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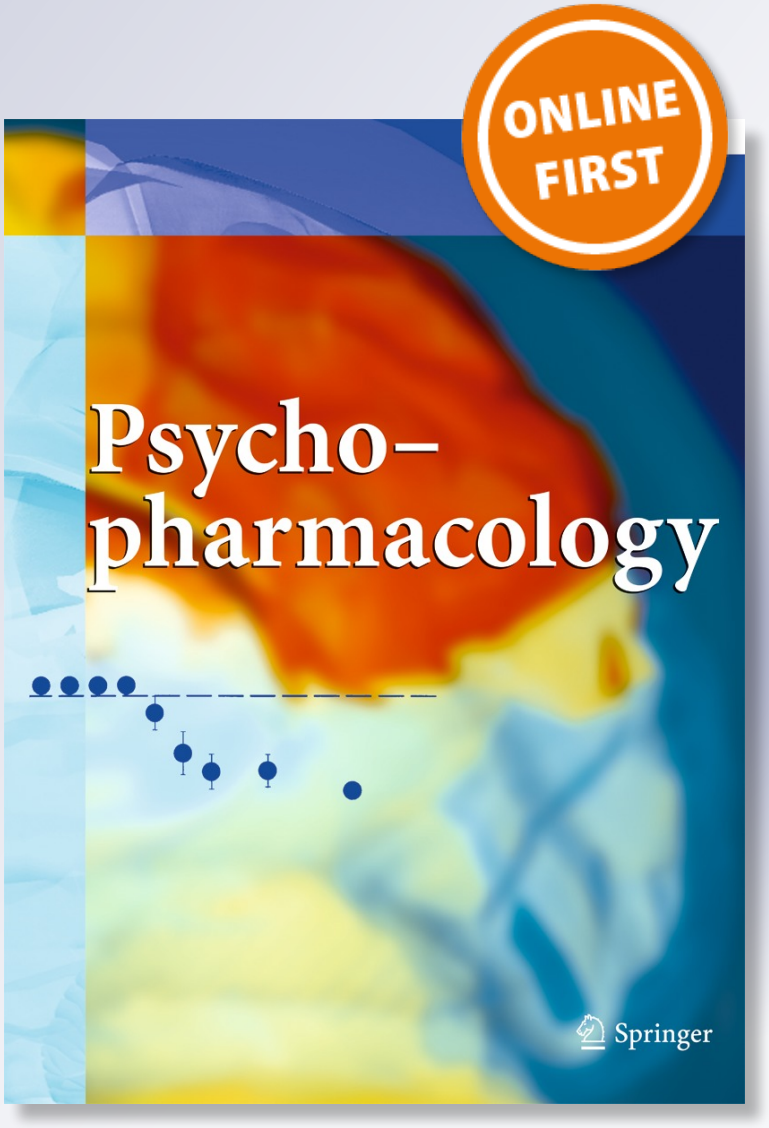
Psychopharmacology

ISSN 0033-3158

Psychopharmacology
DOI 10.1007/s00213-013-3084-y



Springer

Psychopharmacology
DOI 10.1007/s00213-013-3084-y

ORIGINAL INVESTIGATION

Involvement of nNOS/NO/sGC/cGMP signaling pathway in cocaine sensitization and in the associated hippocampal alterations: does phosphodiesterase 5 inhibition help to drug vulnerability?

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Received: 20 December 2012 / Accepted: 20 March 2013

Abstract
Rationale Repeated cocaine administration induces behavioral sensitization in about 50 % of treated animals. Nitric oxide could be involved in the acquisition and maintenance of behavioral cocaine effects, probably by activation of neuronal nitric oxide synthase (nNOS)/NO/soluble guanylyl cyclase (sGC)/cyclic guanosine monophosphate (cGMP) signaling pathway, since inhibition of the nNOS enzyme attenuates development of sensitization in rats. On the other hand, increased cGMP availability by phosphodiesterase 5 inhibitors has been correlated to the misuse and recreational use of these agents and also to the concomitant use with illicit drugs in humans. Hippocampus is an important brain region for conditioning to general context previously associated to drug availability, influencing drug-seeking behavior and sensitization. Moreover, cocaine and other drugs of abuse can affect the strength of glutamate synapses in this structure, lastly modifying neuronal activity in main regions of the reward circuitry.
Objective The objective of this study is to determine whether the pharmacological manipulation of nNOS/NO/sGC/cGMP signaling pathway altered changes induced by repeated cocaine exposure.
Results The present investigation showed a relationship between behavioral cocaine sensitization, reduced threshold to generate long-term potentiation (LTP) in hippocampal dentate gyrus, and increased nNOS activity in this structure. However, when nNOS or sGC were inhibited, the number of sensitized animals was reduced, and the threshold to generate LTP was increased. The opposite occurred when cGMP availability was increased.
Conclusion We demonstrate a key role of the nNOS activity and NO/sGC/cGMP signaling pathway in the development of cocaine sensitization and in the associated enhancement of hippocampal synaptic transmission.

Keywords cGMP · Cocaine · Hippocampus · Long-term potentiation · Nitric oxide · Sensitization

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Introduction

Repeated administration of psychostimulants such as cocaine (COC) causes progressive increases in locomotor activity, called behavioral sensitization, that are correlated to differential neuroadaptations only in a proportion of animals exposed

Published online: 12 April 2013

to the drug, independently of the administration protocol used (Pierce et al. 1996; Boudreau and Wolf 2005). Because sensitization can be context dependent, long lasting, and can increase subsequent drug self-administration (Everitt and Wolf 2002), it is a useful model of relapse in humans (Robinson and Berridge 2000). Furthermore, drug sensitization has been observed in humans and can contribute to enhancement of psychoses with repeated psychostimulant exposure.

The striatum is the major entry into the basal ganglia, receiving inputs from all areas of cortex as well as afferents from the thalamus and limbic structures such us the hippocampus and amygdala (Parent 1990). Particularly, the nucleus accumbens (NAc) is considered as an interface between corticolimbic regions important for motivation and motor regions important for behavioral outputs and thus plays a key role in generating motivated behaviors related to natural rewards as well as drugs of abuse (Kelley 2004). Descending projections from the prefrontal cortex to the NAc and other brain regions exert inhibitory control over reward seeking behaviors. Furthermore, chronic cocaine exposure impairs these inhibitory control mechanisms (Vorel et al. 2001; Jentsch and Taylor 1999).

Interconnections between these areas constitute the main neuronal circuitry involved in the neurobiology of addiction. In fact, the development and persistent expression of addictive behaviors occur through the usurpation of natural learning and memory mechanisms within the limbic system (Robbins et al. 2008; Wolf 2002). A region that has been implicated in context-dependent processes is the hippocampus (HP) that sends projections to NAc through the ventral subiculum. This region has been involved in different context-dependent processes such as drug sensitization (Sinha 2001). A major form of synaptic plasticity in HP is long-term potentiation (LTP) characterized by an enduring increase in the efficacy of glutamatergic synaptic transmission. This phenomenon is accepted as a molecular mechanism for learning and memory in the brain in which contextual cues are relevant (Phillips and LeDoux 1992; Martin et al. 2000). We have recently demonstrated a marked enhancement in the hippocampal dentate gyrus synaptic transmission after sensitization to the locomotive effects of COC, using different COC doses and treatment duration (Perez et al. 2010). Moreover, a modulatory effect on CA1 hippocampal synaptic plasticity was observed after chronic COC treatments (Thompson et al. 2004).

