

# Differential long-term effects of MDMA on the serotonergic system and hippocampal cell proliferation in 5-HTT knock-out vs. wild-type mice

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## Abstract

Although numerous studies investigated the mechanisms underlying 3,4-methylenedioxymethamphetamine (MDMA)-induced neurotoxicity, little is known about its long-term functional consequences on 5-HT neurotransmission in mice. This led us to evaluate the delayed effects of MDMA exposure on the 5-HT system, using in-vitro and in-vivo approaches in both 5-HTT wild-type and knock-out mice. Acute MDMA in-vitro application on slices of the dorsal raphe nucleus (DRN) induced concentration-dependent 5-HT release and 5-HT cell firing inhibition. Four weeks after MDMA administration (20 mg/kg b.i.d for 4 d), a 2-fold increase in the potency of the 5-HT<sub>1A</sub> receptor agonist ipsapirone to inhibit the discharge of DRN 5-HT neurons and a larger hypothermic response to 8-OH-DPAT were observed in MDMA- compared to saline-treated mice. This adaptive 5-HT<sub>1A</sub> autoreceptor supersensitivity was associated with decreases in 5-HT levels but no changes of [<sup>3</sup>H]citalopram binding in brain. Long-term MDMA treatment also induced a 30% decrease in BrdU labelling of proliferating hippocampal cells and an increased immobility duration in the forced swim test suggesting a depressive-like behaviour induced by MDMA treatment. All these effects were abolished in 5-HTT<sup>-/-</sup> knock-out mice. These data indicated that, in mice, MDMA administration induced a delayed adaptive supersensitivity of 5-HT<sub>1A</sub> autoreceptors in the DRN, a deficit in hippocampal cell proliferation and a depressive-like behaviour. These 5-HTT-dependent effects, opposite to those of antidepressants, might contribute to MDMA-induced mood disorders.

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## Introduction

Among illicit substances, the ring-substituted amphetamine, 3,4-methylenedioxymethamphetamine (MDMA, Ecstasy), has become one of the most

widely used during the last few years (Banken, 2004; Maxwell, 2005). Although a great amount of data regarding the neurotoxic potential of MDMA has been accumulated in the relevant literature (Green et al., 1995; McCann et al., 1998; O'Shea et al., 1998), the mechanisms underlying the affective disorders (de Win et al., 2004; McCardle et al., 2004) and cognitive impairments (Kelly, 2000; McCardle et al., 2004; Reneman et al., 2001) induced by this drug have not yet been elucidated. However, results emerging from

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human studies indicate that both cognitive disorders and mood disturbances are among the long-term effects of MDMA exposure (McCardle et al., 2004; Parrott et al., 2002; Parrott and Lasky, 1998). In particular, although recent studies pointed out the contribution of the concomitant use of other drugs to the occurrence of depressive symptomatology in MDMA users (Durdle et al., 2008; Guillot and Greenway, 2006; Medina and Shear, 2007), a relatively high incidence of clinical depression has been consistently reported in later life in former MDMA users (de Win et al., 2004; MacInnes et al., 2001; Peroutka et al., 1988; Thompson et al., 2004).

MDMA is well known to cause the release of all monoamine neurotransmitters but acts preferentially on the serotonergic system (Crespi et al., 1997; Gudelsky and Nash, 1996). Once internalized, MDMA stimulates 5-HT efflux through actions at both the plasma membrane and vesicular membrane transport systems responsible for 5-HT reuptake and storage in presynaptic nerve terminals (Green et al., 1995; Rudnick and Wall, 1992). The plasma membrane 5-HT transporter (5-HTT) plays a key role in the regulation of serotonergic transmission and is the primary target of both selective serotonin reuptake inhibitors (SSRIs), such as fluoxetine, and MDMA (Rudnick and Wall, 1992). However, the behavioural/clinical effects of these two compounds are radically opposed since fluoxetine is a clinically effective antidepressant whereas MDMA can cause depression in humans and depressive-like symptoms in validated animal models (Galineau et al., 2005; Thompson et al., 2004).

In order to unveil the possible causes of the delayed cognitive and mood impairments observed in humans who have consumed ecstasy earlier in life, we used appropriate neurophysiological, biochemical and behavioural approaches to characterize the long-term effects of a subchronic exposure to MDMA in mice. Particular attention was focused on central serotonergic neurotransmission which plays key roles in mood control and cognitive functions (Baumgarten and Göthert, 1997). In addition, cell proliferation in the hippocampal dentate gyrus was also quantified after this treatment since convergent data in the literature showed that depression-like behaviour is associated with a deficit in hippocampal neurogenesis (Joels et al., 2007) whereas, in contrast, antidepressants promote this phenomenon (Jacobs et al., 2000; Malberg et al., 2000). Interestingly, hippocampal neurogenesis is also closely related to 5-HT neurotransmission (Banasar et al., 2004), and 5-HT alterations might have also contributed – at least indirectly – to changes at this levels in MDMA-treated mice. Experiments were

conducted in both wild-type mice and mutants deficient in 5-HTT in order to assess whether or not the effects possibly induced by MDMA treatment were caused through the drug action at its preferred molecular target, i.e. the plasma membrane transporter ensuring 5-HT uptake by 5-HT neurons.

Our data showed, for the first time, a delayed and persistent 5-HT<sub>1A</sub> autoreceptor supersensitivity and decrease in hippocampal cell proliferation occurring 4 wk after a short-term exposure to MDMA. Because these effects appeared to be unrelated to any neurotoxic action of MDMA, our data actually provided new insights into the probable mechanisms causing cognitive and mood disorders in human MDMA users.

## Materials and methods

### Animals

Experiments were performed using homozygous knock-out 5-HTT<sup>-/-</sup> and wild-type 5-HTT<sup>+/+</sup> male littermates born from heterozygous mutants of C57BL/6J genetic background (F10). Genotyping was performed by polymerase chain reaction (PCR) applied to genomic DNA from tail biopsies (Bengel et al., 1998). After weaning and sexing, males were selected and housed in groups of 4–6 animals per cage. Standard laboratory conditions [22 ± 1 °C; 60% relative humidity; 12 h light/dark cycle (lights on 07:00 hours); food and water available ad libitum] were applied, and mice were used at age 2 months when their body weight in each genotype reached around 25 g.

Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies.

### Treatments

Mice were injected intraperitoneally (i.p.) with either saline vehicle (0.9% NaCl, 1 ml/100 g body weight) or (±)-MDMA at a dose of 20 mg/kg twice daily (at 11:00 and 14:00 hours) for four consecutive days. This dosage regimen was chosen because, in rats, it produced sensitization to addictive drugs such as cocaine (Morgan et al., 1997), decrease in hippocampal expression of glucocorticoid receptor (Yau et al., 1994), reduction in 5-HT terminal density and decreased functional activity of 5-HT<sub>1A</sub> receptors (Granoff and Ashby, 2001).

Subsequent experiments (see below) were performed 28 d after the final injection.

### Preparation of dorsal raphe nucleus (DRN) slices

Naive or treated mice were killed by decapitation, and their brains were rapidly removed and immersed in an ice-cold artificial cerebrospinal fluid (aCSF) of the following composition (mM): 126 NaCl, 3.5 KCl, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 1.3 mgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, 25 NaHCO<sub>3</sub>, 11 D-glucose, bubbled continuously with carbogen (95% O<sub>2</sub>/5% CO<sub>2</sub>) to maintain pH value at 7.3.

