Pharmacology, Biochemistry and Behavior 181 (2019) 60–68

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Cocaine-induced behavioral sensitization is greater in adolescent than in adult mice and heightens cocaine-induced conditioned place preference in adolescents

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ARTICLE INFO

Keywords:
Adolescent
Cocaine
Sensitization
Conditioned place preference
Binge

ABSTRACT

Adolescents are more sensitive than adults to the neural and behavioral effects of psychostimulants, and exhibit greater vulnerability to drug abuse, dependence or relapse into these conditions. We have reported that cocaine pretreatment during adolescence promotes the expression of behavioral sensitization to a greater extent than when the pretreatment occurs at adulthood. Behavioral sensitization has been associated to the transition from drug use to addiction and is postulated to indicate heightened sensitivity to the appetitive motivational effects of drugs. The relationship between behavioral sensitization and conventional measures of drug reward, such as conditioned place preference (CPP), has yet to be thoroughly investigated, and little is known about age-related differences in this phenomenon. The present study tested cocaine-induced CPP in adolescent and adult mice exposed to cocaine (or vehicle) pretreatment, either in an intermittent or "binge" (i.e., heavy cocaine use on a single occasion, which increases the likelihood of experiencing cocaine-related problems) fashion. Cocaine administration induced behavioral sensitization to a greater extent in adolescent than in adult mice. Cocaine-induced CPP was fairly similar in vehicle pretreated adolescent and adult mice, yet greater in adolescent vs. adults after cocaine-induced sensitization. The results confirmed the higher sensitivity of adolescent mice to cocaine-induced behavioral sensitization and suggest its association with greater sensitivity to cocaine's rewarding effects.

1. Introduction

The use of psychoactive drugs often begins at adolescence, a developmental period in which the brain is under substantial rearrangement (Giedd et al., 1999; Spear, 2000). An early onset of drug use is Anthony and Petronis, 1995very often associated with a high risk of exhibiting drug dependence later in life (Clark et al., 1998). The mechanisms underlying this association are still under investigation, with some supporting a causal relationship between early onset and greater likelihood of dependence (Pascual et al., 2009) yet others claiming that both are symptoms of a third factor (e.g., pre-existing psychopathology) (Guttmannova et al., 2012).

Pre-clinical studies have helped clarify these issues by analyzing age-related differences in the reactivity to psychostimulants, such as cocaine, and other drugs. The determination of adolescence in rodents has been described in several studies, with the interval between postnatal days (PND) 21 to 35 corresponding to early adolescence, PND 35-46 to periadolescence and PND 46-60 to late adolescence or young

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https://doi.org/10.1016/j.pbb.2019.04.005
Received 12 December 2018; Received in revised form 11 April 2019; Accepted 17 April 2019
Available online 18 April 2019

adulthood (Spear and Brake, 1983; Spear, 2000; Adriani and Laviola, 2000; de Vivo et al., 2014).

Different transmitter systems exhibit receptor peak or pruning at adolescence (Tarazi et al., 1998; Badanich et al., 2006), yet the age-related variability in neurochemical and behavioral responses to acute and chronic cocaine are mainly related to the differential degree of maturation in the dopaminergic (DA) mesocorticolimbic system. During adolescence, the DA system exhibits significant developmental adjustment in the prefrontal cortex (PFC) and *nucleus accumbens* (NAc) (Jernigan et al., 1991; Giedd et al., 1999; Hohn and Wuttke, 1979; Rosenberg and Lewis, 1994). Importantly, the mesocorticolimbic dopamine system, which projects from the ventral tegmental area (VTA) to NAc and PFC, is implicated in drug-induced behavioral sensitization and in the rewarding effects of psychostimulants (Pierce and Kumaresan, 2006).

In preclinical studies, drug treatment regimens vary from intermittent to binge exposures, which may result in specific patterns of neurobehavioral responses (Shippenberg and Heidbreder, 1995). Behavioral sensitization is defined as a progressive increase in a drug-induced response, typically forward locomotion, as a function of the repeated, and often intermittent, exposure to single administrations of the drug (Camarini and Pautassi, 2016). This phenomenon likely reflects the neurochemical changes that accompany the transition from controlled drug use to addiction. Studies have reported age-related differences in cocaine sensitization. Adolescent rats exhibited, when compared to adults, reduced cocaine-induced acute locomotor activation (Laviola et al., 1995) and showed similar or lower cocaine-induced behavioral sensitization (Laviola et al., 1995; Collins and Izenwasser, 2002). Adolescent mice, however, displayed greater behavioral sensitization to cocaine compared to adults (Camarini et al., 2008; Valzachi et al., 2013).

On the other hand, it has been shown that many cocaine users take the drug in binge cycles; i.e., they would engage in repeated drug administration in a single occasion, and only stop when drug resources are depleted (Harzke et al., 2009). This pattern of drug intake, whose prevalence has increased over the past years particularly among adolescents (John and Wu, 2017), is associated with greater frequency of cocaine-induced negative consequences (Harzke et al., 2009). Moreover, binge cocaine administration has been shown to induce deleterious behavioral and neurochemical consequences in the PFC (Bailey et al., 2005) and to be responsible for facilitating cocaine rewarding effects in adults and adolescents (Bailey et al., 2005; Mateos-Garcia et al., 2015).

Ontogenetic studies have demonstrated that adolescent rats at PND 35 are more sensitive to cocaine-induced conditioned place preference (CPP) and showed an earlier onset of cocaine-induced increases of dopamine levels in the NAc, compared to older rats, after repeated cocaine administration (Badanich et al., 2006). CPP is a behavioral assay in which mice or rats are given pairings of a distinctive compartment with pharmacological effects of a drug, and pairings of another compartment with vehicle administration. During the test, the animals can explore both compartments and the level of preference for the drug-paired compartment is considered an index of the rewarding motivational effects of the drug. Adolescent rats have been shown to exhibit delayed extinction of cocaine-induced CPP, compared to adults (Brenhouse et al., 2015).

In the present study we examined cocaine-induced CPP in adolescent and adult mice. After establishing (Experiment 1) that adolescents and adults exhibited similar cocaine-induced CPP, CPP was tested at both ages (Experiments 2 and 3) after two different patterns of cocaine administration. In one of the regimens, mice were given daily injections of either saline or cocaine (10 mg/kg) during an 8-day pretreatment phase. A second, "binge" regimen also exposed the mice to 8 administrations of cocaine, yet concentrated in days 7 and 8 of the pretreatment phase. Locomotor activity was measured before the commencement and on days 1 and 8 of the pre-treatment. We expected the pretreatment – particularly the "binge" regimen - to induce behavioral sensitization and enhance cocaine-induced CPP, to a greater extent in adolescents than in adults.