The diffusible neuromodulator NO is synthesized in the brain mainly by the neuronal NO synthase (nNOS) enzyme from L-arginine, and signals in multiple ways. NO activates soluble guanylyl cyclase (sGC) stimulating the production of cyclic guanosine monophosphate (cGMP; Mustafa et al. 2009; Garthwaite 2010); it also may act through cAMP formation and can react directly with proteins interacting with sulfhydryl groups of cysteine, a phenomenon called protein s-nitrosylation (Garthwaite 2008). Neuronal excitability and synaptic plasticity are modulated by NO in different brain structures including the HP (Prast and Philippu 2001). Furthermore, NO may play a major role in initiating and maintaining the behavioral effects of psychostimulant drugs (Itzhak 1996; Kim and Park 1995). In fact, pharmacological or genetic nNOS activity disruption attenuates the development of sensitization, conditioned place preference, and self-administration of psychostimulants (Itzhak et al. 1998; Itzhak et al. 2010; Orsini et al. 2002). In addition, nNOS inhibition during repeated COC administration prevented the persistent increase in membrane excitability of mPFC pyramidal neurons observed after a short-term withdrawal from COC (Nasif et al. 2011). However, the mechanisms by which NO participate in behavioral sensitization and in the enhanced hippocampal excitability after repeated COC administration are not well understood.

Sildenafil citrate is a drug widely prescribed for erectile dysfunction, among other diseases, and acts by inhibiting phosphodiesterase type-5 (PDE5), resulting in accumulation of cGMP. This accumulation in the central nervous system has been related to long-term memory retention (Boccia et al. 2011), and the rewarding properties of SIL in mice (Tahsili-Fahadan et al. 2006), as well as the development of depression and anxiety in both rodents and humans have been described (Kulkarni and Dhir 2007). These effects are coincident with some symptoms of COC withdrawal (Rogerio and Takahashi 1992). Given that NO is involved in all these processes, the purpose of the present work is to evaluate the participation of nNOS/NO/sGC/GMPc signaling pathway in the development of behavioral sensitization observed after repeated COC administration and in the associated enhancement of hippocampal synaptic transmission previously described.

Materials and methods

Ethics

All procedures were conducted according to the National Institute of Health (NIH) Guide for Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996) and approved by the Animal Care and Use Committee, School of Chemical Sciences, National University of Cordoba. Experiments were made minimizing the number of animals used and their suffering.

Drugs

Cocaine chlorhydrate (15 mg/kg) was purchased from Verardo y Cía., Buenos Aires, Argentina. 7-Nitroindazole (7-NI, 50 mg/kg) from Sigma Aldrich, Buenos Aires, Argentina. Sildenafil citrate (SIL, 5 mg/kg) from Todo Droga,

Argentina. 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 2 mg/kg) from Cayman Chemical, Michigan, USA. All drugs were diluted in Cremophor EL from Sigma Aldrich, Buenos Aires, Argentina, 10 % in 0.9 % NaCl as vehicle (VEH).

Animals and pretreatments

Adolescent male Wistar rats of 5–6 weeks old, age in which subjects are more vulnerable to develop drug addiction (Badanich et al. 2006), were used in this study. Animals were housed in groups in a temperature- and humidity-controlled vivarium under a 12-h light/dark cycle. Food and water were freely available. Rats received, via intraperitoneal (i.p.) injection, VEH+saline (0.9 % NaCl; VEH+SAL); VEH+cocaine (15 mg/kg; VEH+COC); 7-NI (50 mg/kg)+SAL (7-NI+SAL); 7-NI+cocaine (7-NI+COC); SIL (5 mg/kg)+saline (SIL+SAL); SIL+cocaine (SIL+COC); ODQ (2 mg/kg)+saline (ODQ+SAL); and ODQ+cocaine (ODQ+COC). Animals were injected daily, for five consecutive days, with VEH, 7-NI, SIL, or ODQ 30 min before COC or SAL administration. Immediately after drug administration, on days 1 and 5, locomotor activity was evaluated.

Locomotor activity

Locomotor activity was measured using rectangular cages (30.5×19.5×46.5 cm) equipped with two parallel infrared photocell beams located 3 cm above the floor. Interruption of either beam resulted in a single count. Animals were tested under white light in a sound isolated room. On the first treatment day, animals were placed individually in the photocell boxes. After 1 h of habituation, rats were injected with either VEH, 7-NI, SIL, or ODQ. Thirty minutes after, animals were administered with SAL or COC and activity was monitored for 2 h. Animals were considered sensitized when locomotor activity on day 5 increased above 20 % compared to day 1. This criterion was followed considering that differential alterations in excitatory amino-acids release (Pierce et al. 1996) and a lower threshold to generate LTP (Perez et al. 2010) were observed in animals with different patterns of locomotor activity after repeated COC exposure.

Electrophysiology

Electrophysiological experiments were carried out using the in vitro hippocampal slice preparation (Perez et al. 2010). Rats were killed 24 h after the last administration, between 10:00 and 11:00 AM, to prevent variations caused by circadian rhythms or nonspecific stressors (Teyler and DiScenna 1987). The hippocampal formation was dissected, and transverse slices of approximately 400 μm thick were placed in a (BSC-BU Harvard Apparatus) recording chamber, perfused with standard Krebs solution (NaCl, 124.3 mM; KCl, 4.9 mM; $MgSO_4 \cdot 7H_2O$, 1.3 mM; H_2KPO_4, 1.25 mM; $HNaCO_3$, 25.6 mM; glucose, 10.4 mM; $CaCl_2 \cdot 2H_2O$, 2.3 mM; Sigma Chemical Company, St. Louis, MO, USA) saturated with 95 % O_2 and 5 % CO_2. The perfusion rate was 1.6 ml/min, while the bathing solution temperature was kept at 28 °C by a temperature controller (TC-202A Harvard Apparatus) for the duration of the experiment. Field excitatory postsynaptic potentials (fEPSPs) were evoked with a stimulating electrode, made of two twisted wires that were insulated except for the cut ends (diameters, 50 μm), placed in the perforant path (PP), and the recording electrode made with a glass micropipette (10–20 μm tip) was inserted in the dentate granule cell body layer (Fig. 2a). Only slices showing a stable response were included. fEPSP that responded to 0.2 Hz pulses (0.5 ms, 10 mA each) were sampled each 5 min during a 20–30-min period of time (baseline). Once no further changes were observed in the fEPSP amplitude, the stimulation protocol was applied to determine the LTP eliciting frequency threshold. The stimulation protocol consisted of a train pulse (0.5 ms, 10 mA each) of 2 s duration (tetanus), of increasing variable frequency (5–200 Hz) that was delivered to the PP, by an A310 Accupulser Pulse Generator (World Precision Instruments Inc., USA). After the tetanus, a new averaged fEPSP was recorded at 0.2 Hz, and when LTP was not observed, a new stimulation at the next higher frequency was applied. LTP was considered to have occurred when the fEPSP amplitude had increased by at least 30 % from basal fEPSP and persisted for 60 min (Fig. 2b). Once LTP was achieved, no further tetanus was given. For each animal, a second hippocampal slice was used to corroborate the threshold to generate LTP by applying tetanus at the same frequency in which LTP was previously elicited. No differences were observed in LTP generation between slices.

