Involvement of 5-HT2A receptors in MDMA reinforcement and cue-induced reinstatement of MDMA-seeking behaviour

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Abstract

The serotonergic system appears crucial for (±)-3,4-methylenedioxymethamphetamine (MDMA) reinforcing properties. Current evidence indicates that serotonin 5-HT2A receptors (5-HT2ARs) modulate mesolimbic dopamine (DA) activity and several behavioural responses related to the addictive properties of psychostimulants. This study evaluated the role of 5-HT2ARs in MDMA-induced reinforcement and hyperlocomotion, and the reinstatement of MDMA-seeking behaviour. Basal and MDMA-stimulated extracellular levels of DA in the nucleus accumbens (NAc) and serotonin and noradrenaline in the pre-frontal cortex were also assessed. Self-administration of MDMA was blunted in 5-HT2AR knockout (KO) mice compared to wild-type (WT) littermates at both doses tested (0.125 and 0.25 mg/kg per infusion). Horizontal locomotion was increased by MDMA (10 and 20 mg/kg i.p.) to a higher extent in KO than in WT mice. DA outflow in the NAc was lower in KO compared to WT mice under basal conditions and after MDMA (20 mg/kg) challenge. In WT mice, MDMA (5 and 10 mg/kg i.p.) priming did not reinstate MDMA-seeking behaviour, while cue-induced reinstatement was prominent. This cue-induced reinstatement was blocked by administration of the selective 5-HT2AR antagonist, SR46349B (eplivanserin) at a dose of 0.5 mg/kg, but not at 0.25 mg/kg. Our results indicate that 5-HT2ARs are crucial for MDMA-induced reinforcement and cue-induced reinstatement of MDMA-seeking behaviour. These effects are probably due to the modulation of mesolimbic dopaminergic activity.

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Introduction

3,4-Methylenedioxymethamphetamine (MDMA), commonly known as ‘ecstasy’, is widely abused despite its known adverse neuropsychological consequences (Parrott et al. 2000; Parrott, 2001). In humans, MDMA induces an entactogenic state with increased self-confidence, emotional excitation and sensorimotor activation, as well as several physiological changes that include hyperthermia, and increased blood pressure and heart rate (Liechti et al. 2000; Vollenweider et al. 2002). While the reinforcing properties of MDMA are well documented, its addictive potential is still a matter of debate. However, there is evidence showing that a significant proportion of MDMA users meet several DSM-IV criteria for dependence (Cottler et al. 2001; Leung & Cottler, 2008; Stone et al. 2006). Notwithstanding these data, some aspects of MDMA addictive behaviour such as craving and relapse to drug-seeking have not been fully addressed in basic or clinical studies.

Acute MDMA has a complex mechanism of action, which involves an increase in extracellular levels of dopamine (DA), noradrenaline (NE), and serotonin (5-HT) (Green et al. 2003), and differs from other amphetamines in that it primarily affects the serotonergic system by acting on the serotonin transporter (SERT) (Han & Gu, 2006; Trigo et al. 2007). While the
involvement of the 5-HT system in the hyperlocomotor and hyperthermic effects of MDMA is well established, the relative participation of the different 5-HT receptor subtypes in these responses is still not well understood. Both 5-HT1A and 5-HT1B receptors are located pre- and post-synaptically (Depoortere et al. 2010; Peddie et al. 2008), and have been implicated in MDMA-induced hyperlocomotion, sensitization and tolerance (Green et al. 2003). 5-HT2A and 5-HT2C receptors are located post-synaptically in several brain areas of the mesostriatal and mesocorticlimbic systems of humans and rodents (Andree et al. 1998; Ito et al. 1998; Lopez-Gimenez et al. 1997), where they modulate dopaminergic activity (Navailles et al. 2008; Porras et al. 2002). Accordingly, an increasing amount of data points to the participation of 5-HT2A Rs in the addictive properties of psychostimulants. Thus, pre-treatment with the selective 5-HT2AR antagonist, MDL100907 blocked priming-induced (Fletcher et al. 2002a) and cue-induced (Nic Dhonnchadha et al. 2009) reinstatement of cocaine-seeking behaviour in rats, without modifying its reinforcing effects in rats (Fletcher et al. 2002a) and non-human primates (Fantegrossi et al. 2002). In contrast, MDL100907 partially attenuated the response to S(+)-MDMA, and abolished the response to R(−)-MDMA in non-human primates (Fantegrossi et al. 2002). Moreover, studies in humans using the non-selective 5-HT2A/C antagonist ketanserin, report reductions in MDMA-induced perceptual changes and emotional excitation (Liechi et al. 2000). Thus, there is still a lack of knowledge on the exact role of 5-HT2A Rs in MDMA-induced reinforcement and no data are available regarding their involvement in relapse to MDMA-seeking behaviour.