2. Experimental procedures

2.1. Subjects

We employed 94 adolescent and 100 adult Swiss male mice (26–30 or 70–75 days-old, respectively, at the beginning of the experiments; all male), obtained from the animal facility of the Instituto de Ciências Biomédicas (ICB) at the Universidade de São Paulo, Brazil. The subjects were housed 5 per cage in polycarbonate cages, with food and water freely available under a 12-h light/dark cycle with lights on at 07:00 AM. Mice were allowed to acclimate to housing conditions 5–7 days before starting the experiments. All experimental procedures followed the Guide for the Care and Use of Laboratory Animals of NIH (National Research Council, 1996), were approved by the Ethical Committee for Animal Use (CEUA no. 107/2014) at ICB and complied with the ARRIVE guidelines.

2.2. Experimental design and procedures

A schematic diagram of protocols utilized in this study is depicted in Fig. 1.

2.2.1. Experiment 1. CPP in Adolescent and Adult mice without pretreatment

This experiment employed a two-group (adolescent, adults; $n = 8$ in each group) design. The aim was to assess the baseline level of cocaine-induced CPP at both ages (i.e., without drug pre-exposure treatment). The adolescent (PND = 26–28) and adult (PND = 70–72) mice were tested for preference towards a cocaine-paired context (conditioned stimulus - CS+) in a rectangular box (44 L × 13 W × 14 H, cm) divided into three compartments by guillotine type doors. The central compartment was gray with a smooth floor (7 × 13 × 14 cm). The opposite compartments had distinctive visual and tactile cues: one had vertical black and white stripes on the wall and bars on the floor, while the other compartment had horizontal black and white stripes on the wall, and a steel mesh floor.

The CPP protocol included three phases: a single habituation session (15 min), a conditioning phase composed by six drug conditioning sessions of 15 min, and a preference test of 15 min. During the habituation session, the animals received an injection of saline and were positioned in the center of the apparatus with free access to all compartments.

Conditioning sessions were conducted one per day. Three cocaine pairings were alternated with saline pairings. Half of the animals received 10 mg/kg cocaine i.p. and were immediately and individually placed in the compartment with bar floor. The remaining half of the mice was placed in the compartment with steel mesh floor. Half of the animals started the conditioning trials with cocaine and the other half with saline. On the test day, mice did not receive i.p. injections. They were placed into the central compartment and had free access to both compartments for 15 min. The test was videotaped and time spent (s) in each compartment was subsequently measured by trained researcher, unaware of the experimental treatment of the subject. It was considered that mice showed conditioned place preference for cocaine when the time spent in the previously cocaine-paired compartment (further referred to as excitatory conditioned stimulus, CS^+) was significantly higher than the time spent in the saline-paired compartment (CS^-).

2.2.2. Experiment 2. Cocaine-induced Behavioral Sensitization (8-day pretreatment protocol) followed by cocaine-induced CPP, in adolescent and adult mice

