Psychopharmacology (2013) 230:125–135
DOI 10.1007/s00213-013-3142-5

ORIGINAL INVESTIGATION

Cocaine potentiates MDMA-induced oxidative stress but not dopaminergic neurotoxicity in mice: implications for the pathogenesis of free radical-induced neurodegenerative disorders

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Received: 31 January 2013 / Accepted: 30 April 2013 / Published online: 17 May 2013

Abstract
Rationale The drugs of abuse 3,4-methylenedioxymethamphetamine (MDMA; "ecstasy") and cocaine both increase the generation of free radicals, and in the case of MDMA, this increase in oxidative stress is involved in the dopaminergic neurotoxicity produced by the drug in mice. Oxidative stress processes are also involved in the pathogenesis of several neurodegenerative diseases.
Objectives We aimed to determine the consequences of the combined administration of MDMA and cocaine on oxidative stress and dopaminergic neurotoxicity.
Methods Mice received MDMA (20 mg/kg, i.p.; two doses separated by 3 h) followed by cocaine 1, 3, 6, or 24 h after the second MDMA dose. Mice were killed between 1 h and 7 days after cocaine injection.
Results MDMA decreased dopamine transporter density and dopamine concentration 7 days later. Cocaine did not alter this neurotoxicity. MDMA produced an increase in the concentration of 2,3-dihydroxybenzoic acid in striatal microdialysis samples and an increase in lipid peroxidation in the striatum which were potentiated by cocaine. MDMA and cocaine given together also increased nitrate and 3-nitrotyrosine levels compared with either drug given alone. On the other hand, MDMA increased superoxide dismutase activity and decreased catalase activity, changes which were prevented by cocaine administration. In addition, cocaine administration produced an increase in glutathione peroxidase (GPx) activity in both saline-treated and MDMA-treated mice.
Conclusions Cocaine potentiates MDMA-induced oxidative stress but does not produce an increase in the neurotoxicity produced by MDMA, and this lack of potentiation may involve an increase in GPx activity.

Keywords 3,4-Methylenedioxymethamphetamine · Cocaine · Dopamine neurotoxicity · Oxidative stress

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Introduction

3,4-Methylenedioxymethamphetamine (MDMA, "ecstasy") is widely used as a recreational drug by young people, despite having been shown to be a potent neurotoxin in the brain of rodents and nonhuman primates (Green et al. 2003). In the mouse, the neurotoxicity produced by MDMA is dopaminergic specific, reflected by a sustained loss in the concentration of dopamine and its metabolites and in the density of dopamine transporters (DAT), principally in the nigrostriatal pathway (Escobedo et al. 2005; Granado et al. 2008a, b). The neurotoxic effects of the drug are mediated, at least in part, by oxidative stress processes since transgenic mice overexpressing superoxide dismutase (SOD) show protection against the dopaminergic neurotoxicity produced by the drug (Cadet et al. 1995; Jayanthi et al. 1999). In addition, the pattern of dopamine neurotoxicity shows a tight inverse correspondence with the pattern of SOD

distribution in the striatum, with the striosomes showing less SOD activity and more neurotoxicity than the matrix (Granado et al. 2008a). MDMA has been shown to increase the formation of 2,3-dihydroxybenzoic acid (2,3-DHBA) from salicylic acid perfused through a probe implanted in the mouse striatum, indicating that the drug increases free radicals and, in particular, hydroxyl radical formation (Colado et al. 2001; Camarero et al. 2002). MDMA also increases superoxide radical production and lipid peroxidation (Camarero et al. 2002; Simic and Malicevic 2008). Furthermore, there is also some evidence of the involvement of nitric oxide synthase (NOS) in the toxicity produced by the drug since nNOS inhibitors protect against MDMA-induced dopamine depletion (Colado et al. 2001).

Cocaine is the second most used illicit drug in Europe, and an average of 5.9 % of young adults report having used the drug at least once in their lives (EMCDDA 2010). Cocaine is frequently consumed with other drugs, and among cocaine-consuming school students, 53.6 % reported consuming MDMA in the last month (EMCDDA 2009). Studies in experimental animals have shown that cocaine increases reactive oxygen species in the striatum accompanied by an increase in antioxidant enzymes which are thought to mediate an efficient protection, thus preventing cell death despite increases in hydroperoxides and lipid peroxidation (Das and Ratty 1987; Dietrich et al. 2005). In addition, cocaine increases nitric oxide (Bagetta et al. 1999; Sammut and West 2008; Lee et al. 2011), and studies indicate a role for NOS in mediating sensitization to the behavioral effects of cocaine in rodents since NOS inhibitors block the development of psychomotor sensitization (Itzhak 1997; Itzhak et al. 1998).

Oxidative/nitrosative stress processes play an important role in the pathogenesis of diverse neurodegenerative diseases such as Alzheimer's disease, amyotrophic lateral sclerosis, Huntington's disease, Parkinson's disease, and brain ischemia/reperfusion injury. Although the brain damage observed in these diseases is quite varied, alterations in the redox balance, involving increased free radical production or decreased antioxidant defenses, are thought to result in oxidative/nitrosative stress manifested as lipid peroxidation, protein and carbonyl oxidation, and protein nitration (Halliwell 2006), changes which contribute to the development of the diseases. Thus, situations which produce further redox imbalance could lead to a potentiation of brain damage.

Since both drugs of abuse, MDMA and cocaine, have been reported to produce oxidative and/or nitrosative stress, we aimed to use their coadministration as a means of potentiating these stress processes with a view to determining the consequences on the amphetamine-induced dopaminergic lesion. Previous studies have shown that cocaine can protect against MDMA-induced damage by interfering with MDMA uptake into dopaminergic terminals (Peraile et al. 2010). In order to avoid this effect, cocaine was given after MDMA.

Therefore, we determined (1) the formation of hydroxyl radicals and lipid peroxidation following the coadministration of MDMA and cocaine; (2) the concentration of nitrites/nitrates and of nitrotyrosine as markers of NO production and damage following the coadministration of the drugs; (3) the activity of the antioxidant enzymes: catalase, SOD, and glutathione peroxidase (GPx); and (4) the effect of cocaine on the dopaminergic neurotoxicity induced by MDMA.