Pharmacological studies investigating the participation of 5-HT2A Rs in the locomotor-activating effects of MDMA have generated inconsistent results depending on the doses or the animal species used (Banks & Cunningham, 2002; Fantegrossi et al. 2003; Herin et al. 2005; Kehne et al. 1996). Studies using genetically modified mice have shown that locomotor activity induced by amphetamine is enhanced in 5-HT2AR knock-out (KO) mice. This sensitized response was related to an enhanced activation of the noradrenergic system in the prefrontal cortex (PFC) (Salomon et al. 2007).

In the present study, we used a multidisciplinary approach to investigate the contribution of 5-HT2AR Rs on MDMA behavioural and neurochemical effects. Thus, we evaluated the reinforcing properties of MDMA in 5-HT2AR KO and wild-type (WT) littermates using a self-administration paradigm, and examined instrumental responding for food reward. We also evaluated MDMA-induced hyperlocomotion in these mice. Using parallel groups, but from the same litters as the behavioural studies, we performed in-vivo microdialysis experiments to assess extracellular levels of DA in the nucleus accumbens (NAc), as well as NE and 5-HT levels in the PFC before and after MDMA administration in 5-HT2AR KO and WT littermates. In pharmacological experiments, we investigated whether acute blockade of 5-HT2ARs with SR46349B (eplivanserin) was able to modify cue- and priming-induced reinstatement of MDMA-seeking in mice that had previously extinguished MDMA self-administration behaviour.

Material and methods

**Animals**

The 5-HT2AR KO and WT littermates used in our study were originally generated at Columbia University (USA) on a 129S6/SvEv background (Gonzalez-Maeso et al. 2003, 2007). Subsequently, they were backcrossed over at least ten generations onto the inbred C57BL/6J line. Male and female 5-HT2AR KO and WT mice were genotyped as described by (Fiorica-Howells et al. 2002), and only male mice were used in the studies. Pharmacological experiments were performed on C57BL/6J mice (Charles River, France). Mice weighing 25–30 g at the beginning of the experiments were initially housed five per cage in a room with controlled temperature (21 ± 1°C) and humidity (65 ± 10%). The animals were maintained on a 12-h light/dark cycle (lights on 20:00 hours). Food and drug self-administration experiments took place during the dark cycle, while the other experiments were performed during the light phase. Food was restricted during the food-conditioning experiments, but water was available ad libitum. Behavioural tests and animal care were conducted in accordance with the standard ethical guidelines (National Institutes of Health, 1995; European Communities Directive 86/609 EEC) and approved by the local ethical committee (CEEA-PRBB).

**Drugs**

MDMA hydrochloride was obtained from Lipomed, A.G. (Arlesheim, Switzerland). SR 46349B micronized hemifumarate [(1Z,2E)-1-(2-fluoro-phenyl)-3-(4-hydroxyphenyl)-prop-2-en-one-O-(2-dimethylamino-ethyl)-oxime hemifumarate] was generously provided by Sanofi-Aventis (France). All drugs were dissolved in 0.9% physiological saline.
**Locomotor activity**

Locomotor activity was measured using individual locomotor activity boxes (9 x 20 x 11 cm; Imetronic, France). The boxes were equipped with two lines of 14 photocells (2 cm and 6 cm above the floor), and with a fan providing white noise. The boxes were placed in a room with low luminosity (5 lx). Animals received an acute administration of MDMA (10 and 20 mg/kg i.p), and were immediately placed in the locomotor activity boxes. Locomotor activity was measured in 10-min bins for 2 h.

**MDMA self-administration procedure**

See online Appendix (Supplement 1).

**Food-maintained operant behaviour**

See online Appendix (Supplement 2).

**Microdialysis, HPLC analytical procedure and histology**

See online Appendix (Supplement 3).

**Reinstatement of MDMA-seeking behaviour**

C57BL/6J mice were trained to respond for intravenous infusions of MDMA (0.25 mg/kg per infusion) on a FR1 schedule of reinforcement (see above for methodology). Animals that achieved acquisition criteria (as described above) underwent extinction sessions, which lasted until responding on the active hole decreased to < 40% of the stable acquisition mean during two consecutive days, and until no discrimination between active and inactive holes was observed. The cue-light associated with drug self-administration was not present during extinction sessions. After reaching extinction, one group of animals received saline 30 min before each of the following reinstatement conditions according to a within-subject design: (1) a priming injection of saline (control relapse) or MDMA (10 mg/kg i.p.) (priming relapse) without the cue-light, (2) presentation of the cue-light alone (cue relapse), (3) both MDMA-priming plus cue-light (cue + priming relapse). Two additional groups received eplivanserin (at a dose of 0.25 or 0.50 mg/kg i.p.) 30 min before each of the reinstatement conditions described for the first group according to a within-subject design (control relapse, priming relapse, cue relapse, cue + priming relapse). The doses of eplivanserin were chosen based on previous studies in mice using d-amphetamine (Auclair et al. 2004b). Extinction sessions were performed following each reinstatement test, and animals were exposed to the next reinstatement condition only when they reached the same extinction criteria described for the first extinction session.

