Long-Lasting Neuroprotective Effect of Sildenafil Against 3,4-Methylenedioxymethamphetamine-induced 5-Hydroxytryptamine Deficits in the Rat Brain

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Sildenafil, given shortly before 3,4-methylenedioxymethamphetamine (MDMA), affords protection against 5-hydroxytryptamine (5-HT) depletions caused by this amphetamine derivative by an acute preconditioning-like mechanism. Because acute and delayed preconditionings do not share the same mechanisms, we investigated whether sildenafil would also protect the 5-HT system of the rat if given 24 hr before MDMA. For this, MDMA (3 × 5 mg/kg i.p., every 2 hr) was administered to rats previously treated with sildenafil (8 mg/kg p.o.). One week later, 5-HT content and 5-HT transporter density were measured in the striatum, frontal cortex, and hippocampus of the rats. Our findings indicate that sildenafil afforded significant protection against MDMA-induced 5-HT deficits without altering the acute hyperthermic response to MDMA or its metabolic disposition. Sildenafil promoted ERK1/2 activation an effect that was paralleled by an increase in MnSOD expression that persisted 24 hr later. In addition, superoxide and superoxide-derived oxidants, shown by ethidium fluorescence, increased after the last MDMA injection, an effect that was prevented by sildenafil pretreatment. Similarly, MDMA increased nitrotyrosine concentration in the hippocampus, an effect not shown by sildenafil-pretreated rats. In conclusion, our data demonstrate that sildenafil produces a significant, long-lasting neuroprotective effect against MDMA-induced 5-HT deficits. This effect is apparently mediated by an increased expression of MnSOD and a subsequent reduced susceptibility to the oxidative stress caused by MDMA.

Key words: 5-hydroxytryptamine; serotonin; 5-HT; MDMA; preconditioning; PDE5 inhibitor; sildenafil

Single or repeated injections of the ring-substituted amphetamine analogue 3,4-methylenedioxymethamphetamine (MDMA, “ecstasy”) cause long-lasting deficits in neurochemical and histological markers of serotonergic function in the brain of rodents (Goni-Allo et al., 2007) and nonhuman primates (Hatzidimitriou et al., 1999). This effect is evidenced by a decline in the activity of tryptophan hydroxylase; a decrease in the content of 5-HT and its main metabolite, 5-hydroxyindoleacetic acid (5-HIAA); a lower density of [3H]paroxetine-labeled 5-HT transporters (SERT); and the loss of SERT protein in several regions of the brain (for review see Capela et al., 2009). Notably, studies carried out in MDMA abusers have led authors to conclude that MDMA might also be toxic to humans. Thus, 5-HIAA levels in the CSF are reduced, and the pharmacological response to 5-HT agonists or 5-HT-releasing drugs is altered in MDMA abusers (Ricautre et al., 1988; McCann et al., 1999). Furthermore, positron emission tomography or single-photon emission computed tomography used in combination with a SERT ligand also showed a lower density of brain SERT sites in the brains of humans with a history of MDMA abuse (McCann et al., 2005;
de Win et al., 2008). Of interest, ecstasy users also show an altered mood and demonstrate modest deficits on some tests of attention, executive function, and memory associated with SERT decrease (McCann et al., 2008; Kish et al., 2010). Despite these facts, MDMA is widely abused as a recreational drug because of its psychological effects, such as emotional openness, empathy, a decrease in inhibitions, and sexual arousal. Nevertheless, MDMA may also impair sexual performance, making it more difficult for males to achieve an erection. For this reason, nightclub attendees usually combine MDMA with phosphodiesterase 5 (PDE5) inhibitors, such as sildenafil (Viagra), in order to increase sexual performance and functioning, a combination also known as “sextasy,” “trail mix,” or “hammerheading” (Smith and Romanelli, 2005; Mansergh et al., 2006).

