0.01$, Wilcoxon test) in the presence of the other 5-HT$_4$ receptor antagonist, SB-203186 (1 μM), it was also significantly reduced compared to the control ($^{**}p < 0.01$, Dunn's test). Finally, MDMA did not change PSA (5.8 ± 23.5%, $n = 11$ from six animals; nonsignificant, Wilcoxon test) in the presence of β_1 receptor antagonist, CGP-20712 (200 nM), a result significantly different from the control ($^{**}p < 0.01$, Dunn's test). These experiments indicated involvement of both 5-HT$_4$ and β_1 receptors in the PSA increase induced by MDMA.

We next tested if the MDMA-induced PSA increase may be prevented by co-application of 5-HT$_4$ and β_1 receptor antagonists. In the presence of SB-203186 (1 μM) and CGP-20712 (200 nM), the excitatory effect of MDMA was absent and, moreover, an inhibitory component of MDMA response was revealed (Figure 7b). In these experiments, a 30-min application of 10 μM MDMA reduced the PSA by 16.4 ± 21.8% ($n = 12$ from seven animals; significant, $^{***}p < 0.001$, Wilcoxon test). This inhibitory component of the MDMA response was absent in experiments in which Way-100635 (50–100 nM) was included in the antagonist mixture (3.4 ± 18.5% PSA increase, $n = 8$ from five animals; not significant, Wilcoxon test), indicating that the inhibitory component of the MDMA response is mediated by 5-HT$_{1A}$ receptors. Next, we tried to isolate 5-HT$_4$ and β_1 receptor-mediated components of MDMA-induced PSA increase by co-applying mixtures of either β_1 and 5-HT$_{1A}$ receptor antagonists or 5-HT$_4$ and 5-HT$_{1A}$ receptor antagonists, respectively (Figure 7c). In the presence of CGP-20712 (200 nM) and Way-100635 (50 nM), a 30-min perfusion of MDMA (10 μM) increased the PSA by 48.7 ± 29.6% ($n = 32$ from 12 animals; significant $^{***}p < 0.0001$, Wilcoxon test), while in the presence of SB-203186 (0.5–1 μM) and Way-100635 (50 nM), the increase was 16.3 ± 16.0% ($n = 10$ from five animals; significant $^{**}p < 0.01$, Wilcoxon test). These results suggest a predominant role of 5-HT$_4$ receptors in ESP$_{MDMA}$.

In the final set of experiments we wanted to address whether ESP$_{MDMA}$, after being fully induced by 30-min MDMA perfusion, can be reversed to the pre-MDMA level by the addition of 5-HT$_4$ and/or β_1 receptor antagonists (Figure 8). In these experiments, ESP$_{MDMA}$ was not significantly reversed by the 30 min addition of a single 5-HT$_4$ or β_1 receptor antagonist to the perfusate (Wilcoxon test). Reversal of ESP$_{MDMA}$ caused by the addition of RS-23597-190 (10 μM), SB-203186 (1 μM), and CGP-20712 (200 nM) was 3.2 ± 17.3% ($n = 9$ from five animals), 4.6 ± 34.8% ($n = 9$ from five animals), and 2.1 ± 22.4% ($n = 7$ from four animals), respectively (Figure 8c). On the other hand, ESP$_{MDMA}$ was significantly reversed in experiments in which both 5-HT$_4$ and β_1 receptor antagonists were added to the perfusate. Thus, co-application of RS-23597-190 (10 μM) and CGP-20712 (200 nM) reversed the ESP$_{MDMA}$ by 33.1 ± 20.1% ($n = 6$ from three animals; $^{*}p < 0.05$, Wilcoxon test), while co-application of SB-203186 (1 μM) and CGP-20712 (200 nM) reversed it by 53.3 ± 22.0%

($n = 10$ from five animals; $^{**}p < 0.01$, Wilcoxon test; Figure 8c).

