3,4-Methylenedioxymethamphetamine Self-Administration is Abolished in Serotonin Transporter Knockout Mice

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Background: The neurobiological mechanism underlying the reinforcing effects of 3,4-methylenedioxymethamphetamine (MDMA) remains unclear. The aim of the present study was to determine the contribution of the serotonin transporter (SERT) in MDMA self-administration behavior by using knockout (KO) mice deficient in SERT.

Methods: Knockout mice and wild-type (WT) littermates were trained to acquire intravenous self-administration of MDMA (0, .03, .06, .125, and .25 mg/kg/infusion) on a fixed ratio 1 (FR1) schedule of reinforcement. Additional groups of mice were trained to obtain food and water to rule out operant responding impairments. Microdialysis studies were performed to evaluate dopamine (DA) and serotonin (5-HT) extracellular levels in the nucleus accumbens (NAC) and prefrontal cortex (PFC), respectively, after acute MDMA (10 mg/kg).

Results: None of the MDMA doses tested maintained intravenous self-administration in KO animals, whereas WT mice acquired responding for MDMA. Acquisition of operant responding for food and water was delayed in KO mice, but no differences between genotypes were observed on the last day of training. MDMA increased DA extracellular levels to a similar extent in the NAC of WT and KO mice. Conversely, extracellular concentrations of 5-HT in the PFC were increased following MDMA only in WT mice.

Conclusions: These findings provide evidence for the specific involvement of SERT in MDMA reinforcing properties.

Key Words: 5-HT, DA, ecstasy, food and water operant responding, in vivo microdialysis, nucleus accumbens, prefrontal cortex

The psychoactive phenethylamine derivative 3,4-methylenedioxymethamphetamine (MDMA) is widely employed as a substance of recreational abuse. The increased popularity of this drug is associated with the search for its primary subjective effects, which include feelings of openness, euphoria, empathy, and heightened self-awareness (Cami *et al.* 2000) and also with the perception of harmlessness surrounding MDMA intake (Murphy *et al.* 2006). The rewarding properties of this compound have been established in monkeys (Beardsley *et al.* 1986; Fantegrossi *et al.* 2002; Lamb and Griffiths 1987; Lile *et al.* 2005), rats (Bilsky *et al.* 1991; Ratzenboeck *et al.* 2001; Schenk *et al.* 2003), and mice (Robledo *et al.* 2004a, 2004b; Salzmann *et al.* 2003; Trigo *et al.* 2006) by the use of different paradigms, such as intravenous self-administration and conditioned place preference (CPP). However, the exact mechanism by which MDMA exerts its rewarding properties still remains unclear. Indeed, MDMA has a complex neurochemical profile (Green *et al.* 1995), increasing synaptic concentrations of serotonin (5-HT), dopamine (DA), and norepinephrine (Rothman *et al.* 2001), as well as of other neurotransmitters such as gamma-aminobutyric acid (GABA) and acetylcholine (Acquas *et al.* 2001; Bankson and Yamamoto 2004). However, the ability of MDMA to increase DA in the nucleus accumbens (NAC) (Cadoni *et al.* 2005; Camarero *et al.* 2002; Marona-Lewicka *et al.* 1996; Robledo *et al.* 2004b; White *et al.* 1996; Yamamoto and Spanos 1988), together with behavioral studies showing the involvement of the dopaminergic system in MDMA CPP (Bilsky *et al.* 1998) and intravenous self-administration (Daniela *et al.* 2004), support the view that activation of the dopaminergic system plays a crucial role in the rewarding/reinforcing properties of MDMA.

On the other hand, emerging data support a role of 5-HT in MDMA rewarding effects. Using the intravenous self-administration paradigm, it has been shown that 5-HT_{2A} receptors are involved in the reinforcing effects of MDMA in monkeys (Fantegrossi *et al.* 2002). In addition, the 5-HT_3 receptor antagonist MDL 72222 blocked the acquisition of MDMA-induced CPP in rats (Bilsky and Reid 1991). Also, in drug discrimination studies, certain doses of MDMA generalized to serotonergically active compounds, such as fenfluramine and norfenfluramine (Goodwin *et al.* 2003; Schechter 1997). In agreement, MDMA binds with higher affinity to the 5-HT transporter (SERT) than to the DA transporter (DAT) (Han and Gu 2006; Rothman and Baumann 2003) and produces a higher release of 5-HT than DA (Koch and Galloway 1997; Schmidt *et al.* 1987). Also in humans, relevant studies have shown that most of the MDMA effects are markedly reduced after the administration of 5-HT receptor antagonists or 5-HT uptake inhibitors, suggesting that these effects are dependent on SERT-mediated enhancement of 5-HT transmission (Liechti *et al.* 2000a, 2000b).

The main goal of our study was to evaluate the role of SERT in the reinforcing properties of MDMA using the intravenous drug self-administration paradigm in SERT knockout (KO) and wild-type (WT) mice. Operant responding to obtain food and water was also evaluated to determine the possible existence of a general impairment in the acquisition of an operant behavior in KO mice. Finally, in vivo microdialysis studies were performed to

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evaluate the effects induced by acute MDMA in DA and 5-HT extracellular levels.

Methods and Materials

Animals

Experiments were performed using homozygous male SERT KO mice and WT littermates born from heterozygous mutants at the tenth generation (F10) of backcrossing with C57Bl/6J mice. Genotyping was performed as described by Bengel *et al.* (1998). Mice were housed five per cage with ad libitum food and water, except for animals in the food and water operant behavior studies, which followed a deprivation regimen (see below for details), in a temperature (21 ± 1°C) and humidity (65 ± 10%) controlled room with a reversed 12-hour/12-hour light/dark cycle (lights off from 08:00 to 20:00). Mice were maintained under a normal 12-hour/12-hour light/dark cycle (lights on from 08:00 to 20:00) for microdialysis studies. Self-administration studies took place during the dark phase and microdialysis studies during the light phase of the light/dark cycle. Mice were between 6 and 10 weeks old at arrival and weighed 25 to 30 g at the beginning of the experiments. Behavioral tests and animal care were conducted in accordance with the standard ethical guidelines (National Institutes of Health; European Communities Directive 86/609 EEC) and approved by the local ethical committee Comité Etico Experimental Animal-Instituto Municipal de Asistencia Sanitaria-Universitat Pompeu Fabra (CEEA-IMAS-UPF).

Drugs

The MDMA hydrochloride [(+/−) 3,4-methylenedioxymethamphetamine] was obtained from Lipomed, A.G. (Arlesheim, Switzerland) and dissolved in .9% sodium chloride (NaCl).

Drug Self-Administration

Apparatus. Self-administration training and testing occurred in 16 operant chambers (Model ENV-307A-CT, Med-Associates, St. Albans, Vermont) equipped with two holes; one hole was selected as the active hole for delivering the drug and the other as the inactive hole. In half of the operant chambers, the active hole was placed at the right, and in the other half of the operant chambers, it was placed at the left. Acquisition of drug self-administration was performed using a fixed ratio 1 (FR1) schedule of reinforcement such that one nose-poke in the active hole resulted in one MDMA infusion, while nose-poking in the inactive hole had no programmed consequences. A stimulus light, located above the active hole, was paired contingently with the delivery of the reinforcer. Infusions were delivered in a volume of 23.5 μL over 2 sec.