Methods

Animals, drug administration, and experimental design

Adult male NIH/Swiss mice (Harlan Laboratories Models S.L., Barcelona, Spain) weighing 20–30 g were group housed in conditions of constant temperature (21±2 °C) and a 12-h light/dark cycle (lights on at 0800 hours) with ad libitum access to food and water. All experimental procedures were approved by the Animal Welfare Committee of the Universidad Complutense de Madrid (following European Union Directive 2010/63/EU).

(±)-MDMA·HCl (Ultrafine Chemicals Ltd., Manchester, UK) and cocaine HCl (Servicio de Estupefacientes, Ministerio de Sanidad y Consumo, Madrid, Spain) were dissolved in 0.9 % *w/v* NaCl (saline) and injected i.p. in a volume of 10 ml/kg. Doses are quoted in terms of the base. Control mice were given saline.

Mice were treated with MDMA (20 mg/kg, i.p., two injections separated by 3 h) followed by cocaine (30 mg/kg, i.p.) 1, 3, 6, or 24 h after the second MDMA dose. This regimen of MDMA administration has previously been shown to produce approximately 50 % reductions in striatal DAT density and dopamine content in NIH/Swiss mice (Colado et al. 2001), parameters which are considered to reflect neurotoxicity (Colado et al. 2004). For the determination of dopaminergic neurotoxicity, mice were killed 7 days later. Further experiments were carried out with cocaine given 3 h after the second MDMA injection. In these experiments, mice were killed at different times after cocaine injection: at 1 h for nitrate/nitrite and 3-NT levels, lipid peroxidation, striatal levels of MDMA, and catalase and SOD activities, at 3 h for GPx activity, or at 24 h for tyrosine hydroxylase (TH) immunoreactivity and glial fibrillary acidic protein (GFAP) staining.

Measurement of dopamine and metabolites in striatal tissue

Striatal catechol concentration was evaluated as a marker of dopamine neurotoxicity 7 days after MDMA. Mice were killed by cervical dislocation and decapitation, the brains rapidly removed and the striatum dissected out on ice. Striata were

homogenized (0.2 M $HClO_4$ containing 0.1 % cysteine, 0.1 % sodium metabisulfite, and 0.01 % ethylenediaminetetraacetic acid [EDTA]), centrifuged (12,000×*g*; 20 min; 4 °C), and the supernatant assayed by high-performance liquid chromatography (HPLC).

The mobile phase (0.05 M KH_2PO_4, 1 mM octanesulfonic acid, 0.1 mM EDTA, 16 % methanol; pH 3.7) was pumped (Waters 510; 1 ml/min) through a stainless steel reversed-phase column (Spherisorb ODS2, 5 μm, 150×4.6 mm; Waters) and analyzed by coulometric detection (Coulochem II, ESA, USA; 400 mV). The current produced was monitored using an integration software (Clarity, DataApex, Czech Republic).

[^{3}H]WIN 35,428 binding

[^{3}H]WIN 35,428 binding was measured in striatal membranes by modification of the method described by Segal et al. (2003). Striata from individual animals were sonicated in ice-cold sodium phosphate buffer (20 mM; pH 7.4) containing sucrose (0.32 M). The homogenate was centrifuged at 30,000×*g* for 15 min at 4 °C. The supernatant was discarded and the wash procedure repeated twice more. The pellet was finally resuspended in 80 volumes of homogenization buffer.

The assay solution contained [^{3}H]WIN 35,428 (5 nM), desipramine (300 nM), and tissue preparation (approximately 60 μg protein). Nonspecific binding was carried out in the presence of cocaine (30 μM). The reaction mixture was incubated for 90 min at 4 °C. The assay was terminated by rapid filtration and radioactivity was counted by scintillation spectrometry. Protein concentrations were measured by the method of Lowry et al. (1951).

TH and GFAP immunohistochemistry

Twenty-four hours after the administration of MDMA, mice were anesthetized (sodium pentobarbital, 50 mg/kg) and transcardially perfused with 4 % paraformaldehyde. Immunostaining was carried out in free-floating brain sections (30 μm) with standard avidin–biotin immunohistochemical (Granado et al. 2008a, b; Martin et al. 2008; Rodrigues et al. 2007) or immunofluorescence protocols. Sections were incubated overnight with rabbit anti-TH (1:1,000; Chemicon International, Temecula, CA, USA) or rabbit anti-GFAP antibodies (1:1,000; DakoCytomation, Denmark). Next, sections were incubated with biotinylated goat antirabbit secondary antibody for TH (1:500, 1 h; Vector Laboratories) and goat antirabbit Alexa Fluor 488 for GFAP (1:400, 1.5 h; Invitrogen) followed by visualization using the peroxidase reaction developed with diaminobenzidine or fluorescence microscopy (Leica Microsystem, Madrid, Spain). Animals from each of the treatments were processed together.

Quantification of TH expression was performed with the aid of an image analysis system (AIS, Imaging Research Inc., Linton, UK) using a ×5 lens. The data are presented as the proportional stained area (total TH-positive area/scan area) in the striatum. Measurements were carried out in five to six sections per animal.

Microdialysis in vivo for the determination of free radical formation

Mice were anesthetized (sodium pentobarbital, 40 mg/kg) and secured in a Kopf stereotaxic frame (David Kopf Instruments, USA) using a mouse adapter. A guide cannula (CMA/7 Guide Cannula, CMA Microdialysis AB, Sweden) was implanted just above the right striatum +0.70 mm anteroposterior and −1.9 mm mediolateral from the bregma and 2.5 mm below the skull surface (Franklin and Paxinos 1997). The cannula was secured to the skull as described by Izco et al. (2007).

Free radical formation in the brain in vivo was determined by the method described in detail by Camarero et al. (2002) with some modifications. The concentration of 2,3-DHBA was measured in the dialysate of a probe perfused with salicylic acid (Chiueh et al. 1992; Giovanni et al. 1995). 2,3-DHBA is formed by the reaction between salicylic acid and hydroxyl free radicals and is considered a reliable marker of hydroxyl radical formation (Halliwell et al.1991). Probes were perfused with artificial cerebrospinal fluid (KCl, 2.5 mM; NaCl, 125 mM; $MgCl_2{\cdot}6H_2O$, 1.18 mM; $CaCl_2{\cdot}2H_2O$, 1.26 mM; $NaH_2PO_4{\cdot}H_2O$, 0.5 mM; $Na_2HPO_4{\cdot}2H_2O$, 5 mM; pH 6.5) containing salicylic acid (2.5 mM) at a rate of 1 μl/min, and samples were collected from freely moving mice at 30-min intervals.

