# LONG-TERM EFFECTS OF CHRONIC COCAINE EXPOSURE THROUGHOUT ADOLESCENCE ON ANXIETY AND STRESS RESPONSIVITY IN A WISTAR RAT MODEL

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**Abstract—Adolescents display increased vulnerability to engage in drug experimentation. This is often considered a risk factor for later drug abuse. In this scenario, the permanent effects of cocaine exposure during adolescence on anxiety levels and stress responsivity, which may result in behavioral phenotypes prone to addiction, are now starting to be unveiled. Thus, the purpose of the present study was to evaluate the long-lasting effects of chronic cocaine administration during adolescence, on anxiety-like behavior and on stress response. Adolescent male Wistar rats were daily administered 45-mg cocaine/kg of body weight in three equal intraperitoneal doses with 1-h interval, from postnatal day 35 to 50. The effects of cocaine administration on anxiety levels, assessed in the Elevated Plus Maze (EPM), and on social stress response, assessed in the resident–intruder paradigm (R/I), were evaluated 10 days after withdrawal, when rats were reaching the adulthood. The underlying dopaminergic activity, and the corticosterone and testosterone levels were determined. Our results showed that cocaine induced long-lasting alterations in the hypothalamus–pituitary–adrenals (HPA) axis function and in testosterone levels. Such alterations resulted in significant and enduring changes in behavioral responses to environmental challenges, such as the EPM and R/I, including the evaluation of potential threats that may lead to high-risk behavior and low-benefit choices. This was further supported by an altered dopaminergic function in the amygdala and hippocampus. The present findings provide new insights into how the use of cocaine during adolescent development may modulate emotional behavior later in life. Compromised ability to recognize and deal with potential threats is an important risk factor to perpetuate compulsive drug seeking and relapse susceptibility.**

**Key words: adolescence, anxiety, cocaine, corticosterone, dopaminergic system, social stress.**

## INTRODUCTION

The complex and everlasting features of addiction determine its chronic nature, where the risk of relapse is present even after long withdrawal periods. In drug addiction, several risk factors were identified as promoters of relapse during withdrawal periods, and among these, high anxiety levels and stress are commonly reported (Shaham et al., 2000; Brown et al., 2012; Buffalari et al., 2012). As relapse is prevalent in recovery periods, a better understanding of the involved risk factors will promote the development of more effective treatment strategies in drug addiction.

The psychostimulant effects of cocaine are mainly due to its action on the mesocorticolimbic monoaminergic system (Ungless et al., 2001; Nogueira et al., 2006; Gu et al., 2010; Zhang et al., 2012), a neural pathway involved in the processing of affective information and emotional regulation (Kelley and Berridge, 2002). Exposure to cocaine induces a neuroadaptational process that will lead to altered emotional behavior (Young et al., 2011), such as increased anxiety levels and impaired stress responsivity. In cocaine abusers, high levels of anxiety were reported after cessation of cocaine use (Gawin and Kleber, 1986; Satel et al., 1991; Coffey et al., 2000). In animal studies, elevated anxiety levels were shown in withdrawal periods, either in initial stages (Harris and Aston-Jones, 1993; Sarnyai et al., 1995) or after long periods (Sobrian et al., 2003; Salas-Ramirez et al., 2010). Importantly, treatments that target the anxi-

Q2 *Abbreviations:* ANOVA, analysis of variance; DA, dopamine; DAT, dopamine transporter; DOPAC, 3,4-dihydroxyphenylacetic acid; EDTA, ethylenediaminetetraacetic acid; EPM, Elevated Plus Maze; GAPDH, glyceraldahyde-3-phosphate-dehydrogenase; HPA, hypothalamus–pituitary–adrenals; HVA, homovanillic acid/4-hydroxy-3-methoxyphenylacetic acid; Nac, nucleus accumbens; PND, postnatal day; qRT-PCR, quantitative real-time polymerase chain reaction; R/I, Resident– intruder paradigm; TH, tyrosine hydroxylase; VTA/SN, ventral mesencephalon (comprising the ventral tegmental area and substantia nigra).

ety symptoms effectively reduced the risk of cocaine reinstatement (Buffalari et al., 2012), which supports the relevance of anxiety in the etiology of cocaine addiction.

In humans, there is also evidence supporting a link between stress exposure and addiction (Zaslav, 1994; Jacobsen et al., 2001; Mahoney et al., 2012). In rats, prior exposure to stressful stimuli was shown to result in facilitated acquisition (Piazza et al., 1990; Haney et al., 1995; Goeders and Guerin, 1996), maintenance (Miczek and Mutschler, 1996), and reinstatement of cocaine-seeking (Erb et al., 1998; Brown et al., 2012). Cocaine exposure was also shown to modify the stress response (Chaplin et al., 2010; Sithisarn et al., 2011). In agreement, abstinent cocaine-dependent individuals showed enhanced responsivity to stress (Fox et al., 2008). In adult rodents, cocaine-exposure also led to increased stress reactivity (Aujla et al., 2008), as evidenced by enhanced corticosterone response to both the forced swim and restraint stress test during the withdrawal period (Mantsch et al., 2007; Cleck et al., 2008).

Early drug use in the adolescence or young adulthood has been reported as a predictor of later drug abuse (Anthony and Petronis, 1995; Chen et al., 2009). In the adolescent, the undergoing psychophysiological changes (Spear, 2000; Giedd et al., 2009), may lead to increased susceptibility to the consequences of drugs of abuse (Spear, 2000; Andersen, 2003). The adolescent neurodevelopment is characterized by a period of profound structural maturation of the forebrain, associated to increased influence of the motivational drives for novel experiences, in a background of immature inhibitory control due to hypofunctionality of the medial prefrontal cortex (Chambers et al., 2003; Casey and Jones, 2010). This may predispose the adolescent to greater impulsivity and risk-taking, and increase the probability of drug experimentation (Spear, 2000; Chambers et al., 2003; Kuhn et al., 2013). Earlier behavioral studies using locomotor sensitization, conditioned place preference and self-administration paradigms, indicated that adolescents are more vulnerable to the detrimental effects, as well as to the rewarding and reinforcing properties, of addictive drugs (see reviews by Tirelli et al., 2003; Leslie et al., 2004; Barron et al., 2005). Increased vulnerability to cocaine reward during adolescence was recently further supported in an electrophysiological study by Wong et al. (2013), where reward unbalance in this stage is associated with heightened activity of the dopaminergic neurons.

A current major issue yet not fully addressed, is whether the action of addictive drugs in the adolescent developing brain will lead to enduring changes that may compromise adult behavior, increasing vulnerability to addiction (Kuhn et al., 2013). Recent imagiology studies in mice have shown that cocaine induces morphological changes in brain regions implicated in addiction that are more pronounced when exposure occurs in adolescence (Wheeler et al., 2013). The same study has also shown that cocaine-exposure in the adolescent led to increased locomotor sensitization in the adulthood (Wheeler et al., 2013), suggesting that cocaine-induced neuromorphological changes lead to persistent drug-related behaviors. Another recent study, reported a functional impairment of the prefrontal GABAergic network as a consequence of cocaine-exposure in the adolescence, resulting in a lasting disinhibition of the medial prefrontal cortex (Cass et al., 2013). Compromised medial prefrontal cortex function predicts the development of impulsive behavior and impaired decision-making, which are behavioral traits associated with increased susceptibility for substance abuse (Cass et al., 2013; Kuhn et al., 2013).

The present study aims to provide new insights into how the use of cocaine during adolescence may persistently affect stress response and anxiety and modulate behavioral responses later on. Altered response to environmental challenges representing potential threats, may lead to impaired risk-taking behavior and perpetuate compulsive drug seeking and relapse susceptibility. Therefore, in the present work, we used a rat model of chronic cocaine administration throughout adolescence to evaluate anxiety-like behavior and social stress responsivity in the Elevated Plus Maze (EPM) or the resident–intruder paradigm (R/I). The underlying corticosterone response and the testosterone levels were evaluated. The dopaminergic function in relevant brain regions was also assessed. All evaluations were conducted 10 days after the end of the administration period, when rats were reaching the adulthood.

## EXPERIMENTAL PROCEDURES

### Animal model

A total of 68 males born from primiparous three-month-old Wistar female rats acquired from Charles River Laboratories España S.A. (Barcelona, Spain) were used in this study. Animals were kept under stable conditions (20–22 °C, 60% humidity and 12-h light/dark cycle), with water and appropriate food supplied *ad libitum*. Cylindrical plastic tubes and soft paper for nest construction were made available to reduce stress. All procedures used were approved by local ethics committee and by the Portuguese Agency for Animal Welfare, general board of Veterinary Medicine, in compliance with the European Community Council Directive of September 22, 2010 (2010/63/UE). All procedures involving animals were conducted by FELASA C graded researchers, and all efforts were made to minimize the number of animals used and their suffering. Rats were randomly assigned to different experimental groups and treated following a binge pattern administration of cocaine from postnatal day (PND) 35 to PND 50. This administration period was selected to match the onset of mid-adolescence and extend into the late-adolescence period, during which intense reorganization of the mesocorticolimbic dopamine system is occurring (Andersen, 2003; Varlinskaya and Spear, 2008). These animals received three daily administrations of 15-mg cocaine/kg of body weight, administrated in a volume of 1 mL/kg and injected intraperitoneally every hour between 9:00 and 11:00 a.m. This dosing schedule was selected to mimic a frequent pattern of cocaine self-administration in humans (Quinones-Jenab et al., 2000). Moreover, the daily dose

of 3 × 15 mg/kg of cocaine was previously demonstrated to induce neurobiological and behavioral alterations in rats (Tsukada et al., 1996; Sarnyai et al., 1998; Schlussman et al., 2002; Zhou et al., 2005), and conditioned place preference in C57BL/6J mice (Zhang et al., 2002). Control animals received equal doses of NaCl vehicle (0.9% w/v) following the same protocol of administration. Cocaine hydrochloride was supplied by Sigma (St. Louis, MO, USA). The assessment of cocaine effects was performed 10 days after withdrawal, a time-point that matches the beginning of adulthood (Andersen, 2003). Furthermore, persistent effects of exposure to drugs of abuse are often reported after a 10-day withdrawal period (Zhou et al., 1996; Sarnyai et al., 1998; Kimmel et al., 2003).