Surgery. Mice were anesthetized with a ketamine/xylazine mixture (5:1; .10 mL/10 g body weight, intraperitoneal [IP]) and then implanted with an indwelling intravenous (IV) silastic catheter in the right jugular vein, as previously described (Soria *et al.* 2005). After surgery, mice were individually housed. The patency of the catheters was evaluated periodically (once a week) and whenever drug self-administration behavior appeared to deviate dramatically from the one previously observed, by infusing .1 mL of thiopental (5 mg/mL) through the catheter. If prominent signs of anesthesia were not apparent within 3 sec of the infusion, the mouse was removed from the experiment. A total number of 136 mice were operated and 87 mice maintained patent catheters during the whole experimental sequences, which were included in the data analysis and figures.

Drug Self-Administration Procedure. Four days after surgery, different groups of KO and WT control mice were trained to nose-poke under a FR1 schedule of reinforcement to receive one of five different doses of MDMA (0, .03, .06, .125, and .25 mg/kg/infusion) during 10 days, as previously reported (Trigo *et al.* 2006). Daily self-administration started with a priming infusion of MDMA at the selected training dose for that animal, lasted for 120 min, and was conducted 6 days per week. The number of infusions was limited to a maximum of 100 per session, since previous studies showed that when mice were trained at doses lower than .5 mg/kg/infusión, they took close to 100 infusions in 2 hours (Trigo *et al.* 2006). Each infusion was followed by a 30 sec time-out period during which an active nose-poke had no consequence. Stable acquisition of self-administration behavior was achieved when all of the following conditions were met: 1) less than 20% deviation from the mean of the total number of reinforcers earned in three consecutive sessions (80% stability); 2) at least 65% responding on the active hole; and 3) a minimum of eight reinforcers earned per session.

Food-Maintained Operant Behavior. Naive mice were partially deprived of food and trained on a FR1 schedule of reinforcement in the operant cages, as previously described (Soria *et al.* 2005). A 10 sec time-out period was established after each reinforcement, and the session was finished once 100 pellets were delivered or after 1 hour, whichever occurred first. Only those mice achieving the acquisition criteria underwent a progressive ratio (PR) schedule of reinforcement, as previously reported (Soria *et al.* 2005). The PR session lasted for 2 hours or until mice failed to complete the ratio for delivery of one pellet within 1 hour and was performed only once. The breaking point was determined as the maximum ratio completed before the end of the session.

Water-Maintained Operant Behavior. Naive mice were partially deprived of water and trained at the operant cages, as previously described (Soria *et al.* 2005). Water operant responding sessions were conducted as described above for food-maintained responding, except that the food dispenser was changed for a liquid dipper, and one response on the active nose-poke delivered 10 mL of water in 10 sec. Mice were trained under a FR1 schedule of reinforcement with a 20 sec time-out period after each response. The session was terminated after 100 water drinks were delivered or after 1 hour, whichever occurred first. Mice achieving the acquisition criteria underwent a PR schedule of reinforcement, as described above.

Surgery and Microdialysis Procedure

Mice were anesthetized with a ketamine/xylazine mixture (5:1; .1 mL/10g body weight, IP) and placed in a stereotaxic apparatus with a flat skull (Franklin and Paxinos 1997). Evaluation of DA extracellular levels has been previously described (Robledo *et al.* 2004b). Evaluation of 5-HT extracellular levels was conducted in a separate group of mice. A 2 mm analytical probe (CMA/7/2 mm; Microdialysis, Stockholm, Sweden) was implanted directly in the prefrontal cortex (PFC) (antero-posterior (AP): +2.2 mm; medio-lateral (ML): ±.5 mm; dorso-ventral (DV): -3.0 mm from bregma). One day after probe implantation, mice were habituated to the experimental environment overnight. The following morning, a Ringer solution was pumped through the dialysis probe (sodium chloride [NaCl]: 148 mmol/L, potassium chloride [KCl]: 2.7 mmol/L, calcium chloride [$CaCl_2$]: 1.2 mmol/L, and magnesium chloride [$MgCl_2$]: .8 mmol/L, pH 6.0) at a constant rate of 1 μL/min. Baseline samples were taken during 2 hours, and subsequently mice were injected with

MDMA (10 mg/kg, IP). Collection of samples (every 15 min) was continued for 4 hours following injection. The dose of 10 mg/kg (IP) was chosen since it has been reported previously that this dose of MDMA is rewarding in the CPP (Robledo *et al.* 2004a) and increases dopamine levels in the NAC (Robledo *et al.* 2004b). Lower doses of MDMA (3 mg/kg) do not induce CPP or produce significant increments in DA levels in the NAC of mice (Robledo *et al,* unpublished data).

Analytical Procedure

Dialysate samples (15 μL) were injected without any purification into a high-performance liquid chromatography (HPLC) system that consisted of a pump linked to an automatic injector (Agilent 1100, Palo Alto, California), a reverse-phase column (Zorbax SB C18, 3.5 μm, 150 x 4.6 mm, Agilent Technologies), and a coulometric detector (Coulochem II, ESA Inc., Chelmsford, Maryland) with a 5011A analytical cell. Dopamine was quantified as previously described (Robledo *et al.* 2004b). To quantify 5-HT, the first electrode was fixed at -50 mV and the second electrode at +300 mV. The gain of the detector was set at 10 nA. The composition of the mobile phase was 50 mmol/L sodium acetate, .1 mmol/L ethylenediaminetetraacetic acid (EDTA), .65 mmol/L octyl sodium sulfate, and 20% (vol/vol) methanol, pH 5.0. The flow rate in both assays was set at 1 mL/min. The sensitivity of the assay for DA was 1 pg/15 μL and .5 pg/15 μL for 5-HT.

Histology

At the end of the experiments, mice were sacrificed and brains were cut using a cryostat in 20 μm serial coronal sections, which were then processed with Cresyl Violet (Sigma-Aldrich, Madrid, Spain) and observed under a microscope. Only those mice with correct probe placements were used in the study.

Statistical Analysis

Differences in self-administration of MDMA between genotypes were analyzed using a three-way analysis of variance (ANOVA) (GENOTYPE x DAY x HOLE), followed by two-way ANOVAs for differences between the active and the inactive holes, and one-way ANOVAs for comparisons between holes. Food and water maintained operant responding was also analyzed using two-way and one-way ANOVAs for establishing comparisons between holes. The data for the dose response curve and intake were analyzed using one-way ANOVAs followed by the Dunnett and Tukey post hoc tests, respectively. Differences in the breaking points achieved by mice trained with food and water were analyzed using one-way ANOVAs. Basal extracellular levels of DA and 5-HT were analyzed between genotypes using one-way ANOVA, and the effects of MDMA administration on DA and 5-HT outflow were analyzed using two-way ANOVA (GENOTYPE x TIME after injection) followed by one-way ANOVAs comparing genotypes at each time point.

Results

MDMA Self-Administration in SERT WT and KO Mice

Figure 1 shows the acquisition and maintenance of operant responding for different doses of MDMA (.03, .06, .125, and .25 mg/kg/infusion) in WT mice. This behavior was abolished in KO mice. Significance values for two-way and three-way ANOVAs performed for each dose of MDMA are shown on Table 1.

In WT mice trained to self-administer saline (n = 9), only 22.22% reached acquisition criteria with a mean of 3.50 ± .50 days. However, mice trained at the dose of .03 mg/kg/infusion started to discriminate between the active and the inactive holes from the first session, and discrimination was maintained until the last session. At this dose, 55.55% of the animals reached the acquisition criteria with a mean of 4.50 ± .67 days. At the dose of .06 mg/kg/infusion, mice discriminated between the active and the inactive holes from the fourth session, and 66.66% of the mice reached stability criteria with a mean of 3.00 ± .00 days. At the dose of .125 mg/kg/infusion, 70.58% of the mice reached the stability criteria with a mean of 6.00 ± .67 days, and at the dose of .25 mg/kg/infusion, 55.55% of the animals reached stability criteria with a mean of 3.16 ± .47 days (see one-way ANOVA values in Table 2).

