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Long-term effects of repeated social stress on the conditioned place preference induced by MDMA in mice

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ABSTRACT

Previous studies have demonstrated that social defeat stress increases the rewarding effects of psychostimulant drugs such as cocaine and amphetamine. In the present study we evaluated the long-term effects of repeated social defeat (RSD) on the rewarding effects of ±3,4-methylenedioxymethamphetamine (MDMA) hydrochloride in the conditioned place preference (CPP) paradigm. Adolescent and young adult mice were exposed to four episodes of social defeat (on PND 29–40 and PND 47–56, respectively) and were conditioned three weeks later with 1.25 or 10 mg/kg i.p. of MDMA (experiment 1). The long-term effects of RSD on anxiety, social behavior and cognitive processes were also evaluated in adult mice (experiment 2). RSD during adolescence enhanced vulnerability to priming-induced reinstatement in animals conditioned with 1.25 mg/kg of MDMA and increased the duration of the CPP induced by the 10 mg/kg of MDMA. The latter effect was also observed after RSD in young adult mice, as well as an increase in anxiety-like behavior, an alteration in social interaction (reduction in attack and increase in avoidance/flee and defensive/submissive behaviors) and an impairment of maze learning. These results support the idea that RSD stress increases the rewarding effects of MDMA and induces long-term alterations in anxiety, learning and social behavior in adult mice. Thus, exposure to stress may increase the vulnerability of individuals to developing MDMA dependence, which is a factor to be taken into account in relation to the prevention and treatment of this disorder.

1. Introduction

Many people take addictive drugs and do it for different reasons, in different ways and in different contexts (Everitt, 2014). Addiction can be defined as a chronic, relapsing brain disease characterized by a compulsion to seek and take drugs, loss of control over intake, and the emergence of a negative emotional state when access to the drug is prevented (Koob, 2013). The factors that might predispose individuals to lose control over drug use are gradually being defined (Belin et al., 2008; Dalley et al., 2007; Dilleen et al., 2012) leading to the identification of endophenotypes of drug addiction and related neuropsychiatric disorders (Ersche et al., 2010; Everitt, 2014). Adverse life experiences may render individuals more prone to abuse addictive substances and more vulnerable to relapse into drug-seeking after periods of detoxification (Caprioli et al., 2007; Le Moal, 2009; Miczek et al., 2008; Sinha et al., 2011). In experimental animals, it has been demonstrated that exposure to stressors (i.e., social defeat stress, social isolation, maternal separation, immobilization stress, footshock stress, etc.) and activation of neural and hormonal stress mechanisms can produce behavioral and neurochemical adaptations that render animals more vulnerable to the initiation, maintenance and escalation of drug consumption and to the reinstatement of this behavior after extinction (Burke and Miczek, 2014; Koob, 2010; Logrip et al., 2011, 2012; Rodríguez-Arias et al., 2013; Sinha, 2008; Sinha et al., 2011).

Exposure to different procedures of social defeat, considered a stressor of ecological and ethological validity in rodents (Neisewander et al., 2012; Tornatzky and Miczek, 1993), increases the rewarding and reinstating effects of psychostimulant drugs, such as cocaine and amphetamine, in the self-administration and conditioned place preference (CPP) paradigms (for a review see Aguilar et al., 2013; Burke and Miczek, 2014; Miczek et al., 2008; Neisewander et al., 2012). No studies have evaluated the influence of stress on the rewarding effects of ecstasy (±3,4-methylenedioxymethamphetamine, MDMA), with the exception of a previous work carried out in our laboratory in which we observed that exposure to acute social defeat undermined the rewarding effects of MDMA in the CPP paradigm in adult mice (García-Pardo et al., 2014). It is not clear if these results, which diverge from those observed with psychostimulants, were due to differences in the drug tested (MDMA vs cocaine or amphetamine) or in the procedure of social defeat stress. Although MDMA is a less effective reinforcer than

Abbreviations: CPP, Conditioned preference place; MDMA, 3,4 methylenedioxymethylamphetamine; DA, Dopamine; RSD, Repeated social defeat; Pre-C, Pre-conditioning; Post-C, Post-conditioning; PND, Postnatal day; Ado, Adolescent; YA, Young Adult; Ctr, Control; CRF, Corticotrophin release factor; BDNF, Brain derived neurotrophic factor.

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other drugs of abuse, it induces rewarding and reinstating effects in the self-administration and CPP paradigms, which are mainly due to the activation of dopamine (DA) and serotonin neurotransmission (Roger-Sánchez et al., 2013; Schenk, 2009). On the other hand, specific features of the procedure of acute social defeat stress may explain the reduction of MDMA-induced CPP. Firstly, as mice experienced social defeat immediately before each conditioning session with MDMA, the adverse experience of social defeat could reduce the rewarding properties of MDMA. Secondly, only the short-term effects of social defeat stress are evaluated (48 h after the last stress exposure). Thus, in the present study, we aim to evaluate, for the first time, the long-term effects of social defeat exposure on the rewarding effects of MDMA. It is important to note that an increase in the rewarding effects of psychostimulants is generally observed after the absence (for 10 days or more) of repeated social defeat stress in a resident–intruder paradigm (Boyson et al., 2011, 2014; Cruz et al., 2011; Han et al., 2015; Miczek et al., 2008, 2011; Quadros and Miczek, 2009; Yap et al., 2015). Similarly, in a recent study we have demonstrated that mice exposed to intermittent repeated social defeat (RSD) during adolescence display an increase in ethanol consumption and motivation to drink in adulthood. Moreover, we have observed that RSD during adolescence induces depression-like symptoms and social subordination behavior in mice during adulthood, without affecting anxiety-like behavior in the elevated plus maze or cognitive performance in the passive avoidance and Hebb–Williams tests (Rodríguez-Arias et al., 2014). In contrast to this last work, the majority of studies of the effects of RSD on drug vulnerability have been performed in adult mice. However, it is essential to test the effects of social defeat stress in adolescent animals, since adolescence is a highly vulnerable developmental period and adolescent rodents are more vulnerable to stressors than younger or older counterparts (Buwalda et al., 2011; Romeo, 2010; Stone and Quartermain, 1998; Vázquez, 1998).

Thus, in the present work we have evaluated the long-term effects of intermittent RSD on the CPP induced by MDMA in adolescent or young adult mice. We hypothesized that exposure to intermittent RSD stress would induce a long-term increase in the vulnerability of the animals to the rewarding effects of MDMA. In experiment 1, we evaluated the rewarding effects of a low (1.25 mg/kg) and a high (10 mg/kg) dose of MDMA in mice pre-exposed (3 weeks before the initiation of CPP procedure) to four episodes of social defeat stress during adolescence (PND 29–38) or early adulthood (PND 47–56). Corticosterone levels were determined immediately or 30 min after the first and last episodes of RSD, or three weeks after, at the initiation of the CPP procedure. Additionally, we evaluated the long-term effects of this regime of intermittent RSD stress on the behavioral profile of mice (anxiety, social interaction, and cognitive performance). Three weeks after the last social defeat, the behavior of mice in the plus maze, passive-avoidance task, social interaction test and Hebb–Williams maze was evaluated (experiment 2). This experiment was performed only in young adult mice, since the effects of intermittent RSD on these behaviors in adolescent mice have previously been studied (Rodríguez-Arias et al., 2014). In this way, we set out to evaluate if the changes observed in the rewarding effects of MDMA in socially defeated mice are related with behavioral or cognitive alterations induced by RSD.

2. Materials and methods

2.1. Animals

Male OF1 mice (Charles River, Barcelona, Spain) arrived at our laboratory at 21 or 42 days of age (early and late adolescents, respectively). 70 adolescent and 84 young adult mice were employed as experimental subjects in experiment 1, and 30 young adult mice were employed in experiment 2. All mice (except those used as aggressive opponents) were housed in groups of four in plastic cages (25 × 25 × 14.5 cm) for 8 days before the experiments began. To reduce their stress levels in response to experimental manipulations, mice were handled for 5 min per day on each of the 3 days prior to initiation of the behavioral tests. Adult mice used as resident aggressive opponents (n = 85) were individually housed in plastic cages (21 × 32 × 20 cm) for a month prior to experiments in order to induce heightened aggression (Rodríguez-Arias et al., 1998). All mice were housed under the following conditions: constant temperature; a reversed light schedule (white lights on 19:30–07:30 h); and food and water available ad libitum, except during behavioral tests. Procedures involving mice and their care were conducted according to national, regional and local laws and regulations, which are in compliance with the Directive 2010/63/EU.