### EPM

Anxiety-like behavior was evaluated in the EPM, a well validated model, which exploits the conflict between the innate tendency of the rat to explore novel areas and its aversion for heights and open spaces (Rodgers and Cole, 1994). Ten days after withdrawal both cocaine- and saline-treated rats ($n$ = 7/8 per group) were submitted to the EPM. The EPM apparatus consisted of four 46-cm-long and 14-cm-wide arms, two of them open and the other two with 22-cm–high walls. The maze was mounted 74 cm above the floor under fluorescent light (300-Lux). Rats were placed individually on the central portion of the EPM apparatus, facing an open arm, and allowed to explore it for 5 min. The test was recorded using a video camera placed 1 m above the apparatus. Videotapes were analyzed using the Noldus Observer software (Observer 5; Noldus Information Technology, Wageningen, Netherlands). Spatiotemporal and behavioral parameters were recorded. Spatiotemporal measures comprised the frequencies of total, open arm, closed arm and central platform entries, the percentage of open arm entries (open/total × 100) and the time spent in the open, closed and central parts of the maze. Behavioral measures comprised: (1) frequency and duration scores for rearing (vertical movements, animal sustained in posterior paws and with anterior paws against or not to the walls); (2) head dipping (exploratory movement of head and shoulders over the edge of the maze); (3) and grooming (licking the paws, washing movements over the head, fur licking, tail and genitals). Behavioral analysis was performed by a trained observer under blinded conditions.

### R/I

Ten days after the end of the exposure protocol, cocaine- and saline-treated rats (eight per group) were submitted to the R/I as described by Miczek (1979), to evaluate the response to social stress. The test was conducted during the dark phase of the light cycle, with the experimental animal always being the resident and the intruder an unfamiliar, non-experimental age-matched male rat, of similar weight. The resident rat, in its home cage, was allowed to habituate to the test room for 40 min before the unfamiliar intruder was presented. Interaction between subjects was videotaped for 10 min, with red light illumination in the recording area. Sessions were analyzed using the Noldus Observer software (Observer 5 Noldus Information Technology, Wageningen, Netherlands). Frequency and latency data were collected for the following behavioral categories: (1) offensive behaviors, including attacking, biting, pinning, wrestling, chasing and aggressive grooming; (2) defensive behaviors, including flight, contact avoidance and submissive-supine posture; and (3) social investigation behaviors, including sniffing, anogenital sniffing and approach behavior. These behaviors were defined as: attack – the resident lunges at the partner with its forepaws extended outward; pinning – resident places intruder in a supine position; wrestling – the two animals roll and tumble with one another; chasing – the experimental animal rapidly pursues the intruder; aggressive grooming – vigorous grooming of the immobile partner using the teeth and the forepaws; flight– the experimental animal was pursued by the intruder; supine posture – the intruder stands over the exposed ventral area of the resident animal, pressing it against the floor; contact avoidance – resident moved away from the intruder; anogenital sniffing – the resident sniffs the intruder's anogenital region; approach behavior – the resident moves toward the intruder. Behavioral analysis was performed by a trained observer under blinded conditions.

### Neurochemical determinations

Levels of dopamine (DA) and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were evaluated after each behavioral test by high-performance liquid chromatography combined with electrochemical detection (HPLC/EC, Gilson, Inc., Middleton, WI, USA). An analytical column (Supelco Supelcosil LC-18 3 μM; 7.5 cm × 4.6 mm; flow rate: 0.8–1.0 mL/min; Supelco, Bellefonte, PA, USA), was used as previously described (Alves et al., 2009). In order to assess the basal levels both in cocaine- and saline-treated animals, DA, DOPAC and HVA levels were measured 10 days after withdrawal in groups of six animals that did not perform any behavioral test. Rats were killed by decapitation and brains were rapidly removed and dissected on ice following orientation marks provided by Paxinos and Watson (1998). The following regions were collected: nucleus accumbens (Nac), dorsal striatum, hippocampus, amygdala and ventral mesencephalon (comprising the ventral tegmental area and substantia nigra, VTA/SN). Tissue samples were frozen by immersion in 4-methylbutane cooled over dry ice and stored at −70 °C until used. Concentrations of neurotransmitters were calculated using standard curves. Standards were purchased from Sigma (St. Louis, MO, USA). Final results were expressed as monoamine content per amount of protein. Protein content was determined by the Bio-Rad protein assay (Munchen, Germany).

### Quantitative real-time polymerase chain reaction (qRT-PCR)

Ten days after the end of the exposure protocol, the expression levels of mRNA transcripts for tyrosine

hydroxylase (TH) were measured in the VTA/SN and for DA transporter (DAT) were measured in the VTA/SN, Nac, dorsal striatum, hippocampus and amygdala by qRT-PCR, both in cocaine- and saline-treated animals (six animals per group). Rats were killed by decapitation and the brain areas were dissected as described above. Total RNA was isolated using the RNeasy® Lipid Tissue Mini kit (Qiagen, Austin, TX, USA) according to the manufacturer's specifications. RNA purity was estimated from the ratio of absorbance readings at 260 and 280 nm and only a ratio between 1.8 and 2 was accepted. RNA quality was confirmed in an agarose gel and RNA concentration was determined in a NanoDrop spectrophotometer (NanoDrop™ 1000 Spectrophotometer, Thermo Fisher Scientific, Wilmington, DE, USA). One microgram of RNA was then reverse transcribed, using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA), using oligo(dt) primers and following the instructions of the manufacturer. The reference gene, glyceraldahyde-3-phosphate-dehydrogenase (GAPDH) was used as internal standard for normalization. The qRT-PCR reactions, using equal amounts of total RNA from each sample, were performed on the iQ5 Multicolor Real-time PCR Detection System from Bio-Rad (Hercules, CA, USA), using the iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), as follows: one cycle of 95 °C for 15 min followed by 40 cycles of 95 °C for 30 s and 52–65 °C for 1 min (according to the primer annealing temperature), ending with a melting curve analysis to control for the amplification of a single gene product. Product fluorescence was detected at the end of the elongation cycle. Primer design was performed using the software Beacon Designer (Premier Biosoft International, Palo Alto, CA, USA). The primers used were as follows: TH sense primer: 5′-ggc ttc tct gac cag gtg tat c-3′; TH antisense primer: 5′-caa tct ctt ccg ctg tgt att cc-3′; DAT sense primer: 5′-gtc att gtt ctg ctc tac ttc-3′; DAT antisense primer: 5′-gtc cac act gag gta tgc-3′; GAPDH sense primer: 5′-ttc aac ggc aca gtc aag g-3′; GAPDH antisense primer: 5′-ctc agc acc agc atc acc-3'. mRNA quantification was performed by comparative threshold cycle quantification ($\Delta C_t$ method) using GAPDH as a reference gene.

### Western blot analysis

Ten days after the end of the exposure protocol, the protein levels of TH were measured in the VTA/SN ($n$ = 7/6 per group). Rats were killed by decapitation, the VTA/SN was dissected as described above and homogenized in lysis buffer (50 mM Tris/HCl, 150 mM NaCl, 2 mM EDTA, 1% Triton-100, 0.5% NP-40 and 1:500 Protease Inhibitor Cocktail (Sigma St. Louis, MO, USA)). The homogenate was incubated on ice during 1 h and was then centrifuged at 16,000$g$ for 10 min at 4 °C. Twenty-five micrograms of protein supernatant was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a polyvinylidene fluoride membrane (0.2 μm; Bio-Rad). Rabbit anti-TH antibody (1:1000, Millipore, Billerica, MA, USA), mouse anti-GAPDH antibody (1:30,000; HyTest, Turku, Finland), horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:10,000, Sigma, St. Louis, MO, USA) and goat anti-mouse IgG antibodies (1:10,000, Thermo Scientific, USA) were used. Immunoreactive proteins were revealed using enhanced chemiluminescence method (Amersham, ECL Prime Western Blotting Detection Reagent, GE Healthcare, UK). Blots were analyzed with Quantity One 1-D Analysis Software, version 4.6 (Bio-Rad).

