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ORIGINAL INVESTIGATION

Repeated cocaine administration increases nitric oxide efflux in the rat dorsal striatum

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Abstract
Rationale Repeated injections of cocaine alter extracellular nitric oxide (NO) efflux via interactions between dopamine and glutamate receptor-coupled signaling cascades.
Objectives Putative cellular mechanisms underlying changes in NO efflux following repeated cocaine administration were investigated.
Methods Real-time detection of NO efflux using a NO biosensor was mainly performed in the rat dorsal striatum in vivo.
Results Repeated exposure to cocaine (20 mg/kg), once a day for seven consecutive days, increased NO levels. Repeated injections of cocaine also increased the phosphorylation of neuronal nitric oxide synthase (nNOS), and inhibition of nNOS decreased the repeated cocaine-evoked increases in NO levels. Inhibition of protein kinase A, but not protein phosphatases, synergistically increased NO levels elevated by repeated cocaine injections. Blockade of dopamine D1 (D1) receptors or stimulation of dopamine D2 (D2) receptors decreased the repeated cocaine-evoked increases in NO levels. Similarly, blockade of *N*-methyl-D-aspartate (NMDA) receptors and group I metabotropic glutamate receptors (mGluRs) or stimulation of group III mGluRs also decreased the repeated cocaine-evoked increases in NO levels.
Conclusion Stimulation of D1 receptors or group I mGluRs following repeated cocaine administration upregulates NO efflux via an NMDA receptor-evoked Ca^{2+} influx, while stimulation of D2 receptors or group III mGluRs downregulates NO efflux. Dephosphorylation of phosphorylated nNOS by protein phosphatases is necessary for upregulating NO efflux in the dorsal striatum after repeated cocaine administration.

Keywords Biosensor · Caudate · Dopamine · Glutamate · NOS · Psychostimulant

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Introduction

Cocaine is an indirect dopamine agonist and repeated exposure to cocaine can produce enhanced behavioral locomotor response, which is implicated in the development of drug addiction and in drug-induced psychosis (Robinson and Berridge 1993). Considerable evidence suggests that the behavioral and reinforcing effects of cocaine may be mediated by central dopaminergic and glutamatergic systems. For instance, repeated exposure to cocaine upregulates glutamate and dopamine release into the striatum (Cornish and Kalivas 2001; Ito et al. 2002; Kalivas and Duffy 1993; Shin et al. 2007). Increased levels of glutamate caused by repeated cocaine administration upregulate the endoplasmic reticulum stress protein and caspase-12 expression in the dorsal striatum (Shin et al. 2007). Furthermore, *N*-methyl-D-aspartate (NMDA) receptors play an important role in the regulation of the protein

expression (Ahn et al. 2007). These findings suggest that repeated cocaine administration can lead to neuropharmcological, behavioral, or reinforcing responses in the dorsal striatum via NMDA receptor-coupled signaling cascades.

Neuronal nitric oxide synthase (nNOS) and NMDA receptors are structurally associated with PSD95 in the cerebellum (Christopherson et al. 1999). Stimulation of NMDA receptors activates Ca^{2+} signaling cascades, leading to the activation of calcium/calmodulin-dependent protein kinase (CaMK) or protein phosphatases (PPs), which in turn causes NO induction via the alteration of nNOS activity in rat cortical neurons (Hayashi et al. 1999; Nakane et al. 1991). A previous study also demonstrated that protein kinase A (PKA) or protein kinase G can phosphorylate nNOS in the 293-NOS cell lines (Dinerman et al. 1994). Stimulation of dopamine D1 (D1) receptors with the selective D1 receptor agonist, SKF81297, upregulates NO efflux via nNOS activation in the rat dorsal striatum (Sammut et al. 2006). These findings suggest that stimulation of NMDA receptors or D1 receptors are required to produce nNOS activation and NO efflux in the forebrain.

The evidence demonstrates that acute and repeated cocaine administration upregulates NO levels in the medial prefrontal cortex and the dorsal striatum, respectively (Alvin et al. 2008; Sammut and West 2008), which is closely involved in addiction and the development and expression of behavioral sensitization. The behavioral sensitization caused by repeated cocaine exposure is prevented by pretreatment of previously drug-naive animals with the nNOS inhibitor 7-nitroindazole or the pan NOS inhibitor *N*-omega-nitro-L-arginine methyl ester (Byrnes et al. 2000; Haracz et al. 1997).

However, precisely how NO efflux is caused by repeated exposure to cocaine remains elusive, and the putative cellular mechanisms underlying cocaine-mediated behavioral alterations are not well-understood. Therefore, it was hypothesized that repeated injections of cocaine alter extracellular NO efflux by affecting the interactions between dopamine and glutamate receptor-coupled signaling cascades in the dorsal striatum. This hypothesis was investigated by real-time detection of NO efflux using a NO biosensor and altering the function of receptors and transducers affecting the receptor-coupled signaling cascades in the dorsal striatum.

Materials and methods

Animals

Adult male Sprague–Dawley rats (250–300 g) were obtained from Hyo-Chang Science Co. (Taegu, Korea). Rats were individually housed in a controlled environment during all experimental treatments. Food and water were provided ad libitum, and rats were maintained on a 12 h light/dark cycle. On the day of experiments, injections were made in a home cage in a quiet room to minimize stress to the animals. All animal use procedures were approved by the Institutional Animal Care and Use Committee and performed in accordance with the provisions of the NIH Guide for the Care and Use of Laboratory Animals.