Apart from the efficacy of PDE5 inhibitors in the treatment of erectile dysfunction or pulmonary arterial hypertension (Mostafa, 2008), experimental data show that sildenafil enhances angiogenesis and neurogenesis and improves functional outcome during stroke recovery (Ding et al., 2008), improves learning and memory in the Tg2576 model of Alzheimer’s disease and in a senescence accelerated mouse model (Cuadrado-Tejedor et al., 2011; Orejana et al., 2011), and prevents the neurotoxic effects of 3-nitropropionic acid (Puerta et al., 2010a). Moreover, we have recently shown that sildenafil given shortly before MDMA affords complete protection against MDMA-induced 5-HT depletions by opening mitochondrial ATP-sensitive K⁺ channels, a key mechanism involved acute preconditioning paradigms (Puerta et al., 2009b). Preconditioning can be established with at least two temporal profiles, one in which the trigger induces protection within minutes (acute preconditioning; Perez-Pinzon et al., 1997) and one in which the protected state develops after a delay of several hours to days (delayed preconditioning; Kitagawa et al., 1990), which is dependent on de novo protein synthesis (Stenzel-Poore et al., 2003). Although the precise mechanisms underlying acute or delayed preconditioning are not completely understood, attenuation of reactive oxygen species production during lethal challenges is thought to be one of the major end effectors in these processes (see, e.g., Teshima et al., 2003; Arthur et al., 2004; Nagy et al., 2004). This issue is especially relevant in our model, because overwhelming evidence supports the role of oxidative stress in MDMA-induced 5-HT loss (Puerta et al., 2009a). Thus, free radical scavengers and antioxidants attenuate MDMA-induced 5-HT deficits (for review see Capela et al., 2009; Puerta et al., 2009a), providing indirect evidence for the involvement of free radicals in the mechanism of MDMA neurotoxicity. In addition, MDMA has been reported to produce cellular changes, e.g., lipid peroxidation or protein nitration, consistent with the formation of free radicals (Sprague and Nichols, 1995; Colado et al., 1997; Darvesh et al., 2005). Finally, Colado et al. (1997) and Shankaran et al. (1999) demonstrated that MDMA increases the formation of 2,3-dihydroxybenzoic acid from salicylate in hippocampal and striatal dialysates, a conversion that occurs in the presence of a high concentration of free radicals, an effect reversed by ascorbic acid (Shankaran et al., 2001).

The present work investigated whether sildenafil, if given 24 hr prior to MDMA, would also promote a delayed preconditioning-like effect and prevent MDMA-induced 5-HT deficits. Because of the importance of free radicals in MDMA-induced 5-HT deficits, we focused our studies on the attenuation of oxidative stress as a plausible mechanism underlying the long-lasting neuroprotective effects of sildenafil.

**MATERIALS AND METHODS**

**Drugs and Chemicals**

MDMA HCl was a gift from the “Servicio de Restricción de Estupefacientes” (Spanish regulatory body on psychotropic drugs); 1-[4-ethoxy-3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-5-yl)phenylsulphonyl]-4-methylpipеразине citrate (sildenafil citrate, Viagra) was from Pfizer; and 5-HT creatinine sulfate, 5-hydroxyindole-3-acetic acid (5-HIAA), and general reagents were from Sigma (Madrid, Spain) unless otherwise indicated in the text.

**Animals and Treatments**

Experiments were carried out in male Wistar rats (290–340 g; Harlan Ibérica, Barcelona, Spain). Rats (four per cage) were housed under constant conditions of humidity and temperature (22°C ± 1°C) with a 12-hr/12-hr light–dark cycle (lights on at 0700). Food and water were available ad libitum. All the procedures followed in the present work were in compliance with the European Community Council Directive of 24 November 1986 (86/609/EEC) and were approved by the Ethical Committee of the University of Navarra.