Determination of nNOS activity

Three days after the last administration, animals were killed by guillotine decapitation. All groups received a challenge dose of cocaine 20 min prior to decapitation, in order to guarantee the nNOS activation. Brains were removed, and the HP was dissected to measure nNOS activity, using l-[U-14C] arginine. (Bredt et al. 1990). Tissue was placed in a tube with 0.5 ml Krebs–Ringer bicarbonate and incubated at 37 °C in 95 % O_2 and 5 % CO_2 for 8 min. Then, tissue was homogenized in 500 μl of buffer [20 mM Hepes (pH 7.4), 1 mM dithiothreitol, 0.45 mM $CaCl_2$, and 400 μM NADPH] or free Ca^{2+} buffer. Homogenate aliquots of 200 μl were incubated with 0.1 μCi L-[U-14C]-arginine (Amersham Pharmacia Biotech, Buckinghamshire, UK) for 30 min at 37 °C with controlled atmosphere of 95 % O_2 and 5 % CO_2.

Samples were centrifuged at 10,000 rpm at 4 °C for 10 min. Supernatants aliquots (400 μl) were eluted in individual columns of Dowex AG 50W-X8 200–400-mesh sodium form (Fluka), previously stabilized with 20 mM Hepes (pH 7.4) in order to separate L-[14C]-citrulline in the supernatant, and then washed with 3 ml bi-distilled water. L-[14C]-citrulline in the eluent was quantified by liquid scintillation using a β-counter.

Because L-citrulline and NO are generated in equal amounts, and only L-citrulline is stable, quantification of L-citrulline is considered an indirect measurement of NO production, indicating nNOS activity (Knowles and Salter 1998). Data are expressed as picomol of NO generated/milligram of protein. Protein quantification was determined by the Lowry method (Lowry et al. 1951).

Statistics

Data were analyzed using STATISTICA 7.0. Repeated measures analysis of variance (ANOVA) was used for behavior statistical analysis and one-way ANOVA for electrophysiological data. In both cases, post hoc Student–Newman–Keuls (SNK) test was employed. Pearson's correlation was used to analyze the correlation between locomotor activity and NO levels. Two-way ANOVA and least significant difference post hoc were used to analyze nNOS activity determinations. Multiple 2×2 chi-square tests were applied to analyze association between animal treatment and condition (Table 1).

Results

Impact of different enhancers or inhibitors of the nNOS/NO/sGC/cGMP signaling pathway in the development of COC sensitization

It has been demonstrated that repeated COC administration induces sensitization only in a proportion of animals exposed to the drug (Pierce et al. 1996; Boudreau and Wolf 2005; Perez et al. 2010). In the present investigation, we reproduced those results showing that repeated COC administration induced a significant increase in locomotor activity on day 5 compared to day 1 when VEH+COC total group was compared to VEH+SAL group [$F_{(1,85)}$=5.40; p<0.05; post hoc SNK p<0.05 compared to day 1; p<0.05 compared to VEH+SAL] (Fig. 1a). When VEH+COC-treated rats were differentially analyzed, considering if they showed at least 20 % of increase in locomotor activity on day 5 compared to day 1, we observed that 49.02 % of animals met the 20 % criteria (sensitized group, VEH+COC-S; Table 1). Locomotor activity on day 5 was significantly increased compared to day 1 when VEH+COC-S group was compared to VEH+SAL group [$F_{(1,59)}$=92.26; p<0.001; post hoc SNK p<0.05 compared to day 1; p<0.05 compared to VEH+SAL] (Fig. 1b). The remaining animals (50.98 %, Table 1) were considered nonsensitized (VEH+COC-NS). In this group, no significant differences were found in locomotor activity on day 5 compared to day 1 [$F_{(1,60)}$=3.63; p>0.05], but a significant increase in locomotor activity was found when VEH+COC-NS group was compared to VEH+SAL [$F_{(1,60)}$=163.05; p<0.05; post hoc SNK p<0.05] (Fig. 1c).

It has been shown that administration of the selective nNOS inhibitor 7-NI previous to COC administration blocks the development of behavioral sensitization (Itzhak 1997). In the present investigation, we observed that administration of 7-NI 30 min before COC administration prevented development of sensitization, showing no changes in the locomotor activity on day 5 compared to day 1 [$F_{(1,73)}$=1.09; p>0.05]. Although we did not observe sensitization when all animals were included in the statistical analysis, the results showed a significant increase in locomotor activity in 7-NI+COC total group compared to 7-NI+SAL group [$F_{(1,73)}$=22.74; p<0.001; post hoc SNK p<0.05], indicating a modest effect of COC in locomotion even in the presence of the NOS inhibitor (Fig. 1d).

It has been demonstrated that repeated COC administration induces sensitization only in 50–60 % of the treated animals (Pierce et al. 1996; Boudreau and Wolf 2005; Perez et al. 2010). In the present investigation, we analyzed the impact of the 7-NI preadministration in development of sensitization using the 20 % criterion. In this case, we also observed the sensitized (7-NI+COC-S) and the nonsensitized (7-NI+COC-NS) groups. Figure 1e showed a significant increase in locomotor activity on day 5 compared to day 1 when 7-NI+COC-S group was compared to 7-NI+SAL group [$F_{(1,33)}$=22.73; p>0.001; post hoc SNK p<0.05 compared to day 1; p<0.05 compared to 7NI+SAL]. Figure 1f showed a significant decrease in locomotor activity on day 5 compared to day 1 in 7-NI+COC-NS group [$F_{(1,55)}$=11.35; p<0.05; post hoc SNK p<0.05] and a significant increase in 7-NI+COC-NS group compared to 7-NI+SAL [$F_{(1,55)}$=23.55; p<0.001; post hoc SNK p<0.05]. Interestingly, in the 7-NI+COC group, only 31.04 % of animals were sensitized. The chi-square test revealed an association between animal treatment (VEH+COC or 7-NI+COC) and the number of rats in both conditions (sensitized and nonsensitized) [$X^2_{(0.95)}$=3.67; df=1; Table 1].

