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# Contribution of dopamine to mitochondrial complex I inhibition and dopaminergic deficits caused by methylenedioxymethamphetamine in mice

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ABSTRACT

Methylenedioxymethamphetamine (MDMA) causes a persistent loss of dopaminergic cell bodies in the substantia nigra of mice. Current evidence indicates that MDMA-induced neurotoxicity is mediated by oxidative stress probably due to the inhibition of mitochondrial complex I activity. In this study we investigated the contribution of dopamine (DA) to such effects. For this, we modulated the dopaminergic system of mice at the synthesis, uptake or metabolism levels. Striatal mitochondrial complex I activity was decreased 1 h after MDMA; an effect not observed in the striatum of DA depleted mice or in the hippocampus, a dopamine spare region. The DA precursor, L-dopa, caused a significant reduction of mitochondrial complex I activity by itself and exacerbated the dopaminergic deficits when combined with systemic MDMA. By contrast, no damage was observed when L-dopa was combined with intrastriatal injections of MDMA. On the other hand, dopamine uptake blockade using GBR 12909, inhibited both, the acute inhibition of complex I activity and the long-term dopaminergic toxicity caused by MDMA. Moreover, the inhibition of DA metabolism with the monoamine oxidase (MAO) inhibitor, pargyline, afforded a significant protection against MDMA-induced complex I inhibition and neurotoxicity. Taken together, these findings point to the formation of hydrogen peroxide subsequent to DA metabolism by MAO, rather than a direct DA-mediated mitochondrial complex I inhibition, and the contribution of a peripheral metabolite of MDMA, as the key steps in the chain of biochemical events leading to DA neurotoxicity caused by MDMA in mice.



## 1. Introduction

The administration of the amphetamine derivative 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) in animals produces persistent long-term effects in their brains which are considered to reflect neurodegenerative changes (Capela et al., 2009). Of interest, MDMA has a different pharmacology in the mouse compared to other laboratory animals. Thus, it has been repeatedly reported that MDMA is a relatively selective dopamine (DA) neurotoxin in mice, leaving serotonin (5-HT) concentrations intact, in contrast to its selective 5-HT neurotoxicity in rats or non-human primates (Aguirre et al., 1998; Hatzidimitriou et al., 1999; McCann et al., 2005; Schmidt, 1987). Such neurotoxicity is evidenced by a decrease in the content of striatal DA and its main metabolites (Logan et al., 1988; O'Callaghan and Miller, 1994; Stone et al., 1987); the decline in L-tyrosine hydroxylase (TH) and dopamine transporter immunostaining (Granado et al., 2008a); and increased markers of microglial and astrocytic activation in strict anatomical correlation with dopaminergic deficits (Granado et al., 2008b). It is noteworthy that these latter authors were the first to show that MDMA in mice causes a persistent loss of dopaminergic cell bodies in the substantia nigra, indicating that MDMA neurotoxicity in this animal species is not restricted to the loss of neuronal terminals as it is in rats (Granado et al., 2008b; Puerta et al., 2009). Interestingly, a recent study has observed persistent

*Abbreviations:* AMPT, α-methyl-*p*-tyrosine; DA, dopamine; MAO, monoamine oxidase; MDMA, 3,4-methylenedioxymethamphetamine; TH, L-tyrosine hydroxylase; ROS, reactive oxygen species.

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nigrostriatal dopaminergic abnormalities in ex-users of MDMA (Tai et al., 2011), highlighting the importance of a deeper understanding of the mechanisms involved in MDMA-neurotoxicity in mice.

Despite the general agreement that oxidative stress is implicated in the neurotoxicity caused by MDMA (Cadet et al., 1995; Camarero et al., 2002; Colado et al., 2001; Jayanthi et al., 1999; Sanchez et al., 2003), the source of free radicals is still a matter of debate. In this regard, we have recently shown that, akin to other well-known dopaminergic neurotoxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (Nicklas et al., 1985), rotenone (Horgan et al., 1968) or paraquat (Fukushima et al., 1994), systemic administration of MDMA to mice impairs mitochondrial respiration by inhibiting complex I of the electron transport chain. This leads to an increased production of reactive oxygen species (ROS) responsible for oxidative stress (Puerta et al., 2010); however, the exact mechanism(s) by which this may occur remain to be elucidated.

