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# Levodopa prevents the reinstatement of cocaine self-administration in rats via potentiation of dopamine release in the medial prefrontal cortex

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## ABSTRACT

Dopamine agonists have been proposed as therapeutic tools for cocaine addiction. We have recently demonstrated that indirect dopamine agonists, including levodopa (L-DOPA), markedly increase cocaine-induced dopamine release in the medial prefrontal cortex (mPFC) of rats leading to the suppression of cocaine-seeking behavior. This study was aimed to understand the behavioral and neurochemical effects of L-DOPA on cocaine-taking and cocaine-seeking in rats. After reaching a stable pattern of intravenous cocaine self-administration under a continuous fixed ratio (FR-1) schedule of reinforcement, male rats were treated with L-DOPA at different steps of the self-administration protocol. We found that L-DOPA reduced cocaine self-administration under FR-1 schedule of reinforcement and decreased the breaking points and the amount of cocaine self-administered under the progressive ratio schedule of reinforcement. Levodopa also decreased cocaine-seeking behavior both in a saline substitution test and in the cue priming-induced reinstatement test, without affecting general motor activity. Importantly, L-DOPA greatly potentiated cocaine-induced dopamine release in the mPFC of self-administering rats while reducing their cocaine intake. In the same brain area, L-DOPA also increased dopamine levels during cue priming-induced reinstatement of cocaine-seeking behavior. The potentiating effect was also evident in the mPFC but not nucleus accumbens core of drug-naïve rats passively administered with cocaine. Altogether, these findings demonstrate that L-DOPA efficaciously reduces the reinforcing and motivational effects of cocaine likely potentiating dopamine transmission in the mPFC. Its ability to prevent cue priming-induced reinstatement of cocaine-seeking suggests that it might be effective in reducing the risk to relapse to cocaine in abstinent patients.

**Keywords** cocaine, dopamine release, levodopa, medial prefrontal cortex, self-administration.

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## INTRODUCTION

Cocaine dependence is characterized by recurrent relapses, which represent the main problem in its treatment (Simpson *et al.* 1999). Continuing cocaine consumption has been proposed to be a sort of self-medication to dampen dopamine deficiency that occurs during abstinence (Dackis & Gold 1985; Volkow *et al.* 1999). Indeed, while acute euphorigenic effects of cocaine are attributed primarily to its ability to increase the level of dopamine (and other monoamines) in the synaptic cleft of mesolimbic brain areas, chronic use of cocaine leads to striatal dopamine system hypofunctioning (Dackis & Gold 1985; Shearer 2008). A reduced level of dopamine in the striatum has been associated to withdrawal from cocaine and other drugs of abuse (Rossetti *et al.* 1992). Accordingly, animals titrate intake of cocaine to maintain an elevated level of dopamine in the nucleus accumbens (Wise *et al.* 1995), and cocaine-addicted humans display decreased dopamine release in the striatum (Volkow *et al.* 1997).

Building upon these premises, direct and indirect dopamine agonists have received great attention as possible candidates in the search for a valid therapy for

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cocaine addiction (Kosten *et al.* 2002; Pérez-Mañá *et al.* 2011; Verrico *et al.* 2013). We recently demonstrated that dopamine beta-hydroxylase inhibitors, disulfiram and nepicastat, are able to enhance extracellular dopamine levels selectively in the medial prefrontal cortex (mPFC) of rats (Devoto *et al.* 2012, 2014) and suggested that this enhancement could be necessary for the anti-cocaine craving properties of these drugs (Devoto *et al.* 2016). We challenged this hypothesis by using a drug able to increase dopamine levels through a different mechanism, namely the dopamine metabolic precursor levodopa (L-DOPA). This drug was found to reproduce the increasing effect of the DHB inhibitors on extracellular dopamine levels in the mPFC and to markedly inhibit drug-induced cocaine-seeking reinstatement (Devoto *et al.* 2016). Interestingly, L-DOPA has been proposed as a therapeutic tool for cocaine craving, due to its capability to replenish dopamine stores depleted by sustained cocaine consumption (Schmitz *et al.* 2010, 2014). Although some clinical reports on L-DOPA yielded scarce or negative results (Mooney *et al.* 2007; Amato *et al.* 2011), this pharmacotherapy has also provided positive results, especially when associated with behavioral therapy aimed to reduce the reinforcing value of cocaine and to enhance the dopamine-induced salience of contingently delivered reinforcements (Schmitz *et al.* 2008, 2010). More recent findings reported its efficacy in a subset of patients with genetically low beta-hydroxylase activity (Liu *et al.* 2014) and in preventing and reversing the escalation of cocaine intake in rats (Willuhn *et al.* 2014). Importantly, L-DOPA also counteracts the decline in dopamine release observed in the ventromedial striatum of rats that escalate their cocaine intake, but not in non-escalated rats (Willuhn *et al.* 2014), confirming that decreased dopamine level triggers an escalated use of cocaine. Consequently, at present, the use of L-DOPA as substitute therapy for cocaine addiction is still a matter of debate.

Based on these premises, the present study was aimed at deepening our knowledge of the behavioral and neurochemical effects of L-DOPA on cocaine-taking and cocaine-seeking in rats, focusing on self-administration behavior, motivation for cocaine and dopamine release in the mPFC. We demonstrate that L-DOPA efficaciously decreases drug intake in rats self-administering cocaine while potentiating cocaine-induced dopamine release in the mPFC and, expanding upon our previous investigation, is able to reduce cocaine-seeking during acute abstinence from cocaine self-administration and prevent cue-induced relapse in rats. Moreover, L-DOPA significantly lowers the breaking point in the progressive ratio experiment, further strengthening its efficacy in decreasing the incentive value of cocaine.

## MATERIAL AND METHODS

### Animals

A total of 88 male Sprague Dawley rats (Envigo, Italy) weighing 225–250 g on arrival were housed in group of four in a temperature (21 ± 1°C) and humidity (60 ± 10 percent) controlled room and maintained on an inverted 12-hour light/dark cycle (light off at 7:00 a.m.) with food and water freely available. After surgery for the implantation of an intravenous (i.v.) catheter for self-administration procedure, animals were housed individually to avoid damages to the external component of the catheter assembly and allowed to recover for 7 days. Twenty-four hours before starting self-administration training food was restricted to 20 g per day to keep ~85 percent of free feeding weight.