Samples were analyzed by HPLC with coulometric detection. The mobile phase (0.025 M KH_2PO_4, 20 % acetonitrile, and 10 % methanol; pH 3.25) was pumped (Waters 510; 1 ml/min) through a reversed-phase column (250×4.6 mm, 5 μm C8 Ultracarb, Phenomenex), and separated components were measured by coulometric detection (Coulochem 5100A; 5011 analytical cell; 400 mV). The current produced was monitored by an integration software package (Unipoint, Gilson). Upon completion of the experiments, mice were killed with sodium pentobarbital overdose, and the brains were rapidly frozen on dry ice. Coronal sections (25 μm) were cut at the level of the striatum using a cryostat. Sections were stained with cresyl violet to verify correct probe placement. Only data obtained from correctly placed probes were included in the analysis.

Determination of nitrate and nitrite levels

Nitrate and nitrite levels were determined by the Griess method described by Miranda et al. (2001). Briefly, the striatal

samples were deproteinized by homogenization in ethanol. Equal volumes of supernatant and Griess reagent (0.2 % sulfanilamide and 0.1 % *N*-(1-naphthyl)ethylenediamine) were allowed to react for 10 min for nitrite detection (540 nm). Subsequently, aliquots were mixed with vanadium chloride to reduce the nitrates to nitrites and allowed to react as described previously. Nitrite and nitrate levels were obtained by comparison with standard curves.

Measurement of tissue lipid peroxidation

Lipid peroxidation was measured using a modification of the method of Das and Ratty (1987), whereby the thiobarbituric acid reactive substances (TBARS), predominantly malondialdehyde, were quantified using the 2-thiobarbituric acid color reaction. Striatal tissue was homogenized in 0.32 M sucrose buffer and centrifuged at 1,000×*g* for 5 min at 4 °C. The supernatant was centrifuged (15,000×*g*; 15 min) and the pellet resuspended in buffer (Tris 50 mM containing NaCl 120 mM and KCl 5 mM; pH 7.4). Aliquots were heated (15 min; 90 °C) with 5 M HCl, 40 % trichloroacetic acid, and 2 % 2-thiobarbituric acid, centrifuged (10,000×*g*; 4 °C; 10 min) and supernatant absorbance was measured at 532 nm and compared with a standard malondialdehyde curve.

Determination of nitrotyrosine

3-Nitrotyrosine levels were determined with a commercial kit (Nitrotyrosine ELISA Kit, Hycult Biotech, The Netherlands). Briefly, striata were homogenized in PBS buffer containing 0.5 % Nonidet, 0.5 % sodium deoxycholate, 0.1 % SDS, aprotinin (40 μg/ml), leupeptin (4 μg/ml), phenylmethanesulfonyl fluoride (PMSF; 0.5 mM), and pepstatin (0.8 μg/ml). Aliquots were added to wells coated with antinitrotyrosine antibody. After washing, a biotinylated secondary antibody was added, followed by a streptavidin–peroxidase complex and then tetramethylbenzidine. Absorbance was measured at 450 nm.

Determination of antioxidant enzyme activities

SOD constitutes the first line of defense against superoxide radicals which it dismutates to H_2O_2. Striata were homogenized in buffer (HEPES, 20 mM; EGTA, 1 mM; mannitol, 210 mM; sucrose, 70 mM; pH 7.2), centrifuged (1,500×*g*; 5 min; 4 °C), and the supernatant assessed with a commercial kit (Superoxide Dismutase Assay Kit II, Calbiochem, Germany). The addition of xanthine oxidase produces superoxide radicals which are dismutated by SOD to form H_2O_2 which absorbs at 450 nm.

For the activity of catalase which converts H_2O_2 to water and oxygen, striata were homogenized in buffer (KH_2PO_4, 0.05 M; K_2HPO_4, 0.05 M; EDTA, 1 mM), centrifuged (10,000×*g*; 15 min; 4 °C), and the supernatant assayed using a commercial kit (Catalase Assay Kit, Calbiochem, Germany). Catalase oxidizes methanol in the presence of H_2O_2, producing formaldehyde which reacts with a chromogen (5-mercapto-1,2,4-triazole, 4-amino-5-hydrazino-4H-1,2,4-triazole-3-thiol), producing a product with absorption at 540 nm.

GPx activity was determined in the striata of mice killed 3 h after cocaine injection using a commercial kit (Glutathione Peroxidase Activity Kit, Assay Designs, USA). This later time-point was chosen since GPx has been shown not only to eliminate H_2O_2 but also to catalyze reduction of already formed organic hydroperoxides, thus preventing further propagation of radical chain reactions (Cadet and Brannock 1998). Striata were homogenized in buffer containing leupeptin (10 μg/ml), aprotinin (2 μg/ml), and PMSF (0.4 mM), centrifuged (10,000×*g*; 20 min; 4 °C), and the supernatant added to wells containing glutathione reductase, glutathione, and NADPH. Cumene hydroperoxide was added and the enzyme kinetics measured every 30 s for 15 min. The reaction is an indirect measure of GPx activity: GPx oxidizes glutathione to GSSG which is reduced by glutathione reductase with the oxidation of NADPH to $NADP^+$, causing absorption at 340 nm to disappear. All enzyme activities were expressed per milligram of protein determined by the method described by Bradford (1976).

Measurement of MDMA concentration in striatal tissue

Brain concentrations of MDMA were determined following a previously described method with minor modifications (Sanchez et al. 2001). Striatal tissue homogenates (Na_2CO_3–$NaHCO_3$ buffer; pH 11.5) were centrifuged (27,000×*g*; 20 min; 4 °C) and the supernatant applied to a C8 SPE column (International Sorbent Technology, Waters). MDMA was selectively eluted with methanol and assayed by HPLC. The mobile phase (20vmM KH_2PO_4 75 % and acetonitrile 25 %; pH 2.5) was pumped (Waters 510; 0.8 ml/min) through a reversed-phase column (RP 18, 5 μm, 150× 4.6 mm, XTerra), and separated compounds were analyzed by UV detection (Waters 2487; 235 nM). The current produced was monitored using an integration software (Clarity, DataApex).