A block of brainstem containing the DRN was cut into 400- $\mu$ m-thick coronal sections in the same ice-cold aCSF using a vibratome. Brainstem slices were then immediately incubated in oxygenated aCSF at room temperature for at least 1 h before electrophysiological recordings and measurement of 5-HT release.

### Electrophysiological recordings

A single slice with the DRN was then placed on a nylon mesh, completely submerged in a small recording chamber and superfused continuously with oxygenated aCSF (34 °C) at a constant flow rate of 2–3 ml/min (Haj-Dahmane et al., 1991; Lanfumey et al., 1999).

Extracellular recordings of characterized 5-HT<sub>1A</sub> autoreceptor-expressing serotonergic neurons in the DRN (Sotelo et al., 1990) were obtained using glass microelectrodes filled with 2 M NaCl (10–15 M $\Omega$ ) (Lanfumey et al., 1999; Mannoury la Cour et al., 2001). Firing was evoked in the otherwise silent neurons by adding the  $\alpha_1$ -adrenoceptor agonist phenylephrine (3  $\mu$ M) into the superfusing aCSF (Vandermaelen and Aghajanian, 1983). Baseline activity was recorded for 5–10 min prior to the application of drugs via a three-way tap system. The duration of each application of MDMA (0–200  $\mu$ M) or the 5-HT<sub>1A</sub> receptor agonist ipsapirone (0–0.3  $\mu$ M) was 3 min. The effect of a given drug was evaluated by comparing the mean discharge frequency during 2 min prior to its addition to the superfusing aCSF with that recorded at the peak of drug action.

When MDMA or ipsapirone was applied in the presence of the 5-HT<sub>1A</sub> receptor antagonist WAY 100635 (2 nM), the effect of the first compound was compared with the baseline firing rate and the discharge frequency recorded during superfusion with the antagonist alone.

### Measurement of *in vitro* 5-HT release

A single DRN slice was immersed in 100  $\mu$ l of oxygenated aCSF (34 °C) supplemented with phenylephrine (3  $\mu$ M) and MDMA (0–200  $\mu$ M). After a 15 min incubation, the slice was removed and the incubation medium was mixed with HClO<sub>4</sub> (0.1 N final), then supplemented with 0.05% (w/v) Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub> and 0.05%

(w/v) disodium EDTA. Samples were centrifuged (30000 g, 4 °C, 20 min), and supernatants were processed as previously described (Hamon et al., 1988) for the measurement of 5-HT levels by high performance liquid chromatography (HPLC) coupled to electrochemical detection (ED).

### Measurements of 5-HT tissue levels

Mice were decapitated 4 wk after MDMA or saline treatment and their brains were immediately removed at 0 °C. Brain structures (anterior raphe area, hippocampus, striatum and cerebral cortex) were dissected out and weighed. Each sample was homogenized in 250–500  $\mu$ l of 0.1 N HClO<sub>4</sub> supplemented with 0.05% (w/v) Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub> and 0.05% (w/v) disodium EDTA, and subsequent steps for the HPLC-ED measurement of 5-HT tissue levels were as previously described (Hamon et al., 1988).

### [<sup>3</sup>H]Citalopram binding onto brain membranes

Dissected tissues (anterior raphe area, hippocampus, striatum and cerebral cortex) were homogenized in 40 vol (w/v) of ice-cold Tris-salt buffer (50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl; pH 7.4) using a Polytron. Homogenates were centrifuged at 40000 g for 20 min, the pellets were suspended in 40 vol of the same buffer and incubated at 37 °C for 10 min to remove endogenous 5-HT. The membranes were spun down at 40000 g for 20 min and washed another three times by suspension/centrifugation as before. The final pellet was suspended in 10 vol of the same buffer and 25  $\mu$ l aliquots were mixed with 225  $\mu$ l of fresh Tris-salt buffer (pH 7.4), containing [<sup>3</sup>H]citalopram (83.0 Ci/mmol; 0.5 nM final concentration) in an Optiplate 96-well apparatus (PerkinElmer, Wellesley, MA, USA) for 1 h incubation at room temperature. Non-specific binding was determined in the presence of 10  $\mu$ M fluoxetine. After the incubation, the unfilter GF/B plate was drained with a FilterMate harvester (PerkinElmer) and each well was washed three times with 500  $\mu$ l of ice-cold buffer. Microscint-40 scintillation cocktail (30  $\mu$ l) was added to each well of the plate, and radioactivity was counted using a TopCount scintillation counter (PerkinElmer). The specific binding (expressed as cpm/10  $\mu$ g protein) was obtained by subtracting non-specific binding from total binding. Proteins were quantified using the method of Lowry et al. (1951) with bovine serum albumin as standard.

### 8-OH-DPAT-induced hypothermia

Whereas both 5-HT<sub>1A</sub> autoreceptors and post-synaptic 5-HT<sub>1A</sub> receptors mediate the hypothermic effect of

8-OH-DPAT in rats (Thielen and Frazer, 1995), clear-cut evidence has been reported that 8-OH-DPAT-induced hypothermia only reflected 5-HT<sub>1A</sub> autoreceptor activation in mice (Bill et al., 1991; Goodwin et al., 1985). Accordingly, this response was used to assess possible changes in 5-HT<sub>1A</sub> autoreceptor sensitivity in MDMA-treated mice. Core body temperature was measured at ambient temperature of  $23 \pm 1$  °C in gently restrained mice using a thermocouple probe (Betatherm, Galway, Ireland; 1.5 mm diameter) inserted 20 mm into the rectum. Basal value was determined just before subcutaneous injection of the 5-HT<sub>1A</sub> receptor agonist 8-OH-DPAT (0.06–0.3 mg/kg) or vehicle (0.9% NaCl, 1 ml/100 g body weight), and body temperature was measured every 10 min thereafter. The response to 8-OH-DPAT was calculated as the maximum decrease (from baseline) in body temperature during 60-min post injection.

#### ***BrdU labelling and quantification***

Four weeks after the last injection of MDMA or saline, mice were injected with 5'-bromodeoxyuridine (BrdU, 75 mg/kg i.p.). Two hours later, mice were deeply anaesthetized with pentobarbitone (40 mg/kg i.p) and transcardially perfused with 75 ml of anticoagulant solution (0.15 M NaCl supplemented with 15 mM NaNO<sub>2</sub>) followed by 75 ml of 4% paraformaldehyde in 0.1 M PBS (pH 7.4) at 4 °C. The brain was removed and post-fixed in the same solution overnight at 4 °C. Serial coronal sections (40 µm) were collected with a Leica VT1000 vibratome (Leica, Nussloch, Germany) from the entire hippocampus. Every fourth free-floating section was rinsed in 0.1 M PBS, incubated for 5 min in 3% H<sub>2</sub>O<sub>2</sub>/10% methanol in 0.1 M PBS, and further rinsed in 0.1 M PBS. Sections were next incubated in 2 M HCl and 0.1% Triton X-100 in 0.1 M PBS (30 min, 37 °C), then rinsed in 0.1 M sodium tetraborate buffer (pH 8.6), for 30 min, before blocking with 5% normal rabbit serum in 0.1% Triton X-100/0.1 M PBS. Sections were then incubated overnight with the primary BrdU-antibody (monoclonal rat IgG, 1:100, Abcys, Paris, France) at 4 °C, followed by an incubation with the secondary antibody (biotinylated rabbit anti-rat IgG, 1/200, Vector, Burlingame, CA, USA) for 2 h at room temperature, and staining using the ABC staining system (Vectastain ABC Elite kit, Vector). Peroxidase activity was revealed by incubating sections with 0.05% 3,3'-diaminobenzidine and 0.01% H<sub>2</sub>O<sub>2</sub> in 0.1 M PBS for 5 min. After three rinses in PBS, sections were counterstained with Cresyl Violet, dehydrated, mounted, and coverslipped in Eukit medium.