2.2. Apparatus

2.2.1. Place conditioning boxes

For place conditioning, we employed eight identical Plexiglas boxes with two equal-sized compartments (30.7 cm long × 31.5 cm wide × 34.5 cm high) separated by a gray central area (13.8 cm long × 31.5 cm wide × 34.5 cm high). The compartments had different colored walls (black vs white) and distinct floor textures (fine grid in the black compartment and wide grid in the white one). Four infrared light beams in each compartment of the box and six in the central area allowed the recording of the position of the animals and their crossings from one compartment to the other. The equipment was controlled by three IBM PC computers using MONPRE 2Z software (CIBERTEC, SA, Spain).

2.2.2. Elevated plus maze

To evaluate the effects of RSD on anxiety-like behaviors, an elevated plus maze (EPM) was employed. The apparatus consisted of two open arms (30 × 5 × 0.25 cm) and two enclosed arms (30 × 5 × 15 cm). The junction of the four arms formed a central platform (5 × 5 cm). The floor of the maze was made of black Plexiglas and the walls of the enclosed arms of clear Plexiglas. The open arms had a small edge (0.25 cm) to provide additional grip for the animals. The entire apparatus was elevated 45 cm above floor level.

2.2.3. Inhibitory avoidance apparatus

For the passive avoidance test, a step-through inhibitory avoidance apparatus for mice (Ugo Basile, Comerio-Varese, Italy) was employed. This cage, made of sheets of Perspex, is divided into two compartments (15 cm × 9.5 cm × 16.5 cm each one). The safe compartment is white and illuminated by a light fixture (10 W) fastened to the cage lid, whereas the "shock" compartment is dark and made of black Perspex panels. The two compartments are divided by an automatically operated sliding door at floor level. The floor is made of 48 stainless steel bars with a diameter of 0.7 mm and situated 8 mm apart.

2.2.4. Hebb–Williams maze

To perform the Hebb–Williams maze, we used a maze constructed with black plastic and measuring 60 cm wide × 60 cm long × 10 cm high. It contains a start box and a goal box (both 14 cm wide × 9 cm long) positioned at diagonally opposite corners. The maze contains cold water at a wading depth (15 °C, 3.5 cm high), while the goal box is stocked with fresh dry tissue. Several maze designs are produced by fixing different arrangements of barriers to a clear plastic ceiling. This apparatus allows the cognitive process of routed learning and the motivation of water escape to be measured.

2.3. Drugs

Animals were injected intraperitoneally with 1.25 or 10 mg/kg of MDMA (±3,4-methylenedioxymethamphetamine hydrochloride, racemic mixture; Agencia Española del Medicamento, Ministerio de Sanidad, Política Social e Igualdad, Madrid, Spain) in a volume of 0.01 ml/g of weight. Physiological saline (NaCl 0.9%) was used to dissolve the drug. The doses of MDMA we administered were selected on

the basis of previous studies showing that, in late-adolescent mice, 1.25 mg/kg induces CPP but not reinstatement after priming with 0.625 mg/kg of MDMA while 10 mg/kg induces a strong CPP that is reinstated after priming with 5 mg/kg of MDMA (García-Pardo et al., 2014, in press).

2.4. Procedure of social defeat

The experimental group was exposed to a protocol of intermittent RSD consisting of four episodes of social defeat that lasted 25 min each, on days 1, 4, 7, and 10 (Tornatzky and Miczek, 1993). Each episode of social defeat consisted of three phases, which began by introducing the "intruder" (the experimental animal) into the home cage of "resident" (the aggressive opponent) for 10 min. During this initial phase, the intruder was protected from attack, but the wire mesh walls of the cage allowed for social interactions and species-typical threats from the male aggressive resident, serving the function of instigation and provocation (Covington and Miczek, 2001). The wire mesh was then removed from the cage and the confrontation between the two animals began and lasted 5 min. In the third phase, the wire mesh was returned to the cage to separate the two animals once again for another 10 min to allow for social threats by the resident. Intruder mice were exposed to a different aggressor mouse during each episode of social defeat. The criterion used to define an animal as defeated was the adoption of a specific posture signifying defeat, characterized by an upright submissive position, limp forepaws, upwardly angled head, and retracted ears (Miczek et al., 1982; Rodríguez-Arias et al., 1998). All agonistic encounters were videotaped to confirm the presence of social defeat. To evaluate possible differences in social defeat between early- and late-adolescent mice, the first and fourth social encounters of eight mice of each age were videotaped and evaluated (Table 1: corticosterone section, videotaping and behavioral analysis) with a computerized system by an observer who was blind to the treatment (Brain et al., 1989). This custom-developed program allows the time engaged in different broad functional categories of behavior – each of which is characterized by a series of different postures and elements – to be estimated (Rodríguez-Arias et al., 1998). Time spent in threat and attack by resident aggressive mice and in avoidance/flee and submission by experimental mice was measured. Socially defeat-stressed animals were exposed to this protocol of RSD, while the control groups followed the same protocol, without the presence of a "resident" mouse in the cage. Animals were then housed in the vivarium for three weeks, after which they performed the behavioral tests.

2.5. Behavioral testing

2.5.1. Conditioned place preference procedure

This paradigm has been widely used to study the conditioned rewarding effects of addictive drugs (Aguilar et al., 2009; Tzschentke, 1998, 2007).

2.5.1.1. Acquisition. Acquisition of CPP consisted of three phases and took place during the dark cycle following an unbiased procedure in terms of initial spontaneous preference (for a detailed explanation of procedure see Daza-Losada et al., 2007). In brief, during preconditioning (Pre-C) the time spent by the animal in each compartment during a 15-min period was recorded. Nineteen animals showing a strong unconditioned aversion or preference for a given compartment were excluded from the study. In the second phase (conditioning), experimental animals were conditioned with MDMA immediately before being confined to the drug-paired compartment for 30 min on days 4, 6, 8 and 10, and received saline before being confined to the vehicle-paired compartment for 30 min on days 5, 7, 9 and 11. During the third phase, or post-conditioning (Post-C), the time spent by the untreated mice in each compartment was recorded during a 15-min period.

Table 1
Experimental design. Procedures followed in the different experiments. In experiment 1, adolescents (Ado) or young adult (YA) mice were exposed on post-natal day (PND) 29–32–35–38 or PND 47–50–53–56, respectively, to repeated social defeat (RSD) or exploration (EXP) 3 weeks before initiation of the CPP procedure. Pre-conditioning (PRE-C); conditioning with 1.25 or 10 mg/kg of MDMA (M) in the drug-paired compartment on PND 62–64–66–68 in the case of Ado mice and PND 80–82–84–86 in that of YA mice. On alternate days mice were conditioned in the vehicle-paired compartment after administration of saline (PND 63–65–67–69 for Ado mice and PND 81–83–85–87 for YA mice); post-conditioning (POST-C); videotaping: videotaping of encounter for behavior analysis. Blood sampling for corticosterone determinations at 0 min, 30 min or 3 weeks after RSD or EXP (PND 29, 38 and 59 for Ado and PND 47, 56 and 77 for YA mice). In experiment 2, YA mice were exposed to RSD or EXP (on PND 47–50–53–56) and were evaluated in different behavioral tests 3 weeks later: plus maze (PND 77), passive avoidance (PND 79–80), social interaction (PND 81) and Hebb Williams maze (PND 82–89).

