



Exposure of mice to MDPV during adolescence increases the psychostimulant, rewarding and reinforcing effects of cocaine in adulthood

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Abstract

BACKGROUND AND PURPOSE: 3,4-Methylenedioxypyrovalerone (MDPV) is a synthetic cathinone with powerful psychostimulant effects. It selectively inhibits dopamine transporter (DAT) being 10-50-fold more potent as DAT blocker than cocaine, pointing to a high abuse liability. The main objective of the present study was to assess the consequences of an early (adolescence) MDPV exposure on the psychostimulant, rewarding and reinforcing effects induced by cocaine in adult mice.

EXPERIMENTAL APPROACH: 21 days after MDPV pretreatment (1.5 mg·kg⁻¹, s.c., twice daily for 7 days), adult mice were tested to cocaine using locomotor activity, conditioned place preference and self-administration (SA) paradigms. In parallel, D₂ receptor density and the expression of c-Fos and ΔFosB in the striatum were determined.

KEY RESULTS: MDPV treatment enhanced psychostimulant and conditioning effects of cocaine. The acquisition of cocaine SA was unchanged in mice pretreated with MDPV, whereas the breaking point achieved under a progressive ratio program and reinstatement after extinction were higher in this group of mice. MDPV decreased D₂ receptors density but increased 3-fold ΔFosB expression. As expected, acute cocaine increased c-Fos expression but MDPV pretreatment negatively influenced its expression. ΔFosB accumulation declined during MDPV withdrawal, although it remained elevated in adult mice when tested for cocaine effects.

CONCLUSION AND IMPLICATIONS: MDPV exposure during adolescence induced long-lasting adaptive changes related to enhanced responsiveness to cocaine in the adult mice that seems to lead to a higher vulnerability to cocaine abuse. This particular behaviour correlated with increased expression of ΔFosB.

Highlights

- Mice were treated with MDPV during adolescence and tested to cocaine in adulthood.
- Mice exposed to MDPV showed higher locomotor responses to acute cocaine
- MDPV treatment increased reward in the CPP.
- Mice exposed to MDPV achieved higher breaking points under a progressive ratio program
- MDPV treatment decreased D₂ receptors and increased Δ FosB expression

Keywords: MDPV, cocaine, psychostimulant, conditioned place preference, self-administration, Δ FosB

Chemical compounds studied in this article:

- 3,4-methylenedioxypropylamphetamine (PubChem CID: 20111961)
- Cocaine (PubChem CID: 11302220)

Tables of links

TARGETS
GPCRs
D₂ receptor

LIGANDS
Cocaine

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson et al., 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander et al., 2015).

Introduction

In recent years, the illicit drug-market has changed remarkably and several new psychoactive substances (NPS), such as synthetic cathinones, have been identified. The popularity of synthetic cathinones has increased due to their ease of access, price and initial legal status (Bijlsma et al. 2015; 2014; Katselou et al. 2015). 3,4-Methylenedioxypropylvalerone (MDPV) is considered as one of the most abused synthetic cathinone and the main ingredient of “bath salts” (Johnson & Johnson 2014; Zuba & Byrska 2013; 2014), and able of triggering powerful psychostimulant effects (Baumann et al. 2013).

Cocaine is also a powerful psychostimulant and its repeated use could lead to a substance use disorder, and is often associated with other severe psychiatric and medical complications (Pozzi et al. 2008; Walsh et al. 2009). Despite of the irruption of the NPS in the illegal market, the illicit use of cocaine is still a persistent health problem worldwide (UNODC n.d.).

Similarly, MDPV shows cocaine-like properties, and selectively inhibits dopamine (DAT) and norepinephrine transporters, being 10-50-fold more potent as DAT blocker than cocaine (Simmler et al. 2013; Baumann et al. 2013). Furthermore, it shows rewarding and reinforcing effects (King et al. 2014), pointing to a similar abuse liability to that of cocaine.

Although MDPV use could be considered as a transient trend in drug abuse, it is still unknown the long-lasting consequence of its repeated consumption. Considering this, it is relevant to determine whether the use of MDPV will lead to an increased sensitivity and subsequent vulnerability to cocaine abuse. Adolescents and young adults use MDPV as a cheaper and easily alternative to classical psychostimulants; conversely, cocaine is a more widely and currently used psychostimulant, which is generally consumed in adulthood. Consequently, the main objective of the present study was to assess the consequences of early and repeated MDPV exposure on the cocaine psychostimulant responses in adult mice.

A repeated (7 days) moderate dose ($1.5 \text{ mg}\cdot\text{kg}^{-1}$, twice, daily) of MDPV eliciting hyperlocomotion was chosen for this study. After this MDPV schedule, adult mice were tested to cocaine responses. Hence, hyperlocomotion to an acute dose of cocaine was assayed as an indicative of its psychostimulant effect. In a second experiment, we

investigated whether that MDPV schedule could enhance the rewarding effects of cocaine, using the conditioned place preference (CPP). Next, we evaluated the reinforcing cocaine effects in the self-administration (SA) paradigm. Previous studies showed that D₂ receptor plays a role in the development and expression of behavioural sensitization (Thompson et al. 2010). Moreover, the expression of c-Fos and deltaFosB (Δ FosB) in the some brain areas is induced by acute or chronic exposure to virtually all drugs of abuse, and regulates their psychomotor and rewarding effects. Therefore, we have assessed the D₂ receptor density and the expression of c-Fos and Δ FosB in dorsal and ventral striatum.

Hence, we have performed behavioural procedures (hyperlocomotion, CPP and SA) and biochemical analyses that allowed us to characterize the cocaine abuse liability showed by mice pretreated with MDPV.

Methods

Subjects, ethical statement and drugs

Adolescence is a period of particular vulnerability to drug addiction (Cass et al. 2013), being a period of life in which different psychiatric disorders emerge (Paus et al. 2008). For this reason, we used for our study adolescent (PND 41-44) male Swiss CD-1 mice (Charles River, Spain), which are equivalent to the beginning of peri-adolescence (Spear & Brake 1983), and assayed cocaine effects after 21 days of withdrawal, when animals had reached adulthood. CD-1 mouse strain was selected for its optimal sensitivity to the reinforcing and psychostimulating effects of cocaine (McKerchar et al., 2005). Animals were housed four per cage (polycarbonate with wood-derived bedding) at 22±1°C under a 12-h light/dark cycle with free access to food and drinking water. The experimental protocols for the use of animals in this study were approved by the Animal Ethics Committee of the University of Barcelona and PRBB respectively, under the supervision of the Autonomic Government of Catalonia, following the guidelines of the European Community Council (2010/63/EU). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Pure racemic MDPV·HCl was synthesized and characterized in our laboratory as described (Novellas et al., 2015). Cocaine was provided by the Spanish National Institute of Toxicology. MDPV and cocaine solutions for injection were prepared in 0.9% NaCl (saline, pH=7.4) immediately before administration. Mouse monoclonal Δ FosB antibody and the protease inhibitor cocktail were purchased from Abcam (Cambridge, UK) and rabbit polyclonal c-Fos antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA). [3 H]raclopride was obtained from Perkin Elmer Life Sci. (Boston, MA, USA). Ketamine was from Rhône Merieux (Lyon, France). Xylazine, sulpiride and all buffer reagents (analytical grade) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Drug administration protocols and experimental design

In administration regime A, MDPV (1.5 mg·kg⁻¹) or saline (5 mg·kg⁻¹) was injected subcutaneously to mice twice daily (4 h apart) for 3 consecutive days and thereafter,

they were exposed to the cocaine-sensitization protocol (see Fig. 1A). In administration regime B, animals were also treated with MDPV ($1.5 \text{ mg}\cdot\text{kg}^{-1}$) or saline ($5 \text{ ml}\cdot\text{kg}^{-1}$) twice daily for 7 consecutive days and were then housed in their home cages until reaching adulthood (PND 69-72), when they were tested for cocaine-induced horizontal locomotor activity (HLA), CPP and SA experiments as described below (see Fig 1B). This dose is equivalent to a dose in humans of about 6 mg two times in a day (Reagan-Shaw et al. 2008), in which threshold dosages are around 1-5 mg and strong effects are shown with 10-25 mg (2014). Re-dosing is a typical pattern of consumption followed by consumers of such substances to avoid an unpleasant comedown (Ross et al. 2012).