Table 1 Distribution of sensitized and non sensitized animals exposed to different treatments

Treatment	Percent sensitized	Percent nonsensitized
VEH+COC	49.02	50.98
7-NI+COC	31.04[a]	68.96
ODQ+COC	27.78[a]	72.22
SILD+COC	67.44[a]	32.56

[a] Different from VEH+COC (multiple 2×2 chi-square test)

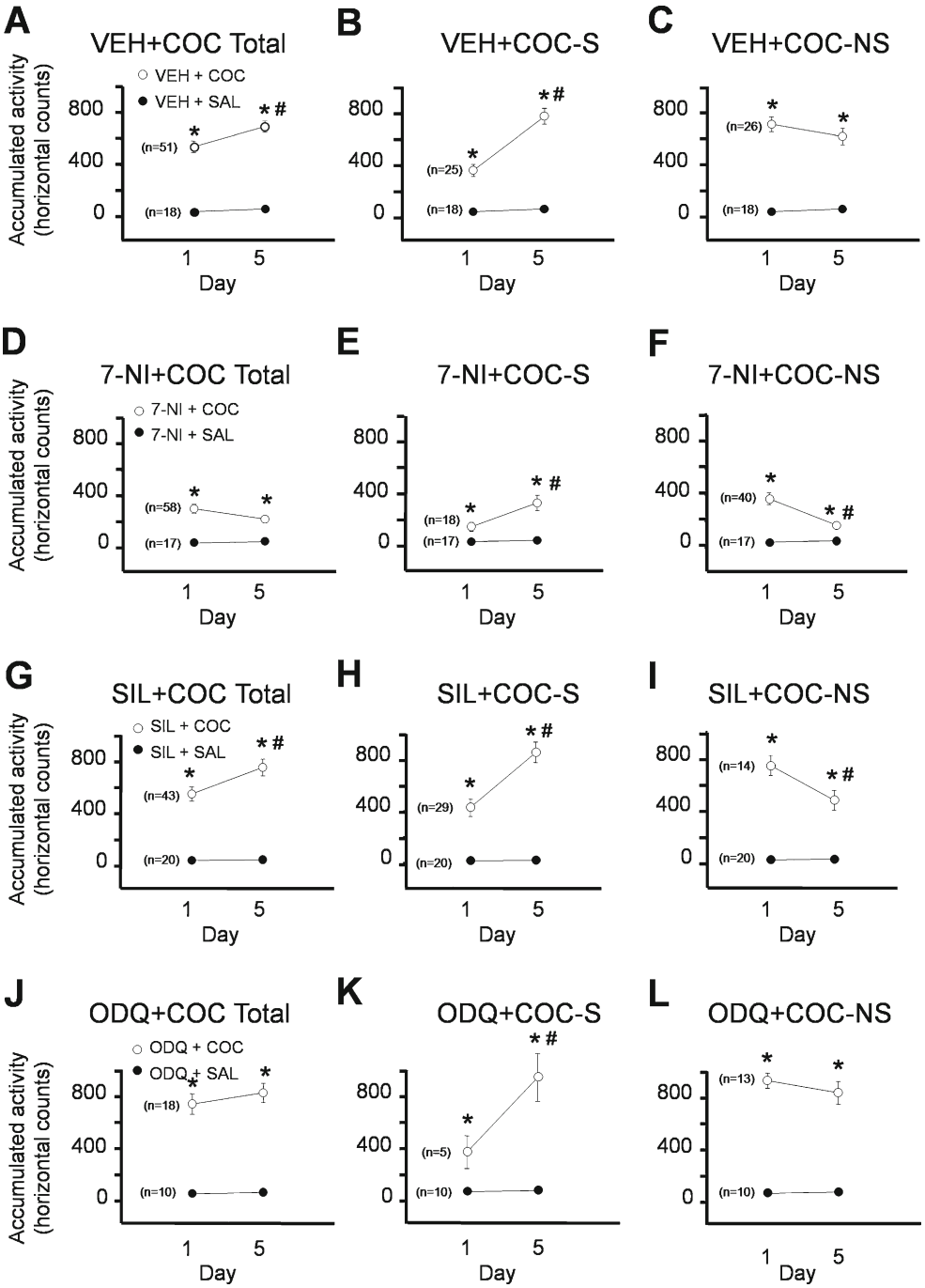

Fig. 1 Pharmacological manipulation of the nNOS/NO/sGC/cGMP pathway affects cocaine sensitization. Behavioral results showing accumulated locomotor activity (50 min) on days 1 and 5 for all treatments. $^{\#}p<0.05$ compared to day 1 for each group; $^{*}p<0.05$ compared to their respective controls on each day. *Circles* represent means±SE. Number of animals is indicated in *parenthesis*

To test whether engagement of the nNOS/NO/sGC/cGMP signaling pathway can contribute to COC sensitization, we impaired cGMP hydrolysis using the PDE5 inhibitor SIL. Administration of SIL 30 min before COC administration induced sensitization, showing significant increases in locomotor activity on day 5 compared to day 1 when SIL+COC total group was compared to SIL+SAL group [$F_{(1,61)}=5.24$; $p<0.05$; post hoc SNK $p<0.05$ compared to day 1; $p<0.05$ compared to SIL+SAL] (Fig. 1g). Under this condition, we also found the sensitized group (SIL+COC-S) and the nonsensitized group (SIL+COC-NS). Figure 1h showed a significant increase in locomotor activity on day 5 compared to day 1 in SIL+COC-S group and when SIL+COC-S group was compared to SIL+SAL group [$F_{(1,47)}=103.09$; $p<0.05$; post hoc SNK $p<0.05$ compared to day 1; $p<0.05$ compared to SIL+SAL]. Figure 1i showed a significant decrease in locomotor activity on day 5 compared to day 1 in SIL+COC-NS group [$F_{(1,32)}=19.07$; $p<0.05$; post hoc SNK $p<0.05$], and a significant increase in SIL+COC-NS group compared to SIL+SAL group [$F_{(1,32)}=113.57$; $p<0.001$; post hoc SNK $p<0.05$]. Surprisingly, under this treatment, 67.44 % of animals showed sensitization. The chi-square test revealed an association between animal treatment (VEH+COC

or SIL+COC) and the number of rats in both conditions (sensitized and nonsensitized) [$X^2_{(0.95)} = 3.24$; df=1; Table 1].

Finally, we evaluated if reduction of cGMP availability had the opposite effect of SIL using a selective, irreversible, and noncompetitive sGC inhibitor (ODQ). ODQ administration 30 min before COC prevented sensitization, showing no changes in locomotor activity on day 5 compared to day 1 [$F_{(1,26)}$=0.42; p>0.05]. Still, a significant increase in locomotor activity in ODQ+COC total group compared to ODQ+SAL group was observed [$F_{(1,26)}$=113.4; p<0.001; post hoc SNK p<0.05] (Fig. 1j). Once more, sensitized (ODQ+COC-S) and nonsensitized (ODQ+COC-NS) groups were detected. Figure 1k showed a significant increase in locomotor activity on day 5 compared to day 1 when ODQ+COC-S group was compared to ODQ+SAL group [$F_{(1,13)}$=22.51; p<0.001; post hoc SNK p<0.05 compared to day 1; p<0.05 compared to ODQ+SAL]. Figure 1l showed no differences in locomotor activity on day 5 compared to day 1 in ODQ+COC-NS group [$F_{(1,21)}$=0.46; p>0.05], but a significant increase in ODQ+COC-NS group compared to ODQ+SAL [$F_{(1,21)}$=211.06; p<0.001; post hoc SNK p<0.05]. As we hypothesized, in the ODQ+COC group, only a 27.78 % of animals showed sensitization. The chi-square test revealed an association between animal treatment (VEH+COC or ODQ+COC) and the number of rats in both conditions (sensitized and nonsensitized) [$X^2_{(0.95)} = 2.44$; df=1; Table 1].

