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ORIGINAL RESEARCH

Behavioral and neurochemical evaluation in rats withdrawn from repeated cocaine treatment and exposed to the forced swimming and open field tests

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ABSTRACT

Objective: We aimed to investigate possible behavioral alterations in the forced swimming (FST) and open field tests (OFT), and monoamine level changes in rats withdrawn for one and seven days from repeated cocaine administration. **Methods:** Animals were treated once a day during a week with cocaine 20 mg kg^{-1} or saline (control), intraperitoneally. Following one or seven days of cocaine administration, animals were submitted to the FST or OFT, while another one was decapitated and their brain striatum was removed for determination of dopamine (DA) and serotonin (5-HT) and their metabolites levels determined by HPLC analysis. **Results:** Cocaine was able to induce a significant decrease in the immobility time of animals in FST and a significant increase in the exploratory activity in OFT after both withdrawal times. During the abstinence periods, monoamine levels were significantly increased, while DA and 5-HT turnovers were decreased. **Conclusions:** Results indicate that 20 mg kg^{-1} of cocaine administered during 7 consecutive days to rats were not sufficiently able to promote depression-like symptoms 24 h or 7 days after treatment discontinuation.

Key words

Animal models; Biogenic monoamines; Drug withdrawal symptoms

RÉSUMÉ

Objectif: Investigation des altérations possibles du comportement lors des tests de natation forcée (TNF) et de champ ouvert (TCO) et du taux des monoamines chez des rats un et sept jours après administration répétée de cocaïne. **Méthodes:** Les animaux ont reçu une fois par jour pendant une semaine 20 mg kg^{-1} de cocaïne ou du sérum physiologique (témoin) en i.p. Après un ou sept jours d'administration de cocaïne les animaux ont été soumis au TNF ou au TCO, ou ont été décapités pour analyse par HPLC des taux de dopamine (DA), de sérotonine (5-HT) et de leurs métabolites dans le striatum. **Résultats:** La cocaïne entraîne une réduction significative du temps d'immobilité des animaux lors du TNF et une augmentation significative de l'activité exploratrice lors du TCO, lors des deux périodes de sevrage. Lors des périodes d'abstinence le taux des monoamines est significativement augmenté et le renouvellement de DA et de 5-HT est réduit. **Conclusions:** L'administration de 20 mg kg^{-1} de cocaïne à des rats pendant 7 jours consécutifs n'est pas suffisante pour produire des symptômes de dépression 24 h ou 7 jours après l'arrêt du traitement.

Mots clés

Modèles animaux; Monoamines biogéniques; Symptômes de sevrage

RESUMEN

Objetivo: Investigar las posibles alteraciones conductuales posibles en las pruebas de natación forzada (forced swimming test; FST) y de campo abierto (open field test; OFT) así como también en los niveles de monoaminas en ratas a las cuales se les retiró el consumo de cocaína después de uno y siete días de administración repetida de cocaína. **Métodos:** Los animales recibieron diariamente una dosis única de cocaína (20 mg kg^{-1}) o solución salina (control), intraperitonealmente. Después de uno o siete días de la última administración de cocaína, diferentes grupos de animales fueron sometidos a la FST o a la OFT. Otro grupo fue sacrificado y el cerebro (cuerpo estriado) fue removido para cuantificación de dopamina (DA) y serotonina (5-HT) y sus metabolitos por HPLC. **Resultados:** La administración de cocaína indujo una disminución significativa en el tiempo de inmovilidad de los animales en la FST y un aumento significativo en la actividad exploratoria en la OFT tanto al día uno como al séptimo post-administración de cocaína. Durante los períodos de abstinencia, los niveles de monoaminas aumentaron significativamente, mientras que el metabolismo de DA y 5-HT disminuyó. **Conclusión:** Los resultados indican que 20 mg kg^{-1} de cocaína administrada durante 7 días consecutivos en ratas, no fueron suficientes para producir síntomas de depresión 24 h ó 7 días después de descontinuar el tratamiento.