Slides were examined under 10× and 20× objectives (Leica microscope), and BrdU-labelled cells were counted bilaterally on each section, within the subgranular layer of the dentate gyrus, using a computer image analysis system (Lucia, Laboratory Imaging, Praha, Czech Republic).

#### ***Tail suspension test (TST)***

The apparatus consists of a suspension unit of three cages (ID-Tech-Bioseb, Chaville, France). Each mouse was suspended by the tail using adhesive tape to a hook connected to a strain gauge. The strain gauge picks up all movements of the mouse, which are transmitted to a central unit for signal digitalization. The distance from hook to floor of apparatus is 200 mm. Care was taken to use mice of same weight ( $25 \pm 1$  g) in this test (Cryan et al., 2005).

On the test day, mice were moved to the testing laboratory where they stayed undisturbed for at least 3 h. Mice were then suspended by the tail in the TST apparatus according to a randomization scheme generated by the computer's program. The duration of immobility over a 6-min test session was measured and expressed in seconds. Each mouse was used only once for each experimental session.

#### ***Forced swim test (FST)***

Mice were dropped individually into glass cylinders (height 25 cm, internal diameter 10 cm) containing 10 cm water, maintained at 21–23 °C. The apparatus consisted of two Plexiglas cylinders placed side by side in a Makrolon cage (38 × 24 × 18 cm). Two mice were tested simultaneously for a 6-min period but a non-transparent screen placed between the two cylinders prevented the mice from seeing each other. The immobility time was measured by an observer who was blinded to the drug treatment and the mouse genotype. A mouse was judged to be immobile when it remained floating in water, making only the necessary movements to keep its head above water. Each mouse was used only once for each experimental session.

#### ***Statistical analyses***

Statistical analyses were performed using Prism 4.0 software (GraphPad Software Inc., San. Diego, CA, USA). For all the experiments, a two-way ANOVA was used with treatment and genotype as main factors. In case of significant effect, the two-way ANOVA was followed by a Bonferroni post-hoc test to determine specific group differences. In all cases, the significance level was set at  $p < 0.05$ .

## Drugs and Chemicals

[<sup>3</sup>H]Citalopram was purchased from GE Healthcare Europe (Orsay, France). BrdU, (±)-MDMA (HCl salt) and (±)-8-OH-DPAT (HBr salt) were from Sigma (St Louis, MO, USA). Other compounds were WAY 100635 (Wyeth-Ayerst, Princeton, NJ, USA) and ipsapirone (Bayer-Tropon, Cologne, Germany).

## Results

### Acute in-vitro effects of MDMA in 5-HTT<sup>+/+</sup> and 5-HTT<sup>-/-</sup> mice

#### Spontaneous firing of DRN 5-HT neurons

DRN serotonergic neurons recorded in brainstem slices from C57BL/6J wild-type mice displayed the characteristic slow (all values are given as means ± s.e.m.) ( $2.07 \pm 0.20$  spikes/s,  $n = 15$ ) and regular pattern of discharge as previously described in rodents (Lanfumeu et al., 1999; Vandermaelen and Aghajanian, 1983). No differences in both in-vitro firing frequency ( $1.87 \pm 0.16$  spikes/s,  $n = 20$ ) and discharge pattern of DRN serotonergic neurons were observed in 5-HTT<sup>-/-</sup> mutants compared to wild-type mice, in agreement with previous recordings in the same mouse lines under in-vitro conditions (Mannoury la Cour et al., 2001, 2004).

#### MDMA-induced inhibition of DRN 5-HT neurons

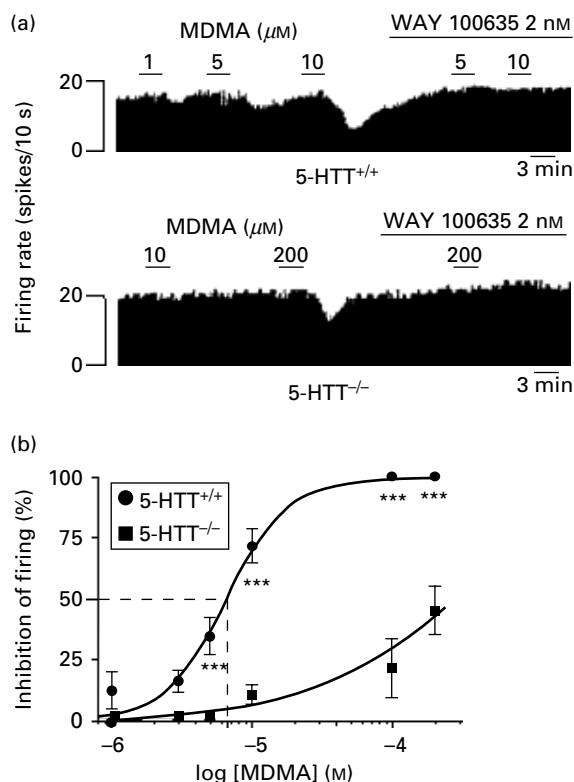
In 5-HTT<sup>+/+</sup> mice, addition of MDMA (0–200 μM) into the aCSF superfusing brainstem slices resulted in a concentration-dependent inhibition of the firing of DRN 5-HT neurons ( $EC_{50} 6.53 \pm 0.63$  μM,  $n = 15$ ) (Figure 1a,b), which was prevented by the selective 5-HT<sub>1A</sub> receptor antagonist WAY 100635 (2 nM).

In 5-HTT<sup>-/-</sup> mice, MDMA (0–200 μM) was essentially inactive on the in-vitro firing of DRN 5-HT neurons (Figure 1a,b). Only the highest concentration of the drug (200 μM) produced a clear-cut inhibition of firing in the knock-out mice ( $-45.20 \pm 10.13\%$ ,  $n = 15$ ,  $p < 0.05$ ) (Figure 1b). This effect was also prevented by WAY 100635 at 2 nM (Figure 1a).