Effect of different enhancers or inhibitors of the nNOS/NO/sGC/cGMP signaling pathway on hippocampal synaptic plasticity

Previous results demonstrated that repeated COC administration reduced the threshold to generate hippocampal LTP (Perez et al. 2010). In the present study, we

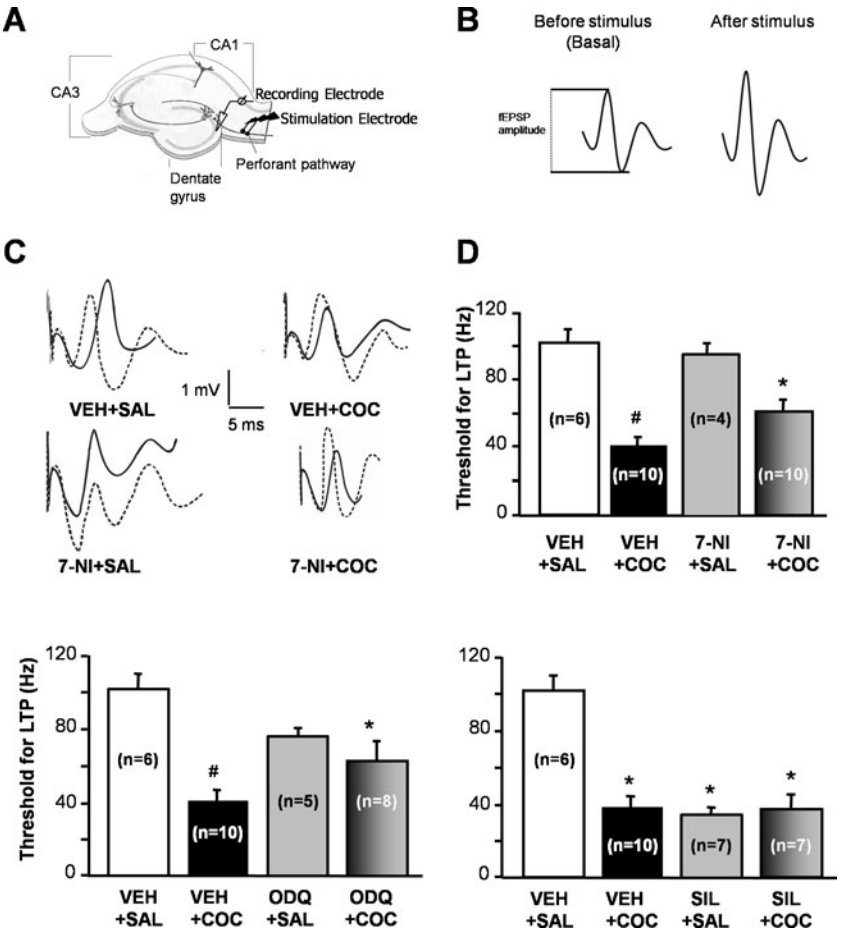

Fig. 2 Facilitated LTP generation induced by repeated COC administration is affected by manipulation of the nNOS/NO/sGC/cGMP signaling pathway. **a** Hippocampal slice cartoon indicating the position of stimulation and recording electrodes. **b** fEPSP sample traces showing how measurements of fEPSP are taken. **c** fEPSP sample traces for VEH+SAL, VEH+COC, 7-NI+SAL, and 7-NI+COC groups before (*full line*) and after (*dotted line*) effective tetanus. *Bar graphs* showing threshold to generate LTP in **d** VEH+COC and 7-NI+COC treated animals (and their respective controls). $^{\#}p$<0.05 compared to VEH+SAL group; *p<0.05 compared to 7-NI+SAL; **e** VEH+COC and ODQ+COC treated animals (and their respective controls). $^{\#}p$<0.05 compared to VEH+SAL group; *p<0.05 compared to VEH+COC; **f** VEH+COC and SIL+COC treated animals (and their respective controls). *p<0.05 compared to VEH+SAL group. *Bars* represent means±SE. Number of animals is indicated in *parenthesis*

reproduced those results, and when nNOS inhibitor (7-NI) was administered before COC, we observed a significant increase in the threshold to generate LTP when compared to VEH+COC group [$F_{(3,30)}$=17.01; p<0.001; post hoc SNK p<0.05] (Fig. 2d). When cGMP availability was reduced using a GC inhibitor (ODQ), similar results were found between VEH+COC and ODQ+COC groups [$F_{(3,25)}$=8.78; p<0.001; post hoc SNK p<0.05] (Fig. 2e). Conversely, when cGMP hydrolysis was prevented using a PDE5 inhibitor (SIL), no differences in the threshold to generate LTP between SIL+COC and VEH+COC was observed. Interestingly, repeated SIL administration (SIL+SAL group) induced a reduction in the threshold to generate LTP when compared to VEH+SAL group [$F_{(3,26)}$=18.24; p<0.001; post hoc SNK p<0.05] (Fig. 2f). Furthermore, a significant reduction in the threshold to generate LTP was observed with acute SIL administration (data not shown).

Hippocampal nNOS activity in COC sensitization

It has been demonstrated that COC sensitization is associated with increases in nNOS activity in different mice brain regions (Bhargava and Kumar 1997). In the present investigation, we observed a significant increase in NO levels in VEH+COC-S, which was prevented by 7-NI administration previous to COC (7-NI+COC-S) [$F_{(2,35)}$=9.49; p<0.05; post hoc SNK p<0.05] (Fig. 3a). Figure 3b showed the percentage of NO levels in animals that received an acute COC administration (VEH+COC-Acute) or animals that sensitized after repeated COC (VEH+COC-S) with respect to their controls (VEH+SAL-acute and VEH+SAL), a significant increase in NO levels was observed in VEH+COC-S compared to VEH+COC-acute [$F_{(1,13)}$=11.36; p<0.05]. Interestingly, when we analyzed the relationship between NO levels and locomotor activity on day 5 in VEH+COC-S and VEH+COC-NS groups, a positive correlation was observed (Pearson's