Measurement of rectal temperature

Immediately before and up to 6 h after the first MDMA administration, temperature was measured by use of a digital readout thermocouple (BAT-12 Thermometer, Physitemp Instruments, Clifton, NJ, USA) attached to a RET-3 Rodent Sensor inserted 2 cm into the rectum of the mouse.

Data analysis and statistical analysis

Neurochemical and biochemical data were analyzed using a one-way analysis of variance (ANOVA) followed by the Newman–Keuls test. MDMA concentrations and baseline microdialysis concentrations were analyzed using a *t* test. Temperature and microdialysis data were analyzed by two-way ANOVA with repeated measures (BMDP/386 Dynamic, BMDP Statistical Solutions, Ireland).

Results

Effect of cocaine on MDMA-induced striatal dopamine neurotoxicity

Seven days after treatment, MDMA (20 mg/kg; two doses separated by 3 h) decreased striatal [^{3}H]-WIN 35,428 binding (57 %, $P<0.001$; Fig. 1a) and concentration of dopamine (44 %, $P<0.001$; Fig. 1b) and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) (41 and 34 %, respectively, $P<0.001$; Table 1). Cocaine (30 mg/kg), administered 1 h after the second MDMA injection, attenuated the MDMA-induced decrease in dopaminergic parameters ($P<0.05$ for [^{3}H]-WIN 35,428 binding and HVA concentration; $P<0.01$ for dopamine and DOPAC concentration). In fact, [^{3}H]-WIN 35,428 binding in the MDMA+cocaine-treated group was similar to that in the cocaine only-treated group. However, when cocaine was administered from 3 h onwards after MDMA treatment, no modification in MDMA-induced dopaminergic damage was observed.

The results obtained when cocaine was administered 1 h after MDMA confirm the ability of cocaine to block DAT and, as a consequence, to protect against MDMA-induced dopaminergic neurotoxicity (O'Shea et al. 2001; Peraile et al. 2010). More importantly, the time–response curve allowed us to determine the disappearance of cocaine-induced neuroprotection and establish the time-point of 3 h as suitable for further studies.

Twenty-four hours after MDMA treatment, TH expression was markedly reduced in the striatum compared with saline-treated mice (48 %, $P<0.01$; Fig. 2a, b). Cocaine administered 3 h after MDMA did not modify the TH staining compared with animals treated only with MDMA, thereby further confirming that, 3 h after MDMA, cocaine no longer has the ability to block MDMA (or metabolite) entry into the terminal and produce neuroprotection.

Effect of cocaine on the MDMA-induced increase in striatal GFAP staining

Twenty-four hours after MDMA (20 mg/kg; two doses separated by 3 h) treatment, an increase in GFAP expression was found in the striatum (Fig. 2c). GFAP is an astrocytic marker, and an increase in its expression indicates astrogliosis which accompanies neuronal damage (Thomas et al. 2004; Pekny et al. 2007; Granado et al. 2008a). Cocaine (30 mg/kg) administration 3 h after the second injection of MDMA did not modify the MDMA-induced increase in GFAP expression. Cocaine alone did not change GFAP expression.

Free radicals contribute to MDMA-induced neurotoxicity, and since cocaine also increases free radicals, we studied the consequences of the combination of both drugs on hydroxyl radical formation and lipid peroxidation. In addition, the effects of both drugs on body temperature and antioxidant status were examined since these factors may play a determining role in the extent of neurotoxic damage.

Effect of cocaine on MDMA-induced hyperthermia

MDMA (20 mg/kg; two doses separated by 3 h) produced a hyperthermic response peaking (1.5 °C) 30 min after each injection ($P<0.0001$; Fig. 1c). Three hours after the second injection of MDMA, mice exhibited a marked hypothermia which was maintained for at least a further 1 h ($P<0.0001$). Cocaine (30 mg/kg), administered 3 h after the second MDMA injection, attenuated the hypothermic response of MDMA ($P<0.0001$) without altering the temperature of saline-treated mice ($P=0.23$).

Effect of cocaine on MDMA-induced free radical generation

MDMA (20 mg/kg; two doses separated by 3 h) increased striatal dialysate concentration of 2,3-DHBA starting 1 h after the first administration which was maintained for up to 3 h after the second MDMA injection ($P<0.01$; Fig. 3a). Cocaine (30 mg/kg, i.p.) administration 3 h after the second MDMA injection further increased this MDMA-induced hydroxyl radical formation 30 min after administration (58 %; $P<0.05$) but did not produce a significant effect in saline-treated mice.

Striatal nitrate concentration was quantified 1 h after cocaine injection (4 h after the second MDMA injection). MDMA (20 mg/kg; two doses separated by 3 h) did not modify nitrate levels compared with saline-treated animals (Fig. 3b). Cocaine, given 1 h earlier, increased nitrate levels in the striatum of MDMA-treated mice (79 %, $P<0.05$) and showed a tendency to increase nitrate concentration in saline-treated mice.

None of the treatments modified nitrite concentration (100±3 pmol/mg tissue for saline, 102±3 pmol/mg tissue for MDMA, 108±3 pmol/mg tissue for MDMA+cocaine, and 100±2 pmol/mg tissue for cocaine).