#### MDMA-induced 5-HT release from brainstem slices

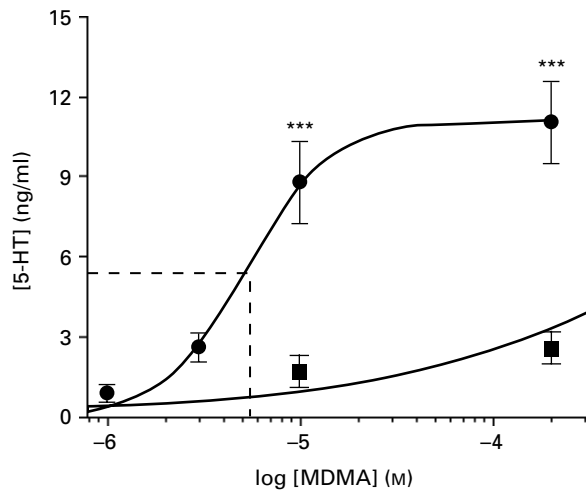
Under basal conditions, spontaneous 5-HT outflow from brainstem slices was  $0.01 \pm 0.01$  and  $1.23 \pm 0.44$  ng/ml ( $n = 7-8$ ,  $p < 0.05$ ) in 5-HTT<sup>+/+</sup> and 5-HTT<sup>-/-</sup> mice, respectively.

MDMA (1–200 μM) produced a concentration-dependent ( $EC_{50} 5.24 \pm 1.66$  μM,  $n = 10$ ) increase in 5-HT outflow from 5-HTT<sup>+/+</sup> slices, the maximum



**Figure 1.** Acute effects of MDMA on the firing of dorsal raphe nucleus (DRN) serotonergic neurons in 5-HTT<sup>+/+</sup> and 5-HTT<sup>-/-</sup> mice. (a) MDMA-induced inhibition and prevention by WAY 100635. Integrated firing rate histograms (in spikes/10 s) showing the effect of increasing concentrations of MDMA on the electrical activity of a DRN 5-HT neuron, in a 5-HTT<sup>+/+</sup> and a 5-HTT<sup>-/-</sup> mouse. Generally, the onset of the response occurred after 1–2 min of tissue superfusion with MDMA, and the peak effect was observed during the first few minutes following the drug application. Repeated applications of a fixed concentration of MDMA (10 μM for 3 min, with 10–15 min drug-free intervals) to the same cell produced similar responses (not shown). (b) Concentration–response curves of MDMA-induced inhibition of the firing of DRN 5-HT neurons in brainstem slices. MDMA-induced inhibition is expressed as a percentage of the baseline firing rate. Each point is the mean ± s.e.m. of data obtained from 15 individual cells. The dotted lines illustrate the  $EC_{50}$  value of MDMA in 5-HT<sup>+/+</sup> mice (abscissa).

outflow ( $11.03 \pm 1.56$  ng/ml) being obtained with 200 μM MDMA (Figure 2). 5-HT release induced by 10 μM MDMA was not significantly modified by adding WAY 100635 (2 nM) into the incubation medium ( $8.75 \pm 1.60$  and  $9.42 \pm 2.75$  ng/ml in the absence and the presence of WAY 100635, respectively;  $n = 10$ , n.s.). In contrast, in 5-HTT<sup>-/-</sup> mice, incubation of brainstem



**Figure 2.** Acute effects of MDMA on 5-HT outflow from brainstem slices from 5-HTT<sup>+/+</sup> (●) and 5-HTT<sup>-/-</sup> (■) mice. Concentration–response curves of MDMA-induced 5-HT overflow from brainstem slices. MDMA-induced overflow is expressed as ng/ml 5-HT. Each point is the mean  $\pm$  S.E.M. of 10 independent determinations. \*\*\*  $p < 0.001$  compared to the respective basal 5-HT outflow. The dotted lines illustrate the EC<sub>50</sub> value of MDMA in 5-HT<sup>+/+</sup> mice (abscissa).

slices with MDMA (1–200  $\mu$ M) did not significantly increase 5-HT outflow (Figure 2).

#### Long-term effects of MDMA treatment in 5-HTT<sup>+/+</sup> and 5-HTT<sup>-/-</sup> mice

##### DRN 5-HT neuron firing after MDMA treatment

**5-HTT<sup>+/+</sup> mice.** Four weeks after the last injection of MDMA, DRN serotonergic neurons recorded in brainstem slices from wild-type mice displayed a lower frequency ( $1.32 \pm 0.12$  spikes/s,  $n = 19$ ,  $p < 0.01$ ) than that determined in saline-treated mice ( $2.05 \pm 0.16$  spikes/s,  $n = 24$ ) (Figure 3a). However, the spontaneous DRN 5-HT cell firing in MDMA-treated mice returned to the control basal values in the presence of the 5-HT<sub>1A</sub> receptor antagonist WAY 100635 (10 nM), suggesting that 5-HT<sub>1A</sub> autoreceptors remained tonically activated 4 wk after MDMA treatment (Figure 3a).

The addition of ipsapirone into the aCSF superfusing brainstem slices induced a stronger inhibition of the firing of DRN 5-HT neurons in MDMA-treated wild-type mice than in saline-treated controls. EC<sub>50</sub> values of the 5-HT<sub>1A</sub> receptor agonist were significantly different ( $p < 0.001$ ) in the two groups,  $26.7 \pm 1.7$  nM ( $n = 19$ ) for MDMA-treated wild-type mice and  $52.1 \pm 2.2$  nM ( $n = 24$ ) for paired saline-treated mice

(Figure 4c). However, the stronger ipsapirone-induced inhibition observed in MDMA-treated mice was not related to the spontaneous activity of 5-HT neurons recorded in this group since it was also found on the few neurons recorded from MDMA-treated mice (6/19 neurons), which spontaneous activity was not different from that recorded in saline-treated mice (Figure 3a).

**5-HTT<sup>-/-</sup> mice.** In contrast to that found in 5-HTT<sup>+/+</sup> mice, MDMA treatment had no influence on the spontaneous firing rate of DRN 5-HT neurons in brainstem slices from 5-HTT<sup>-/-</sup> mutants ( $1.89 \pm 0.13$  and  $1.95 \pm 0.14$  spikes/s for saline- and MDMA-treated 5-HTT<sup>-/-</sup> mice, respectively,  $n = 13$ –18) (Figure 3b). As already described (Mannoury la Cour et al., 2001), higher concentrations of ipsapirone were needed for inhibiting 5-HT neuron discharge in 5-HTT<sup>-/-</sup> mice, and the EC<sub>50</sub> value of the 5-HT<sub>1A</sub> receptor agonist was about 100-fold higher in saline-treated mutants than in saline-treated wild-type mice (EC<sub>50</sub>  $5.6 \pm 1.1$   $\mu$ M,  $n = 18$  vs.  $52.1 \pm 2.2$  nM,  $n = 24$ ,  $p < 0.001$ ) (Figure 3c, d). Interestingly, MDMA treatment did not significantly modify the inhibitory potency of ipsapirone on 5-HT neuron firing in 5-HTT<sup>-/-</sup> mutants (EC<sub>50</sub>  $9.3 \pm 2.2$   $\mu$ M,  $n = 10$ ) (Figure 3d).