Sensitization to the locomotor responses induced by a repeated cocaine administration

Locomotor activity was evaluated by placing the mice individually in the actimeter boxes ($24\times 24\times 24 \text{ cm}$) (LE881 IR, Panlab, Barcelona, Spain) provided with 14 axes (Y and X) in a low luminosity room. Animals were treated with saline ($n = 10$) or MDPV ($n = 11$), according to the administration regime A. The sensitization procedure consisted of 3 phases along 14 days: habituation, treatment and challenge. In the habituation phase (Days 6-7), mice were placed on the actimeter boxes for 30 min immediately after an i.p. saline injection. Treatment phase consisted of 5 sessions (Days 8-12). In each session, mice received daily an i.p. injection of cocaine ($7.5 \text{ mg}\cdot\text{kg}^{-1}$) immediately before being placed in the apparatus for 15 min. Finally, following a 7 days drug-free period after the last cocaine injection, mice were tested (Day 19 – challenge phase) with a cocaine injection ($7.5 \text{ mg}\cdot\text{kg}^{-1}$, i.p.) in the same actimeter boxes and the locomotor activity was registered for 15 min.

Cocaine-induced horizontal locomotor activity

HLA response induced by a single cocaine injection was video-monitored (Smart 3.0, Panlab, s.l.u., Barcelona, Spain) for 15 min in a black Plexiglass open field arena ($25\text{cm} \times 25\text{cm} \times 40\text{cm}$) under low-light conditions. Two days before testing, the animals were handled for 10 min and placed in the black arena for habituation. Two groups of animals ($n=12/\text{group}$) were pretreated in their home cage according to administration regime B and, after 21 days of withdrawal, when they reached adulthood (PND 69-72),

both groups were challenged with saline (5 ml·kg⁻¹, i.p.) (Day 27) and cocaine (7.5 mg·kg⁻¹ i.p.) (Day 28), and locomotor activity was registered.

Cocaine-induced conditioned place preference

The cocaine potential to induce approaching behaviours toward drug-related stimuli was determined using an unbiased place conditioning paradigm, as described by Soria et al. (2006). The apparatus consisted of two main conditioning compartments (30×29×35 cm) connected by a smaller, central compartment (Cibertec S.A., Madrid, Spain). The conditioning compartments were disposed with differences in visual and tactile cues. All the compartments were equipped with infrared emitter/detector pairs along the length of the box.

Saline- and MDPV-pretreated animals according to administration regime B ($n = 16$ /group) were subjected to the CPP procedure after a 21 days long drug-free period. During the preconditioning phase (day 28), initial unconditioned preference for the stimulus alternatives was determined. In this test, mice were placed in the central compartment and had free access to both compartments of the apparatus for 18 min. During the conditioning phase (days 29-34), mice received an i.p. injection of cocaine 10 mg·kg⁻¹ immediately before being placed into one of the two conditioning compartments for 20 min on days 29, 31 and 33. On the alternate days (30, 32 and 35), mice were placed in the other compartment for 20 min after being administered with a saline injection. Treatments were counterbalanced as much as possible between compartments. Control animals received saline every day. The preference test was conducted exactly as the preconditioning phase. A CPP score was calculated for each subject as the difference between times spent in the drug-paired and the saline-paired compartments during the pre-conditioning and the preference tests.

Cocaine operant self-administration

Cocaine self-administration acquisition

SA experiments were carried out in 8 mouse operant chambers (Model ENV-307A-CT, Med Associates, Inc. Cibertec S.A., Madrid, Spain), as previously described by (Soria et al. 2006). Saline- and MDPV-pretreated mice according to administration regime B ($n = 16$ /group) were trained for 2 h per day to nose-pokes in order to receive 1 mg/kg cocaine infusions on 10 consecutive days under a fixed ratio 1 (FR1).

Surgical implantation of the catheter into the jugular vein was performed following anaesthesia with a mixture of ketamine ($100 \text{ mg}\cdot\text{ml}^{-1}$) and xylazine ($20 \text{ mg}\cdot\text{kg}^{-1}$). The anaesthetics solution was injected in a volume of 0.15 ml per 10 g body weight, i.p. (Tourino et al. 2012; Soria et al. 2006). Mice were housed individually and allowed to recover for at least 3 days. During recovery, mice were treated daily with an analgesic (meloxicam $0.5 \text{ mg}\cdot\text{kg}^{-1}$, injected in a volume of 0.1 ml per 10 g body weight, i.p.) and an antibiotic solution (enrofloxacin $7.5 \text{ mg}\cdot\text{kg}^{-1}$, injected in a volume of 0.03 ml per 10 g body weight, i.p.). The home cages were placed upon thermal blankets to avoid post-anaesthesia hypothermia.

SA procedures started 21 days after the last day of administration regime B (Day 28). Active and inactive nosepokes were assigned randomly. Cocaine was delivered in a 20 μl injection for 2 s via a syringe mounted on a microinfusion pump (PHM-100A, Med-Associates, Georgia, VT, USA) connected to single-channel liquid swivel (375/25, Instech Lab, Plymouth Meeting, PA, USA) and the mouse's intravenous catheter. All FR1 sessions started with a cocaine priming infusion. When mice responded on the active hole, the stimulus lights (one located inside the nosepoke and the other above it) lit up for 4s and a cocaine infusion was delivered automatically. Each infusion was followed by a 30s time-out period in which a nosepoke on the active hole had no consequences. Mice were considered to have acquired stable SA behaviour when the following criteria were met in 2 consecutive FR1 sessions: *a*) 80% stability in reinforcements (the number of reinforces in each day deviated by $< 20\%$ from the mean number of reinforces in 2 consecutive days); *b*) $\geq 65\%$ of responses were received at the active hole; and *c*) a minimum of 5 responses in the active hole. After 10 days of training (Day 38), mice that achieved the acquisition criteria ($n = 9/\text{group}$) were moved to a progressive ratio session (PR). In the PR session (2h), the response requirement to earn an injection escalated throughout the following series: 1-2-3-5-12-18-27-40-60-90-135-200-300-450-675-1000.

Extinction and reinstatement

All the animals that reached the acquisition criteria were subjected to an extinction phase. The extinction procedure was adapted from Soria et al. (2008). Nosepokes in the active hole produced neither cocaine infusion nor stimulus light presentation. Extinction sessions (2h) were conducted once a day, 5 days per week until reaching the extinction

criteria. These criteria were achieved when mice made a mean number of responses in two consecutive extinction sessions of less than 40% of the responses performed during the last day of the cocaine-training phase. 24h after achieving the extinction criteria, mice underwent a cocaine-primed reinstatement session, as previously described (Soria et al. 2008). In order to recover the extinguished cocaine-seeking behaviour, saline- and MDPV-pretreated mice ($n = 9/\text{group}$) were confined to the operant chambers for 2h immediately after receiving an i.p. injection of cocaine 10 mg/kg. Nosepokes had no consequences in any of the holes.