Palabras clave

Modelos animales; Monoaminas biogénicas; Síntomas de abstinencia a drogas

INTRODUCTION

Cocaine addiction has been a serious medical and social problem in recent years and intensive research has been carried out in an attempt to explain the behavioral and neurobiological bases of its development [1] [2] [3]. Previous studies using animal and human models have shown that cocaine withdrawal is often accompanied by a collection of symptoms including depression, anxiety, fatigue, and psychomotor retardation or agitation [4]. As a result, there is a high likelihood of relapse following prolonged periods of withdrawal [4] [5]. Such behavioral consequences have been credited to induced changes in neurotransmitters, with the dopaminergic system represented as the most responsible for the adverse effects of abstinence to cocaine [6] [7] [8]. Nevertheless, the serotonergic system also fulfills an important role in functional cocaine effects [1] [9] [10] [11].

Neurobiological studies in literature about withdrawal from cocaine administration are still conflicting. Results obtained through a combination of behavioral animal models and microdialysis have demonstrated that cocaine administration to rats concomitantly produced a progressive augmentation in motor activity (known as behavioral sensitization) and elevated DA and 5-HT release in the nucleus accumbens after discontinuing daily treatments [1] [12] [13].

On the other hand, evidence for decreased dopaminergic and serotonergic function during the withdrawal syndromes are also associated with cocaine use [14]. In contrast, in a study by Alburgues et al. [6], the concentration of monoamines was monitored (using HPLC-ECD) in rat central nervous system, following a dose schedule of 5, 10, 15, 20 and 25 mg kg^{-1}, intraperitoneally (i.p.), for 21 days; 12 h after the last cocaine injection, cortical and striatal concentrations of monoamines and their metabolites were not significantly different in saline versus cocaine-treated animals.

Since cocaine withdrawal can be associated with symptoms of depression, some studies have also evaluated the behavioral effects of animals treated with cocaine following forced swimming test [15] [16]. Among all animal models, the forced swimming test remains one of the most used tools for evaluate antidepressants drug effects [17]. In this test, drugs with established antidepressant activity, such as imipramine, reduce the time during which the animals remain immobile, while depressant drugs exert opposite effects [18].

Hayase et al. [15], using forced swimming test (FST), demonstrated that cocaine administered acutely, at high doses (75 mg kg^{-1} i.p), promoted an acceleration of behavioral despair in the Porsolt test similar to that observed in the imipramine group after the disappearance of the acute toxic symptoms (5 h after treatment).

In a subsequent study, Hayase et al. [16] verified increased immobility in the FST (depression behavior) 12 h after large doses of cocaine (60 mg kg^{-1}, i.p.) were acutely administered. In addition, Grasing & Ghosh [14] have reported similar increase of immobility duration in the FST during withdrawal from long-term amphetamine treatment, a psychostimulant drug similar to cocaine. These behavioral effects were also observed in the animals receiving large doses of amphetamine. Therefore, studies using the FST to investigate possible changes occurring after long-lasting withdrawal periods from repeated cocaine at lower doses are scarce in literature.

Thus, it is hoped that additional preclinical investigation about behavioral and neurobiological changes that persist after cocaine discontinuation can be fundamental to understand the mechanisms involved in addiction and craving. In the present study we investigated possible behavioral and monoamine-

level changes in rats one and seven days after cocaine withdrawal.

MATERIALS AND METHODS

Animals

Male Wistar rats (180-200 g) were used in each experiment and were maintained at a controlled temperature (23 ± 1°C) with a 12 h dark/light cycle and free access to water and food. Animals were treated in accordance to the current law and the NIH Guide for Care and Use of Laboratory Animals and the study was performed under the consent and surveillance of the Committee of Ethics in Animal Research, Department of Physiology and Pharmacology, Faculty of Medicine, Federal University of Ceará, Brazil.