##### Effects of MDMA treatment on brain 5-HT levels

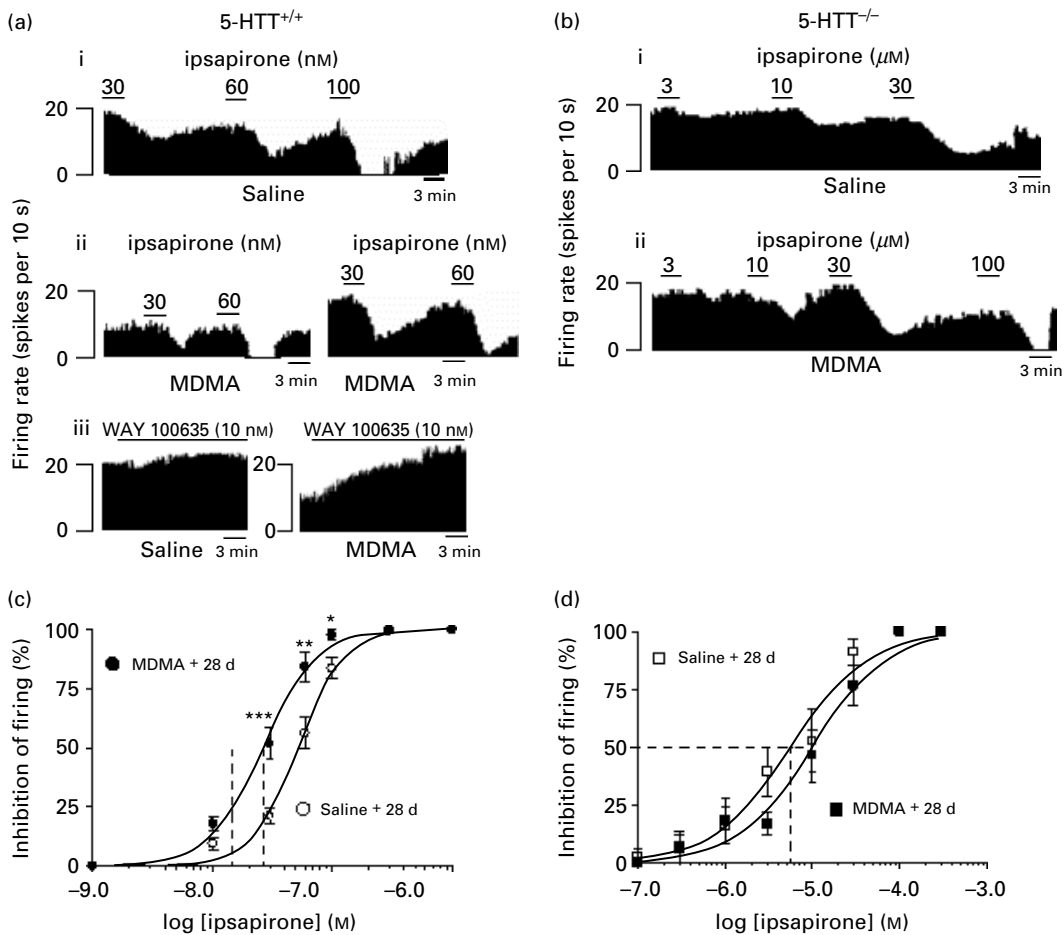
In wild-type mice, MDMA produced a limited but significant decrease of the indolamine levels in the anterior raphe area (–12%), hippocampus (–8%), and striatum (–14%). In contrast, under the same treatment conditions, MDMA did not significantly affect 5-HT levels in these brain regions in 5-HTT<sup>-/-</sup> mice. However, in line with previous data (Bengel et al., 1998; Fabre et al., 2000), in both MDMA-treated and saline-treated groups, 5-HT levels in 5-HTT<sup>-/-</sup> mice were only ~20% of those measured in 5-HTT<sup>+/+</sup> mice (Table 1).

##### Effects of MDMA treatment on [<sup>3</sup>H]citalopram binding

As indicated in Table 1, the 4-d treatment with MDMA did not significantly affect [<sup>3</sup>H]citalopram binding in all the brain areas studied in wild-type mice. In 5-HTT<sup>-/-</sup> mutants, as expected, [<sup>3</sup>H]citalopram specific binding was close to nil in both saline- and MDMA-treated groups (Table 1).

##### 8-OH-DPAT-induced hypothermia in MDMA-treated wild-type mice

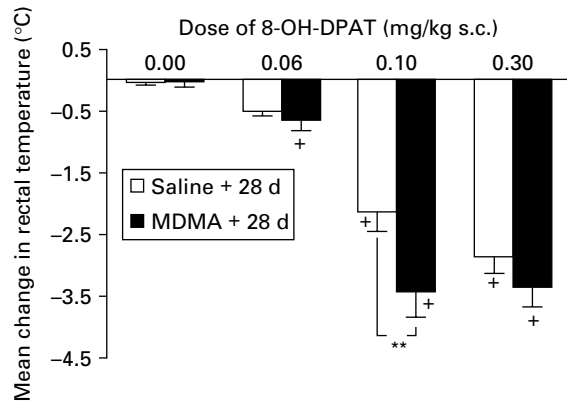
Baseline temperature values were similar in both saline-treated ( $37.57 \pm 0.08$  °C,  $n = 43$ ) and MDMA-treated



**Figure 3.** Long-term (+28 d) effects of MDMA treatment (20 mg/kg i.p., twice a day for 4 d) on 5-HT<sub>1A</sub> receptor-mediated control of the firing of dorsal raphe nucleus (DRN) serotonergic neurons in 5-HTT<sup>-/-</sup> mutants compared to 5-HTT<sup>+/+</sup> wild-type mice. (a, b) Integrated firing rate histograms (in spikes/10 s) showing the inhibitory effect of increasing concentrations of the 5-HT<sub>1A</sub> receptor agonist, ipsapirone, on the electrical activity of DRN 5-HT neurons in 5-HTT<sup>-/-</sup> mutants (right) compared with paired 5-HTT<sup>+/+</sup> mice (left: i = saline, ii = MDMA). Application of the 5-HT<sub>1A</sub> receptor antagonist WAY 100635 (10 nM) increased 5-HT neuron firing only in MDMA-treated 5-HTT<sup>+/+</sup> mice (a, iii). (c, d) Concentration–response curves of ipsapirone-induced inhibition of the firing of DRN 5-HT neurons in brainstem slices from 5-HTT<sup>+/+</sup> (left) and 5-HTT<sup>-/-</sup> (right) mice treated with saline or MDMA. Recordings were carried out 28 d after saline or MDMA treatment. Ipsapirone-induced inhibition is expressed as a percentage of the baseline firing rate. Each point is the mean ± S.E.M. of data obtained from 14–24 individual cells. The dotted lines illustrate the EC<sub>50</sub> values of ipsapirone (abscissa). \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  compared to the respective inhibition in control saline-treated mice (two-way ANOVA followed by a Bonferroni post-hoc test).

( $37.44 \pm 0.08$  °C,  $n = 40$ ) mice. In saline-treated mice, acute administration of 8-OH-DPAT (0.06–0.3 mg/kg s.c.) induced a dose-dependent decrease in rectal temperature, which peaked at 20 min with a maximum decrease of  $-2.14 \pm 0.30$  °C after treatment with 0.10 mg/kg 8-OH-DPAT (Figure 4). At the same dose, 8-OH-DPAT-induced decrease in rectal temperature was ~60% larger in MDMA-treated mice than in

those treated with the vehicle (Figure 4), with a maximum decrease of  $-3.43 \pm 0.42$  °C ( $p < 0.01$ , two-way ANOVA followed by a Bonferroni post-hoc test). Graphical determination (not shown) of 8-OH-DPAT EC<sub>50</sub> values also showed a greater sensitivity to the hypothermic effect of the 5-HT<sub>1A</sub> receptor agonist in MDMA-treated (EC<sub>50</sub> 0.062 mg/kg) than in saline-treated (EC<sub>50</sub> 0.081 mg/kg) wild-type mice.