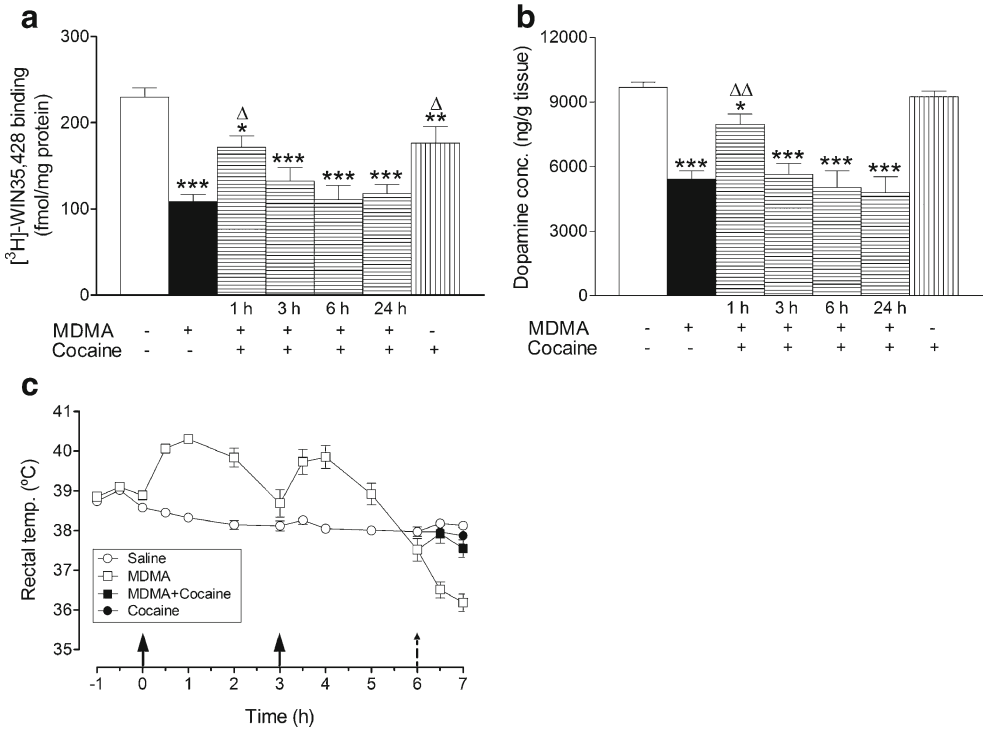

Fig. 1 Effect of cocaine on MDMA-induced dopaminergic neurotoxicity and hyperthermia. Mice were given MDMA (20 mg/kg, i.p.; two doses separated by 3 h) followed by cocaine (30 mg/kg, i.p.) at different times after the second MDMA injection (1, 3, 6, or 24 h) and killed 7 days later. DAT density (**a**) and dopamine concentration (**b**) in the striatum and rectal temperature (**c**; *solid arrows* mark the time of MDMA injections and the *broken arrow* marks the time of cocaine injection). Results are shown as the mean±SEM, n=5–13. For **c**, two-way ANOVA revealed that MDMA produced an increase in rectal temperature between 0.5 and 6 h after injection ($F(1,40)=94.16$, $P<0.0001$) followed by a hypothermia between 6.5 and 7 h ($F(1,22)=66.21$, $P<0.0001$) compared with saline-treated mice. Cocaine given 3 h after the second MDMA injection attenuated the hypothermic effect ($F(1,17)=21.20$, $P<0.0001$) but did not modify the temperature of saline-treated mice ($F(1,21)=1.33$, $P=0.23$). *$P<0.05$, **$P<0.01$, ***$P<0.001$, different from saline; $^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.01$, different from MDMA

Effect of cocaine and MDMA on nitrotyrosine formation

A consequence of NO production is the nitration of the tyrosine residues of intracellular proteins. Neither MDMA (20 mg/kg; two doses separated by 3 h) nor cocaine (30 mg/kg) given alone modified 3-nitrotyrosine in the striatum compared with saline-treated mice (Fig. 3c). Interestingly, both drugs given together produced an increase in this parameter measured 1 h after cocaine (61 %, $P<0.05$).

Table 1 Effect of cocaine on MDMA-induced dopaminergic neurotoxicity

	DOPAC	HVA
Saline	946±20	1057±22
MDMA	561±37***	697±30***
MDMA+cocaine (1 h)	764±41*,*****	861±41**,****
MDMA+cocaine (3 h)	712±53***	773±50***
MDMA+cocaine (6 h)	537±63***	663±62***
MDMA+cocaine (24 h)	529±55***	651±57***
Cocaine	896±35	998±36

Mice were given MDMA (20 mg/kg, i.p.; two doses separated by 3 h) followed by cocaine (30 mg/kg, i.p.) at different times after the second MDMA injection (1, 3, 6, or 24 h) and killed 7 days later. Data are expressed as nanograms per gram tissue. Results shown as the mean± standard error of the mean (SEM), n=5–12

*$P<0.05$, **$P<0.01$, ***$P<0.001$, different from saline; ****$P<0.05$, *****$P<0.01$, different from MDMA

Effect of cocaine and MDMA on lipid peroxidation

Increases in ROS/NOS generally result in damage to cell membranes which can be evaluated as lipid peroxidation. MDMA (20 mg/kg; two doses separated by 3 h) produced a slight increase (14 %, $P<0.05$) in lipid peroxidation 4 h after the second dose compared with saline-treated mice (Fig. 3d). Cocaine (30 mg/kg) potentiated this increase in MDMA-treated mice (26 %, $P<0.001$) but did not affect the lipid peroxidation in saline-treated mice.

Effect of cocaine and MDMA on antioxidant enzyme activities

Since cocaine increased both MDMA-induced free radical generation and lipid peroxidation and both drugs together