Experiments 2A and 2B employed a 2 (Age: adolescents, adults) x 2

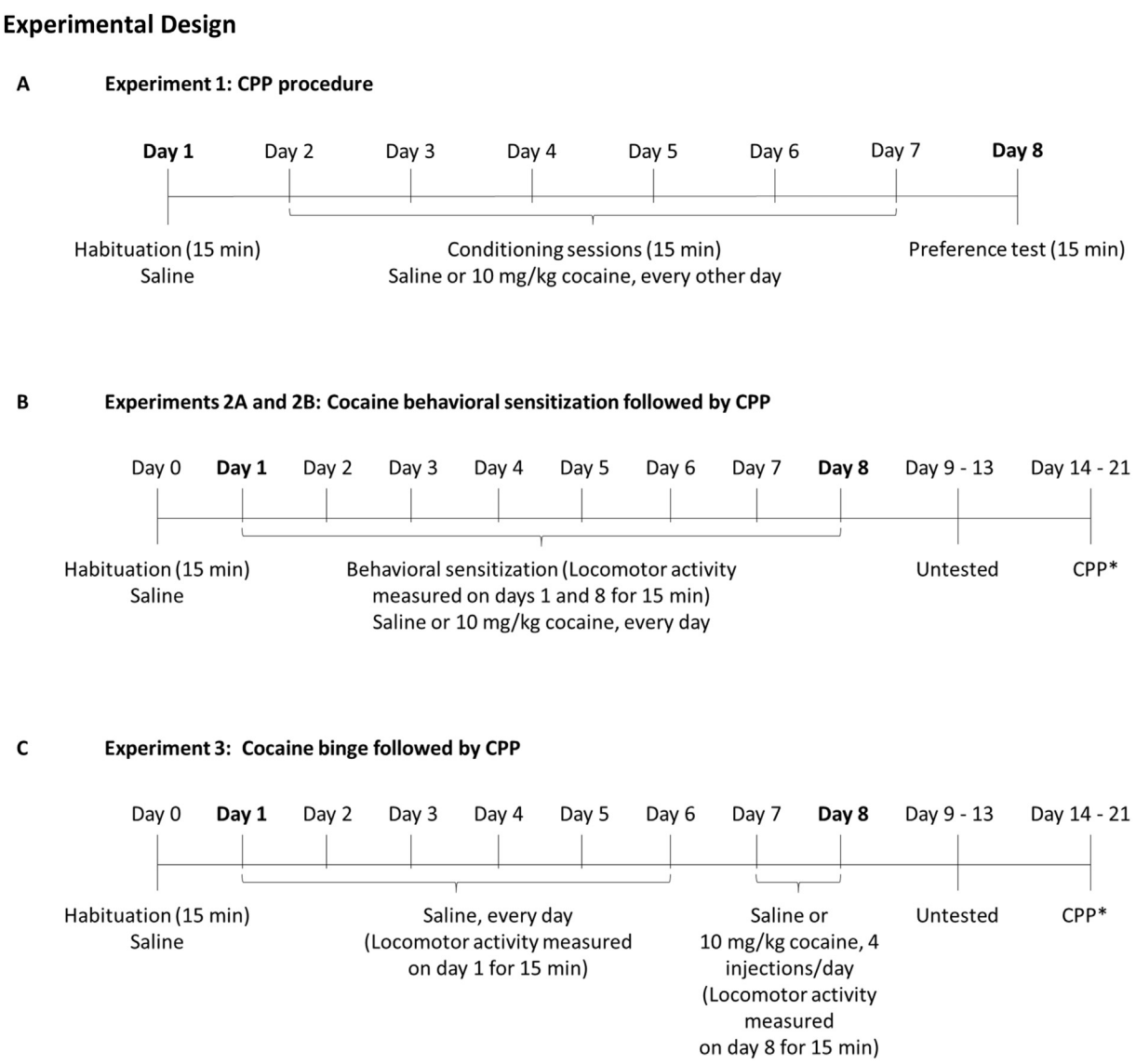

Fig. 1. Experimental designs of Experiments 1, 2A and 2B, and 3. (A) Conditioned place preference (CPP) procedure employed to induce cocaine conditioned preference. (B) In Experiments 2A and 2B the mice were exposed to a protocol to induce cocaine behavioral sensitization, which was followed by cocaine-induced CPP*. The latter procedure was run as described in (A), except that in Experiment 2B the mice were given pairings of the CS^+ with 5 mg/kg cocaine. The mice remained in their home cage on days 9–13. (C) In Experiment 3 the mice were exposed to a cocaine "binge" protocol, which was followed by cocaine-induced CPP*, conducted as described in (A).

(drug administered during the 8-day pretreatment: 0.0 or 10 mg/kg cocaine) factorial design, with 9–12 animals in each of the groups. During the CPP all mice were given pairings of vehicle in the CS− and pairings of 10 mg/kg (Experiment 2A) or 5 mg/kg (Experiment 2B) cocaine in the CS+.

A habituation session (Experimental day 0) was conducted 24 h before the pretreatment. The mice were given a saline injection and then assessed for locomotor activity during 15 min in a 40-cm diameter open field arena, which was surrounded by a 50-cm wall. The aim of this test was to minimize novelty responses to the open-field apparatus and to the injection, and also to obtain a measure of basal locomotor activity at both ages. Distance travelled was measured via Ethovision XT (Noldus, The Netherlands).

During the behavioral sensitization protocol, the adolescent and adult mice were repeatedly treated with saline or 10 mg/kg cocaine. Specifically, every day for 8 days (Experimental days 1 to 8) they were given one injection of saline or cocaine. Locomotor sensitization was assessed for 15 min, on days 1 and 8, by individually placing each mouse in the open field arena, immediately after the cocaine or vehicle administration.

After the pretreatment, all mice remained in their home cage for 5 days (Experimental days 9–13) before commencement of the CPP, which was conducted as described above. The rationale for using 5 mg/kg in Experiment 2B was that all mice, regardless of age, exhibited CPP to 10 mg/kg cocaine in Experiment 2A. Therefore, Experiment 2B assessed the possibility that age-related differences in cocaine-induced CPP emerged after using a lower (5 mg/kg) dose. It is important to emphasize that the younger mice were tested for cocaine-induced CPP during late adolescence (PND = 41–45).

2.2.3. Experiment 3. Cocaine "Binge" in Adolescent and Adult mice and CPP

Experiment 3 employed a 2 (Age) × 2 (pre-exposure treatment: vehicle or cocaine) factorial, similar to that of Experiment 2. Saline pretreated groups were composed by 12 subjects, whereas cocaine pretreated groups had 18 (adolescent) or 20 (adult) subjects.

The mice were habituated to the open field, as described before. During the pretreatment, the mice received the same amount of saline

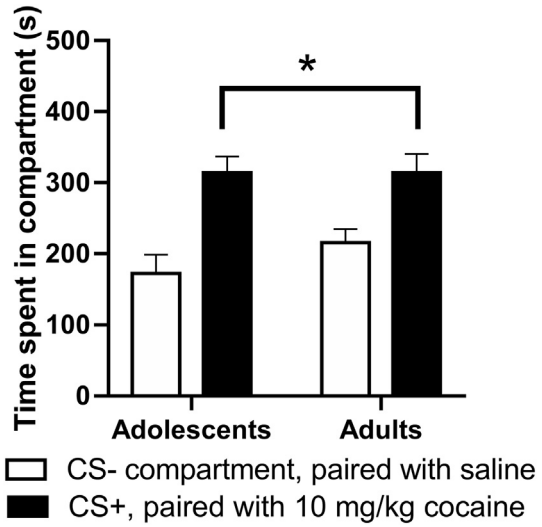

Fig. 2. Conditioned place preference to 10 mg/kg cocaine in adolescent and adult mice. Data are shown as time spent (s) in the cocaine or saline-paired sides, which are referred to as excitatory and inhibitory conditioned stimulus (CS+ and CS−, respectively; mean ± S.E.M). The asterisk sign (*) indicates that both adolescents and adults exhibited a significant preference for the CS+ over the CS−, thus expressing cocaine-induced CPP.

or cocaine injections as they did in Experiment 2, yet via a "binge" procedure. Although the term "binge" usually defines a behavioral pattern related to intake or consumption of a specific drug, in the present study we denominated "binge" a protocol of multiple doses administered 1 h apart. Specifically, on days 1 to 6 all mice received daily injections of saline in their home cage. On days 7 and 8, half of the adolescent and adult mice received 4 injections/day of 10 mg/kg cocaine whereas the remaining half (controls) received 4 injections/day of vehicle. The injections were administered 1 h apart, similarly to protocol used by Schlussman et al. (2005) and Unterwald et al. (1994). Locomotion was evaluated on day 1 and immediately after the last saline or cocaine administration of day 8, for 15 min. The mice remained in their home cage on days 9–13, before commencement of the CPP procedure, which employed 10 mg/kg cocaine and followed the protocol described in Exp. 1.

2.3. Statistical analyses

CPP scores in Experiment 1 were analyzed via a two-way mixed ANOVA [between factor: age (adolescent, adult); within-factor: time spent (s) on CS+ or CS− sections)]. In Experiments 2A, 2B and 3, CPP scores were analyzed via a three-way ANOVA that considered Age and Pretreatment (saline, cocaine) as between factors, and time spent (seconds) on CS+ or CS− sections as the within-subject measure.