Surgery and intrastriatal drug infusion

The rats were anesthetized with intraperitoneal (i.p.) injection of chloral hydrate (6 ml/kg) and placed in a stereotaxic apparatus. Under aseptic conditions, a 23-gauge stainless steel guide cannula (0.29 mm inner diameter, 10 mm in length) was implanted 1 mm anterior to the bregma, 2.5 mm right of the midline, and 5 mm below the surface of the skull. The guide cannula was sealed with a stainless steel wire of the same length. The rats were allowed at least 3 days to recover from surgery. On the day of the experiment, the inner steel wire was replaced by a 30-gauge stainless steel injection cannula (0.15 mm inner diameter, 12.5 mm in length) that protruded 2.5 mm beyond the guide cannula. Throughout the experiments, rats were randomly divided into four groups (n=4–5 per group): vehicle+repeated saline, vehicle+repeated cocaine, drug+repeated saline, and drug+repeated cocaine. Drugs were infused unilaterally into the central part of the right dorsal striatum 5 min prior to the final injection of cocaine or saline in a volume of 1 μl at a rate of 0.2 μl/min in freely moving rats. The progress of the injection was monitored by observing the movement of a small air bubble along the length of precalibrated PE-10 tubing inserted between the injection cannula and a 2.5-μl Hamilton microsyringe. After the completion of the injection, the injector was left in place for additional 5 min to reduce any possible backflow of the solution along the injection tract. Cocaine (Belgopia, Louvain-La-Neuve, Belgium) was dissolved in physiological saline (0.9% sodium chloride, NaCl), and each rat received cocaine (20 mg/kg, i.p.) once daily for seven consecutive days. Parallel to each drug injection, dimethylsulfoxide (DMSO)/artificial cerebrospinal fluid (aCSF) containing (mM) 123 NaCl, 0.86 $CaCl_2$, 3.0 KCl, 0.89 $MgCl_2$, 0.50 NaH_2PO_4, and 0.25 Na_2HPO_4 aerated with 95% O_2/5% CO_2, pH 7.2–7.4 or NaCl was injected into either the center of the dorsal striatum or the peritoneum 5 min before the final injection of cocaine or saline in each experiment, as a control. All drugs, except cocaine used in this study, were purchased from Tocris Cookson (Ellisville, MO, USA), and the drug solutions were all adjusted to pH 7.2–7.4 with 1 N NaOH if necessary. The doses of drugs used in the experiments were determined in previous studies (Choe and Wang 2001,

2002a, b; Choe et al. 2004, 2005, 2006; Mao and Wang 2000; Resstel and Correa 2006; Wang 1998; Wang and McGinty 1995) as well as in our own preliminary studies. The physical accuracy of the injection was verified by the reconstruction of microinjection placements as demonstrated previously (Choe and Wang 2001). The possibility of gliosis caused by the implantation of guide cannula and the infusion of drugs dissolved in DMSO/aCSF was verified using Nissl staining.

Preparation of the NO microbiosensor

The NO microbiosensor was prepared as described previously (Alvin et al. 2008) and cleaned by cycling the applied potential between +1.4 and −0.2 V for ten cycles at a scan rate of 500 mV/s in 0.5 M H_2SO_4 solution, followed by washing with distilled water. Subsequently, the Pt microelectrode was coated with a conductive polymer (CP) through an electropolymerization reaction with a carboxylic acid group, 5,2′:5,2″-terthiophene-3′-carboxylic acid (TTCA) monomer in a 0.1 M tetrabuthylammonium perchlorate/CH_2Cl_2 solution. This was accomplished by cycling the potential between 0.0 and 1.5 V, two times at the scan rate of 50 mV/s. After this, the electrode was washed with CH_2Cl_2 to remove the excess monomer. The CP-coated Pt microelectrode was then immersed for 12 h in a 0.1 M phosphate-buffered saline (PBS, pH 7.0) containing 20 mM 1-ethyl-3 [3-(dimethylamino)-propyl] carbodimide (EDC) to activate the carboxylic acid groups of the CP. At this point, the EDC-treated CP-modified microelectrode was washed with buffer solution and subsequently incubated for 48 h in 5 mM PBS solution containing 6 mg/mL cyt *c* at 4°C. By this procedure, cyt *c* was covalently bound through its amine groups to the carboxylic groups on the poly-TTCA, forming amide bonds. The cyt *c*/poly-TTCA microelectrode was dipped in 1% Nafion solution (diluted with ethanol) for 2 min. The Nafion film was then dried for 1 h in a calcium chloride atmosphere. It was found that Nafion films dried in a low-humidity atmosphere, provided by calcium chloride pellicles in a sealed container, produced an increase in stability.

Real-time detection of NO

On the day of the experiment, rats were anesthetized with 8% chloral hydrate and placed in a Stoelting stereotaxic apparatus. Under aseptic conditions, the enzyme-coated electrode and the reference electrode of the NO microbiosensor were implanted at the coordinates of 1 mm anterior of the bregma, 2.5 mm right of the midline, and 5 mm below the surface of the skull. The sensor tip was inserted unilaterally into the central part of the right dorsal striatum. The sensor electrode was connected to a Potentiostat/Galvanostat, Model PT-1 (Kosentech, Pusan, Korea), which can read and record the electrochemical signal (Fig. 1A). Confirmation of the location of recording electrodes was verified by the reconstruction of placements (Fig. 1B). The sensor was calibrated daily with a series of NO standard solutions during pre- and post experimental measurements. During this study, a 30-min time point was selected based on a time course of repeated cocaine injections in which NO levels were shown to peak at 30-min relative to control groups.

Western immunoblotting

Rats were anesthetized deeply with 8% chloral hydrate and decapitated 30 min after the final injection of saline or cocaine. The brains were removed, frozen in isopentane at −70°C, and stored in a deep freezer until used. Serial sections were cut in a cryostat, and the overall dorsal striatum was removed with a steel borer (inner diameter, 2 mm) as described previously (Ahn et al. 2007). All tissue samples were transferred into homogenization buffer containing (mM) 10 Tris–HCl, pH 7.4, 5 NaF, 1 Na_3VO_4, 1 EDTA, and 1 EGTA. The samples were then sonicated for 30 s on ice and centrifuged at 13,000 rpm for 30 min at 4°C, and the pellet containing mainly nuclei and large debris was discarded. The supernatant was again centrifuged at 15,600×*g* for 30 min at 4°C and was resolved using 8% sodium dodecyl phosphate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was blocked with a blocking buffer containing 5% skim milk. The membrane was probed with a rabbit primary antiserum (Millipore Bioscience Research, Billerica, MA, USA) against phosphorylated nNOS (p-nNOS) (ser1417) at 1:1,000 overnight at 4°C with shaking. The membrane was then incubated with a goat anti-rabbit secondary antiserum (KPL, Gaithersburg, MD, USA) at 1:1,000 for 1 h at room temperature with shaking. Next, immunoreactive protein bands were detected by enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and exposed to X-ray films. Unphosphorylated proteins were probed after stripping the same membrane that was confirmed to contain phosphorylated proteins. The same membrane was also probed for β-tubulin to normalize the blots. Immunoreactive protein bands, as visualized on X-ray films, were semi-quantified using NIH Image 1.62 software as previous described (Shin et al. 2007). Briefly, the film background was measured and saved as a "blank field" to correct for uneven illumination. The upper limit of the density-slice option was set to eliminate any background, and this value was used to measure all images. The lower limit was set at the bottom of the lookup table scale. The immunoreactive protein bands were measured using a rectangle enclosing each individual band.