Groups		Procedures				
Experiment 1	(n=)	RSD/EXP	PRE-C	Conditioning	POST-C	Reinstatement
Adolescent mice		PND 29–32–35–38	PND 59–60–61	PND 62–64–66–68	PND 70	PND ±75–91
Group 1: RSD + Ado1	17	RSD		M 1.25		M 0.625
Group 2: Ctr + Ado1	15	Exp		M 1.25		M 0.625
Group 3: RSD + Ado10	13	RSD		M 10		M 5
Group 4: Ctr + Ado10	11	Exp		M 10		M 5
Young adult mice		PND 47–50–53–56	PND 77–78–79	PND 80–82–84–86	PND 88	PND ±103–167
Group 5: RSD + YA1	20	RSD		M 1.25		M 0.625
Group 6: Ctr + YA1	19	Exp		M 1.25		M 0.625
Group 7: RSD + YA10	13	RSD		M 10		M 5
Group 8: Ctr + YA10	15	Exp		M 10		M 5
Corticosterone determinations	(n=)	RSD/EXP	Blood sampling			
Adolescent mice		PND 29–32–35–38	PND 29/38			
Group 1: RSD	8	RSD	0 min	Videotaping and behavioral analysis		
Group 2: Ctr	8	Exp	0 min			
Group 3: RSD	8	RSD	30 min	Blood sampling PND 59		
Group 4: Ctr	8	Exp	30 min	Blood sampling PND 59		
Young adult mice		PND 47–50–53–56	PND 47/56			
Group 5: RSD	8	RSD	0 min	Videotaping and behavioral analysis		
Group 6: Ctr	8	Exp	0 min			
Group 7: RSD	8	RSD	30 min	Blood sampling PND 77		
Group 8: Ctr	8	Exp	30 min	Blood sampling PND 77		
Experiment 2	(n=)	RSD/EXP	Behavioral testing			
Young adult mice		PND 47–50–53–56	PND 77	PND 79–80	PND 81	PND 82–89
Group 1: RSD	15	RSD	Plus maze	Passive avoidance	Social interact	Hebb Williams
Group 2: Ctr	15	Exp	Plus maze	Passive avoidance	Social interact	Hebb Williams

2.5.1.2. Extinction. After CPP had been confirmed in the Post-C session, mice were exposed to the extinction procedure by which they underwent an extinction session every three days (all the mice performed each extinction session on the same day). This consisted of placing them in the apparatus for 900 s until the time spent in the drug-paired compartment was similar to that of the Pre-C phase (no significant difference revealed by the Student's t test between extinction and Pre-C sessions). Additionally, with the objective of determining whether differences in the duration of extinction between groups were statistically significant, extinction was measured in each mouse when the animal spent less time, or until 10 s more, in the drug paired compartment than in Pre-C (for details see Ribeiro Do Couto et al., 2012).

2.5.1.3. Reinstatement. The reinstatement tests were the same as for Post-C (free ambulation for 900 s), except that animals were administered MDMA 15 min before the test. In the reinstatement phase, the dose administered was half that given during the conditioning phase, and was given in a different room to that of the conditioning sessions. The aim of this procedure was to administer the drug in a non-contingent way with respect to conditioning, so that the animal did not associate the contextual cues of the experimental room with the drug.

After this first reinstatement test with half of the MDMA dose used in the conditioning phase, the groups that demonstrated reinstatement — i.e. a positive significant difference (Student's t test) between the time spent in the drug-paired compartment in the reinstatement and Pre-C tests — were re-tested until a new extinction was confirmed. The following day, the effects of the priming – a quarter of the dose used for conditioning – on reinstatement of place preference were evaluated following the procedure described earlier. This procedure was repeated with progressively lower priming doses until a non-effective priming injection was determined.

2.5.2. Elevated plus maze

In order to facilitate adaptation, mice were transported to the dimly illuminated laboratory 1 h prior to testing. At the beginning of each trial, subjects were placed on the central platform so that they were facing an open arm, and were allowed to explore for 5 min. The maze was thoroughly cleaned with a damp cloth after each trial. The behavior displayed by the mice was videorecorded and later analyzed by a 'blind' observer using a computerized method. The measurements recorded during the test period were frequency of entries and time and percentage of time spent in each section of the apparatus. An arm was considered to have been visited when the animal placed all four paws on it. The details of the procedure and measurements are described in Daza-Losada et al. (2009a). Number of open arm entries, time spent in open arms and percentage of open arm entries are generally used to characterize the anxiolytic effects of drugs (Pellow and File, 1986; Rodgers et al., 1997).

2.5.3. Passive avoidance

Tests were carried out essentially following the procedure described in Aguilar et al. (2000). On the day of training, each mouse was placed in the illuminated compartment facing away from the dark compartment. After a 60 s habituation period, the door leading to the dark compartment was opened. When the animal had placed all four paws in the dark compartment a footshock (0.5 mA, 3 s) was delivered and the animal was immediately removed from the apparatus and returned to its home cage. The time taken to enter the dark compartment (step-through latency) was recorded. Retention was tested 24 h later following the same procedure but without the shock. The maximum step-through latency was 300 s.

2.5.4. Social interaction test

This test consisted of confronting an experimental animal with a standard opponent in a neutral cage (61 × 30.5 × 36 cm) for 10 min following a 1 min adaptation period prior to the encounter. Standard opponents were rendered temporarily anosmic by intranasal lavage with a 4% zinc sulfate solution 1 day before testing (Smoothy et al., 1986). This kind of mouse induces an attack reaction in its opponent but does not outwardly provoke or defend itself, since it cannot perceive a pheromone that is present in the urine of the experimental animals and functions as a cue for eliciting aggressive behavior in mice with a normal sense of smell (Brain, 1981; Mugford and Nowell, 1970). Behavior was videotaped under white illumination and videotapes were analyzed using the previously mentioned program (Brain et al., 1989). The following functional categories of behavior were recorded: body care, digging, non-social exploration, explore from a distance, social investigation, threat, attack, avoidance/flee and defensive/submissive behavior. In addition, the unit of social investigation (total time spent in social investigation/number of social investigations) was calculated. A detailed description of the different postures and elements that characterized each category of behavior can be found in Rodríguez-Arias et al. (1998).

2.5.5. Hebb–William maze

The procedure we followed was based on that employed by Galsworthy et al. (2005), in which mice must navigate the maze and cross from the wet start box to the dry goal box in order to escape the cold water. Animals underwent a 5 min habituation period (dry sand, no barriers) on day 1 and undertook problem A on day 2 and problem D on day 3 (4 trials/day) (practice mazes). Mice were subsequently submitted to mazes 1, 5, 3, 4 and 8 on separate days on which 8 trials took place (see Rabinovitch and Rosvold, 1951, for all maze designs). Maze-type was always presented in the same order: however, to avoid any training effects, easier (1, 3 and 4) and harder mazes (5 and 8) were alternated. The time limit for reaching the goal box was 5 min, after which the mouse was guided to the box. As in previous studies carried out in our laboratory (Rodríguez-Arias et al., 2014; Vidal-Infer et al., 2012), the following measurements were recorded: acquisition criterion score, considered to be completion of the task in less than 60 s in two consecutive trials; total latency score (the sum of the latencies in all the problem trials in each maze); and error scores, for which a similar total was used, where "error" was considered as entering the error zone specified by Galsworthy et al. (2005).

2.6. Procedure of corticosterone measurement

Blood sampling for corticosterone determination was performed by the tail-nick procedure, in which the animal is wrapped in a cloth and a 2-mm incision is made at the end of the tail artery. The tail is then massaged until 50 μl of blood is collected in an ice-cold Microvette® CB 300 capillary tube (Sarstedt, Germany). Blood samples were kept on ice, and plasma was separated from whole blood by centrifugation (5 min, 5000 *g*) and transferred to sterile, 2 ml microcentrifuge tubes. Plasma samples were stored at −80 °C until determination of corticosterone. On the day of the assay, samples were diluted, in a proportion of ~1:40, in the Steroid Displacement Reagent mix provided with the kit. Corticosterone levels in diluted plasma were then analyzed using a corticosterone EIA kit (Enzo® Life Sciences, catalog no. ADI-900-097, 96 well kit), according to the manufacturer's instructions, and an iMark microplate reader (Bio-Rad) and Microplate Manager 6.2. software. The optical density was read at 405 nm, with 590 nm correction.