Locomotion (distance travelled, cm) on the habituation day (Experiments 2A, 2B and 3) and on Day 1 of Experiment 3 was analyzed by a Student *t*-test (comparative factor between groups: age). Locomotor activity on day 1 and day 8 of the pre-treatment phase of Experiments 2A and 2B was analyzed via a three-way mixed ANOVA, that considered age (adolescent, adult) and pre-treatment (saline, cocaine) as between factors and Days (1, 8) as the repeated measure. A factorial ANOVA (between factors: Pretreatment and Age) was conducted on the locomotor activity registered at the end of the "binge" cocaine pretreatment (i.e., after the last cocaine injection on Day 8) of Experiment 3.

The locus of the significant interactions yielded by ANOVAs was analyzed through follow-up ANOVAs, Tukey's *post-hoc* tests or, when supported by a priori hypotheses, planned comparisons. Effect sizes are reported using the partial eta-squared (η^2p) and interpreted using the following guidelines [small (η^2p = 0.01–0.05), medium (η^2p = 0.06–0.13), and large (η^2p = ≥0.14)]. Data were analyzed by Statistica 7 (Tulsa, OK, USA). The significance level for all comparisons was set at $\alpha = 0.05$. All data are presented as mean ± standard error of the mean (S.E.M). Please note that, given the difficulty of representing significant main effects or significant interactions that span several groups, some of the significant, main effects or group-to-group differences may have not been depicted in the figures (via asterisk, pounds or other signs). Instead, a brief description of these significant effects and differences can be found in each figure legend.

3. Results

3.1. Preliminary experiment: Validation of the unbiased apparatus

It was possible that either the cocaine pretreatment or the age of conditioning was associated with a bias towards the steel mesh or bar sides of the compartments. Therefore, in a preliminary experiment adolescent and adult mice were pretreated with saline or 10 mg/kg cocaine (one daily injection for eight days). Each of the four groups was composed by 6–7 animals.

The mice were subsequently trained and tested in the CPP procedure described for Experiment 1, yet received saline throughout all six conditioning sessions. A three-way mixed ANOVA [between factors: age (adolescence, adulthood) and pretreatment (saline, cocaine); within-factor: time spent (s) on the grid or bar sections] analyzed time spent in each section. No significant main effects nor significant interactions were observed (all $p > 0.10$). The lack of a significant side preference or side × age or side × pretreatment interactions suggested that the apparatus was unbiased. Thus, for the following experiments, the CPP procedure only entailed interspersed cocaine/saline administration during conditioning sessions. In other words, "saline-only" groups were not employed in Experiments 1, 2 or 3.

3.2. Experiment 1. CPP in Adolescent and Adult mice – no pretreatment

The two-way mixed ANOVA revealed a significant main effect of CS [$F_{1,14} = 18.86$, $p < 0.001$; η^2p = 0.57], with an effect size that surpassed the threshold for being considered large. Both adolescents and adults exhibited a significant preference for the CS+ over the CS-, thus expressing cocaine-induced CPP. These results are depicted in Fig. 2.

3.3. Experiment 2. Cocaine-induced Behavioral Sensitization in Adolescent and Adult mice and CPP

Adolescent and adult mice exhibited similar locomotor activity during the habituation trial, either in Experiment 2A ($t = -0.41$, $p = 0.69$) or in Experiment 2B ($t = 0.23$, $p = 0.82$) (descriptive data not shown).

3.3.1. Experiment 2A

The ANOVA for locomotor activity on days 1 and 8 of the pretreatment phase yielded significant main effects of pretreatment and Days [$F_{1,32} = 75.43$, η^2p = 0.57 and $F_{1,32} = 48.83$, η^2p = 0.60; $p < 0.05$] and significant interactions between Days and Age [$F_{1,32} = 14.67$, η^2p = 0.31; $p < 0.001$] and between Days and Pretreatment [$F_{1,32} = 44.63$, η^2p = 0.58; $p < 0.001$]. The three-way interaction Day × Age × Pretreatment also achieved significance [$F_{1,32} = 7.18$, η^2p = 0.18; $p < 0.001$], with a large effect size. Tukey post hoc tests revealed that cocaine administration induced significant acute behavioral activation in day 1, i.e., the mice, adolescents or adults, given 10 mg/kg cocaine exhibited significantly greater locomotor activity than saline-treated counterparts. Cocaine-induced acute motor activity at day 1 did not differ between adolescent and adults. On the other hand, cocaine-induced locomotor activity was greater at Day 8 than at Day 1, both in adolescents and in adults. This result, which