Tissue sample preparations

Mice pretreated according to the administration regime B were sacrificed by cervical dislocation 24 h after the treatment (Day 8) or after saline/cocaine challenge (Day 28) for the analysis of ΔFosB expression and D_2 receptor density or 2h after saline/cocaine challenge for the determination of c-Fos expression. Including a saline challenge group let us to study also the long-term effects of MDPV treatment. Ventral (including NAcc), dorsal or the whole striatum, when appropriate, were quickly dissected out and stored at -80°C until use.

Tissues samples for Western blot analysis were processed as described (Pubill et al., 2013), with minor modifications. Briefly, for nuclear c-Fos Western blot analysis, dorsal striatum tissue samples were homogenized at 4°C in 400 μL of buffer (5 mM Tris-HCl, 320 mM sucrose) with the protease inhibitor cocktail. The homogenates were centrifuged at $1,000 \times g$ for 15 min at 4°C and the pellets were resuspended in buffer (Tris-HCl 50 mM) with the protease inhibitor cocktail.

For ΔFosB Western blot analysis, ventral striatum tissue samples were thawed and homogenized at 4°C in 20 volumes of lysis buffer (20mM Tris-HCl, pH=8, 1% NP40, 137mM NaCl, 10% glycerol, 2mM EDTA) with the protease inhibitor cocktail. The homogenates were shaken and rolled for 120 min at 4°C , and centrifuged at $15,000 \times g$ for 30 min at 4°C . Aliquots of resulting supernatants (total lysate) were stored at -80°C until use.

For [^3H]raclopride binding assays, crude membrane preparation from the whole striatum was prepared as described (Martínez-Clemente et al. 2012). Briefly, tissue samples were thawed and homogenized at 4°C in 20 volumes of buffer (5 mM Tris-HCl, 320 mM sucrose) with the protease inhibitor cocktail. The homogenates were

centrifuged at 15,000 x g for 30 min at 4°C. The pellets were resuspended in buffer and incubated at 37°C for 5 min to remove endogenous neurotransmitters. The protein samples were recentrifuged and the final pellets were resuspended in the appropriate buffer and stored at -80 °C until use. Protein content was determined using the Bio-Rad Protein Reagent (BioRad, Inc., Madrid, Spain).

Western blotting and immunodetection

Δ FosB and c-Fos Western blot analysis were performed as described by Buenrostro-Jáuregui et al. (2016) with minor modifications. Briefly, for each sample, 10 μ g or 20 μ g of protein was mixed with loading buffer (0.5M Tris-HCl, pH=6.8, 10% glycerol, 2% (w/v) SDS, 5% (v/v) 2- β -mercaptoethanol, 0.05% bromophenol blue), boiled for 5 min, and loaded onto a 10% acrylamide gel. Proteins were then transferred to polyvinylidene fluoride (PVDF) sheets (Immobilion-P, Millipore). PVDF membranes were blocked 1h at room temperature with 5% defatted milk in Tris-buffer plus 0.05% Tween-20 and incubated overnight at 4°C with mouse primary antibody anti-FosB (1:250) or rabbit primary antibody anti-c-Fos (1:200). After washing, membranes were incubated for 1h at room temperature with a peroxidase-conjugated (1:2500) antimouse or antirabbit (1:2000) IgG antibody. Immunoreactive protein was visualized using a chemoluminescence-based detection kit (Immobilion Western, Millipore) and a BioRad ChemiDoc XRS gel documentation system (BioRad, Inc., Madrid, Spain). Scanned blots were analyzed using BioRad Image Software and dot densities were expressed as a proportion of those taken from control. As a control for load, β -tubulin (1:2500) or GAPDH (1:5000) antibody was used.

D₂ receptor density

The density of D₂ receptor in striatal membranes was measured by [³H]raclopride binding assays as described (Martínez-Clemente et al. 2014). Assays were performed in tubes containing 2 nM [³H]raclopride and 50 μ g of membranes. Incubation was carried out at 25°C for 1 h in a Tris-HCl buffer. Sulpiride (300 μ M) was used to determine non-specific binding. The incubation was finished by rapid filtration under vacuum through Whatman GF/B glass fiber filters. The radioactivity in the filters was measured by liquid scintillation spectrometry.

Normalization, randomization, blinding and statistical analysis

Data from biochemical analyses were normalized with 100% defined as the mean of the technical replicates in the control group, and the SEM was normalized appropriately. Animals were randomly assigned to an experimental group. During the behavioural manipulations, researchers were not aware of the pretreatment that each animal previously received.

The data and statistical analysis in this study comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al. 2015). Data were expressed as mean \pm standard error of the mean (SEM). Differences between groups were compared using two-way analysis of variance (ANOVA) or Student's test for independent samples where appropriate. The α error probability was set at 0.05. Significant differences ($p < 0.05$) were analysed using the Tukey's post hoc test for multiple comparison measures (InVivoStat software package) only when no significant variance inhomogeneity was observed. The exact group size for the individual experiments is shown in the corresponding figure legends. To analyse the acquisition of cocaine self-administration during the 10-day training, extinction of the operant behaviour and reinstatement, a three-way ANOVA was calculated, with nosepoke (active or inactive), treatment with MDPV or saline, and day (or session) as factors of variation. Subsequent Tukey's post-hoc tests were calculated when required. Breaking point achieved at the end of the PR sessions was analysed using the non-parametric Mann-Whitney *U*-test.

Results

Sensitization to the hyperlocomotor responses induced by a repeated cocaine administration.

To evaluate the behavioural sensitization to cocaine 5 days after MDPV pretreatment (regime A), we assessed the hyperlocomotion induced by repeated cocaine administration (Fig 2). During the treatment phase (Fig 2 inset), cocaine induced an acute hyperlocomotion that increased with repeated daily exposure. Two-way ANOVA revealed effect of the *day* ($F_{4,76} = 8.791, p < 0.05$) and the *pretreatment* factor ($F_{1,19} = 6.025, p < 0.05$) without *interaction* between factors.

When analysing the differences between the first day of the treatment (Day 8) and the cocaine challenge (Day 19) (Fig 2), two-way ANOVA also demonstrated a significant effect of the *day* ($F_{1,19} = 9.909, p < 0.05$) and the *pretreatment* factor ($F_{1,19} = 9.504, p < 0.05$) without the *interaction between pretreatment and day*.

Interestingly, mice pretreated with MDPV showed a significant increase in the hyperlocomotor activity induced by cocaine on both, the first day of cocaine administration (Day 8) and the challenge day (Day 19) compared to saline-pretreated mice. Overall, these results indicate that pre-exposure to MDPV is able to enhance the response to cocaine, without affecting the acquisition of sensitization.

Cocaine-induced horizontal locomotor activity

HLA was monitored for 15 min after a single saline ($5 \text{ mg}\cdot\text{kg}^{-1}$) or cocaine (7.5 mg/kg) i.p. injection to mice previously pretreated with saline or MDPV according to administration regime B (Fig 3). This dose of cocaine elicited a significant psychostimulant effect in saline- ($p < 0.05$) and MDPV-pretreated animals ($p < 0.05$), although a higher hyperlocomotor response was observed in the group pretreated with the psychostimulant ($p < 0.05$). Two-way ANOVA revealed significant effect of the cocaine challenge ($F_{1,44} = 53.60, p < 0.05$) and *interaction between challenge and pretreatment* ($F_{1,44} = 4.58, p < 0.05$), but no effect of the *pretreatment* factor was found. This means that MDPV pre-treatment, by itself, is not capable of favouring an increase in locomotor activity when mice are challenged with saline but only with cocaine.