Drugs and Experimental Protocol

Cocaine (donated from the Federal Police of Ceará State and checked for purity by the Chemistry Department of Federal University of Ceará) was dissolved in distilled water and administered i.p. to the test groups. Imipramine (IMP) 10 mg kg^{-1} (Geigy) and diazepam (DZP) 2 mg kg^{-1} (União Química/Brazil) were dissolved in distilled water and injected i.p. prior the FST and OFT, respectively, as standard drugs (Table 1). All the control animals received saline.

Forty rats were treated once a day for 7 consecutive days with cocaine i.p. 20 mg kg^{-1} and 40 other rats were treated with saline i.p. (control). On day 1 and 7 after the last cocaine administration, two distinct groups of animals (n= 10, each group) were submitted to the FST or OFT (Table 1). Similar procedures were performed to the rats treated with saline. Imipramine (10 mg kg^{-1}) or diazepam (2 mg kg^{-1}) was administered i.p. 30 minutes prior to the respective FST or OFT tests.

Another group of rats was similarly treated with cocaine, and 24 h (n= 8) or 7 days (n= 8) after the last administration, animals were decapitated and their brain striatum removed for determination of monoamines and metabolites levels (Table 1). Similar procedures were performed to another set of rats treated with saline.

Table 1 Experimental Protocol

Treatments	Treatment period	Behavioral tests			
		Forced Swimming Test[a]		Open Field Test[b]	
		Group 1 (n= 10)	Group 2 (n= 10)	Group 3 (n= 10)	Group 4 (n= 10)
		24 h after the last administration	7 d after the last administration	24 h after the last administration	7 d after the last administration
Saline (control)	7 days	X	X	X	X
Cocaine (20 mg kg^{-1})	7 days	X	X	X	X

Animals received either [a]imipramine (10 mg kg^{-1}) or [b]diazepam (2 mg kg^{-1}) i.p., 30 min prior the tests

Treatments	Treatment period	Monoamines and metabolites levels[c]	
		Group 5 (n= 8)	Group 6 (n= 8)
		24 h after the last administration	7 d after the last administration
Saline (control)	7 days	X	X
Cocaine (20 mg kg^{-1})	7 days	X	X

[c]Animals were decapitated and monoamine and metabolite levels were obtained in the brain striatum.

Forced Swimming Test

The Porsolt et al. [18] swimming test includes two exposures to a water tank, spaced 1 day apart. For the experiments, a vertical glass cylinder (diameter 22.5 cm and height 60 cm) containing about 35 cm water at 25ºC was used. During the first exposure, rats were placed in the cylinder and monitored for 15 min. During the second exposure (test session), animals were placed in the tank and monitored for 5 min during which immobility time was registered.

A rat was considered immobile when it remained floating in the water, without struggling, making only very slight movements necessary to keep its head above water. This prolonged floating time (immobility time) during the second exposure to the tank has been interpreted by Porsolt as reflecting the animal's state of despair [17], elicited by the inescapable nature of the tank, which was learned during pretest. Drugs with antidepressant activity reduce that parameter (immobility time), while depressant ones exert opposite effects.

The animals were divided into four groups (Control, COC-24h, COC-7d and IMI-10) of 8-10 animals per group. Each animal was used only once.

Open Field Test

The open-field area was made of acrylic, with transparent walls, black floor and divided into nine squares of equal area. This apparatus was used to evaluate the exploratory activity of the animal [19]. The observed parameter was the number of squares crossed (with the four paws) during five minutes.