In KO mice, a significant effect of day appeared at the doses of .06 mg/kg/infusion, .125 mg/kg/infusion, and .25 mg/kg/infusion (see Table 1 for two-way ANOVA values). No significant discrimination between holes was observed at any of the doses tested (see Table 2 for one-way ANOVA values). Only one KO mouse trained to self-administer saline (n = 12) reached acquisition criteria (8.33%) at day 6, and one (16.66%) reached acquisition criteria at day 8 for .06 mg/kg/infusion of MDMA.

MDMA Dose-Dependent Effects and Intake

Figure 2 shows the number of infusions obtained and the amount of drug consumed on day 10 by KO and WT mice at the different doses of MDMA tested (0, .03, .06, .125, and .25 mg/kg/infusion). Day 10 was chosen for this analysis to be sure of the acquisition of a constant and reliable operant responding to self-administer MDMA. One-way ANOVA revealed a significant main effect of dose in WT mice trained with MDMA at the dose of .06 mg/kg/infusion [$F(4,42) = 2.696$, $p < .05$]. Wild-type mice exhibited a higher rate of responding when compared with the saline group in the last training session (Dunnett post hoc test, $p < .05$). For MDMA intake, one-way ANOVA showed significant differences [$F(3,33) = 4.304$, $p < .05$] between the doses of .25 and .03 mg/kg in WT mice at day 10 (Tukey post hoc test, $p < .05$). Conversely, in KO mice, no significant effects of dose were observed for the number of infusions obtained or for intake of MDMA.

Food Maintained Operant Behavior in SERT KO and WT Mice

Knockout and WT control mice were trained to acquire operant responding for food pellets under a FR1 schedule of reinforcement for 13 days (Figure 3). Discrimination along the different sessions between holes was analyzed in each genotype by using two-way ANOVAs. In WT mice, significant effects of hole [$F(1,10) = 241.295$, $p < .001$], day [$F(12,120) = 7.616$, $p < .001$], and interaction between these two factors [$F(12,120) = 15.261$, $p < .001$] were revealed. Wild-type mice started to discriminate between the active and the inactive holes from the first training session (Figure 3A), and this discrimination was maintained during the next nine operant responding sessions (see Table 3 for one-way ANOVA values). All the WT mice tested reached the stability criteria, and the mean time required to reach it in this group was 4.16 ± 1.24 days.

Two-way ANOVA in KO mice showed a significant effect of hole [$F(1,8) = 10.450$, $p < .05$], day [$F(12,96) = 5.451$, $p < .001$], and interaction between these two factors [$F(12,96) = 4.242$, $p < .001$]. Knockout mice started to discriminate from session 6, and discrimination continued until the last training session (Figure 3B). In this group, the stability criteria were achieved by five out of six mice tested, and the mean time required to reach it was 6.80 ± 1.24 days (see Table 3 for one-way ANOVA values).

Figure 3C shows the number of pellets earned by KO and WT mice during the 13 days of training. Differences in the number of

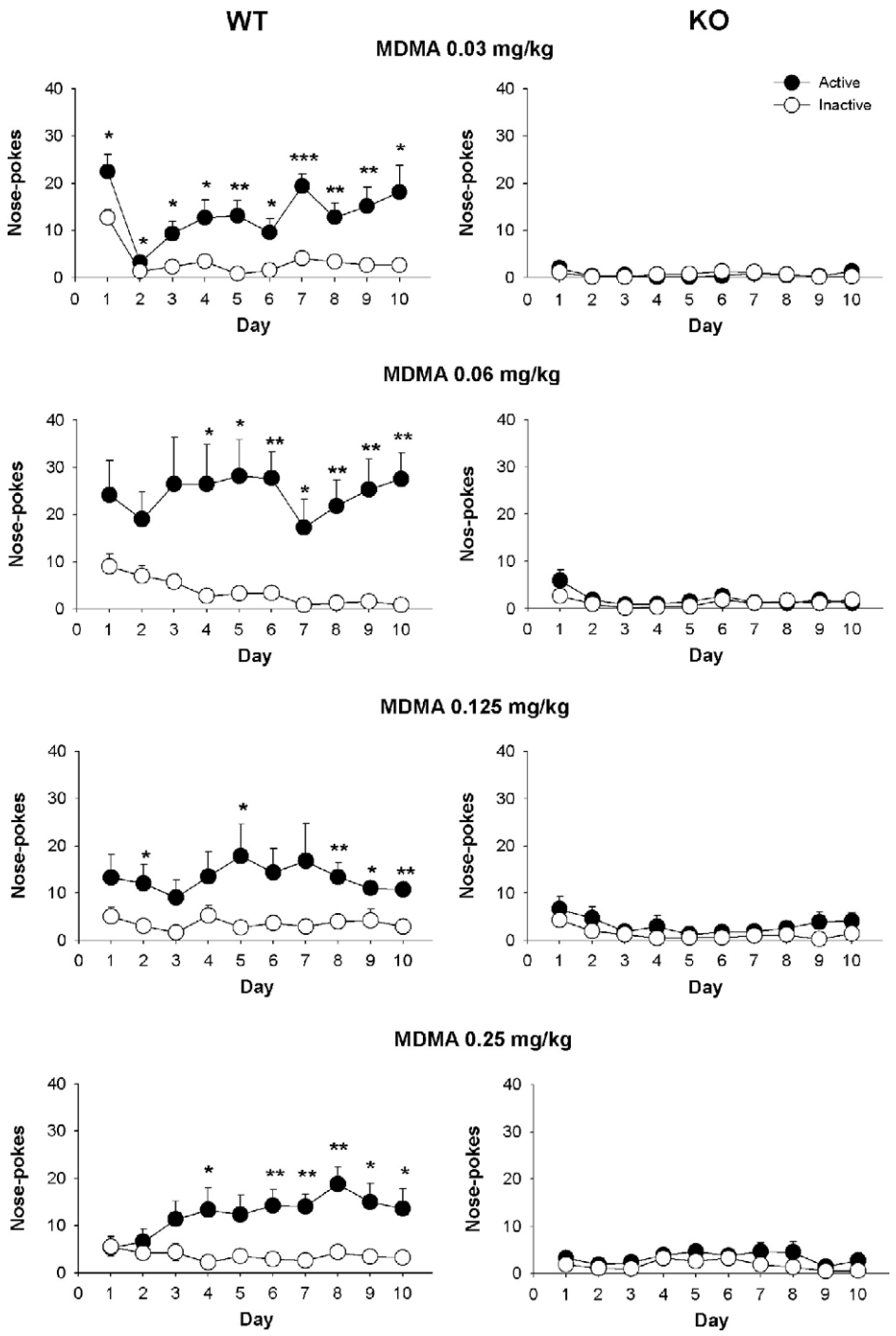

Figure 1. Acquisition of intravenous MDMA self-administration in wild-type (WT) and knockout (KO) drug naïve mice. Average number of nose-pokes +SEM in both the active (filled circles) and the inactive (empty circles) holes in 2-hour sessions during 10 days of training for each dose tested; .03 mg/kg/infusion WT ($n = 9$), KO ($n = 8$); .06 mg/kg/infusion WT ($n = 6$), KO ($n = 6$); .125 mg/kg/infusion WT ($n = 10$), KO ($n = 10$); .25 mg/kg/infusion WT ($n = 9$), KO ($n = 8$). *$p < .05$, **$p < .01$, ***$p < .001$ active versus the inactive hole. MDMA, 3,4-methylenedioxymethamphetamine; WT, wild-type; KO, knockout.

responses to obtain food pellets in both genotypes were analyzed using two-way ANOVA. Significant effects of day [$F(12,108) = 13.541$, $p < .001$], genotype [$F(1,9) = 19.555$, $p < .01$], and interaction between these two factors [$F(12,108) = 2.798$, $p < 0.01$] were observed. Subsequent one-way ANOVA confirmed significant differences between KO and WT mice in the number of pellets on days 1, 5 to 10, and 12 (see Table 3 for one-way ANOVA values).

