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Reduced D-serine levels in the nucleus accumbens of cocaine-treated rats hinder the induction of NMDA receptor-dependent synaptic plasticity

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Cocaine seeking behaviour and relapse have been linked to impaired potentiation and depression at excitatory synapses in the nucleus accumbens, but the mechanism underlying this process is poorly understood. We show that, in the rat nucleus accumbens core, D-serine is the endogenous coagonist of *N*-methyl-D-aspartate receptors, and its presence is essential for *N*-methyl-D-aspartate receptor-dependent potentiation and depression of synaptic transmission. Nucleus accumbens core slices obtained from cocaine-treated rats after 1 day of abstinence presented significantly reduced D-serine concentrations, increased expression of the D-serine degrading enzyme, D-amino acid oxidase, and downregulated expression of serine racemase, the enzyme responsible for D-serine synthesis. The D-serine deficit was associated with impairment of potentiation and depression of glutamatergic synaptic transmission, which was restored by slice perfusion with exogenous D-serine. Furthermore, *in vivo* administration of D-serine directly into the nucleus accumbens core blocked behavioural sensitization to cocaine. These results provide evidence for a critical role of D-serine signalling in synaptic plasticity relevant to cocaine addiction.

Keywords: nucleus accumbens; D-serine; cocaine; synaptic plasticity; NMDA receptors

Abbrevations: AMPA = 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid; BsGO = recombinant *Bacillus subtilis* glycine oxidase; DAAO = D-amino acid oxidase; EPSC = excitatory postsynaptic current; EPSP = excitatory postsynaptic potential; LTD = long term depression of synaptic transmission; LTP = long term potentiation of synaptic transmission; NMDA = *N*-methyl-D-aspartate

Introduction

Cocaine addiction is a pathological learned behaviour characterized by compulsive drug seeking and high vulnerability to relapse even after prolonged abstinence (Mendelson and Mello, 1996). A large body of evidence has linked the development and expression of this addictive behaviour to neuroadaptations in the mesocorticolimbic system. The interconnected brain regions that make

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up this system include the prefrontal cortex, the ventral tegmental area and the ventral striatum (also known as the nucleus accumbens), which has been critically implicated in the expression of a variety of addiction-related behavioural alterations (Koob and Volkow, 2010).

Cocaine-induced neuroadaptations in the nucleus accumbens involve changes in gene transcription, membrane excitability, neuronal morphology, and other parameters as well, but those affecting the function and plasticity of glutamatergic synapses appear to be crucial for the development of addictive behaviour (Kauer and Malenka, 2007; Lüscher and Malenka, 2011).

Activity-dependent long-term potentiation (LTP) and long-term depression (LTD) of synaptic transmission are the two principal forms of synaptic plasticity that permit strengthening or weakening of synapses in neural circuits. As in other brain regions, excitatory synapses in the nucleus accumbens are subject to several forms of synaptic plasticity, including *N*-methyl-D-aspartate (NMDA)-dependent long term potentiation and depression, endocannabinoid-dependent LTD, and a presynaptic form of LTD mediated by metabotropic glutamate receptors (mGluRs). Chronic cocaine exposure can activate or 'hijack' some of these synaptic plasticity mechanisms, as well as influence the subsequent generation of synaptic plasticity thus leading to the development of addictive behaviour (Lüscher and Malenka, 2011). In particular, a growing body of evidence support the notion of an impairment of NMDA receptor-dependent LTP and LTD at glutamatergic synapses in the nucleus accumbens of animal models of cocaine addiction (Martin *et al*., 2006; Moussawi *et al*., 2009; Kasanetz *et al*., 2010).

NMDA receptors are heteromeric protein complexes composed of at least one GluN1 subunit in combination with GluN2A and/or GluN2B subunits. The capacity of these receptors to mediate ion flux depends on their simultaneous interaction with glutamate and a coagonist (originally thought to be exclusively glycine) that binds the glycine site on the NR1 subunit. Recent data have shown that D-serine is also a physiologically relevant glycine-site ligand in several brain areas, including the hippocampus, hypothalamus and prefrontal cortex (Panatier *et al*., 2006; Fossat *et al*., 2011). Free D-serine is abundant in the mammalian brain (Hashimoto and Oka, 1997). It is generated by the activity of the proteolipid protein-containing enzyme serine racemase, which catalyses the reversible conversion of L- into D-serine (Wolosker *et al*., 1999). Its catabolism is also mediated by the activity of serine racemase and by that of the flavin adenine dinucleotide-dependent enzyme D-amino acid oxidase (DAAO) (Pollegioni and Sacchi, 2010).

D-serine is crucial for the activation of synaptic NMDA receptors and NMDA receptor-dependent synaptic plasticity mechanisms. Its degradation by exogenous DAAO suppresses the NMDA component of excitatory postsynaptic currents (EPSCs) and prevents LTP and LTD in different brain areas, including the hippocampus and supraoptic nucleus (Yang *et al*., 2003; Panatier *et al*., 2006). More recently, the induction of NMDA receptor-dependent LTP at excitatory hippocampal synapses has been shown to depend on the availability of NMDA receptors provided by the Ca^{2+}-dependent release of D-serine from astrocytes (Henneberger *et al*., 2010).

D-Serine's emerging role as an active modulator of synaptic transmission and plasticity prompted us to investigate its physiological role in the nucleus accumbens and its potential involvement in the cocaine-induced changes in synaptic plasticity that occur in this brain region. Although the core and shell subregions of the nucleus accumbens are both involved in cocaine-induced behavioural changes, we focused on the nucleus accumbens core, which appears to be crucial for the control of behaviour by salient stimuli such as those associated with cue- and stress-induced reinstatement of drug seeking (Kalivas and McFarlard, 2003) and for the induction and expression of cocaine-induced behavioural sensitization (Kalivas *et al*., 2009). We found that in this subregion of the rat brain, endogenous D-serine is essential for NMDA receptor-dependent LTP and LTD, and in rats undergoing early withdrawal from passive cocaine exposure, levels of this D-amino acid are decreased, thereby impairing the processes of NMDA receptor-dependent synaptic plasticity. Furthermore, we found that *in vivo* administration of D-serine blocks locomotor sensitization to cocaine. Thus, we propose a new role for D-serine signalling as molecular correlate for cocaine-induced changes in synaptic plasticity and locomotor sensitization.

Materials and methods

Slice preparation and electrophysiology

Coronal slices (300–400 μm thick) containing the nucleus accumbens were prepared using standard procedures (D'Ascenzo *et al*., 2009). Briefly, the animals were anaesthetized with halothane (Sigma) and decapitated. The brains were rapidly removed and placed in ice-cold cutting solution containing in mM: 124 NaCl, 3.2 KCl, 1 NaH_2PO_4, 26 $NaHCO_3$, 2 $MgCl_2$, 1 $CaCl_2$, 10 glucose, 2 Na-pyruvate and 0.6 ascorbic acid (pH 7.4, 95% O_2/5% CO_2). Slices were cut with a vibratome (VT1000S, Leica Microsystems) and incubated in the cutting solution at 32°C for at least 1 h and then at room temperature until use. For patch-clamp recordings slices were transferred to a submerged recording chamber and continuously perfused with artificial CSF bubbled with 95% O_2 5% CO_2 (pH 7.4). The artificial CSF contained (in mM): 124 NaCl, 3.2 KCl, 1 NaH_2PO_4, 26 $NaHCO_3$, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose. The flow rate was kept at 1.5 ml/min with a peristaltic pump (Minipuls 3, Gilson) and bath temperature was maintained at 30–32°C by an inline solution heater and temperature controller (TC-344B, Warner Instruments). Picrotoxin (50 μM) was present in the artificial CSF throughout the experiment to block GABAA receptor-mediated inhibitory postsynaptic potentials and currents. Medium spiny neurons within the nucleus accumbens core subregion were identified with a ×40 water-immersion objective on an upright microscope equipped with differential interface contrast optics under infrared illumination (BX5IWI, Olympus) and video observation. Voltage-clamp recordings were made with a MultiClamp 700B amplifier (Molecular Devices). Electrodes were made from borosilicate glass micropipettes (Warner Instruments) prepared with a P-97 Flaming-Brown micropipette puller (Sutter Instruments). Excitatory postsynaptic potentials (EPSPs) and EPSCs were evoked with a bipolar tungsten electrode connected to a high-voltage isolator. The stimulating electrode was positioned 200–300 μm dorsal to the recording electrode. Most whole-cell patch-clamp EPSC recordings were performed with pipettes (resistance of 3–5 MΩ) filled with internal solution containing (in mM): 120 caesium methanesulphonate, 20 HEPES, 0.4 EGTA, 2.8 NaCl, 5 TEA-Cl, 2.5 MgATP and 0.25 NaGTP, pH 7.2–7.3 (280–290