2.7. Experimental design

2.7.1. Experiment 1: effect of repeated social defeat in adolescent and young adult mice on the acquisition and reinstatement of the CPP induced by MDMA

To evaluate the long-term effects of RSD on the rewarding effects of MDMA, eight groups of animals were used. Four groups of adolescent or young adult mice experienced RSD on PND 29–38 (Ado-RSD groups) or

PND 47–56 (YA-RSD groups). The other four groups were control mice of the same age that only performed exploration without an opponent (Ado-Ctr and YA-Ctr groups). Three weeks later, half of the groups were conditioned with 1.25 mg/kg (RSD-Ado1, Ctr-Ado1, RSD-YA1, Ct-YA1) and the other half with 10 mg/kg (RSD-Ado10, Ctr-Ado10, RSD-YA10, Ctr-YA10) of MDMA (see Table 1). After the Post-C test, all groups underwent extinction sessions. When extinction had been confirmed, the animals received a priming dose of MDMA (half of the dose used during conditioning) and performed a reinstatement test. Groups showing reinstatement underwent successive extinction/reinstatement tests (as described in Section 2.5.1). We did not include any saline-treated control groups because of a previous study in which we demonstrated that a social encounter resulting in defeat for experimental mice (even when administered immediately before CPP) did not induce effects in adolescent or young adult mice conditioned with saline (García-Pardo et al., 2014).

2.7.2. Effect of social defeat stress on corticosterone levels

Different blood samples were obtained from the mice in experiment 1 in order to evaluate the effect of social defeat on adolescent or young adult mice. As can be seen in Table 1, blood samples were obtained from mice of each age group (randomly chosen) at the following times ($n = 8$): immediately or 30 min after the first and last social defeat (RSD1st-0 min, RSD1st-30 min, RSD4th-0 min and RSD4th-30 min) or exploration of the cage (Ctr1st-0 min, Ctr1st-30 min, Ctr4th-0 min and Ctr4th-30 min), and 3 weeks after social defeat or exploration (SD3w, Ctr3w). In the first and fourth episodes of defeat, the social defeat behavior of one group of adolescent mice and one group of young adult mice was videotaped and analyzed, as shown in Table 1.

2.7.3. Experiment 2: effect of repeated social defeat in young adult mice on anxiety, social interaction, learning and memory

This experiment was performed in young adult mice only, since the effects of this kind of RSD on these behaviors in adolescent mice have been evaluated in a previous study by our group (Rodríguez-Arias et al., 2014). Two groups of mice were employed: one experienced RSD (PND 47–56) and the other performed exploration without an opponent during this period (control group). Three weeks after the last episode of RSD mice underwent the different behavioral tests: plus maze (PND 77), passive-avoidance (PND 79–80), social interaction (PND 81) and Hebb–Williams (PND 82–89).

2.8. Statistical analysis

The data of the time that experimental mice and their aggressive opponents spent engaged in different behavioral categories during the social defeat episodes were compared by means of a two-way ANOVA with one between-subjects variables – "Age", with two levels (Ado or YA) – and a within-subject variable: "Days" with two levels (1st and 4th). The data of the time spent in the drug-paired compartment during Pre- and Post-C tests after conditioning with each dose of MDMA were analyzed with a mixed three-way ANOVA with two between-subjects variables – "Age" and "Stress", with two levels (RSD or Ctr) – and a within-subjects variable: "Days", with two levels (Pre-C and Post-C). Extinction and reinstatement values were analyzed by means of a Student's t test. The time required for preference to be extinguished in each animal was analyzed by means of the Kaplan–Meier test, with Breslow (generalized Wilcoxon) comparisons when appropriate. Corticosterone data were analyzed with a mixed ANOVA with the two abovementioned between-subjects variables and a within-subjects variable: "Time", with five levels (1st-0 min, 1st-30 min, 4th-0 min, 4th-30 min and 3w). The data relating to social interaction and the elevated plus maze were analyzed by a one-way ANOVA with the variable "Stress". The data of the passive avoidance test were analyzed by a two-way ANOVA, with the variable "Stress" and one within-subjects variable: "Days", with two levels (training and test). The data of Hebb Williams maze were analyzed by a two-way ANOVA, with the variable "Stress" and one within-subjects variable: "Maze", with five levels (mazes 1, 3, 4, 5 and 8). Latency values in the Hebb Williams maze were transformed to log scores in order to normalize the data. Maximum latencies were scored by individuals unable to complete the task within the time limit. All post-hoc comparisons were performed with Bonferroni tests.

3. Results

3.1. Behavioral characterization of repeated social defeat in adolescent and young adult mice

The times spent in the behavioral categories by experimental and opponent mice are shown in Table 2. The time spent by aggressive mice (resident opponents) engaged in aggressive behaviors when confronted with adolescents differed to when they were confronted with young adults: they devoted less time to threat and attack and showed a longer latency to initiate threat ($ps < 0.05$). Consequently, the behavior of adolescent and young adult mice exposed to social defeat also differed. With respect to young adult mice, adolescents spent less time in avoidance/flee behavior ($p < 0.05$). Moreover, the time spent in submissive/defensive behaviors was longer and the latency to perform this behavior was lower in the fourth than in the first episode of defeat ($ps < 0.05$).

3.2. Effect of repeated social stress on corticosterone levels

Blood concentrations of corticosterone (pg/ml) are shown in Table 3. The ANOVA revealed a significant effect of the variables "Time" [$F(4,112) = 19.389$; $p < 0.001$], "Age" [$F(1,28) = 39.286$; $p < 0.001$] and "Stress" [$F(1,28) = 41.693$; $p < 0.001$], and the Interactions "Time × Age" [$F(4,112) = 8.070$; $p < 0.001$], "Time × Stress" [$F(4,112) = 5.113$; $p < 0.001$], "Age × Stress" [$F(1,28) = 14.011$; $p < 0.001$] and "Time × Age × Stress" [$F(4,112) = 3.509$; $p < 0.01$]. Post-hoc comparisons revealed higher corticosterone levels in socially defeated mice than in controls, with this difference being significant at all the time points measured (except 3 weeks) in young adult mice and 0 min after the fourth defeat in adolescent mice ($ps < 0.01$). In young adult mice, levels of corticosterone were higher after the first and fourth episodes of defeat than 3 weeks later ($ps < 0.01$), and were higher 0 min than 30 min after the fourth encounter ($p < 0.05$). In adolescent mice, levels of corticosterone were higher after the fourth than after the first episode or 3 weeks later ($ps < 0.01$). A significant difference in the levels of corticosterone between socially defeated adolescent and young adult mice was observed after the first and fourth episodes of defeat, with older mice showing higher levels ($ps < 0.01$). Conversely, 3 weeks after defeat, young adult mice presented lower levels of corticosterone than adolescents ($p < 0.05$).

3.3. Experiment 1: effect of repeated social stress in adolescent and young adult mice on the acquisition and reinstatement of the CPP induced by MDMA

3.3.1. CPP induced by 1.25 mg/kg of MDMA

Only the variable "Days" [$F(1,69) = 36.508$; $p < 0.001$] and the Interaction "Days × Age" [$F(1,69) = 4.754$; $p < 0.05$] were significant. Post-hoc comparison revealed that all the groups spent more time in the drug-paired compartment in Post-C than in the Pre-C test ($p < 0.05$, for RSD-Ado1 and Ctr-Ado1; and $p < 0.001$, for RSD-YA1 and Ctr-YA1) (see Fig. 1).

All the groups underwent extinction sessions until the CPP was extinguished: 2 sessions (RSD-Ado1), 3 sessions (Ctr-Ado1), 10 sessions (RSD-YA1) and 4 sessions (Ctr-YA1). There are not significant differences in the duration of extinction between groups. Reinstatement of CPP after a priming dose of MDMA (0.625 mg/kg) was observed only

Table 2
Behavior of mice during agonistic encounters. Mean cumulative times (±S.E.M.) spent in different behavioral categories by adolescent and young adult experimental mice (avoidance/flee, defence/submission and latency to initiate these behaviors) and by aggressive opponents confronted with adolescent and young adult experimental mice (threat, attack and latency to initiate these behaviors), during the first (1) and the fourth (4) agonistic encounter.