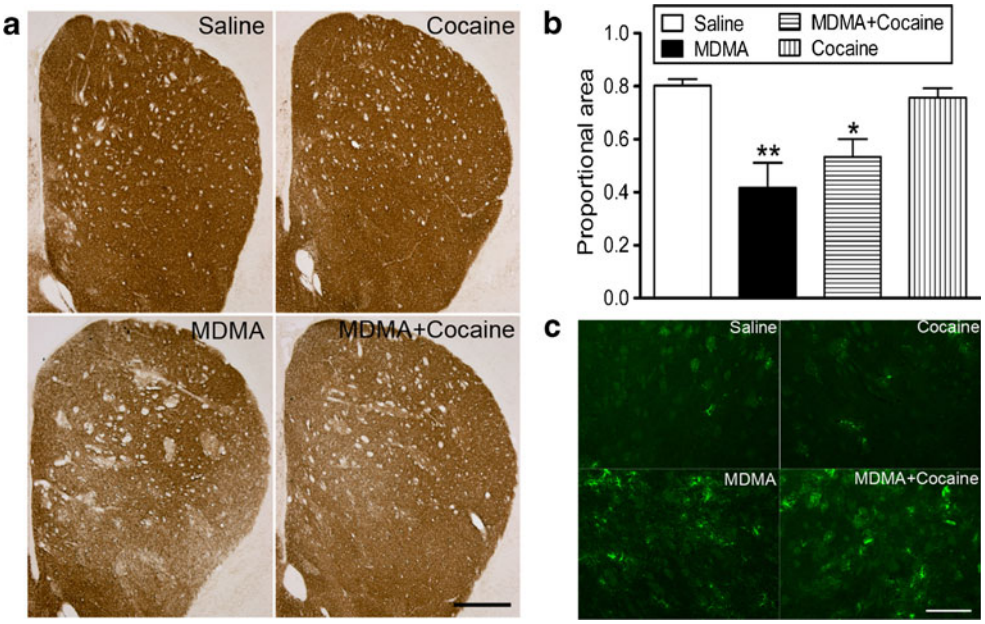

Fig. 2 Effect of cocaine on MDMA-induced reduction in TH immunoreactivity and astrogliosis. Mice were given MDMA (20 mg/kg, i.p.; two doses separated by 3 h) followed by cocaine (30 mg/kg, i.p.) 3 h after the second MDMA injection and killed 24 h later. TH immunoreactivity (**a**), TH immunoreactivity quantification (**b**), and GFAP staining (**c**) in the striatum. Scale bar=500 μm for **a** and 200 μm for **c**. Results are shown as the mean±SEM, n=5–6. *P<0.05, **P<0.01, different from saline

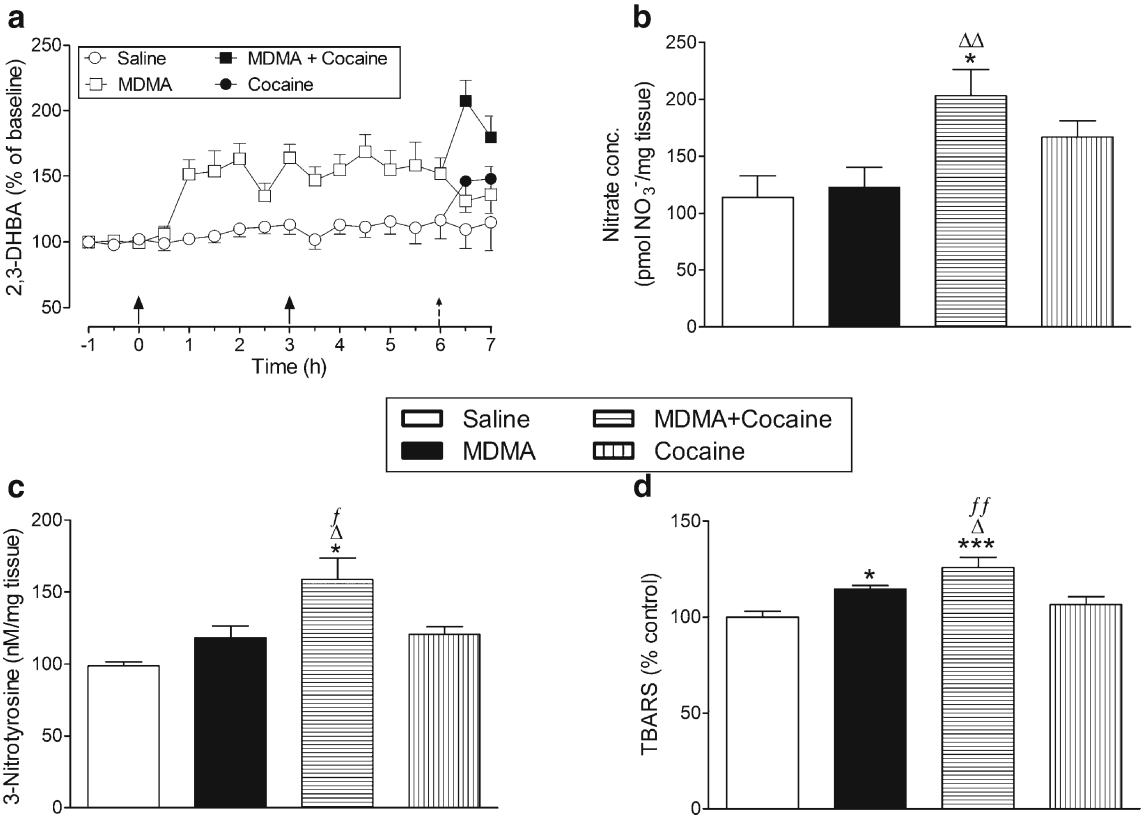

Fig. 3 Effect of cocaine on MDMA-induced oxidative/nitrosative stress. Mice were given MDMA (20 mg/kg, i.p.; two doses separated by 3 h) followed by cocaine (30 mg/kg, i.p.) 3 h after the second MDMA injection. Concentration of 2,3-DHBA in striatal microdialysates (**a**) and nitrate levels (**b**), 3-nitrotyrosine levels (**c**), and TBARS (**d**) expressed as percentage of controls representing lipid peroxidation in the striatum 1 h after cocaine injection. Results are shown as the mean±SEM, n=8–10. For **a**, two-way ANOVA revealed that MDMA produced an increase in 2,3-DHBA concentration in the striatal dialysate (F(1,17)=30.4, P<0.01). Cocaine given 3 h after the second MDMA injection increased the 2,3-DHBA concentration in MDMA-treated (P<0.05; one-way ANOVA) but not saline-treated mice 30 min after administration. *Solid arrows* mark the time of MDMA injections and the *broken arrow* marks the time of cocaine injection. Data are expressed as the percentage of three basal samples (basal concentration=6.2±0.42 pg/μl). *P<0.05, ***P<0.001, different from saline; $^{\Delta}P$<0.05, $^{\Delta\Delta}P$<0.01, different from MDMA; ^{f}P<0.05, ^{ff}P<0.01, different from cocaine

increased protein nitration but did not increase MDMA-induced dopaminergic toxicity, it may be that a cellular defense mechanism, such as the antioxidant response, is activated to protect the cell.

MDMA (20 mg/kg; two doses separated by 3 h) increased SOD activity 4 h after the second dose (34 %, $P<0.05$) compared with saline-treated mice. By contrast, cocaine (30 mg/kg) given to saline-treated mice reduced the activity of the enzyme (33 %, $P<0.05$), while the combination of both drugs did not modify SOD activity compared with saline-treated mice (Fig. 4a). MDMA decreased catalase activity compared with saline-treated mice (16 %, $P<0.05$), while mice receiving cocaine or a combination of MDMA and cocaine did not show any change in the activity of the enzyme (Fig. 4b).