All procedures and experiments were carried out in an animal facility according to Italian (D.L. 26/2014) and European Council directives (63/2010) and in compliance with the animal policies approved by the local Ethical Committee for Animal Experiments (CESA, University of Cagliari) and by the Italian Department of Health. All possible efforts were made to minimize animal pain and discomfort and to reduce the number of experimental subjects.

### Drugs

Cocaine hydrochloride (MacFarlan Smith Ltd., Edinburgh, UK) was dissolved in heparinized (0.1 percent) sterile saline solution at the dose of 0.5 mg/kg per 0.1 ml/infusion and was filtered through a 0.20-μm syringe filter prior to be intravenously infused. For microdialysis experiments, cocaine (10 mg/kg) was dissolved in sterile saline solution and administered intraperitoneally (i.p.) 20 minutes after L-DOPA or vehicle (saline) administration.

Levodopa (L-3,4-dihydroxyphenylalanine, Sigma-Aldrich, Italy) was administered at the dose of 50 mg/kg, on the basis of previous pilot experiment and results (Devoto *et al.* 2016). It was dissolved in a volume of 2 ml/kg saline and injected i.p. 20 minutes before starting the experimental session, always in association with the peripheral DOPA-decarboxylase inhibitor benserazide (10 mg/kg, Sigma-Aldrich, Italy) to prevent peripheral breakdown of L-DOPA.

#### *Intravenous catheter implantation*

After 7 days of acclimation, animals were deeply anaesthetized with isoflurane 2 percent and surgically implanted with home-made silastic chronic indwelling catheter into the right jugular vein (Fattore *et al.* 2001). After surgery, animals were allowed to recover

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for a minimum of 7 days and received a daily subcutaneous (s.c.) injection (0.1 ml) of enrofloxacin (Baytril, Bayer HealthCare) as antibiotic therapy.

### Intravenous cocaine self-administration apparatus and procedure

Cocaine self-administration training was performed in 12 operant chambers (Med Associates, St Albans, VT, USA), each housed in a ventilated, light- and sound-attenuating box. Each chamber (29.5 × 32.5 × 23.5 cm) was equipped with two retractable levers (4 cm wide and extending 1.5 cm into the chamber) located 12 cm apart and 8 cm above the grid floor, with a white visual stimulus light (cue light, 2.5 W, 24 V) placed between the two levers and with a yellow house light (2.5 W, 24 V) located on the top of the opposite wall. Outside each box, a 10-ml syringe mounted upon an infusion pump (Med Associates, USA) delivered 0.1-ml cocaine solution (at a rate of 20 µl/second over 5 seconds) contingently to each active lever press. Plastic tubing connected the syringe with a counter-balance swivel system that was linked to the animal's catheter through additional plastic tubing shielded with a metal spring thus permitting free movements to the animal. During each session of self-administration, rat locomotor activity within the operant chambers was constantly monitored and recorded through two series of four photocells (placed at regular intervals above the grid floor). The number of photocell beam breaks was recorded and served as a measure of general horizontal locomotor activity, and in particular to assess potential non-specific motor effects induced by the experimental manipulation. Programming of experimental parameters, data collection and storage were controlled by a computer-integrated system using Med Associates software (Med PC IV).

As previously described (Devoto *et al.* 2016), cocaine self-administration training started 7 days following i.v. surgery and was performed in daily 2-hour sessions, 7 days per week, approximately at the same time every day during the dark phase of the cycle, under a continuous fixed ratio (FR-1) schedule of reinforcement and lever-pressing as operant behavior.

At the beginning of each self-administration session, the two levers were extended into the experimental chamber, and the yellow house light was turned on. At the end of each session, the house light was turned off, and both levers retracted. A response on the lever identified as active induced the retraction of both levers for 20 seconds and the activation of the syringe pump for 5 seconds that resulted in a contingent i.v. infusion of cocaine (0.5 mg/kg/100 µl). Each cocaine infusion was accompanied by the simultaneous illumination of the white cue light to incentivize the salience of drug-paired cue. Responses on the other lever, identified as inactive, were recorded but had no scheduled consequences.

After reaching the criterion for the acquisition phase of self-administration (typically within 4–7 days of training), *i.e.* when animals showed accurate discrimination (≈70 percent) between the two levers and the number of responses on the active lever was ≥20 for at least 3 consecutive days, rats continued self-administration training to stabilize their cocaine intake pattern during the maintenance phase (Fattore *et al.* 2009; Devoto *et al.* 2016). After 12 days of stable cocaine intake, i.e. variation in response numbers ≤15 percent, animals were split into six different groups to receive an i.p. injection of L-DOPA (50 mg/kg + benserazide 10 mg/kg) or vehicle (saline, 2 ml/kg) 20 minutes before starting the session at different time points (Fig. 1). Three days prior to testing, rats were given daily saline injections (2 ml/kg, i.p.) as habituation for future treatments.

The first group was tested at the end of the maintenance phase to evaluate L-DOPA effect on cocaine-taking behavior. Thus, 20 minutes before starting the FR-1 session, each animal received one injection of L-DOPA (50 mg/kg + benserazide 10 mg/kg, i.p.) and one injection of vehicle (2 ml/kg, i.p.) in a counterbalanced order, each separated by at least 3 days of cocaine training to avoid carryover effects.

A second group underwent *in vivo* microdialysis experiments, while a third group was switched to a progressive ratio (PR) schedule of reinforcement for 5 days to evaluate the effect of L-DOPA on the motivation for cocaine. Under this schedule, the response : infusion ratio increased exponentially within the session after each infusion of cocaine according to the following series: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402, 492, 603, etc. This PR series derived from the equation: *Response ratio* = $[5^{e\ (n \times 0.2)}] - 5$, where $n$ corresponded to the number of cocaine infusions (Richardson & Roberts 1996). The breaking point, defined as the final ratio completed during each daily self-administration session, i.e. the maximum effort that an animal expends to obtain the drug, was analyzed. After five PR sessions, animals were injected with either L-DOPA (50 mg/kg + benserazide 10 mg/kg, i.p.) or vehicle (saline, 2 ml/kg, i.p.) 20 minutes before the session, and breaking points were measured for each animal.

A fourth group underwent a saline substitution test. On the day after the last FR-1 session of cocaine self-administration, animals were tested in a session in which cocaine solution was replaced by saline solution (while maintaining the other experimental parameters unvaried) to evaluate the effect of L-DOPA on cocaine-seeking 24 hours after the last cocaine infusion.