Experimental animals	Behavioral categories (time spent in seconds)							
	Avoidance/flee		Defence/submission		Latency A/flee		Latency D/submission	
Encounter	1	4	1	4	1	4	1	4
Adolescent mice (n = 8)	15.12 (±3.5)	10.18 (±1.6)	37.23 (±7.44)	61.63^{+} (±8.1)	9.62 (±5.45)	5.11 (±1.01)	13.85 (±6.4)	1.76^{+} (±0.7)
Young adult mice (n = 8)	23.01* (±2.48)	25.23* (±7)	41.61 (±6)	60.7^{+} (±10.16)	1.9* (±6.19)	2.87* (±1.09)	9.41 (±8.4)	0.08^{+} (±0.24)
Aggressive opponents	Threat		Attack		Latency threat		Latency attack	
Encounter	1	4	1	4	1	4	1	4
Confronted to adolescents (n = 8)	1.85 (±0.55)	1.82 (±0.5)	16.16 (±5.321)	25.34 (±1.7)	28.16 (±10)	21.4 (±7)	9.42 (±5.6)	1.8 (±0.5)
Confronted to young adults (n = 8)	5.13* (±1.35)	2.16* (±1.25)	36.01* (±4.6)	56.23* (±14.32)	7.75* (±2.4)	11.3* (±8)	0.12 (±0.1)	1.05 (±1)

* $p < 0.05$, significant difference with respect to early-adolescent mice.
$^{+}$ $p < 0.05$, significant difference with respect to the first encounter.

in the group of mice socially defeated during adolescence (RSD-Ado1) (see Fig. 1).

3.3.2. CPP induced by 10 mg/kg of MDMA

Only the variable "Days" was significant [F (1,57) = 26.790; p < 0.001]. Thus, all groups spent more time in the drug-paired compartment in Post-C than in Pre-C (ps < 0.001), irrespective of the exposure to stress or the age at which this exposure took place (Fig. 2a).

There were significant differences between the RSD and control groups with respect to the number of days needed for the CPP to be extinguished [X^2 (3) = 10,690, p < 0.05] (see Fig. 2b). Post-hoc comparison showed that adolescent and young adult mice exposed to RSD required more extinction sessions than their respective control groups (7 sessions for RSD-Ado10 vs 4 sessions for Ctr-Ado10, X^2 = 4.215, p < 0.05; 15 sessions for RSD-YA10 vs 7 sessions for Ctr-YA10, X^2 = 3.932, p < 0.05). There were no significant differences in the time required for extinction between adolescent and adult mice in either control (X^2 = 1.320, p < 0.251) or RSD (X^2 = 0.390, p < 0.532) groups.

Reinstatement was achieved in all groups of mice after they received a priming dose of 5 mg/kg of MDMA (Fig. 2a) (ps < 0.001, significant difference with respect to previous extinction value). However, reinstatement after priming with 2.5 mg/kg was observed only in the two groups of young adult mice (RSD-YA10 and Ctr-YA10) (ps < 0.001, significant difference with respect to previous extinction value), irrespective of the exposure to social defeat.

3.4. Experiment 2: effect of repeated social defeat in young adult mice on anxiety, social interaction, learning and memory

3.4.1. Elevated plus maze (see Table 4)

Socially defeated animals spent less time [F (1,26) = 6.135; p < 0.05] and percentage of time [F (1,26) = 6.104; p < 0.05] and performed a lower number of entries in the open arms [F (1,26) = 4.123; p < 0.05] than control mice.

3.4.2. Passive avoidance test

Only the variable "Days" was significant. Both groups presented longer step-through latencies in the test than in the training session [F (1,28) = 37.916; p < 0.001] (data not shown).

3.4.3. Social interaction test (see Table 5)

In comparison with the control group, mice exposed to RSD spent less time in Attack behavior [F (1,28) = 7.558; p < 0.05] and in each contact with the standard opponent (Unit of Social Investigation) [F (1,28) = 3.920; p < 0.05], but more time in Avoidance/Flee [F (1,28) = 7.558; p < 0.05], Defence/Submission [F (1,28) = 9.604; p > 0.05] and exploration of the opponent from a distance [F (1,28) = 98.451; p < 0.05].

3.4.4. Hebb–Williams maze

The ANOVA for the total latency score (see Fig. 3a) revealed an effect of the variable "Maze" [F (4,100) = 8.669; p < 0.05], as the time employed to reach the goal in the five mazes varied according to the level of difficulty of each maze. Post-hoc analysis showed differences between the performance in maze 1 and mazes 3 and 5 (p < 0.05). The performance in maze 5 (the most difficult maze) differed from that in mazes 1, 4 and 8 (p > 0.05). The ANOVA also revealed an effect of "Stress" [F (1,25) = 9.203; p < 0.05], because the group of socially defeated mice employed more time to reach the goal than the control group in all the mazes. There was also an effect of the Interaction "Maze × Stress" [F (4,100) = 3.279; p < 0.05]. Socially defeated mice took more time to perform the mazes than the control group, and the time spent to reach the goal varied with the difficulty of the maze. There were significant differences between the groups in their

Table 3
Effect of repeated social stress on corticosterone levels. Mean corticosterone levels (±S.E.M.) in blood (pg/ml) of adolescent and young adult mice after exploration (control) or repeated social defeat exposure (defeated), 0 or 30 min after the first (1st-0, 1st-30) and fourth (4th-0, 4th-30) social defeat or exploration and 3 weeks after.

		Measurements (pg/ml, ± S.E.M.) after social encounters				
		1st-0 min	1st-30 min	4th-0 min	4th-30 min	3 weeks
Adolescent mice	Control	1180 (±260)	1475 (±168)	1628 (±225)	1777 (±187)	1134 (±75)
	Defeated	1401 (±267)$^{++}$	1172 (±204)$^{++}$	4233 (±475)**, $^{++}$	2432 (±405)$^{++}$	1303 (±154) $^{+}$
Young adult mice	Control	1726 (±366)	2497 (±586)	2848 (200)	2561 (±357)	676 (±138)
	Defeated	4778 (±796)**	6560 (±1318)**	6380 (±765)**	4280 (±552)**	893 (±164)

** $p < 0.01$, significant difference with respect to controls of the same age.
$^{++}$ $p < 0.01$, significant difference with respect to young adult mice.
$^{+}$ $p < 0.05$, significant difference with respect to young adult mice.

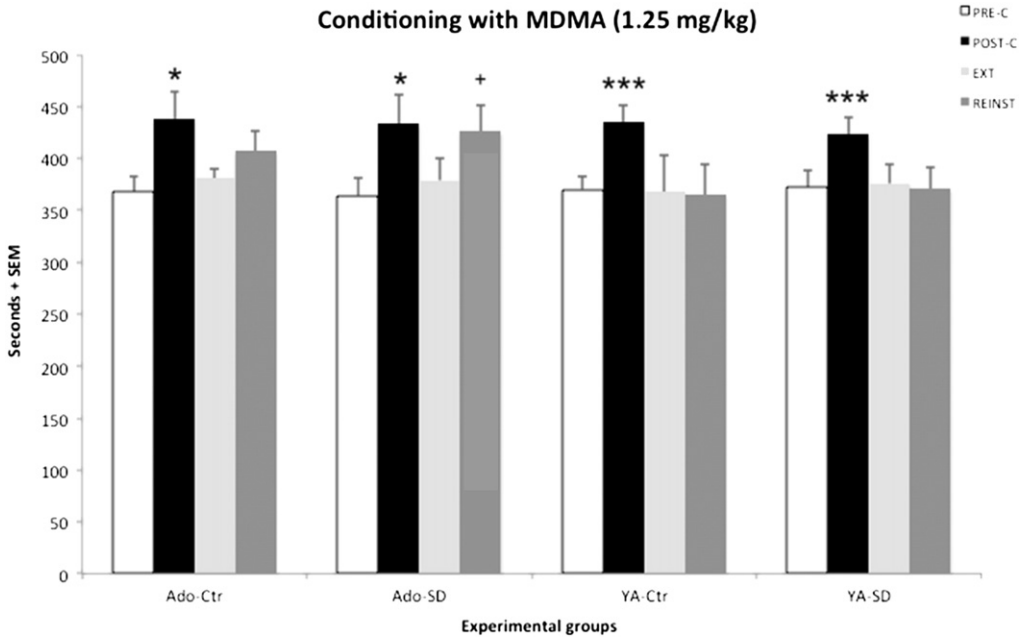

Fig. 1. Effects of repeated social defeat (RSD) on the acquisition of CPP induced by 1.25 mg/kg of MDMA and reinstatement after extinction. Adolescent or young adult mice were exposed to exploration (Ado-Ctr, YA-Ctr) or repeated social defeat (Ado-SD, YA-SD) three weeks before initiation of the CPP procedure. All groups were conditioned with 1.25 mg/kg of MDMA. The bars represent the time (in seconds) spent in the drug-paired compartment before conditioning sessions in the pre-conditioning (PRE-C) test (white bars) and after conditioning sessions in the post-conditioning (POST-C) test (black bars), in the last extinction (EXT) session (light gray bars), and in the reinstatement (REINST) test (dark gray bars). *$p < 0.05$ and ***$p < 0.001$, significant difference in the time spent in the drug-paired compartment in PRE-C vs POST-C. $^{+}p < 0.05$, significant difference in the time spent in the drug-paired compartment in REINST vs EXT.