**Statistical analysis**

The locomotor, MDMA self-administration and food-maintained operant responding data were analysed using three-way repeated-measures ANOVA followed by post-hoc tests. Discrimination between holes during acquisition and differences between genotypes in the breaking points were analysed using one-way ANOVA. Basal and stimulated extracellular levels of DA, NE and 5-HT were analysed between genotypes using one- and two-way ANOVA followed by post-hoc analysis. The reinstatement data were analysed using three-way repeated-measures ANOVA with phase and hole as within-subjects factors and treatment (saline or eplivanserin) as between-subjects factors, followed by post-hoc analysis. In order to compare the different reinstatement conditions in each within-subjects design, a repeated-measures ANOVA followed by pairwise comparisons was applied. A two-way between-subjects ANOVA (treatment x hole) was additionally used to analyse dose-related effects of eplivanserin on each reinstatement phase.

**Results**

**MDMA-induced locomotor activity in 5-HT2A KO and WT mice**

The acute administration of MDMA increased horizontal activity in WT and KO mice at both doses tested (10 and 20 mg/kg) with respect to saline injection. However, this effect was significantly greater in 5-HT2A KO mice than in WT littermates (see Fig. 1 for details).

**MDMA self-administration in 5-HT2A KO and WT littermates**

MDMA self-administration behaviour for the doses of 0.125 and 0.25 mg/kg per infusion in WT and KO mice is shown in Fig. 2. Three-way repeated-measures ANOVA (genotype x hole x day) for 0.125 mg/kg per infusion revealed a significant main effect of hole (\(F_{1,14} = 16.690, p < 0.001\)) and day (\(F_{9,126} = 2.660, p < 0.01\)), and a significant two-way genotype x hole interaction (\(F_{1,14} = 5.091, p < 0.05\)). Subsequent comparisons between active and inactive responding in WT mice revealed significant differences in discrimination from day 5 to day 10 of training (Fig. 2a). No significant effects of genotype or genotype x day interaction were observed, revealing no differences.
Fig. 1. Dose-related effects of MDMA on locomotor activity in wild-type (WT) and 5-HT<sub>2A</sub>R knockout (KO) mice. Mice were treated with 10 mg/kg MDMA [WT MDMA 10 (n = 15), KO MDMA 10 (n = 12)], 20 mg/kg MDMA [WT MDMA 20 (n = 9), KO MDMA 20 (n = 10)] or saline [WT Saline (n = 16), KO Saline (n = 14)]. Horizontal locomotion (average ± S.E.M. photocell counts during 120 min) was increased in KO mice to a greater extent than in WT mice. Three-way repeated-measures ANOVA (dose × genotype × time) revealed a significant main effect of time (F<sub>11,770</sub> = 131.766, p < 0.001), dose (F<sub>2,70</sub> = 60.654, p < 0.001), and genotype (F<sub>1,70</sub> = 8.964, p < 0.01). Significant interactions were revealed for treatment × time (F<sub>22,770</sub> = 18.492, p < 0.001), genotype × time (F<sub>11,770</sub> = 7.877, p < 0.001), genotype × dose (F<sub>2,70</sub> = 5.391, p < 0.01), and between these three factors (F<sub>22,770</sub> = 4.207, p < 0.001). The symbols denote significant differences between WT and KO mice at doses of 10 mg/kg (** p < 0.01) and 20 mg/kg (*** p < 0.001) (estimation of parameters).

Fig. 2. Acquisition of intravenous MDMA self-administration at 0.125 mg/kg per infusion in (a) wild-type (WT) (n = 8) and (b) knockout (KO) (n = 8) mice, and at 0.25 mg/kg per infusion in (c) WT (n = 9) and (d) KO (n = 10) mice. At 0.125 mg/kg per infusion, 10 WT and 13 KO mice started self-administration training. Two WT and five KO mice were discarded due to loss of catheter patency. Five out of eight WT mice (62.5%) and 2/8 KO mice (25%) met the acquisition criteria. At 0.25 mg/kg per infusion, nine WT and 12 KO mice started self-administration training. Two mice in the KO group and none in the WT group were discarded due to loss of catheter patency. Eight out of nine WT mice (88.8%) and 2/8 KO mice (20%) met the acquisition criteria. The data represent the average number of nose-pokes ± S.E.M. in the active and inactive holes in 2-h sessions during 10 d of training. * p < 0.05, ** p < 0.01, *** p < 0.001 active vs. inactive hole (estimation of parameters).
between WT and KO mice in the reinforcing effects of MDMA at this dose.

Three-way repeated-measures ANOVA (genotype × hole × day) for 0.25 mg/kg per infusion revealed a significant main effect of genotype \((F_{1,16} = 13.112, p < 0.01)\), hole \((F_{1,16} = 47.840, p < 0.001)\), and a significant two-way genotype × hole interaction \((F_{1,16} = 17.380, p < 0.001)\), hole × day interaction \((F_{9,144} = 4.654, p < 0.05)\), and genotype × day interaction \((F_{9,144} = 2.124, p < 0.05)\). Comparisons between active and inactive responding for WT mice revealed significant differences in discrimination from day 3 to day 10 of training (Fig. 2). In contrast, KO mice showed significant discrimination between holes only on day 7 (Fig. 2).