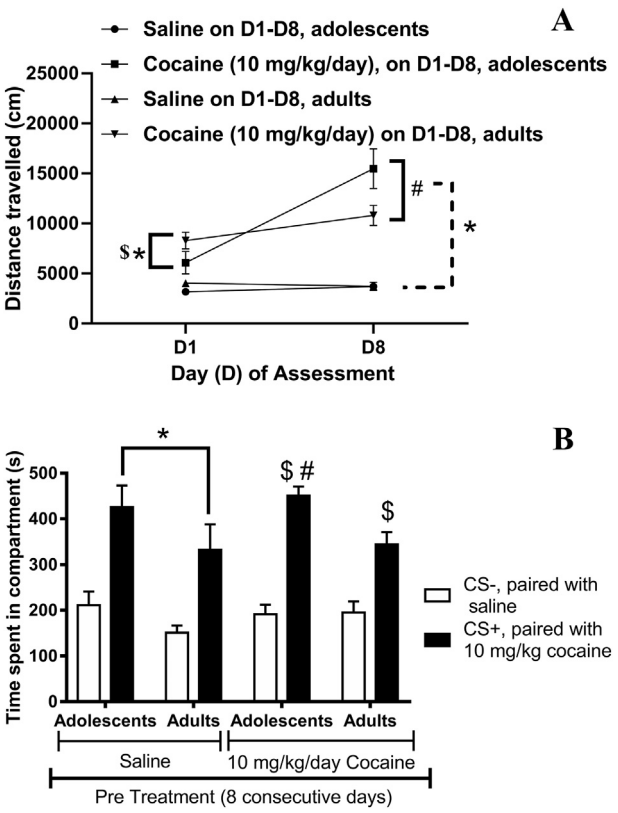

Fig. 3. (A) Distance travelled (mean ± S.E.M) of adolescent and adult mice in response to saline or cocaine (10 mg/kg). Mice were treated for 8 consecutive days and the locomotor activity was assessed for 15 min on days 1 and 8. The asterisk signs (*) indicates that, on day 1 or 8, adolescents or adults given cocaine exhibited significantly greater cocaine-induced activation than their controls. The $ sign indicates that cocaine induced-locomotion on Day 1 was significantly different from Day 8, in both adolescents and adults. The pound (#) sign indicates that cocaine induced-locomotion on Day 8 was significantly greater in adolescents than in adults. (B) Conditioned place preference to 10 mg/kg cocaine in adolescent and adult mice pretreated with daily 10 mg/kg cocaine or saline injections during 8 days. Data are shown as time spent (s) in the cocaine or saline-paired sides, which are referred to as excitatory and inhibitory conditioned stimulus (CS+ and CS−, respectively; mean ± S.E.M). The asterisk sign (*) indicates that, among saline-pretreated mice, both adolescents and adults exhibited a significant preference for the CS+ over the CS− (i.e., a significant main effect of CS), thus expressing cocaine-induced CPP. The $ sign indicates that either adolescent or adult mice pretreated with cocaine had a significant preference for the CS+ over the CS. The pound sign (#) indicates that, among cocaine-pretreated mice, adolescents not only had a significant preference for the CS+ over the CS− but also spent greater time on the cocaine-paired side (CS+) than adults did. Thus, cocaine-induced CPP after cocaine pre-exposure was significantly greater in adolescents than in adults.

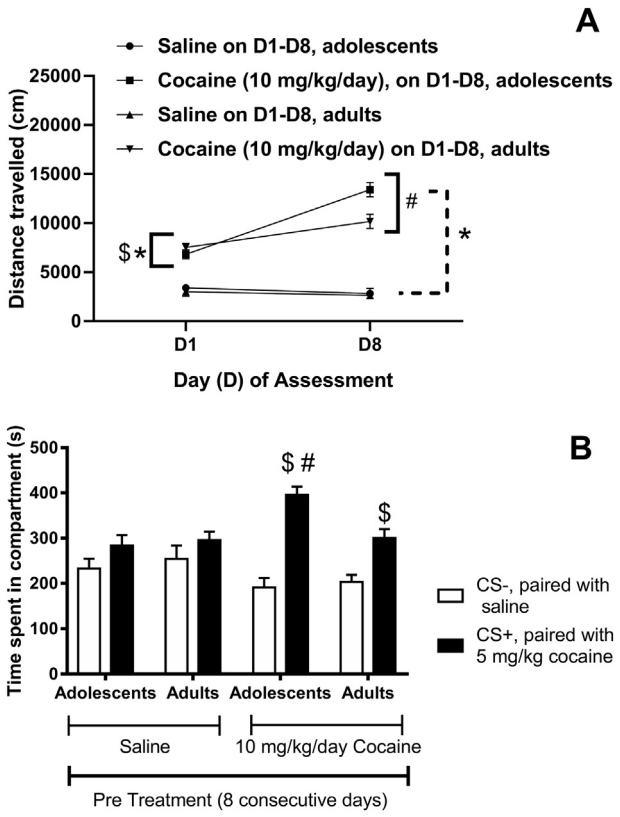

Fig. 4. (A) Distance travelled (mean ± S.E.M) of adolescent and adult mice in response to saline or cocaine (10 mg/kg). Mice were treated for 8 consecutive days and the locomotor activity was assessed for 15 min on days 1 and 8. The asterisk signs (*) indicates that, on day 1 or 8, adolescents or adults given cocaine exhibited significantly greater cocaine-induced activation than their controls. The $ sign indicates that cocaine induced-locomotion on Day 1 was significantly different from Day 8, in both adolescents and adults. The pound (#) sign indicates that cocaine induced-locomotion on Day 8 was significantly greater in adolescents than in adults. (B) Conditioned place preference to 5 mg/kg cocaine in adolescent and adult mice pretreated with daily 10 mg/kg cocaine or saline injections. Data are shown as time spent (s) in the cocaine or saline-paired sides, which are referred to as excitatory and inhibitory conditioned stimulus (CS+ and CS−, respectively; mean ± S.E.M). The $ sign indicates that either adolescent or adult mice pretreated with cocaine had a significant preference for the CS+ over the CS. The pound sign (#) indicates that, among cocaine-pretreated mice, adolescents not only had a significant preference for the CS+ over the CS− but also spent greater time on the cocaine-paired side (CS+) than adults did. Thus, cocaine-induced CPP after cocaine pre-exposure was significantly greater in adolescents than in adults.

indicates the development of cocaine-induced behavioral sensitization, was more robust in adolescents than in adults. Specifically, the Tukey *post-hoc* tests indicated greater cocaine-induced locomotor activity in adolescents vs. adults, on Day 8. These data are depicted in Fig. 3A.

The ANOVA for CPP scores (descriptive data in Fig. 3B) yielded significant main effects of Age [$F_{1,32} = 14.57$, $\eta^2p = 0.31$; $p < 0.001$] and CS [$F_{1,32} = 55.22$, $\eta^2p = 0.63$; $p < 0.001$]. The mice, both adolescents and adults, exhibited greater time spent on the CS+ than in the CS−, a result indicative of the development of cocaine-induced CPP. Guided by our a priori hypotheses we conducted separate two-way ANOVAs for each pre-treatment condition. The ANOVA for saline-pretreated groups revealed a significant main effect of Age [$F_{1,15} = 7.93$, $\eta^2p = 0.35$; $p < 0.05$] and a significant main effect of CS [$F_{1,15} = 15.65$, $\eta^2p = 0.51$; $p < 0.005$], yet the interaction between Age and CS did not reach significance. The *post-hoc* tests indicated that, regardless of age, all mice showed cocaine-induced CPP. In sharp contrast, the ANOVA conducted in cocaine-pretreated mice revealed significant main effects of Age [$F_{1,17} = 6.35$, $\eta^2p = 0.27$; $p < 0.05$] and CS [$F_{1,17} = 66.74$, $\eta^2p = 0.80$; $p < 0.001$], and a significant interaction between Age and CS which achieved a large effect size [$F_{1,17} = 4.93$, $\eta^2p = 0.22$; $p < 0.05$]. The post hoc tests revealed that cocaine-induced CPP was significantly greater in adolescents than in adults. Specifically, among cocaine-pretreated mice, adolescents and adults spent similar time on the CS− side, yet adolescents spent greater time on the cocaine-paired side (CS+) than adults did.