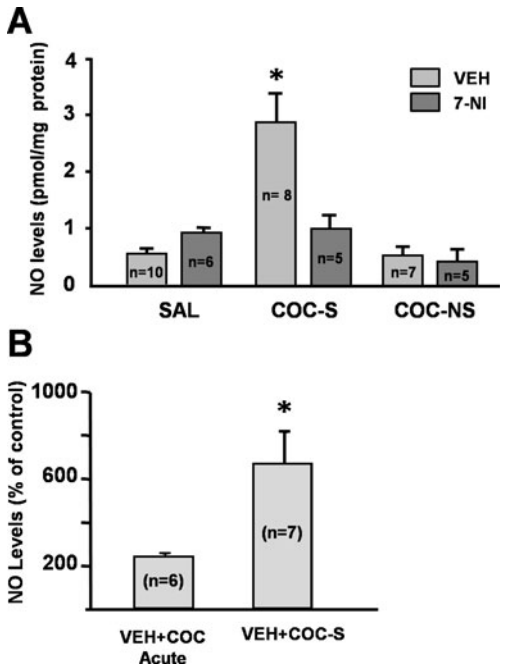

Fig. 3 Cocaine sensitization increases nNOS activity in hippocampus. *Bars graph* indicating NO levels in **a** sensitized and non-sensitized groups with or without nNOS inhibition and their controls, *p<0.05 compared to other groups. Results are expressed as means of NO picomol per milligram protein±S.E.; **b** acute treated animals (VEH+COC acute) and sensitized rats (VEH+COC-S), *p<0.05. Results are expressed as means of percentage of their respective controls (VEH+SAL acute and VEH+SAL)±SE. Number of animals are indicated in *parenthesis*

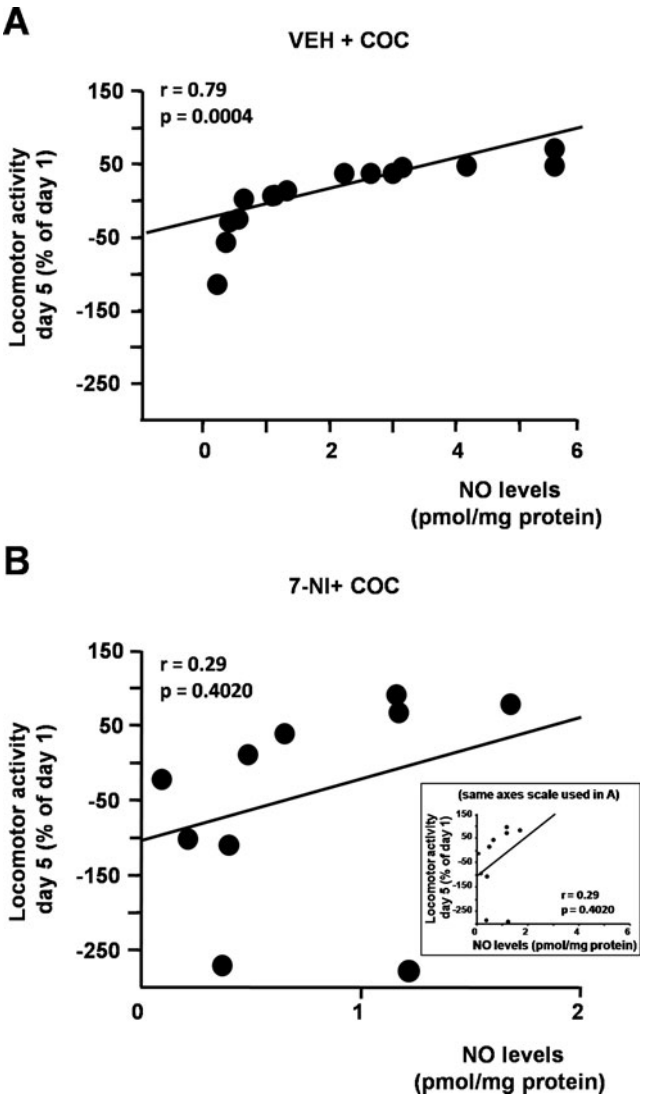

Fig. 4 Development of sensitization correlates with high levels of NO production in hippocampus. Graphs showing correlation between the percent of increase in locomotor activity (day 5 respect to day 1) and NO levels (pmol/mg protein) in **a** VEH+COC (sensitized and non-sensitized) treated rats and **b** 7-NI+COC (sensitized and nonsensitized) treated rats. The correlation coefficients (r) are indicated in *bold*

correlation r=0.79; p=0.0004; Fig. 4a). Administration of 7-NI prior to COC (7-NI+COC-S and 7-NI+COC-NS) weakened this correlation (Pearson's correlation r=0.29; p=0.4020; Fig. 4b).

Discussion

The results from the present investigation showed a relationship between behavioral COC sensitization, reduced threshold to generate LTP in hippocampal dentate gyrus, and increased nNOS activity in this structure. However, when nNOS was inhibited, the number of sensitized animals was reduced, and the threshold to generate LTP was increased. Similar results in behavior and hippocampal LTP were observed when sGC was inhibited during repeated COC administration. Oppositely, when availability of cGMP was increased by PDE5 blockade, the number of sensitized animals was increased and the lower threshold to generate LTP was preserved. This study indicates a key role of the nNOS/NO/sGC/cGMP signaling pathway in the development of sensitization and in the associated hippocampal synaptic transmission induced by repeated COC administration.

Sensitization is thought to underlie drug craving and relapse to many drugs of abuse, including COC (Steketee 2005; Belujon and Grace 2011). Although different protocols of repeated COC administration have been proved to develop sensitization (Pierce et al. 1996; Yamaguchi et al. 2005), this phenomenon occurs only in a proportion of treated animals, even when the protocols used varied in duration of repeated cocaine administration (5 vs. 14 vs. 7 days) and COC doses (15 vs. 20 vs. 30 mg kg^{-1} day^{-1}; Perez et al. 2010; Boudreau and Wolf 2005; Pierce et al. 1996). Based on previous data, it seems that the proportion of sensitized and nonsensitized animals is independent of the administration protocol used. As reported in a previous paper (Perez et al. 2010), here, we observed that nonsensitized animals had higher locomotor activity after the first COC exposure compared with the sensitized animals, in accordance to the observations of Pierce et al. (1996). The mechanisms underlying these differential responses between sensitized and nonsensitized rats are still unknown.

The results of the present investigation demonstrated that the proportion of sensitized animals increases with activation of nNOS/NO/sGC/cGMP pathway, whereas blockade of this pathway significantly reduced this proportion. A further observation supporting the participation of nNOS/NO/sGC/cGMP pathway is that sGC inhibition by ODQ prevented COC sensitization without affecting the hyperlocomotor effect, while upstream inhibition of this pathway (nNOS inhibition) not only prevented COC sensitization but also reduced the hyperlocomotor effect. Oppositely, the enhanced cGMP availability by SIL increased the proportion of sensitized animals. These results support the possible involvement of nNOS/NO/sGC/cGMP pathway in the vulnerability to develop COC sensitization.