To evaluate whether KO and WT mice showed differences in the motivation to obtain food, a PR schedule of reinforcement was performed in mice achieving the acquisition criteria during the fixed ratio training. No significant differences were observed

Table 1. Three (Hole × Genotype × Day) and Two-Way ANOVAs (Hole × Day) for Mice Self-Administering MDMA

	MDMA .03 mg/kg		MDMA .06 mg/kg		MDMA .125 mg/kg		MDMA .25 mg/kg	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Three-way ANOVA								
Genotype	$F_{(1,15)} = 66.282$	.001	$F_{(1,10)} = 16.904$	.01	$F_{(1,18)} = 13.594$	.01	$F_{(1,15)} = 11.898$	.01
Hole	$F_{(1,15)} = 27.320$	.001	$F_{(1,10)} = 22.024$	.01	$F_{(1,18)} = 9.422$	.01	$F_{(1,15)} = 9.922$	.01
Day	$F_{(9,135)} = 6.674$	.001	$F_{(9,90)} = .660$	ns	$F_{(1,162)} = .668$	ns	$F_{(9,135)} = 1.806$	ns
Genotype × Day	$F_{(9,135)} = 4.778$	.001	$F_{(9,90)} = .468$	ns	$F_{(1,162)} = .833$	ns	$F_{(9,135)} = 1.382$	ns
Hole × Day	$F_{(9,135)} = 1.435$	ns	$F_{(9,90)} = .600$	ns	$F_{(1,162)} = .338$	ns	$F_{(9,135)} = 2.727$	.01
Genotype × Hole	$F_{(1,15)} = 26.916$	.001	$F_{(1,10)} = 19.794$	.01	$F_{(1,18)} = 4.341$	ns	$F_{(1,15)} = 4.844$	.05
Genotype × Hole × Day	$F_{(9,135)} = 1.357$	ns	$F_{(9,90)} = .803$	ns	$F_{(1,162)} = .824$	ns	$F_{(9,135)} = 2.054$	.05
Two-way ANOVA								
WT								
Hole	$F_{(1,16)} = 31.542$	.001	$F_{(1,10)} = 16.565$	.01	$F_{(1,18)} = 8.758$	.01	$F_{(1,16)} = 12.201$	.01
Day	$F_{(9,144)} = 6.593$	.001	$F_{(9,90)} = .540$	ns	$F_{(9,162)} = .566$	ns	$F_{(9,144)} = 1.602$	ns
Hole × Day	$F_{(9,144)} = 1.591$	ns	$F_{(9,90)} = .581$	ns	$F_{(9,162)} = .470$	ns	$F_{(9,144)} = 2.668$	.01
KO								
Hole	$F_{(1,14)} = .012$	ns	$F_{(1,10)} = .767$	ns	$F_{(1,18)} = 1.860$	ns	$F_{(1,14)} = 1.361$	ns
Day	$F_{(9,126)} = 1.870$	ns	$F_{(9,162)} = 4.298$	.001	$F_{(9,162)} = 3.498$	.01	$F_{(9,126)} = 4.163$	.001
Hole × Day	$F_{(9,126)} = 1.056$	ns	$F_{(9,162)} = 1.086$	ns	$F_{(9,162)} = .515$	ns	$F_{(9,126)} = .886$	ns

ANOVA, analysis of variance; MDMA, 3,4-methylene dioxymethamphetamine.

in the breaking points reached by KO versus WT mice under this PR schedule (Figure 3D).

Water Maintained Operant Behavior in SERT KO and WT Mice

Additional groups of KO and WT control mice were trained to acquire operant behavior for water under a FR1 schedule of reinforcement for 13 days (Figure 4). Discrimination between holes was analyzed in each genotype by using two-way ANOVA. In WT mice, a nonsignificant effect of day [$F(12,120) = 1.126$, ns] but significant effects of hole [$F(1,10) = 84.424$, $p < .001$] and interaction between these two factors [$F(12,120) = 4.423$, $p < .001$] were revealed. Wild-type mice started to discriminate between the active and the inactive holes from the first training session (Figure 4A) and discrimination was maintained during the next nine sessions (see Table 3 for one-way ANOVA values). All the animals tested achieved the stability criteria with a mean of 2.66 ± .55 days.

In KO mice, significant effects of day [$F(12,120) = 2.164$, $p < .05$], hole [$F(1,10) = 22.894$, $p < .01$], and interaction between these two factors [$F(12,120) = 4.962$, $p < .001$] were observed.

Table 2. One-way ANOVAs for Active Versus Inactive Hole Discrimination in SERT WT and KO Mice Self-Administering MDMA

	MDMA .03 mg/kg		MDMA .06 mg/kg		MDMA .125 mg/kg		MDMA .25 mg/kg	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*
WT								
Day 1	$F_{(1,17)} = 5.793$	.05	$F_{(1,11)} = 3.771$	ns	$F_{(1,19)} = 2.506$	ns	$F_{(1,17)} = .005$	ns
Day 2	$F_{(1,17)} = 4.983$	.05	$F_{(1,11)} = 4.748$	ns	$F_{(1,19)} = 4.528$	.05	$F_{(1,17)} = .573$	ns
Day 3	$F_{(1,17)} = 6.759$	.05	$F_{(1,11)} = 4.526$	ns	$F_{(1,19)} = 3.671$	ns	$F_{(1,17)} = 2.681$	ns
Day 4	$F_{(1,17)} = 5.516$	.05	$F_{(1,11)} = 7.141$	.05	$F_{(1,19)} = 2.078$	ns	$F_{(1,17)} = 5.585$	.05
Day 5	$F_{(1,17)} = 14.791$	.01	$F_{(1,11)} = 8.900$	.05	$F_{(1,19)} = 4.768$	.05	$F_{(1,17)} = 3.956$	ns
Day 6	$F_{(1,17)} = 7.649$	.05	$F_{(1,11)} = 13.468$	.01	$F_{(1,19)} = 4.075$	ns	$F_{(1,17)} = 10.978$	.01
Day 7	$F_{(1,17)} = 28.920$	.001	$F_{(1,11)} = 7.652$	.05	$F_{(1,19)} = 3.039$	ns	$F_{(1,17)} = 16.538$	.01
Day 8	$F_{(1,17)} = 9.708$	.01	$F_{(1,11)} = 14.006$	.01	$F_{(1,19)} = 8.529$	.01	$F_{(1,17)} = 14.286$	.01
Day 9	$F_{(1,17)} = 9.270$	.01	$F_{(1,11)} = 17.473$	.01	$F_{(1,19)} = 5.673$	.01	$F_{(1,17)} = 8.064$	.05
Day 10	$F_{(1,17)} = 7.268$	.05	$F_{(1,11)} = 17.872$	.01	$F_{(1,19)} = 17.273$	.01	$F_{(1,17)} = 5.671$	.05
KO								
Day 1	$F_{(1,15)} = .728$	ns	$F_{(1,11)} = 2.041$	ns	$F_{(1,19)} = .652$	ns	$F_{(1,15)} = .812$	ns
Day 2	$F_{(1,15)} = .737$	ns	$F_{(1,11)} = 1.106$	ns	$F_{(1,19)} = .994$	ns	$F_{(1,15)} = .633$	ns
Day 3	$F_{(1,15)} = 1.615$	ns	$F_{(1,11)} = 2.353$	ns	$F_{(1,19)} = .348$	ns	$F_{(1,15)} = 1.829$	ns
Day 4	$F_{(1,15)} = .615$	ns	$F_{(1,11)} = .571$	ns	$F_{(1,19)} = .983$	ns	$F_{(1,15)} = .114$	ns
Day 5	$F_{(1,15)} = .978$	ns	$F_{(1,11)} = 1.200$	ns	$F_{(1,19)} = .729$	ns	$F_{(1,15)} = 1.457$	ns
Day 6	$F_{(1,15)} = 1.367$	ns	$F_{(1,11)} = .179$	ns	$F_{(1,19)} = .982$	ns	$F_{(1,15)} = .086$	ns
Day 7	$F_{(1,15)} = .093$	ns	$F_{(1,11)} = .041$	ns	$F_{(1,19)} = .566$	ns	$F_{(1,15)} = 1.774$	ns
Day 8	$F_{(1,15)} = .089$	ns	$F_{(1,11)} = .220$	ns	$F_{(1,19)} = .729$	ns	$F_{(1,15)} = 1.742$	ns
Day 9	$F_{(1,15)} = 1.273$	ns	$F_{(1,11)} = .563$	ns	$F_{(1,19)} = 2.811$	ns	$F_{(1,15)} = 1.750$	ns
Day 10	$F_{(1,12)} = .982$	ns	$F_{(1,11)} = .240$	ns	$F_{(1,19)} = 2.094$	ns	$F_{(1,15)} = 1.789$	ns