DISCUSSION

In experiments in which population responses in the CA1 region of the ventral hippocampus were evoked by stimulation of the Schaffer collateral/commissural pathway, MDMA potentiated the fEPSP–PS coupling without affecting the fEPSP slope. Since the fEPSP slope, recorded at the level of afferent synapses, principally reflects synaptic transmission while PS also depends on postsynaptic neuronal processing, these results indicate that MDMA modulates signal processing in the CA1 region without affecting synaptic input from the CA3 region. This net excitatory effect of MDMA was also observed in the experiments in which contribution of interneuronal activity to CA1 signal processing was blocked by antagonism of $GABA_A$ receptors, suggesting that the change in intrinsic excitability of pyramidal cells is principally responsible for ESP_{MDMA}.

N-methyl-3,4-methylenedioxyamphetamine was clearly effective at 1 μM, the lowest concentration tested, and induced a nearly maximal response when applied at a concentration of 3 μM. This dose range corresponds to the plasma MDMA concentration reached in humans following the consumption of a typical MDMA dose, ie 100–150 mg (de la Torre *et al*, 2000; Irvine *et al*, 2006), suggesting that our observed increase in excitability of CA1 pyramidal cells may be relevant to the psychoactive effects of the drug in humans.

Neuromodulators Mediating MDMA Action

N-methyl-3,4-methylenedioxyamphetamine, besides releasing endogenous monoamine neuromodulators by acting on their plasma membrane transporters, has relatively high affinity ($K_i < 6$ μM) for 5-HT_2, α_2-adrenergic, H-1 histamine and M-1 muscarinic receptors (Battaglia *et al*, 1988). MDMA also activates a trace amine receptor with an EC_{50} of 1.7 μM (Bunzow *et al*, 2001) and, in cultured rat hippocampal neurons, increases excitability by direct block of a K^+ channel with an IC_{50} at 11.8 μM (Premkumar and Ahern, 1995). Therefore, we sought to determine whether the observed MDMA effect was mediated by the release of endogenous monoamines or by direct activation of pyramidal cell receptors. To test for the involvement of the serotonergic system, MDMA was applied following selective block of SERT by 1 μM citalopram. In these experiments, the MDMA-induced PSA increase was significantly reduced when compared to the control experiments, but was significant, indicating that 5-HT release contributes, but is not solely responsible for MDMA action in the CA1 region of the ventral hippocampus. The involvement of the noradrenergic system was tested in