The NAc works as an interface between limbic and motor systems (Kelley 1999; Groenewegen et al. 1999), and drug-seeking behavior depends on glutamate transmission in this structure (Di et al. 2001). HP is one of the glutamate-projecting afferents to NAc (French and Totterdell 2002; Kelley and Domesick 1982), and synaptic changes occurring in this structure can affect the level of neuronal activity within the NAc. Hippocampal LTP has long been postulated to underlie learning and memory processes and may play a role in the complex associative learning that contributes to drug-seeking behavior and relapse (Wolf, 2002). In fact, repeated COC administration enhances hippocampal LTP (Thompson et al. 2002), and theta burst stimulation of hippocampal ventral subiculum reinstates COC-seeking behavior in rats (Vorel et al. 2001). Previously, we reported that COC sensitization induced an increased hippocampal synaptic transmission (observed as a reduction in the threshold to generate LTP; Perez et al. 2010). In the present investigation, we observed that this enhancement was prevented by nNOS inhibition. Similar results were observed when sGC was inhibited by ODQ administration during repeated COC. On the other hand, when cGMP availability was increased using SIL, the COC-facilitated synaptic transmission was maintained. This facilitation could indicate an increased susceptibility to stimuli able to strength glutamate synapses. Then, activation of nNOS/NO/sGC/cGMP pathway by repeated COC may facilitate the LTP generation in HP, activating glutamate containing pathways to NAc, finally responsible for motor execution of goal-directed behavior such as sensitization. These results are in accordance to the effects of cGMP upregulation on corticostriatal synaptic transmission in vivo (Sammut et al. 2010). Surprisingly, the acute or repeated sildenafil treatment (SIL+SAL group) facilitated synaptic transmission in hippocampal dentate gyrus, indicating a possible enhancement in the strength of glutamate synapses by sildenafil exposure. These results are in accordance with previous reports showing that sildenafil induces long-term memory retention and reconsolidation (Puzzo et al. 2008; Boccia et al. 2011) and rescues synaptic plasticity in an Alzheimer's disease mouse model (Puzzo et al. 2009).

It has been demonstrated that a COC single exposure increases NO release in mPFC, HP, and striatum (Sammut and West 2008; Bagetta et al. 1999), and repeated administration enhances nNOS activity and NO production in many brain

regions (Bhargava and Kumar 1997). In our study, we have observed that nNOS activity was increased in HP only in sensitized animals (VEH+COC-S). Furthermore, the percentage of nNOS activity increase in this group was considerably greater than in acute COC group. These results may indicate that nNOS enzyme evidenced a neuroadaptative process of "sensitization" after repeated administration, which is associated to behavioral sensitization and elevated efficiency of hippocampal synaptic transmission (Perez et al. 2010). We also showed that inhibition of nNOS during repeated COC administration (7-NI+COC-S) prevented the "sensitization" of the enzyme activity observed in sensitized animals (VEH+COC-S). These results are further supported by the correlation analysis between locomotor activity on day 5 and NO levels in both sensitized and nonsensitized groups, in which high locomotor activity is positively correlated with elevated NO levels. However, correlation was weakened when nNOS inhibitor was administered concomitantly to COC.

Systemic administration of different nNOS/NO/sGC/cGMP modulators, such as 7-NI, ODQ, or SIL, affects NO or cGMP availability in the whole central nervous system. Thus, changes observed in the HP can be due to a primary effect of such inhibitors in brain areas related to the reward circuit that project to HP, such as the ventral tegmental area, modulating neuronal activity. A local effect in the HP cannot be ruled out, since single or repeated COC administration induces increments in nNOS activity in this area. In summary, this work demonstrates a key role of the nNOS/NO/sGC/cGMP signaling pathway in the development of COC sensitization and in the associated enhancement of hippocampal synaptic transmission, since manipulation of this signaling pathway can significantly affect the proportion of sensitized animals or the threshold to generate LTP. However, the mechanisms by which this pathway is differentially engaged in sensitized or nonsensitized animals remain to be elucidated.

In the present investigation, sildenafil was used as a tool to increase cGMP availability during repeated COC administration, but interestingly, a misuse and recreational use of PDE5 inhibitors have been described in different human populations (Smith and Romanelli 2005; Tahsili-Fahadan et al. 2006) and linked to the illicit use of drugs of abuse (McCambridge et al. 2006). In addition, sildenafil is used under prescription for chronic medical conditions, including lung hypertension, coronary cardiopathies, and erectile dysfunction, among others, even in patients under drug detoxification programs. Considering the results of the present investigation, we can speculate that upregulation of the nNOS/NO/sGC/cGMP signaling pathway in different brain areas could initiate, contribute, or exacerbate addictive behaviors in humans, PDE5 inhibitors being potential candidates for these actions because they may increase vulnerability to drug abuse. It will be appropriate to strictly supervise the use of this drug considering previous patient history or to recommend the use of PDE5 inhibitors that do not cross the brain blood barrier.

Acknowledgment We thank Kerstin Ford who assisted us with the manuscript edition.

Funding source Funding for this study was provided by SECyT (PICT number 2476), CONICET (PIP number 6381), and MINCyT (PID 100). The authors declare no conflict of interest.

References

Badanich KA, Adler KJ, Kirstein CL (2006) Adolescents differ from adults in cocaine conditioned place preference and cocaine-induced dopamine in the nucleus accumbens septi. Eur J Pharmacol 550:95–106

Bagetta G, Rodino P, Arabia A, Massoud R, Paoletti AM, Nistico R, Passantino L, Preziosi P (1999) Systemic administration of cocaine, given alone or in combination with sensory stimuli, differentially affects L-arginine-nitric oxide metabolism in discrete regions of the brain of rat. Neurosci Lett 266:153–156

Belujon P, Grace AA (2011) Hippocampus, amygdala and stress: interacting systems that affect susceptibility to addiction. Ann N Y Acad Sci 1216:114–21

Bhargava HN, Kumar S (1997) Sensitization to the locomotor stimulant activity of cocaine is associated with increases in nitric oxide synthase activity in brain regions and spinal cord of mice. Pharmacology 55:292–298

Boccia MM, Blake MG, Krawczyk MC, Baratti CM (2011) Sildenafil, a selective phosphodiesterase type 5 inhibitor, enhances memory reconsolidation of an inhibitory avoidance task in mice. Behav Brain Res 220:319–324

Boudreau AC, Wolf ME (2005) Behavioral sensitization to cocaine is associated with increased AMPA receptor surface expression in the nucleus accumbens. J Neurosci 25:9144–9151

Bredt DS, Hwang PM, Snyder SH (1990) Localization of nitric oxide synthase indicating a neural role for nitric oxide. Nature 347:768–770

Di CP, Cardinal RN, Cowell RA, Little SJ, Everitt BJ (2001) Differential involvement of NMDA, AMPA/kainate, and dopamine receptors in the nucleus accumbens core in the acquisition and performance of pavlovian approach behavior. J Neurosci 21:9471–9477

Everitt BJ, Wolf ME (2002) Psychomotor stimulant addiction: a neural systems perspective. J Neurosci 22:3312–3320

French SJ, Totterdell S (2002) Hippocampal and prefrontal cortical inputs monosynaptically converge with individual projection neurons of the nucleus accumbens. J Comp Neurol 446:151–165

Garthwaite J (2008) Concepts of neural nitric oxide-mediated transmission. Eur J Neurosci 27:2783–2802

Garthwaite J (2010) New insight into the functioning of nitric oxide-receptive guanylyl cyclase: physiological and pharmacological implications. Mol Cell Biochem 334:221–232

Groenewegen HJ, Wright CI, Beijer AV, Voorn P (1999) Convergence and segregation of ventral striatal inputs and outputs. Ann N Y Acad Sci 877:49–63