HPLC Analysis

The striatum of control animals (n= 10) and cocaine group (n= 7) were used for the preparation of a 10% homogenate (10%, w/v). Brain tissue samples were sonicated in 0.5–1 mL of 0.1 M perchloric acid ($HClO_4$, Qeel, SP, Brazil) for 30 s and centrifuged for 15 min at 26.000 *g*, at 4°C. Then, a 20 µL supernatant aliquot was injected directly into the high performance liquid chromatography (HPLC) column. For the monoamines analyses, a CLC-ODS (M) Shimadzu column was used. The mobile phase was 0.163 M citric acid (Vetec, RJ, Brazil), pH 3.0, containing 0.02mM of ethylenediaminetetraacetic acid (EDTA, Vetec, RJ, Brazil), with 0.69mM sodium octanesulfonic acid (SOS-Sigma, St. Louis, MO, USA), as ion pairing reagent, 4% (v/v) acetonitrile (Carlo Erba Reagenti, MI, Italy) and 1.7% (v/v) tetrahydrofurane (Sigma). Dopamine (DA), 4-hydroxy-3-methoxyphenylacetic acid (DOPAC), serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA) and homovanilic acid (HVA) were electrochemically detected using an amperometric detector (Model L-ECD-6A; Shimadzu Corp., Japan) by oxidation on a glassy carbon electrode at 0.85V relative to an Ag–AgCl reference electrode.

Amounts of neurotransmitters and metabolites in the supernatants were calculated by comparing their peak heights with that of standards determined the same day. Additionally, DA turnover was expressed as the ratio of DOPAC/DA (intracellular DA turnover) or HVA/DA (extracellular DA turnover), while 5-HT turnover as the ratio of 5-HT/5-HIAA.

Statistical analyses

All results are presented as mean ± S.E.M. Data were analyzed by ANOVA followed by Student-Newman-Keuls post-hoc test or by Student-t test. Results were considered significant at $p<0.05$.

RESULTS

Cocaine was able to induce a significant decrease in the immobility time in rats, as compared to control group (Figure 1). Imipramine, as expected of an antidepressive drug, promoted similar effects [$F(3,33)= 41.1$; $P <0.001$].

In relation to monoamine levels (Figure 2), there was a significant 42% [$T(10)= 3.2$; $P <0.01$] and 37% [$T(12)= 4.9$; $P <0.001$] increase in DA levels one and seven days after the last cocaine administration, respectively, as compared to control animals. A decrease of 28% [$T(10)= 4.0$; $P <0.01$] and 32% [$T(11)= 5.3$; $P <0.001$] was observed in DOPAC levels, while HVA was decreased by 30% [$T(10)= 3.3$; $P <0.01$] and 44% [$T(11)= 4.7$; $P <0.001$] following both withdrawals periods, respectively. Similar to the DA, 5-HT levels increased by 102% [$T(9)= 3.2$; $P <0.01$] and 346% ($T(9)= 12.9$; $P <0.001$) one and seven days after the last cocaine administration, as compared to control, respectively. Its metabolite, 5-HIAA, also increased 45% [$T(10)= 3.7$; $P <0.01$] and 31% [$T(9)= 3.2$; $P <0.05$], respectively, after both periods. Table 2 shows the DA and 5-HT turnover; the DOPAC/DA ratio was decreased 50% [$T(8)= 5.6$; $P <0.001$] from control following one abstinence day and 51% [$T(10)= 6.9$; $P <0.001$] after seven abstinence days. A decrease of 53% [$T(8)= 5.4$; $P <0.001$ $t=5.4$ $df=8$] and 58% [$T(10)= 6.5$; $P <0.001$] was observed in the HVA/DA ratio following both withdrawal times, respectively. 5-HIAA/5-HT ratio was decreased by 32% [$T(9)= 2.4$; $P <0.5$] and 73% [$T(9)= 5.5$; $P <0.001$] following one and seven days after the last cocaine administration, respectively.