3.3.2. Experiment 2B

The three-way mixed ANOVA on locomotion activity at Day 1 and 8 revealed a similar pattern to that found in Experiment 2A (Fig. 4A). There were significant main effects of pretreatment, Age and Days [$F_{1,43} = 300.17$, $\eta^2p = 0.87$; $F_{1,43} = 4.32$, $\eta^2p = 0.09$ and $F_{1,43} = 36.52$, $\eta^2p = 0.46$, respectively; $p < 0.05$] and significant

interactions between Days and Age [$F_{1,43} = 7.53$, $\eta^2p = 0.15$; $p < 0.01$] and between Day and Pretreatment [$F_{1,43} = 55.66$, $\eta^2p = 0.56$; $p < 0.001$]. The interaction comprising Days, Age and Pretreatment was also significant and achieved a large effect size [$F_{1,43} = 9.23$, $\eta^2p = 0.18$; $p < 0.005$]. As shown in Fig. 4A and confirmed by the Tukey post hoc tests, the mice exhibited acute cocaine-induced behavioral activation and cocaine-induced behavioral sensitization. Similar to Experiment 2A, the acute activating effect of cocaine was similar in adolescents and adults, yet cocaine-induced behavioral sensitization was significantly greater in adolescents than in adults (i.e., the *post-hoc* tests revealed significantly greater locomotor activity at Day 8 in cocaine-treated adolescents vs. adult counterparts).

In this Experiment, the mice were conditioned with a lower (5 mg/kg) cocaine dose than that used in Experiment 2A (10 mg/kg). CPP scores yielded by this dose are depicted in Fig. 4B. The ANOVA indicated significant main effects of CS [$F_{1,43} = 28.89$, $\eta^2p = 0.41$; $p < 0.001$] and significant interactions between Pretreatment and Age [$F_{1,43} = 6.39$, $\eta^2p = 0.13$; $p < 0.05$], and between pretreatment and CS [$F_{1,43} = 8.40$, $\eta^2p = 0.16$; $p < 0.01$]. The *post-hoc* tests indicated that the pretreatment affected CPP scores in adolescents but not in adults, and that cocaine-induced CPP was observed after cocaine pre-exposure but not after repeated saline treatment. Guided by these interactions and by our a priori hypotheses we conducted separate two-way (Age × CS) ANOVAs for each pre-treatment condition. The ANOVA for saline-pretreated groups did not yield significant main effects or significant interactions. In sharp contrast, the ANOVA for cocaine-pretreated mice yielded significant main effects of Age [$F_{1,22} = 12.02$, $\eta^2p = 0.35$; $p < 0.005$] and CS [$F_{1,22} = 40.85$, $\eta^2p = 0.65$; $p < 0.001$], and a significant interaction between Age and CS, which surpassed the threshold for a large effect size [$F_{1,22} = 5.18$, $\eta^2p = 0.19$; $p < 0.001$]. The *post-hocs* indicated that both adolescents and adults spent greater time in the CS+ than in the CS−, a pattern suggestive of cocaine-induced CPP. The *post-hoc* tests also showed that time spent in the CS+ was greater in adolescent than in adult mice, a result indicating that cocaine-induced CPP was – among these cocaine-pretreated animals – greater in adolescent, compared to adult, mice.

3.4. Experiment 3. Cocaine "binge" in Adolescent and Adult mice and CPP

The locomotor activity of adolescents did not differ from that observed in adults, neither on habituation day ($t = -1.29$, $p = 0.20$, descriptive data not shown) nor on Day 1 ($t = -0.30$, $p = 0.76$). During these days all mice (adolescent and adult) received an injection of saline.

A factorial ANOVA (between factors: Pretreatment and Age) conducted on locomotor activity registered after the last cocaine injection on Day 8 yielded significant main effects of Pretreatment [$F_{1,59} = 57.08$, $\eta^2p = 0.50$; $p < 0.01$] and Age [$F_{1,59} = 11.7$, $\eta^2p = 0.17$; $p < 0.01$]. The interaction between these factors almost reached significance [$F_{1,59} = 3.47$, $\eta^2p = 0.06$; $p = 0.06$]. The *post-hoc* tests revealed similar locomotor activity in adolescent or adult mice that had been treated with saline, yet among mice treated with cocaine locomotion was significantly greater in adolescents than in adults. This pattern is depicted in Fig. 5A.

The ANOVA for CPP scores (descriptive data depicted at Fig. 5B) revealed a significant and large main effect of CS [$F_{1,59} = 31.79$, $\eta^2p = 0.35$; $p < 0.001$] and a borderline interaction between CS and Pretreatment [$F_{1,59} = 3.83$, $\eta^2p = 0.06$; $p = 0.055$]. All mice, regardless of age, spent significantly more time in the CS+ than in the CS- compartment. Moreover, the *post-hoc* tests suggested that this effect, indicative of cocaine-induced CPP, was restricted to those animals that had been given "binge" cocaine pre-exposure. Specifically, significantly greater CS+ vs CS− preference was found in animals treated with cocaine, but not in those given saline, during the pre-exposure treatment.

Guided by these results and by our a priori hypotheses we conducted separate follow-up ANOVAs for each age. The follow-up ANOVA for adolescent mice revealed a significant main effect of CS [$F_{1,28} = 12.47$, $\eta^2p = 0.31$; $p < 0.005$]. Both saline- and cocaine-pretreated adolescent mice exhibited significant preference for the CS+ vs. the CS−, thus indicating development of cocaine-induced CPP. The follow-up ANOVA for adult mice revealed a significant main effect of CS [$F_{1,31} = 21.39$, $\eta^2p = 0.41$; $p < 0.001$], and a significant interaction between CS and Pretreatment [$F_{1,31} = 6.23$, $\eta^2p = 0.18$; $p < 0.05$]. The effect size of this interaction was large and the *post-hoc* tests indicated that cocaine-pretreated, but not saline-pretreated, adult mice exhibited significant preference for the CS+ vs. the CS-. This indicates that, in adult mice, cocaine-induced CPP was only observed after cocaine pretreatment.