Itzhak Y (1996) Attenuation of cocaine kindling by 7-nitroindazole, an inhibitor of brain nitric oxide synthase. Neuropharmacology 35:1065–1073

Itzhak Y (1997) Modulation of cocaine- and methamphetamine-induced behavioral sensitization by inhibition of brain nitric oxide synthase. J Pharmacol Exp Ther 282:521–527

Itzhak Y, Ali SF, Martin JL, Black MD, Huang PL (1998) Resistance of neuronal nitric oxide synthase-deficient mice to cocaine-induced locomotor sensitization. Psychopharmacology (Berl) 140:378–386

Itzhak Y, Roger-Sanchez C, Kelley JB, Anderson KL (2010) Discrimination between cocaine-associated context and cue in a modified conditioned place preference paradigm: role of the nNOS gene in cue conditioning. Int J Neuropsychopharmacol 13:171–180

Jentsch JD, Taylor JR (1999) Impulsivity resulting from frontostriatal dysfunction in drug abuse: implications for the control of behavior by reward-related stimuli. Psychopharmacology 146:373–90

Kelley AE (1999) Neural integrative activities of nucleus accumbes subregions in relation to learning and motivation. Psychobiology 27:198–213

Kelley AE (2004) Ventral striatal control of appetitive motivation: role in ingestive behavior and reward-related learning. Neurosci Biobehav Rev 27:765–776

Kelley AE, Domesick VB (1982) The distribution of the projection from the hippocampal formation to the nucleus accumbens in the rat: an anterograde- and retrograde-horseradish peroxidase study. Neuroscience 7:2321–2335

Kim HS, Park WK (1995) Nitric oxide mediation of cocaine-induced dopaminergic behaviors: ambulation-accelerating activity, reverse tolerance and conditioned place preference in mice. J Pharmacol Exp Ther 275:551–557

Knowles RG, Salter M (1998) Measurement of NOS activity by conversion of radiolabeled arginine to citrulline using ion-exchange separation. Methods Mol Biol 100:67–73

Kulkarni SK, Dhir A (2007) Possible involvement of L-arginine-nitric oxide (NO)-cyclic guanosine monophosphate (cGMP) signaling pathway in the antidepressant activity of berberine chloride. Eur J Pharmacol 569:77–83

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275

Martin SJ, Grimwood PD, Morris RG (2000) Synaptic plasticity and memory: an evaluation of the hypothesis. Annu Rev Neurosci 23:649–711

McCambridge J, Mitcheson L, Hunt N, Winstock A (2006) The rise of Viagra among British illicit drug users: 5-year survey data. Drug Alcohol Rev 25:111–113

Mustafa AK, Gadalla MM, Snyder SH (2009) Signaling by gasotransmitters. Sci Signal 2:68

Nasif FJ, Hu XT, Ramirez OA, Perez MF (2011) Inhibition of neuronal nitric oxide synthase prevents alterations in medial prefrontal cortex excitability induced by repeated cocaine administration. Psychopharmacology (Berl) 218:323–330

Orsini C, Izzo E, Koob GF, Pulvirenti L (2002) Blockade of nitric oxide synthesis reduces responding for cocaine self-administration during extinction and reinstatement. Brain Res 925:133–140

Parent A (1990) Extrinsic connections of the basal ganglia. Trends Neurosci 13:254–8

Perez MF, Gabach LA, Almiron RS, Carlini VP, de Barioglio S, Ramirez OA (2010) Different chronic cocaine administration protocols induce changes on dentate gyrus plasticity and hippocampal dependent behavior. Synapse 64:742–753

Phillips RG, LeDoux JE (1992) Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. Behav Neurosci 106:274–285

Pierce RC, Bell K, Duffy P, Kalivas PW (1996) Repeated cocaine augments excitatory amino acid transmission in the nucleus accumbens only in rats having developed behavioral sensitization. J Neurosci 16:1550–1560

Prast H, Philippu A (2001) Nitric oxide as modulator of neuronal function. Prog Neurobiol 64:51–68

Puzzo D, Sapienza S, Arancio O, Palmeri A (2008) Role of phosphodiesterase 5 in synaptic plasticity and memory. Neuropsychiatr Dis Treat 4:371–387

Puzzo D, Staniszewski A, Deng SX, Privitera L, Leznik E, Liu S, Zhang H, Feng Y, Palmeri A, Landry DW, Arancio O (2009) Phosphodiesterase 5 inhibition improves synaptic function, memory, and amyloid-beta load in an Alzheimer's disease mouse model. J Neurosci 29:8075–8086

Robbins TW, Ersche KD, Everitt BJ (2008) Drug addiction and the memory systems of the brain. Ann N Y Acad Sci 1141:1–21

Robinson TE, Berridge KC (2000) The psychology and neurobiology of addiction: an incentive-sensitization view. Addiction 95(Suppl 2):S91–117

Rogerio R, Takahashi RN (1992) Anxiogenic properties of cocaine in the rat evaluated with the elevated plus-maze. Pharmacol Biochem Behav 43:631–633

Sammut S, West AR (2008) Acute cocaine administration increases NO efflux in the rat prefrontal cortex via a neuronal NOS-dependent mechanism. Synapse 62:710–713

Sammut S, Threlfell S, West A (2010) Nitric oxide-soluble guanylyl cyclase signaling regulates corticostriatal transmission and short-term synaptic plasticity of striatal projection neurons recorded in vivo. Neuropharmacology 58:624

Sinha R (2001) How does stress increase risk of drug abuse and relapse? Psychopharmacology 158:343–59

Smith KM, Romanelli F (2005) Recreational use and misuse of phosphodiesterase 5 inhibitors. J Am Pharm Assoc 45:63–72

Steketee JD (2005) Cortical mechanisms of cocaine sensitization. Crit Rev Neurobiol 17:69–86

Tahsili-Fahadan P, Yahyavi-Firouz-Abadi N, Orandi AH, Esmaeili B, Basseda Z, Dehpour AR (2006) Rewarding properties of sildenafil citrate in mice: role of the nitric oxide-cyclic GMP pathway. Psychopharmacology (Berl) 185:201–207

Teyler TJ, DiScenna P (1987) Long-term potentiation. Annu Rev Neurosci 10:131–161

Thompson AM, Gosnell BA, Wagner JJ (2002) Enhancement of long-term potentiation in the rat hippocampus following cocaine exposure. Neuropharmacology 42:1039–1042

Thompson AM, Swant J, Gosnell BA, Wagner JJ (2004) Modulation of long-term potentiation in the rat hippocampus following cocaine self-administration. Neuroscience 127:177–185

Vorel SR, Liu X, Hayes RJ, Spector JA, Gardner EL (2001) Relapse to cocaine-seeking after hippocampal theta burst stimulation. Science 292:1175–1178

Wolf ME (2002) Addiction: making the connection between behavioral changes and neuronal plasticity in specific pathways. Mol Interv 2:146–157

Yamaguchi M, Suzuki T, Seki T, Namba T, Liu J, Arai H, Hori T, Shiga T (2005) Decreased cell proliferation in the dentate gyrus of rats after repeated administration of cocaine. Synapse 58:63–71