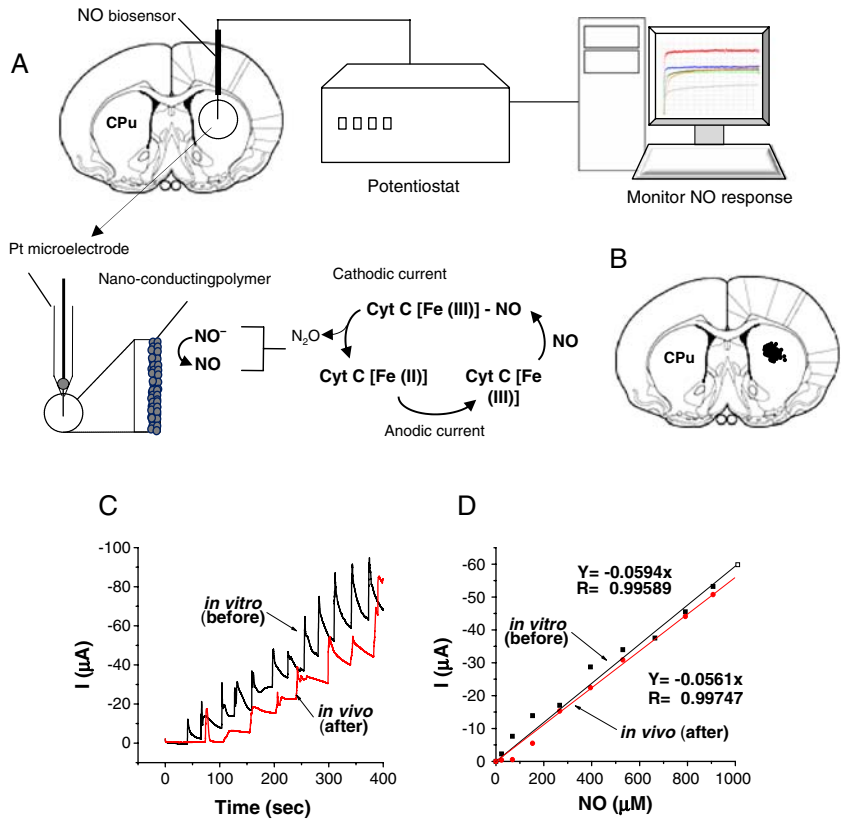

Fig. 1 Schematic diagram illustrating a NO microbiosensor system and its working model for the detection of altered extracellular NO levels evoked by cocaine administration (**A**) and reconstruction of recording electrode placements (**B**) in the dorsal striatum. Current–time plots obtained upon the addition of a series of NO standard solutions in the 0.1 M PBS at pH 7.2 (**C**). Calibration plots of current–NO concentrations detected by the NO microbiosensor (**D**)

Statistics

Data from NO measurements were recorded as currents, which were converted to concentrations, and immunoblotting were expressed as the number of immunoreactive pixels per measured area. Statistical significance between groups was determined using one- or two-way ANOVA followed by a Tukey's honestly significant difference test in GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA). Data were expressed in terms of mean ± SEM for each group (n=4–5 per group). The level of statistical significance was assigned to $p<0.05$.

Results

Performance of the microbiosensor

Before the NO measurement in the dorsal striatum, the microbiosensor was calibrated (Fig. 1C, D), and the control experiment was then carried out in the dorsal striatum. Under optimized conditions, the steady-state currents exhibited a linear relationship with the NO concentration in the range of 0–55.0 μM. The sensitivity of the NO microbiosensor was 0.117±0.006 μA/μM. The detection limit of NO was determined to be 13±3 nM based on five times measurement for the standard deviation of the blank noise (95% confidence level, k=3, n=5). The Nafion film was found to have pore size less than 50 nm, which could prevent peroxide and oxygen from diffusing through. However, NO gas can diffuse somewhat easily through Nafion. In addition, the Nafion layer also prevents microelectrode fouling due to the nonspecific adsorption of proteins and other biological materials present in the brain. In order to remove interference from oxygen, superoxide, and hydrogen peroxide, a thin Nafion film was coated onto the cyt *c*/poly-TTCA surface of the electrode. The selectivity of the cyt *c*/poly-TTCA-modified electrode with Nafion coating was evaluated with cyclic voltamograms in the presence of oxygen, other reactive oxygen species, such as hydrogen peroxide and superoxide, catecholamine, and ascorbate. The sensor exhibited no significant response to these electroactive species (Alvin et al. 2008).

Repeated injections of cocaine increased NO levels in the dorsal striatum

This experiment was conducted to determine if repeated injections of cocaine alter NO efflux in the dorsal striatum; real-time amperometric NO responses were measured for 120 s before and 5 min after the final injection of saline or cocaine up to 60 min. A 10-min time interval was chosen for the measurements of the NO levels in the dorsal

striatum following repeated injections of saline or cocaine. Amperometric response after repeated cocaine injections significantly increased NO levels when compared with repeated saline injections (Fig. 2A, B). Semiquantitation confirmed that 7 days of repeated cocaine, but not repeated saline, injections increased NO levels at 20 min after the final injection and was prolonged up to 60 min in the dorsal striatum (Fig. 2C). To determine if the increase in NO levels was indeed induced by repeated cocaine administration, we measured the levels of NO efflux in four groups (repeated saline–acute saline challenge, repeated saline–acute cocaine challenge, repeated cocaine–acute saline challenge, and repeated cocaine–acute cocaine challenge) of rats at a 20-min time point. A group of rats treated with six daily injections of saline followed by acute cocaine challenge at the seventh day did not produce a significant increase in NO levels when compared with repeated saline–acute saline challenge group (Fig. 2D). However, a group of rats treated with six daily injections of cocaine followed by acute cocaine, but not acute saline, challenge at the seventh day caused a significant increase in NO levels when compared with repeated saline–acute saline challenge group (Fig. 2D), indicating that changes in the levels of NO efflux result from repeated cocaine administration. No gliosis was observed by the implantation of guide cannula and the infusion of drugs dissolved in DMSO/aCSF (data not shown).