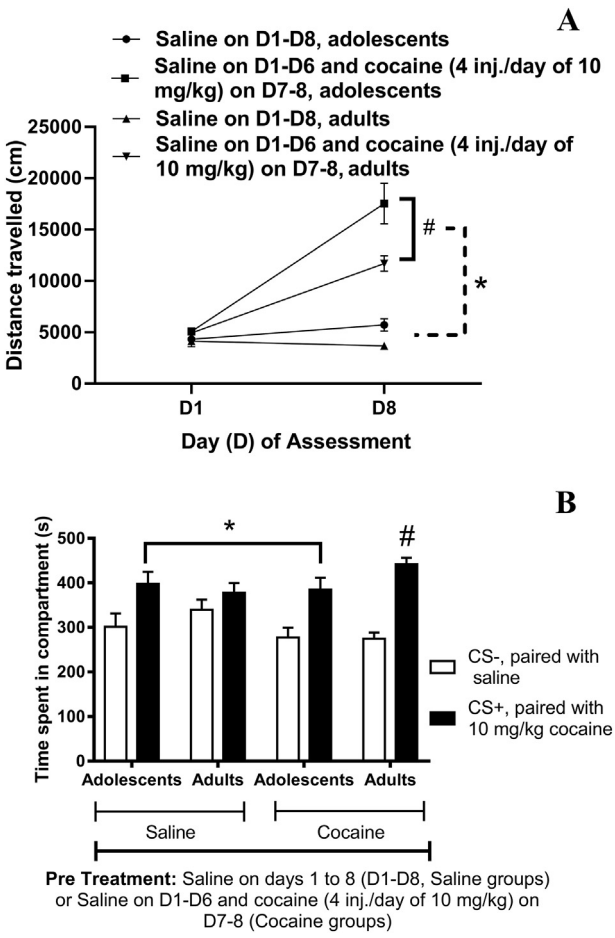

Fig. 5. (A) Distance travelled (mean ± S.E.M) of adolescent and adult mice in response to saline or cocaine (10 mg/kg). Mice were given daily saline injections from days 1 to 6, and on days 7 and 8, cocaine-treated mice received 4 injections/day of 10 mg/kg cocaine, while the controls received vehicle. The locomotor activity was assessed for 15 min on day 1 and for 15 min after the last injection of day 8. The asterisk (*) sign indicates a significant difference from same-age saline-treated mice, within the same treatment day. The pound (#) sign indicates a significant difference from cocaine-treated adult mice, within the same treatment day. (B) Conditioned place preference to 10 mg/kg cocaine in adolescent and adult mice pretreated with 4 doses of cocaine (10 mg/kg per dose) on 2 consecutive days ("binge" treatment) or saline injections. Data are shown as time spent (s) in the cocaine or saline-paired sides, which are referred to as excitatory and inhibitory conditioned stimulus (CS+ and CS−, respectively; mean ± S.E.M). The asterisk (*) sign indicates that both saline- and cocaine-pretreated adolescent mice exhibited significant preference for the CS + vs. the CS−, thus indicating development of cocaine-induced CPP. The pound (#) sign indicates that cocaine-pretreated, but not saline-pretreated, adult mice exhibited significant preference for the CS+ vs. the CS−.

4. Discussion

The present study indicates that adolescents are significantly more sensitive to both locomotor sensitization and rewarding effects of cocaine than adults. More in detail, regardless of the pretreatment received, daily or "binge" treatment, adolescents showed a higher locomotor activity at the end of the repeated treatment with cocaine, when compared to adults. The finding of greater cocaine-induced behavioral sensitization in adolescents vs. adults is consistent with previous reports in mice (Camarini et al., 2008) and with some, yet not all (see Collins and Izenwasser, 2002) reports in rats (Rowson et al., 2018). For instance, it has been recently shown that adolescent, but not adult, female Wistar rats sensitized to repeated and intermittent administrations of 15 mg/kg cocaine (Rowson et al., 2018) and Camarini et al. (2008) found that daily treatment for 9 days with 10 mg/kg cocaine increased distance travelled in the open field, both at the end of the daily treatment and in a challenge test conducted a week later, to a greater extent in adolescent than in adult DBA/2J mice.

The main new result of the present study was that adolescents and adults exhibited fairly similar cocaine-induced CPP after saline pretreatment, yet adolescents exhibited significantly greater cocaine-induced CPP than adults after daily cocaine pretreatment. Interestingly, the behavioral sensitization induced by the daily cocaine treatment was greater in adolescent than adult mice, which suggests the cocaine-induced behavioral sensitization might have influenced the cocaine-induced CPP.

We may not have detected age-differences in cocaine-induced CPP in "binge"-cocaine pretreated mice because of the short period of exposure to the drug. Most of the binge protocols involve several cycles of exposures to the drug, including escalation of dose, which lacked in this experiment (Bailey et al., 2005). The rationale for doing only 8 administrations across two days, in the present study, was to equate the number of cocaine administrations between both pretreatment protocols.

The findings, mainly derived from the results of Experiments 2A and 2B, have important implications. Before further discussing these implications it is important to address that, in Experiment 3, greater cocaine-induced CPP in adolescent vs. adults was found also in the no-preexposure, control condition. Unlike Experiments 2A/2B, in which the mice were given one injection a day, in Experiment 3 the mice were handled repeatedly during days and 7 and 8, and injected 4 times each day. It can be proposed that the stress associated with the repeated injections promoted a heightened expression of cocaine-induced CPP in adolescents, or blunted the sensitivity of adults. Indeed, adolescents and adults exhibit different, and sometimes opposite, drug-related effects when exposed to stress. For instance, a relatively brief exposure to restraint stress (120 min/day for five days) enhanced subsequent ethanol intake in adolescent, but reduced the intake of this drug in adult rats (Wille-Bille et al., 2017). Moreover, Song et al. (2007) found that chronic footshock promoted the acquisition of ethanol-induced CPP in adolescent, but had no effect in adult mice.

Previous studies conducted in adult rats have shown that behavioral sensitization to psychostimulants, such as amphetamine and cocaine (and also to morphine), increased the conditioned rewarding effects of these drugs, as measured by conditioned place preference (Lett, 1989; Shippenberg and Heidbreder, 1995). In the present study, such an amplified response in the adults was detected after using a relatively low dose of cocaine (5 mg/kg) during conditioning, which did not induce CPP in saline-pretreated adult mice. Similarly, adults did not show cocaine-induced CPP in Experiment 3, unless exposed to the "binge"-like cocaine pre-exposure treatment. An age-related difference in CPP with 10 mg/kg cocaine (Exp. 2A) was not detected in the saline pretreated animals, probably because this dose reached a ceiling effect.