We initially studied the effects of sildenafil on MDMA-induced 5-HT deficits in rats. For this, rats were orally administered saline or sildenafil (8 mg/kg) 24 hr before saline or MDMA (3 × 5 mg/kg i.p. every 2 hr). The dose of sildenafil was chosen to simulate that of a 70-kg patient after orally taking a 100-mg tablet of Viagra according to Reagan-Shaw et al. (2008). This dose is in the range of doses previously used by other authors (see Ding et al., 2008). Sildenafil solution was prepared by grinding Viagra tablets into powder and dissolving the powder in distilled water. Before oral administration, the drug solutions were filtered (0.45-μm pore size) to remove insoluble inactive ingredients contained in the Viagra tablets. Seven days later, animals were killed by decapitation; brains were rapidly removed and placed on ice; and the striatum, hippocampus, and frontal cortex were dissected free, frozen on dry ice; and stored at −80°C until HPLC, binding, or Western blot analysis.

**Temperature Measurements**

Rectal temperature of the rats was measured at an ambient temperature of 22°C ± 1°C with a lubricated digital thermometer (ph 0331; Panlab, Barcelona, Spain) inserted 3 cm into the rectum, the rat being lightly restrained by holding in the hand. Temperature was recorded before any drug treatment and
thereafter every 60 min up to 8 hr. Probes were reinserted from time to time until the temperature stabilized.

Determination of MDMA and Its Main Metabolites

MDMA, 3,4-methylenedioxyamphetamine (MDA), 4-hydroxy-3-methoxymethamphetamine (HMMA), or 4-hydroxy-3-methoxyamphetamine (HMA) concentrations in brain and plasma samples were determined following a previously described method (Goñi-Allo et al., 2008b). Briefly, animals were decapitated and brains were rapidly removed from the skull. One hemisphere was homogenized in 10 volumes of perchloric acid (0.2 N) containing cysteine (0.1% w/v) and sodium metabisulphite (0.1% w/v) and EDTA (0.01% w/v). Homogenates were centrifuged at 12,000 g for 20 min at 4°C. After centrifugation, supernatants were transferred to sterile Eppendorf tubes and were frozen and stored at −20°C until analysis. For plasma measurements, trunk blood was collected into Microvette CB 300LH tubes and centrifuged immediately. After centrifugation, plasma was transferred to sterile Eppendorf tubes containing sodium metabisulfite (2 g, 0.001% w/v). Samples were frozen and stored at −20°C until analysis. Samples were hydrolyzed enzymatically with β-glucuronidase at pH 5.2 (incubation for 16 hr at 37°C). For cytoplasmic protein determination of total extracellular signal-regulated kinases (ERK), phosphorylated-ERK, thioreredoxin, manganese superoxide dismutase (MnSOD), and heme oxygenase-1, tissues were homogenized in 10 vol of lysis buffer containing 200 mM NaCl, 100 mM HEPES, 10% glycerol, 200 mM NaF, 2 mM Na,P2O7, 5 mM EDTA, 1 mM EGTA, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, and 1:100 of phosphatase inhibitors cocktail set II (Calbiochem, Darmstadt, Germany). Lysates were left for 30 min on ice with shaking and were then centrifuged at 4°C for 20 min at 12,000g. The pellet was discarded, and protein concentration was determined in the supernatant by Bradford protein assay (Bio-Rad). We focused our studies on these proteins because they have been repeatedly implicated in the mechanisms underlying the protective effects of different preconditioning paradigms known to protect neurons from several neurotoxic insults (see, e.g., Andoh et al., 2003; Lee et al., 2003; Das, 2004; Chiueh et al., 2005a,b; Abdul and Butterfield, 2007; Zeynalov et al., 2009).