With regard to GPx, the activity of this enzyme was increased in the striatum of mice receiving cocaine only (94 %, $P<0.001$) and in those receiving a combination of MDMA and cocaine (64 %, $P<0.001$) compared with saline-treated mice, while the activity in those receiving only MDMA was not different from that of saline-treated mice (Fig. 4c).

Effect of cocaine on striatal MDMA levels

In order to rule out that the alterations observed might be due to changes in brain MDMA levels, the concentration of MDMA in the striatum was measured 4 h after the second injection (1 h after cocaine injection). Striatal MDMA levels were similar in mice receiving only MDMA and those receiving a combination of MDMA and cocaine (for MDMA alone and MDMA+cocaine, 14.8±1.3 vs 15.4±1.4 nmol/g tissue).

Discussion

We have demonstrated for the first time that cocaine given to MDMA-treated mice potentiates the MDMA-induced increase in free radical formation but does not increase the neurotoxicity produced by amphetamine. The combination of drugs produces changes in several oxidative parameters and antioxidant enzyme activities such that the end result is a defensive response which prevents a potentiation of the neurotoxicity produced by MDMA.

Cocaine is a DAT blocker (Yorgason et al. 2011) that prevents MDMA (or its neurotoxic metabolite) entry into the dopamine neuron (Escobedo et al. 2005). Supporting this is evidence that cocaine or GBR 12909 given before MDMA protects against MDMA-induced neurotoxicity (O'Shea et al. 2001; Peraile et al. 2010). This protection may involve either a downregulation of surface expression of DAT (Peraile et al. 2010) or possibly a direct occupation of the transporter (Camarero et al. 2002). In this study, we have found that the window for protection against MDMA-induced dopaminergic neurotoxicity extends to 1 h after the second dose of MDMA, presumably reflecting the time required for the distribution and metabolism of the drug. Interestingly, occupancy of the transporter by cocaine only blocks the entry of the toxic metabolite arising from the second dose of MDMA, while that originating from the first dose would have had access to the terminal. This supports previous observations that one dose of MDMA is not sufficient to produce neurotoxic damage in the mouse striatum but rather that at least two doses are required (Colado et al. 2001). Cocaine given 3 h following the second dose no longer protects against neurotoxicity, indicating that, at this

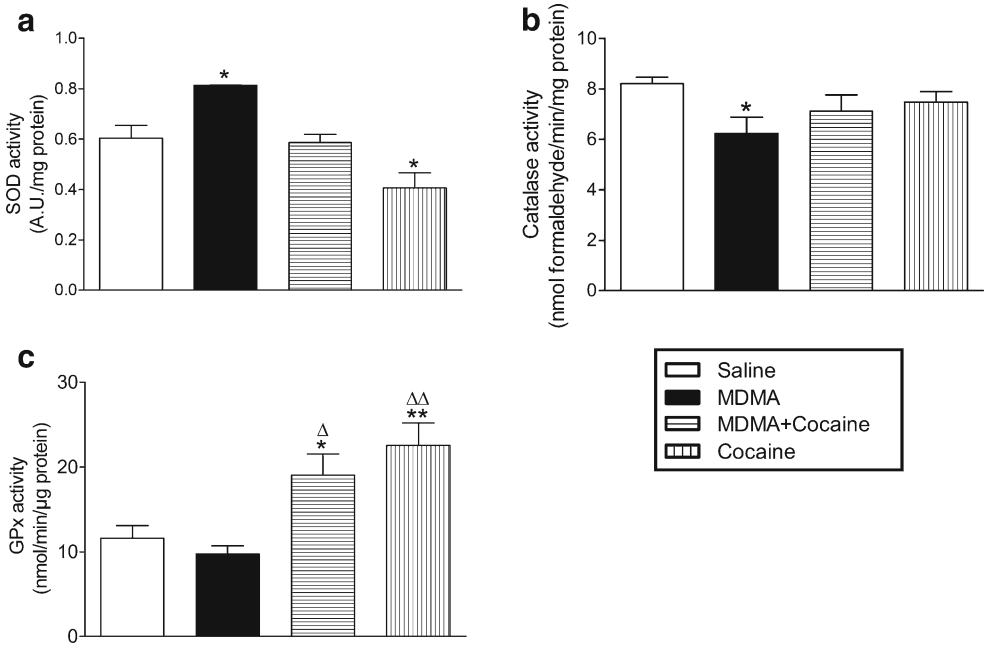

Fig. 4 Effect of cocaine on MDMA-induced changes in antioxidant enzyme activities. Mice were given MDMA (20 mg/kg, i.p.; two doses separated by 3 h) followed by cocaine (30 mg/kg, i.p.) 3 h after the second MDMA injection. SOD (**a**) and catalase (**b**) activities in the striatum 1 h after cocaine injection and striatal GPx activity (**c**) 3 h after cocaine injection. Results are shown as the mean±SEM, $n=5–8$. *$P<0.05$, **$P<0.01$, different from saline; $^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.01$, different from MDMA

time, the toxic metabolite has entered the dopamine terminal. Thus, this administration time of cocaine was used in the remainder of the studies.

Free radicals have been implicated in the pathogenesis of neurodegenerative diseases including Alzheimer's disease, amyotrophic lateral sclerosis, Huntington's disease, Parkinson's disease, and brain ischemia/reperfusion injury. They have also been associated with the neuronal damage induced by toxins such as MPTP, some pesticides, and amphetamine derivatives affecting, in particular, the dopaminergic system. The evidence suggests that there is a tilting of the pro/antioxidant balance towards increased oxidative stress (Cadet and Brannock. 1998; Halliwell 2006). The reasons for this imbalance may be the result of either increases in free radical production or a lowering of the antioxidant defense mechanisms. Among the most common oxyradicals produced in aerobic organisms are superoxide, H_2O_2, and hydroxyl radicals. Of particular interest is the superoxide radical which is converted to H_2O_2 by SOD. H_2O_2 can react with transition metals such as iron (Fenton reaction), producing the more reactive and highly damaging hydroxyl radical. With regard to MDMA, various sources for these superoxide radicals have been proposed, including the metabolism and/or autoxidation of dopamine or MDMA metabolites. In line with this, MDMA produces an increase in hydroxyl radicals (Colado et al. 2001; Camarero et al. 2002) and SOD activity is increased 4 h after MDMA, reflecting an increase in superoxide formation. Moreover, MDMA exposure increases H_2O_2 production in brain synaptosomes (Barbosa et al. 2012).