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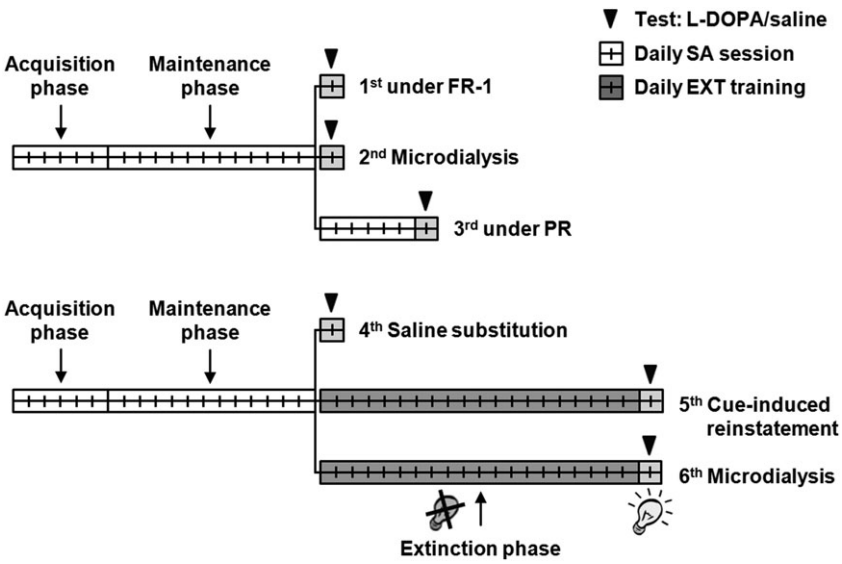


**Figure 1** Schematic representation of the experimental design

Finally, two last groups (fifth and sixth) were switched to extinction condition, obtained by replacing cocaine with saline and omitting (1) the illumination of the cue light; (2) the retraction of both levers; and (3) the activation of the infusion pump, so that presses of the active lever had no more consequences. Briefly, from the first day of the extinction training, rats were placed into the box and attached to the swivel system, the house light and ventilation fan were on, levers were extended into the box, but the drug-associated light (visual cue) and the lever retraction system were switched off (Chauvet *et al.* 2009). Under these conditions, cue priming-induced reinstatement testing took place once drug-reinforced behavior was extinguished, i.e. when the cumulative number of active + inactive lever-presses was ≤10. The conditions during reinstatement testing were similar to those of extinction, i.e. the house light and ventilation fan were on, and cue light remained deactivated. As the extinction criterion was reached, animals of both groups received an i.p. injection of L-DOPA (50 mg/kg + benserazide 10 mg/kg) or vehicle (saline, 2 ml/kg) 20 minutes before starting the session and were exposed for 5 seconds to the cocaine-paired cue light immediately before starting the cue priming-induced reinstatement test session. Each animal received one injection of L-DOPA and one injection of vehicle in a counterbalanced order, each separated by at least 3 days of extinction training (to avoid carryover effects of drug priming).

*In vivo microdialysis*

The day before microdialysis experiments, rats were anesthetized with an i.p. injection of Equithesin (5 ml/kg; 0.97 g pentobarbital, 2.1 g $MgSO_4$, 4.25 g chloral hydrate, 42.8 ml propylene glycol, 11.5 ml 90 percent ethanol, distilled water up to 100 ml). Home-made vertical microdialysis probes (AN 69-HF membrane, Hospal-Dasco, Bologna, Italy; cut-off 40 000 Dalton, 3 mm dialyzing membrane length) were implanted in the mPFC (AP: +3.0, L: ±0.6, V: −6.5 from bregma) or in the nucleus accumbens core (AP: +1.7, L: ±1.6, V: −7.8 from bregma) according to the coordinates of the atlas by Paxinos & Watson (1997) as previously described (Devoto *et al.* 2016). Rats were given antibiotic therapy and allowed to recover for 24–48 hours before microdialysis experiment.

Microdialysis experiments were aimed to evaluate the effect of L-DOPA on extracellular dopamine levels in the mPFC during the maintenance phase of cocaine self-administration, i.e. after 12 days of stable cocaine intake (Fig. 1, second group), and during cue priming-induced reinstatement of cocaine-seeking behavior, i.e. once reached the abovementioned criteria for extinction (Fig. 1, sixth group). In addition, dopamine levels were measured in the mPFC and nucleus accumbens core of a separate group of drug-naïve rats treated with cocaine and L-DOPA, alone or in association, to verify whether L-DOPA effect was specific to the mPFC and evident also when cocaine was passively administered and not self-administered. An artificial cerebrospinal fluid (147 mM NaCl, 4 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, pH 6–6.5) was pumped through the dialysis probe at a constant rate of 1.1 µl/minute via a CMA/100 microinjection pump (Carnegie Medicine, Stockholm, Sweden). Samples were collected every 20 minutes and immediately analyzed for dopamine content by HPLC with electrochemical detection, as previously described (Devoto *et al.* 2003). When a stable baseline was obtained (three consecutive samples with a variance not exceeding 15 percent), L-DOPA (50 mg/kg + benserazide 10 mg/kg) or vehicle (saline, 2 ml/kg) was administered i.p. and after 20 minutes (i.e. after

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collection of one more sample), rats were placed in the self-administration chamber. Sample collection continued during the 2-hour self-administration session, for a total of six samples collected and analyzed. Drug-naïve animals were injected with L-DOPA (50 mg/kg + benserazide 10 mg/kg, i.p.) or vehicle (saline, 2 ml/kg, i.p.); after 20 minutes, they received cocaine (10 mg/kg, i.p.) or its vehicle (saline 1 ml/kg, i.p.) and sampling continued in their home cage for 2 hours. Microdialysis data were calculated as pg/20 μl dialysate, and treatment-induced changes were expressed as percent of mean basal level. On completion of testing, rats were sacrificed by Equithesin overdose, the brains removed and sectioned by a cryostat (Leica CM3050 S) in 40-μm-thick coronal slices to verify locations of dialysis probes. Animals with errant location of the device were excluded from analysis.