Cocaine-induced conditioned place preference

As shown in Figure 4A, saline- and MDPV-pretreated mice (regime B) presented no preference for any of the compartments (Fig 4A). Only one animal from the saline-pretreated group was withdrawn from the study due to an initial preference for one of the compartments (> 65% of the total session time spent in one compartment). The repeated administration of cocaine (10 mg·kg⁻¹, i.p.) produced a preference for the cocaine-paired compartment (Fig 4B). Two-way ANOVA demonstrated a significant effect of the *pretreatment* factor ($F_{1,58} = 15.0$, $p < 0.05$), *treatment* effect ($F_{1,58} = 114.4$, $p < 0.05$), and *interaction between treatment and pretreatment* ($F_{1,58} = 7.075$, $p < 0.05$). Accordingly, MDPV-pretreated mice showed an increased expression of the cocaine-induced CPP compared to the saline-pretreated group ($p < 0.05$) (Fig 4B).

Self-administration reinforced with cocaine

Acquisition of cocaine self-administration

The effect of the MDPV pretreatment (regime B) on the reinforcing properties of cocaine was evaluated in the SA procedure. First, both saline- and MDPV pretreated mice were trained to self-administer cocaine (1 mg·kg⁻¹ per infusion) during 10 days under a FR1 schedule of reinforcement. The criteria acquisition rates were equal for both groups: 56%. All the statistical analyses and data representations were performed only with mice that acquired the learning criteria. Three-way ANOVA (*pretreatment* × *day of training* × *nosepoke*) of infusions on both nosepokes given along the FR1 sessions yielded no significant effects of the *pretreatment* factor ($F_{1,9} = 0.56$, $p > 0.05$) (Fig 5A), thus indicating a lack of effect of MDPV pretreatment on the acquisition of cocaine SA behaviour. The operant procedure produced the acquisition of cocaine self-administration as revealed by the *day of training* factor ($F_{9,9} = 2.138$, $p < 0.05$). Animals were able to discriminate between active and inactive nosepokes as indicated by the *nosepoke* factor ($F_{1,9} = 108.8$, $p < 0.05$). An interaction between *day of training* and *nosepoke* factors was also found ($F_{9,9} = 4.45$, $p < 0.05$). No other interactions were found. A significant effect of MDPV pretreatment was revealed in the breaking point achieved on the PR session (Mann-Whitney $U = 15.5$, $p < 0.05$) (Fig 5B), indicating that MDPV pretreatment increased the value of cocaine as reinforcer.

Extinction and cocaine-primed reinstatement

Three-way ANOVA (*pretreatment* x *extinction day* x *nosepokes*) showed that groups extinguished cocaine SA behaviour among the extinction sessions, as the factor *extinction day* had a significant effect ($F_{11,11} = 14.78, p < 0.05$) (Figure 6A). However, the factor *pretreatment* had no significant influence over nosepokes along the extinction phase ($F_{1,11} = 0.057, p > 0.05$). Animals were able to discriminate between *nosepokes* ($F_{1,11} = 284.6, p < 0.05$) but, as the interaction between this factor and *pretreatment* revealed ($F_{1,11} = 16, p < 0.05$), both groups stopped to discriminate between nosepokes in different days. Subsequent post-hoc analyses indicated that MDPV-pretreated animals discriminated between nosepokes until day 7 (Tukey, $p > 0.05$), whereas SAL-pretreated animals, discriminated until day 6 (Tukey, $p > 0.05$). A significant interaction between *extinction day* and *nosepokes* ($F_{11,11} = 7,645, p < 0.05$) was found. No other interactions were found.

After extinction of cocaine SA, mice that acquired the extinction criteria were submitted to a cocaine-primed (10 mg/kg), drug-induced reinstatement session (Fig 6B). Three-way ANOVA (*pretreatment* x *session* x *nosepokes*) showed a significant effect of *day* ($F_{3,3} = 8.263, p < 0.05$), and *nosepokes* ($F_{1,3} = 116.9, p < 0.05$), without *pretreatment effect* ($F_{1,3} = 0.477, p < 0.05$). Significant interactions were found between *days* and *nosepokes* ($F_{3,3} = 6.128, p < 0.05$), and *pretreatment* and *nosepokes* ($F_{1,3} = 3.979, p < 0.05$). Post-hoc analyses indicated that both groups of pretreatments did not differ in the number of active nosepokes in the reinstatement session. However, MDPV-pretreated mice, unlike SAL-pretreated, reinstated their previously extinguished cocaine-SA behaviour after being drug-primed (cocaine 10 mg/kg). Therefore, SAL-pretreated mice did not reinstate previous extinguished cocaine-SA behaviour (Tukey, $p > 0.05$). Interestingly, MDPV-pretreated mice achieved a significant difference between active nosepokes in reinstatement session versus the last day of extinction (Tukey, $p < 0.05$) (Fig 6B).

c-Fos and Δ FosB expression

Since we had seen that the pretreatment with MDPV modified the response to cocaine, we wanted to determine how the MDPV pretreatment influenced the expression of certain factors that are known to be associated to cocaine effects, such as c-Fos and Δ FosB. Expression of the transcription factor Δ FosB in the brain also controls the responsiveness of an animal to the rewarding and locomotor-activating

effects of cocaine. Following regime B, we investigated the expression of Δ FosB 24 h after saline or MDPV pretreatment (Day 8) (Fig 7A) and after saline or cocaine challenge (Day 29) (Fig 7B) and also c-Fos 2 h after saline or cocaine challenge (Day 28) (Fig 7C).

As shown in Figure 7A, mice pretreated with MDPV (regime B) showed a Δ FosB expression by 300% ($p < 0.05$) compared to saline treated mice, when measured 24 h after finishing the treatment. At day 29, 24 h after receiving the saline/cocaine challenge, the statistical analysis showed a significant effect of the *pretreatment factor* ($F_{1,18} = 5.976$, $p < 0.05$, see Fig 7B). Therefore, and although the high Δ FosB expression declined during withdrawal, this factor still remained apparently elevated (132% animals pretreated with MDPV and challenged with saline). In addition, cocaine-challenge also produced an increase in Δ FosB expression compared to saline injection ($F_{1,18} = 4.699$, $p < 0.05$).

The c-Fos expression might be used as a marker for neuronal activity. In this context, two-way ANOVA revealed effect of the *challenge factor* ($F_{1,18} = 54.21$, $p < 0.05$) (Fig 7C). Cocaine induced a significant increase in c-Fos expression in both groups, saline- and MDPV-pretreated mice (320%, $p < 0.05$ and 220%, $p < 0.05$, respectively). Accordingly, statistical analysis also evidenced the effect of the *pretreatment factor* ($F_{1,18} = 8.497$, $p < 0.05$), thus, MDPV pretreatment reduces the cocaine-induced expression of this marker ($p < 0.05$). Moreover, after saline-challenge, MDPV-pretreated mice showed a decrease (52 %) in c-Fos expression compared to SAL-pretreated mice, although such difference did not reach statistical significance. Therefore, it seems that when increasing Δ FosB, c-Fos expression was reduced.

D₂ receptor density

To determine the involvement of striatal DA D₂ receptors, we measured [³H]raclopride binding in this brain area 24 h after treatment (Fig 8A) or saline- and cocaine-challenge (Fig 8B). 24 hours after MDPV pretreatment a significant decrease in D₂ receptors density was evidenced ($t_{18} = 2.613$; $p < 0.05$). However, 24 h after saline or cocaine challenge, two-way ANOVA only revealed differences of the *challenge factor* ($F_{1,30} = 4.179$, $p < 0.05$). However, post-hoc analysis did not evidence statistical significance.