Preparation of Whole-Cell Lysates/Protein Extraction for Immunoblotting

For cytoplasmic protein determination of total extracellular signal-regulated kinases (ERK), phosphorylated-ERK, thioreredoxin, manganese superoxide dismutase (MnSOD), and heme oxygenase-1, tissues were homogenized in 10 vol of lysis buffer containing 200 mM NaCl, 100 mM HEPES, 10% glycerol, 200 mM NaF, 2 mM Na,P2O7, 5 mM EDTA, 1 mM EGTA, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, and 1:100 of phosphatase inhibitors cocktail set II (Calbiochem, Darmstadt, Germany). Lysates were left for 30 min on ice with shaking and were then centrifuged at 4°C for 20 min at 12,000g. The pellet was discarded, and protein concentration was determined in the supernatant by Bradford protein assay (Bio-Rad). We focused our studies on these proteins because they have been repeatedly implicated in the mechanisms underlying the protective effects of different preconditioning paradigms known to protect neurons from several neurotoxic insults (see, e.g., Andoh et al., 2003; Lee et al., 2003; Das, 2004; Chiueh et al., 2005a,b; Abdul and Butterfield, 2007; Zeynalov et al., 2009).

5-HT Transporter Density

[3H]paroxetine binding studies to the 5-HT transporter were performed according to the procedure described by Hervias et al. (2000). These experiments were carried out in rats treated with sildenafil (8 mg/kg i.p.) alone or in combination with MDMA and killed 7 days later.
antibody and washed with TBS-Tween buffer (TBS and 0.01% Tween 20), membranes were incubated with polyclonal goat peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:5,000; DakoCytomation, Glostrup, Denmark) in blocking buffer for 2 hr at 22°C. Blots were visualized using a chemiluminescence ECL Western blotting detection reagent (Amer sham, Buckinghamshire, United Kingdom), and band intensity was estimated densitometrically on a GS-800 calibrated densitometer (Bio-Rad One).

**In Situ Detection of Superoxide Production**

In situ visualization of superoxide production was assessed by hydroethidine histochemistry as previously described (Kim and Chan, 2002). Thirty minutes after the last injection of MDMA, rats were anesthetized with pentobarbital (60 mg/kg i.p.), and 200 μl of phosphate-buffered saline (PBS) containing 1 μg/μl hydroethidine (Molecular Probes, Invitrogen, Carlsbad, CA) and 1% DMSO was administered through the carotid artery. Brains were harvested 15 min later and were frozen on dry ice. Four hippocampal sections (25 μm thick) at approximately –3.8 mm relative to bregma according to the atlas of Paxinos and Watson (1997) were cut on a cryostat and were mounted onto gelatin-coated glass slides. Sections were incubated with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Merck, Darmstadt, Germany) in PBS for 15 min in a dark chamber and then were rinsed in distilled H2O and mounted with Aquamount (Shandon, Pittsburgh, PA). Hydroethidine’s oxidation product, ethidium accumulation, was examined by fluorescence microscopy (excitation 510 nm, emission 580 nm) and was quantified using the image analysis software AnalySISD 5.0 (Soft Imaging System, Olympus, Münster, Germany).

**Nitrotyrosine Measurement**

Hippocampal homogenates were prepared in PBS containing a protease inhibitor cocktail set (Calbiochem), 0.5% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS. The homogenates were centrifuged at 10,000 × g for 10 min, and the supernatants were assayed for nitrotyrosine content using a Nitrotyrosine ELISA Kit (Hycult Biotechnology b.v., Uden, The Netherlands) according to the manufacturer’s instructions. Briefly, microtiter wells coated with antibodies recognizing nitrotyrosine were incubated with standards and samples (100 μl) for 60 min at room temperature. Once washed, samples were incubated with a biotinylated tracer antibody that will bind to captured nitrotyrosine. After washing, 100 μl of streptavidin-peroxidase conjugate was incubated with samples for an additional 60 min. Samples were washed once again and incubated with tetramethylbenzidine for 30 min. The enzyme reaction was stopped by the addition of oxalic acid, and absorbance was measured at 450 nm in a Victor X3 Multilabel Reader (Perkin Elmer, Madrid, Spain). Data are expressed as nitrotyrosine levels in nanomoles per milligram protein.