### Quantification of corticosterone and testosterone plasma levels

Corticosterone levels were measured after the EPM or R/I tests. Additionally, to assess the basal levels both in cocaine- and saline-treated rats, corticosterone and testosterone plasma levels were also measured 10 days after treatment, in animals that did not perform any behavioral test. Animals were killed by decapitation and trunk blood was collected into lithium heparin covered tubes and centrifuged at 1600$g$ for 15 min to obtain the plasma fraction. The plasma was kept at −70 °C until hormonal assays. Hormonal levels were measured by enzyme immunoassay, using commercially available kits for corticosterone (Immunodiagnostic Systems Ltd, Boldon, UK) and for testosterone (Cayman Chemical Company, Ann Arbor, MI, USA). Gonadal index was considered as the weight of the gonads as a percent of the total body weight.

### Statistic analyses

Weight evolution was analyzed using an analysis of variance (ANOVA) with repeated measures. Student's $t$-test was used to compare behavioral data, testosterone levels, gonadal weight, as well as TH protein and mRNA expression levels and DAT mRNA expression levels between cocaine- and saline-treated animals. A two-way ANOVA (behavior test × treatment) was used to compare corticosterone levels between groups and post hoc comparisons were performed using Tukey's test. For the neurochemical data, since variables did not pass the homogeneity of variance test and did not follow a normal distribution, the analysis was performed using the non-parametric Kruskal–Wallis test followed by the Mann–Whitney $U$ Test to assess statistically significant differences between cocaine- and saline-treated animals, within each behavioral test group or within the basal group. Differences were considered at the significant level of $p < 0.05$. All data are expressed as mean ± S.E.M. Statistical analyses were performed using the software SPSS 14.0 (SPSS INC. Chicago, Illinois, USA) and GraphPad-Prism, version 6.00 for Mac Os X (San Diego, CA, USA).

## RESULTS

### Cocaine exposure did not affect weight gain

As represented in Fig. 1, the ANOVA with repeated measures performed to evaluate body weight gain throughout the experimental period, revealed no differences between cocaine-treated rats and controls. In addition, no death, seizures or other signs of pain

and discomfort were observed during the entire experimental period.

### Cocaine exposure altered the response to environmental challenges (EPM and R/I)

In order to evaluate the impact of cocaine exposure throughout adolescence on the anxiety-like behavior and on the ability to cope with social stress, the rats were submitted to the EPM or to the R/I, 10 days after the end of the exposure protocol (early adulthood). Cocaine-treated rats showed a decrease in the time spent on the central platform of the EPM apparatus, as compared to controls [$t(13) = -2.988$; $p < 0.05$] (Table 1). No differences were observed in other behavioral parameters (Table 1).

During the R/I, cocaine-treated rats showed lower frequency [$t(14) = -2.430$; $p < 0.05$] and higher latency [$t(7.493) = 2.346$; $p < 0.05$] of flight behavior (Table 2). Simultaneously, these animals spent more time investigating the intruder through anogenital sniffing [$t(14) = 2.474$; $p < 0.05$]. No other behavioral differences were observed (Table 2).

### Cocaine exposure led to altered dopaminergic transmission in response to environmental challenges

In order to investigate alterations in the dopaminergic system subjacent to the behavioral response to the EPM or to the R/I, the levels of DA and its metabolites DOPAC and HVA were measured immediately after each behavioral test, and compared to the basal levels in rats exposed to the same experimental protocol that did not perform any behavioral test. Data analysis revealed that in response to the EPM, cocaine-treated rats showed lower levels of DOPAC in the amygdala, as compared to controls (Kruskal–Wallis [$H(5) = 12.06$, $p < 0.05$], Mann–Whitney $U$ test [$Z = -3.009$ (2-tailed) $p < 0.01$]) (Table 3). According to our results, after the EPM test, the levels of DOPAC were increased in the saline control group when compared to the control rats that did not perform the test (Mann–Whitney $U$ test [$Z = -3.000$ (2-tailed) $p < 0.05$]). This effect was not observed in the cocaine-treated rats. The higher levels of DOPAC observed in the saline-treated rats were reflected in a higher DOPAC/DA turnover ratio after the EPM as compared to basal levels (Kruskal–Wallis [$H(5) = 14.40$, $p < 0.05$], Mann–Whitney $U$ test [$Z = -2.143$ (2-tailed) $p < 0.05$]). This may be related to the fact that the basal levels of DA were lower in cocaine-treated rats (Kruskal–Wallis [$H(5) = 11.24$, $p < 0.05$], Mann–Whitney $U$ test [$Z = -2.562$ (2-tailed) $p < 0.05$]). In addition, the amygdalar basal levels of DAT mRNA expression were found to be decreased in the cocaine-treated rats [$t(10) = 2.810$; $p < 0.05$] (Fig 2A).

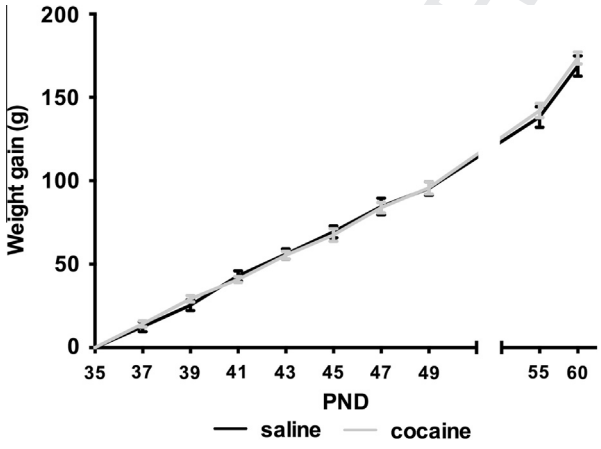


**Fig. 1.** Effects of chronic cocaine administration on body weight gain during exposure period and withdrawal. Results are reported as mean ± S.E.M., for $n = 7$ in control and cocaine-treated animals. PND, postnatal day.

In rats that performed the R/I, we observed that the test itself affected the DA levels in the hippocampus, as both cocaine-treated and control rats displayed higher levels of DA after the R/I as compared to the basal levels (Kruskal–Wallis [$H(5) = 30.61$, $p < 0.01$]], Mann–Whitney $U$ test [$Z = -3.098$ (2-tailed) $p < 0.01$] Mann–Whitney $U$ test [$Z = -3.098$ (2-tailed) $p < 0.01$], respectively) (Table 3). Associated to this, lower DOPAC/DA turnover ratio was observed in both groups after the test as compared to basal levels (Kruskal–Wallis [$H(5) = 30.77$, $p < 0.01$], Mann–Whitney $U$ test [$Z = -3.098$ (2-tailed) $p < 0.01$], Mann–Whitney $U$ test [$Z = -3.098$ (2-tailed) $p < 0.01$], respectively). Importantly, upon the effect of the behavioral test in the DA levels, the R/I test also led to lower DA levels in cocaine-treated rats when compared to the control group (Kruskal–Wallis [$H(5) = 30.84$, $p < 0.01$], Mann–Whitney $U$ test [$Z = -2.521$ (2-tailed) $p < 0.05$], indicating that the R/I–triggered augment in DA levels was less intense in the cocaine-treated rats. In addition, the levels of DOPAC were also lower in cocaine-treated animals as compared to controls (Kruskal–Wallis [$H(5) = 13.07$, $p < 0.05$], Mann–Whitney $U$ test [$Z = -2.521$ (2-tailed) $p < 0.05$], respectively). As observed for DA levels, the DOPAC levels between saline and cocaine-treated rats after the R/I test, also indicate that in the cocaine-treated rats the hippocampal dopaminergic system seems to be less responsive to the test effect (Kruskal–Wallis [$H(5) = 13.07$, $p < 0.05$], Mann–Whitney $U$ test [$Z = -2.32$ (2-tailed) $p < 0.05$].

The effect of cocaine in the basal activity of dopaminergic mesolimbic and nigrostriatal pathways was also evaluated. In the dorsal striatum, we observed only an increase in the HVA levels of the cocaine-treated group (Kruskal–Wallis [$H(5) = 12.45$, $p < 0.05$], Mann–Whitney [$Z = -2.082$ (2-tailed) $p < 0.05$]) (Table 4). However, in VTA/SN, analysis of the neurochemical data revealed that in this set of rats, the levels of DOPAC were increased in the cocaine-treated rats as compared to controls (Kruskal–Wallis [$H(5) = 17.30$, $p < 0.01$], Mann–Whitney $U$ test [$Z = -2.082$ (2-tailed) $p < 0.05$]) (Table 4), while the increase in the DA levels did not reach significance ($p = 0.068$). We evaluated also the TH mRNA expression levels in this region, which were shown to be increased in the

**Table 1.** Long-term effects of chronic cocaine administration throughout adolescence on the behavior of rats placed on EPM for 5 min

| | Saline | Cocaine |
|---|---|---|
| Latency to enter open arms (s.) | 14.92 ± 0.35 | 14.16 ± 4.59 |
| Time in open arm (s) | 99.78 ± 18.38 | 103.59 ± 37.11 |
| Time in closed arm (s) | 151.36 ± 20.36 | 165.00 ± 35.57 |
| Time in central platform | 50.06 ± 3.73 | 31.43 ± 5.13* |
| Entries into open arms | 6.12 ± 0.67 | 4.43 ± 0.75 |
| Entries into closed arms | 7.38 ± 0.84 | 6.57 ± 1.51 |
| Number of rearing | 16.25 ± 2.10 | 13.43 ± 2.67 |
| Number of head dipping | 5.50 ± 1.40 | 3.57 ± 1.56 |
| Grooming time (s) | 8.01 ± 2.21 | 10.29 ± 4.36 |

Q6 Data are expressed as mean ± S.E.M. (from eight animals in the control group and seven animals in the cocaine group). Significant differences between groups were assessed using Student's *t*-test.