Repeated injections of cocaine increased p-nNOS immunoreactivity and inhibition of nNOS activity decreased NO levels in the dorsal striatum

Since repeated cocaine injections increased NO levels in the dorsal striatum, the involvement of nNOS in the regulation of NO efflux was investigated further. Repeated cocaine injections significantly increased p-nNOS immunoreactivity in the dorsal striatum (Fig. 3A). Inhibition of nNOS with the nNOS inhibitor, N^{ω}-propyl-L-arginine (N^{ω}-propyl) (0.01 nmol), significantly decreased the repeated cocaine-induced increases in NO levels when compared with

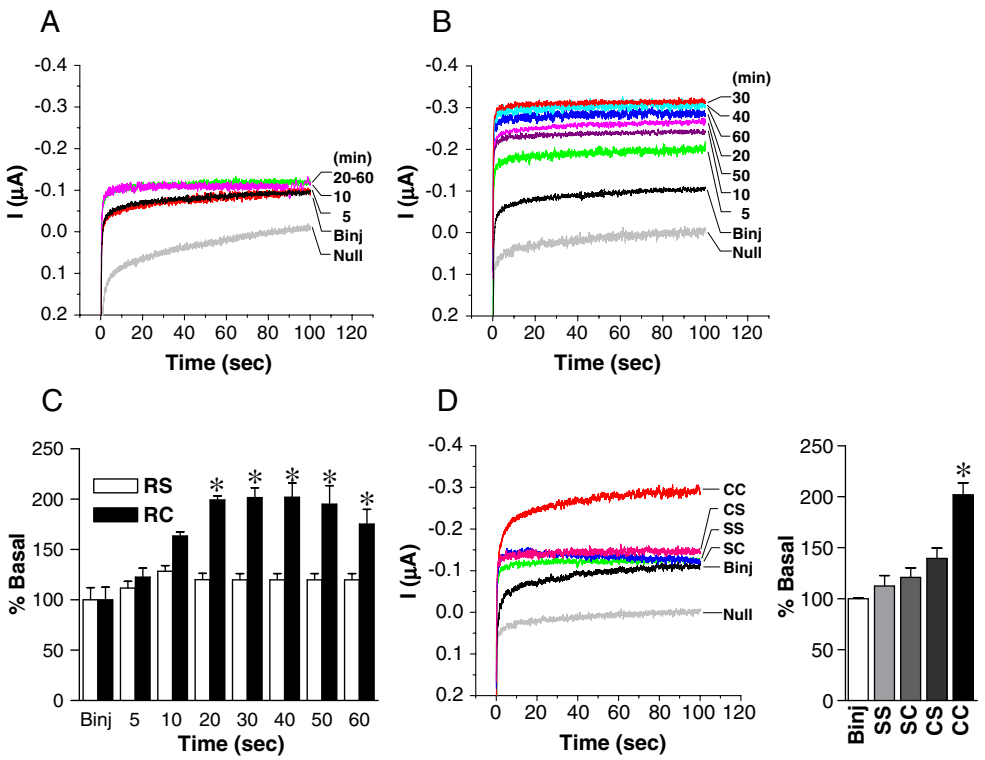

Fig. 2 Alterations of real-time NO responses by cocaine using the NO microbiosensor in the dorsal striatum. Current–time plots detected by NO microbiosensor at before and 5, 10, 20, 30, 40, 50, and 60 min after the final injection of repeated saline (**A**) or cocaine (**B**) administration. Quantitation shows that NO concentrations were increased 20 min after repeated cocaine injections and were prolonged by 60 min (**C**, F_C=21.99). A group of rats treated with six daily injections of cocaine followed by the final injection of cocaine, but not saline, at the seventh day caused a significant increase in NO levels when compared with repeated saline–acute saline challenge group (**D**, F_D=19.01). *Null* null (reference) sensor, *Binj* before injection, *RS* repeated saline injections, *RC* repeated cocaine injections, *SS* repeated saline (6 days)+saline challenge, *SC* repeated saline (6 days)+cocaine challenge, *CS* repeated cocaine (6 days)+saline challenge, *CC* repeated cocaine (6 days)+cocaine challenge. *p<0.05 vs. repeated saline, repeated saline–saline challenge, repeated saline–cocaine challenge, or repeated cocaine–saline challenge group

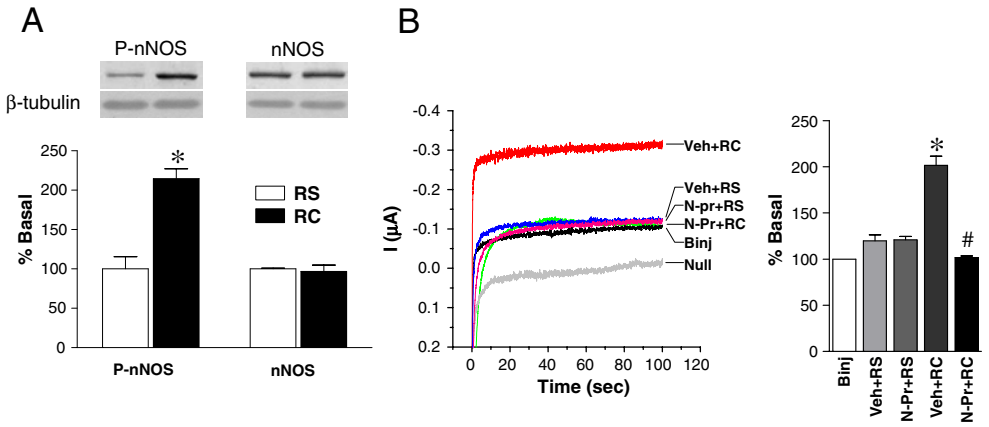

Fig. 3 Inhibition of nNOS decreases the NO efflux in the dorsal striatum after repeated injections of cocaine. P-nNOS immunoreactivity was increased and reached a peak at 30 min after repeated injections of cocaine, when compared to repeated saline injections (**A**, F_A=34.31). Increased NO levels after repeated cocaine injections were decreased by the pretreatment of the nNOS inhibitor N^{ω}-propyl (N-Pr) (**B**, F_B=57.12). *p<0.05 vs. repeated saline group; $^{\#}p$<0.05 vs. repeated cocaine group

repeated saline injections (Fig. 3B). However, inhibition of N^{ω}-propyl alone did not alter the NO levels as compared with repeated saline group. Except for the increased p-nNOS immunoreactivity, neither iNOS nor p-eNOS immunoreactivity was detected in the dorsal striatum after repeated injections of cocaine (data not shown).