### Statistics

Statistical significance of data from cocaine self-administration (FR-1) and cue priming-induced reinstatement experiments was calculated by separate analyses of active and inactive responses using one-way analysis of variance (ANOVA) followed by *post-hoc* multiple comparisons Tukey's test. One-way ANOVA followed by Tukey's test was also used for analyzing data from PR experiments. Two-way ANOVA followed by Sidak's *post-hoc* test was used to analyze the number of responses on both levers during saline substitution session. Two-way ANOVA with repeated measures followed by Sidak's *post-hoc* test was used for microdialysis data. *T*-test with Welch's correction was used for analyzing active lever-pressing activity during cocaine self-administration in rats undergoing microdialysis experiments. Statistical analyses were performed by means of GraphPad Prism 7.0 (San Diego, CA, USA) software. For all analyses, $P < 0.05$ was considered statistically significant.

## RESULTS

Locomotor activity (mean ± SEM of photocell beam breaks) during all phases of cocaine self-administration and reinstatement test sessions was not altered by acute administration of L-DOPA (50 mg/kg + benserazide 10 mg/kg, i.p.), thus ensuring the absence of any non-specific effect on response (Supporting Information Fig. 1). Similarly, acute exposure to the cue light did not affect spontaneous motor activity in any group.

### Effect of levodopa on cocaine-taking behavior under FR-1 schedule of reinforcement

After the acquisition phase and maintenance of a stable FR-1 pattern of cocaine self-administration, rats were pretreated with L-DOPA (50 mg/kg + benserazide 10 mg/kg, i.p.) or saline (2 ml/kg, i.p.) 20 minutes before being placed in the self-administration chamber (Fig. 1, first group). During the maintenance phase of cocaine self-administration, the mean daily responding on the active lever varied from 26.8 ± 0.9 to 36 ± 1.3 (Basal intake, Fig. 2a). Saline pretreatment did not affect cocaine-taking with respect to basal level of drug intake. Conversely, pretreatment with L-DOPA (50 mg/kg, i.p.) significantly reduced responding for cocaine. No significant differences were found in the inactive lever activity among groups.

One-way ANOVA evidenced significant differences in the responding rate on the active [$F_{(2,21)} = 47.47$, $P < 0.0001$] but not the inactive lever. *Post-hoc* multiple comparisons Tukey's test revealed a significant difference of 'Basal intake' versus 'L-DOPA' ($P < 0.0001$) but not versus 'Saline' groups. This latter group was significantly different from 'L-DOPA' group ($P < 0.0001$).

The response patterns of operant behavior during the 2-hour self-administration sessions were further examined (Fig. 2, panels b–d). Analysis of individual response patterns indicated that the time interval between two consecutive drug infusions tended to be rather regular throughout the session (Fig. 2b). Quite similar was the mean inter-response interval after pretreatment with saline (Fig. 2c). After L-DOPA pretreatment, rats maintained a rather regular intake throughout the 2-hour session but with longer intervals between two consecutive responses and an overall decreased number of infusions self-administered (Fig. 2d).

### Effect of levodopa on extracellular dopamine concentration in medial prefrontal cortex during cocaine self-administration

After stable maintenance of cocaine self-administration paradigm, rats were subjected to microdialysis probe insertion and again exposed to self-administration protocol (Fig. 1, second group). Once verified the stability of dopamine basal levels, rats were injected i.p. with L-DOPA (50 mg/kg) or saline (2 ml/kg, i.p.) and, after 20 minutes, placed into the self-administration box, as described above for the previous experiment. Microdialysis sampling continued every 20 minutes during cocaine access (2 hours). Extracellular dopamine basal levels were not significantly different between groups (Student's *t* test, $P > 0.1$) and the mean ± SEM were 1.50 ± 0.36 and 0.76 ± 0.29 pg/sample for saline and L-DOPA group, respectively ($n = 6$ each).

In control (saline-treated) animals, cocaine self-administration increased extracellular dopamine levels to about 400 percent of basal value starting from the first sample collected after the beginning of the session (time

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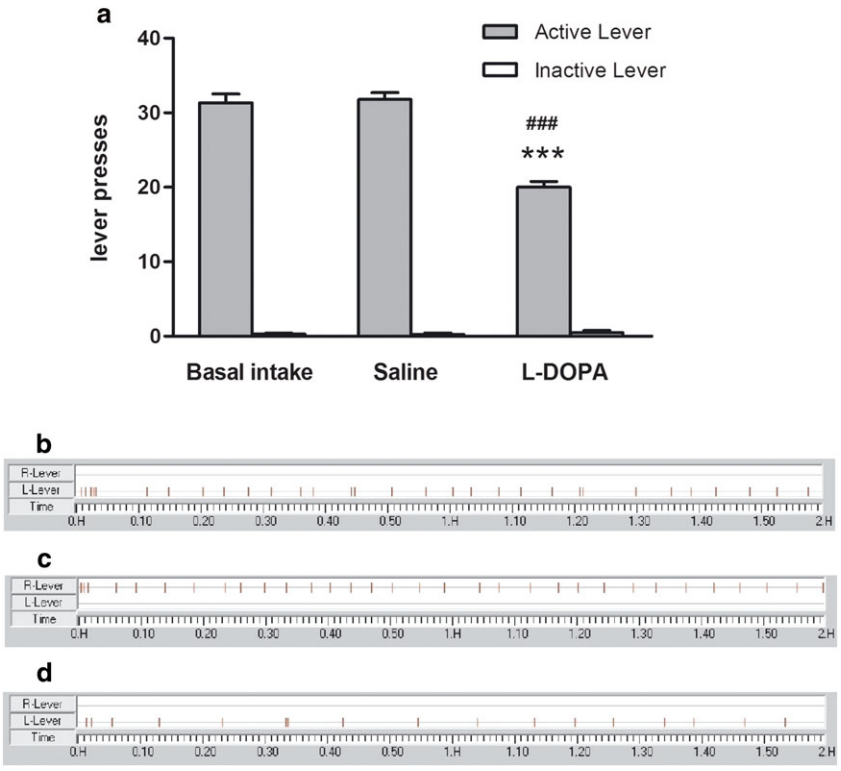


**Figure 2** (a) Effect of L-DOPA on cocaine-taking behavior during 2-hour self-administration session under FR-1 schedule. Bars represent the mean ± SEM of active and inactive lever presses during the five consecutive self-administration sessions before ('Basal intake'), and after pretreatment with vehicle ('Saline') or 'L-DOPA' ($n$ = 8/group). (b–d) Representative individual patterns of responding during the maintenance phase, before (b) and after treatment with saline (c) or L-DOPA (d). Each record represents cumulative responses within the 2-hour test session, while each vertical upward line corresponds to a press on the active lever. $^{***}P < 0.001$ versus 'Basal intake'; $^{###}P < 0.001$ versus 'Saline'

interval (T): T100) and remained roughly constant during the entire session (Fig. 3). Conversely, pretreatment with L-DOPA strongly potentiated cocaine-induced dopamine release up to 3100 percent of basal value and contextually decreased cocaine intake to about 54 percent of control group (Fig. 3, inset; $P < 0.01$, Student's $t$ test). Two-way ANOVA for repeated measures, conducted on microdialysis data with L-DOPA and saline as treatment factors and time as repeated measure, evidenced a significant main effect of treatment [$F_{(1,10)} = 10.68$, $P < 0.01$]. Sidak's multiple comparisons test confirmed that the increase in extracellular dopamine level induced by cocaine was significantly greater in L-DOPA- than in saline-pretreated rats at all time points from T120 to T200 minutes ($P < 0.05$).