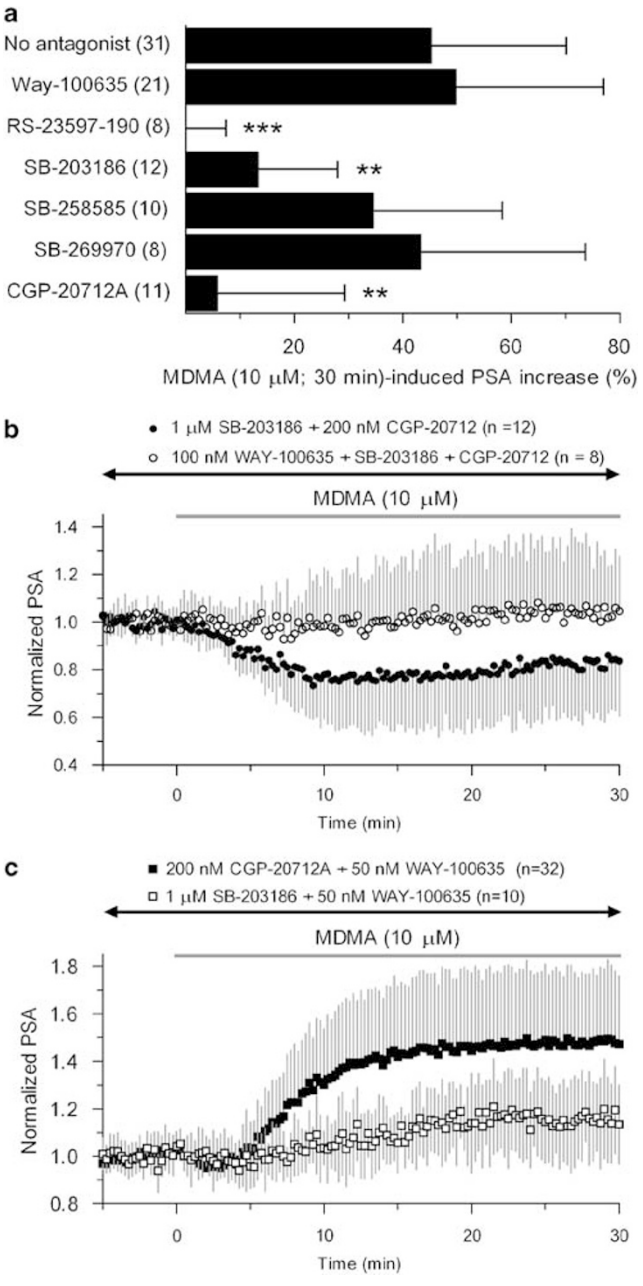

Figure 7 Receptor pharmacology of the *N*-methyl-3,4-methylenedioxyamphetamine (MDMA) response. (a) Bar chart shows the effect of the presence of single neurotransmitter receptor antagonists on the increase in population spike amplitude (PSA) induced by 10 μM MDMA. In the presence of 5-HT_4 receptor antagonists, RS-23597-190 (10 μM) or SB-203186 (1 μM), or the β_1 receptor antagonist, CGP-20712 (200 nM), the effect of a 30-min application of 10 μm MDMA was significantly reduced compared to the control group (Kruskal–Wallis test $^{***}p < 0.001$; Dunn's multiple comparisons *post hoc* test, $^{***}p < 0.001$; $^{**}p < 0.01$), indicating that 5-HT_4 and β_1 receptors both mediate the MDMA action. The effect of MDMA did not significantly change in the presence of single 5-HT_{1A}, 5-HT_6, or 5-HT_7 receptor antagonists, Way-100635 (50 nM), SB-258585 (200–500 nM), and SB-269970 (500 nM). *n* values are indicated. (b) Summary time–course (mean ± SD) of the MDMA effect on PSA in the presence of both 1 μM SB-203186 and 200 nM CGP-20712 reveals an inhibitory component of the MDMA effect (filled circles). This inhibitory effect was prevented in experiments in which Way-100635 (50–100 nM) was added to the antagonist mixture (open circles). (c) Superimposition of summary time–courses (mean ± SD) of the MDMA effect on PSA in the presence of indicated antagonist pairs shows excitatory components of the MDMA effect mediated by 5-HT_4 receptors (filled squares) and by β_1 receptors (open squares).