Inhibition of PKA had a synergistic effect on increased NO levels evoked by repeated injections of cocaine; however, inhibition of PPs decreased NO levels in the dorsal striatum

Since repeated cocaine injections increased NO levels by the activation of nNOS, the involvement of protein kinases and PPs in the regulation of NO efflux was investigated further. Intrastriatal infusion of the protein kinase C (PKC) inhibitor, GF109203X (5 nmol), alone significantly increased NO levels as compared with repeated saline injections (Fig. 4A). Although increased NO levels by repeated cocaine injections were synergistically increased by the infusion of the PKC inhibitor, the NO levels were not different from the results driven by the infusion of GF109203X alone. Intrastriatal infusion of the PKA inhibitor, KT5720 (5 nmol), synergistically increased NO levels by repeated cocaine injections, while KT5720 infusion alone did not alter the NO levels (Fig. 4B). Increased NO levels evoked by the repeated cocaine injections were not altered by the inhibition of CaMK with KN62 (20 nmol) or MEK with SL327 (150 nmol; data not shown). In contrast, intrastriatal infusion of the PP1/2A inhibitor, okadaic acid (0.05 nmol), or the calcineurin (PP2B) inhibitor, cyclosporin A (0.005 nmol), significantly decreased the repeated cocaine-evoked increases in NO levels in the dorsal striatum, while okadaic acid or cyclosporin A alone did not alter the NO levels (Fig. 4C, D).

Blockade of D1 receptors or stimulation of D2 receptors decreased the repeated cocaine-evoked increases in NO levels in the dorsal striatum

This experiment was conducted to investigate the involvement of dopamine receptors in the regulation of NO efflux in the dorsal striatum after repeated injections of cocaine because D1 receptor-stimulated PKA and PKC were demonstrated to be involved in the regulation of NO efflux. Intrastriatal infusion of the D1 receptor antagonist, SCH23390 (7.5 nmol), significantly decreased the repeated cocaine-evoked increases in NO levels in the dorsal striatum (Fig. 5A). Similarly, intrastriatal infusion of the D2 receptor agonist, quinpirole (5 nmol), also significantly decreased the repeated cocaine-evoked increases in NO levels in the dorsal striatum (Fig. 5B). However, SCH23390 or quinpirole alone did not alter NO levels as compared with the repeated saline group.

Blockade of NMDA receptors decreased the repeated cocaine-evoked increases in NO levels in the dorsal striatum

In addition to D1 receptor stimulation, protein kinases or PPs are also activated by the stimulation of NMDA receptors in the striatum and ventral tegmental area of rats (Borgland et al. 2006; Choe et al. 2005; Schilstrom et al. 2006). This experiment was conducted to investigate the involvement of NMDA receptors in the regulation of NO efflux after repeated injections of cocaine. Intrastriatal infusion of the NMDA receptor antagonists, MK801 (2 nmol) and AP5 (2 nmol), significantly attenuated the repeated cocaine-evoked increases in NO levels in the dorsal striatum (Fig. 6). However, MK801 or AP5 alone did not alter NO levels as compared with the repeated saline group.

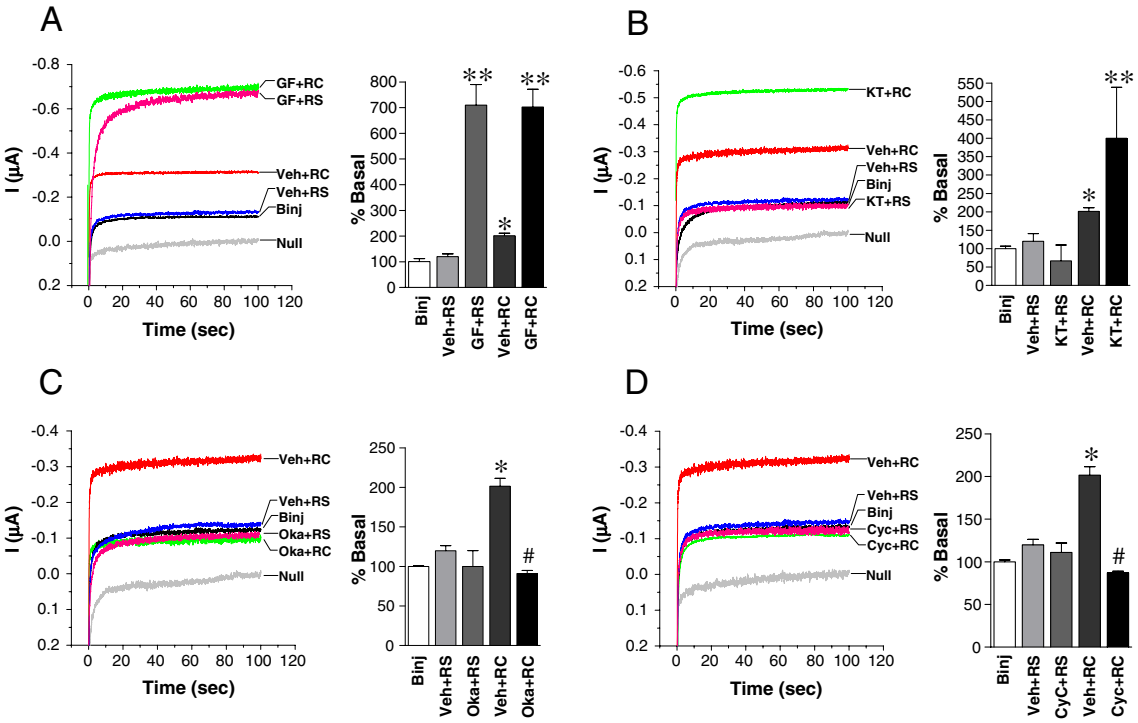

Fig. 4 Inhibition of protein kinases and PPs differentially regulates the NO efflux in the dorsal striatum evoked by repeated injections of cocaine. Quantitation shows that the PKC inhibitor GF109203X (GF) alone (**A**, F_A=203.6) and pretreatment with the PKA inhibitor KT5720 (KT) (**B**, F_B=15.53) synergistically increased NO levels elevated by repeated cocaine injections. However, pretreatment with the PP1/2A inhibitor okadaic acid (Oka) (**C**, F_C=27.50) or the PP2B inhibitor cyclosporin A (Cyc) (**D**, F_D=42.47) decreased the repeated cocaine-evoked increases in NO levels in the dorsal striatum. *p<0.05 vs. repeated saline group; **p<0.05 vs. repeated cocaine group; $^{\#}p$< 0.05 vs. repeated cocaine group

Blockade of group I metabotropic glutamate receptors (mGluRs) or stimulation of group III mGluRs decreased the repeated cocaine-evoked increases in NO levels in the dorsal striatum