Discussion

Cocaine abuse represents a heavy burden of disease in many countries, becoming a global problem. Any factor that increases the vulnerability to cocaine abuse must be carefully evaluated. In the present study, we demonstrate that MDPV enhances the responsiveness to cocaine in all tested aspects.

In a first study (regime A) we investigated the influence of a short-time MDPV exposure in the behavioural sensitization induced by cocaine. MDPV produced a gradual increase of cocaine hyperlocomotor effects and a high reactivity to a cocaine challenge after 7 days of cocaine withdrawal. However, mice exposed to MDPV showed a higher response to cocaine when they received this psychostimulant for the first time, and this increased hyperlocomotor effect was maintained throughout all the sensitization procedure.

In a second set of experiments, we carried out a repeated treatment (regime B) with a moderate dose of MDPV, in adolescent mice, and we investigated the influence on the acute effects of cocaine in adulthood, including motor responses, rewarding effects in the CPP, and the cocaine-induced reinforcing effects in the SA paradigm.

We found that mice exposed to MDPV were more reactive to cocaine but not to saline injection. So, this observed response was independent on the environmental context because MDPV treatment was administered in the home cages. Accordingly, a MDPV treatment with low doses (0.3 or 0.5 mg·kg⁻¹) during 5-7 days increased responsiveness to acute cocaine (5 -10 mg·kg⁻¹) after a 10-11 days drug-free period (Berquist et al. 2016; Buenrostro-Jáuregui et al. 2016).

We also sought to find out if MDPV exposure resulted in increased responsiveness to cocaine in reward and reinforcing effects in mice. Our results revealed that repeated administration of MDPV in adolescent mice led to a long-term increase of cocaine-induced behavioural adaptations, related to its abuse potential, when tested into adulthood in the CPP. This paradigm is used to reflect the degree by which drugs of abuse establish approaching behaviours toward drug-related stimuli (Bardo & Bevins 2000). The observed increase in the robustness of the conditioned responses in the CPP expression seems to reflect an increase in the abuse liability of cocaine after exposure to MDPV. Thus, our findings demonstrate that MDPV exposure during adolescence can effectively potentiate cocaine abuse liability in adulthood by sensitizing the neural circuitry underlying associations between cocaine and its related stimuli. To our

knowledge, this is the first study that evaluates the long-term effects of a MDPV treatment on cocaine-induced CPP. However, recent evidences have demonstrated similar effects of MDPV pretreatment on the development of psychostimulants-induced reinforcing effects. For instance, MDPV ($1.8 \text{ mg}\cdot\text{kg}^{-1}$ during 5 days) attenuated the taste avoidance induced by cocaine ($18 \text{ mg}\cdot\text{kg}^{-1}$), but not those induced by LiCl, in rats (Woloshchuk et al. 2016). As above mentioned, MDPV also sensitized to cocaine locomotor response after a drug washout period (Berquist et al. 2016; Buenrostro-Jáuregui et al. 2016). Moreover, an intermittent, repeated exposure to MDPV ($1 \text{ mg}\cdot\text{kg}^{-1}$ during 5 days) produced sensitization to methamphetamine challenge ($0.5 \text{ mg}\cdot\text{kg}^{-1}$) in rats (Watterson et al. 2016). Nevertheless, any study has yet evaluated global MDPV effects on cocaine-induced abuse liability, in particular, evaluating its effects on cocaine-induced reinforcing effects in the self-administration paradigm as we present in the present study. In fact, mice repeatedly exposed to MDPV during adolescence displayed higher effort to obtain a cocaine infusion in a PR schedule of reinforcement when tested in adulthood. PR schedule of reinforcement requires behaviours specially linked to motivational functions, such as instrumental learning, execution of efforts and sustained engagement (Randall et al. 2012). The lack of differences between mice exposed to MDPV or to saline during the acquisition phase of SA could be attributed to the fact that this phase was performed under a FR1 schedule of reinforcement, that seems not to be appropriated to determine the reinforcement efficiency (Richardson & Roberts 1996). In this sense, it is a common feature of rodent cocaine self-administration studies to find a certain degree of inconsistency between results on FR1, PR schedules and reinstatement sessions depending on the experimental conditions (España et al. 2011; Homberg et al. 2002; Morgan et al. 2005; Zhang et al. 2005).

In addition, MDPV-pretreated mice delayed extinction after acquisition of the operant behaviour to cocaine and reinstated after a cocaine priming injection. Reinstatement of SA behaviour reflects the reinforcing properties of drugs and its pharmacological manipulations (Shaham et al. 2003; Soria et al. 2008; Bossert et al. 2013). We have also found a drug-seeking reinstatement behaviour in mice previously exposed to MDPV, suggesting a higher level of craving than in control mice. Thus, we suggest that MDPV exposure during adolescence will invigorate execution of effort to obtain cocaine in the adulthood, and this effect leads to an enhanced vulnerability to reinstate cocaine SA behaviour once extinguished.

These results, lead us to hypothesize that MDPV administration induces long-lasting adaptive changes, leading to a greater cocaine responses. Δ FosB, c-Fos and D_2 receptors are factors involved in the acute and long lasting effects of cocaine (Larson et al. 2010; Lee et al. 2013). For instance, studies performed using positron emission topography have consistently shown that drug abuse is accompanied by a decrease in striatal D_2 receptor availability. Furthermore, studies in drug naive non-human primates suggest that D_2 receptor availability is predictive of drug seeking behaviour (Nader et al. 2006). In the present study, we have found that repeated MDPV exposure was associated to a decrease in striatal density of D_2 receptor, probably reflecting a neuroadaptive effect in response to MDPV dopaminergic stimulation, but this effect is only transient since did not remain after 21 days of withdrawal, when D_2 receptor population was observed to return to initial values. Additionally, we determined D_2 receptor levels after the challenge of cocaine. As expected, an acute dose of this drug resulted to be a factor that modulated D_2 receptor although without influence of MDPV pre-exposure.

There is growing evidence for an important role of Δ FosB in animal models of drug addiction (Nestler 2008). The Fos family of proteins is rapidly and transiently induced in the striatum after acute administration of several drugs of abuse (Graybiel et al. 1990; Hope et al. 1992; Young et al. 1991). Although most of them are highly unstable, Δ FosB is progressively accumulated after repeated drug exposure. This accumulation has been linked to cocaine-induced reward, locomotor sensitization, and self-administration behaviour (Colby et al. 2003; Kelz et al. 1999; McClung et al. 2004), which together suggest a role in the neural mechanisms involved in transitioning between recreational use and abuse phenomenon. In the present study, an increased expression of Δ FosB was found 24h after finishing MDPV exposure, and levels of this factor remained raised throughout the period of abstinence. Therefore, it is reasonable to propose that the increased level of this transcription factor responded to MDPV treatment, leading to an increased responsiveness to cocaine effects. Changes in Δ FosB seem to extend the regulation of drug sensitivity towards more complex behaviours (Colby et al., 2003). Thus, accordingly with our findings, mice overexpressing Δ FosB work harder to self-administer cocaine in PR schedule of reinforcement in SA assays, suggesting that Δ FosB may sensitize animals to the incentive motivational properties of cocaine and thereby leading to a propensity for relapse after drug withdrawal.