* $p < 0.05$, used for cocaine significant difference from saline control.

**Table 2.** Long-term effects of chronic cocaine administration throughout adolescence on the behavior of rats during the R/I

| | | | Saline | Cocaine |
|---|---|---|---|---|
| Aggressive behavior | Attack | Frequency | 8.87 ± 1.82 | 6.50 ± 3.29 |
| | | Latency (s) | 76.91 ± 15.70 | 144.65 ± 66.41 |
| | Biting | Frequency | 2.25 ± 1.45 | 0.37 ± 0.18 |
| | | Latency (s) | 435.49 ± 76.97 | 437.12 ± 84.62 |
| | Pinning | Frequency | 6.37 ± 1.81 | 5.50 ± 2.00 |
| | | Latency (s) | 204.49 ± 51.78 | 203.59 ± 71.35 |
| | Wrestling | Duration (s) | 82.27 ± 16.37 | 46.86 ± 12.91 |
| | | Latency (s) | 70.43 ± 12.74 | 128.04 ± 59.73 |
| Defensive behavior | Flight | Duration (s) | 8.00 ± 1.85 | 2.75 ± 1.11* |
| | | Latency (s) | 87.34 ± 16.73 | 300.06 ± 89.12 * |
| | Supine posture | Frequency | 10.25 ± 3.00 | 8.12 ± 2.45 |
| | | Latency (s) | 104.30 ± 32.86 | 188.82 ± 60.20 |
| Social investigation behavior | Anogenital sniffing | Duration (s) | 3.28 ± 1.98 | 13.02 ± 3.40* |
| | | Latency (s) | 313.12 ± 108.51 | 184.67 ± 90.27 |
| | Approach behavior | Frequency | 74.62 ± 6.38 | 58.62 ± 6.43 |
| | | Latency (s) | 11.29 ± 4.31 | 16.73 ± 3.31 |

Data are expressed as the mean value ± S.E.M. (from eight animals per group). Significant differences between groups were assessed using Student's *t*-test.

* $p < 0.05$, used for cocaine significant difference from saline control.

cocaine-treated rats [$t(4.372) = -3.089$; $p < 0.05$] (Fig. 2B). This increase was further confirmed assessing TH protein levels, that were also increased in the Q3 cocaine-treated rats [$t(11) = 2.366$; $p < 0.05$] (Fig. 2B).

### Cocaine exposure induce long-term sensitization in the corticosterone response to the EPM

In order to assess the effects of cocaine treatment in the adrenal response to environmental challenges, the corticosterone levels were measured after each behavioral test. The effects of cocaine in the basal levels were also assessed.

A two-way ANOVA, with behavioral test and treatment as factors, revealed a main effect of the behavioral test, showing that the corticosterone levels after the behavioral challenging were increased when compared to the basal levels ([$F(1,26) = 51.26$; $p < 0.01$] and [$F(1,25) = 184.3$; $p < 0.01$], respectively) (Fig. 3). An interaction between the behavioral test and the treatment was also seen [$F(1,26) = 4.492$; $p < 0.05$]. Further post hoc testing showed that after the EPM test, cocaine-treated rats presented higher corticosterone levels than the respective controls ($p < 0.05$, Tukey's test). Regarding the corticosterone response to the R/I test, no differences were observed between cocaine- and saline-treated animals (Fig. 3).

### Cocaine exposure induced long-term decrease in testosterone plasma levels

During puberty the hypothalamic–pituitary–gonadal axis undergoes important dynamic changes leading to sexual maturation, and this on-going process may enhance its vulnerability. In order to explore a possible correlation between testosterone and the behavior on the R/I, testosterone plasma levels were measured 10 days after the exposure protocol. As represented in Fig. 4, the testosterone plasma levels were lower in cocaine-treated rats when compared to the control group [$t(10) = -2.366$; $p < 0.05$]. This was accompanied by a lower relative gonadal weight in the cocaine-treated group [$t(10) = -2.931$; $p < 0.05$] (Fig. 4).

## DISCUSSION

The present study shows that chronic cocaine exposure throughout adolescence induced long-lasting alterations

**Table 3.** Levels of DA and its metabolites (DOPAC and HVA) and turnover rates in the amygdala and hippocampus of rats exposed to cocaine or saline, measured after the EPM or R/I

| | | Basal | After EPM | After R/I |
|---|---|---|---|---|
| *Amygdala* | | | | |
| DA | Saline | 77.61 ± 7.57 | 68.53 ± 6.56 | 286.51 ± 116.54 |
| | Cocaine | 48.21 ± 3.69* | 55.50 ± 15.20 | 496.73 ± 199.84 |
| DOPAC | Saline | 13.36 ± 2.39 | 21.68 ± 2.82# | 13.78 ± 2.33 |
| | Cocaine | 16.19 ± 6.12 | 14.60 ± 1.26* | 14.02 ± 2.34 |
| HVA | Saline | 4.66 ± 2.69 | 8.03 ± 3.03 | – |
| | Cocaine | 3.68 ± 0.79 | 7.94 ± 2.76 | – |
| DOPAC/DA | Saline | 0.17 ± 0.02 | 0.33 ± 0.05# | 0.16 ± 0.06 |
| | Cocaine | 0.34 ± 0.13 | 0.36 ± 0.07 | 0.10 ± 0.04 |
| HVA/DA | Saline | 0.11 ± 0.04 | 0.17 ± 0.06 | – |
| | Cocaine | 0.08 ± 0.02 | 0.15 ± 0.06 | – |
| *Hippocampus* | | | | |
| DA | Saline | 7.04 ± 1.75 | 6.87 ± 1.03 | 239.07 ± 43.87## |
| | Cocaine | 8.65 ± 1.52 | 6.41 ± 1.63 | 86.40 ± 24.32*,## |
| DOPAC | Saline | 1.67 ± 0.44 | 2.49 ± 0.34 | 5.67 ± 1.02# |
| | Cocaine | 2.48 ± 2.11 | 1.92 ± 0.11 | 2.60 ± 0.81* |
| HVA | Saline | – | – | – |
| | Cocaine | – | – | – |
| DOPAC/DA | Saline | 0.24 ± 0.05 | 0.39 ± 0.06 | 0.024 ± 0.002## |
| | Cocaine | 0.33 ± 0.05 | 0.42 ± 0.10 | 0.03 ± 0.002## |
| HVA/DA | Saline | – | – | – |
| | Cocaine | – | – | – |

Data are expressed as the mean value ± S.E.M.; levels of DA and its metabolites are expressed as ng/mg of protein for six animals per group. DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; EPM, elevated plus maze; HVA, homovanillic acid; R/I, resident-intruder paradigm.

* $p < 0.05$ used for cocaine significant difference from saline control.

\# $p < 0.05$ used for significant difference from basal.

\## $p < 0.01$ used for significant difference from basal.



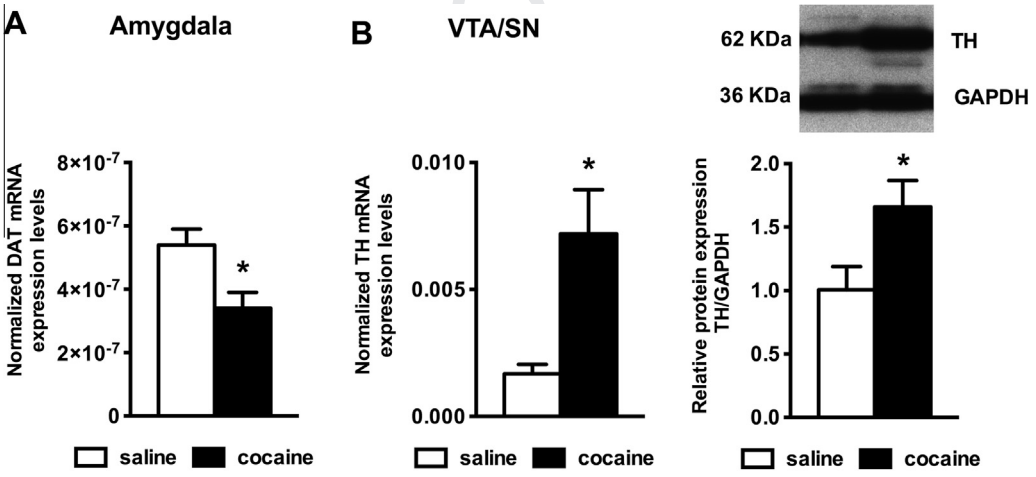


**Fig. 2.** Chronic cocaine administration throughout adolescence caused a decrease in the DAT mRNA expression levels in the amygdala and an increase in the mRNA and protein levels of TH in the VTA/SN. The DAT and TH mRNA expression levels were evaluated by qRT-PCR, and TH protein levels by western blot, in rats exposed to cocaine (3 × 15 mg/kg/day from PND 35 to PND 50) or saline (isovolumetric saline), 10 days after the end of administration protocol. Columns represent the mean + S.E.M. of normalized mRNA or protein expression, from six animals per group. DAT, dopamine transporter; TH, tyrosine hydroxylase. Significant differences between groups are represented as: $^{*}p < 0.05$ (Student's *t*-test).

in the ability to cope with environmental challenges. The behavioral changes observed when rats exposed to cocaine throughout adolescence were tested in the EPM or in the R/I were accompanied by altered dopaminergic function in brain regions essential for processing emotional information, and paralleled by enhanced hypothalamus–pituitary–adrenals (HPA) axis activity in response to an anxiogenic stimulus.