Cocaine pretreatment, regardless of the regimen (daily or "binge", Exp. 2 and 3, respectively), sensitized the rewarding effects of cocaine in both adolescents and adults, yet this effect was significantly greater in adolescents. Chronic cocaine can induce tolerance or sensitization to its locomotor-activating effects, depending on a diversity of variables, such as the schedule of administration. In general, continuous administration leads to tolerance while intermittent administration results in behavioral sensitization (Reith et al., 1987). Moreover, experimenter-administered injections have been linked to development of sensitization in adult rats, which was in turn associated with increases in dopamine transporter density (DAT) in the caudate putamen nucleus (Collins and Izenwasser, 2002). By contrast, self-administration procedure usually leads to development of tolerance to both behavioral and DAT-inhibiting effects of cocaine (Ferris et al., 2011; Calipari et al., 2014; Siciliano et al., 2016). Repeated cocaine administration, using a binge-like schedule, has been shown to induce behavioral sensitization or lack of effect (tolerance or sensitization). Our study agrees with Unterwald et al. (1994), which showed development of locomotor sensitization during cocaine administration in rats, although lack of tolerance or sensitization to behavioral stereotypy has also been reported in mice (Schlussman et al., 2005). Overall, these data fit the incentive-sensitization theory (Robinson and Berridge, 2008; Berridge and Robinson, 2016). Under the tenets of this theory a response to a drug may become more salient or amplified with repeated exposure, and the neural changes underlying this behavioral sensitization are more evident after a period of discontinuation of the drug exposure. In our study, the conditioned responses to cocaine were investigated after a period of withdrawal of repeated cocaine administration. This suggests that there may be a specific mechanism dictated by sensitized neural mechanisms that regulate the distinctly higher cocaine rewarding effect in adolescents compared to adults, as found in the present study. Remarkably, Berridge and Robinson (2016) have proposed that incentive-sensitization theory is linked to neural circuit adaptations promoting excessive wanting of a reward, especially involving the dopaminergic circuitry.

The length of cocaine withdrawal after cocaine pretreatment is one of the factors that can change subsequent cocaine outcomes. While 1-day cocaine may mitigate subsequent cocaine-induced CPP, a longer withdrawal interval induces CPP, suggesting blunted cocaine rewarding effects in early withdrawal. In the present study, we used a 5-day withdrawal period to avoid any potential ceiling effect of a robust CPP expression, masking potential differences between adolescent and adult mice.

The impact of daily, intermittent cocaine exposure on dopamine levels in the NAc of adolescent rodents has been evaluated in other studies. Dopamine levels increase after acute or repeated cocaine administration in the NAc, regardless of age (Badanich et al., 2006; Camarini et al., 2008). However, after a cocaine pretreatment, the basal dopamine levels diminished in adolescent but not in adult rats (Badanich et al., 2006). Peaks in dopamine levels also differ between adolescent and adult rodents. In general, dopamine peaks induced by cocaine are faster in younger rodents as compared to older counterparts (Philpot and Kirstein, 1999; Badanich et al., 2006; Camarini et al., 2008). A "binge" cocaine regimen lowered basal and absolute levels of dopamine in the NAc compared to saline controls in adult mice, although the percentage relative to basal levels was higher in cocaine binge-treated mice than in cocaine acute-treated mice (Zhang et al., 2003). Due to scarcity of studies measuring extracellular dopamine after binge cocaine in adolescents, to our knowledge, it is speculative but tempting to suggest that ontogenetic differences in dopamine levels following specific regimens of cocaine may be determinant of the expression to cocaine reward.

The lack of dopamine, or any other biological measurements is certainly a limitation of the present study. Another serious limitation is that we did not employ female rats. An increasing number of reports indicate that males and females react differently to drugs, which has led to explicit suggestions of increasing sex representativeness in pre-clinical studied (McCullough et al., 2014). Another potential concern is that we employed a single cocaine dose to induce sensitization and only two

doses to induce CPP. It is conceivable that different outcomes might have been observed had we implemented a dose-response curve. It should be noted, however, that some have claimed that CPP is not particularly sensitive to changes in drug dose (Bardo and Bevins, 2000). Specifically, while some drugs (e.g., morphine or amphetamine) may yield graded dose-response curves, other substances (notably cocaine, Bardo et al., 1995) are more likely to produce all-or-none outcomes. Some of the findings may appear modest, yet it is important to remark that most of the relevant significant main effects or significant interactions described, including the age-related interactions, had large effect sizes.

It is interesting to remark that adolescent mice or rats are insensitive, or at least significantly less sensitive, to the rewarding effects to non-psychostimulant drugs, such as ethanol, when compared to adults. However, when adolescents are pre-exposed to different stimuli (e.g., stress, repeated ethanol injections, or even an enriched housing environment), they acquire conditioned preferences for ethanol (Song et al., 2007; Carrara-Nascimento et al., 2014; Pautassi et al., 2017). These studies, and ours, suggest that the adolescent stage – perhaps for being a period of relevant neural maturation and remodeling – renders the individual sensitive to several types of environmental stimuli, including the pharmacological effects of drugs.

5. Conclusion

The conditioned-place preference is thought to measure affective, rewarding effects of drugs of abuse (Prus et al., 2009), which play an important role in drug use. Therefore, the main implication of the present study is that the greater sensitivity of adolescents to cocaine-induced behavioral sensitization, which in turn promoted or at least was associated with greater sensitivity to cocaine-induced conditioned preference may represent another age-related difference in drug responsiveness (for review and references, see Doremus-Fitzwater and Spear, 2016) that put adolescents at risk for initiation and escalation into drug use.

Acknowledgements

Funding for this study was provided by grant #2012/10260-7, São Paulo Research Foundation, Brasil (FAPESP) to R. Camarini. This study was financed in part by the Coordination for the Improvement of Higher Education Personnel - Brasil (CAPES) - Finance Code 001. Camarini and Marcourakis are researcher fellows of the National Council for Scientific and Technological Development (CNPq) of Brazil. Ricardo Pautassi is a researcher of the National Council of Research (CONICET) of Argentina, and his work in this project was possible thanks to a scholarship from the Programa Professor Visitante do Exterior (PVE) from the Coordination for the Improvement of Higher Education Personnel (CAPES). L. Hoffmann was recipient of scholarship from FAPESP (#2017/11987-1). M. Rae was recipient of scholarships from CAPES and CNPq.

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