In the progressive ratio task, only those mice that acquired the self-administration behaviour were tested. For the dose of 0.125 mg/kg per infusion, the breaking point in WT mice was 14.4 in KO mice. For the dose of 0.25 mg/kg per infusion, the breaking point in WT mice was 14.4 tested. For the dose of 0.125 mg/kg per infusion, the that acquired the self-administration behaviour were significant effects of genotype \((F_{1,13} = 3.995, n.s.)\), dose \((F_{1,13} = 0.293, n.s.)\), and dose × genotype interaction \((F_{1,13} = 0.551, n.s.)\).

Food-maintained operant behaviour in 5-HT_{2A}R KO and WT littermates

The specificity of the effects observed in the MDMA self-administration paradigm was confirmed since both genotypes acquired responding for food in a similar manner. [For details see online Appendix (Supplement 4, Fig. S1).]

In-vivo microdialysis in 5-HT_{2A}R KO and WT littermates

Histological analysis of the brains showed that the probes in the NAc were placed between +1.34 mm and +1.54 mm from bregma (Fig. 3a), and those in the PFC were placed between +2.30 mm and +2.8 mm from bregma (Fig. 3b).

Mean (five samples) basal extracellular levels of DA in the NAc and 5-HT and NE in the PFC are shown in Fig. 4. DA levels were significantly lower in KO compared to WT mice, while no differences between genotypes were observed for 5-HT or NE in the PFC. Repeated-measures ANOVA between genotypes for each basal DA sample revealed a significant main effect of group \((F_{1,13} = 48.985, p < 0.001)\), and subsequent estimation of parameters showed significant differences \((p < 0.01)\) for all basal DA samples (Fig. 5a).

A robust increase in extracellular levels of DA in the NAc, and of 5-HT and NE in the PFC was observed in WT and KO mice following an acute challenge with MDMA (20 mg/kg i.p.; Fig. 5). The peak increase in DA was observed 30 min after injection in WT \((8.89 ± 1.6 \, \text{pg/sample})\) and KO \((4.39 ± 0.46 \, \text{pg/sample}, \text{Fig. 5a})\) mice. Two-way repeated-measures ANOVA (genotype × time after injection) revealed a significant main effect of time \((F_{11,143} = 29.117, p < 0.001)\), genotype \((F_{1,13} = 206.839, p < 0.001)\), and interaction between these factors \((F_{11,143} = 4.547, p < 0.001)\). Comparisons between genotypes at each time-point following MDMA administration indicated significantly less DA outflow in KO mice from 15 to 75 min and from 135 to 180 min after MDMA injection \((p < 0.05–0.01)\). However, when MDMA-evoked
stimulation was calculated as a percent of baseline, WT and KO mice showed a similar increase in DA levels in the NAc (Fig. 5b).

Changes in the extracellular levels of 5-HT in the PFC following MDMA administration were not significantly different between genotypes. The peak increase in 5-HT was observed 30 min after injection in WT (4.28 ± 0.27 pg/sample) and KO (4.03 ± 0.65 pg/sample) mice (Fig. 5c). Two-way repeated-measures ANOVA revealed a significant main effect of time after injection (F(1,1,143) = 41.284, p < 0.001), but no significant effect of genotype (F(1,13) = 0.013, n.s.) or genotype × time interaction (F(11,1,143) = 0.440, n.s.). No significant differences were observed between genotypes with respect to percent of baseline stimulation (Fig. 5d).

MDMA induced a similar increase in NE levels in the PFC of WT and KO mice (Fig. 5c). The maximum increase was observed 20 min after injection in WT (4.62 ± 0.92 pg/sample) and KO (3.48 ± 0.64 pg/sample) mice. Repeated-measures ANOVA revealed a significant main effect of time after injection (F(9,90) = 8.353, p < 0.001), but no significant main effect of genotype (F(1,10) = 0.946, n.s.), or genotype × time interaction (F(9,90) = 0.565, n.s.). No significant differences were observed between genotypes with respect to percent of baseline stimulation (Fig. 5f).

**Effects of 5-HT2AR blockade on the reinstatement of MDMA-seeking behaviour in WT mice**

Three groups of mice were first trained to respond for MDMA infusions at a dose of 0.25 mg/kg per infusion (group 1: 17 mice initiated training, two mice were discarded due to loss of catheter patency, 11/15 mice (73.3%) met acquisition criteria. Group 2: 18 mice initiated training, two mice were discarded due to loss of catheter patency, 13/16 mice (81.3%) met acquisition criteria. Group 3: 13 mice initiated training, none lost their catheter patency, 10/13 mice (76.9%) met acquisition criteria.