mOsm). Access resistance was monitored throughout the recording and was typically <15 MΩ. The 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid (AMPA)/NMDA ratio was measured as the ratio of the peak amplitude of the EPSCs recorded at -60 mV over the peak amplitude of the EPSCs recorded at +40 mV in the presence of the AMPA receptor antagonist NBQX (30 μM). In a set of experiments, we recorded pure NMDA currents in the presence of NBQX (30 μM) at a holding potential of -60 mV. The electrodes were filled with internal solution containing (in mM): 145 K-gluconate, 2 $MgCl_2$, 0.1 EGTA, 2 Na_2ATP, and 10 HEPES (pH 7.2 with KOH; 290 mOsm). Spontaneous unitary excitatory postsynaptic currents (miniature EPSCs) were collected in the presence of tetrodotoxin (TTX, 0.5 μM), and and D-2-amino-5-phosphonopentanoate (D-AP5, 50 μM) to suppress spontaneous action potential-driven release and NMDA miniature EPSCs. Cells were clamped at -70 mV and AMPA miniature EPSPs were recorded for at least 3 min with K-gluconate-based internal solution. Paired-pulse ratios were derived from two consecutively evoked AMPA receptor-mediated EPSCs at a holding potential of −70 mV. The interstimulus intervals ranged from 20 to 200 ms. Data acquisition and stimulation were performed with a Digidata 1440A Series interface and pClamp 10 software (Molecular Device). Data were filtered at 1 kHz, digitized at 10 kHz and were analysed off-line with pClamp 10 software. The amplitudes and frequency of miniature EPSCs for each cell were evaluated in 180-s recordings. Amplitudes are expressed as absolute values. Field EPSPs were recorded in the nucleus accumbens core in the current clamp-mode with an artificial CSF-filled pipette. After 20 min of stable baseline responses to test stimulations delivered once every 20 s, LTP was induced with an established high frequency stimulation protocol (100 Hz train of stimuli, 0.5 s duration repeated four times at 20 s intervals) (Schotanus and Chergui, 2008). In a subset of experiments LTP was induced with a different high frequency stimulation consisting of 100 Hz train of stimuli, 3 s duration repeated four times at 20 s intervals (Caria *et al*., 1996; Hernandez *et al*., 2005). The stimulation intensity that elicited 50% of the maximal response was used for test pulses and high frequency stimulation. The LTD-induction protocol consisted in moderate frequency stimulation (10 Hz for 10 min or 20 min; Mockett *et al*., 2002; Wang, 2008). Test pulses and moderate frequency stimulation were delivered at intensities that produced 50% and 90% of the maximal response, respectively. Peak field EPSP amplitude and slope were expressed as percentages of the mean baseline values observed during the 10 min (100%) preceding high or moderate frequency stimulation delivery. Each data point on the plot represents the mean of three consecutive observations. LTP and LTD magnitude were assessed by measuring field EPSP amplitude and slope 30 min after the high and moderate frequency stimulation induction. When not specified the magnitude of LTP and LTD was expressed as per cent changes in amplitude.

Immunohistochemistry

Rats were anaesthetized with a cocktail of ketamine (100 mg/ml) and medetomidine (1 mg/ml) and transcardially perfused with Ringer's solution followed by 4% paraformaldehyde fixative solution. The brains were removed, post-fixed overnight at 4°C, cut into coronal sections (40-μm thick) and floated in ice-cold PBS. Sections were blocked for 45 min at room temperature in 1% bovine serum albumin solution containing 10% goat serum and 0.5% Triton™ X-100 (Sigma-Aldrich). The sections were incubated for 72 h at 4°C with one of the following primary antibodies: goat anti-SR (1:100; Santa Cruz Biotechnology Inc.); rabbit anti-D-serine (1:1000, Gemac Bio); rabbit anti-DAAO (1:1000, Nordic Immunological). They were then incubated with the appropriate secondary antibody: Alexa Fluor® 488 rabbit anti-goat (1:300) or Alexa Fluor® 488 donkey anti-rabbit (1:300) (Invitrogen).

Slices were also double-labelled with primary antibody plus mouse monoclonal anti-NeuN antibody (1:150; Chemicon) or mouse monoclonal anti-glial fibrillary acidic protein (GFAP) antibody (1:200; Cell Signaling Technology Inc.). Both markers were then visualized with Alexa Fluor® 546 donkey anti-mouse (1:200; Invitrogen). Nuclei were counterstained with 4′6-diamidino-2-phenylindole (DAPI; 0.5 μg/ml; Invitrogen), and the sections were mounted on glass slides and cover-slipped with ProLong® Gold antifade reagent (Invitrogen). Control sections from all specimens were obtained by omitting the primary antibody. No specific signals were ever detected.

Images of brain slices were obtained with a confocal laser scanning system (TCS-SP2, Leica). DAPI staining was imaged by two-photon excitation performed with Ti:Sapphire laser (Chameleon, Coherent, Inc.).

Animal treatment

Male Wistar rats (26–32 days old) received five once-daily injections (intraperitoneal) of either saline or saline with 15 mg/kg cocaine hydrochlorate (Tocris Bioscience). Brain slices used for electrophysiological experiments, as well as, for real-time reverse transcription PCR, western blot and HPLC analysis, were obtained 1 day after the last injection. All animal procedures were approved by the Ethics Committee of the Catholic University and were fully compliant with Italian and European Union legislation on animal research.

Enzymes

Recombinant *Rhodotorula gracilis* D-amino acid oxidase (RgDAAO, EC 1.4.3.3) was overexpressed in *Escherichia coli* cells and purified to homogeneity. The final enzyme preparation had a specific activity of 100 ± 15 U/mg protein on D-serine as substrate. Recombinant *Bacillus subtilis* glycine oxidase (BsGO, EC 1.4.3.19) was also overexpressed in *E. coli* (Job *et al*., 2002): the enzyme preparation had a specific activity of 0.9 ± 0.2 U/mg protein on glycine as substrate. Rg*DAAO* and Bs*GO* specifically degrade D-serine and glycine respectively, as demonstrated by the calculated apparent kinetic efficiency (Rg*DAAO* k_{cat}/K_m ratio values are 3.0 and 0.058 mM/s on D-serine and glycine, respectively; Bs*GO* k_{cat}/K_m ratio values are 0.00025 and 0.867 mM/s on D-serine and glycine, respectively). The affinity of both proteins for their best substrate ($K_m \approx$ 0.7–0.8 mM) is substantially lower than the affinity of D-serine and glycine for the NMDA receptor glycine-site. To selectively degrade D-serine or glycine, slices were incubated for at least 30 min and then continuously perfused with artificial CSF containing RgDAAO (0.2-0.4 U/ml) or BsGO (0.2 U/ml), respectively.

Quantitative real-time reverse transcriptase polymerase chain reaction

Coronal slices containing the nucleus accumbens were obtained from saline- ($n = 6$) and cocaine-injected ($n = 6$) rats (as described above for electrophysiological experiments). After slice recovery at 32°C for 1 h, the nucleus accumbens core subregions were dissected bilaterally and immediately frozen in liquid nitrogen. RNA was extracted by using a Paris™ Kit (Ambion Inc.). Reverse transcription reactions were performed on equal amounts of RNA with a high-capacity complementary DNA reverse transcription kit (Applied

Biosystems). Real-time reverse transcription PCR experiments were performed using inventoried TaqMan® probes (Applied Biosystems) specifically designed to recognize messenger RNAs of serine racemase and DAAO.

Expression levels for genes of interest were normalized to levels measured for the TATA-box-binding protein (tbp) housekeeping gene. Three independent experiments were performed and each measure was assessed in quadruplicate with the ABI 7500 Sequence Detection System Analyzer for reverse transcriptase-PCR (Applied Biosystems). The threshold cycle number furnished by the analyser was normalized to the housekeeper and used to calculate fold changes in gene expression. Variations in gene expression induced by cocaine treatment were expressed as logged fold changes compared to saline-injected rats.

Protein extraction and western immunoblot analysis

Nucleus accumbens core subregions, obtained from saline-treated ($n = 5$) and cocaine-treated rats ($n = 5$), were homogenized in ice-cold RIPA buffer solution supplemented with 1 mM Na_3VO_4, 1 mM NaF and protease (Sigma-Aldrich). After centrifugation (14 000 *g* for 15 min at 4°C), measured aliquots of each sample were used to determine the protein concentration (microBCA kit, Pierce). SDS-PAGE reducing sample buffer was added to protein lysates and samples were loaded onto a 12% SDS-polyacrylamide gel. Colorburst™ Electrophoresis markers (Sigma-Aldrich) were used as molecular mass standards. Proteins were then transferred onto nitrocellulose membranes, at 37 V overnight at 4°C. After staining with Ponceau-S (Sigma-Aldrich), membranes were blocked for 1 h with a solution containing 10% non-fat dry milk in Tris-buffered saline plus 0.1% Tween-20 and incubated overnight at 4°C with primary antibodies directed against serine racemase (1:500), DAAO (1:4,000) and tubulin (1:1000; Sigma-Aldrich). The membranes were then incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies. The bands were visualized with an enhanced chemiluminescence detection kit (Pierce). Densitometric analysis of protein expression was performed with TINA software.

Cell surface protein biotinylation

Plasma membrane proteins were biotinylated to be easily distinguished after immunoprecipitation. Nucleus accumbens tissues isolated from coronal brain slices of saline- ($n = 4$) and cocaine-treated rats ($n = 4$) were incubated in artificial CSF at 32°C for recovery and then linked to 1 mg/ml NHS-LC-biotin (Pierce). After 30 min at 4°C the biotinylation treatment was blocked with lysine (500 μg/ml for 5 min), followed by three washes in cold artificial CSF. The biotinylated nucleus accumbens tissues were then lysed in a RIPA buffer solution and the protein concentration was determined as described above. Measured aliquots of each samples were then incubated with streptavidin beads (Pierce) for 12 h at 4°C under rotation. Bound material, corresponding to the surface fraction, was washed and then eluted with Laemmli loading buffer. Samples were subjected to western immunoblot analysis with the following antibodies: mouse monoclonal anti-NR1 (1:1000; BD Pharmingen); rabbit polyclonal anti-NR2A (1:500; Millipore); mouse monoclonal anti-NR2B (1:1000; BD Transduction Laboratories); mouse monoclonal anti-GLUR1 (1:100; Millipore) and mouse monoclonal anti-tubulin (1:1000; Sigma-Aldrich).

High-performance liquid chromatography analysis

Nucleus accumbens core slices were obtained from cocaine- and saline-treated rats ($n = 3$ for each group) as for electrophysiological experiments. After slices recovery at 32°C for 1 h, the nucleus accumbens core subregions were dissected bilaterally under light microscopy and tissues were immediately transferred to liquid nitrogen in an Eppendorf tube and then stored at -80°C. The tissues were pooled and sonicated in 5 vol of ice-cold 10% trichloroacetic acid and centrifuged (20 min; 13 000 rpm; 4°C) to remove proteins. Soluble fraction was then extracted with water-saturated ether and neutralized with NaOH before pre-column derivatization with *o*-phthaldialdehyde/*N*-acetyl-L-cysteine in borate buffer (0.1 M, pH 10.4). Diastereoisomer derivatives were then resolved on an Adsorbosphere XL C8 300A 5 μm (Alltech, 4.6 × 250 mm) reversed phase column in isocratic conditions. The mobile phase consisted in sodium acetate buffer (0.1 M, pH 6.2) added with 1% tetrahydrofuran (1 ml/min flow rate). After every single run a washing step in 0.1 M sodium acetate buffer, 3% tetrahydrofuran and 47% acetonitrile was performed. Identification and quantification of the D- and L- isomer of serine was based on retention times and peak areas, compared to those associated with D- and L-serine external standards. Standards were prepared as 10 mM stock solution in Milli-Q water. Calibration curves were built by injecting increasing amount of standards (2–200 pM). The identity of D-serine peak was also confirmed by a pre-column treatment with RgDAAO: the samples were incubated with 20 μg of RgDAAO at room temperature for 1 h and then derivatized.