As intact mitochondria can accumulate dopamine, and dopamine per se is capable of inhibiting complex I activity (Brenner-Lavie et al., 2009, 2008), one could hypothesize that, under conditions of increased availability of cytoplasmic dopamine, the potential for dopamine-induced effects on mitochondria would increase. Such conditions are thought to exist following a toxic dosage regimen of MDMA, resulting in the redistribution of dopamine from vesicular storage to the cytoplasm (Sabol and Seiden, 1998). On the other hand, since MDMA is metabolized to catechol compounds capable of crossing the blood–brain barrier (Escobedo et al., 2005), another possibility is that further oxidation of these compounds could also contribute to disruption of mitochondrial function, free radical formation and final long-term neurotoxicity. The present study was therefore undertaken to investigate which, if any of these factors (DA, MDMA and/or their metabolism), are responsible for the acute mitochondrial complex I inhibition and ROS formation produced by the systemic administration of MDMA and for its long-term neurotoxic effects.

## 2. Methods

### 2.1. Drugs and chemicals

MDMA-HCl was a gift from the "Servicio de Restricción de Estupefacientes" (Spanish regulatory body on psychotropic drugs); The following reagents were purchased from Sigma (Madrid, Spain): 2,3-dimethoxy-5-methyl-6-(3-methyl-2-butenyl)-1,4-benzoquinone (Coenzyme Q1), 3,4-dihydroxyphenylacetic acid (DOPAC), α-methyl-*p*-tyrosine (AMPT), β-nicotinamide adenine dinucleotide, reduced form (NADH), DA, homovanillic acid (HVA), KCN, pargyline, reserpine and rotenone. GBR 12909 dihydrochloride was obtained from Tocris Bioscience (Ellisville, MO). High-performance liquid chromatography (HPLC)-grade methanol, acetic acid (glacial), sodium acetate, ammonia solution, ethyl acetate, potassium hydrogen phosphate, and potassium dihydrogen phosphate were obtained from Merck (Darmstadt, Germany). Ultrapure water was obtained using a Milli-Q purification system (Milli-pore, Molsheim, France).

### 2.2. Animals

Male C57BL/6J mice (Harlan Interfauna Ibérica S.L., Barcelona, Spain), weighing 25–30 g, were housed in groups of 5 in constant conditions of humidity and temperature (22 ± 1 °C) with a 12-h/12-h light–dark cycle (lights on at 07:00 h). Food and water were available *ad libitum*. The experiments were performed after approval of the protocol by the institutional Ethics Committee, in accordance with the law in force (European Directive 86/609/EEC and Real Decreto 1201/2005), following the Research Council's Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments.

### 2.3. Drug treatments and experimental design

The first set of experiments were designed to test the role of DA in the inhibitory effect of MDMA on mitochondrial complex I activity. For this, a group of mice was treated with reserpine (5 mg/kg i.p.) in combination with AMPT (150 mg/kg i.p., at 5-h intervals × 2). Reserpine was given 24 h before the first injection of MDMA (10, 20, 30 mg/kg i.p. every 2 h) or saline, while AMPT was administered 4 h before and 1 h after the first dose of MDMA or saline. The reason these drugs were utilized was not to target particular pools of brain DA, but to effectively deplete all possible sources of DA that might contribute to mitochondrial complex I inhibition (Yuan et al., 2001). The dosage regime for MDMA used was based on a previous study showing that it produces significant and reproducible dopaminergic deficits in mice (Puerta et al., 2010). Mitochondrial complex I activity was analyzed in the striatum of mice 1 h after the last dose of MDMA and in the hippocampus of mice treated only with saline or MDMA.