Reinstatement of MDMA-seeking behaviour evaluated in the different groups of mice treated with saline or epiluvanserin (0.25 and 0.5 mg/kg i.p.) is shown in Fig. 6. An overall three-way repeated-measures ANOVA (treatment × hole × phase) revealed significant main effects of treatment (F(1,30) = 16.078, p < 0.001), hole (F(1,240) = 227.586, p < 0.001), and phase of study (F(5,240) = 51.873, p < 0.001). Significant two-way interactions were observed between hole × treatment (F(2,240) = 20.132, p < 0.001), phase × treatment (F(16,240) = 15.878, p < 0.001), and hole × phase (F(8,240) = 49.362, p < 0.001). A significant three-way interaction between factors (F(16,240) = 14.123, p < 0.001) was also observed.

Active hole responding was compared within phases (control relapse, priming relapse, cue relapse, cue + priming relapse) of each study by repeated-measures ANOVA, followed by pairwise comparisons.

In saline-treated mice (Fig. 6a), a significant main effect of phase was revealed (F(8,30) = 21.073, p < 0.001). Extinction of responding was achieved in 11 mice after a mean of 13.1 ± 1.2 extinction sessions. On the last day of extinction, a significant decrease in active responding was observed compared to the mean of the 3 d meeting the acquisition criteria (p < 0.01). Most importantly, no discrimination between active and inactive nose-pokes was observed, indicating that extinction had taken place. No significant differences were observed for active responding between the four different extinction sessions performed before each reinstatement session. Saline administration (control relapse) significantly decreased active responding with respect to the previous extinction session (p < 0.01). Similarly, MDMA (10 mg/kg) administration (priming relapse) significantly reduced active responding with respect to the previous extinction session.
session ($p < 0.05$), indicating that under these experimental conditions, MDMA priming at a dose of 10 mg/kg does not reinstate MDMA-seeking behaviour. In order to examine whether another dose of MDMA could induce reinstatement of MDMA-seeking behaviour, we performed an additional experiment evaluating the effects of MDMA at a dose of 5 mg/kg. The results showed that this dose was also ineffective in producing MDMA-seeking behaviour in C57BL/6J mice (Fig. S2, online).

In contrast, the presentation of the cue-light previously paired with MDMA self-administration (cue relapse) or the combination of cue presentation and the priming dose of MDMA (cue + priming relapse) significantly increased active hole responding with respect to the previous extinction session ($p < 0.001$ and $p < 0.01$, respectively). These results show that the cue presentation is an effective stimulus to induce reinstatement of MDMA-seeking behaviour in C57BL/6J mice.

In mice treated with 0.25 mg/kg eplivanserin (Fig. 6b), a significant main effect of phase was revealed ($F_{8,64} = 88.92, p < 0.001$). Extinction of responding in this group of mice ($n = 10$) was achieved after a mean of $13.4 \pm 1.7$ d. On the last day of extinction, a significant decrease in active responding was observed compared to the mean of 3 d meeting the acquisition criteria ($p < 0.01$), and no discrimination between active and inactive nose-pokes was observed. No significant differences were revealed for active

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Fig. 5. Basal and stimulated extracellular levels of monoamines in 5-HT$_{2A}$R knockout (KO, –■–) and wild-type (WT, –□–) mice following an acute administration of MDMA (20 mg/kg i.p.; black arrow). (a) Dopamine (DA) levels (pg/sample) in the nucleus accumbens were significantly lower in KO compared to WT mice. Neither (c) serotonin (5-HT) nor (e) noradrenaline (NE) levels (pg/sample) in the prefrontal cortex were significantly different between KO and WT mice. The data are expressed as the average ± S.E.M. pg/sample (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, WT vs. KO mice). In terms of percent of baseline, the stimulation produced by MDMA was not significantly different between genotypes for (b) DA, (d) 5-HT or (f) NE.
responding between the four different extinction sessions performed before each reinstatement phase. Administration of 0.25 mg/kg eplivanserin to mice receiving a saline (control relapse) or a MDMA (priming relapse) injection did not significantly modify responding in the active hole with respect to the previous extinction sessions. A significant increase in active hole responding was observed following pre-treatment with eplivanserin before cue relapse and before priming + cue relapse with respect to each previous extinction session (p < 0.001). These results indicate that 0.25 mg/kg eplivanserin does not block cue- or cue + priming-induced reinstatement of MDMA-seeking behaviour.

In mice treated with 0.50 mg/kg eplivanserin (Fig. 6c), a significant main effect of phase was revealed (F(8,96) = 18.102, p < 0.001). Extinction of responding in this group of mice (n = 13) was achieved...
with a mean of 12.8±1.0 d. On the last day of extinction, a significant decrease in active responding was observed compared to the mean of 3 d meeting the acquisition criteria (p < 0.001), and no discrimination between active and inactive nose-pokes was observed. No significant differences were revealed for active responding between the four different extinction sessions performed before each reinstatement phase. Administration of 0.5 mg/kg eplivanserin to mice receiving saline challenge (control relapse) significantly decreased active responding with respect to the previous extinction phase (p < 0.01). The combination of eplivanserin and MDMA (priming relapse) also significantly decreased responding in the active hole with respect to the previous extinction session (p < 0.01). On the other hand, eplivanserin pre-treatment before the presentation of the cue-light, or before a priming dose of MDMA plus the presentation of the cue-light, did not significantly change active responding with respect to each previous extinction session. These results reveal that 0.5 mg/kg eplivanserin blocks cue-induced and cue + priming-induced reinstatement of MDMA-seeking behaviour.