### Effect of levodopa on cocaine-taking behavior under progressive ratio schedule of reinforcement

Under the PR schedule of reinforcement (Fig. 1, third group), animals reached a mean breaking point of 172.8 ± 14.4 and a mean number of cocaine infusions of 17.6 ± 0.40. These two parameters were not changed after pretreatment with saline. On the contrary, pretreatment with L-DOPA (50 mg/kg, i.p.) decreased the mean breaking points and the number of infusions

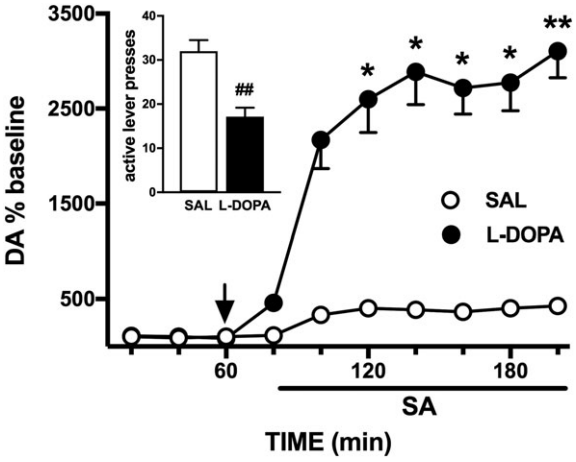


**Figure 3** Effect of L-DOPA on extracellular dopamine (DA) levels in the medial prefrontal cortex during 2-hour cocaine self-administration (SA) session (from minutes 80 to 200) under FR-1 schedule. Data are expressed as percentage of mean dopamine basal level and are the mean ± SEM of 6 rats/group. The arrow indicates the time of L-DOPA/Saline (SAL) administration. The inset shows the mean ± SEM number of active responses made by the same rats during self-administration session. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus corresponding 'Saline' time point (Sidak's multiple comparisons test). Inset: $^{##}P < 0.01$ versus 'Saline' (Unpaired $t$ test with Welch's correction)

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to 81.2 ± 14.0 and 13.6 ± 0.8, respectively (Fig. 4). One-way ANOVA revealed significant effect of treatment on breaking points among groups [$F_{(2,27)} = 9.84$, $P < 0.001$]. *Post-hoc* Tukey's multiple comparison test evidenced significant differences of 'L-DOPA' versus 'Basal intake' and 'Saline' groups ($P < 0.001$ and $P < 0.01$, respectively). No significant differences were detected between 'Basal intake' and 'Saline' groups.

### Effect of levodopa on cocaine-seeking in the saline substitution test

At the end of the maintenance phase, the effect of L-DOPA on cocaine-seeking behavior was investigated in a saline substitution test (Fig. 1, fourth group), in which cocaine solution was replaced with saline (i.e. in absence of contingent drug reward). As expected, the activity on both levers was increased, resembling the very first day of extinction training during which animals react to the lack of the rewarding stimulus by increasing their activity. Pretreatment with L-DOPA (50 mg/kg + benserazide 10 mg/kg, i.p.) attenuated responding on both the active and inactive lever with respect to saline-treated group (Fig. 5). Two-way ANOVA evidenced significant effect of treatment [$F_{(1,20)} = 14.08$, $P < 0.01$] and levers [$F_{(1,20)} = 105$, $P < 0.0001$]; *post-hoc* Sidak's multiple comparisons test indicated a significant difference between active levers ($P < 0.01$) but not between the inactive ones.

### Effect of levodopa on cue priming-induced reinstatement of cocaine-seeking behavior

Once reached the established criterion for extinction (Fig. 1, fifth group), animals were tested for cue priming-induced reinstatement of cocaine-seeking behavior (Fig. 6). When re-exposed to the cocaine-paired cue immediately before starting the session, animals readily resumed active lever-pressing activity to previous basal level of cocaine intake (Fig. 6a) and slightly increased activity on the inactive lever (Fig. 6b). However, pretreatment with L-DOPA (50 mg/kg, i.p.) efficaciously reverted this effect by significantly decreasing responding on both levers.

One-way ANOVA revealed significant differences in the mean number of active lever presses [$F_{(3,40)} = 19.18$, $P < 0.0001$]. *Post-hoc* multiple comparison Tukey's test evidenced a significant difference between 'Basal intake' versus 'L-DOPA' and 'Extinction' ('EXT') groups ($P < 0.0001$), but not between 'Basal intake' and 'Saline' groups (Fig. 6a). This latter group was significantly different from 'EXT' ($P < 0.0001$) and 'L-DOPA' groups ($P < 0.001$). One-way ANOVA revealed statistical differences in the mean number of responses on the