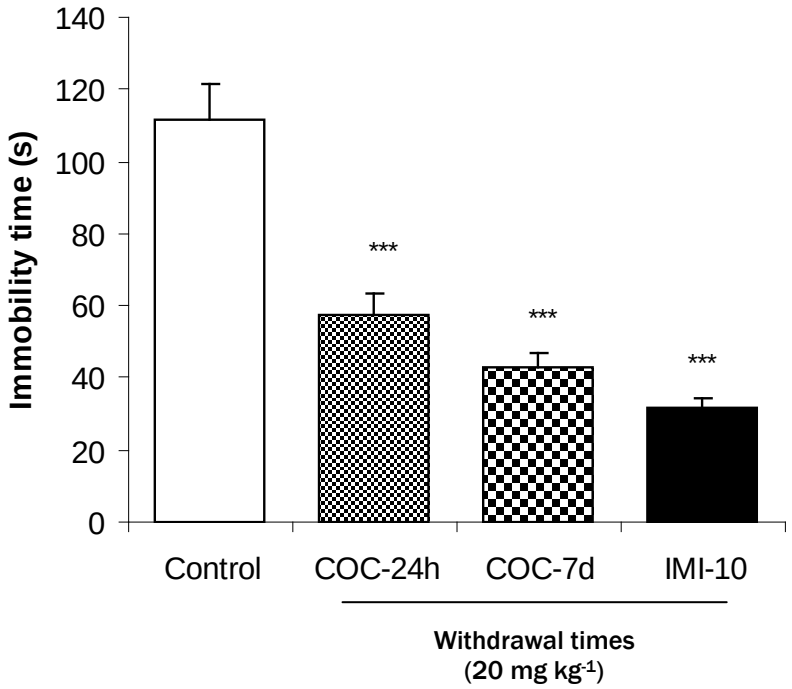

Figure 1 **Forced swimming of groups of rat that received saline solution (control), cocaine 20 mg kg^{-1} (COC-24h and COC-7d) or imipramine 10 mg kg^{-1} (IMI-10). Animals received cocaine (20 mg kg^{-1}) during 7 days and were withdrawn one (COC-24h) or seven (COC-7d) days after the last cocaine administration. The figure shows immobility time (seconds) and results are presented as mean ± S.E.M. Significant difference compared with control: ***P<0.001; ANOVA followed by *post-hoc* Student-Newman-Keuls test.**

Table 2 **Determination of DA and 5-HT turnover from monoamines levels and their metabolites**

	Monoamines turnover one and seven days after repeated cocaine (20 mg kg^{-1}) discontinuation			
Ratio of Monoamines and their metabolites	**Control**	**COC-24h**	**Control**	**COC-7d**
DOPAC/DA	**0.92 ± 0. 075**	**0.45 ± 0.037****	**0. 91 ± 0.059**	**0.44 ± 0.031****
HVA/DA	**0.43 ± 0.041**	**0.20 ± 0.010****	**0.42 ± 0.036**	**0.17 ± 0.010****
5-HIAA/5-HT	**4.95 ± 0.685**	**3.34 ± 0.199***	**4.49 ± 0,653**	**1.20 ± 0.037****

DA turnover was expressed as the ratio of DOPAC/DA (intracellular DA turnover) or HVA/DA (extracellular DA turnover), while 5-HT turnover as the ratio of 5-HT/5-HIAA.
DA= dopamine, DOPAC= acid dihidroxifenilacetic, HVA= acid homovanilic, 5-HT= serotonin, 5-HIAA= acid 5-hidroxiindolacetic.
The results are presented as mean ± S.E.M. *P<0.05; **P<0.001; significant difference compared with control (Student t test).