ANOVA, analysis of variance; SERT, serotonin transporter; WT, wild-type; KO, knockout; MDMA, 3,4-methylene dioxymethamphetamine.

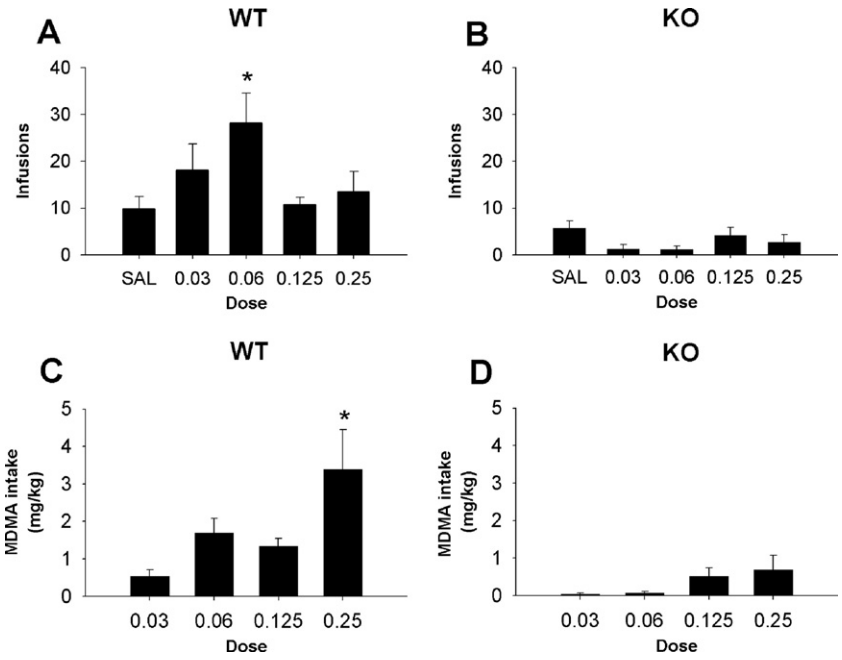

Figure 2. MDMA dose-dependent effects and intake. The data represent the average number of infusions of MDMA (0, .03, .06, .125, and .25 mg/kg/infusion) obtained by wild-type (WT) **(A)** and knockout (KO) **(B)** mice and the total MDMA intake (mg/kg) in WT **(C)** and in KO **(D)** animals on day 10 of training +SEM. In **(A)** $*p < .05$ versus saline (Dunnett post hoc test), in **(C)** $*p < .05$ versus .03 mg/kg/infusion (Tukey post hoc test). MDMA, 3,4-methylenedioxymethamphetamine; WT, wild-type; KO, knockout.

Knockout mice started to discriminate from session 1 until the last training session except for session 2 (Figure 4B). In this group, all the animals tested achieved the stability criteria and the mean time required to reach it was 4.83 ± 1.77 days.

Figure 4C shows operant water self-administration in WT and KO mice under a FR1 schedule of reinforcement during 13 days of training. Differences in the number of the operant responses to obtain water in both genotypes were analyzed using two-way ANOVA. A significant effect of day [$F(12,120) = 4.366$, $p < .001$] and significant interaction between genotype and day [$F(12,120) = 2.102$, $p < .05$] were revealed, but no significant effect of genotype [$F(1,10) = 3.814$, ns] was found. One-way ANOVA showed significant differences between KO and WT control mice in the number of operant responses to obtain water on days 1, 2, 3, 4, and 8 (see Table 3 for one-way ANOVA values).

To evaluate whether KO and WT mice presented differences in the motivation to obtain water, a PR schedule of reinforcement was performed in mice achieving acquisition criteria. No differences between KO and WT mice were observed in the breaking point achieved under this PR schedule (Figure 4D).

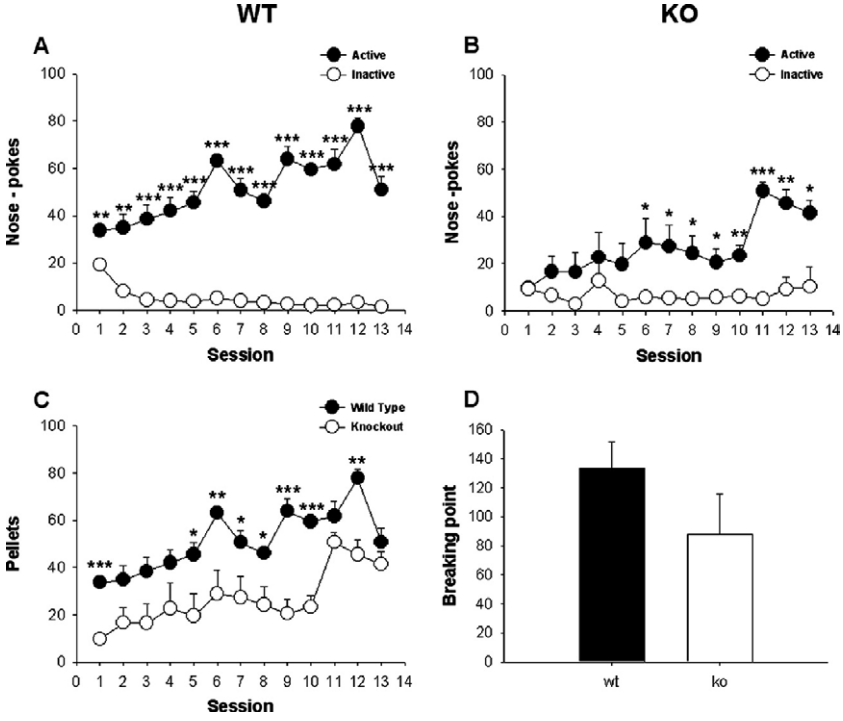

Figure 3. Acquisition of operant responding for food in wild-type (WT) and knockout (KO) mice. Average number of nose-pokes +SEM in both the active (filled circles) and the inactive (empty circles) holes in 1-hour sessions. The 13 days of training are represented for WT ($n = 6$) **(A)** and KO ($n = 6$) mice **(B)**. In **(C)** average number of pellets +SEM obtained by WT (filled circles) and KO (empty circles) mice under a FR1 schedule. In **(D)** the breaking points achieved by WT and KO mice under a progressive schedule of reinforcement are shown. In **(A)** and **(B)** $*p < .05$, $**p < .01$, $***p < .001$ active versus the inactive hole. In **(C)** $*p < .05$, $**p < .01$, $***p < .001$ WT versus KO mice. WT, wild-type; KO, knockout; FR1, fixed ratio 1.