**Figure 4.** Long term (+28 d) effects of MDMA treatment (20 mg/kg i.p., twice a day for 4 d) on 8-OH-DPAT-induced hypothermia in 5-HTT<sup>+/+</sup> mice. Maximal change (in °C) in rectal temperature relative to baseline values (immediately before subcutaneous injection) was determined generally 20 min after subcutaneous injection of vehicle (0.00 on abscissa) or 8-OH-DPAT (0.06, 0.10 or 0.30 mg/kg). The lowest dose of 8-OH-DPAT (0.06 mg/kg) produced a significant hypothermia only in MDMA-treated mice. In contrast, at the highest dose of 8-OH-DPAT (0.30 mg/kg), the resulting hypothermia did not significantly differ in MDMA-treated vs. saline-treated mice. Each bar is the mean  $\pm$  S.E.M. of 7–10 determinations in each group. \*\*  $p < 0.01$  (two-way ANOVA followed by a Bonferroni post-hoc test), +  $p < 0.05$  compared to vehicle (0.00 on abscissa) in respective saline-treated or MDMA-treated group.

#### Effects of MDMA treatment on hippocampal cell proliferation

The BrdU-labelled cells were located in the subgranular zone of the dentate gyrus, where they frequently made clusters, in both wild-type and 5-HTT<sup>-/-</sup> mice (Figure 5a). The total number of BrdU-positive cells was not significantly different in 5-HTT<sup>-/-</sup> compared to 5-HTT<sup>+/+</sup> mice, with a mean ( $\pm$  S.E.M.) value per hippocampus of  $1031 \pm 61$  ( $n=4$ ) and  $1017 \pm 39$  ( $n=6$ ), respectively (Figure 5b). Four weeks after MDMA treatment, hippocampal cell proliferation was significantly decreased in wild-type mice (approx. -30%;  $F_{1,16} = 11.95$ ,  $p < 0.01$ ), with a total of  $746 \pm 59$  ( $n=5$ ) BrdU-positive cells per hippocampus. In contrast, MDMA pre-treatment exerted no significant effect on hippocampal cell proliferation in 5-HTT<sup>-/-</sup> mutants (Figure 5b).

#### Effects of MDMA treatment on mouse behaviour in the TST

In both wild-type and 5-HTT<sup>-/-</sup> mice, there was no significant effect of the MDMA treatment ( $F_{1,36} = 0.03$ ,

$p > 0.05$ ) on immobility time in the TST. However, we found a significant effect of the genotype in this test ( $F_{1,36} = 173$ ,  $p < 0.001$ ). Saline-treated 5-HTT<sup>-/-</sup> mice were about four times less immobile than their wild-type controls:  $50 \pm 8$  s ( $n=13$ ) vs.  $190 \pm 11$  s ( $n=9$ ), respectively (Figure 6).

#### Effects of MDMA treatment on mouse behaviour in the FST

As shown in Figure 7, a significant effect of the genotype was found in the FST ( $F_{1,38} = 26$ ,  $p < 0.001$ ). The duration of immobility of saline-treated 5-HTT<sup>-/-</sup> mice was about twice of that of wild-type controls ( $252 \pm 12$  s,  $n=9$ , vs.  $139 \pm 16$  s,  $n=12$ , respectively).

MDMA treatment significantly increased immobility duration in wild-type mice (+56%;  $F_{1,38} = 6.7$ ,  $p < 0.05$ ), but had no significant influence on this parameter in the 5-HTT<sup>-/-</sup> mutants (Figure 7).

## Discussion

The purpose of the present study was to investigate whether a subchronic treatment with MDMA might exert effects on the 5-HT system in mice and to evaluate whether or not such effects were induced by MDMA interaction with its main molecular target, the 5-HTT.

We first saw evidence that an acute in-vitro application of MDMA on mouse DRN slices induced a concentration-dependent 5-HT overflow and inhibition of 5-HT neuron firing. Interestingly, both effects occurred within the same range of MDMA concentrations, suggesting that 5-HT cell firing inhibition induced by MDMA was caused by the endogenous 5-HT released, which bound to inhibitory 5-HT<sub>1A</sub> autoreceptors. As expected from this interpretation, blockade of the latter receptors by WAY 100635 prevented the inhibitory effect of MDMA on 5-HT cell firing. Similarly, Sprouse et al. (1989) provided evidence that, in rat brainstem slices, MDMA-induced inhibition of 5-HT cell firing indirectly resulted from the release of 5-HT evoked by the drug. That MDMA-induced effects were directly linked to the drug action on 5-HTT was confirmed by the fact that this drug, up to 0.1 mM, induced neither 5-HT release nor 5-HT neuron firing inhibition in 5-HTT<sup>-/-</sup> mice. However, in the latter mutants, a higher concentration (0.2 mM) of MDMA induced an inhibition of 5-HT cell firing, which could be prevented by WAY 100635. Since, at this concentration, MDMA did not evoke 5-HT release from 5-HTT<sup>-/-</sup> tissues, these data suggest that MDMA could directly activate 5-HT<sub>1A</sub> inhibitory autoreceptors, but with a very low potency (Battaglia et al., 1988).



**Table 1.** Effects of MDMA treatment on 5-HT levels and [<sup>3</sup>H]citalopram specific binding in the anterior raphe area, hippocampus, striatum and cortex in 5-HTT<sup>+/+</sup> and 5-HTT<sup>-/-</sup> mice

Area	Genotype	[5-HT] tissue levels (ng/g tissue)		[ <sup>3</sup> H]citalopram binding (cpm/10 μg protein)	
		Saline	MDMA	Saline	MDMA
Anterior raphe	WT	1220 ± 39	1073 ± 48 <sup>##</sup>	131.1 ± 5.5	116.3 ± 5.4
	KO	216 ± 12 <sup>***</sup>	222 ± 14	2.0 ± 0.5 <sup>***</sup>	1.6 ± 0.6
Hippocampus	WT	529 ± 11	489 ± 21 <sup>#</sup>	93.1 ± 4.8	92.6 ± 3.2
	KO	105 ± 5 <sup>***</sup>	94 ± 6	2.2 ± 1.0 <sup>***</sup>	1.5 ± 0.5
Striatum	WT	458 ± 14	395 ± 16 <sup>##</sup>	79.9 ± 3.2	72.9 ± 7.3
	KO	116 ± 5 <sup>***</sup>	98 ± 9	5.0 ± 0.8 <sup>***</sup>	5.3 ± 1.2
Cortex	WT	558 ± 26	529 ± 19	99.7 ± 7.3	95.1 ± 7.5
	KO	117 ± 12 <sup>***</sup>	110 ± 9	2.6 ± 0.5 <sup>***</sup>	0.3 ± 0.8

WT, Wild-type; KO, knock-out.