Numerous putative targets for Δ FosB have been identified in brain, and some of these target genes have been related to the cellular and behavioural effects of this transcription factor (McClung et al. 2004). One of these target genes is *c-Fos*. Induction of c-Fos protein is considered an early marker of neural activation, and it is also important for behavioural responses to cocaine (Zhang et al. 2006). This factor is dramatically activated by acute psychostimulants administration, but only weakly after repeated exposure (Persico et al. 1993; Hope et al. 1992), when levels of Δ FosB are high. In this sense, Renthal et al (2008) demonstrated that Δ FosB mediates epigenetic desensitization of the *c-Fos* gene after chronic exposure to a psychostimulant. In our study, a significant increase of c-Fos expression appeared short time after cocaine challenge that undoubtedly reflects neuronal activation by the drug. However, this protein expression was related to MDPV pretreatment thus, levels of c-Fos seemed to be inversely associated to MDPV pretreatment, when Δ FosB is significantly expressed. Results demonstrate a negative association between both factors, in accordance with (Renthal et al. 2008).

To sum up, MDPV increased the most of behaviour responses related to cocaine effects, including locomotor sensitization, reward and the strength of cocaine as reinforcer in a SA procedure. It is noteworthy that MDPV increased capability to reinstate cocaine SA behaviour once extinguished, presumably indicating increased of craving after MDPV exposure during adolescence. These behaviour alterations were associated to an accumulation of Δ FosB, a sustained molecular switch for cocaine addiction, providing a possible mechanism by which molecular changes induced by MDPV can persist for weeks after withdrawal and, supporting the deleterious effects of MDPV on cocaine abuse liability. **Therefore, these results suggest that consumption of MDPV during adolescence induces long-lasting adaptive changes leading to a higher response to cocaine in the adulthood, predisposing to a higher vulnerability to abuse of this drug. From a clinical point of view, this feature represents a basic step to provide new knowledge about factors involved in the vulnerability to cocaine addiction.**

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Author contributions

OV and EE were responsible for the study concept and design. RLA, MAL and LDC carried out the experimental studies. JC participated in the data analysis and DP in immunoassays methodology. RLA and MAL drafted the manuscript. JC and DP participated in the interpretation of findings. OV and EE provided a critical revision of the manuscript for intellectual content. All authors critically reviewed the content and approved the final version for publication.

Conflict of interest:

The authors declare that they have no conflict of interest.

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For Peer Review

Legends

Figure 1. Drug exposure protocol and experimental design. In administration regime A, animals were treated with MDPV or saline twice daily for 3 days and, 5 days after, they were exposed to a cocaine sensitization protocol. In administration regime B animals were treated with MDPV or saline twice daily for 7 days and, 21 days after, cocaine-induced HLA, CPP and SA experiments were performed.

Figure 2. Effect of MDPV treatment (regime A) on cocaine-induced sensitization. Bars represent mean (\pm SEM) of cumulative breaks/animal after cocaine ($7.5 \text{ mg}\cdot\text{kg}^{-1}$, i.p.) injection on the first day of the sensitization protocol (Day 8) and the challenge day (Day 19) (saline pretreated group, $n = 10$ and MDPV pretreated group, $n = 11$). $*p < 0.05$ compared to the saline-pretreated group. **Inset:** Cumulative breaks/animal during the treatment phase of the cocaine-induced sensitization protocol in saline- and MDPV-pretreated mice.

Figure 3. Effect of MDPV treatment (regime B) on cocaine-induced HLA. Bars represent mean (\pm SEM) of the distance travelled after a single saline ($5 \text{ ml}\cdot\text{kg}^{-1}$, i.p.) or cocaine ($7.5 \text{ mg}\cdot\text{kg}^{-1}$, i.p.) injection 21 days after treatment ($n = 12/\text{group}$). $*p < 0.05$ compared to the corresponding saline injection. $\#p < 0.05$ compared to the saline-pretreated group.

Figure 4. Effect of MDPV treatment (regime B) on cocaine-induced CPP. Bars represent mean (\pm SEM) of CPP score (see methods for details) during preconditioning ($n = 16/\text{group}$) (Panel A) and preference test ($n = 16/\text{group}$) (Panel B). $*p < 0.05$ compared to saline-pretreated group, $\#p < 0.05$ compared to its respective control group (conditioned with saline).

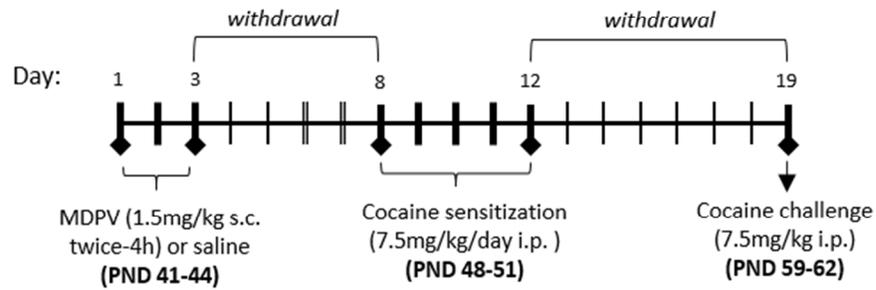
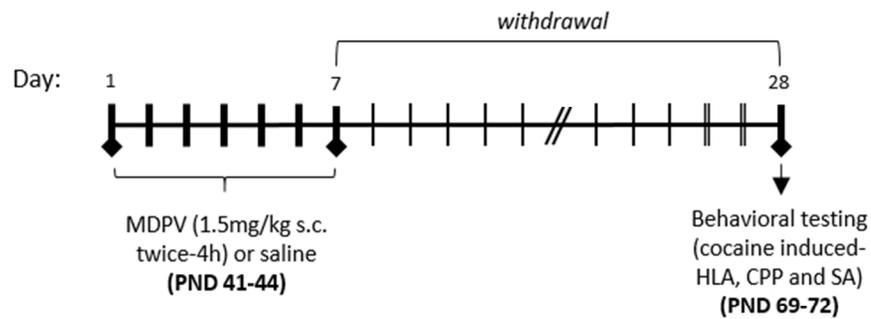
Figure 5. (Panel A) Effect of MDPV treatment (regime B) on the acquisition of cocaine- SA behaviour for 2 h daily FR1 sessions during 10 days of training ($n = 9/\text{group}$). **(Panel B)** Effect of MDPV treatment (regime B) on self-administration behaviour breaking point in a PR schedule of reinforcement ($n = 9/\text{group}$). Data

represent the mean (\pm SEM) of the last ratio achieved in the PR session during 2h. * $p < 0.05$ compared to the saline-pretreated group.

Figure 6. (Panel A) Extinction of cocaine self-administration behaviour for 2 h daily sessions during 12 days ($n = 9/\text{group}$). * $p < 0.05$ compared to the inactive nose-pokes performed by the same group in the same day of extinction phase. **(Panel B)** Cocaine-primed, drug-induced reinstatement of cocaine self-administration behaviour ($n = 9/\text{group}$). The different phases of the experiment are showed in the X axis. * $p < 0.05$ compared to the active nose-pokes performed by the same group in the last day of extinction. # $p < 0.05$ compared to the active nose-pokes of the same group in the same experimental phase. Data represent the mean of nose-pokes \pm SEM in the active and inactive holes.