Intra-nucleus accumbens injections and locomotor activity

Rats (150–200 g) were implanted with bilateral cannulae directed at the nucleus accumbens core. The following coordinate with lambda and bregma in the same horizontal plane were used: anterior to bregma 1.8 mm; lateral to midline ±1.3 mm; ventral from the dura −2.3 mm; according to Franklin and Paxinos). Following recovery from surgery (1 week), spontaneous locomotor activity was measured by using an open field apparatus (a square chamber, 90 × 90 × 45 cm). Rats were allowed to habituate to the chamber for 15 min on two consecutive days during which the animals received only intraperitoneal saline injections. On the following days (Days 1–5) rats received intra-core nucleus accumbens D-serine (4 μg/0.3 μl) or vehicle (0.3 μl) and were tested for locomotor activity after receiving intraperitoneal injection of cocaine. Control rats received intraperitoneal injection of saline and inta-core nucleus accumbens administration of either D-serine or vehicle. Locomotor activity was tested for 15 min. Videotracking software (Smart, Panlab, Harvard Apparatus) was used to measure locomotor activity that has been expressed as travelled distance (cm).

Statistical analysis

Data are expressed as means ± standard errors of the means (SEM). Statistical significance was assessed with the following tests: (i) Student's *t*-test (when used, paired *t*-test has been indicated in the text); (ii) one-way ANOVA for multiple groups comparison (with Bonferroni *post hoc* test); (iii) two-way ANOVA; and (iv) two-way ANOVA for repeated measures. The level of significance was set at 0.05.

Results

D-serine is the endogenous co-agonist of NMDA receptors in the nucleus accumbens core

To identify the endogenous co-agonist(s) of synaptic NMDA receptors in the nucleus accumbens core, we performed whole-cell patch-clamp recordings on gamma-aminobutyric acidergic medium spiny neurons, which represent >95% of the cell population in the nucleus accumbens, in slices incubated (30–45 min) with recombinant *R. gracilis* DAAO (RgDAAO) or recombinant *B. subtilis* glycine oxidase (BsGO), which selectively degrade D-serine and glycine, respectively (Job *et al*., 2002; Panatier *et al*., 2006; Papouin *et al*., 2012). Analysis of the peak amplitudes of AMPA receptor- and NMDA receptor-mediated EPSCs revealed a significantly increased AMPA/NMDA ratio in slices treated with RgDAAO (0.2 U/ml) [6.01 ± 1.07 ($n = 10$) versus 1.33 ± 0.15 ($n = 8$) in controls; one-way ANOVA $F_{(2,22)} = 12.4$; $P < 0.001$; Fig. 1A and B]. Input-output curves revealed consistent differences in NMDA receptor currents between control and RgDAAO-treated slices in the entire range of stimulation intensities [Fig. 1C; $n = 6$; two-way ANOVA; $F_{(1,70)} = 206.7$; $P < 0.0001$]. The effects of RgDAAO were not due to a deleterious action exerted by D-serine oxidation byproducts since control experiments, performed on 6 of the 10 cells recorded from RgDAAO-treated slices, showed that bath application of D-serine (100 μM) increased the NMDA receptor-mediated current, bringing the AMPA/NMDA ratio close to control values (from 6.65 ± 1.74 to 1.52 ± 0.4; $n = 6$; paired *t*-test; $P > 0.05$). Furthermore, RgDAAO did not affect the amplitude and the frequency of AMPA miniature EPSCs nor the paired-pulse ratio of AMPA current, a standard paradigm to test for changes in glutamate release probability (Supplementary Fig. 1), thus confirming its specific action on NMDA receptor. BsGO slice treatment (0.2 U/ml), instead, had no significant effect on the AMPA/NMDA ratio [1.80 ± 0.31 ($n = 7$); one-way ANOVA; $F_{(2,22)} = 12.4$; $P > 0.05$ versus controls; Fig. 1A and B]. The efficacy of BsGO in degrading glycine in our experimental condition was confirmed by control experiments showing that the same treatment increased the AMPA/NMDA ratio in neurons from the nucleus of the tractus solitarius (Supplementary Fig. 2) a brainstem region where glycine is known to act as co-agonist of NMDA receptor (Miyazaki *et al*., 1999; Panatier *et al*., 2006).

Taken together these evidences demonstrate that the amino acid D-serine, not glycine, is the endogenous coagonist of NMDA receptors in the nucleus accumbens core.

Previous immunohistochemical studies in various brain structures selectively localized D-serine and serine racemase in the astrocyte population, supporting the view that D-serine is a gliotransmitter (Wolosker *et al*., 1999; Panatier *et al*., 2006), but more recent data indicate that D-serine and serine racemase are also found in neurons (Wolosker, 2011). We performed double immunofluorescence experiments to explore the cellular distribution, in the nucleus accumbens core, of D-serine and the enzymes primarily responsible for its synthesis and degradation (serine racemase and DAAO). As shown in Supplementary Figs 3, 4 and 5, all three were found in both astrocytes and neurons (labelled with glial fibrillary acidic protein and neuronal nuclei, respectively).

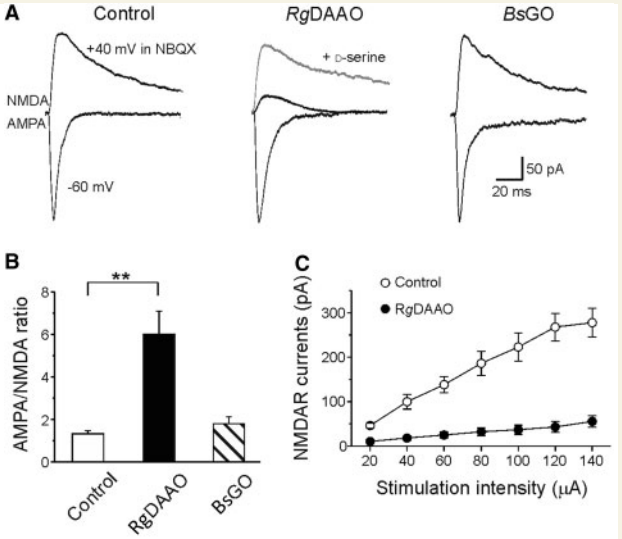

Figure 1 D-serine control of NMDA receptor activity at nucleus accumbens glutamatergic synapses. (**A**) Examples of AMPA receptor- and NMDA receptor-mediated EPSCs in cells from control slices (*left*) and in cells from slices treated with RgDAAO (0.2 U/ml; *middle*) or BsGO (0.2 U/ml; *right*). Traces are the average of at least five consecutive EPSCs. The AMPA/NMDA ratio was obtained by measuring the peak AMPA receptor-mediated EPSCs (holding potential = -60 mV) and peak NMDA receptor-mediated EPSCs in the presence of 30 μM NBQX (holding potential = +40 mV). (**B**) Bar graph of the AMPA/NMDA ratio in cells from control slices ($n = 8$) and in cells from slices treated with RgDAAO ($n = 10$) or BsGO ($n = 8$). (**C**) Input-output curves of NMDA receptor-mediated EPSCs in control and RgDAAO-treated slices ($n = 6$). Input-output curves were generated by plotting peak NMDA current amplitudes against the intensities of presynaptic stimulation. Error bars indicate SEM, $*P < 0.05$, $**P < 0.001$ in this and the following figures.

NMDA receptor-dependent long-term potentiation and depression at excitatory synapses depend on the availability of D-serine

The previous experiments demonstrate the presence of D-serine in the nucleus accumbens core and its active involvement in the regulation of synaptic NMDA receptor activity. The next set of experiments focused on its possible role in NMDA receptor-dependent LTP and LTD in this brain area. As shown in Fig. 2, we measured the amplitude of field EPSPs before and after high-frequency stimulation of glutamatergic afferent fibres, which is known to induce LTP in this area (Schotanus and Cherugi, 2008). After 30 min from high frequency stimulation