**Table 4.** Basal levels of DA and its metabolites (DOPAC and HVA), and turnover rates in the VTA/SN, Nac and dorsal striatum of rats exposed to cocaine or saline, measured 10 days after last administration

| | | VTA/SN | Nac | Dorsal striatum |
|---|---|---|---|---|
| DA | Saline | 261.02 ± 101.08 | 3970.23 ± 725.03 | 2451.91 ± 864.13 |
| | Cocaine | 587.61 ± 123.55 | 3187.41 ± 454.67 | 4793.50 ± 1310.23 |
| DOPAC | Saline | 46.41 ± 12.62 | 1106.40 ± 207.245 | 484.41 ± 174.19 |
| | Cocaine | 84.69 ± 10.58* | 814.50 ± 108.70 | 907.95 ± 310.29 |
| HVA | Saline | 22.82 ± 8.46 | 320.25 ± 87.28 | 122.08 ± 41.63 |
| | Cocaine | 37.53 ± 9.46 | 208.95 ± 27.73 | 346.47 ± 126.18* |
| DOPAC/DA | Saline | 24.29 ± ± 5.70 | 0.28 ± 0.03 | 0.21 ± 0.01 |
| | Cocaine | 16.20 ± 2.08 | 0.27 ± 0.03 | 0.18 ± 0.02 |
| HVA/DA | Saline | 8.98 ± 1.94 | 0.08 ± 0.01 | 0.05 ± 0.01 |
| | Cocaine | 7.45 ± 1.85 | 0.07 ± 0.01 | 0.07 ± 0.01 |

Data are expressed as the mean value ± S.E.M.; levels of DA and its metabolites are expressed as ng/mg of protein for six animals per group. DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; Nac, nucleus accumbens; VTA/SN, ventral mesencephalon.

* $p < 0.05$, used for cocaine significant difference from saline control.

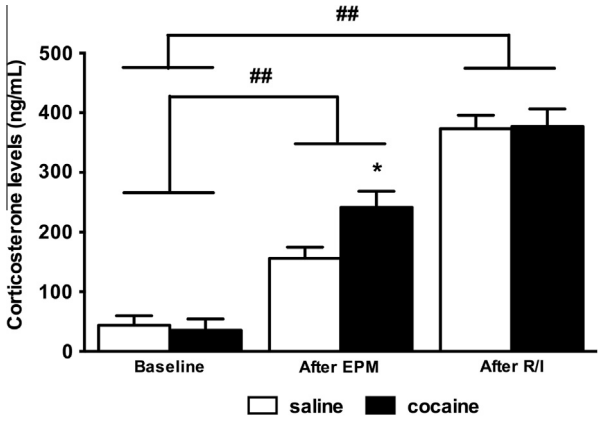


**Fig. 3.** Chronic cocaine administration throughout adolescence induced long-lasting sensitization in the corticosterone response to the EPM. Levels of corticosterone were assessed in the plasma obtained 10 days after cocaine withdrawal (3 × 15 mg/kg/day from PND 35 to PND 50) or saline (isovolumetric saline) to determine the basal levels, and following the EPM or R/I. Columns represent the mean + S.E.M. (from six animals per group). EPM, elevated plus maze test; R/I, resident intruder paradigm. Significant differences between groups are represented as: $^{\#\#}p < 0.01$ used for significant difference from basal and $^{*}p < 0.05$ used for cocaine significant difference from saline control (Two-way ANOVA followed by Tukey's test as post hoc).

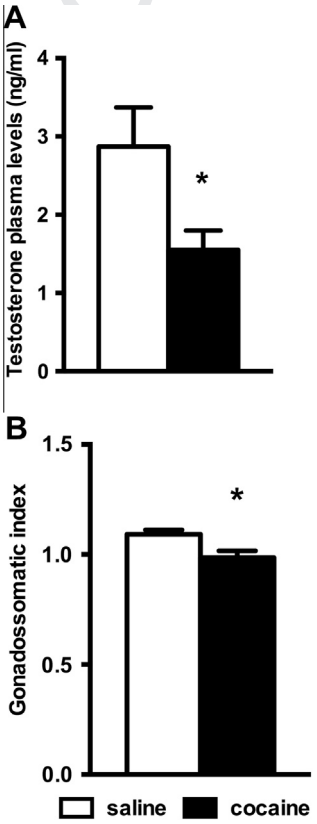


**Fig. 4.** Chronic cocaine administration throughout adolescence induced a decrease in the levels of testosterone, and long-term reduction in the gonadosomatic index. Levels of testosterone were assessed in plasma obtained 10 days after cocaine withdrawal (3 × 15 mg/kg/day from PND 35 to PND 50) and saline (isovolumetric saline). Gonads' weight was registered at the same time point. Columns represent the mean + S.E.M. (from six animals per group). Significant differences in the testosterone levels between groups are represented as: $^{*}p < 0.05$ (Student's *t*-test). At 10 days after withdrawal, cocaine-treated rats presented significantly lower relative testes weight, as compared to controls ($^{*}p < 0.05$, Student's *t*-test).

The EPM is the most common test to assess anxiety-related behavior in rodents (Pellow et al., 1985). We did not observe significant differences in the number of arm entries or in the time spent in the different arms, and therefore no evidence of long-term effects on anxiety after exposure to cocaine in the adolescence. However, in addition to the traditional use of the number of open arm entries and the time spent in the open arms as measures of anxiety, Rodgers and Johnson (1995) showed through a factor analysis of the EPM behavioral measures, that the time spent in the central platform reflects decision-making processes, most probably related to the approach/avoidance conflict. Importantly, our results show that cocaine-treated animals spent less time in the central platform. Therefore, despite the unaltered anxiety-like behavior, cocaine exposure during adolescence may have decreased the time spent in decision-making process, which suggests reduced evaluation before taking action.

Of note, in the present study, regardless of the unaltered classic anxiety-like EPM measures after cocaine administration, the corticosterone response to this anxiogenic test was increased, suggesting a sensitization of the HPA axis activity. Dissociation between HPA axis activation and the anxiety-like behavior in the EPM has been reported (Munoz-Abellan et al., 2008). Despite a clear implication of corticosterone (either stress-induced or exogenously administered) in the anxiety-like behavior displayed in the EPM (e.g., Shepard et al., 2000; Myers et al., 2005; Adamec et al., 2006; Matuszewich et al., 2007), it was shown that the HPA axis activation after a short period in the EPM may reflect arousal rather than anxiety (Munoz-Abellan et al., 2008). As such, the sensitization of the HPA axis responsiveness we observed in the cocaine-exposed group may underlie the decreased time spent in approach/avoidance conflict. This is further supported by recent published data reporting a positive correlation between increased corticosterone levels and impaired decision-making function (Koot et al., 2013).

The response of the amygdalar dopaminergic system to the EPM was also modified by cocaine exposure. At basal conditions cocaine-treated animals displayed lower amygdalar DA levels, concurrent with down regulation of the DAT mRNA levels. Since our results did not evidence increased DA turnover, the lower DA levels could result from a decreased number of dopaminergic terminals. Repeated cocaine administration is known to increase dendritic arborization by cytoskeletal RhoGTPases regulation in regions like the Nac (Dietz et al., 2012), however, to our knowledge there are no data exploring the impact of those regulatory mechanisms in the amygdala. Importantly at early withdrawal stages there is evidence of synaptic depression (Shen et al., 2009; Dietz et al., 2012), which together with a study in cocaine-addicted subjects that revealed a significant reduction in the amygdalar volume (Makris et al., 2004), favors the hypothesis that our observation may result from a decreased amygdalar dopaminergic innervation.

Amygdala is a critical structure of the neural network, necessary for the decision-making and implementation of advantageous choices, which is also involved in the attachment of an emotional valence to an event (i.e., reward and punishment) (Bechara et al., 1999; Gupta et al., 2011). In that sense, the decreased time spent in approach/avoidance conflict displayed by cocaine-treated rats in the EPM, could result from an inability to adequately attribute an emotional value to the stimuli provided by the EPM, which in turn may be a consequence of an anomalous dopaminergic activity in this region.