Dose-related effects were evaluated using between-subjects ANOVA (treatment × hole) in all conditions (Table 1), followed by post-hoc tests. No significant differences were observed in active or inactive responding in mice treated with either dose of eplivanserin with respect to saline administration during the control or the priming relapse phases. In contrast, 0.50 mg/kg eplivanserin, significantly blocked cue-induced reinstatement [significant differences in active (p < 0.001), and inactive (p < 0.05) responding vs. saline], and cue + priming-induced reinstatement of MDMA-seeking behaviour (significant differences for the active hole vs. saline, p< 0.01). A significant difference in active responding was also observed during cue + priming-induced relapse in mice treated with 0.25 mg/kg eplivanserin + MDMA compared to mice treated with saline + MDMA (p < 0.001).

**Discussion**

This study shows that the reinforcing properties of MDMA are blunted in 5-HT2AR KO mice. In addition, the selective 5-HT2AR antagonist eplivanserin, blocked cue-induced reinstatement of MDMA-seeking behaviour in a dose-related manner. Interestingly, our microdialysis data showed lower extracellular levels of DA in the NAc of KO mice before and after a MDMA challenge, whereas extracellular levels of 5-HT and NE in the PFC were similar in KO and WT littermates. These results suggest that a modulatory effect of 5-HT2ARs on mesolimbic dopaminergic activity could be involved in the behavioural responses induced by MDMA. Conversely, MDMA-induced hyperactivity is potentiated in KO mice, suggesting a differential role for 5-HT2ARs in the control of locomotor and reinforcing effects of MDMA.

5-HT2AR KO mice failed to acquire operant behaviour to self-administer two different doses of MDMA (0.125 and 0.25 mg/kg per infusion). These results reveal the involvement of 5-HT2ARs in MDMA reinforcement. Different data have been obtained with other psychostimulants. For instance, MDL100907 did not affect the ability of d-amphetamine to reduce the threshold required to sustain rewarding brain stimulation in the ventral tegmental area (VTA) (Moser et al. 1996), nor did it modify the reinforcing potency of cocaine in rats (Filip et al. 2006; Fletcher et al. 2002a) or non-human primates (Fantegrossi et al. 2002). The contrasting results observed in the studies with MDMA compared to amphetamine or cocaine suggest that serotonergic mechanisms mediated via 5-HT2ARs play a prominent role in the reinforcing properties of MDMA. Indeed, MDMA releases 5-HT more potently than cocaine or amphetamine (Han & Gu, 2006), and it has been shown that 5-HT is crucial for MDMA reinforcement processes (Trigo et al. 2007). Moreover, unlike other psychostimulants, MDMA can also directly stimulate 5-HT2ARs, an effect that has been

Table 1. Two-way ANOVA values for the effects of eplivanserin treatment on responses in the active and inactive holes for relapse phase

<table>
<thead>
<tr>
<th>Hole</th>
<th>F value</th>
<th>p value</th>
<th>Treatment</th>
<th>F value</th>
<th>p value</th>
<th>Hole × treatment</th>
<th>F value</th>
<th>p value</th>
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<tbody>
<tr>
<td>Control relapse</td>
<td>F1,30 = 4.578</td>
<td>&lt; 0.05</td>
<td></td>
<td>F1,30 = 2.620</td>
<td>n.s.</td>
<td>F1,30 = 0.470</td>
<td>n.s.</td>
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<td>Priming relapse</td>
<td>F1,30 = 26.168</td>
<td>&lt; 0.001</td>
<td></td>
<td>F1,30 = 8.085</td>
<td>&lt; 0.05</td>
<td>F1,30 = 1.875</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>Cue relapse</td>
<td>F1,30 = 128.322</td>
<td>&lt; 0.001</td>
<td></td>
<td>F1,30 = 27.617</td>
<td>&lt; 0.001</td>
<td>F1,30 = 30.389</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>Cue + priming relapse</td>
<td>F1,30 = 109.894</td>
<td>&lt; 0.001</td>
<td></td>
<td>F1,30 = 27.242</td>
<td>&lt; 0.001</td>
<td>F1,30 = 26.389</td>
<td>&lt; 0.001</td>
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</table>
associated with its slight hallucinogenic action (Nichols & Oberlender, 1989).