**Data Analysis**

For the neurochemical analysis, differences in brain monoamine concentrations and [3H]paroxetine binding were analyzed by one-way ANOVA. Temperatures were analyzed by two-way ANOVA for repeated measures. In this case, treatment was used as the between-subjects factor and time as the repeated measure. When appropriate, group means at individual time points were compared by one-way ANOVA. Multiple pairwise comparisons were performed using the Student-Newman-Keuls test. Treatment differences were considered statistically significant at \( P < 0.05 \). Data analyses were performed in the Statistical Program for the Social Sciences (SPSS for Windows 15.0).

**RESULTS**

**Effect of Sildenafil on MDMA-Induced Hyperthermia and 5-HT Deficits**

As shown in Figure 1, sildenafil (8 mg/kg p.o.) administered 24 hr before MDMA (3 × 5 mg/kg i.p., given 2 hr apart) partially prevented the loss of 5-HT and 5-HIAA caused by MDMA in the striatum, the frontal cortex, and the hippocampus 1 week after drug treatment. Rats treated with MDMA also showed a substantial loss of [3H]paroxetine binding in all three brain regions examined (Fig. 2A), an effect fully prevented by sildenafil (8 mg/kg). Similar findings were obtained by Western blot analysis (Fig. 2B).

It is well known that MDMA-induced hyperthermia plays a key modulator role in the mechanisms underlying toxicity (Goñi-Allo et al., 2008a). We therefore analyzed whether sildenafil pretreatment affected MDMA-induced hyperthermia. As demonstrated by our data, sildenafil’s protection was independent of any effect on MDMA-induced rise in the rat core body temperature (Fig. 3). Thus, rectal temperature analysis using two-way ANOVA for repeated measures over time revealed a significant interaction treatment × time \( F(27,378) = 16.542, P < 0.001 \). Single-time-point comparisons did not show any significant difference between MDMA-treated rats previously treated with sildenafil or saline. These data are in agreement with a recent report showing no difference in the hyperthermic response to a 5-HT-depleting regimen of MDMA in rats that had received saline or a preconditioning MDMA treatment 24 hr earlier (Bhide et al., 2009).

**Effect of Sildenafil on the Metabolic Disposition of MDMA**

MDMA metabolism into toxic compounds has been suggested to participate in the mechanisms underlying 5-HT depleting effects of MDMA (Puerta et al., 2009a). To investigate the possible effect of sildenafil on the metabolic disposition of MDMA, rats were given sildenafil (8 mg/kg p.o.) 24 hr before MDMA (3 × 5 mg/kg i.p., 2 hr apart) and concentrations of MDMA and its main metabolites, MDA, HMMA, and HMA, were measured 60 min later in plasma and in the hippocampus. This time was chosen based on a previous study (Goñi-Allo et al., 2008a). As shown in Table I, there were no differences in MDMA, MDA, HMMA, or
HMA levels between rats pretreated with sildenafil and those injected with saline.

**Sildenafil Induces the Phosphorylation of ERK1/2**

The Ras/Erk pathway is a hierarchical cascade that typically originates with the recruitment of the p21\(^{\text{Ras}}\) GTPase. Ras engages the serine/threonine kinase Raf, which activates MEK (MAPK/ERK kinase). MEK, in turn, phosphorylates and activates p42 and p44 ERKs. Recently, Gonzalez-Zulueta et al. (2000), using a model of oxygen-glucose deprivation in primary cortical cultures, proved that activation of the MAPK cascade composed by Ras/Raf/MEK/ERK is a critical mechanism in the development of oxygen-glucose deprivation tolerance. Accordingly, we examined the levels of ERK1/2 phosphorylation in hippocampal homogenates at different times after sildenafil. As can be seen in Figure 4, Western blot analysis revealed a significant increase in ERK1/2 phosphorylation produced by sildenafil in a time-dependent manner. p-ERK1/2 levels were significantly increased by 1.5 hr after sildenafil and remained above control levels up to 12 hr later. Consistent with a posttranscriptional regulation of ERK1/2 protein levels remain unaltered (Fig. 4).