The data obtained in the EPM were strengthened by the behavioral response of cocaine-treated rats in the R/I, which is compatible with decreased cautious behavior. In the R/I, cocaine-treated rats presented lower frequency and higher latency of flight behavior, as well as increased social investigation, as shown by an increased duration of anogenital sniffing, while no evidence of increased offensive behaviors was observed. Flight is a behavioral strategy to avoid a source of harm (Dixon, 1998) and reduce provocative interactions between animals, particularly in the case of conspecific agonistic relations (Shuhama et al., 2007). The observed decreased flight behavior indicates that cocaine exposure decreased defensive behaviors, suggesting that cocaine-treated rats were less cautious in response to a social threat. This hypothesis seems to be further supported by increased social investigation, a non-adaptive strategy in the R/I circumstances. Social investigation is regarded as a discriminative learning process for establishing individual recognition (Yamamuro, 2006). Thus, the augmented anogenital sniffing by cocaine-treated rats can be associated to impaired individual recognition. Regardless of the underlying causes, the increase in social investigation uncovers a failure to attribute an adequate threat value to the intruder. To date, animal studies on the long-lasting effects of cocaine on aggressive behavior as a response to social stress were mainly conducted in prenatal exposure models (reviewed by Sobrian and Holson, 2011). Within these studies, contradictory results have been reported, i.e. either no effects (Estelles et al., 2005) or increased aggressive behavior was observed (Wood and Spear, 1998; Chae and Covington, 2009). In this context, the present study is the first to report that adolescent cocaine exposure seems to lead to long-lasting imprudent behavior in face of a possible social threat. This may also imply a persistent impairment in the ability to cautiously explore a potentially dangerous environment.

Paralleling the behavioral response in the R/I, we found decreased testosterone levels in the cocaine-treated animals. Cocaine exposure was reported to affect testes morphology (Barroso-Moguel et al., 1994; George et al., 1996), and decrease the levels of testosterone both acutely and after chronic exposure (Chin et al., 2002; Festa et al., 2003; Yang et al., 2006). The persistent decrease in the plasma levels of testosterone after cocaine administration during adolescence observed in our study may result from an abnormal maturation process of the gonads along the exposure period. Supporting this, a decrease in the relative gonadal weight of the cocaine-treated rats was also observed. Testosterone has been suggested to play an important role in social-emotional behavior. Particularly, its role in aggressive behavior has long been explored in different paradigms, such as oriented aggression and social status defense (Eisenegger et al., 2011). In R/I models, testosterone is known to facilitate offensive aggression (Delville et al., 1996). Recently, testosterone was also found to be positively associated with social vigilance in response to status threats (Bos et al., 2012). Based on this knowledge, the low plasma levels of testosterone observed in our study are probably underlying both the increase in social investigation and the concomitant decrease in behaviors oriented toward social status defense observed in the R/I test.

In opposition to the EPM, in the R/I there were no differences on the HPA axis response between cocaine-treated and control rats. It is known that R/I induces a very large adrenocortical stress response both in resident and intruder rats (Schuurman, 1980). Our data corroborate this finding, as both control and treated rats

exhibited approximately a sevenfold increase of plasma corticosterone in response to the R/I. This considerable HPA axis response, in both groups may, however, be masking the cocaine effects.

Along with the behavioral response to the R/I, changes in the hippocampal dopaminergic system were also observed. Our data indicate that the R/I per se modified the dopaminergic activity in this brain region, leading to increased DA content. The involvement of the dopaminergic system in the response to the R/I was previously described (van Erp and Miczek, 2000). However, in the cocaine group this dopaminergic response to the R/I was less extensive as reflected by the DA and DOPAC levels when compared to the control group. This adds to our data supporting an enduring effect of cocaine exposure through adolescence in the dopaminergic system.

In addition to its involvement in memory processes, the hippocampus, more specifically the ventral hippocampal portion, is involved in the adequate cautious exploration of a potential dangerous environment, indicating its relevant role in information processing and subsequent behavioral regulation (reviewed by Bannerman et al., 2004; Bertoglio et al., 2006). Hippocampal lesions were reported to cause an increase in investigative behavior (Bannerman et al., 2001) and reduction of defensive behavior when facing both predatory and painful threat stimuli (Pentkowski et al., 2006). Based on these reports and on the relevance of the dopaminergic regulation in the hippocampus (Hansen and Manahan-Vaughan, 2012; Hernandez et al., 2013), our observation of altered dopaminergic function in the hippocampus may underlie the non-adaptive strategy displayed by cocaine-treated animals in response to the R/I.

Taken as a whole, our behavioral data evidence long-lasting impaired evaluation of potential threats after cocaine exposure during adolescence. This is often associated with reduction of impulse control. Recent data showing the disinhibition of the medial prefrontal-cortex after cocaine exposure during adolescence, endured through adulthood, further reinforces the hypothesis of more impulsive behavior and impaired decision-making (Cass et al., 2013; Kuhn et al., 2013).

Importantly, at the basal level, i.e. before any challenge was presented, the activity of the dopaminergic system was already altered in the cocaine rats. Our results revealed an increase in the levels of DOPAC in VTA/SN. As the DA turnover was not affected, the increase in the DOPAC levels could be associated to enhanced ability to synthesize DA. In fact, although the increase in DA did not reach significance, our data showed a trend to the increase in the DA levels. This was supported by increased levels of TH in the VTA/SN of cocaine-treated rats. Increased dopaminergic activity after long-term withdrawal may render individuals more susceptible to the reward effects of cocaine, and therefore more vulnerable to drug use reinstatement. Our results indicating a long-lasting increase in the dopaminergic activity add to the data from Catlow and Kirstein (2007) showing an enhanced response of the dopaminergic system to naturally reinforcing substances in rats pre-treated with cocaine during adolescence.

## CONCLUSION

The present findings provide new insights into how cocaine administration throughout adolescence may modulate emotional behavior later on. Our data showed that chronic cocaine administration during this developmental period induced long-lasting alterations in the HPA axis response and testosterone levels, which led to altered behavioral response to environment challenges that seem to support a long-lasting impaired evaluation of potential threats. This was supported by altered dopaminergic function in relevant brain regions. A compromised ability to recognize and deal with potential threats, may lead to high-risk and low-benefit choices. This is an important risk factor for enhanced susceptibility to the maintenance of drug use and relapse.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## AUTHORS CONTRIBUTION

Authors Alves, Tavares, Monteiro and Summavielle designed the study and were responsible for its implementation. Alves, Magalhães and Sousa collected and analyzed the behavioral data. Sousa designed the statistical analysis. Alves and Melo performed neurochemical and hormonal assays. Author Alves wrote the first draft of the manuscript. Magalhães, Monteiro and Summavielle contributed to the final manuscript.

*Acknowledgments—This research project was funded by Programa Operacional Factores de Competitividade (COMPETE) and by National Funds through FCT – Fundação para a Ciência e Tecnologia, Reference FCOMP-01-0124-FEDER-011320 (FCT POCTI/ESP/43630/1999 and PTDC/MHC-PAP/5304/2012). Teresa Summavielle was supported by Fundo Social Europeu e ao Programa Operacional Potencial Humano (POPH) through Ciencia 2007 and Investigador FCT programs. Cecília Juliana Alves, Ana Magalhães and Pedro Melo were granted by FCT (Alves CJ-SFRH/BD/17195/2004 and SFRH/BPD/63618/2009; Magalhães A-SFRH/BPD/19200/2004 and PTDC/MHC-PAP/5304/2012; Melo P SFRH/BPD/26477/2006).*

## REFERENCES

Adamec R, Head D, Blundell J, Burton P, Berton O (2006) Lasting anxiogenic effects of feline predator stress in mice: sex differences in vulnerability to stress and predicting severity of anxiogenic response from the stress experience. Physiol Behav 88:12–29.

Alves E, Binienda Z, Carvalho F, Alves CJ, Fernandes E, de Lourdes Bastos M, Tavares MA, Summavielle T (2009) Acetyl-L-carnitine provides effective in vivo neuroprotection over 3,4-methylenedioximethamphetamine-induced mitochondrial neurotoxicity in the adolescent rat brain. Neuroscience 158:514–523.