Figure 7. Effect of MDPV treatment (regime B) in Δ FosB expression 24 h after treatment ($n = 6/\text{group}$) **(Panel A)** or saline- and cocaine-challenge (saline pretreated group, $n = 5/\text{group}$ and MDPV pretreated group, $n = 6/\text{group}$) **(Panel B)**. * $p < 0.05$ compared to the saline-pretreated group. \$ $p < 0.05$, two-way ANOVA *pretreatment factor* ($F_{1,18} = 5.976$). **(Panel C)** Effect of MDPV treatment (regime B) in c-Fos expression 2 h after saline- and cocaine challenge (saline pretreated group, $n = 5/\text{group}$ and MDPV pretreated group, $n = 6/\text{group}$). * $p < 0.05$ compared to its corresponding saline-challenge group. # $p < 0.05$ compared to the saline-pretreated group. Results are expressed as mean \pm SEM.

Figure 8. Effect of MDPV treatment (regime B) in D_2 receptor density 24 h after treatment (saline pretreated group, $n = 9$ and MDPV pretreated group, $n = 11$). **(Panel A)**, and after saline- or cocaine-challenge (saline pretreated, $n = 8/\text{group}$ and MDPV pretreated, $n = 9/\text{group}$) **(Panel B)**. D_2 receptor density was measured as [^3H]raclopride bound in the mouse striatum. Results are expressed as mean \pm SEM. * $p < 0.05$ compared to the saline-pretreated group.

A**B**

|| Habituation days
(saline injection)

Figure 1

117x104mm (150 x 150 DPI)

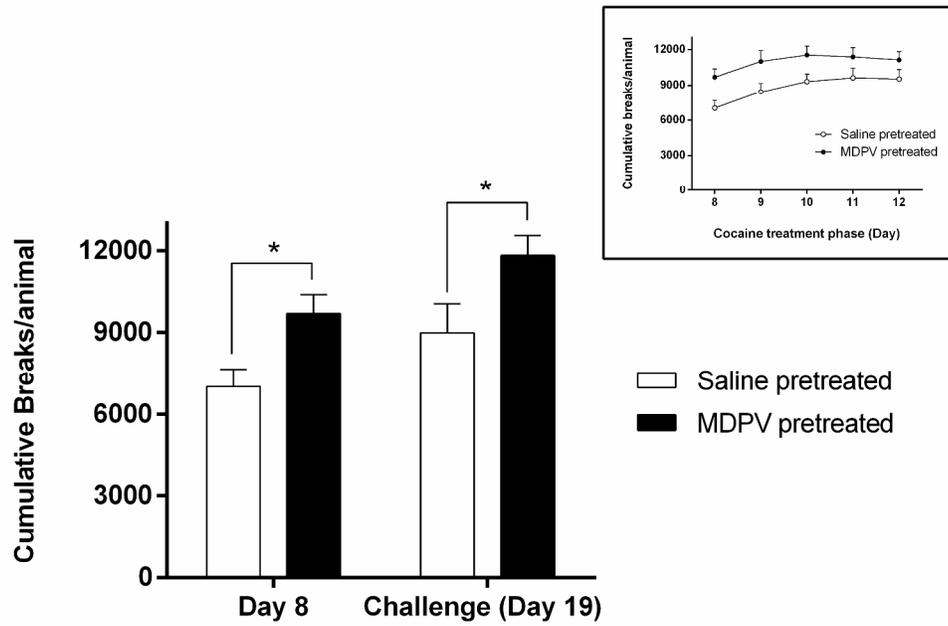


Figure 2

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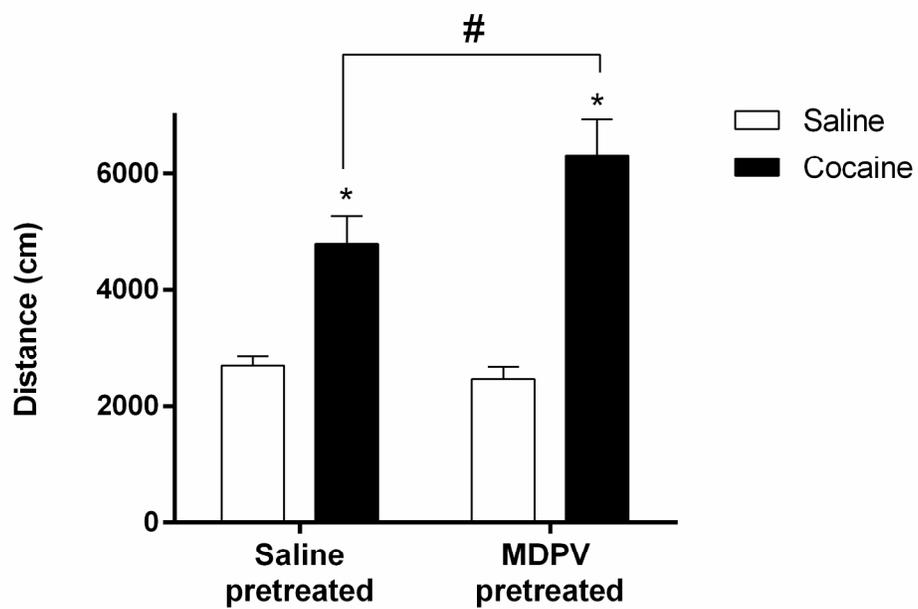


Figure 3

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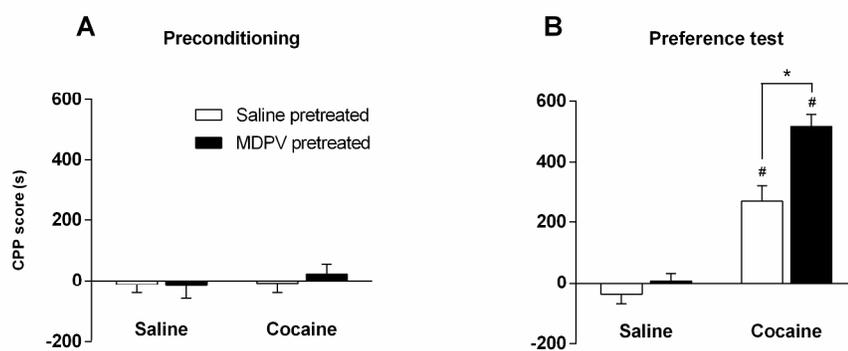


Figure 4

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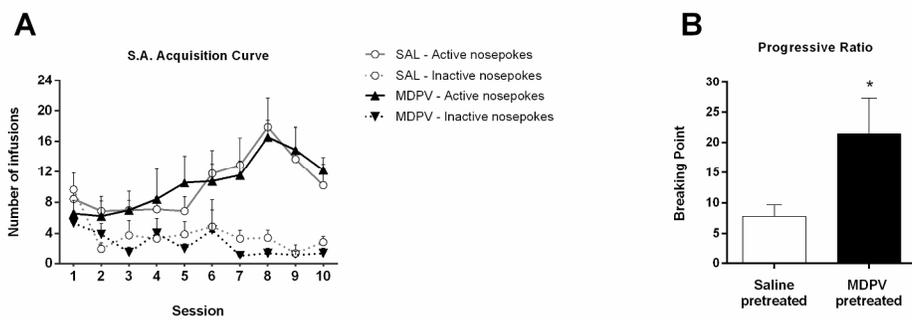