performance in maze 3 ($p < 0.05$), maze 4 ($p < 0.05$), maze 5 ($p < 0.05$) and maze 8 ($p < 0.05$). No significant differences were found for maze 1, as it was the easiest maze.

Regarding the total number of errors made in each maze, there was an effect of "Maze" [$F(4,104) = 15.502$; $p < 0.05$], but not of the variable "Stress", since the average of errors in all the mazes was similar in both groups (Fig. 3b).

The ANOVA of the Acquisition criterion score revealed an effect of the variable "Maze" [$F(4,112) = 9.736$; $p < 0.01$], as all the mice employed more time in the most difficult maze (number 5) than in the mazes 1, 4 and 8 (ps < 0.01). The ANOVA also showed an effect of the variable "Stress" [$F(1,28) = 11.687$; $p < 0.01$], as socially defeated mice needed more trials to complete the task than control mice (Fig. 3c). There was an effect of the Interaction "Maze × Stress" [$F(4,112) = 3.803$; $p < 0.01$]. Socially defeated mice needed more trials than controls to acquire learning in maze 3 ($p < 0.01$), maze 4 ($p < 0.05$), maze 5 ($p < 0.01$) and maze 8 ($p < 0.05$), while there were no significant differences between groups in maze 1.

4. Discussion

The original contributions of the present study to the knowledge of the effects of social defeat on the rewarding properties of psychostimulant drugs are based on the following two aspects: the study of the influence of RSD on the effects of MDMA in the CPP paradigm, and the evaluation of the long-term effects of RSD in both adolescent and young adult mice. We demonstrate for the first time that stress induced by intermittent episodes of social defeat induces a long-term increase in the effects of MDMA in the CPP paradigm. Mice exposed to RSD during adolescence showed an increase in vulnerability to reinstatement of the CPP induced by a low dose of MDMA and an increase in the duration of the CPP induced by an effective dose of this drug when they were adults. Young adult mice exposed to RSD also presented an increase in the duration of CPP during adulthood, in addition to other long-lasting behavioral changes, such as an enhancement in anxiety, alterations in social interaction (a reduction of attack and an increase in defensive and avoidance behaviors) and an impairment of learning.

As we hypothesized, exposure to RSD seems to increase the rewarding effects of MDMA in the CPP paradigm, which is in accordance with the results of previous studies demonstrating that RSD enhances amphetamine and cocaine CPP (Burke et al., 2011; Hymel et al., 2014; McLaughlin et al., 2006) and intake of these drugs (Boyson et al., 2011, 2014; Cruz et al., 2011; Han et al., 2015; Miczek et al., 2008, 2011; Quadros and Miczek, 2009; Yap et al., 2015) or alcohol (Croft et al., 2005; Norman et al., 2015; Rodríguez-Arias et al., 2014). A recent work by our group demonstrated that the same procedure of RSD enhances the rewarding and reinstating effects of sub-threshold doses of cocaine and increased the duration of the CPP induced by effective doses (Rodríguez-Arias et al., submitted). In the present work, the stress-induced increase in the rewarding effects of MDMA was expressed as an enhanced vulnerability to reinstatement (in mice exposed to RSD during early adolescence) and as an increase in the duration of CPP (resistance to extinction). Irrespective of exposure to RSD, all groups exhibited a similar CPP, even with the low dose of MDMA. This may have been due to the fact that all the mice were adults when they underwent the CPP procedure three weeks after RSD (PND 59–70 or PND 77–88). This result, in line with that of a previous study in which the low dose of MDMA was ineffective in adolescents but induced CPP in young adult mice (García-Pardo et al., 2014), suggests that adolescent mice are less sensitive to the rewarding effects of MDMA, conversely to that observed with cocaine (Schramm-Sapyta et al., 2009). A stress-induced potentiation of CPP across a variety of drugs of abuse, including cocaine and nicotine, has been reported by numerous studies (Schindler et al., 2010, 2012; Smith et al., 2012). Activation of the CRF and kappa opioid receptor systems seems to play a major role in the pro-addictive effects of stress (for a review see Bruchas et al., 2010; Ehrich et al., 2014), and kappa receptor stimulation has been shown to induce reinstatement of drug seeking through stress-like effects (Wee and Koob, 2010). However, in the present study we did not observe a potentiation of MDMA CPP after RSD exposure. A possible reason for this is that the rewarding effects of MDMA represented an