Andersen SL (2003) Trajectories of brain development: point of vulnerability or window of opportunity? Neurosci Biobehav Rev 27:3–18.
Anthony JC, Petronis KR (1995) Early-onset drug use and risk of later drug problems. Drug Alcohol Depend 40:9–15.
Aujla H, Martin-Fardon R, Weiss F (2008) Rats with extended access to cocaine exhibit increased stress reactivity and sensitivity to the anxiolytic-like effects of the mGluR 2/3 agonist LY379268 during abstinence. Neuropsychopharmacology 33:1818–1826.
Bannerman DM, Lemaire M, Beggs S, Rawlins JN, Iversen SD (2001) Cytotoxic lesions of the hippocampus increase social investigation but do not impair social-recognition memory. Exp Brain Res 138:100–109.
Bannerman DM, Rawlins JN, McHugh SB, Deacon RM, Yee BK, Bast T, Zhang WN, Pothuizen HH, Feldon J (2004) Regional dissociations within the hippocampus-memory and anxiety. Neurosci Biobehav Rev 28:273–283.
Barron S, White A, Swartzwelder HS, Bell RL, Rodd ZA, Slawecki CJ, Ehlers CL, Levin ED, Rezvani AH, Spear LP (2005) Adolescent vulnerabilities to chronic alcohol or nicotine exposure: findings from rodent models. Alcohol Clin Exp Res 29:1720–1725.
Barroso-Moguel R, Mendez-Armenta M, Villeda-Hernandez J (1994) Testicular lesions by chronic administration of cocaine in rats. J Appl Toxicol 14:37–41.
Bechara A, Damasio H, Damasio AR, Lee GP (1999) Different contributions of the human amygdala and ventromedial prefrontal cortex to decision-making. J Neurosci 19:5473–5481.
Bertoglio LJ, Joca SRL, Guimaraes FS (2006) Further evidence that anxiety and memory are regionally dissociated within the hippocampus. Behav Brain Res 175:183–188.
Bos PA, Panksepp J, Bluthe RM, van Honk J (2012) Acute effects of steroid hormones and neuropeptides on human social-emotional behavior: a review of single administration studies. Front Neuroendocrinol 33:17–35.
Brown ZJ, Kupferschmidt DA, Erb S (2012) Reinstatement of cocaine seeking in rats by the pharmacological stressors, corticotropin-releasing factor and yohimbine: role for D1/5 dopamine receptors. Psychopharmacology 224:431–440.
Buffalari DM, Baldwin CK, See RE (2012) Treatment of cocaine withdrawal anxiety with guanfacine: relationships to cocaine intake and reinstatement of cocaine seeking in rats. Psychopharmacology 223:179–190.
Casey BJ, Jones RM (2010) Neurobiology of the adolescent brain and behavior: implications for substance use disorders. J Am Acad Child Adolesc Psychiatry 49:1189–1201. quiz 1285.
Cass DK, Thomases DR, Caballero A, Tseng KY (2013) Developmental disruption of gamma-aminobutyric acid function in the medial prefrontal cortex by noncontingent cocaine exposure during early adolescence. Biol Psychiatry 74:490–501.
Catlow BJ, Kirstein CL (2007) Cocaine during adolescence enhances dopamine in response to a natural reinforcer. Neurotoxicol Teratol 29:57–65.
Chae SM, Covington CY (2009) Biobehavioral outcomes in adolescents and young adults prenatally exposed to cocaine: evidence from animal models. Biol Res Nurs 10:318–330.
Chambers RA, Taylor JR, Potenza MN (2003) Developmental neurocircuitry of motivation in adolescence: A critical period of addiction vulnerability. Am J Psychiatry 160:1041–1052.
Chaplin TM, Freiburger MB, Mayes LC, Sinha R (2010) Prenatal cocaine exposure, gender, and adolescent stress response: a prospective longitudinal study. Neurotoxicol Teratol 32:595–604.
Chen CY, Storr CL, Anthony JC (2009) Early-onset drug use and risk for drug dependence problems. Addict Behav 34:319–322.
Chin J, Sternin O, Wu HB, Burrell S, Lu D, Jenab S, Perrotti LI, Quinones-Jenab V (2002) Endogenous gonadal hormones modulate behavioral and neurochemical responses to acute and chronic cocaine administration. Brain Res 945:123–130.
Cleck JN, Ecke LE, Blendy JA (2008) Endocrine and gene expression changes following forced swim stress exposure during cocaine abstinence in mice. Psychopharmacology 201:15–28.
Coffey SF, Dansky BS, Carrigan MH, Brady KT (2000) Acute and protracted cocaine abstinence in an outpatient population: a prospective study of mood, sleep and withdrawal symptoms. Drug Alcohol Depend 59:277–286.
Delville Y, Mansour KM, Ferris CF (1996) Testosterone facilitates aggression by modulating vasopressin receptors in the hypothalamus. Physiol Behav 60:25–29.
Dietz DM, Sun H, Lobo MK, Cahill ME, Chadwick B, Gao V, Koo JW, Mazei-Robison MS, Dias C, Maze I, Damez-Werno D, Dietz KC, Scobie KN, Ferguson D, Christoffel D, Ohnishi Y, Hodes GE, Zheng Y, Neve RL, Hahn KM, Russo SJ, Nestler EJ (2012) Rac1 is essential in cocaine-induced structural plasticity of nucleus accumbens neurons. Nat Neurosci 15:891–896.
Dixon AK (1998) Ethological strategies for defence in animals and humans: their role in some psychiatric disorders. Br J Med Psychol 71(Pt 4):417–445.
Eisenegger C, Haushofer J, Fehr E (2011) The role of testosterone in social interaction. Trends Cogn Sci 15:263–271.
Erb S, Shaham Y, Stewart J (1998) The role of corticotropin-releasing factor and corticosterone in stress- and cocaine-induced relapse to cocaine seeking in rats. J Neurosci 18:5529–5536.
Estelles J, Rodriguez-Arias M, Maldonado C, Aguilar MA, Minarro J (2005) Prenatal cocaine exposure alters spontaneous and cocaine-induced motor and social behaviors. Neurotoxicol Teratol 27:449–457.
Festa ED, Jenab S, Chin J, Gazi FM, Wu HB, Russo SJ, Quinones-Jenab V (2003) Frequency of cocaine administration affects behavioral and endocrine responses in male and female Fischer rats. Cell Mol Biol (Noisy-le-grand) 49:1275–1280.
Fox HC, Hong KI, Siedlarz K, Sinha R (2008) Enhanced sensitivity to stress and drug/alcohol craving in abstinent cocaine-dependent individuals compared to social drinkers. Neuropsychopharmacology 33:796–805.
Gawin FH, Kleber HD (1986) Abstinence symptomatology and psychiatric diagnosis in cocaine abusers. Clinical observations. Arch Gen Psychiatry 43:107–113.
George VK, Li H, Teloken C, Grignon DJ, Lawrence WD, Dhabuwala CB (1996) Effects of long-term cocaine exposure on spermatogenesis and fertility in peripubertal male rats. J Urol 155:327–331.
Giedd JN, Lalonde FM, Celano MJ, White SL, Wallace GL, Lee NR, Lenroot RK (2009) Anatomical brain magnetic resonance imaging of typically developing children and adolescents. J Am Acad Child Adolesc Psychiatry 48:465–470.
Goeders NE, Guerin GF (1996) Role of corticosterone in intravenous cocaine self-administration in rats. Neuroendocrinology 64:337–348.
Gu H, Salmeron BJ, Ross TJ, Geng X, Zhan W, Stein EA, Yang Y (2010) Mesocorticolimbic circuits are impaired in chronic cocaine users as demonstrated by resting-state functional connectivity. Neuroimage 53:593–601.
Gupta R, Koscik TR, Bechara A, Tranel D (2011) The amygdala and decision-making. Neuropsychologia 49:760–766.
Haney M, Maccari S, Le Moal M, Simon H, Piazza PV (1995) Social stress increases the acquisition of cocaine self-administration in male and female rats. Brain Res 698:46–52.
Hansen N, Manahan-Vaughan D (2012) Dopamine D1/D5 receptors mediate informational saliency that promotes persistent hippocampal long-term plasticity. Cereb Cortex. Q5
Harris GC, Aston-Jones G (1993) Beta-adrenergic antagonists attenuate withdrawal anxiety in cocaine- and morphine-dependent rats. Psychopharmacology 113:131–136.
Hernandez VS, Luquin S, Jauregui-Huerta F, Corona-Morales A, Medina MP, Ruiz-Velasco S, Zhang L (2013) Dopamine receptor dysregulation in hippocampus of aged rats underlies chronic pulsatile L-Dopa treatment induced cognitive and emotional alterations. Neuropharmacology.
Jacobsen LK, Southwick SM, Kosten TR (2001) Substance use disorders in patients with posttraumatic stress disorder: a review of the literature. Am J Psychiatry 158:1184–1190.