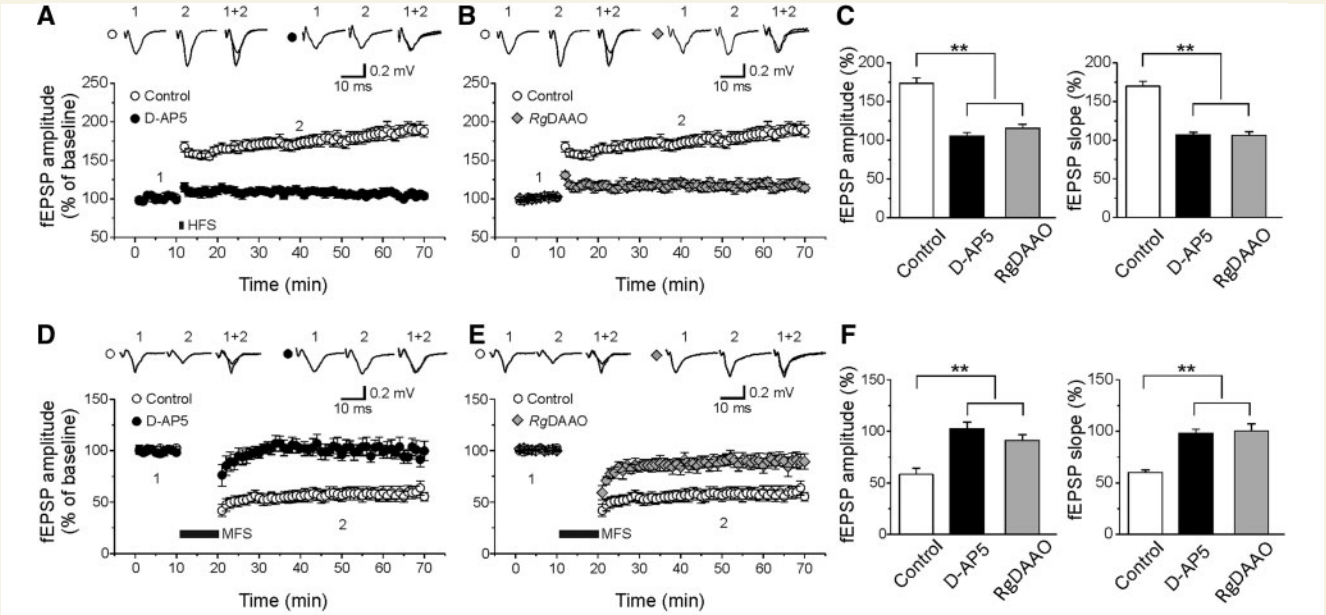

Figure 2 Endogenous D-serine is required for NMDA receptor-dependent LTP and LTD in the nucleus accumbens. (**A**) High frequency stimulation (HFS; four trains of 1 s; 100 Hz; 15 s intertrain) potentiated evoked field EPSPs (fEPSP) in the nucleus accumbens (open circles). LTP induction was blocked by D-AP5 (50 μM; black circles), reflecting its NMDA receptor-dependence. Insets: Representative traces showing field EPSPs before and after (30 min) LTP induction under each condition. The thick horizontal line indicates high frequency stimulation duration. Each point of the plots represents the average of three successive observations. (**B**) LTP could not be induced in slices preincubated (30 min) and continuously perfused with RgDAAO (0.2 U/ml). For comparison, control LTP is also shown (open circles, same recordings shown in **A**). (**C**) Bar graph comparing average field EPSP amplitude and slope measured 30 min after LTP induction expressed as a percentage of the baseline amplitude (100%) under control conditions ($n = 22$), in D-AP5-treated ($n = 7$) and DAAO-treated slices ($n = 7$). (**D**) Moderate frequency stimulation (MFS; 10 Hz; 10 min) decreased evoked field EPSP amplitudes in the nucleus accumbens (open circles). LTD induction was blocked by D-AP5 (black circles). (**E**) In RgDAAO-treated slices, LTD amplitude was markedly reduced. (**F**) Bar graph comparison of average field EPSP amplitude and slope values measured 30 min after LTD induction in controls ($n = 15$), in D-AP5- ($n = 7$) and RgDAAO-treated slices ($n = 8$). See also Supplementary Fig. 6.

the amplitudes of field EPSPs were significantly increased (173.7 ± 7.0% of baseline; $n = 22$; $P < 0.001$; Fig. 2A and C). When field EPSPs were recorded in the presence of the selective NMDA receptor antagonist D-AP5 (50 μM) no modification of the synaptic strength could be observed [105.0 ± 4.7% of baseline; $n = 7$; $P > 0.05$; one-way ANOVA; $F_{(3,37)} = 16.2$; $P < 0.00001$ versus control group; Fig. 2A, C], thus confirming the NMDA receptor-dependency of this LTP.

We then tested the effect of RgDAAO treatment on the magnitude of NMDA receptor-dependent LTP. It is known that this form of synaptic plasticity is related to the number of NMDA receptors activated during afferent stimulation and the Ca^{2+} influx triggered by this activation (Cummings *et al*., 1996). Therefore, on the basis of our findings (Fig. 1), we expected that LTP in the nucleus accumbens core would be diminished by RgDAAO-catalysed degradation of D-serine. Indeed, as shown in Fig. 2B and C, when slices were preincubated and continuously perfused during recordings with RgDAAO, field EPSP amplitude was not significantly increased by high frequency stimulation [115.3 ± 5.2% of baseline; $n = 7$; $P > 0.05$; one-way ANOVA; $F_{(3,37)} = 16.2$; $P < 0.0005$ versus control group]. In contrast, after slice treatment with BsGO (0.2 U/ml), the LTP magnitude was almost identical to that observed under control conditions [171.7 ± 8.0% of baseline, $n = 5$; $P < 0.001$; one-way ANOVA; $F_{(3,37)} = 16.2$; $P > 0.05$ versus control group; Fig. 2C and Supplementary Fig. 6].

These findings indicate that D-serine is necessary for NMDA receptor-dependent LTP in the nucleus accumbens core. To test whether D-serine is also involved in the induction of NMDA receptor-dependent LTD in nucleus accumbens we measured the magnitude of LTD in control and RgDAAO-treated slices. As in a previous study (Wang, 2008), moderate-frequency stimulation of glutamatergic fibres (10 Hz; 10 min) reliably induced LTD in the nucleus accumbens core slices (58.2 ± 5.9% of baseline; $n = 15$; $P < 0.001$; Fig. 2D and F). Similarly to LTP, LTD was blocked by the application of D-AP5 (50 μM) (102.8 ± 7.7% of baseline; $n = 7$, $P > 0.05$; one-way ANOVA $F_{(3,31)} = 14.3$; $P < 0.0005$ versus control group; Fig. 2D and F], confirming its dependence on NMDA receptor activation.

In line with our working hypothesis, LTD could not be induced in slices preincubated for 30 min and continuously perfused with RgDAAO (0.2 U/ml) [90.9 ± 7.7% of baseline; $n = 8$; $P > 0.05$; one-way ANOVA $F_{(3,31)} = 14.3$; $P < 0.005$ versus control group; Fig. 2E and F]. In contrast, in slices treated with BsGO the LTD magnitude was similar to that observed in control conditions [38.1 ± 11.2% of baseline; $n = 5$; $P < 0.001$; one-way ANOVA

$F_{(3,31)} = 14.3$; $P > 0.05$ versus control group; Supplementary Fig. 6]. These results demonstrate that D-serine is also fundamental for the induction of LTD in the nucleus accumbens core.

Exogenous D-serine restores long-term potentiation and depression in slices from cocaine-treated rats

Next, we investigated whether D-serine signalling plays a role in the neuradaptations of glutamatergic synaptic plasticity occurring in the nucleus accumbens core of rats chronically treated with cocaine (Kauer and Malenka, 2007; Lüscher and Malenka, 2011).

For these experiments, we used nucleus accumbens slices from rats chronically exposed to cocaine according to a standard paradigm (five once-daily injections; 15 mg/kg; intraperitoneally; Fig. 3A) that produces behavioural sensitization (Thomas *et al*., 2001; Kourrich *et al*., 2007).

First, we studied LTP and LTD (induced as described above; Fig. 2) in slices obtained from cocaine- and saline-treated rats the day after the treatment protocol was completed. As shown in Fig. 3B and C, high frequency stimulation-induced LTP was significantly smaller in slices from cocaine-treated animals [one-way ANOVA; $F_{(3,31)} = 6.6$; $P < 0.005$ versus saline]. Thirty minutes after high frequency stimulation, field EPSP amplitude was $168.1 \pm 13.1\%$ of baseline value ($n = 7$; $P < 0.001$) in slices from saline-treated rats and $130.5 \pm 5.5\%$ of baseline value ($n = 11$; $P < 0.001$) in cocaine-treated slices. These results indicate that chronic exposure to cocaine caused a marked LTP deficit in the nucleus accumbens core.

As for LTD, moderate frequency stimulation stimulation of excitatory fibres induced robust LTD in slices from saline-treated rats ($55.6 \pm 7.2\%$ of baseline; $n = 8$; $P < 0.001$; Fig. 3D, E), whereas in slices from cocaine-treated rats LTD was absent [$92.4 \pm 6.3\%$ of baseline, $n = 7$; $P > 0.05$; one-way ANOVA $F_{(3,27)} = 10.3$; $P < 0.005$ versus saline group Fig. 3D and E]. The results suggest that during early withdrawal from cocaine exposure NMDA receptor-dependent LTD is also significantly attenuated in the rat nucleus accumbens core.

We hypothesized that the impaired NMDA receptor-dependent synaptic plasticity we found in cocaine-treated rats might be due, at least in part, to diminished D-serine concentrations at synaptic level. A deficit of this type would decrease the number of NMDA receptors available for activation, reducing Ca^{2+} entry into medium spiny neurons to levels incompatible with the induction of LTP and LTD. To test this hypothesis, we evaluated the magnitude of LTP and LTD in slices from cocaine-treated rats exposed to saturating concentrations of exogenous D-serine. If the cocaine-associated impairment of LTP and LTD was indeed related to a D-serine deficit, bath application of exogenous D-serine would be expected to reverse it.

As shown in Fig. 3, in slices from cocaine-treated rats exposed to (10 min before stimulation) and continuously perfused with 100 μM of D-serine, our protocols induced LTP ($160.5 \pm 6.0\%$ of baseline, $n = 10$; $P < 0.001$; Fig. 3B and C) and LTD ($42.9 \pm 7.8\%$ of baseline; $n = 8$; $P < 0.001$; Fig. 3D and E) similar to those observed in the saline-treated group [one-way ANOVA; $F_{(3,31)} = 6.6$; $P = 0.72$ and $F_{(3,27)} = 10.3$; $P = 0.98$ for LTP and LTD respectively]. It is important to note that in saline-treated animals, exposure to exogenous D-serine had no effect on LTP or LTD amplitude (Supplementary Fig. 7). Moreover, in slices from cocaine-treated rats the application of stronger induction protocols elicited LTP and LTD similar to those observed in the saline-treated group following standard high frequency and moderate frequency stimulation protocols (Supplementary Fig. 8). In light of these findings, it would seem that the level of NMDA receptor glycine site occupancy achieved with physiological levels of endogenous D-serine is sufficient to elicit both types of synaptic plasticity, whereas in cocaine-treated rats, endogenous D-serine levels in the nucleus accumbens core are probably decreased, and this alteration might explain the impaired LTP and LTD observed in these animals.

NMDA receptor hypofunction in nucleus accumbens slices from cocaine-treated rats depends on decreased levels of D-serine

To strengthen the above evidence we performed whole-cell patch-clamp recordings and compared the amplitude of synaptic NMDA receptor currents recorded in slices from saline- and cocaine-treated rats. If the above hypothesis was correct, we reasoned that exposure to saturating concentrations of exogenous D-serine would increase synaptic NMDA receptor currents in slices from cocaine-treated rats, to a greater extent than that observed in the saline-treated rats. We, therefore, performed whole-cell patch-clamp experiments and recorded these currents before and after bath application of D-serine (100 μM).