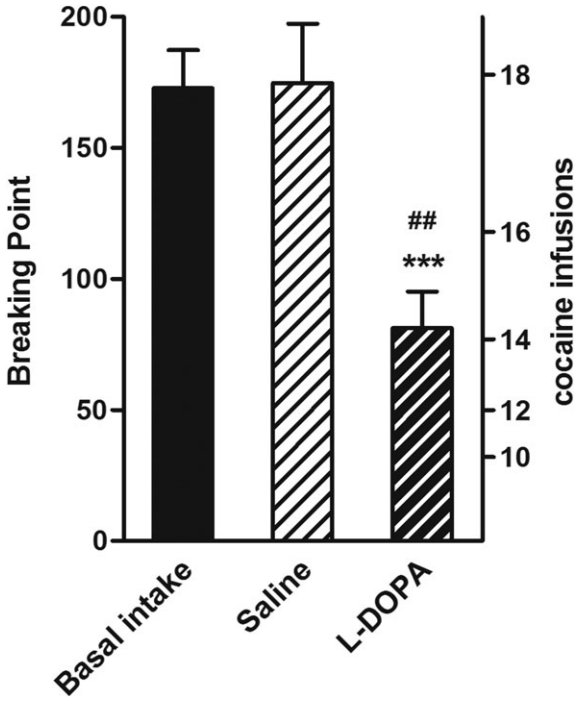


**Figure 4** Effect of L-DOPA on cocaine-taking during 2-hour self-administration session under PR schedule. Bars represent the mean ± SEM of breaking points during the last three consecutive self-administration sessions before ('Basal intake', $n = 15$), and after pretreatment with vehicle ('Saline', $n = 6$) or 'L-DOPA' ($n = 9$). $^{***}P < 0.001$ versus 'Basal intake'; $^{\#\#}P < 0.01$ versus 'Saline'

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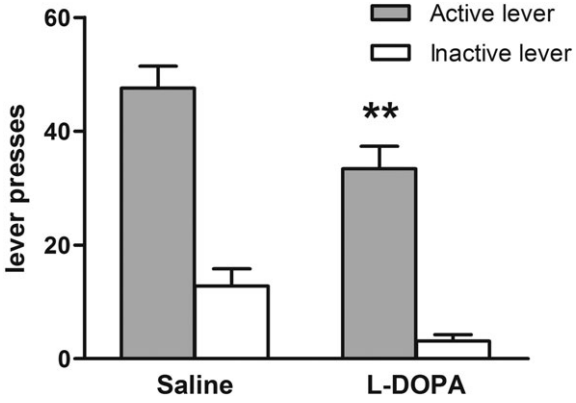


**Figure 5** Effect of L-DOPA on cocaine-seeking in the saline substitution test. Data show mean ± SEM of active (grey bars) and inactive (white bars) lever presses ('Saline', $n = 5$; 'L-DOPA', $n = 7$). $^{**}P < 0.01$ versus 'Saline' active lever (Sidak's multiple comparison test)

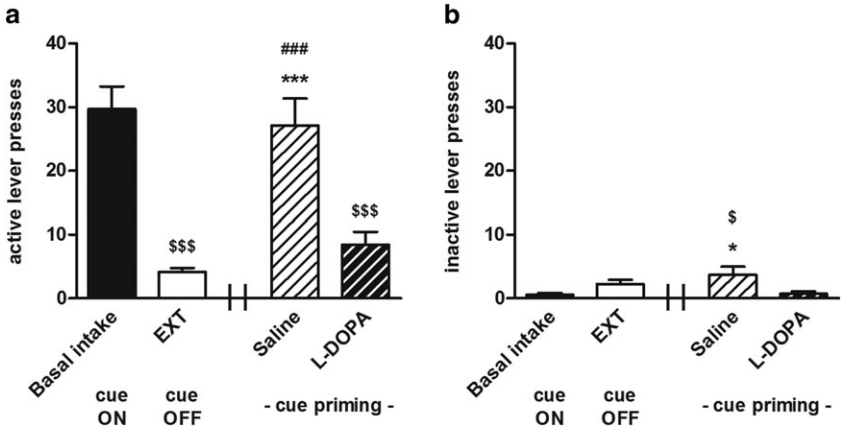


**Figure 6** Effect of L-DOPA on cue priming-induced reinstatement of cocaine-seeking behavior. Bars represent the mean ± SEM number of active (a) and inactive (b) lever presses during the five consecutive self-administration sessions before ('Basal intake' and 'EXT'), and after pretreatment with vehicle ('Saline') or 'L-DOPA' ($n = 11$/group). A: $^{\$\$}P < 0.0001$ versus 'Basal intake'; $^{***}P < 0.0001$ versus 'EXT'; $^{\#\#\#}P < 0.0001$ versus 'L-DOPA'. B: $^{*}P < 0.05$ versus 'Basal intake'; $^{\$}P < 0.05$ versus 'L-DOPA' (Tukey's multiple comparison test). EXT, extinction

inactive lever [$F_{(3,40)} = 3.942$, $P < 0.02$]. Tukey's test evidenced a significant difference ($P < 0.05$) between 'Saline' versus 'Basal intake' and 'L-DOPA' groups (Fig. 6b).

### Effect of levodopa on extracellular dopamine concentration in medial prefrontal cortex during cue priming-induced reinstatement of cocaine-seeking behavior

As in the fifth group, L-DOPA pretreatment inhibited cocaine-seeking behavior (Fig. 7, inset; $P < 0.001$, Student's $t$ test). Contextually, extracellular dopamine levels increased up to a maximum of about 700 and 300 percent in L-DOPA- and saline-pretreated rats, respectively (Fig. 7). Two-way ANOVA for repeated measures indicated a significant effect of treatment [$F_{(1,8)} = 16.5$, $P < 0.01$], and time × treatment interaction [$F_{(5,40)} = 3.17$, $P < 0.05$]. *Post-hoc* analysis indicated L-DOPA-treated rats were significantly different from controls from T160 to T200 (Sidak's multiple comparisons test).

### Effect of levodopa plus cocaine administration on extracellular dopamine in medial prefrontal cortex and nucleus accumbens core of naïve rats

In a separate group of drug-naïve rats, L-DOPA and cocaine (50 and 10 mg/kg, i.p., respectively) were administered, alone or in combination, soon after the collection of a stable baseline, and extracellular dopamine variations were monitored by microdialysis in the mPFC or nucleus accumbens core (Fig. 8). Mean basal values were 1.8 ± 0.39 and 10.73 ± 2.32 pg/sample for mPFC and nucleus accumbens, respectively. As previously observed (Devoto *et al.* 2016), when given alone, L-DOPA increased extracellular dopamine level to about 190 and 300 percent of basal values in the nucleus accumbens and mPFC, respectively, while cocaine produced an increment to about 400 percent in the nucleus accumbens and 370 percent in the mPFC. However, their co-administration produced a clear potentiation of their effect up to 1300 percent of the basal value in the mPFC (i.e. a synergistic effect), but just a modest increment, which magnitude corresponds to the sum of the single