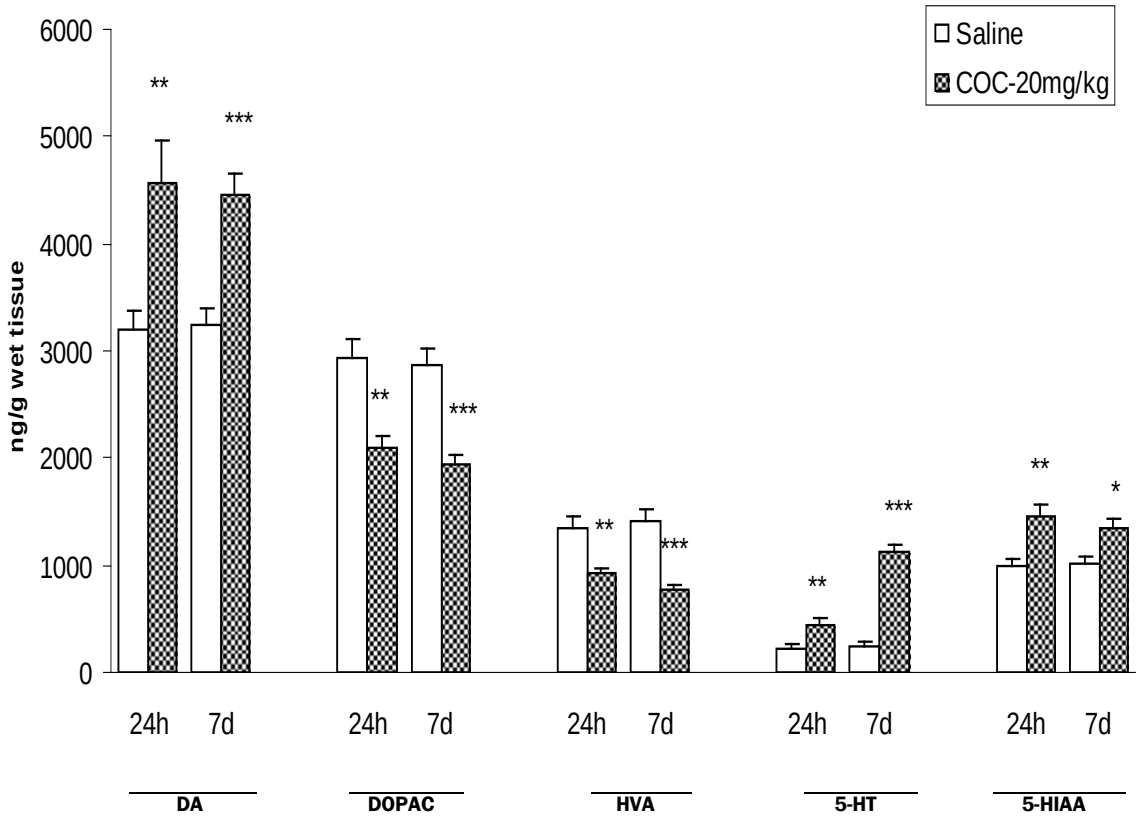

Figure 2 **Determination of monoamine levels and their metabolites in striatum of rats 24h and 7 days after the last cocaine administration. Animals received cocaine (20 mg kg^{-1}) or saline, i.p., during seven days. DA: dopamine, DOPAC: acid dihidroxifenilacetic, HVA: acid homovanilic, 5-HT: serotonin, 5-HIAA: acid 5-hidroxiindolacetic.**
The results are presented as mean ± S.E.M. *P<0.05; **P<0.01; ***P<0.001; significant differences compared with control (Student t test).

Effects of cocaine or diazepam in the OFT are presented in Figure 3. Both withdrawal times (one and seven days) from repeated cocaine treatment increased the number of crossings compared to controls; this parameter decreased in animals treated with diazepam (2 mg kg^{-1}) [F(3,49)= 41.0, P <0.05].