Table 3. One-Way ANOVAs for Active Versus Inactive Hole Discrimination for Food and Water Operant Maintained Behavior in SERT KO and WT Mice (Hole) and for Differences in the Number of Reinforcers Obtained Between Genotypes (Genotype)

	WT Hole		KO Hole		Genotype			WT Hole		KO Hole		Genotype	
Food	*F*	*p*	*F*	*p*	*F*	*p*	Water	*F*	*p*	*F*	*p*	*F*	*p*
Day 1	$F_{(1,11)} = 13.294$	.01	$F_{(1,11)} = .128$	ns	$F_{(1,11)} = 39.938$	.001	Day 1	$F_{(1,11)} = 19.945$	.01	$F_{(1,11)} = 2.503$	ns	$F_{(1,11)} = 8.123$	.05
Day 2	$F_{(1,11)} = 20.810$	.01	$F_{(1,11)} = 2.387$	ns	$F_{(1,11)} = 4.195$	ns	Day 2	$F_{(1,11)} = 7.258$	.05	$F_{(1,11)} = 2.710$	ns	$F_{(1,11)} = 5.964$	.05
Day 3	$F_{(1,11)} = 33.339$	.001	$F_{(1,11)} = 2.956$	ns	$F_{(1,11)} = 4.602$	ns	Day 3	$F_{(1,11)} = 90.699$	.001	$F_{(1,11)} = 8.845$	.05	$F_{(1,11)} = 10.377$	.01
Day 4	$F_{(1,11)} = 47.236$	.001	$F_{(1,11)} = .529$	ns	$F_{(1,11)} = 2.557$	ns	Day 4	$F_{(1,11)} = 87.511$	.001	$F_{(1,11)} = 8.873$	.05	$F_{(1,11)} = 6.465$	.05
Day 5	$F_{(1,11)} = 71.220$	.001	$F_{(1,11)} = 3.091$	ns	$F_{(1,11)} = 6.166$	.05	Day 5	$F_{(1,11)} = 58.892$	.001	$F_{(1,11)} = 15.624$	.01	$F_{(1,11)} = 3.317$	ns
Day 6	$F_{(1,11)} = 297.650$	.001	$F_{(1,11)} = 5.162$	.05	$F_{(1,11)} = 10.281$	.01	Day 6	$F_{(1,11)} = 31.431$	.001	$F_{(1,11)} = 23.236$	.01	$F_{(1,11)} = .727$	ns
Day 7	$F_{(1,11)} = 87.982$	.001	$F_{(1,11)} = 5.800$	.05	$F_{(1,11)} = 5.078$	.05	Day 7	$F_{(1,11)} = 38.019$	.001	$F_{(1,11)} = 19.697$	.01	$F_{(1,11)} = 1.436$	ns
Day 8	$F_{(1,11)} = 206.721$	.001	$F_{(1,11)} = 6.994$	.05	$F_{(1,11)} = 7.210$	.05	Day 8	$F_{(1,11)} = 126.859$	.001	$F_{(1,11)} = 16.420$	.01	$F_{(1,11)} = 6.358$	.05
Day 9	$F_{(1,11)} = 122.279$	.001	$F_{(1,11)} = 6.238$	.05	$F_{(1,11)} = 28.533$	.001	Day 9	$F_{(1,11)} = 61.738$	.001	$F_{(1,11)} = 18.550$	.01	$F_{(1,11)} = 1.471$	ns
Day 10	$F_{(1,11)} = 859.142$	.001	$F_{(1,11)} = 13.305$	.01	$F_{(1,11)} = 52.826$	.001	Day 10	$F_{(1,11)} = 44.606$	.001	$F_{(1,11)} = 15.845$	.01	$F_{(1,11)} = 1.175$	ns
Day 11	$F_{(1,11)} = 93.597$	.001	$F_{(1,11)} = 103.814$	.001	$F_{(1,11)} = 2.315$	ns	Day 11	$F_{(1,11)} = 80.480$	.001	$F_{(1,11)} = 15.655$	.01	$F_{(1,11)} = .539$	ns
Day 12	$F_{(1,11)} = 393.875$	.001	$F_{(1,11)} = 21.505$	.01	$F_{(1,11)} = 20.684$	.01	Day 12	$F_{(1,11)} = 83.935$	.001	$F_{(1,11)} = 30.308$	.001	$F_{(1,11)} = 2.108$	ns
Day 13	$F_{(1,11)} = 75.226$	.001	$F_{(1,11)} = 10.379$	.05	$F_{(1,11)} = 1.395$	ns	Day 13	$F_{(1,11)} = 84.391$	.001	$F_{(1,11)} = 49.256$	.001	$F_{(1,11)} = .649$	ns

ANOVA, analysis of variance; SERT, serotonin transporter; KO, knockout; WT, wild-type.

Extracellular Concentrations of DA and 5-HT

Figure 5 shows representative probe placements in the NAC (Figure 5C) and the PFC (Figure 5D) of mice used in the microdialysis experiments. Most probes aimed at the NAC were placed between 1.70 mm and .86 mm from bregma and between 2.10 mm and 1.78 mm from bregma in the prefrontal cortex. Basal extracellular levels of DA in the NAC did not differ significantly between KO and WT mice. Mean ± SEM values were 2.86 ± .80 pg/sample for WT ($n = 6$) and 2.57 ± .60 pg/sample for KO ($n = 9$). Basal extracellular levels of 5-HT in the PFC were significantly higher in KO mice (2.83 ± .30 pg/sample, $n = 7$) than in WT mice (1.18 ± .20 pg/sample, $n = 7$) [$F(1,13) = 20.416$, $p < .001$], as previously reported (Shen *et al.* 2004).

Changes in the extracellular concentrations of DA in the NAC following MDMA (10 mg/kg, IP) administration are shown in Figure 5A. The MDMA induced a similar increase in DA levels in the NAC of WT and KO mice. The maximum percent increase was observed 40 min after injection for both WT (183.39 ± 47.32) and KO mice (178.68 ± 27.25). Repeated-measures ANOVA revealed a significant main effect of time after injection [$F(11,143) = 2.099$, $p < .05$] but no significant main effect of genotype [$F(1,13) = .395$, ns] or interaction between these two factors [$F(11,143) = .481$, ns]. Changes in the extracellular concentrations of 5-HT in the PFC following MDMA (10 mg/kg, IP) administration are shown in Figure 5B. The MDMA (10 mg/kg, IP) induced a markedly larger increase in 5-HT levels in the PFC of WT mice (409.02 ± 71.35%) than in KO mice (130.37 ± 9.34%). Repeated-measures ANOVA revealed a significant effect of time after injection [$F(11,132) = 8.798$, $p < .001$], genotype [$F(1,12) = 8.386$, $p < .05$], and interaction between these two factors [$F(11,132) = 9.663$, $p < .001$]. One-way ANOVA comparing genotypes at each time point following MDMA administration indicated significant differences between groups from 20 to 140 min following injection ($p < .01$–$.05$).