During these experiments, neurons were kept at holding potential of -60 mV in a modified artificial CSF containing 0.1 mM Mg^{2+} supplemented with 30 μM NBQX, and using K-gluconate-based internal solution to prevent any possible time-dependent run-down of the currents.

As shown in Fig. 4A–C, the addition of D-serine significantly increased the evoked NMDA current amplitudes in neurons of slices from saline-treated rats ($+78.2 \pm 9.2\%$ of control, $n = 9$; $P < 0.001$), but a significantly larger increase ($+196.0 \pm 41.1\%$ of control, $n = 8$; $P < 0.001$) was seen in neurons from the cocaine-treated rats ($P < 0.05$ versus saline-treated slices, Fig. 4A and B). These findings further support that nucleus accumbens D-serine levels are decreased in these animals, and as a result, fewer NMDA receptors are recruited during synaptic stimulation.

To verify this, we used HPLC and measured D-serine concentrations in the nucleus accumbens slices. In both cocaine- and saline-treated samples, HPLC analysis revealed peaks at retention times of 21.4 ± 0.7 and 23.5 ± 0.8 min, which corresponded to D-serine and L-serine, respectively.

The identity of the peaks was confirmed by addition of known amount of D- and L-serine as internal standards to the samples, as well as, by incubating the samples with RgDAAO before derivatization and column analysis (Fig. 4D and E). The latter pretreatment yielded to the complete depletion of D-serine peak, as shown in Fig. 4D and E. Interestingly, the amount of D-serine decreased

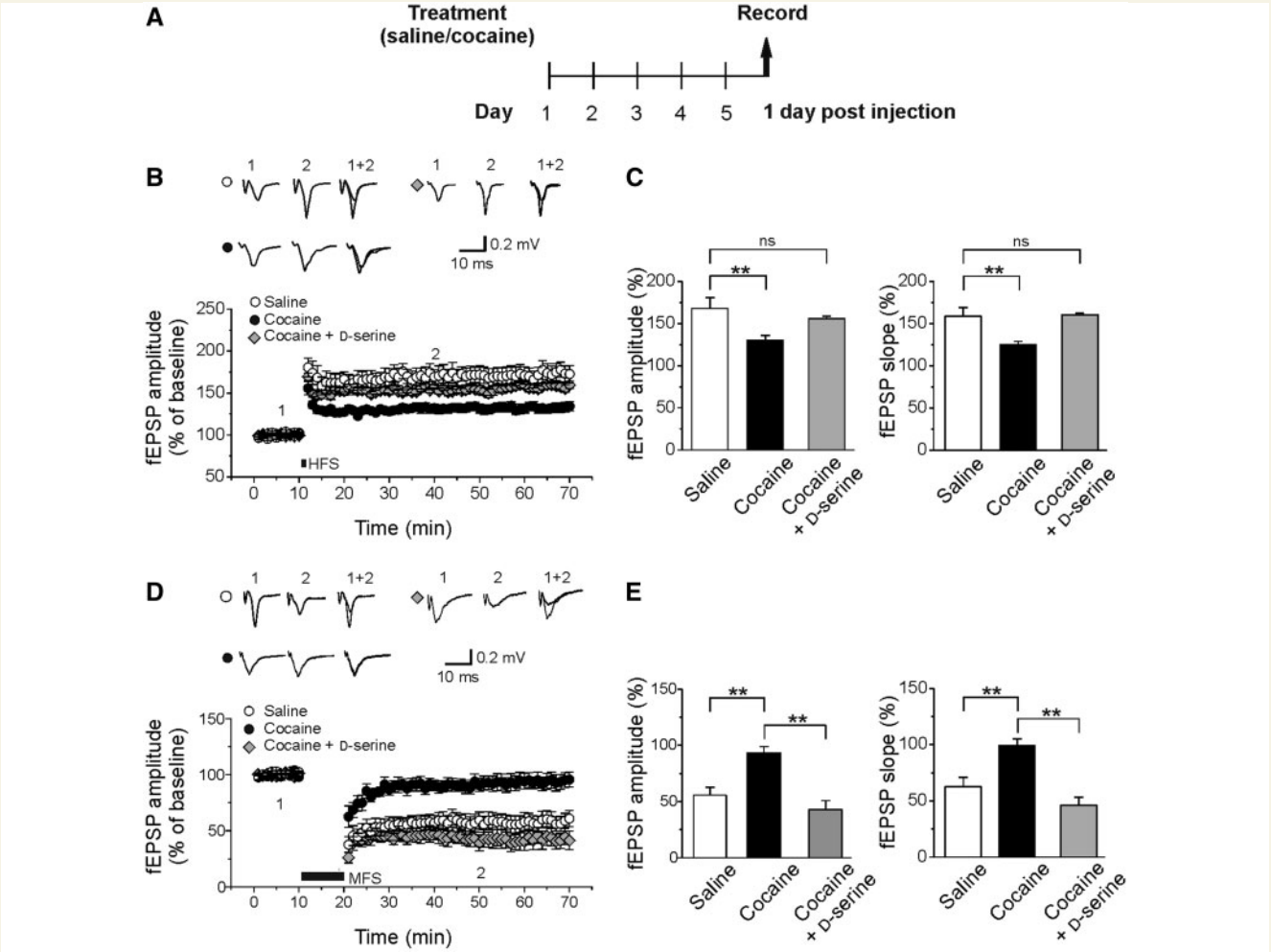

Figure 3 The impairment of LTP and LTD in rats undergoing early withdrawal from passive cocaine exposure is restored by exogenous D-serine. (**A**) Experimental timeline of cocaine-treatment protocol (15 mg/kg; intraperitoneal). (**B**) Exogenous D-serine (100 μM) restored the ability to induce LTP in slices from cocaine treated-rats. (**C**) Mean amplitude and slope of field EPSP recorded 30 min after high frequency stimulation under the experimental conditions shown in **B** (saline: $n = 7$; cocaine: $n = 11$; cocaine + D-serine $n = 10$). (**D**) Exogenous D-serine also restored LTD inducibility in slices from cocaine-treated rats. (**E**) Field EPSP amplitude and slope values under the experimental conditions reported in **D** (saline: $n = 8$; cocaine: $n = 7$; cocaine + D-serine $n = 8$). See also Supplementary Figs 7 and 8.

significantly from 4.63 ± 0.93 pmol/μg of protein (saline-treated rats) to 2.77 ± 0.40 pmol/μg of protein (cocaine-treated rats; $P < 0.05$; $n = 4$; Fig. 4F). These results are fully in agreement with our electrophysiological evidences and support our hypothesis for a reduced D-serine signalling in the nucleus accumbens core of cocaine-treated rats.

An alternative possible explanation for the NMDA receptor hypofunction would involve an altered expression of NMDA receptor subunits. However, western blot and surface biotinylation analysis demonstrated that cocaine treatment does not affect overall nucleus accumbens expression levels of GluN1, GluN2A, and GluN2B (Supplementary Fig. 9A and B) or their insertion in the plasma membrane (Supplementary Fig. 9A and C). These findings are in agreement with previous reports (Schumann and Yaka, 2009), and strongly support our attribution of the cocaine-associated impairment of NMDA receptor-dependent synaptic plasticity to reduced D-serine levels.

We next tested whether cocaine treatment affected the AMPA/NMDA ratio in the nucleus accumbens core. As shown in Fig. 4G and H, the AMPA/NMDA ratio was significantly increased in cocaine-treated animals compared with saline treated rats (cocaine: 2.76 ± 0.48; saline 1.23 ± 0.39; $P < 0.05$), in agreement with our findings of cocaine-induced reduction of D-serine levels and NMDA receptor hypofunction. In order to exclude the possibility that altered AMPA/NMDA ratio was due to changes in AMPA receptor-mediated synaptic transmission we recorded AMPA miniature EPSCs and response to paired-pulse stimulation in both saline- and cocaine-treated animals. As shown in Supplementary Fig. 10, no changes were found in miniature EPSC amplitude and frequencies, as well as in paired-pulse ratios, arguing against altered AMPA receptor function and glutamate release probability in cocaine-treated rats. Accordingly, expression level of AMPA receptor GluA1 subunit was not affected by cocaine administration (Supplementary Fig. 11). All these