**Effect of Sildenafil on MnSOD Expression, Superoxide Production, and Nitrotyrosine Formation**

MnSOD has been proposed to be one of the main final targets of the MAPK cascade Ras/Raf/MEK/ERK1/2. Indeed, this pathway has been shown to activate MnSOD expression and activity (Scorciello et al., 2007). In light of this evidence, we investigated whether sildenafil would also increase MnSOD expression. As shown in Figure 5A, MnSOD expression increased in a time-dependent manner \(F(4,29) = 9.074; P < 0.001\). Importantly, MnSOD expression was still significantly elevated 24 hr after sildenafil \((P < 0.05 \text{ vs. control})\).

Because MDMA increases the formation of superoxide radicals (Puerta et al., 2010b), which are dismutated by MnSOD, we next studied whether sildenafil pretreatment would reduce the production of superoxide radicals determined by using hydroethidine in situ detection 1 hr after MDMA. In saline-injected rats, hippocampal superoxide and superoxide-derived oxidant production was minimal (Fig. 5B). In contrast, in MDMA-treated rats, hippocampal production of superoxide and superoxide-derived oxidants shown by ethidium fluorescence was increased at 1 hr after the last MDMA injection, an effect that was significantly prevented by sildenafil pretreatment.

On the other hand, it has been shown that MDMA increases nitrotyrosine formation, which is a stable marker of peroxynitrite formation (Darvesh et al., 2005), a highly reactive anion formed in the reaction of nitric oxide (NO) with superoxide radicals (Ishiropolous and al-Mehdi., 1995). Accordingly, we next studied the effects of sildenafil pretreatment on nitrotyrosine formation caused by MDMA \((3 \times 5 \text{ mg/kg i.p.})\). As depicted in Figure 5C, the concentration of nitrotyrosine in the hippocampus of MDMA-treated rats was significantly greater than that found in vehicle-treated rats 24 hr before \([F(3,25) = 4.573; P < 0.05]\). By contrast, those animals previously treated with sildenafil \((8 \text{ mg/kg p.o.})\) 24 hr before MDMA showed nitrotyrosine levels similar to those found in control rats. We also measured the expression levels of two other antioxidant enzymes, thioredoxin and heme oxygenase-1, in the hippocampus at different times after sildenafil but no significant change.
DISCUSSION

The present study demonstrates that the PDE5 inhibitor sildenafil affords significant protection against 5-HT deficits caused by the systemic administration of MDMA given 24 hr later. This protective effect is independent of changes in MDMA-induced hyperthermia or its metabolic disposition and appears to be related to an increased expression of MnSOD levels and an attenuated superoxide and superoxide-derived oxidant production, possibly preventing the subsequent oxidative stress responsible for 5-HT deficits. Sildenafil also prevented the formation of nitrotyrosine found after MDMA, but these was observed at any time in either protein (data not shown).
effects were not enough to prevent fully the loss of 5-HT content caused by MDMA (Fig. 1), suggesting that other mechanisms may be generating the remaining 5-HT depletion. Sildenafil also increased the expression levels of p-ERK1/2 but not those of two other antioxidant enzymes, thioredoxin and heme oxygenase-1.