The mesocorticolimbic DA system, which has been widely related with the reinforcing properties of drugs of abuse (Hyman et al. 2006) including MDMA (Bilsky et al. 1998; Daniela et al. 2004), is known to be regulated by serotonergic neurotransmission (De Deurwaerdère et al. 1998; Gobert & Millan, 1999; Schmidt et al. 1992). 5-HT2ARs are localized in several brain structures of the reward circuit such as the VTA, the NAc and the PFC, where they modulate DA activity (Bortolozzi et al. 2005; Porras et al. 2002). However, acute blockade of 5-HT2ARs does not affect basal DA extracellular efflux in the striatum or NAc of rats (De Deurwaerdère & Spampinato, 1999; Porras et al. 2002; Schmidt et al. 1992). In contrast, our in-vivo microdialysis experiments revealed that mice constitutively lacking 5-HT2ARs show lower basal levels of DA in the NAc (43.6% less), while no significant differences in basal levels of 5-HT or NE in the PFC of WT and KO mice were observed. Previous evaluation of the total monoamine content in 5-HT2AR KO mice did not reveal any differences in basal levels of monoamines compared to WT mice, probably due to the fact that the experiments were carried out in brain homogenates (Weisstaub et al. 2006). Supporting our data, it has been reported that chronic treatment with MDL100907 reduces the activity of nigrostriatal DA neurons (Sorensen et al. 1993). In addition, our results show that a challenge administration of MDMA (20 mg/kg) produces a similar percent of baseline increase in DA, 5-HT and NE levels in WT and KO mice. However, in terms of estimated extracellular levels, KO mice show lower DA values in the NAc than WT mice. These results suggest that the changes observed in DA function following chronic blockade of 5-HT2ARs may participate in the impairment of MDMA reinforcement. We have previously shown that SERT KO mice are insensitive to the 5-HT-releasing actions of MDMA and do not self-administer this drug, while MDMA-enhanced DA levels in the NAc are comparable to WT controls (Trigo et al. 2007). The fact that neither SERT (Trigo et al. 2007) nor 5-HT2AR KO mice self-administer MDMA (present study) at doses sustaining operant responding in WT mice argues for the critical involvement of a concurrent 5-HT–DA stimulation in the complete expression of the reinforcing properties of MDMA.

In order to verify that the effects observed on MDMA self-administration were not due to unspecific motor or learning deficits, we tested 5-HT2AR WT and KO mice in an operant food-reinforced task. 5-HT2AR KO mice showed lower active responding for food pellets than WT mice only during the first 3 d of acquisition. Similar levels of responding were observed between genotypes during the remaining training period, and all KO mice tested reached the acquisition criteria. Moreover, breaking points for food reward evaluated in a PR task were not altered in KO mice. These findings indicate that a constitutive deletion of 5-HT2ARs does not disrupt instrumental responding or the motivation for food reward, and support a specific role of 5-HT2ARs in MDMA reinforcement.

Interestingly, the hyperactivity induced by an acute administration of MDMA at both doses tested (10 and 20 mg/kg) was potentiated in 5-HT2AR KO mice. These findings are in line with studies showing that an acute administration of d-amphetamine also enhances locomotor responses in mice with a constitutive deletion of the 5-HT2AR compared to WT control mice (Salomon et al. 2007). In that study, the behavioural response to d-amphetamine was paralleled by increased extracellular levels of NE in the PFC (Salomon et al. 2007). These authors proposed that 5-HT2ARs and a1b-adrenergic receptors in the PFC regulate each other under normal conditions to modulate psychostimulant-induced locomotor activity, as well as DA levels in the NAc (Auclair et al. 2004a, b). In mice lacking 5-HT2ARs, a partial ‘constitutive’ sensitization to d-amphetamine occurs, related to heightened NE levels in PFC, possibly leading to higher levels of DA in the NAc (Salomon et al. 2007). In our study, the stimulation produced by MDMA in terms of percent of baseline was similar in WT and KO mice for NE and 5-HT in the PFC, and for DA in the NAc. Although the estimated basal and stimulated values of DA were lower in KO than in WT mice. These results suggest that distinct neurochemical mechanisms may be involved in the enhanced hyperlocomotor effects induced by d-amphetamine and MDMA in 5-HT2AR KO mice. Our behavioural data showed no differences in locomotor activity between WT and KO mice under basal conditions, whereas an enhanced response to MDMA was observed in KO mice. These behavioural findings diverge from our microdialysis results, and suggest that in the absence of 5-HT2ARs, a complex monoaminergic dysregulation could take place eliciting singular basal and MDMA-evoked locomotor responses. Most pharmacological studies using acute administration of 5-HT2AR antagonists have shown a reduction in MDMA-induced locomotor activation in several animal species, although contradictory results have also been reported (Ball & Rebec, 2005; Bankson & Cunningham, 2002; Fantegrossi et al. 2003; Herin et al. 2005; Kehne et al. 1996). Thus, compensatory mechanisms in KO mice leading to opposite changes
with respect to acute blockade of these receptors cannot be completely ruled out.