Since stimulation of mGluRs are found to modulate Ca^{2+}-dependent protein kinases in the dorsal striatum (Choe and Wang 2002a, b), the involvement of mGluRs in the regulation of NO efflux after repeated cocaine injections was investigated. Intrastriatal infusion of the pan mGluR antagonist, MCPG (10 nmol), significantly decreased NO levels in the dorsal striatum increased by repeated cocaine injections (Fig. 7A). Similarly, the group I mGluR antagonist, PHCCC (25 nmol), the group I mGluR subtype

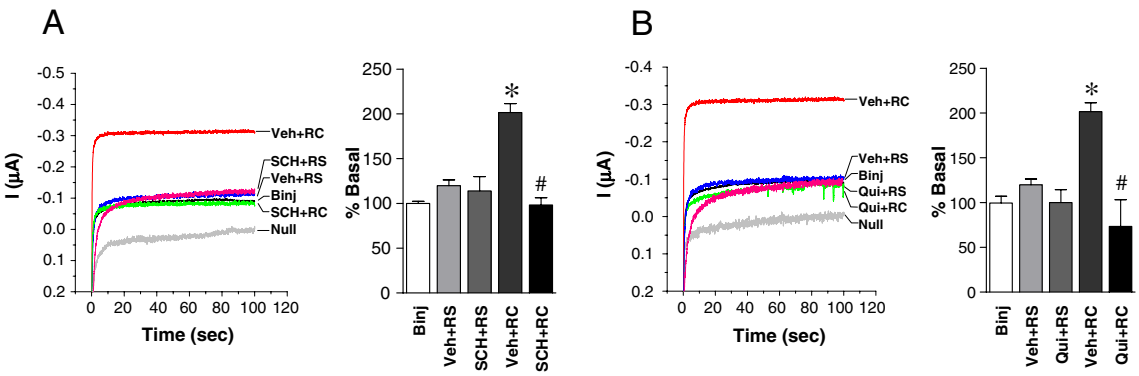

Fig. 5 Blockade of D1 receptors or stimulation of D2 receptors results in a decrease of the NO efflux in the dorsal striatum after repeated injections of cocaine. Quantitation shows that pretreatment with the D1 receptor antagonist SCH23390 (SCH) (**A**, F_A=30.94) or the D_2 receptor agonist quinpirole (Qui) (**B**, F_B=14.75) decreased the repeated cocaine-evoked increases in NO levels in the dorsal striatum. *p<0.05 vs. repeated saline group; $^{\#}p$<0.05 vs. repeated cocaine group

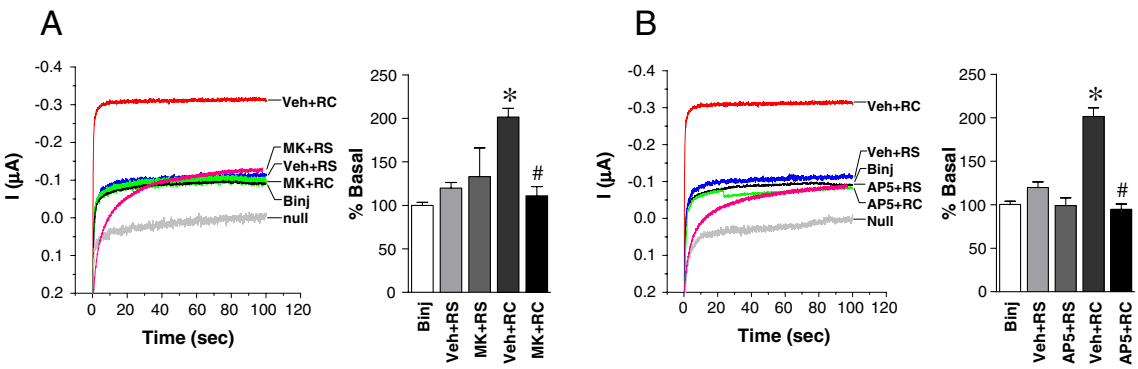

Fig. 6 Blockade of NMDA receptors results in a decrease of NO efflux in the dorsal striatum after repeated injections of cocaine. Quantitation shows that pretreatment of the NMDA antagonist MK801 (MK) (**A**, F_A=11.22) or AP5 (**B**, F_B=49.24) decreased the repeated cocaine-induced increases in NO levels in the dorsal striatum. *p<0.05 vs. repeated saline group; $^{\#}p$<0.05 vs. repeated cocaine group

1 (mGluR1) antagonist, CPCCOEt (5 nmol), or the group I mGluR subtype 5 (mGluR5) antagonist, MPEP (0.5 nmol), also decreased NO levels in the dorsal striatum increased by repeated cocaine injections (Fig. 7B–D). In addition, the involvement of group III mGluRs in the regulation of NO efflux evoked by repeated injections of cocaine was investigated. Stimulation of group III mGluRs with AP4 (100 nmol) significantly decreased the repeated cocaine-evoked increases in NO levels in the dorsal striatum (Fig. 7E). However, the antagonist or agonist alone did not alter NO levels as compared with the repeated saline group. The real-time changes of actual NO levels caused by the infusion of the drugs in all experiments are summarized in Table 1.

Discussion

The present data demonstrate that repeated injections of cocaine upregulate NO efflux, which is regulated by interactions between the dopamine and glutamate receptor-coupled signaling cascades in the dorsal striatum. In this study, repeated injections of cocaine increased NO levels as well as p-nNOS immunoreactivity in the dorsal striatum. Inhibition of nNOS and PPs decreased NO levels that were elevated by repeated injections of cocaine. These data suggest that the cocaine-evoked NO efflux in the dorsal striatum is regulated by the activation of nNOS via dephosphorylation of p-nNOS. Although evidence concerning the mechanisms underlying NO efflux is limited, nNOS is activated by NMDA receptor stimulation in the brain (Dawson and Snyder 1994). It is well known that activation of NMDA receptor-coupled Ca^{2+} signaling cascades in the dorsal striatum is regulated by the integration of dopamine and glutamate receptor stimulation in the dorsal striatum. A recent study shows that striatal NO efflux induced by the electrical or pharmacological stimulation of the substantia nigra is regulated by D1 receptor stimulation (Sammut et al. 2006). Our data also demonstrated that increased levels of NO in the dorsal striatum caused by repeated cocaine injections are decreased by the blockade of D1 receptors or stimulation of D2 receptors. These data may indicate that D1 receptor stimulation is required for an NMDA receptor-evoked Ca^{2+} influx, whereas presynaptic D2 receptor stimulation is required for the downregulation of AC/cAMP cascades by inhibiting the release of dopamine in the dorsal striatum (Cepeda et al. 1998; Cepeda and Levine 1998; Scott et al. 2002).