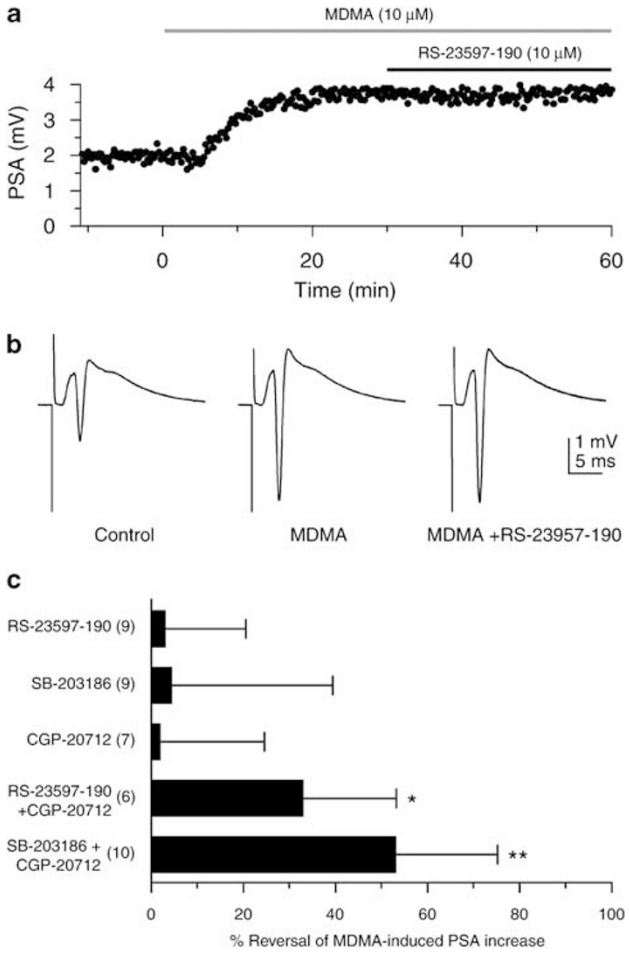

Figure 8 Persistence of ESP_{MDMA} is only partially due to tonic activation of 5-HT_4 and β_1 receptors. (a) Time–course plot of a representative experiment in which reversal of *N*-methyl-3,4-methylenedioxyamphetamine (MDMA)-induced PSA increase was attempted by the addition of the 5-HT_4 receptor antagonist, RS-23597-190 (10 μM). (b) Data traces from the above experiment. Traces were averaged over the last 20 sweeps recorded in control (left), in MDMA (middle) and in MDMA + RS-23597-190 (right). (c) Bar graph showing the reversal of MDMA-induced population spike amplitude (PSA) increase (mean ± SD) following 25 to 30-min addition of 5-HT_4 and/or β_1 receptor antagonists. In individual experiments, data were normalized so that 0 and 100% reversals correspond to average PSA values obtained during 25–30 min of MDMA application and during the last 5 min before MDMA application, respectively. *n* values are indicated. $^*p<0.05$, $^{**}p<0.01$ (Wilcoxon test).

experiments in which MDMA was applied in the presence of the NRI, nisoxetine. In these experiments the effect of MDMA, although on the average smaller, was not significantly different from control, suggesting a lack of involvement of the noradrenergic system. However, in experiments in which both SERT and NET were blocked by co-application of citalopram and nisoxetine, MDMA produced no significant effect, suggesting that release of both neuromodulators contributes to the observed effect. The finding that MDMA did not appreciably change PSA in conditions where both transporters were inhibited (Figure 5, citalopram + nisoxetine group), besides indicating involvement of both SERT and NET, also suggests a lack of direct action of MDMA on pyramidal cells. The lag of several minutes before the onset of MDMA effect in the absence of pharmacological pretreatment (Figure 2) is also consistent with an indirect mode of MDMA action.

N-methyl-3,4-methylenedioxyamphetamine-induced increase in the extracellular 5-HT concentration might be, in part, caused by SERT-independent and therefore citalopram-resistant mechanisms, ie by MDMA effects on serotonergic vesicles (Rudnick and Wall, 1992; Schuldiner *et al*, 1993; Mlinar and Corradetti, 2003) and/or on monoamine oxidase type A (Leonardi and Azmitia, 1994; Scorza *et al*, 1997). To additionally test whether the observed effect of MDMA has a nonserotonergic component, we carried out experiments on brain slices obtained from animals in which 5-HT was depleted by PCPA-pretreatment. In these experiments MDMA still significantly increased PSA (~20%, Figure 6), confirming a nonserotonergic component of MDMA effect in the CA1 region of the ventral hippocampus. The finding that MDMA acts via release of both 5-HT and NE is consistent with observations that MDMA is essentially equipotent at 5-HT and NE release from rat-brain slices (EC_{50} values = 4.5 and 1.9 μM, respectively; Fitzgerald and Reid, 1993) and from rat-brain synaptosomal preparations (IC_{50} values = 56.6 and 77.4 nM, respectively; Rothman *et al*, 2001). Our results further extend the findings of these studies, demonstrating the contribution of NE release to functional MDMA effects. Importantly, in humans, NE release might contribute to MDMA effects even more significantly since MDMA has a higher affinity ratio for human NET *vs* human SERT (Verrico *et al*, 2007).