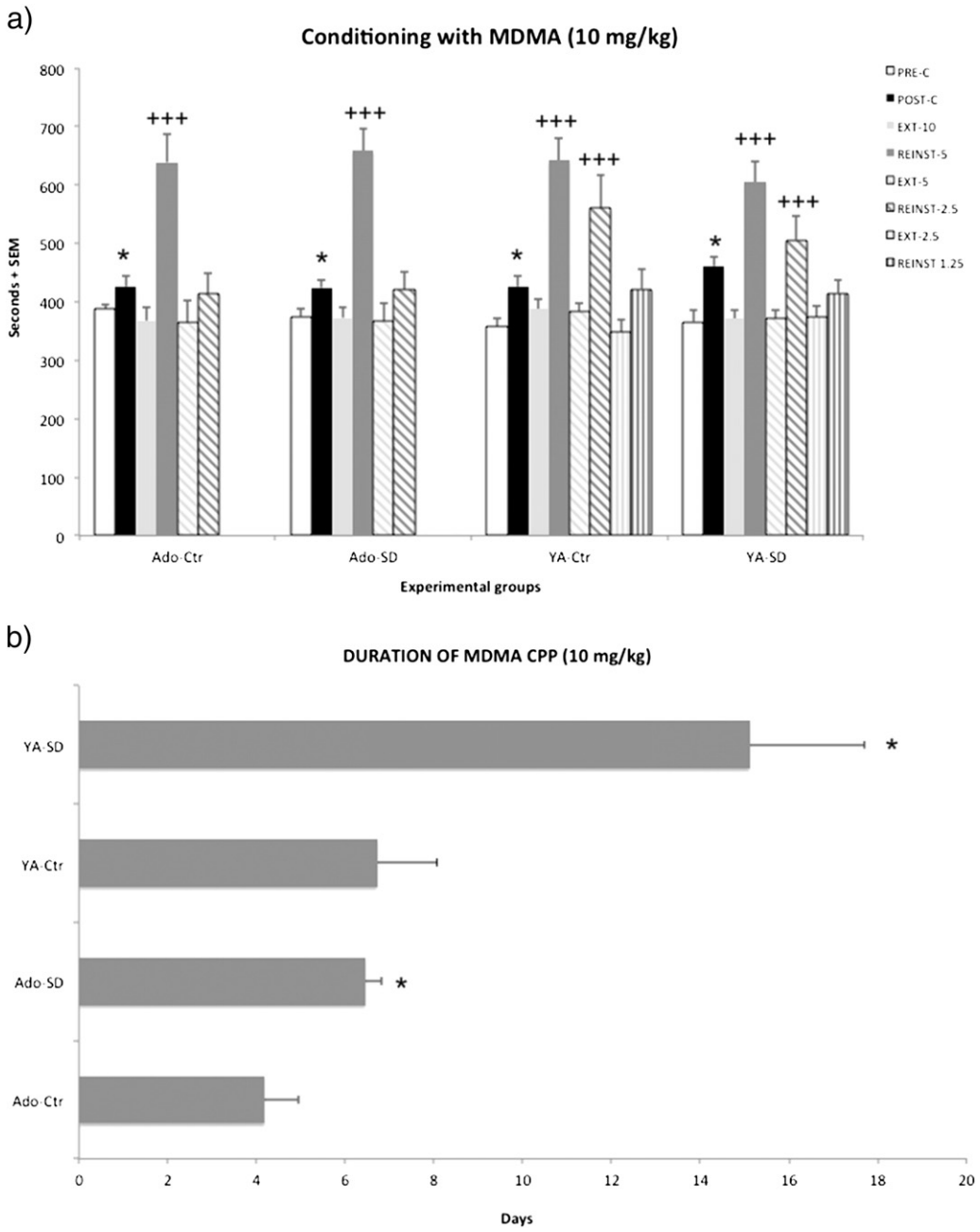

Fig. 2. Effects of repeated social defeat (RSD) on the CPP induced by 10 mg/kg of MDMA. a) Effects of RSD on the acquisition and reinstatement of MDMA CPP. Adolescent or young adult mice were exposed to exploration (Ado-Ctr, YA-Ctr) or repeated social defeat (Ado-SD, YA-SD) three weeks before initiation of the CPP procedure. All groups were conditioned with 10 mg/kg of MDMA. The bars represent the time (in seconds) spent in the drug-paired compartment before conditioning sessions in the pre-conditioning (PRE-C) test (white bars) and after conditioning sessions in the post-conditioning (POST-C) test (black bars), in the last extinction (EXT) session (light gray bars), and in the reinstatement (REINST) test (dark gray bars). *$p < 0.05$, significant difference in the time spent in the drug-paired compartment in PRE-C vs POST-C. $^{+++}p < 0.001$, significant difference in the time spent in the drug-paired compartment in REINST vs EXT. b) Effects of RSD on the extinction of MDMA CPP. Mean number of days needed to achieve complete extinction of CPP in the following groups: Ado-Ctr and Ado-SD, mice exposed during adolescence to exploration or repeated social defeat, respectively; YA-Ctr and YA-SD, mice exposed during late-adolescence to exploration or repeated social defeat, respectively. After conditioning with 10 mg/kg of MDMA all groups showed CPP in the Post-C test. Both groups exposed to repeated social defeat needed more extinction sessions to achieve complete extinction of CPP than mice non-exposed to stress. *$p < 0.05$, significant difference with respect to the control group of the same age.

inverted U curve in function of the dose administered, with only medium doses proving to be rewarding (Daza-Losada et al., 2007; Schenk, 2009). Thus, the stress-induced increase in the sensitivity of mice to the effects of MDMA was not reflected by a greater CPP. In previous studies carried out in our laboratory, we have observed that the enhancement in the rewarding effects of MDMA induced by adolescent drug pre-exposure, which is not evident in the CPP test, can be detected by measuring the time needed to achieve extinction of CPP (Daza-Losada et al., 2009b; Do Couto et al., 2011; Ribeiro Do Couto et al., 2012). It is well established that the duration of CPP is dependent on the dose used (Aguilar et al., 2009), and the period required for preference to be extinguished is a marker of the motivational properties of a drug (Pulvirenti, 2003). According to this, we observed that the CPP induced by the low dose of MDMA was extinguished faster than that induced by the high dose (mean of all groups, 3.5 vs 8.2 days needed to extinguish the CPP induced by 1.25 and 10 mg/kg of MDMA, respectively). Most importantly, our results demonstrate that mice exposed to RSD have an enhanced resistance to the extinction of the CPP induced by 10 mg/kg of MDMA, which can be interpreted as an increased susceptibility to the motivational properties of this drug. In agreement

Table 4
Long-term effects of RSD exposure on anxiety-like behavior in the elevated plus maze. Mice were exposed to RSD (social defeat) or exploration (control) during late-adolescence and were tested in the elevated plus maze 3 weeks later. Data are presented as mean values ± S.E.M.

Elevated plus maze measurements	Control	Social defeat
Time in open arms (s)	86.3 ± 8.7	52.3 ± 10.5*
Time in central platform (s)	78.52 ± 6.4	88.1 ± 5.9
Time in closed arms (s)	138.64 ± 9.5	161.77 ± 9.5
% time in open arms	38.4 ± 3.6	23.9 ± 4.6*
Entries in open arms	9 ± 1.2	5.6 ± 1.1*
Entries in closed arms	14.7 ± 1.1	15.3 ± 1.2
Total entries	23.7 ± 1.6	20.9 ± 1.7
% open entries	36.5 ± 3.6	25.8 ± 4

* $p < 0.05$, significant difference with respect to control.

with this idea, it has been suggested that adolescent defeat can enhance behavioral responses to amphetamine and seeking of drug-associated cues in adulthood (Burke et al., 2013). In addition, social stress increases vulnerability to reinstatement in mice exposed to RSD during adolescence and conditioned with the low dose of MDMA in adulthood. Although acute exposure to an episode of social defeat immediately before the test induces the reinstatement of morphine (Ribeiro Do Couto et al., 2006) and cocaine CPP (Land et al., 2009; Titomanlio et al., 2013) and increases priming-induced cocaine CPP (Ribeiro Do Couto et al., 2009), this is the first study to demonstrate that RSD induces a long-term increase in vulnerability to reinstatement. It is particularly significant the fact that adolescent mice showed weaker behavioral and hormonal responses (less time in avoidance/flee behavior and lower corticosterone levels) during exposure to social defeat than young adult mice. This could be due to the fact that aggressive opponents confronted with adolescent mice spent less time in threat and attack behaviors. Consequently, young adult mice exposed to RSD presented higher corticosterone levels than controls at all the time points measured (0 and 30 min after the first and fourth episodes of defeat), while mice exposed to RSD during adolescence only showed higher levels of corticosterone immediately after the fourth episode of defeat. These age differences in behavior and corticosterone response have been observed in previous studies in our laboratory after both acute and repeated social defeat (García-Pardo et al., 2014; Rodríguez-Arias et al., 2014). In future studies it would be interesting to modify some parameters of the social interaction of adolescent and young adult mice with an aggressive resident opponent in order to equal the level of stress induced by social defeat in both age groups. This would allow us to determine whether the effects of social stress exposure on the rewarding effects of MDMA differ in adolescent and adult mice.

As discussed previously, the effects of RSD on the rewarding properties of MDMA have not been evaluated previously, but a previous study carried out in our laboratory demonstrated that acute social defeat before each conditioning session with MDMA reduces the CPP induced

Table 5
Long-term effects of RSD exposure on social interaction with conspecifics. Means of accumulated times (in seconds) with S.E.M. allocated to different categories of spontaneous behavior from the social interaction test in mice exposed to RSD (social defeat, n = 15) or exploration (control, n = 15) three weeks before social interaction test.

Behavioral categories (time in seconds)	Control	Social defeat
Body care	17.8 ± 3.2	14.5 ± 2.6
Digging	16.2 ± 2.2	16.8 ± 2.8
Non-social exploration	443.9 ± 9.9	434.4 ± 7.2
Explore from a distance	7.4 ± 1.1	41.1 ± 3.3*
Social investigation	52.8 ± 7.9	50.1 ± 6.4
Unit of social investigation	2.5 ± 0.2	2 ± 0.2*
Threat	28 ± 7.9	14.3 ± 6.1
Attack	19.1 ± 5.8	2.8 ± 1.5*
Avoidance/flee	0 ± 0	4 ± 1*
Defensive/submissive	0 ± 0	10.8 ± 3.5*

* $p < 0.05$, significant difference with respect to exploration mice.