Figure 5

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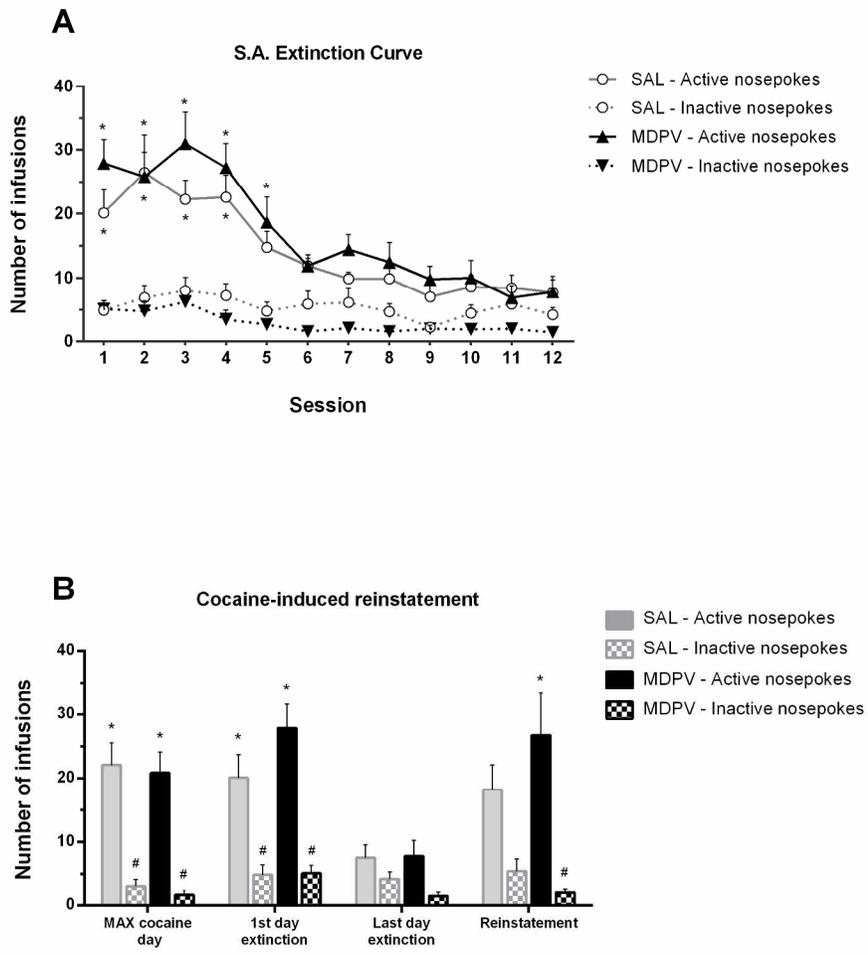


Figure 6

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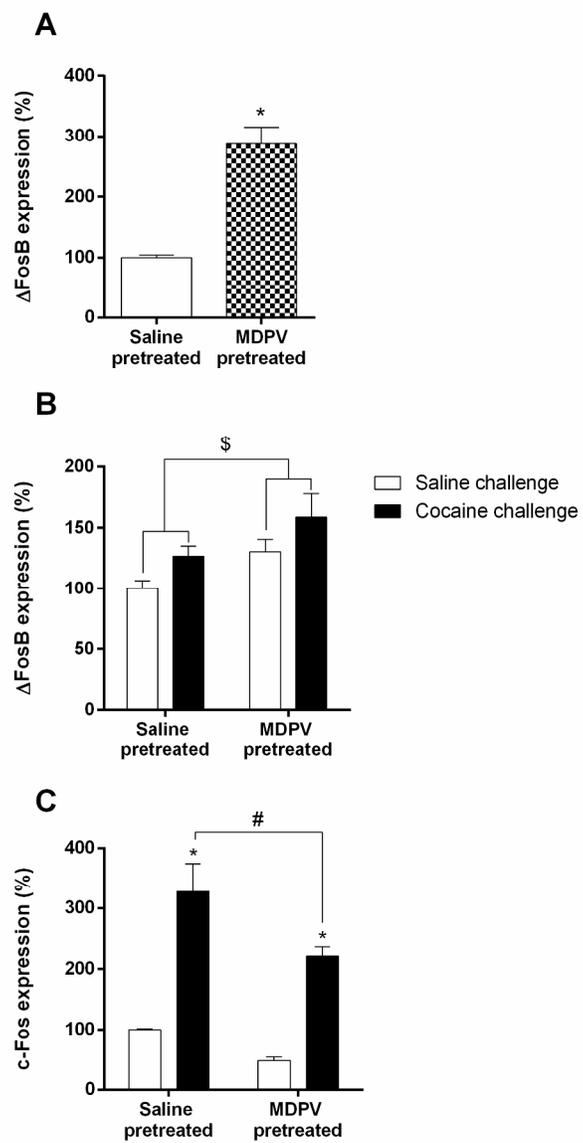


Figure 7

144x272mm (300 x 300 DPI)

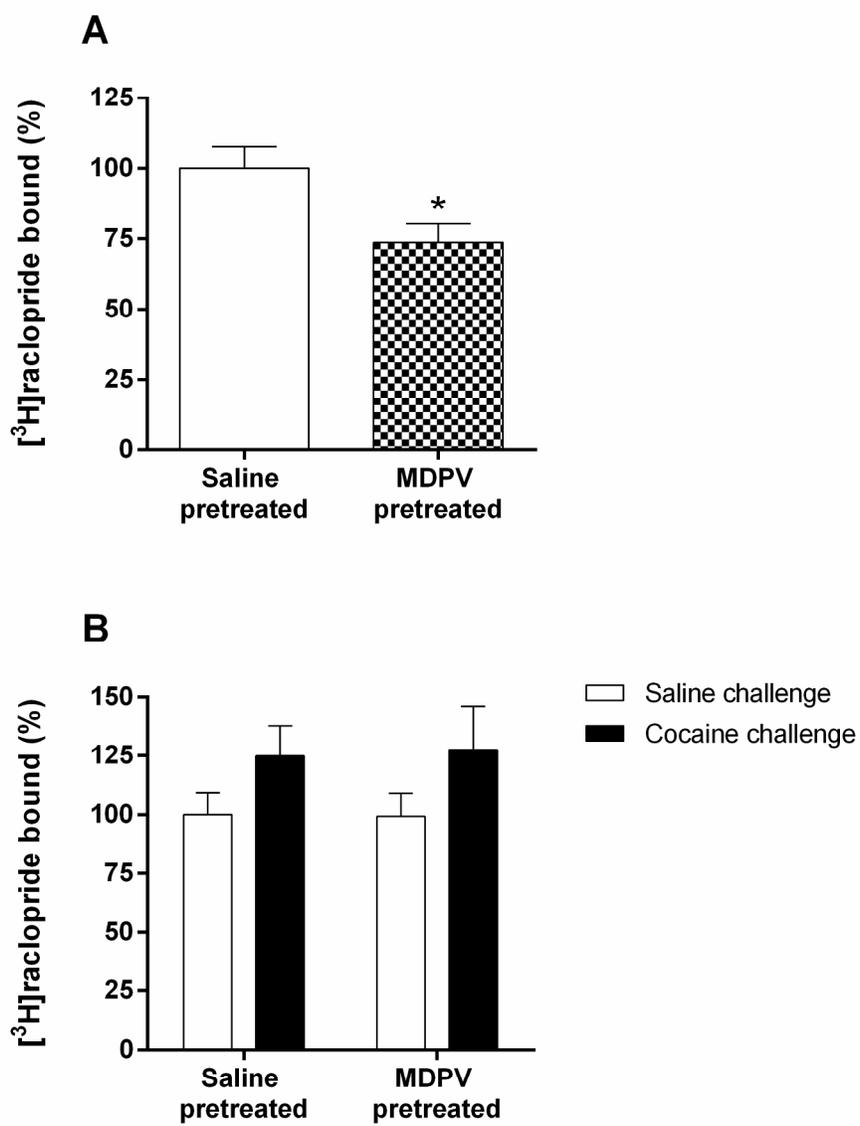


Figure 8

187x239mm (300 x 300 DPI)