Receptor Pharmacology of MDMA Effects

Activation of several subtypes of 5-HT and NE receptors expressed in CA1 pyramidal cells may increase intrinsic neuronal excitability (Mueller *et al*, 1981; Madison and Nicoll, 1986; Mongeau *et al*, 1997; Andrade, 1998). Experiments in which individual receptor antagonists were applied before MDMA indicate involvement of 5-HT_4 and β_1 receptors in MDMA action. Interestingly, block of either receptor was sufficient to prevent MDMA-induced PSA increase (Figure 7a), suggesting that activation of both receptors is required for induction of PSA increase by MDMA. That this is not the case was revealed in the experiments in which both receptors were blocked by co-application of their antagonists. In these experiments (Figure 7b), application of MDMA caused small, but significant PSA reduction, suggesting involvement of at least one additional receptor. The addition of selective 5-HT_{1A} receptor antagonist, Way-100635, to the antagonist mixture prevented PSA reduction by MDMA, revealing that activation of 5-HT_{1A} receptors mediates this inhibitory component of MDMA action. Thus, the increase in PSA induced by MDMA is caused by opposing actions mediated by simultaneous activation of two excitatory (5-HT_4 and β_1), and one inhibitory (5-HT_{1A}) receptor. The overall effect of MDMA on electrical properties of CA1 pyramidal cells is even more complex since the release of 5-HT induced by MDMA, in addition to the effect on fEPSP–PS coupling also activates 5-HT_{1B} receptors in pyramidal cells, thus inhibiting their output (Mlinar and Corradetti, 2003). In summary, our pharmacological study shows that in the CA1 region of the ventral hippocampus, MDMA induces SERT- and

NET-dependent release of 5-HT and NE, which in turn activate 5-HT and NE receptors, producing an overall increase in pyramidal cell excitability. The excitatory component of MDMA action is due to converging effects of 5-HT_4 and β_1 receptor activation with a predominant role of 5-HT4 receptor.

The Characteristics of ESP_{MDMA}

The most interesting aspect of MDMA action observed in this study is that ESP_{MDMA} persists for at least 1 h after removal of MDMA. As shown in Figure 3, ESP_{MDMA} slowly reversed ($\sim$13% in 1 h; $\sim$80% in 2 h) upon prolonged washout of MDMA.

The reversal was not due to depletion of endogenous neuromodulators since the second application of MDMA, following a 2-h washout, was still able to induce an effect similar to that of the first application (Figure 3a and b). Several mechanisms might account for the persistence of MDMA effect after removal of the drug from the superfusing ACSF. One potential mechanism is by entrapment of MDMA inside serotonergic and noradrenergic axons where it could continue to release 5-HT and NE. Alternatively, acute activation of 5-HT_4 and β_1 receptors by 5-HT and NE, released during the MDMA application, could induce the observed long-lasting effects in pyramidal cells by processes downstream of 5-HT_4 and β_1 receptors. We attempted to discriminate between these two possibilities by applying 5-HT_4 and β_1 receptor antagonists after inducing the potentiation. Thus, if entrapment of MDMA in monoaminergic axon terminals underlies ESP_{MDMA}, application of 5-HT_4 and β_1 receptor antagonists after inducing ESP_{MDMA}, should reverse the effect of MDMA. On the other hand, if processes downstream of 5-HT_4 and β_1 receptors are responsible for persistence of the effect, application of the antagonists after induction of ESP_{MDMA} should not reverse the MDMA effect. As shown in Figure 8, individual application of neither 5-HT_4 nor β_1 receptor antagonist reversed the effect of MDMA, suggesting the involvement of processes downstream of 5-HT_4 and β_1 receptors. However, co-application of 5-HT_4 and β_1 receptor antagonists partially reversed the effect of MDMA, indicating that entrapment of MDMA in monoaminergic axon terminals may also contribute to persistence of the effect. The finding that 5-HT_4 and β_1 receptor antagonists, when individually applied, do not affect ESP_{MDMA}, but when co-applied, partially reverse it, further indicates that activation of either receptor type is sufficient to produce ESP. Overall, our experiments suggest that ESP_{MDMA} is caused both by processes in monoaminergic axons and by processes in pyramidal cells, downstream of 5-HT_4 and β_1 receptors.