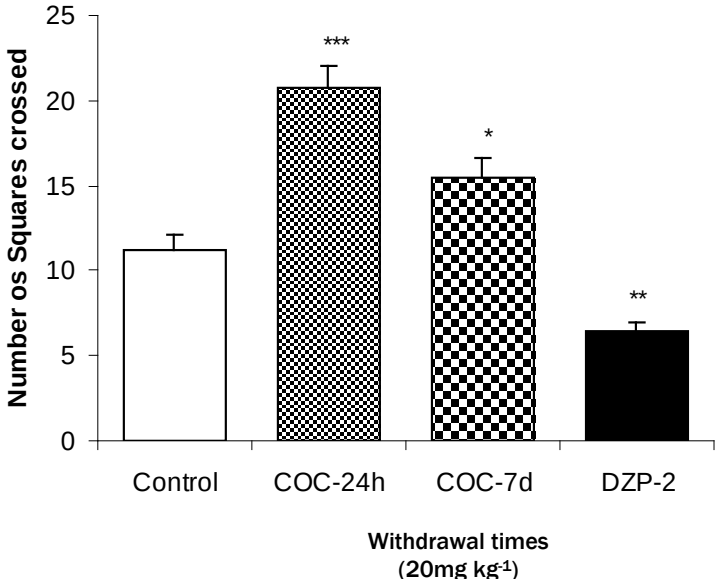

Figure 3 Open-field test of groups of rat which received saline (control), cocaine 20 mg kg^{-1} (COC-24h and COC-7d) or DZP (2 mg kg^{-1}). Animals received cocaine (20 mg kg^{-1}) during 7 days and were withdrawn one (COC-24h) or seven (COC-7d) days after the last cocaine administration. The results are presented as mean ± S.E.M. Significant difference compared with control: *P <0.05; **P <0.01; *** P <0.001; ANOVA followed by *post-hoc* Student-Newman-Keuls test.

DISCUSSION

It is well established that cocaine exerts its action through binding with monoamine presynaptic plasma membrane transporters, and blocking their uptake [20]. Therefore, behavioral effects of drug use have been related to the increase in extraneuronal levels of DA and 5-HT in central nervous system (CNS) [21] [22]. On the other hand, both preclinical and human data suggest that depression symptoms from repeated cocaine withdrawal are the most aggravated, indicating a drop or disruption of DA and 5-HT neurotransmission secondary to cocaine withdrawal [1] [23].

In this way, the present study investigated whether significant behavioral or monoamine level changes could be observed in rats treated with cocaine, at 20 mg kg^{-1} dose, following two different withdrawal times.

FST was developed by Porsolt in rats and mice and is the most widely used tool for assessing antidepressant activity in a preclinical setting [24]. In addition, this test has been exploited for assessing animal models of depression induced by pharmacological manipulation [25]. Our results from FST showed that rats treated with cocaine (20 mg kg^{-1}) during seven consecutive days, displayed significantly increased mobility (i.e. spent more time swimming) one day after drug abstinence, demonstrating a nonspecific anti-immobility effect related to hyperactivity.

These findings did not agree with those showed by Hayase and collaborators [16], who verified depressive behavior in FST 12h after cocaine treatment. However, those authors used a much higher drug dose (60 mg kg^{-1} i.p) as compared to that used in present study, and treatments using high doses may induce severe behavioral, neurochemical, and anatomical adaptations leading to a more pronounced withdrawal syndrome, including measurable depression-like behavioral changes [26].

Although our results oppose the general assumption that cocaine withdrawal induces behavioral markers of depression-like states, they are in accord with those of Erhardt et al. [27], who reported that mice acutely treated with cocaine 5, 10 and 20 mg kg^{-1} presented higher locomotion in FST 1, 5 and 24h after the treatment. Also, similar results were observed between our findings and those reported by Hédou and collaborators [26]. These authors demonstrated that 2 days of withdrawal from the repeated administration of cocaine over a 5-day period were not able to induce depressive-like behavior in rats in FST.

Grasing and Ghosh [14] showed that opiate withdrawal increased immobility in response to a forced warm water swim test performed during the second and third weeks of abstinence following the withdrawal. Such behavioral alterations were related to long-term changes in dopaminergic function following opiate withdrawal.

As both cocaine and opiates present similar action and addiction mechanisms, we decided to investigate whether a larger period of cocaine abstinence would also be able to promote behavioral changes in animals submitted to FST, as observed with opiates [14]. Similar to initial findings in this study after one day of abstinence, animals also significantly increased mobility in the FST following seven days of cocaine discontinuation. Taken together, our results suggest that, possibly, 20 mg kg^{-1} of cocaine administered during 7 consecutive days were not sufficiently able to promote depression-like symptoms 24h or 7 days after treatment discontinuation.