Discussion

In this study, we demonstrated the implication of the SERT in the acquisition and maintenance of intravenous MDMA self-administration. The SERT KO mice did not acquire intravenous MDMA self-administration, even though MDMA induced a similar increase in DA outflow in the NAC of WT and KO mice. Nevertheless, MDMA-induced changes in PFC 5-HT release were smaller in KO mice as compared with WT littermates. These data point to the specific involvement of the serotonergic system in the reinforcing properties of MDMA.

In the self-administration studies, WT mice trained to self-administer MDMA at different doses (0, .03, .06, .125, and .25 mg/kg/infusion) acquired and maintained the highest response rates at the dose .06 mg/kg/infusion. The same dose was not found to be reinforcing in CD1 mice under similar experimental conditions (Trigo *et al.* 2006). This leftward shift in the dose-response curve reveals a higher sensitivity to the reinforcing properties of MDMA in C57Bl/6J mice, the background strain of the WT mice used in this study, and supports findings pointing out differences in sensitivity to diverse drugs of abuse depending on the strain of mice (Elmer *et al.* 1987; Grahame and Cunningham 1995; Rocha *et al.* 1998; Thomsen and Caine 2006).

In contrast to WT mice, KO mice did not respond above saline levels at any of the MDMA doses tested, demonstrating the relevance of the 5-HT transporter in the reinforcing properties of

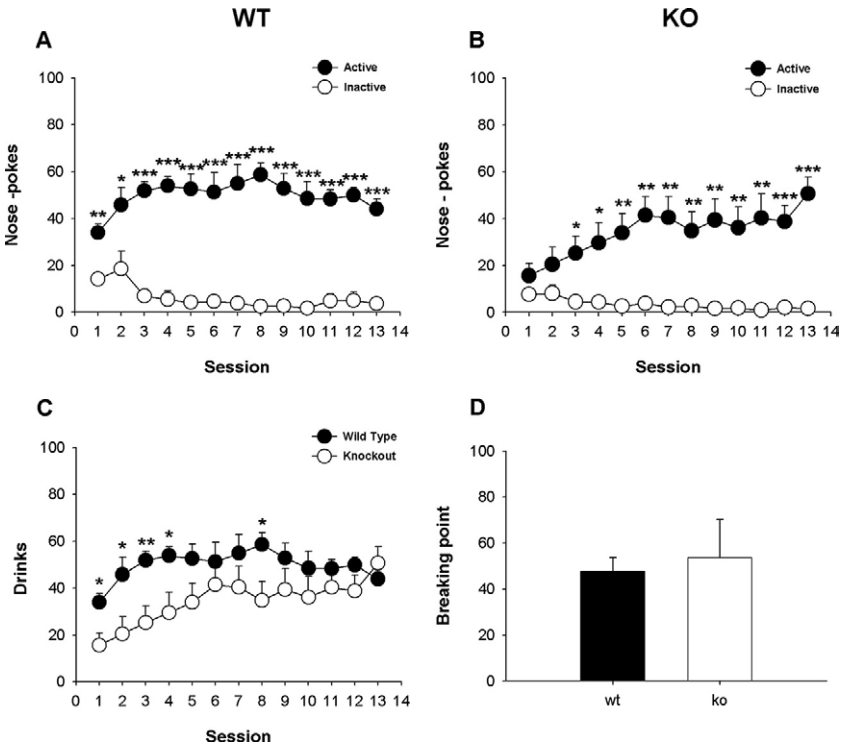

Figure 4. Acquisition of operant responding for water in wild-type (WT) and knockout (KO) mice. Average number of nose-pokes +SEM in both the active (filled circles) and the inactive (empty circles) holes in 1-hour sessions. The 13 days of training are represented for WT ($n = 6$) **(A)** and KO ($n = 6$) mice **(B)**. In **(C)** average number of water drinks +SEM obtained by WT (filled circles) and KO (empty circles) mice under a FR1 schedule. In **(D)** the breaking points achieved by WT and KO mice under a progressive schedule of reinforcement are shown. In **(A)** and **(B)** $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ active versus the inactive hole. In **(C)** $^{*}p < .05$, $^{**}p < .01$ WT versus KO mice. WT, wild-type; KO, knockout; FR1, fixed ratio 1.

MDMA. These findings parallel those obtained by Kelaï *et al.* (2003), showing a decrease in alcohol intake in SERT KO mice. The absence of MDMA self-administration in SERT KO mice could be attributed to possible unspecific learning or motor deficits produced by the SERT deletion impairing the acquisition of operant behavior. However, this contention is unlikely since both genotypes acquired and maintained stable operant responding for food and water. Nonetheless, some differences between KO and WT mice were observed in the acquisition of operant responding for these two natural reinforcers. Although KO mice finally acquired operant behavior for food, the time required to achieve the criteria was longer than in WT mice, and furthermore, the number of food pellets was lower during most of the training sessions. This decrease in the reinforcing ability of food reward could be due to the increased brain levels of 5-HT in the SERT KO mice (Fabre *et al.* 2000; Shen *et al.* 2004, and the present study), since an inverse relationship between brain extracellular 5-HT concentrations and food intake has been reported (Foltin 2005, 2006; LeSage *et al.* 2004). Alternatively, the anxiogenic-like phenotype of these KO mice (Holmes *et al.* 2003a, 2003b, 2003c) can also account for the differences observed between genotypes in operant responding for natural rewards and for MDMA. Indeed, KO mice showed lower rates of operant responding for water than WT mice only at the beginning of training, probably due to increased anxiety in the novel environment and not to deficits in drinking behavior (Kelaï *et al.* 2003). In agreement with this interpretation, the reduced operant responding for water was transient; once responding was acquired, no differences between genotypes were observed on a FR1 schedule. With respect to operant responding maintained by food, the anxiogenic-like phenotype of KO mice, together with the existing impairment in food intake, could contribute to the prolonged delay observed in the acquisition of this operant response. Following stable acquisition, however, KO and WT mice showed no differences in the motivation to seek these two natural rewards, as evidenced by the similar breaking points obtained in the PR schedule, therefore ruling out the possible existence of an anhedonic state.

The possible contribution of the anxiogenic phenotype of SERT KO mice in the lack of MDMA self-administration in these animals cannot be discarded, since anxiety-like effects can influence the reinforcing properties of other psychostimulants such as cocaine (David *et al.* 2001). However, SERT KO mice trained to self-administer cocaine at 1 mg/kg/infusion under similar experimental conditions significantly discriminated between holes from the eighth session of training and reached a reliable cocaine self-administration behavior (Trigo *et al,* unpublished observations), suggesting a dissociation between the anxiogenic phenotype and the suppression of MDMA self-administration in these mutants. All these data support the selective involvement of SERT in the reinforcing properties of MDMA with respect to food, water, and cocaine.