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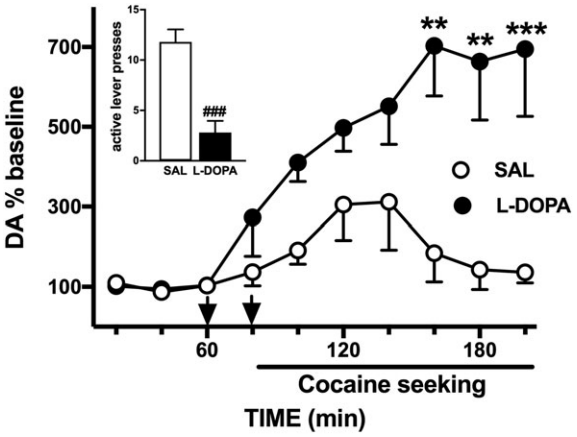


**Figure 7** Effect of L-DOPA on extracellular dopamine (DA) levels in the medial prefrontal cortex during cue priming-induced reinstatement testing (from minutes 80 to 200). Data are expressed as percentage of mean dopamine basal level and are the mean ± SEM of 5 rats/group. The first arrow indicates the time of L-DOPA/Saline (SAL) administration, the second one the time of cocaine-paired cue exposure. The inset shows the mean ± SEM number of active responses made by the same rats during cue priming-induced reinstatement session. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus corresponding 'Saline' time point (Sidak's multiple comparisons test). Inset: $^{###}P < 0.001$ versus 'Saline' (Unpaired *t* test with Welch's correction)

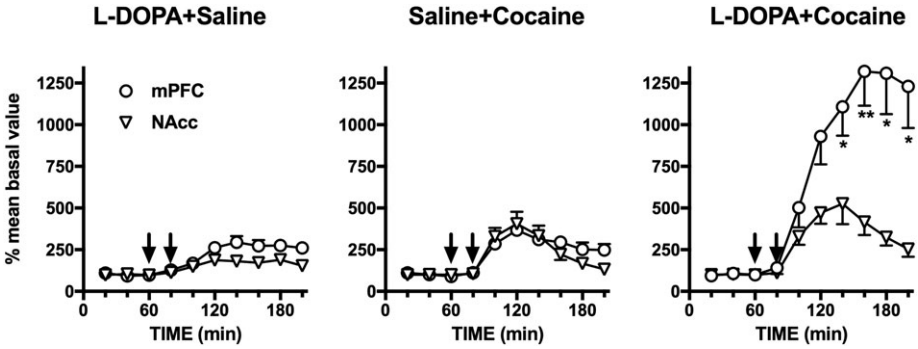


**Figure 8** Effect of L-DOPA and/or cocaine administration on extracellular dopamine levels in the medial prefrontal cortex (mPFC) and nucleus accumbens core (NAcc) of drug-naïve rats. Data are expressed as percentage of mean dopamine basal level and are the mean ± SEM of 4–6 rats/group. The first arrow indicates the time of L-DOPA/Saline administration, the second one the time of cocaine/vehicle administration. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus corresponding 'NAcc' time point (Two-way ANOVA with Sidak's multiple comparisons test as *post hoc*)

drug effects (i.e. an additive effect), in the nucleus accumbens core. Two-way ANOVA for L-DOPA alone effect indicated a significant effect of area [$F_{(1,35)} = 15.5$, $P < 0.001$] and no effect of time [$F_{(4,35)} = 2.02$, $P > 0.1$]; Sidak's *post-hoc* test evidenced no difference between time points. Two-way ANOVA for cocaine alone effect showed a significant effect of time [$F_{(4,45)} = 5.28$, $P < 0.01$] but no effect of area [$F_{(1,45)} = 0.167$, $P > 0.5$]. For L-DOPA + cocaine-treated rats, two-way repeated measure ANOVA indicated a significant effect of time [$F_{(5,48)} = 3.39$, $P < 0.05$] and area [$F_{(1,48)} = 44$, $P < 0.0001$], while Sidak's *post-hoc* test evidenced significant differences between curves from T140 to T200.

## DISCUSSION

In the present study, we showed that L-DOPA administration counteracts spontaneous intake of cocaine under a continuous schedule of reinforcement, suggesting its efficacy in decreasing the reinforcing effects of cocaine. In support to this, prenatal L-DOPA exposure significantly lowered cocaine-induced conditioned place preference in adult animals (Ren *et al.* 2011) without inducing rewarding effects by itself (Zengin-Toktas *et al.* 2013). The uniformity in the individual patterns of responding of rats that received saline and L-DOPA pretreatment ensures about the absence of aspecific (e.g. motor) effects in animals' behavior. Analysis of motor activity during self-administration training confirms the lack of motor side effects in animals undergoing cocaine self-administration during the different test sessions. Levodopa also significantly reduces breaking points and cocaine intake under the progressive schedule of reinforcement, showing to be effective in decreasing the incentive value of cocaine and the motivation to work for obtaining it. These first two experiments demonstrate that L-DOPA consistently reduces cocaine-taking behavior in rats, in apparent contrast with clinical studies showing little evidence of therapeutic effects resulting from administration of L-DOPA to cocaine-dependent patients in the usual therapeutic range of doses (Amato *et al.* 2011; Minozzi *et al.* 2015). Yet, clear

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evidence of safety and tolerability of L-DOPA pharmacotherapy in cocaine addicts has been reported (Mooney *et al.* 2007), and a clear benefit was observed when the dopamine precursor was associated to a robust behavioral intervention (Schmitz *et al.* 2008, 2014). Because chronic cocaine use might result in dopamine depletion (Mateo *et al.* 2005; Koob 2008) and dopaminergic agonist medications for cocaine abuse are effective in rodent (Cheung *et al.* 2012, 2013) and primate (Czoty *et al.* 2016) models of cocaine self-administration, including L-DOPA (Willuhn *et al.* 2014, Devoto *et al.* 2016, present data), search for successful pharmacological interventions targeted at stimulating dopamine neurotransmitter system in cocaine-dependent patients surely deserves further consideration (Herin *et al.* 2010).