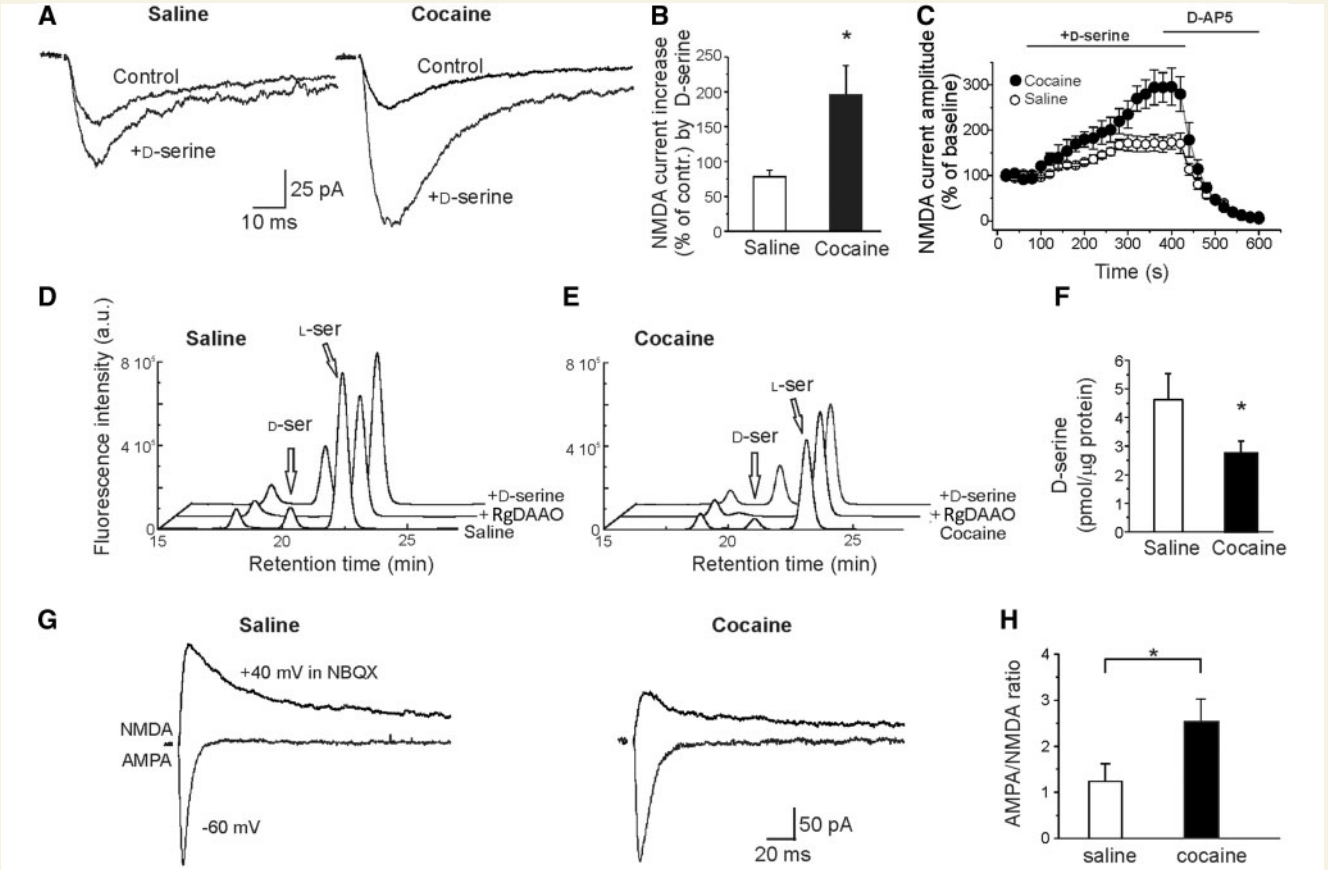

Figure 4 NMDA receptor activation is impaired in nucleus accumbens slices from cocaine–treated rats. (**A**) Representative traces showing the effect of D-serine application (100 µM) on NMDA currents in nucleus accumbens slices from saline- (*left*) and cocaine- (*right*) treated rats. Recordings were made in 0.1 mM Mg^{2+} artificial CSF containing the AMPA receptor antagonist NBQX (30 µM). (**B**) Summary bar graph showing facilitation of NMDA current by exogenous D-serine (saline: $n = 9$; cocaine $n = 8$; $P < 0.05$). (**C**) Time course of the NMDA current recorded in slices from saline- (open circles) and cocaine- (filled circles) treated rats before and after application of D-serine ($n = 9$). (**D** and **E**) HPLC chromatograms of nucleus accumbens samples from saline- and cocaine-treated rats. The identity of the D-serine peak (retention time: 21.4 ± 0.7 min) was verified by adding RgDAAO or D-serine as an internal standard. (**F**) Summary bar graph depicting D-serine contents (pmoles/µg). (**G**) Examples of AMPA receptor- and NMDA receptor-mediated EPSCs in cells from saline- (*left*) and cocaine-treated rats (*right*). (**H**) AMPA/NMDA ratio values from nucleus accumbens core neurons in saline- ($n = 10$) and cocaine-treated rats ($n = 13$; $P < 0.05$). See also Supplementary Fig. 10.

findings demonstrate that cocaine-treatment, by reducing D-serine levels in the nucleus accumbens core, leads to NMDA receptor hypofunction and impairment of NMDA receptor-dependent synaptic plasticity.

To investigate whether the reduced D-serine levels in the nucleus accumbens core was associated with a decreased expression of serine racemase and/or with an increased expression of DAAO, we performed real-time reverse transcription PCR and western blot analyses of nucleus accumbens extracts from saline- and cocaine-treated rats. This set of experiments revealed that DAAO messenger RNA and protein levels were significantly increased in the nucleus accumbens core of cocaine-treated rats (+68.5%; $n = 5$; $P < 0.05$ and +195.7%; $n = 3$; $P < 0.05$, respectively; Fig. 5B–D). Differently, we observed a significant decrease in serine racemase protein levels (-50.6%; $n = 3$; $P < 0.05$; Fig. 5C and D), although the corresponding messenger RNA transcript level was not modified ($n = 3$; $P > 0.05$; Fig. 5A). These data indicate that the D-serine deficit observed in the nucleus accumbens core of cocaine-treated rats is likely due to its reduced biosynthesis and increased degradation. This cocaine-induced alteration in D-serine levels is conceivably maintained in slice preparation despite some degree of washout by artificial CSF perfusion, since DAAO and serine racemase are active in acute brain slices, as demonstrated by previously published reports in other brain regions (Panatier *et al.*, 2006; Turpin *et al.*, 2011).

Exogenous D-serine blocks the development of locomotor sensitization to cocaine

A hallmark feature associated with chronic exposure to cocaine in rodents is locomotor sensitization (usually measured as increased locomotor responses to repeated administration of cocaine) that

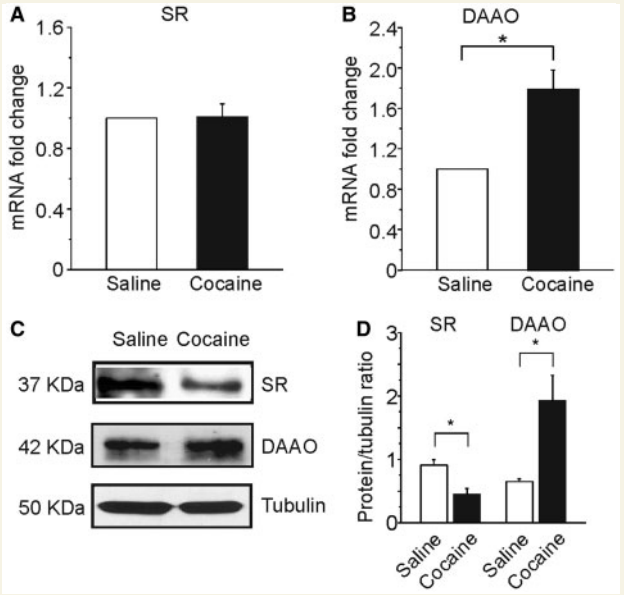

Figure 5 D-serine biosynthesis/catabolism is altered in the nucleus accumbens of cocaine-treated rats. Messenger RNA levels for serine racemase (**A**) and *DAAO* (**B**) in nucleus accumbens extracts from saline- and cocaine-treated rats, as documented by real-time reverse transcription-PCR analysis ($n = 6$ rats for each group; $P < 0.05$). (**C**) Western immunoblots for serine racemase, DAAO in the nucleus accumbens of saline- and cocaine-treated rats; 50 μg of total proteins were loaded in each lane. (**D**) Bar graphs depicting quantification of immunoreactivity for serine racemase and DAAO normalized to tubulin protein levels ($n = 5$ rats for each group; $P < 0.05$).

has been related to impairment of synaptic plasticity in the nucleus accumbens (Thomas *et al*., 2001). To determine whether the impaired D-serine signalling in the nucleus accumbens of cocaine-treated rats played a role in cocaine-induced locomotor sensitization we compared the locomotor activity in rats treated with cocaine and with cocaine plus D-serine. We hypothesized that if the decreased D-serine levels in the nucleus accumbens contribute to cocaine-induced locomotor sensitization, microinjection of D-serine directly into the nucleus accumbens core would reduce this behavioural response.

First, we evaluated the locomotor activity in rats receiving intra-nucleus accumbens microinjection of vehicle (0.3 μl) and exposed to cocaine according to our paradigm (five once-daily injections; 15 mg/kg; intraperitoneal). After 2 days of saline injection to habituate the animals to the open-field chamber, the immediate locomotor response in rats receiving intra-nucleus accumbens vehicle before cocaine or saline injection were measured for 15 min each day. As expected, cocaine-induced robust sensitization [Fig. 6A and B; locomotor activity was higher on Days 2–5 relative to Day 1; ANOVA, $F_{(4,28)} = 17.17$; $P < 0.0001$]. The mean distance travelled at Day 5 was 13615 ± 1366 cm; $n = 8$; whereas at Day 1 was 5450 ± 734 cm; $n = 8$; $P < 0.005$. Interestingly, when rats were intra-nucleus accumbens microinjected with D-serine prior to cocaine treatment, the development of sensitization was blocked as the distances travelled on Days 2–5 were not significantly different from Day 1 [Fig. 6; distances travelled at Day 1 and Day 5 were 5317 ± 1079 cm and 5843 ± 1047 cm, respectively ($n = 8$); ANOVA, $F_{(4,28)} = 0.46$; $P = 0.77$]. Of note, rats exposed to saline and receiving either intra-nucleus accumbens D-serine or vehicle exhibited similar locomotor activity (Fig. 6A). In order to better correlate the effect of D-serine on locomotor activity with our data obtained from electrophysiological recordings we tested the locomotor activity in rats receiving a single intra-nucleus accumbens D-serine injection prior to the fifth cocaine injection. As shown in Fig. 6D in a group of animals developing locomotor sensitization [on Days 1–4; ANOVA, $F_{(3,9)} = 7.71$; $P = 0.007$] a single intra-nucleus accumbens injection of D-serine at Day 5 reverted the effect of cocaine on locomotor activity. Indeed, a significant decrease in the distance travelled was observed at Day 5 (6805 ± 1340; $n = 6$;) compared with Day 4 of cocaine injection (11062 ± 950 cm; $n = 6$; $P < 0.05$).

Because the nucleus accumbens is critically involved in the expression of locomotor sensitization (Kalivas, 2004; Kauer and Malenka, 2007) these results suggest a potential role for cocaine-induced impairment of D-serine signalling as a molecular correlate for cocaine-induced locomotor sensitization.