Like dopamine receptors, stimulation of mGluRs is also necessary for the regulation of Ca^{2+} influx in the dorsal striatum via NMDA receptor stimulation (Choe et al. 2006). For instance, stimulation of group I mGluRs upregulates NMDA NR1 phosphorylation in the dorsal striatum, while stimulation of group II/III mGluRs shows the opposite effect on NMDA receptor phosphorylation (Choe and Wang 2002a, b). Our data demonstrate that blockade of group I mGluRs or stimulation of group III mGluRs decreased NO levels in the dorsal striatum elevated by repeated cocaine injections. These data suggest that stimulation of group I mGluRs after repeated cocaine administration is able to increase NO efflux via NMDA receptor-coupled Ca^{2+}-signaling cascades. However, stimulation of group III mGluRs after repeated cocaine administration downregulates NO levels by inhibiting the release of glutamate in the dorsal striatum similar to the presynaptic action of D2 receptors discussed above. Our findings that blockade of NMDA receptors with MK801 or AP5 decreases repeated cocaine-evoked increases in NO levels in the dorsal striatum support the idea that NMDA receptor-coupled Ca^{2+} cascades may influence NO efflux after repeated cocaine administration. This idea is further supported by the previous findings that systemic injection of the NMDA receptor antagonist, MK801, decreases striatal NO efflux evoked by the electrical stimulation of the frontal cortex (Sammut et al. 2007).

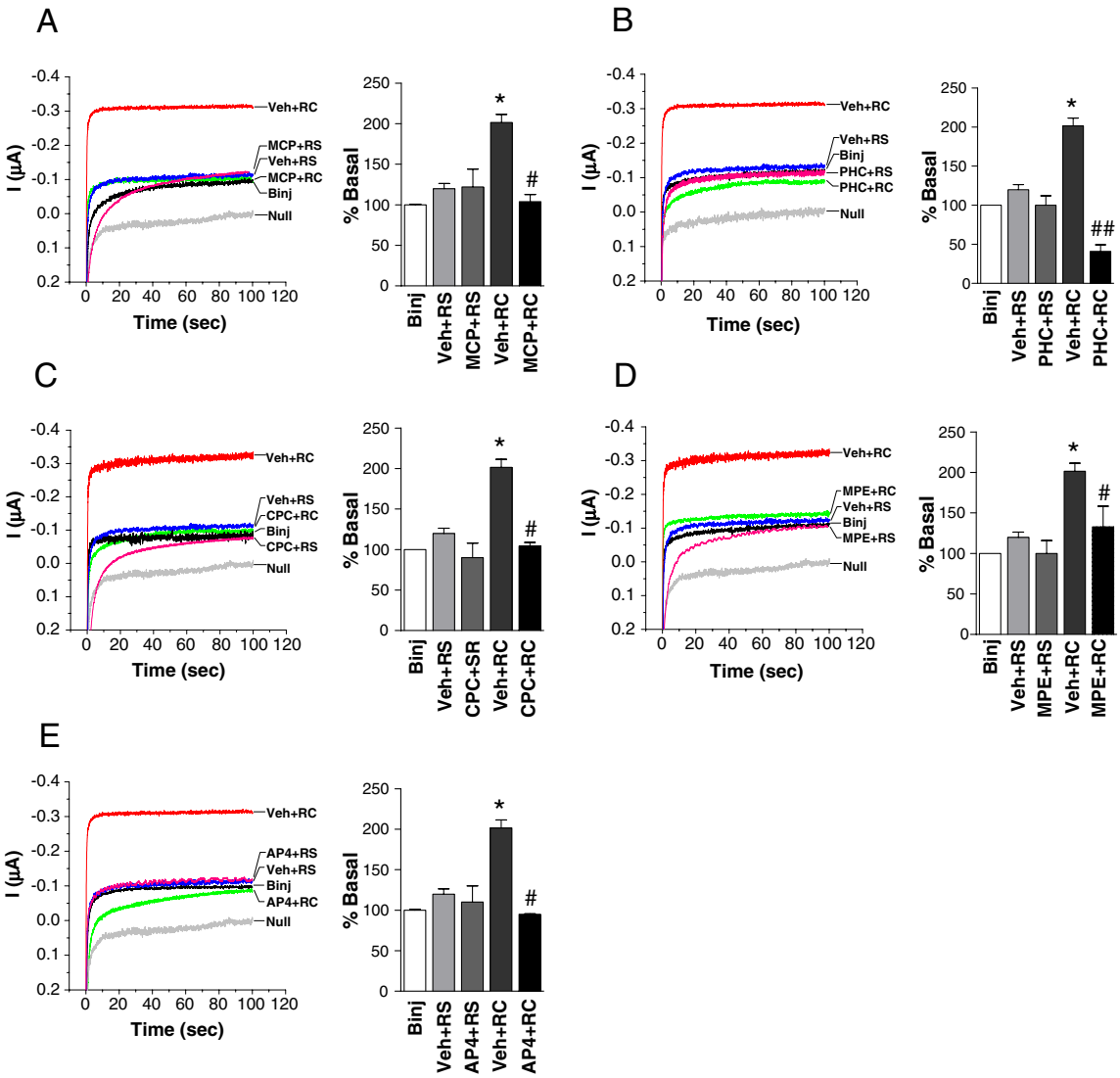

Fig. 7 Blockade of group I mGluRs or stimulation of group III mGluRs results in a decrease of NO efflux in the dorsal striatum after repeated injections of cocaine. Quantitation shows that pretreatment of the pan mGluRs antagonist MCPG (MCP) (**A**, F_A=21.10), the group I mGluR antagonist PHCCC (PHC) (**B**, F_B=44.19), the mGluR1 antagonist CPCCOEt (CPC) (**C**, F_C=34.02), the mGluR5 antagonist MPEP (MPE) (**D** F_D=34.95) or the group III mGluR agonist AP4 (**E** F_E=25.43) decreased the repeated cocaine-induced increases in NO levels in the dorsal striatum. *p<0.05 vs. repeated saline group; $^{\#}p$<0.05 vs. repeated cocaine group; $^{\#\#}p$<0.05 vs. repeated saline group

Ca^{2+} levels, altered by the stimulation of either dopamine or glutamate receptors in striatal neurons, can activate Ca^{2+} signaling cascades and subsequently increase NO production via nNOS activation. The data presented here showed that inhibition of PKC alone increases NO levels, and inhibition of PKA, but not PPs, synergistically increases the levels of NO in the dorsal striatum after repeated cocaine injections, suggesting the contribution of PKC and PKA in the activation of nNOS in the dorsal striatum. It is well-known that stimulation of D1 receptors and group I mGluRs activates AC/cAMP (or PKA) and PLC-coupled PKC cascades, respectively. These in turn phosphorylate nNOS, as discussed earlier. Like protein kinases, PPs are also activated by Ca^{2+} cascades via dopamine or glutamate receptor stimulation in the striatum (Nishi et al. 2005). A previous study demonstrates that activation of PP1 or PP2A is necessary for the induction of NO caused by dephosphorylating p-nNOS in cortical cell culture (Rameau et al. 2003). Our data also show that inhibition of either PP1/2A or PP2B decreases NO levels in the dorsal striatum. These data suggest that activation of nNOS, by the dephosphorylation of p-nNOS, is required for the production of NO in striatal neurons.