Consistent with the involvement of postsynaptic mechanisms in ESP_{MDMA} are previous observations of similar long-lasting effects induced by activation of β_1 and 5-HT_4 receptors by direct agonist application (Heginbotham and Dunwiddie, 1991; Mlinar *et al*, 2005). The present study extends these findings by showing that ESP can also be induced by the release of endogenous 5-HT and NE. Interestingly, the potentiation induced by MDMA, was reversed almost completely upon 2-h washout while, in most experiments where 5-HT_4 and β_1 receptors were directly activated by agonists, ESP was reversed significantly less over the same period. Presently, available data do not indicate the causes of this discrepancy. We speculate that activation of 'physiological' 5-HT_4 and β_1 receptors, which are normally stimulated by endogenous 5-HT and NE and, in this study, indirectly activated following MDMA application, produces a more reversible response than activation of 'pharmacological' 5-HT_4 and β_1 receptors which are activated by exogenous agonist application.

Neuropsychological Implications of Acute MDMA Action in Ventral Hippocampus

The MDMA-induced long-lasting ESP in pyramidal cells observed in the present study likely translates to an increase in ventral hippocampal activity *in vivo*. Consistently, hippocampal activation following oral administration of 1 mg/kg MDMA was recently observed in conscious monkeys (Brevard *et al*, 2006). Ventral hippocampus activity could be relevant to the psychoactive effects of MDMA since hippocampal activation has been shown in mood- and emotion-related behaviors. Thus, positive social stimuli activate the hippocampus in normal subjects, but not in depressed patients (Schaefer *et al*, 2006) and sustained hippocampal activation occurs during a period of subjective emotion (Garrett and Maddock, 2006). Increase in hippocampal activity has been associated to encoding of emotional stimuli, which implicates interaction between the amygdala and the hippocampus (Dolcos *et al*, 2004; Richardson *et al*, 2004) and involves stimulation of hippocampal β_1-adrenergic receptors (Strange and Dolan, 2004). Finally, 5-HT4 receptor polymorphism has been implicated in vulnerability to mood disorders (Ohtsuki *et al*, 2002). It is, therefore, conceivable that MDMA-induced coactivation of β_1 and 5-HT_4 receptors in the ventral hippocampus contributes to acute psychoactive drug effects in humans such as positive mood changes, enhanced empathy, and increased social interaction.

In addition, the activation of the ventral hippocampus could contribute to motivational and reinforcing effects of MDMA. In fact, local stimulation of the ventral hippocampus by *N*-methyl-D-aspartate infusion in rats activates dopamine transmission to the medial prefrontal cortex (Peleg-Raibstein *et al*, 2005), produces an acute increase in extracellular dopamine levels (Peleg-Raibstein and Feldon, 2006), and stimulates c-Fos expression (Bardgett and Henry, 1999) in the nucleus accumbens shell, a region playing a key role in motivational behavior and in the reinforcing effects of stimulant drugs (Robbins and Everitt, 1996).

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DISCLOSURE/CONFLICT OF INTERESTS

The authors have no potential conflict of interests to disclose.

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