In addition to its conversion to the hydroxyl radical, superoxide can react with nitric oxide, giving rise to peroxynitrite (Halliwell 2006). Interestingly, peroxynitrite may react with salicylate, giving rise to 2,3-DHBA (Narayan et al. 1997), and may be relevant in some protocols of MDMA administration where neuronal nitric acid synthase inhibition reduces 2,3-DHBA formation and attenuates MDMA-induced neurotoxicity (Colado et al. 2001). However, in this study, although it cannot be ruled out, we did not find evidence of nitric oxide participation in the MDMA-induced effects since the drug given alone did not increase nitrite/nitrate levels. In the case of cocaine given alone, we found a nonsignificant increase in nitrate levels (46 %) which may indicate a tendency for increased nitric oxide formation, explaining the slight, though not significant, increase in 2,3-DHBA. In the case of both drugs given together, there was an increase in both nitrate and 2,3-DHBA possibly due to an additive effect.

MDMA increases lipid peroxidation, reflecting damage to cellular membranes produced by reactive oxygen species such as hydroxyl radicals (Camarero et al. 2002). The present results are in line with these previous studies, as MDMA increased lipid peroxidation 4 h after its administration. Cocaine alone did not increase this parameter but potentiated that produced by MDMA. In addition, 3-nitrotyrosine, a marker of nitrosative stress, was increased by both drugs given together but not by either drug given alone.

Ultimately, the damaging effects produced by oxidative/nitrosative stress on cell membranes and proteins depend not only on the increases in oxygen-derived and nitric oxide-derived radicals but also on the availability of an effective antioxidant defense mechanism. The increased oxidative stress produced by MDMA has been widely implicated in the neurotoxicity produced by the drug (Jayanthi et al. 1999; Cadet et al. 2001; Camarero et al. 2002) and deficiencies in antioxidant defense mechanisms produced by low vitamin E or selenium diets increase the neurotoxicity produced by the drug (Johnson et al. 2002; Sanchez et al. 2003), whereas transgenic mice overexpressing human Cu/Zn SOD are partially protected against the neurotoxic effects of the drug (Cadet et al. 1995). Moreover, striatal striosomes, which express lower levels of SOD compared with the matrix, are more susceptible to MDMA-induced dopaminergic neurotoxicity (Granado et al. 2008a).

MDMA increases SOD activity and decreases catalase activity. This could lead to increased production of H_2O_2 and decreased detoxification to H_2O and O_2, a situation favoring the Fenton reaction and production of hydroxyl radicals (Mao et al. 1993). A previous study reported decreased SOD activity following MDMA (Jayanthi et al. 1999); however, this was observed following a higher dose and at a much later time-point (16 h) which may reflect a use-dependent depletion. In our case, the greater activity may reflect increased superoxide production by MDMA.

Both drugs given together did not modify SOD or catalase activities but did elevate oxidative/nitrosative stress and cell damage and yet dopamine neurotoxicity was unaltered. In order to find a possible explanation for this, we studied the activity of GPx, an important element in the antioxidant defense mechanism. GPx activity was unaltered by MDMA but clearly increased by cocaine given alone, and this increase was maintained in mice treated with both drugs. GPx catalyses the reduction of peroxynitrite (Sies et al. 1997, 1998) and is involved in the reduction of lipid hydroperoxides (Imai and Nakagawa 2003). Thus, this could explain why, despite the increased lipid peroxidation, there is no increase in dopamine neurotoxicity; a hypothesis that is in line with the importance of Se-containing enzymes in MDMA-induced dopaminergic neurotoxicity (Sanchez et al. 2003).

We do not think that cocaine-induced changes in the MDMA temperature response participate in the observed effects (Colado et al. 2004). The only protection observed occurred when cocaine was administered 1 h after the second MDMA injection, and similar to the effects observed when cocaine was given 3 h after, cocaine attenuated the

hypothermic effect produced by MDMA. It is unlikely that this reversal of the hypothermic effect would contribute to protecting against MDMA neurotoxicity. Interestingly, this effect of cocaine on MDMA-induced hypothermia is in line with evidence that both dopamine D1 and D2 receptors and α1-adrenoceptor and α2-adrenoceptor are involved in MDMA-induced changes in body temperature through central and peripheral mechanisms (Docherty and Green 2010; Granado et al. 2011) and that cocaine can increase temperature through several mechanisms including vasoconstriction, with the response being more pronounced at lower basal temperatures (Kiyatkin 2013). In addition, we can rule out a role for changes in the brain levels of MDMA since mice treated with MDMA and those treated with MDMA and cocaine had similar levels of MDMA.

In conclusion, our studies show that the combination of MDMA and cocaine augments oxidative/nitrosative stress in the mouse striatum, leading to an increase in protein nitration and lipid peroxidation but does not potentiate dopaminergic neurotoxicity. This lack of neurotoxicity potentiation may involve the cocaine-induced increase in GPx activity, a key cellular antioxidant defense mechanism. This observation may have important implications for diseases whose pathogenesis involves oxidative/nitrosative stress as it highlights a possible endogenous neuroprotective mechanism.

Acknowledgments This work was supported by grants from Ministerio de Ciencia e Innovación, Ministerio de Sanidad y Política Social, ISCIII, and Universidad Complutense-Comunidad de Madrid: SAF2006-07045, SAF2010-21529, PI070892, PNSD 3SI/04/01, Retics RTA RD06/0001/0006 and RD06/0001/1011, CIBERNED CB06/05/0055, and UCM-CAM 910258. IP and ET received predoctoral grants from Universidad Complutense de Madrid and Ministerio de Educación y Ciencia. NG was funded by a Juan de la Cierva postdoctoral contract from Ministerio de Ciencia e Innovación.

Conflict of interest None.

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