The in vivo microdialysis studies showed similar basal extracellular concentrations of DA in the NAC of KO and WT mice, while basal 5-HT extracellular concentrations in the PFC were higher in KO than in WT mice, substantiating previous results in these mice using conventional (Shen *et al.* 2004) or zero-net-flux methods of quantitative microdialysis (Mathews *et al.* 2004). An acute administration of MDMA (10 mg/kg, IP) increased DA levels in the NAC to a similar extent in both genotypes. These results showing a dissociation between DA outflow in the NAC and MDMA self-administration further illustrate that reward/reinforcement processes cannot be reduced to the mesolimbic DA system and that other neurotransmitters may also play a role. In this sense, MDMA did not produce a significant elevation in the extracellular concentrations of 5-HT in the PFC of KO mice. Although this result was expected from the lack of SERT in these mice (Bengel *et al.* 1998), we cannot rule out the possibility that a ceiling effect

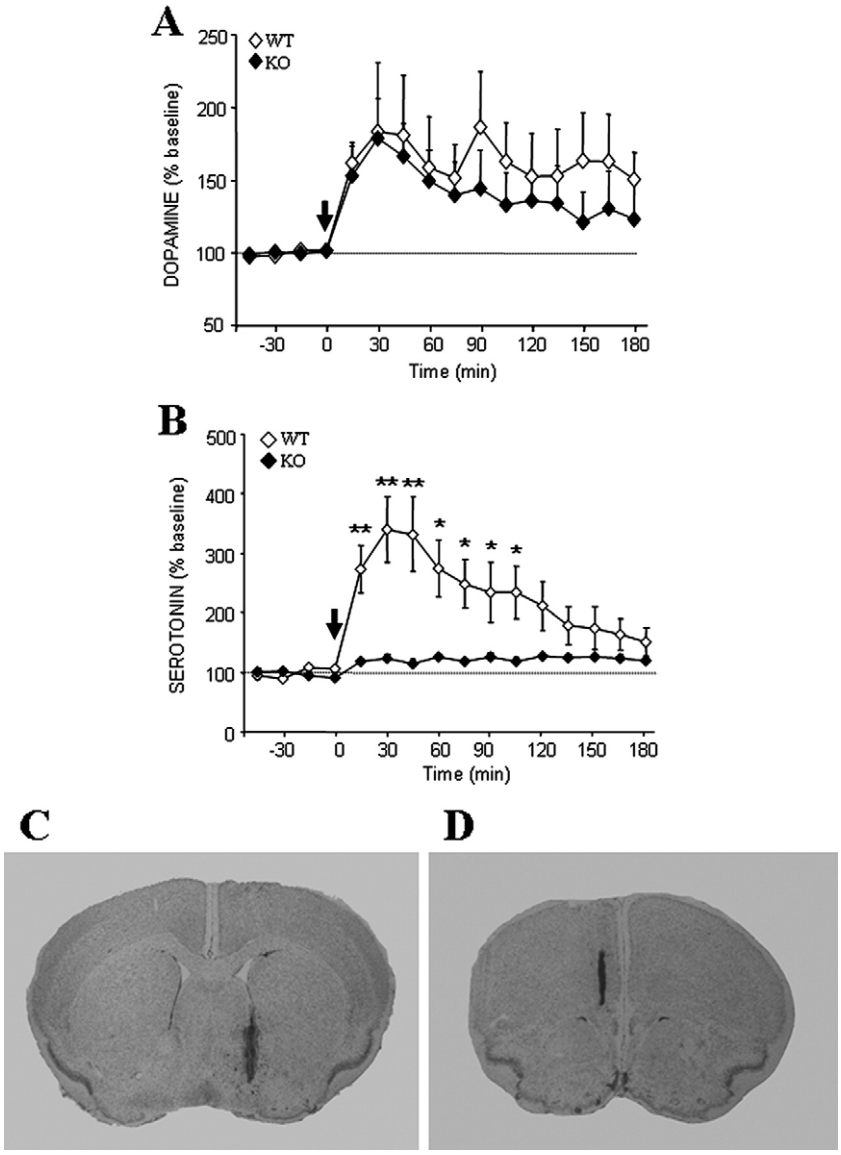

Figure 5. In vivo microdialysis in the nucleus accumbens (NAC) and prefrontal cortex (PFC) of SERT wild-type (WT) and knockout (KO) mice and representative mouse brain coronal sections (20 μm) stained with Cresyl Violet. Effects of MDMA (10 mg/kg, IP) administration at time 0 (black arrow) on dopamine release in the NAC **(A)** and on serotonin release in the PFC **(B)** of WT and KO mice. Each point is the mean +SEM of six to nine determinations expressed as percentage of basal values, $*p < .05$, $**p < .01$ WT versus KO. In **(C)**, the placement of the entire 1 mm probe is shown in the nucleus accumbens (bregma +1.10). In **(D)**, the extremity of the 2 mm probe in the prefrontal cortex is shown (bregma +1.98). NAC, nucleus accumbens; PFC, prefrontal cortex; WT, wild-type; KO, knockout; MDMA, 3,4-methylenedioxymethamphetamine.

could have masked any further MDMA-induced 5-HT increase in KO mice since these animals show higher basal 5-HT levels than WT mice. Administration of MDMA potently and rapidly increased 5-HT levels in the PFC of WT mice. However, the absolute concentrations of 5-HT reached following MDMA administration were virtually the same in both genotypes (WT: 3.20 ± .35 pg/15 μL, KO: 3.65 ± .40 pg/15 μL), since WT mice show lower levels of 5-HT than KO mice. This observation suggests that one important factor mediating the reinforcing properties of MDMA appears to be the fast change in neurotransmitter release in the PFC rather than the "absolute levels" of 5-HT attained in this structure.

The MDMA-induced hyperlocomotion also seems to be, in part, under serotonergic control. Thus, the administration of compounds preventing MDMA-induced 5-HT release blocked MDMA hyperlocomotion in rats (Callaway *et al.* 1990; Hekmatpanah and Peroutka 1990) and mice (Fantegrossi *et al.* 2005; Scearce-Levie *et al.* 1999). Similarly, SERT KO mice were insensitive to the locomotor effects of MDMA, while the response to amphetamine was not altered (Bengel *et al.* 1998). These data are in line with studies demonstrating that MDMA shows a higher affinity to bind to SERT than to DAT (Han and Gu 2006) and underpin the importance of the serotonergic system in the behavioral effects of MDMA. However, the possible involvement of the dopaminergic system cannot be completely ruled out since there is also evidence demonstrating its role in MDMA rewarding effects (Bilsky *et al.* 1998; Daniela *et al.* 2004; White *et al.* 1996). Under normal conditions, both DA and 5-HT systems interact and reciprocally influence their activity level (Benloucif *et al.* 1993; Blandina *et al.* 1989; Ferré and Artigas 1993; Ferré *et al.* 1994) to modulate the actions of psychostimulant drugs (Auclair *et al.* 2004a, 2004b; Carta *et al.* 2006; Schmidt *et al.* 1994). Thus, it is plausible that the fine-tuning of this interaction, allowing an appropriate ratio between DA/5-HT in reward circuits, may be necessary for the complete perception of MDMA reward. In contrast to this hypothesis, for other psychostimulants such as cocaine, an exchangeable role of the dopaminergic and serotonergic systems has been proposed (Rocha 2003) to explain the preserved cocaine self-administration (Rocha *et al.* 1998) and CPP (Sora *et al.* 1998) in mice lacking DAT and SERT, respectively, and the absence of cocaine CPP in combined DAT/SERT KO mice

(Sora *et al.* 2001). Therefore, it is still not clear how the compensatory changes that occur in SERT KO mice, including increased extracellular levels of 5-HT (Fabre *et al.* 2000; Mathews *et al.* 2004; Shen *et al.* 2004) and altered expression of 5-HT_{1A}, 5-HT_{1B} (Fabre *et al.* 2000), 5-HT_{2A}, and 5-HT_{2c} receptors (Gainetdinov and Caron 2003; Li *et al.* 2003; Rioux *et al.* 1999), could differentially impinge on the rewarding/reinforcing effects of MDMA and cocaine.

The present results provide evidence for a key role of the serotonergic system in MDMA rewarding effects. Since the particular patterns of MDMA consumption in young adults suggest that this drug of abuse may lead to addiction (Cottler *et al.* 2001), the SERT can be an interesting target to develop effective treatments for MDMA abuse, as has been suggested for methamphetamine dependence in humans (Piasecki *et al.* 2002).

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