Our studies indicate that locomotor and reinforcing effects induced by MDMA are modulated differently in 5-HT2A(R) KO mice. These distinct responses could be attributed to the different dosing regimens of MDMA (contingent low intravenous doses of MDMA in self-administration vs. non-contingent high intraperitoneal doses in the locomotor studies), or to acute (locomotor activity) vs. repeated (self-administration) exposure to MDMA. In addition, possible compensatory mechanisms may have differentially influenced MDMA-induced locomotion and reinforcement. Indeed, 5-HT1B and 5-HT2B,2C receptors have been shown to participate in distinctive aspects of MDMA-related effects (Doly et al. 2009; Fantegrossi et al. 2002; Fletcher et al. 2002b; Kehne et al. 1996; Ramos et al. 2005; Scearce-Levie et al. 1999). Although no changes in mRNA expression for the different 5-HT receptors have been observed in 5-HT2AR KO mice (Weistaub et al. 2006), a possible 5-HT2BR hyposensitivity has been reported in these mutants probably related to receptor desensitization (Popa et al. 2005). This finding is relevant because a high dose of MDMA induces locomotor hyperactivation in 5-HT2BR KO mice, while MDMA-conditioned place preference is abolished (Doly et al. 2009). Therefore, even if 5-HT2BRs are discretely expressed in the adult brain (Leysen, 2004), a possible change in the sensitivity of these receptors could have also contributed to the enhancement of MDMA locomotor responses observed in 5-HT2AR KO mice.

In our pharmacological experiments using WT mice, a priming injection of MDMA at two different doses (5 and 10 mg/kg i.p.) was unable to reinstate MDMA-seeking behaviour, while the presentation of a cue previously paired with MDMA self-administration did. The result showing that an acute challenge with MDMA failed to reinstate MDMA-seeking was surprising since we had previously shown that MDMA could reinstate cocaine-seeking behaviour in mice (Trigo et al. 2009). Differences in the mechanisms involved in cocaine and MDMA reinstatement or in mice strains used (CD1 vs. C57BL/6J) could possibly explain these discrepant results. In addition, the lack of effectiveness of a priming administration of MDMA on reinstatement of MDMA-seeking behaviour is in line with a number of studies showing inconsistent results with intraperitoneal priming drug injections for reinstating extinguished drug-seeking behaviour in mice (see Yan & Nabeshima, 2009). On the other hand, our results showing that cue presentation reinstates MDMA-seeking behaviour agree with previous studies performed in rats, where cue presentation alone (Ball et al. 2007) as well as, cue + MDMA priming also reinstates MDMA-seeking behaviour (Schenk et al. 2008). More interestingly, we demonstrate for the first time that selective blockade of 5-HT2ARs can effectively prevent cue-induced reinstatement of MDMA-seeking behaviour. The ability of eplivanserin, at a dose of 0.5 mg/kg, to block cue-induced reinstatement cannot be explained by changes in locomotion, as previous studies have shown that even higher doses than those used in our study do not modify basal locomotion in mice (1 mg/kg i.p.; Auclair et al. 2004b). Although our results show that 0.5 mg/kg eplivanserin alone, has a tendency to decrease responding in the inactive hole, this tendency was similar to the one observed after saline administration. Therefore, the suppression of cue-induced reinstatement of MDMA-seeking behaviour by eplivanserin is probably due to a selective 5-HT2AR antagonism. In agreement, recent data show that MDL100907 prevented cue-induced cocaine-seeking (Nic Dhonnchadha et al. 2009). Our reinstatement studies were carried out after an extended period of extinction in mice which had self-administered MDMA during 10 d. In recent studies, rats withdrawn from acute or chronic treatment with MDMA (Reneman et al. 2002) or cocaine (Carrasco & Battaglia, 2007; Carrasco et al. 2007) show up-regulation of 5-HT2ARs. Furthermore, imaging studies in MDMA users report an increase in 5-HT2ARs after 30 d of abstinence (Reneman et al. 2002). Thus, the blockade by eplivanserin of cue-induced reinstatement of MDMA-seeking behaviour, may be related to a 5-HT2AR hyperactivity produced after repeated MDMA self-administration in structures involved in reinstatement processes, such as the medial PFC (Bradberry & Rubino, 2004; Ciccocioppo et al. 2001; See, 2005; Van den Oever et al. 2009). Indeed, 5-HT2ARs are highly expressed in the PFC (Bortolozzi et al. 2005; Jiang et al. 2009; McDonald & Mascagni, 2007; Vazquez-Borsetti et al. 2009), where they enhance the excitatory cortical output to the VTA, activating DA neurons and increasing DA release in the NAc (Bortolozzi et al. 2005; Pehek et al. 2006; Vazquez-Borsetti et al. 2009).

In summary, we show that 5-HT2AR KO mice do not acquire MDMA self-administration behaviour; while they do learn an instrumental response to obtain food pellets, supporting the specific involvement of these receptors in MDMA reinforcing properties. These effects were associated to lower basal and MDMA-stimulated levels of DA in the NAc of 5-HT2AR KO mice. Conversely, MDMA-induced
role of 5-HT2ARs in MDMA reinforcement and seeking. Considering the crucial results also showed that eplivanserin potently blocked MDMA-seeking behaviour. The rats, suggesting a different involvement of these receptors in locomotor effects of MDMA. 5-HT2AR antagonists may be a therapeutic option for reducing craving and preventing relapse in patients with addiction to MDMA.

Note

Supplementary material accompanies this paper on the Journal’s website (http://journals.cambridge.org/pnp).

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Statement of Interest

None.

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