Discussion

The data presented in this study demonstrate that D-serine metabolism and signalling are impaired in the nucleus accumbens core of rats exposed to cocaine resulting in a deregulation of synaptic plasticity in this brain area. Four experimental evidences support these conclusions: (i) D-serine is the endogenous coagonist of synaptic NMDA receptor in the nucleus accumbens core of rats; (ii) D-serine is required for NMDA receptor-dependent LTP and LTD in this brain region; (iii) D-serine concentration is reduced in cocaine-treated rats leading to downregulation of NMDA current and impairment of NMDA receptor-dependent LTP and LTD; and (iv) alterations in the biosynthesis and catabolism of D-serine are responsible for the lowering of D-serine levels in cocaine-treated rats.

Our demonstration that D-serine is the endogenous co-agonist of synaptic NMDA receptors in the nucleus accumbens core is based on electrophysiological data showing marked increases in the AMPA/NMDA ratio at excitatory synapses in brain slices treated with RgDAAO, which selectively degrades endogenous D-serine. In contrast, enzymatic degradation of glycine with BsGO had no effect on this ratio (Fig. 1). It should be pointed out that these enzymes have been widely used to demonstrate D-serine's role as an endogenous ligand of synaptic NMDA receptors in several brain regions, including the supraoptic nucleus, hippocampus and prefrontal cortex (Yang *et al*., 2003; Panatier *et al*., 2006; Fossat *et al*., 2011).

In our experimental conditions, RgDAAO treatment did not completely abolish the NMDA receptor component of the evoked EPSCs (Fig. 1). This residual activity may reflect incomplete degradation of D-serine in our slices, similarly to that observed in a previous study showing residual D-serine levels in RgDAAO-treated slices (Panatier *et al*., 2006). Alternatively in the presence

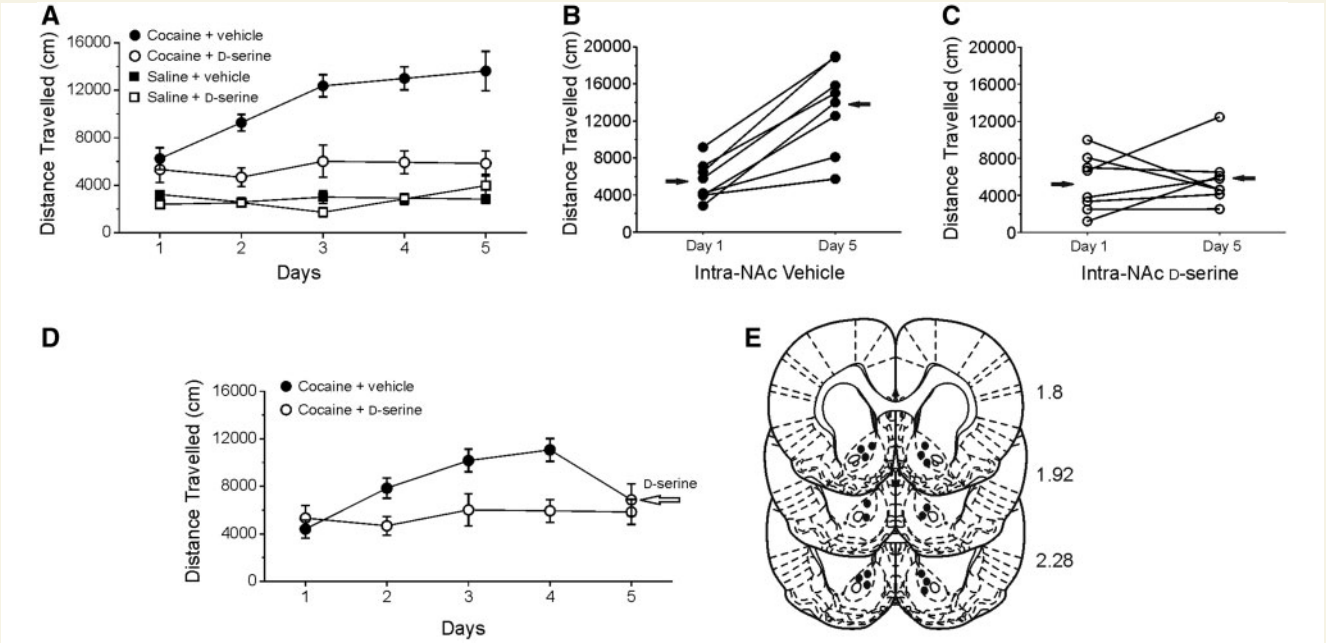

Figure 6 Intra-nucleus accumbens core microinjection of D-serine blocks the development of locomotor sensitization to cocaine. (**A**) Rats received intra-nucleus accumbens core D-serine (4 μg/0.3 μl; open symbols) or vehicle (0.3 μl; closed symbols) and were tested for locomotor activity after receiving intraperitoneal injection of cocaine (circles: D-serine, $n = 8$; vehicle, $n = 8$) on Days 1-5. Control rats received intraperitoneal injection of saline (square: D-serine, $n = 6$; vehicle $n = 6$). (**B** and **C**) Individual locomotor activity for cocaine-treated rats on Days 1 and 5 in the absence (**B**) or presence (**C**) of intra-nucleus accumbens D-serine. Arrows indicate mean travelled distances. (**D**) Graph showing locomotor activity of rats treated with cocaine and receiving intra-nucleus accumbens core D-serine at Day 5 (arrow). For comparison, locomotor activity of cocaine-treated rats receiving daily intra-nucleus accumbens D-serine are shown (same data presented in **A**). (**E**) Coronal slice sections showing reconstructed D-serine injection sites, in the nucleus accumbens core, from animals receiving cocaine. Distance from bregma is shown (in mm).

of a markedly reduced D-serine concentrations, glycine may serve as surrogate co-agonist in a subpopulation of NMDA receptors.

The fact that BsGO treatment had no effect on the AMPA/NMDA ratio, besides supporting the notion of D-serine as co-agonist of NMDA receptors, also suggests that glycine levels at synapses may be below the range of NMDA receptor affinity for the coagonist (Laurie and Seeburg, 1994).

Our immunohistochemical results (Supplementary Figs 3–5) clearly demonstrate the presence of D-serine, and the enzymes involved in its biosynthesis and catabolism (serine racemase and DAAO, respectively), in both neurons and astrocytes of the nucleus accumbens core. There has been considerable debate on the types of cells that express serine racemase and D-serine in the CNS. Early studies localized both to the astrocyte populations of various brain structures, giving rise to the view that D-serine is a gliotransmitter (Wolosker *et al*., 1999; Panatier *et al*., 2006). The cellular localization of D-serine we found in the nucleus accumbens core is consistent with a recent model of D-serine dynamics proposed by Wolosker (2011) that takes into account the notion that D-serine is primarily made in neurons and explains why drugs blocking astrocyte metabolism affect D-serine extracellular levels (Zhang *et al*., 2008; Henneberger *et al*., 2010). This so-called 'serine shuttle model' proposes that astrocytes synthesize and export L-serine to neurons to fuel the synthesis of D-serine by the neuronal serine racemase. Once synthesized and released by neurons, D-serine can be taken up by astrocytes for storage and activity-dependent release (Wolosker, 2011).

It should be pointed out that, although most of nucleus accumbens core neurons (>80%) displayed immunostaining for D-serine and its related enzymes, the intensity of the signals among the neuronal cells was different, likely reflecting different level of expression. It is known that there are two main types of medium spiny neurons: those containing D1-like receptors and those that express D2-like dopamine receptors (Podda *et al*., 2010). It is tempting to speculate that these two neuronal subtypes differentially express D-serine machinery.

In this study we demonstrated that D-serine is required for NMDA receptor-dependent LTP and LTD in the nucleus accumbens core. Indeed, enzymatic D-serine degradation by RgDAAO prevented the induction of both forms of synaptic plasticity in our nucleus accumbens slices, and these findings are consistent with recent reports identifying D-serine as a fundamental player in LTP induction in the supraoptic nucleus and hippocampus (Panatier *et al*., 2006; Henneberger *et al*., 2010).

Our data take on particular relevance in light of the fact that chronic cocaine exposure can activate or 'hijack' LTP and LTD thus leading to addictive behaviour (Kauer and Malenka, 2007; Lüscher and Malenka, 2011). Several studies have demonstrated impairment of these forms of synaptic plasticity in different models of cocaine-addiction. *In vivo* experimental evidences showed that

after long-term withdrawal from cocaine self-administration LTP cannot be induced in the rat nucleus accumbens core (Moussawi *et al*., 2009). The impairment of LTP reported in this study has been attributed to a pre-existing LTP-like state, developed during cocaine treatment and/or withdrawal, in which the synaptic strength is maximized. Therefore, if the synaptic strength is saturated further attempts to induce LTP cannot cause additional upregulation. Several findings are consistent with the hypothesis that excitatory synapses in the nucleus accumbens undergo a LTP-like potentiation after cocaine exposure, including increased AMPA/NMDA ratio of synaptic currents (Kourrich *et al*., 2007), increased surface expression of AMPA receptors (Conrad *et al*., 2008) and increased behavioural responsiveness to microinjection of AMPA (Pierce *et al*., 1996).

Moussawi *et al*. (2009) also observed loss of LTD inducibility in the rat nucleus accumbens after long-term withdrawal from self-administered cocaine, an effect that could not be explained by pre-existing synaptic potentiation. The authors linked it to reduced stimulation of mGluR5 receptors.

Impairment of LTD has also been reported by other authors in rats with a history of cocaine in both *in vivo* and *ex vivo* (i.e. brain slices) preparations (Martin *et al*., 2006; Kasanetz *et al*., 2010). In particular, Martin *et al*. (2006) showed that cocaine self-administration (2 h/day for 14–19 days) abolishes LTD in the nucleus accumbens core and shell after 1 day of abstinence. However, after 21 days of abstinence LTD was abolished exclusively in the core slices, suggesting that long-lasting alteration of glutamatergic plasticity in this subregion might contribute to cocaine-seeking behaviour. The finding of a persistent impairment of LTD only in the core could reflect pre-existing LTD-like state. However, no data are available supporting the notion of a reduced AMPA receptor levels (i.e. pre-existing LTD-like state) in the nucleus accumbens after the cocaine-self administration regimen used by Martin *et al*. (2006). Moreover, in contrast with the above reported hypothesis, Conrad *et al*. (2008) found an increased number of synaptic AMPA receptors in the nucleus accumbens following a different self-administration protocol (6 h/day) and longer withdrawal (45 days).