We have recently shown that sildenafil, given shortly before MDMA, prevented the long-term 5-HT deficits caused by MDMA in rats by a preconditioning-like mechanism (Puerta et al., 2009b). Minoxidil afforded protection against MDMA-induced 5-HT depletion by a similar mechanism (Goñi-Allo et al., 2008c). Not only acute but also delayed chemical preconditioning has been shown to protect the rat brain 5-HT neurotransmitter system from MDMA (Piper et al., 2006, 2010; Bhide et al., 2009). In this regard, 3-nitropropionic acid given 24 hr before a toxic MDMA treatment completely prevented 5-HT depletions by a mechanism involving NO production (Puerta et al., 2010c). These data were in accordance with a sizable amount of evidence indicating that increased NO production is involved in various preconditioning paradigms (Huang, 2004; Pignataro et al., 2009).

The physiological actions of NO are mediated primarily through stimulation of soluble guanylate cyclase, which results in accumulation of cGMP and subsequent activation of cGMP-dependent protein kinase (PKG; for review see Schlossmann et al., 2003). Sildenafil, by inhibiting enzymatic hydrolysis of cGMP, also leads to downstream activation of PKG (Corbin and Francis, 1999). Accordingly, we speculated that sildenafil could share some of the mechanisms that have been proposed to mediate NO-induced preconditioning. Among them, NO activation of the Raf/MEK/ERK pathway has been shown to play a key role in various ischemic preconditioning models (Shamloo et al., 1999; Gonzalez-Zulueta et al., 2000; Jones and Bergeron, 2004). Therefore, we analyzed the phosphorylation levels of ERK1/2 at different times after sildenafil administration (Fig. 4).

### Table I. Effect of Sildenafil on Brain and Plasma Concentrations of MDMA and Its Main Metabolites*

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<th>MDMA concentration (ng/ml)</th>
<th>Brain concentration (µg/g tissue)</th>
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<td>MDMA</td>
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<td>572.8 ± 30.5</td>
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<tr>
<td>Sildenafil + MDMA</td>
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<td>508.8 ± 38.6</td>
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*Statistical analysis revealed no differences between groups.

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**Fig. 3.** Effect of sildenafil on MDMA-induced hyperthermia. Rat temperatures were recorded at baseline, immediately before the first injection of MDMA (t = 0 hr), and then every hour up to 8 hr. Values are means ± SEM (n = 8–10).

**Fig. 4.** Sildenafil up-regulates ERK1/2 phosphorylation in the hippocampus of rats. Representative Western blots (A) and relative densitometry (B) showing that sildenafil administration results in the phosphorylation of Erk1/2 at Thr202/Tyr204 at 1.5, 3, 6, and 12 hr later (n = 5 per group). *P < 0.05, **P < 0.01 vs. control.
ent time points after sildenafil and found that, by 1.5 hr, p-ERK levels were significantly increased in the hippocampus, an effect that was still present up to 12 hr after sildenafil.

It is widely accepted that delayed preconditioning depends on de novo protein synthesis (Stenzel-Poore et al., 2003), which includes various antioxidant enzymes (Chiueh et al., 2005a,b). In this regard, Scorziello et al. (2007) recently demonstrated that the Ras/Raf/MEK/ERK1/2 pathway increases MnSOD expression and activity, promoting neuroprotection during preconditioning. Furthermore, attenuation of reactive oxygen species production during lethal challenges is thought to be one of the major end effectors in this process (Teshima et al., 2003; Arthur et al., 2004; Nagy et al., 2004). This issue is especially relevant in our model, because a large amount of evidence indicates that increased free radical formation is responsible for MDMA-induced 5-HT depletions (for review see Puerta et al., 2009a). We analyzed MnSOD levels at various times after sildenafil, based on these reports. As demonstrated by our data, sildenafil increased MnSOD levels. In agreement with previous reports, the kinetic study demonstrated that phosphorylation of Erk1/2 preceded the changes in MnSOD expression caused by sildenafil (Borrás et al., 2006; Scorziello et al., 2007; Li et al., 2010).