A different set of animals were treated with saline or three increasing doses of MDMA (10, 20, 30 mg/kg i.p. every 2 h) alone or in combination with the DA precursor L-dopa (100 mg/kg p.o.), the dopamine uptake inhibitor, GBR 12909 (10 mg/kg i.p.) or the MAO inhibitor pargyline (15 mg/kg i.p.). These compounds were administered 30 min prior to the first and second doses of MDMA or saline (except for GBR 12909, which was administered 30 min before each of three MDMA or saline injections). Treatment regimens of L-dopa, GBR 12909 or pargyline, were chosen based on previous findings showing an increased vesicular concentration of DA (Cleren et al., 1999); a blockade of the dopamine transporter (Camarero et al., 2002) and an effective inhibition of mouse brain MAO-A/B activity (Youdim et al., 2006; Watanabe et al., 2004) respectively. All drugs were dissolved in saline (0.9% NaCl). L-dopa solutions were prepared by grinding Madopar® (Roche) tablets into powder, dissolved in saline and filtered (0.45 μm pore size). Seven days after drug administrations, mice were killed by decapitation; brains were rapidly removed, placed on ice and striatal tissue was dissected out, frozen on dry ice and stored at −80 °C until chromatographic or western-blot analysis. Similar treatments were administered to a different set of animals that were sacrificed 1 h later in order to analyze mitochondrial complex I activity.

Furthermore, we also studied the effect of intrastriatal and subtoxic systemic administrations of MDMA in combination with L-dopa. For this, mice were treated with a subtoxic (3 × 10 mg/k i.p. every 2 h) treatment of MDMA either alone or in combination with L-dopa (100 mg/kg p.o., given 30 min before the first and second doses of MDMA). In the second set, MDMA (3 × 100 μM/2 μL, every 2 h) was administered into the striatum of mice either alone or in combination with a systemic administration of L-dopa (100 mg/kg p.o., given 30 min before the first and second doses of MDMA). The concentration of MDMA in the solution injected intrastriatally was chosen based upon a previous study showing that the extracellular concentration of MDMA under these experimental conditions is similar to that found after systemic neurotoxic doses of MDMA (Breier et al., 2006; Escobedo et al., 2005; Esteban et al., 2001). Seven days after drug administrations, mice were killed by decapitation and striatal tissue was prepared for chromatographic or western-blot analysis.

### 2.4. Surgical procedures

The effect of direct administration of MDMA into the striatum alone or in combination with L-dopa (i.p) was assessed. For this, mice were anaesthetized with pentobarbital sodium (50 mg/kg) and secured in a Kopf stereotaxic frame with the tooth bar at 3.3 mm below the interaural line. The skull was exposed, and the guide cannula (CMA/11, Sweden) was implanted into the right striatum (0.2 mm anterior, 3.0 mm lateral from bregma). After surgery, the animals were housed individually with free access to food and water. Mice were allowed to recover from surgery and the experiments were carried out 5 days later.

On the day of the experiment, solutions were microinjected into the right striatum using a stainless steel 33-gauge internal cannula (C315I; Plastics1), connected to PE-20 tubing leading to a 10 μL Hamilton syringe. The internal cannula extended 1 mm below the guide cannula and a volume of 2 μL was delivered over a period of 2 min. Probes were perfused with MDMA (3 × 100 μM, every 2 h) at a flow rate of 1 μL/min. Finally, the internal cannula was allowed to remain in place for an additional 2 min following the injection.

### 2.5. Measurement of rectal temperature

Temperature measurement was performed using a TMP 812 thermometer, with digital readout (Panlab, Barcelona, Spain) and a lubricated YSI 451 rectal semi-flexible probe for mice. Each mouse was lightly restrained by hand, for approximately 10 s, whilst the probe was inserted into its rectum and a steady reading was obtained.

### 2.6. Elevation of body temperature with a homeothermic blanket

When MDMA was administered intrastriatally, body temperature was maintained elevated to near that seen in mice given MDMA i.p. This was achieved by placing the mice in a cage with a Harvard Homeothermic Blanket (Model 50-7087) covering the base.

### 2.7. Determination of mitochondrial complex I activity

Mice were sacrificed by decapitation 1 h after the last MDMA injection. Striata or hippocampi were dissected free and homogenized in 20 mM HEPES buffer (pH 7.8) to obtain a final tissue concentration of 2 μg/μL. Complex I activity was determined as described (Puerta et al., 2010; Ratner et al., 2009). Briefly, samples were freeze-thawed twice and 50 μL aliquots were transferred to 150 μL of the assay medium containing 1 mM KCN, 100 μM NADH and 40 μM Coenzyme Q1. Oxidation of NADH was followed at 340 nm spectrophotometrically in a Multiskan Spectrum (Thermo,