In the nucleus accumbens core slices from our cocaine-treated rats, LTP and LTD were clearly impaired (Fig. 3) and our data indicate that reduced NMDA receptor function is largely responsible for both changes. Our conclusions are apparently in contrast with the abovementioned hypothesis of LTP impairment due to occlusion by pre-existing state of cocaine-induced LTP-like potentiation. However, it should be pointed out that cocaine-related LTP impairment reported by Moussawi *et al*. (2009), as well as evidences supporting their hypothesis, such as increased AMPA/NMDA ratio and surface expression of AMPA receptor, (Kourrich *et al*., 2007; Wolf and Tseng, 2012) were all observed following long-term withdrawal from chronic cocaine treatment. Our observation, instead, refers to an early withdrawal period (1 day after last cocaine injection) and, therefore, we propose that reduced D-serine levels by cocaine represents an additional mechanism underling cocaine impairment of synaptic plasticity in the nucleus accumbens core.

It is well known that NMDA receptor-dependent LTP and LTD both require the activation of NMDA receptors, which leads to postsynaptic Ca^{2+} influx. LTP is mediated by large, short-lived increases in the intracellular Ca^{2+} concentration (i.e. high-level NMDA receptor activation), whereas smaller increases (i.e. moderate NMDA receptor activation) are involved in the induction of LTD. Such different regulation of intracellular Ca^{2+} levels are supposed to trigger different subsets of Ca^{2+}-dependent intracellular signalling molecules required for both forms of synaptic plasticity. NMDA receptor hypofunction with reduced Ca^{2+} influx at the postsynaptic level could conceivably explain the impairment of both LTP and LTD in our cocaine-treated rats. Possible changes in AMPA receptor number at postsynaptic levels (i.e. LTP- or LTD-like state) occurring following cocaine treatment, cannot be responsible at the same time for the alterations of LTP and LTD reported here.

The cocaine-associated changes we observed in NMDA receptor function and synaptic plasticity do not appear to be caused by alterations involving the subunit composition of the NMDA receptors (Supplementary Fig. 9). These data are in agreement with a previous report demonstrating no changes in NMDA receptor subunit expression in the nucleus accumbens core following 24–72 h withdrawal (Loftis and Janowsky, 2000). However, it should be acknowledged that the same study reported in the shell subregion bidirectional changes in GluNB expression at 1 and 14 days of withdrawal. Moreover, in the nucleus accumbens shell increased expression of GluNB containing NMDA receptors has been demonstrated at silent synapses during the course of repeated cocaine injections (2–5 days; Huang *et al*., 2009). In another study, performed in the same nucleus accumbens subregion, an increase in the NMDA receptor subunit expression was seen only after 21 days withdrawal from cocaine but not after 1 day (Schumann and Yaka, 2009). Our data provide novel evidences that cocaine–induced impairment of NMDA receptor-dependent synaptic plasticity is related to drug-induced deficits in D-serine at the synaptic level. Indeed, HPLC analysis revealed D-serine levels in nucleus accumbens extracts from cocaine-treated rats that were appreciably lower than those found in extracts from saline-treated rats (Fig. 4D–F). Above all, while slice exposure to saturating levels of exogenous D-serine had no effect on synaptic plasticity in slices from saline-treated rats, it fully restored LTP and LTD inducibility in slices from cocaine-treated rats (Fig. 3). Likewise, exogenous D-serine substantially facilitated NMDA-induced currents in slices from cocaine-treated rats, but its effects were much less evident in control slices (Fig. 4A–C).

Our contention for the involvement of NMDA receptor hypofunction in mediating changes in synaptic plasticity observed in our cocaine treatment paradigm is fully supported by our data demonstrating an increase in AMPA/NMDA ratio in cocaine-treated rats which was not associated with changes in AMPA receptor function and expression, as also demonstrated by others studies (Boudreau and Wolf, 2005; Wolf and Tseng, 2012). Changes in the AMPA/NMDA ratio in the nucleus accumbens have been explored by different studies as correlate for cocaine-induced alteration in glutamate function. Contingent (self-administration) and non-contingent (passive) cocaine exposure are known to differently affect glutamate transmission (Bowers *et al*., 2010; Wolf and Tseng, 2012). Moreover, the effect of

cocaine exposure on AMPA/NMDA ratio is also influenced by the duration of withdrawal, the age and species of animal used, as well as the nucleus accumbens subregion and medium spiny neuron population studied. By using a non-contingent protocol, similar to that used in our study, Kourrich *et al*. (2007) reported a decreased AMPA/NMDA ratio in the nucleus accumbens shell of mice after 24 h of withdrawal. However, after a longer abstinence period (>10 days) the AMPA/NMDA ratio was increased due to increased AMPA receptor-mediated transmission. The issue of cocaine-induced changes in the AMPA/NMDA ratio has been less explored in the nucleus accumbens core subregion. To our knowledge there is only one study that reports no changes after short-term withdrawal from cocaine exposure, although in the shell the same cocaine paradigm elicited a decreased AMPA/NMDA ratio (Thomas *et al*., 2001). Our study adds complexity to this scenario by indicating that in the core of rats the AMPA/NMDA ratio is decreased. The reason for the discrepancy with the previous study remains unclear, and it is unlikely that it resides only on the different animal model used in our study (rats versus mice).

Correlation between downregulated serine racemase expression, decreased D-serine levels, and impaired NMDA receptor-dependent LTP has already been demonstrated in hippocampal slices from aged rats (Mothet and Billard, 2006). More recently, decreased hippocampal levels of D-serine and downregulated expression of serine racemase (at the transcriptional level) have also been demonstrated in a rat strain characterized by accelerated aging and diminished LTP (Turpin *et al*., 2011). Decreased D-serine concentrations and NMDA receptor hypofunction have also been implicated in the aetiology of schizophrenia (Fuchs *et al*., 2011). Patients with this disease show reduced CSF and serum levels of D-serine (Bendikov *et al*., 2007) and increased DAAO expression and activity in the cerebellum, parietal cortex and hippocampus (Verrall *et al*., 2007). These findings have led some investigators to propose that increased DAAO expression and activity in specific brain areas, and its impact on D-serine levels, contribute to NMDA receptor hypofunction and schizophrenia (Pollegioni and Sacchi, 2010).

Our findings also indicate that repeated cocaine use can alter the biosynthesis and catabolism of D-serine. Nucleus accumbens core slices from cocaine-treated rats exhibited reduced serine racemase expression and upregulated DAAO expression. The latter alteration was evident at both the messenger RNA and protein levels, suggesting both transcriptional and post-transcriptional modifications. By contrast, post-transcriptional mechanisms are more likely to be the cause of the diminished serine racemase expression, which was observed exclusively at the protein level. Many messenger RNAs display altered expression in the nucleus accumbens after chronic cocaine exposure, suggesting that transcription of individual genes is differentially regulated in this condition, thus contributing substantially to addictive phenotype (Robison and Nestler, 2011).

The present study also demonstrates that D-serine administered directly in the nucleus accumbens core blocks the development of locomotor sensitization to cocaine (Fig. 6). Furthermore, our data indicate that a single intranucleus accumbens D-serine injection is able to revert the cocaine-induced enhancement of locomotor activity. This locomotor sensitization paradigm has provided a major impetus to explore the neuroplasticity that may occur during the transition from drug use to addiction (Koob and Volkow, 2010) and several studies have demonstrated the essential role played by the nucleus accumbens in both the induction and expression phases of this cocaine-induced behavioural alteration (Kalivas *et al*., 2009). It has long been known that pharmacological manipulation at the various sites of the NMDA receptor complex can alter cocaine-induced behavioural changes (Witkin, 1993). In this regard it is worth noting that R-(+)-HA966, a glycine-site partial agonist, prevented the locomotor sensitization to a subsequent challenge dose of cocaine (Khan and Shoaib, 1996). More recently, it has been shown that D-cycloserine, another partial agonist of the NMDA receptor acting at its glycine modulatory site, facilitates the extinction of a cocaine-induced conditioned place preference (Thanos *et al*., 2009), reduces reacquisition of cocaine self-administration by enhancing extinction learning (Nic Dhonnchadha *et al*., 2010), and attenuates the reinstatement of cocaine-seeking in a context-independent manner (Torregrossa *et al*., 2010). In addition, local administration of D-cycloserine directly into the nucleus accumbens shell lowered cocaine-induced locomotor activity without affecting basal responses (Huang *et al*., 2008). Furthermore, D-serine itself has been shown to affect cocaine-induced behavioural changes. In particular, it has been shown that D-serine reduces the expression of cocaine-induced conditioned place preference (Yang *et al*., 2010), and reduces cocaine-primed reinstatement following extended access to cocaine self-administration (Kelamangalath and Wagner, 2010). Recently a key role of D-serine signalling in the development of behavioural sensitization following repeated methamphetamine administration has also been suggested by using serine racemase knock-out mice (Horio *et al*., 2012). All these findings strongly support the notion that NMDA receptor-associated glycine recognition site plays an important role in the cocaine-induced behavioural changes. Our results extend these findings indicating that the cocaine-induced impairment of nucleus accumbens D-serine signalling may be a molecular correlate for cocaine-induced behavioural sensitization. Since it is generally believed that the cellular and molecular mechanisms mediating behavioural sensitization also contribute to cocaine-induced emotional and motivational states characteristic of cocaine addiction (Robinson and Berridge, 2008) it is tempting to speculate that D-serine-dependent impairment of synaptic plasticity we found in the nucleus accumbens might contribute also to abnormal cocaine-seeking behaviours.

In conclusion, data reported here indicate that D-serine is essential for NMDA receptor-dependent LTP and LTD in the nucleus accumbens core of rats and that chronic cocaine treatment alters the metabolism of D-serine in this brain region leading to reduced D-serine levels, NMDA receptor hypofunction, and impairment of NMDA receptor-dependent LTP and LTD. Furthermore, our data suggest that the impairment of the D-serine signalling in the nucleus accumbens contributes to the development of locomotor sensitization. Thus, our study highlights the potential role of glycine site of NMDA receptors as pharmacotherapeutic target for cocaine addiction.

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Supplementary material

Supplementary material is available at *Brain* online.

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