The two neurotransmitter systems that may be most critically involved in the association between depression and stimulant dependence may be serotonin and dopamine [11], and it is well known that the striatum is the main site of cocaine's action in the brain [8]. Therefore, to corroborate the behavioral changes observed in the forced swimming test, we decided to also quantify monoamine (DA and 5-HT) and metabolite (DOPAC, HVA and 5-HIAA, respectively) levels in brain striatum of rats submitted to the same treatment regime and period of withdrawal previously described. In order to avoid possible monoamine level alterations related to stress from FST [28], we resolved to use a distinct animal group for quantifying monoamines.

Results obtained by HPLC analysis showed an increase in DA and 5-HT levels in rats' brain striatum one and seven days after the cocaine discontinuation, while a decrease was seen in DOPAC and HVA metabolites in both abstinence periods, and 5-HIAA was increased. Our findings are in disagreement with studies which present evidence for decreased dopaminergic and serotonergic function during the withdrawal syndromes [14], as well as to those that report lack of change in basal monoamines after cessation of cocaine treatment [6]. On the other hand, our results are in accordance with some studies which have assumed that DA and 5-HT release is elevated in the nucleus accumbens after long-lasting cocaine discontinuation [12] and also corroborate with the behavioral alterations initially observed in the present study in FST.

The major theory of depressive disorder, the monoamine hypothesis, proposes that decreasing the levels of one or more of the brain monoamine neurotransmitters, such as 5-HT, noradrenaline, or DA, can produce such disturbances [24]. Therefore, the increase of monoamines observed in present study one and seven days after cocaine discontinuation supports our hypothesis generated from FST, that cocaine at relatively low doses is not enough to promote depression symptoms after short abstinence periods.

In order to understand, at least in part, the potential mechanism that may underlie the increased monoamine levels measured by HPLC analysis, we determined the intracellular and extracellular DA turnover, as well as 5-HT turnover. Our results showed decreased DA and 5-HT turnover in both withdrawal times observed. Such decrease in monoamine turnover can begin to explain the increased monoamine levels detected in present study. It is known that cocaine blocks the uptake of monoamines during administration and, therefore, there is an increase in extracellular levels of these neurotransmitters [21]. However, prolonged blockade of reuptake may cause depletion of monoamines [6].

Thus, it is reasonable to hypothesize that, at the pharmacological scheme evaluated herein, a decreased monoamine degradation could be occurring in the abstinence periods as a compensatory mechanism to the blockade in the reuptake of neurotransmitters promoted by repeated administrations of cocaine.

Data from literature show that drugs that increase general motor activity may provide significantly less immobility in FST [29]. In order to test our previous hypothesis, we decided to study the effects of early (one day) and long last (seven days) withdrawal from repeated cocaine intake by monitoring the number of squares crossed on open-field test, a classical animal model used to evaluate autonomic effects of drugs and general activity of animals [30].

Our study showed that in both abstinence times cocaine significantly increased the motor activity in animals, differently from DZP (2 mg kg^{-1}), which decreased this parameter. Therefore, the effects previously observed in FST are based on the stimulation of general motor activity, suggesting that animals stayed stimulated one and seven days following the cocaine abstinence, probably due to elevated monoamines levels observed in both withdraw periods.

CONCLUSION

In summary, animals did not present depressant-like behavior in FST and OFT and monoamine levels remained increased 24 h and 7 days after discontinuation from cocaine (20 mg kg^{-1}) sub-chronic treatment, suggesting that such therapeutic scheme and abstinence periods were not enough to promote depression symptoms in animals. Thus, the critical dosage and treatment duration to induce a withdrawal syndrome detectable by the FST and OFT requires further investigation.

AUTHORS' PARTICIPATION

MI GS, MC dOC & FC FdS designed the study; MI GS, MR AN, BA M, MM dFF & D SM performed the behavioral experiments; MI GS, MC dOC, J FdA, SM MdV & P FdV performed the neurochemical experiments; MI GS drafted the manuscript; GS dBV & FC FdS guided and reviewed the manuscript.

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CONFLICT OF INTERESTS/DISCLAIMERS

FC FdS is member of the Editorial Board. There are no other conflicts of interests to declare.

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