Kelley AE, Berridge KC (2002) The neuroscience of natural rewards: relevance to addictive drugs. J Neurosci 22:3306–3311.
Kimmel HL, Carroll FI, Kuhar MJ (2003) Withdrawal from repeated cocaine alters dopamine transporter protein turnover in the rat striatum. J Pharmacol Exp Ther 304:15–21.
Koot S, Baars A, Hesseling P, van den Bos R, Joels M (2013) Time-dependent effects of corticosterone on reward-based decision-making in a rodent model of the Iowa Gambling Task. Neuropharmacology 70:306–315.
Kuhn CM, Wilson W, Swartzwelder S (2013) Enduring effects of adolescent drug exposure. Biol Psychiatry 74:480–481.
Leslie FM, Loughlin SE, Wang R, Perez L, Lotfipour S, Belluzzia JD (2004) Adolescent development of forebrain stimulant responsiveness: insights from animal studies. Ann N Y Acad Sci 1021:148–159.
Mahoney 3rd JJ, Newton TF, Omar Y, Ross EL, De La Garza 2nd R (2012) The relationship between lifetime stress and addiction severity in cocaine-dependent participants. Eur Neuropsychopharmacol.
Makris N, Gasic GP, Seidman LJ, Goldstein JM, Gastfriend DR, Elman I, Albaugh MD, Hodge SM, Ziegler DA, Sheahan FS, Caviness Jr VS, Tsuang MT, Kennedy DN, Hyman SE, Rosen BR, Breiter HC (2004) Decreased absolute amygdala volume in cocaine addicts. Neuron 44:729–740.
Mantsch JR, Cullinan WE, Tang LC, Baker DA, Katz ES, Hoks MA, Ziegler DR (2007) Daily cocaine self-administration under long-access conditions augments restraint-induced increases in plasma corticosterone and impairs glucocorticoid receptor-mediated negative feedback in rats. Brain Res. 1167:101–111.
Matuszewich L, Karney JJ, Carter SR, Janasik SP, O'Brien JL, Friedman RD (2007) The delayed effects of chronic unpredictable stress on anxiety measures. Physiol Behav 90:674–681.
Miczek KA (1979) A new test for aggression in rats without aversive stimulation: differential effects of d-amphetamine and cocaine. Psychopharmacology 60:253–259.
Miczek KA, Mutschler NH (1996) Activational effects of social stress on IV cocaine self-administration in rats. Psychopharmacology 128:256–264.
Munoz-Abellan C, Andero R, Nadal R, Armario A (2008) Marked dissociation between hypothalamic-pituitary-adrenal activation and long-term behavioral effects in rats exposed to immobilization or cat odor. Psychoneuroendocrinology 33:1139–1150.
Myers DA, Gibson M, Schulkin J, Greenwood Van-Meerveld B (2005) Corticosterone implants to the amygdala and type 1 CRH receptor regulation: effects on behavior and colonic sensitivity. Behav Brain Res 161:39–44.
Nogueira L, Kalivas PW, Lavin A (2006) Long-term neuroadaptations produced by withdrawal from repeated cocaine treatment: role of dopaminergic receptors in modulating cortical excitability. J Neurosci 26:12308–12313.
Paxinos G, Watson C (1998) The rat brain in stereotaxic coordinates. fourth ed. San Diego: Academic Press.
Pellow S, Chopin P, File SE, Briley M (1985) Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Methods 14:149–167.
Pentkowski NS, Blanchard DC, Lever C, Litvin Y, Blanchard RJ (2006) Effects of lesions to the dorsal and ventral hippocampus on defensive behaviors in rats. Eur J Neurosci 23:2185–2196.
Piazza PV, Deminiere JM, le Moal M, Simon H (1990) Stress- and pharmacologically-induced behavioral sensitization increases vulnerability to acquisition of amphetamine self-administration. Brain Res 514:22–26.
Quinones-Jenab V, Krey LC, Schlussman SD, Ho A, Kreek MJ (2000) Chronic 'binge' pattern cocaine alters the neuroendocrine profile of pregnant rats. Neurosci Lett 282:120–122.
Rodgers R, Cole C (1994) The elevated plus maze: pharmacology, methodology and ethology. In: Cooper S, Hendrie C, editors. Ethology and psychopharmacology. John Wiley & Sons. p. 9–44.
Rodgers RJ, Johnson NJ (1995) Factor analysis of spatiotemporal and ethological measures in the murine elevated plus-maze test of anxiety. Pharmacol Biochem Behav 52:297–303.
Salas-Ramirez KY, Frankfurt M, Alexander A, Luine VN, Friedman E (2010) Prenatal cocaine exposure increases anxiety, impairs cognitive function and increases dendritic spine density in adult rats: influence of sex. Neuroscience 169:1287–1295.
Sarnyai Z, Vecsernyés M, Julesz J, Bíró E, Gardi J, Telegdy G (1995) Brain corticotropin-releasing factor mediates 'anxiety-like' behavior induced by cocaine withdrawal in rats. Brain Res 675:89–97.
Sarnyai Z, Dhabhar FS, McEwen BS, Kreek MJ (1998) Neuroendocrine-related effects of long-term, 'binge' cocaine administration: Diminished individual differences in stress-induced corticosterone response. Neuroendocrinology 68:334–344.
Satel SL, Price LH, Palumbo JM, McDougle CJ, Krystal JH, Gawin F, Charney DS, Heninger GR, Kleber HD (1991) Clinical phenomenology and neurobiology of cocaine abstinence: a prospective inpatient study. Am J Psychiatry 148:1712–1716.
Schlussman SD, Nyberg F, Kreek MJ (2002) The effects of drug abuse on the stress responsive hypothalamic–pituitary–adrenal axis and the dopaminergic and endogenous opioid systems. Acta Psychiatr Scand Suppl:121–124.
Schuurman T (1980) Hormonal correlates of agonistic behavior in adult male rats. Prog Brain Res 53:415–420.
Shaham Y, Erb S, Stewart J (2000) Stress-induced relapse to heroin and cocaine seeking in rats: a review. Brain Res Rev 33:13–33.
Shen HW, Toda S, Moussawi K, Bouknight A, Zahm DS, Kalivas PW (2009) Altered dendritic spine plasticity in cocaine-withdrawn rats. J Neurosci 29:2876–2884.
Shepard JD, Barron KW, Myers DA (2000) Corticosterone delivery to the amygdala increases corticotropin-releasing factor mRNA in the central amygdaloid nucleus and anxiety-like behavior. Brain Res 861:288–295.
Shuhama R, Del-Ben CM, Loureiro SR, Graeff FG (2007) Animal defense strategies and anxiety disorders. An Acad Bras Cienc 79:97–109.
Sithisarn T, Bada HS, Dai H, Randall DC, Legan SJ (2011) Effects of perinatal cocaine exposure on open field behavior and the response to corticotropin releasing hormone (CRH) in rat offspring. Brain Res 1370:136–144.
Sobrian SK, Holson RR (2011) Social behavior of offspring following prenatal cocaine exposure in rodents: a comparison with prenatal alcohol. Front Psychiatry/Front Res Found 2:66.
Sobrian SK, Marr L, Ressman K (2003) Prenatal cocaine and/or nicotine exposure produces depression and anxiety in aging rats. Prog Neuropsychopharmacol Biol Psychiatry 27:501–518.
Spear L (2000) The adolescent brain and age-related behavioral manifestations. Neurosci Biobehav Rev 24:417–463.
Tirelli E, Laviola G, Adriani W (2003) Ontogenesis of behavioral sensitization and conditioned place preference induced by psychostimulants in laboratory rodents. Neurosci Biobehav Rev 27:163–178.
Tsukada H, Kreuter J, Maggos CE, Unterwald EM, Kakiuchi T, Nishiyama S, Futatsubashi M, Kreek MJ (1996) Effects of binge pattern cocaine administration on dopamine D1 and D2 receptors in the rat brain: An in vivo study using positron emission tomography. J Neurosci 16:7670–7677.
Ungless MA, Whistler JL, Malenka RC, Bonci A (2001) Single cocaine exposure in vivo induces long-term potentiation in dopamine neurons. Nature 411:583–587.
van Erp AM, Miczek KA (2000) Aggressive behavior, increased accumbal dopamine, and decreased cortical serotonin in rats. J Neurosci 20:9320–9325.
Varlinskaya EI, Spear LP (2008) Social interactions in adolescent and adult Sprague-Dawley rats: impact of social deprivation and test context familiarity. Behav Brain Res 188:398–405.
Wheeler AL, Lerch JP, Chakravarty MM, Friedel M, Sled JG, Fletcher PJ, Josselyn SA, Frankland PW (2013) Adolescent cocaine

exposure causes enduring macroscale changes in mouse brain structure. J Neurosci 33:1797–1803a.
Wong WC, Ford KA, Pagels NE, McCutcheon JE, Marinelli M (2013) Adolescents are more vulnerable to cocaine addiction: behavioral and electrophysiological evidence. J Neurosci 33:4913–4922.
Wood RD, Spear LP (1998) Prenatal cocaine alters social competition of infant, adolescent, and adult rats. Behav Neurosci 112:419–431.
Yamamuro Y (2006) Social behavior in laboratory rats: applications for psycho-neuroethology studies. Anim Sci J 77:386–394.
Yang GS, Wang W, Wang YM, Chen ZD, Wang S, Fang JJ (2006) Effect of cocaine on germ cell apoptosis in rats at different ages. Asian J Androl 8:569–575.
Young KA, Gobrogge KL, Wang Z (2011) The role of mesocorticolimbic dopamine in regulating interactions between drugs of abuse and social behavior. Neurosci Biobehav Rev 35:498–515.
Zaslav MR (1994) Psychology of comorbid posttraumatic stress disorder and substance abuse: lessons from combat veterans. J Psychoactive Drugs 26:393–400.
Zhang Y, Mantsch JR, Schlussman SD, Ho A, Kreek MJ (2002) Conditioned place preference after single doses or ''binge'' cocaine in C57BL/6J and 129/J mice. Pharmacol Biochem Behav 73:655–662.
Zhang Y, Schlussman SD, Rabkin J, Butelman ER, Ho A, Kreek MJ (2012) Chronic escalating cocaine exposure, abstinence/withdrawal, and chronic re-exposure: effects on striatal dopamine and opioid systems in C57BL/6J mice. Neuropharmacology.
Zhou Y, Spangler R, LaForge KS, Maggos CE, Ho A, Kreek MJ (1996) Corticotropin-releasing factor and type 1 corticotropin-releasing factor receptor messenger RNAs in rat brain and pituitary during ''binge''-pattern cocaine administration and chronic withdrawal. J Pharmacol Exp Ther 279:351–358.
Zhou Y, Bendor JT, Yuferov V, Schlussman SD, Ho A, Kreek MJ (2005) Amygdalar vasopressin mRNA increases in acute cocaine withdrawal: evidence for opioid receptor modulation. Neuroscience 134:1391–1397.



Please cite this article in press as: Alves CJ et al. Long-term effects of chronic cocaine exposure throughout adolescence on anxiety and stress responsivity in a Wistar rat model. Neuroscience (2014), http://dx.doi.org/10.1016/j.neuroscience.2014.07.008