Mice received either saline or MDMA (20 mg/kg i.p. × 2; 3 h apart) daily for 4 d and were sacrificed 28 d after the last injection. 5-HT levels are expressed as ng/g tissue, and [<sup>3</sup>H]citalopram specific binding as cpm/10 μg protein.

Values are the means ± s.e.m. of independent determinations in 9–11 mice of each genotype.

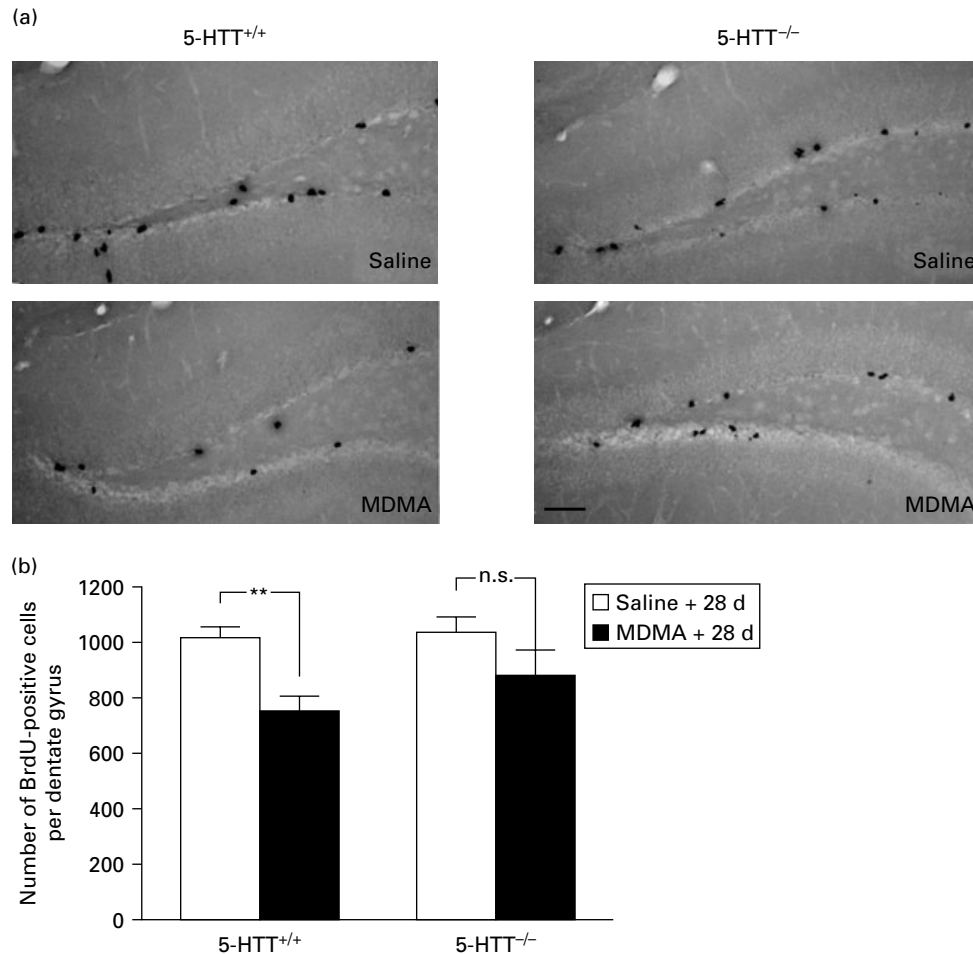
#*p* < 0.05, ##*p* < 0.01, compared to saline-treated mice of the same genotype, \*\*\**p* < 0.001 compared to saline-treated 5-HTT<sup>+/+</sup> mice.

The second part of our study was dedicated to exploring the delayed effects (after 28 d) of a 4-d treatment with MDMA at 20 mg/kg twice daily.

In wild-type mice, the spontaneous in-vitro firing of DRN 5-HT neurons was significantly reduced 4 wk after MDMA treatment. Interestingly, a lower baseline firing rate of DRN 5-HT neurons has also been observed in a validated animal model of depression (Kinney et al., 1997). In MDMA-treated mice, the long-lasting reduction of DRN 5-HT neuron firing was associated with a higher potency of the 5-HT<sub>1A</sub> receptor agonist ipsapirone to inhibit cell discharge, indicating that this MDMA treatment led to 5-HT<sub>1A</sub> autoreceptor supersensitivity, and, consequently, to a reduction in brain 5-HT tone. Interestingly, a 14-d treatment with cocaine was also shown to supersensitize 5-HT<sub>1A</sub> autoreceptors in rats (Cunningham et al., 1992), and chronic alcohol intake was reported to produce the same effect in C57BL/6J mice (Kelai et al., 2005). In contrast, 5-HT<sub>1A</sub> autoreceptor desensitization has been repeatedly observed after a 2–3 wk treatment with SSRIs (Blier and de Montigny, 1983; Blier et al., 1998; Le Poul et al., 1995, 2000), although both these drugs and MDMA share the same molecular target, i.e. the 5-HTT. As already shown for the in-vitro effects of MDMA, the delayed in-vivo changes evoked by MDMA were clearly related to the drug action onto 5-HTT since no 5-HT<sub>1A</sub> autoreceptor supersensitivity was noted in MDMA-treated 5-HTT<sup>-/-</sup> mice.

In the mouse, the involvement of 5-HT<sub>1A</sub> autoreceptors in 8-OH-DPAT-induced hypothermia is clearly established (Bill et al., 1991; Goodwin et al., 1985). Interestingly, the hypothermic response to 8-OH-DPAT was enhanced in wild-type mice that had been treated with MDMA 4 wk previously, thereby confirming the occurrence of 5-HT<sub>1A</sub> autoreceptor supersensitivity in these animals. However, an MDMA-induced sensitization of 5-HT<sub>7</sub> receptors cannot be excluded since the involvement of these receptors in 8-OH-DPAT-induced hypothermia has also been reported in mice (Hedlund et al., 2004). Whether such an adaptive change occurs under MDMA treatment conditions used here is an interesting question to be addressed in future studies.