Notably, MDMA-induced 5-HT depletions in the rat is dependent on formation of peroxynitrite (Darvesh et al., 2003; Arthur et al., 2004; Nagy et al., 2004). Statistical analysis revealed significant differences [F(3,62) = 33.68, \( P < 0.001 \)]; *\( P < 0.001 \) vs. control and \( P < 0.001 \) vs. MDMA. Bottom: Representative photomicrographs showing fluorescent ethidium signals (white) in the hippocampus 1 hr after saline or MDMA last injection. C: Effect of sildenafil on MDMA-induced nitrotyrosine formation in the hippocampus. Results are mean ± SEM, n = 6–8.

### Statistical Analysis

- **A:** Representative Western blots and relative densitometry showing the time course expression levels of MnSOD after sildenafil. Data are means ± SEM (n = 6 animals for each group). Data analyzed by one-way ANOVA revealed significant differences \([F(4,29) = 9.074, P < 0.001]\). *\( P < 0.05 \); **\( P < 0.01 \) vs. control.
- **B:** Effect of sildenafil on MDMA-induced superoxide production. Top: Quantification of fluorescence intensity. Data are means ± SEM (n = 4 animals for each group). Statistical analysis revealed significant differences \([F(3,62) = 33.68, P < 0.001]\); *\( P < 0.001 \) vs. control and \( P < 0.001 \) vs. MDMA. Bottom: Representative photomicrographs showing fluorescent ethidium signals (white) in the hippocampus 1 hr after saline or MDMA last injection. C: Effect of sildenafil on MDMA-induced nitrotyrosine formation in the hippocampus. Results are mean ± SEM, n = 6–8. Statistical analysis yielded the following result: \([F(3,28) = 5.82; P < 0.01]\). Different from the corresponding saline group: *\( P < 0.05 \). Different from MDMA-only animals: †\( P < 0.05 \).
et al., 2005), a highly reactive anion formed in the reaction of NO with superoxide radicals (Ischiropoulos and al-Mehdi, 1995). Because MnSOD is a key mitochondrial antioxidant enzyme responsible for the dismutation of superoxide radicals, we next analyzed whether sildenafil reduced the superoxide production caused by MDMA. Our data confirmed that sildenafil pretreatment not only attenuated superoxide production but also decreased nitrotyrosine concentrations found 24 hr after a 5-HT-depleting treatment with MDMA, which provides a plausible mechanism by which sildenafil protected the 5-HT neurotransmitter system against MDMA.

A recent study demonstrated that sildenafil increased the expression of thioredoxin and heme oxygenase-1 in human coronary arteriolar endothelial cells (Vidavalur et al., 2006). These two enzymes play a pivotal role in neuroprotection against oxidative stress caused by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, serum deprivation, ischemia, or 4-hydroxy-2-nonenal (Andoh et al., 2003; Lee et al., 2003; Das, 2004; Chiueh et al., 2005a,b; Abdul and Butterfield, 2007; Zeynalov et al., 2009). Accordingly, we also measured the expression levels of these two important antioxidant enzymes after sildenafil, but we could not detect any significant change in the expression levels of either enzyme in the hippocampus, suggesting that their role in our model is not so relevant.

In conclusion, our data demonstrate that sildenafil produces a significant, long-lasting protective effect against MDMA-induced 5-HT deficits that is apparently mediated by an increased expression of MnSOD and a subsequent reduction of free radical formation. Future studies using other neurotoxins or animal models of neurodegenerative diseases are warranted to exploit the potential utility of sildenafil and other PDE5 inhibitors, not only in protecting the heart from ischemic damage, as suggested elsewhere (Raja, 2006), but also in neuroprotection (Puerta et al., 2010a). Because sildenafil, given shortly before MDMA, also prevents the long-term 5-HT deficits caused by this amphetamine derivative, it is also important to address whether acute and long-lasting neuroprotective effects of sildenafil are mediated by independent mechanisms and effector systems, or, by contrast, acute protective mechanisms are essential to trigger the cellular pathways responsible for delayed protection.

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