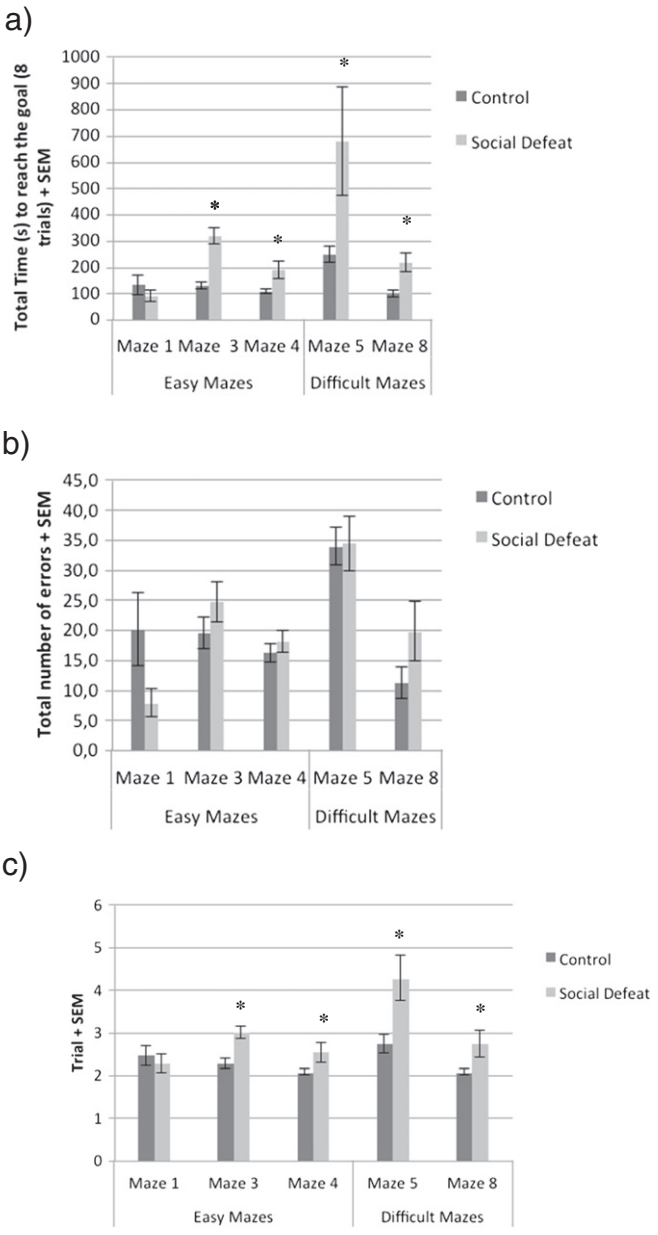

Fig. 3. Long-term effects of repeated social defeat (RSD) during late-adolescence in the Hebb–Williams maze. On PND 47–56 mice were exposed to social defeat or exploration (control). On PND 82–89 they were tested in the Hebb–Williams maze. The mazes were classified as easy (1, 3 and 4) or difficult (5 and 8). a) Long-term effects of RSD on total latency score in the Hebb–Williams maze. Data of the total time (in seconds) needed to reach the goal in the 8 trials performed in each maze are presented as mean values ± S.E.M. *$p < 0.05$, significant difference with respect to the performance of control group. b) Long-term effects of RSD on the total number of errors in the Hebb–Williams maze. Data of the total number of errors to reach the goal in the 8 trials are presented as mean values ± S.E.M. c) Long-term effects of RSD on the acquisition criterion in the Hebb–Williams maze. Data of the acquisition criterion are presented as mean values ± S.E.M. *$p < 0.05$ and ***$p < 0.001$, significant difference with respect to the control group.

by this drug (García-Pardo et al., 2014). Thus, different schedules of social defeat exposure have opposite results on MDMA-induced CPP. From our point of view, the main contributing factor of these divergent results is that, in our previous study, the adverse experience of social defeat was suffered by mice immediately before conditioning with MDMA, which probably reduced its rewarding properties when evaluated 48 h after the last episode of social defeat. The importance of timing in the effects induced by social stress has been reviewed by Neisewander et al. (2012); for example, a decrease of alcohol intake is observed immediately after social defeat, conversely to the increase observed when

there is a delay between social defeat and self-administration (Caldwell and Riccio, 2010), that is consistent with the suppressant effects of stressors on ongoing behavior (Meerlo et al., 1996). Considered together, the results obtained by the two MDMA studies confirm that acute and RSD alter the rewarding effects of MDMA in opposite ways.

Our results also demonstrate that young adult mice exposed to RSD suffer long-term behavioral consequences, exhibiting an increase in anxiety and impaired social behavior and cognitive processes in adulthood. The behavioral profile observed in young adult mice exposed to RSD (anxiety and cognitive impairment) may have been related with the longer duration of CPP observed in these mice. Our study shows an increase in anxiety in socially defeated mice in the elevated plus maze (less time, lower percentage of time and fewer entries into the open arms) 21 days after RSD. It is very difficult to compare the different studies because of variability in the methodology used to induce social defeat and to evaluate its effects (acute vs chronic, short- vs long-term, etc.); however, generally, an increase in anxiety has been observed with different procedures of acute and repeated social defeat in adult (Heinrichs et al., 1992, 1995; Jin et al., 2015; Kinn Rød et al., 2012; Patki et al., 2013, 2014; Rodgers and Cole, 1993) and adolescent (Huang et al., 2013; Iñiguez et al., 2014; Kinsey et al., 2007) rodents. In some studies this effect was not evident 1–3 weeks after social defeat (Korte and de Boer, 2003; Watt et al., 2009), while in others it was observed until 14 days after defeat (Jacobson-Pick et al., 2013; Ruis et al., 1999). An anxiolytic effect of MDMA in socially defeated adult mice with enhanced anxiety could increase the acquisition of CPP and enlarge the duration of this effect. With respect to the effects of social defeat on learning and memory, there are many studies reporting important structural and functional alterations of the hippocampus produced by social defeat, such as decreased cell proliferation (Yap et al., 2006) and decreased BDNF levels (Miczek et al., 2011). Most studies have reported a deficit in spatial learning in socially defeated animals (Colas-Zelin et al., 2012; Jin et al., 2015; Novick et al., 2013; Patki et al., 2013, 2014), although a lack of effects has also been observed 3 weeks after social defeat (Buwalda et al., 2005). The advantage of the Hebb–Williams Maze used in the present study, with respect to other learning tasks, is that it provides information about the acquisition process. Although all animals learned how to reach the goal, socially defeated mice needed more time to exit the maze in the first trials, suggesting that the acquisition of learning had been slowed down by RSD exposure. These effects on learning can also help to explain why young adults exposed to RSD were so resistant to the extinction of CPP, which is considered an acquisition of a new learning rather than the simple debilitation of previously learned information.

Social defeat stress seriously affects social behavior with conspecifics, inducing social avoidance and producing consequences that persist for a long time. Studies in rats report a decrease in aggressive behavior in socially defeated rats and an increase in escape and avoidance behaviors (Blanchard et al., 1995, 2001). Similarly, social defeat stress induces significant social avoidance in the social interaction test in adult and adolescent mice (Goto et al., 2014; Iñiguez et al., 2014). Consequently, in the present work, we have observed that socially defeated mice spend less time in attack and unit of social investigation and display an increase in avoidance and defence behaviors. Similar results have observed previously in mice exposed to our RSD procedure during adolescence (Rodríguez-Arias et al., 2014). As an increase in the rewarding effects of MDMA and social avoidance is consistently observed in adolescent and adult mice exposed to RSD, we believe that these effects are related. The protective influence of social interaction on vulnerability to the effects of drugs of abuse is well known; in the CPP, social interaction is rewarding and can reduce preference for the drug-paired context (Aguilar et al., 2013; Bardo et al., 2013; Neisewander et al., 2012; Ribeiro Do Couto et al., 2009). Thus, it can be hypothesized that mice with poor social attachment will show an enhanced response to MDMA administration, expressed as an increase in the duration of the CPP.

In conclusion, the present study demonstrates that exposure to social stress induces a long-term increase in the rewarding effects of MDMA, thereby enhancing vulnerability to the development of dependence. Impairments in social behavior and alterations of monoamine levels induced by RSD might account for this enhanced vulnerability to the effects of MDMA. Future studies should evaluate the effects of pharmacological strategies that prevent such alterations and establish new targets for the treatment of subjects with MDMA dependence.

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