In addition to its effect on 5-HT<sub>1A</sub> autoreceptors, MDMA treatment also induced a delayed, limited, but significant, decrease in 5-HT levels in most brain areas examined in wild-type mice. Because MDMA can cause the degeneration of 5-HT axons and terminals (Green et al., 1995; McCann et al., 1998; O'Shea et al., 1998, 2001; Xie et al., 2006), it might be proposed that such an effect occurred under the dose regimen used, and that the reduction in brain 5-HT levels reflected a loss of 5-HT fibres and terminals. However, MDMA treatment did not significantly alter [<sup>3</sup>H]citalopram binding in the same areas as those where 5-HT levels were decreased, allowing the inference that the latter changes could not be ascribed to some loss of 5-HT fibres and terminals. Accordingly, the decreased 5-HT

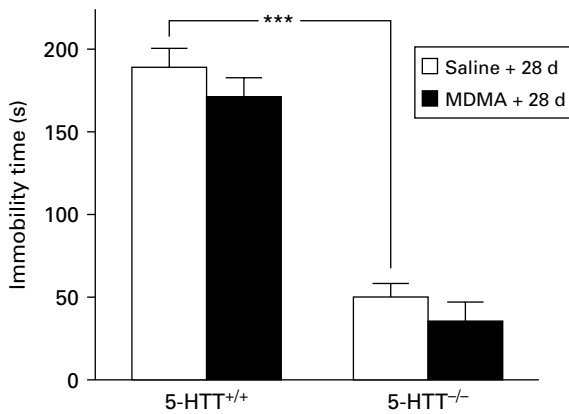


**Figure 5.** Long-term (+28 d) effects of MDMA treatment (20 mg/kg i.p., twice a day for 4 d) on cell proliferation within the granule cell layer in the dentate gyrus of both 5-HTT<sup>+/+</sup> and 5-HTT<sup>-/-</sup> mice. (a) Photomicrographs of BrdU-positive cells examined under  $\times 20$  magnification in the dentate gyrus of 5-HTT<sup>+/+</sup> (left) and 5-HTT<sup>-/-</sup> (right) mice treated with saline or MDMA. (b) Number of BrdU-positive cells 28 d after MDMA vs. saline treatment, in 5-HTT<sup>-/-</sup> mutants compared to 5-HTT<sup>+/+</sup> mice. The number of cells actually counted in sections was multiplied by four (see Materials and methods) to obtain an estimate of the total number of BrdU-positive cells in the entire dentate gyrus (from Bregma  $-0.94$  to  $-3.80$ ). Each bar is the mean  $\pm$  S.E.M. of data obtained in 4–6 mice in each group. \*\*  $p < 0.01$ , n.s., non-significant (two-way ANOVA followed by a Bonferroni post-hoc test). Scale bar, 200  $\mu$ m.

levels most probably reflected a decrease in 5-HT synthesis possibly linked to 5-HT<sub>1A</sub> autoreceptor supersensitivity, thereby further contributing to lower brain 5-HT tone in MDMA-treated mice.

Because both hippocampal cell proliferation and mouse depressive-like behaviour are clearly dependent on the brain 5-HT tone, through the stimulation of specific 5-HT receptors (Banasr et al., 2004; O'Leary et al., 2007; Radley and Jacobs, 2002; Santarelli et al., 2003), quantification of these two relevant parameters was performed in order to further assess the functional consequences of MDMA-induced changes in brain

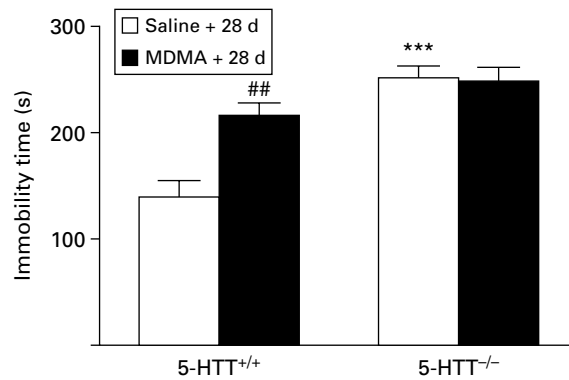
5-HT neurotransmission. Four weeks after the 4-d treatment with MDMA, cell proliferation was significantly reduced within the subgranular zone and the hilus of the dentate gyrus. However, this effect was not observed in 5-HTT<sup>-/-</sup> mice, further confirming that MDMA-induced functional alterations resulted from drug action at the 5-HT transporter. Interestingly, this effect of MDMA, also found under other treatment conditions (Cho et al., 2007; Hernandez-Rabaza et al., 2006), closely resembled that of other drugs of abuse such as cocaine, methamphetamine, heroin and nicotine (Abrous et al., 2002; Crews et al., 2006; Eisch



**Figure 6.** Long-term (+28 d) effects of MDMA treatment (20 mg/kg i.p, twice a day for 4 d) on immobility time (in seconds) in 5-HTT<sup>+/+</sup> and 5-HTT<sup>-/-</sup> mice subjected to the tail suspension test. No significant effect of MDMA was noted in both 5-HTT<sup>+/+</sup> and 5-HTT<sup>-/-</sup> mice. Each bar is the mean  $\pm$  S.E.M. of determinations in 9–13 mice per group. \*\*\*  $p < 0.001$  (two-way ANOVA followed by a Bonferroni post-hoc test).

et al., 2000; Teuchert-Noodt et al., 2000; Yamaguchi et al., 2005), but was opposite to that of SSRIs, which have been consistently shown to enhance hippocampal cell proliferation in rodents (Malberg et al., 2000; Santarelli et al., 2003).

Together with the convergent data which all showed MDMA-induced changes in 5-HT neurotransmission globally opposite to those occurring after chronic treatment with SSRI antidepressants, we observed an increase in FST immobility time in MDMA-pretreated 5-HTT<sup>+/+</sup> mice. Using the same paradigm, Thompson et al. (2004) reported a similar depression-like behaviour 2 months after MDMA treatment in rats. However, inconsistent data were obtained using the TST since we did not observe any significant change in TST immobility duration after MDMA treatment in 5-HTT<sup>+/+</sup> mice. These discrepant data further corroborate those repeatedly reported in the literature (Cryan et al., 2005) with a large body of evidence showing that the neurochemical pathways mediating animal performance in these two tests are clearly distinct (Bai et al., 2001; Porsolt and Lenegre, 1992). Indeed, discrepancies were even noted regardless of MDMA treatment because we found that vehicle-treated 5-HTT<sup>-/-</sup> mutants were much less immobile than 5-HTT<sup>+/+</sup> mice in the TST, whereas the reverse was observed in the FST. Interestingly, these behavioural data completely agreed with previous findings in 5-HTT<sup>-/-</sup> vs. wild-type mice raised on Sv129 genetic background (Holmes et al., 2002).



**Figure 7.** Long term (+28 d) effects of MDMA treatment (20 mg/kg i.p, twice a day for 4 d) on immobility time (in seconds) in 5-HTT<sup>+/+</sup> and 5-HTT<sup>-/-</sup> mice subjected to the forced swim test. MDMA treatment induced an increase of immobility time only in 5-HTT<sup>+/+</sup> mice. Each bar is the mean  $\pm$  S.E.M. of determinations in 8–13 mice per group. ##  $p < 0.01$ , compared to saline-treated mice of the same genotype, \*\*\*  $p < 0.001$  compared to saline-treated 5-HTT<sup>+/+</sup> mice (two-way ANOVA followed by a Bonferroni post-hoc test).

In conclusion, our data showed that a subchronic exposure to MDMA induced strong and persistent long-term negative effects on the 5-HT system, independently of its neurotoxicity, that might contribute to the mood and cognitive disorders observed in former MDMA users. Indeed, these MDMA-induced neurobiological and behavioural effects are globally opposite to those induced by chronic treatment with SSRI antidepressants. However, the molecular mechanisms by which 5-HT<sub>1A</sub> autoreceptors become supersensitive in MDMA-treated mice remain to be elucidated. In particular, the possible role of the corticotrope system, which exerts marked influence on 5-HT<sub>1A</sub> receptor expression and signaling mechanisms (Lopez et al., 1999; Ou et al., 2001), should be addressed in further studies.

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**Statement of Interest**

None.

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