Interestingly, in this study, we showed a significant reduction in the responding rate also in L-DOPA-treated animals during saline substitution test, in which the rewarding stimulus (i.e. cocaine) was replaced with a neutral stimulus (i.e. saline solution) on the day after the last cocaine self-administration session. Thus, in animals with a history of cocaine self-administration, L-DOPA showed its efficacy in reducing not only cocaine-taking but also cocaine-seeking behavior. Dopamine deficiency status, as that occurring following chronic exposure to cocaine, has been proposed as a condition that triggers relapse (Blum *et al.* 2015). In our study, L-DOPA diminished the reinstatement of cocaine-seeking behavior induced by a drug-associated cue presentation, in line with its ability to prevent responding for cocaine in abstinent animals exposed to a drug priming following complete extinction (Devoto *et al.* 2016). In the same animals, L-DOPA increased extracellular dopamine level in the mPFC, supporting the idea that a decreased cocaine-seeking behavior is linked to an excessive stimulation of dopamine receptors in the prelimbic cortex that might inhibit their functionality (Devoto *et al.* 2016). At a translational level, these findings strengthen the hypothesis that L-DOPA may have therapeutic efficacy under conditions of reduced cocaine intake or abstinence (Schmitz *et al.* 2008). It has been previously demonstrated that extinction training of cocaine self-administration induces plasticity in glutamate receptor expression (Ghasemzadeh *et al.* 2011) and that cue priming-induced reinstatement of cocaine-seeking critically involves the glutamatergic transmission (Uys & LaLumiere 2008). Thus, in the reinstatement test, L-DOPA exerts its effect likely by also affecting glutamatergic neurotransmission besides the dopaminergic one.

Finding that L-DOPA disturbs behaviors that necessitate dopamine neuron phasic bursts is not surprising (Grace 2000). Animal studies have consistently shown that acute L-DOPA administration stimulates tonic dopamine neurotransmission (Butcher & Engel 1969) while significantly affecting the firing of dopamine neurons under conditions of compromised dopamine functionality (Harden & Grace 1995)—as in the case of chronic cocaine self-administration. Levodopa is a dopamine precursor, and consequently its administration increases dopamine concentrations in the brain. Here, we showed that this effect is potentiated in the rat mPFC both when cocaine is self-administered by animals and when animals are primed with the cocaine-associated cue, and that extracellular dopamine level increases dramatically in L-DOPA-treated group although in these animals cocaine intake and cocaine-seeking are halved with respect to rats pretreated with saline. Notably, such an effect is evident also when L-DOPA is co-administered with cocaine to drug-naïve rats while is absent in the nucleus accumbens core, indicating a regional specificity for the combined cocaine + L-DOPA effect on dopamine release in the mPFC. We previously suggested that excessive stimulation of dopamine D1 receptors in the prelimbic cortex might inhibit their functionality, thus decreasing cocaine-seeking behavior (Devoto *et al.* 2016). Indeed, the reinstatement of cocaine-seeking in rats is thought to depend on activation of the prelimbic cortex, which facilitates the activity of glutamatergic neurons projecting to the nucleus accumbens core and thereby restores cocaine-seeking behavior (McFarland & Kalivas 2001; McGlinchey *et al.* 2016). Accordingly, inactivation of prelimbic mPFC with GABA receptor agonists, tetrodotoxin or dopaminergic antagonists blocks cocaine-, cue- and stress-primed reinstatement of cocaine-seeking in rodents (reviewed in McFarland & Kalivas 2001; Kalivas & O'Brien 2008). In cocaine-addicted state, the PFC is no more able to influence behavioral responding adaptation necessary to cope with changes in stimulus–reinforcement association (Goldstein & Volkow 2002), thus failing to control drug craving. The inhibitory control is lost, resulting in compulsive stimulus-driven behaviors, such as the perseverative and useless seeking for cocaine following exposure to the drug-associated cue (Jentsch & Taylor 1999). Our results showed that L-DOPA reinstates the correct behavioral response to a not-reinforced stimulus (e.g. in the absence of the contingent presentation of a reward), likely through a dopamine increase particularly into the mPFC (Devoto *et al.* 2016). Human brain imaging studies suggested that the cognitive control exerted through PFC could play a fundamental role in downregulating drug craving by inhibiting sub-cortical regions related to drug-seeking (Kober *et al.* 2010; Hanlon *et al.* 2015). Indeed, Kober *et al.* (2010) demonstrated that the ability to control craving is higher when PFC activity is increased and ventral striatum activity is reduced. Active inhibitory control of cocaine-seeking is reliant on neuronal

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activation of precise cortical areas also in rats engaged in discriminative stimulus-regulated cocaine self-administration (Mihindou *et al.* 2013; Navailles *et al.* 2015). Thus, by improving dopaminergic signaling selectively in the PFC, L-DOPA might facilitate activity in those areas deemed at inhibiting sub-cortical craving-related structures. This phenomenon possibly involves D1 receptors located on cortical pyramidal neurons, as activation of $G_{i/o}$ coupled DREADD receptors located on cortico-striatal pyramidal neurons in the mPFC (which decreases the activity of mPFC projections to the nucleus accumbens) diminishes drug-induced reinstatement of cocaine-seeking (Kerstetter *et al.* 2016). Alternatively, or at the same time, it could involve also the dopaminergic stimulation of GABAergic interneurons, which in turn could inhibit pyramidal neuron activity.

In conclusion, based on the rationale that cocaine use can profoundly alter dopaminergic functioning through dopamine depletion and changes in receptor functioning, and that L-DOPA refills dopamine stores, our findings support the hypothesis that L-DOPA pharmacotherapy may be helpful in reducing cocaine use and relapse rate, although further studies will be necessary to assess the efficacy of L-DOPA in animal models at lower concentrations that better mimic clinical dosages.

## Role of funding source

The funding source had no involvement in any stage of the study.

## Authors Contribution

S.A. was responsible for the acquisition and analysis of behavioral data and participated in study design and manuscript drafting. P.S. conducted microdialysis experiments. W.F. and G.L.G. participated in the study design and provided critical revisions of the manuscript. L.F. was responsible for the behavioral study design, supervised the acquisition and analysis of behavioral data and drafted the manuscript. P.D. was responsible for the study concept and for designing and analyzing the experiments as well as drafting the manuscript. All authors reviewed content and approved the final version for publication.

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## Conflict of Interest

The authors have no conflict of interest to declare.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

**Figure S1**. Horizontal motor activity in L-DOPA- or saline-treated rats during 2-hour self-administration session under FR-1 (a) and PR schedule of reinforcement (b), and during the saline substitution (c) and cue priming-induced reinstatement (d) tests. Motor activity is expressed as mean ± SEM of photocell beam breaks. No significant difference in motor activity was detected in any condition between L-DOPA and saline group (Unpaired *t* test with Welch's correction).

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