Several studies have shown that NO plays an important role in cocaine-induced increases in behavioral locomotor activity. For instance, NOS inhibitors have been shown to

Table 1 The real-time changes of NO efflux by the infusion of drugs following repeated exposure to cocaine in the dorsal striatum

Group	Before injection	30min
Vehicle+repeated saline	1.778±0.217	2.089±0.146
Vehicle+repeated cocaine	1.778±0.226	3.650±0.630[a]
Repeated saline+acute saline	1.626±0.042	1.806±0.162
Repeated saline+acute cocaine	1.632±0.012	1.698±0.012
Repeated cocaine+acute saline	1.650±0.054	2.238±0.270
Repeated cocaine+acute cocaine	1.715±0.055	3.460±0.140[a]
Quinpirole+repeated cocaine	1.843±0.137	1.359±0.317[b]
AP4+repeated cocaine	2.038±0.014	1.937±0.014[b]
SCH23390+repeated cocaine	1.518±0.036	1.489±0.126[b]
MK801+repeated cocaine	1.677±0.058	1.861±0.178[b]
AP5+repeated cocaine	1.652±0.064	1.561±0.101[b]
PHCCC+repeated cocaine	2.226±0.000	0.910±0.188[c]
CPCCOEt+repeated cocaine	1.836±0.000	1.915±0.080[b]
MPEP+repeated cocaine	1.764±0.000	2.342±0.448[b]
MCPG+repeated cocaine	1.937±0.014	2.009±0.173[b]
Nω-propyl+repeated cocaine	1.749±0.000	1.778±0.029[b]
GF109203X+repeated cocaine	1.710±0.400	11.984±1.171[d]
Okadaic acid+repeated cocaine	1.850±0.014	1.684±0.080[b]
Cyclosporin A+repeated cocaine	1.923±0.043	1.677±0.043[b]
KT5720+repeated cocaine	2.349±0.152	9.389±3.260[d]

Values represent mean ± SEM (μM)

[a] Increase NO levels as compared with repeated saline, repeated saline + acute saline, repeated saline + acute cocaine, or repeated cocaine + acute saline

[b] Decrease NO levels as compared with repeated cocaine

[c] Decrease in NO levels as compared with repeated cocaine and repeated saline

[d] Increase in NO levels as compared with repeated cocaine and repeated saline

block cocaine-induced behavioral effects (Pudiak and Bozarth 1993). It is well-known that the neuronal glutamate–NO pathway modulates several important physiological processes, such as drug dependence, and NO is an intermediary in the action of glutamate (Fedele and Raiteri 1999). NO has been suggested as a retrograde neurotransmitter and may diffuse from the postsynaptic membrane to the presynaptic membrane (Snyder 1992). Thus, repeated stimulation of postsynaptic receptors by cocaine may produce increases in the release of presynaptic neurotransmitters including dopamine and glutamate. Since NOS inhibitors are shown to block cocaine or methamphetamine-induced dopamine release in the striatum (Bowyer et al. 1995; Inoue et al. 1996), it is possible that inhibition of NO formation reduces development of locomotor activity to cocaine by modulating dopamine release or activation of postsynaptic dopamine receptors in the striatum or nucleus accumbens. The present results strongly suggest that activation of nNOS by dephosphorylating p-nNOS has an important role in NO-mediated behavioral and reinforcing effects produced by cocaine.

Summary

Repeated exposure to cocaine upregulates NO efflux into the dorsal striatum and requires nNOS activation via NMDA receptor-coupled Ca^{2+} cascades. As proposed in Fig. 8, stimulation of D1 receptors or group I mGluRs appears to upregulate NO efflux by influencing NMDA

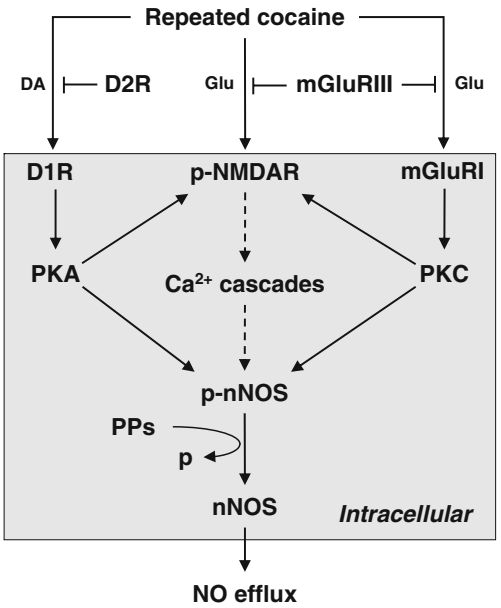

Fig. 8 A postulated diagram illustrating intracellular mechanisms underlying repeated cocaine-regulated NO efflux in the dorsal striatum. Putative interactions are discussed in detail in the text. *DA* dopamine, *Glu* glutamate, *D1R* and *D2R*, dopamine D1 and D2 receptors, *mGluRI* and *mGluRIII* groups I and III mGluRs, *p* phosphorylation. *Solid arrows* and *blunt arrows* represent stimulation and blockade of downstream targets, respectively, while *broken arrows* represent indirect stimulation of p-nNOS via NMDA receptor-coupled Ca^{2+} signaling cascades

receptor-evoked Ca^{2+} influx, while stimulation of presynaptic D2 receptors or group III mGluRs downregulates NO efflux in the dorsal striatum by inhibiting the release of dopamine or glutamate. Conversion of p-nNOS into nNOS by PPs is necessary for repeated cocaine administration to produce a NO efflux in the dorsal striatum in vivo.

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