Ecstasy induces apoptosis via 5-HT\textsubscript{2A}-receptor stimulation in cortical neurons

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Abstract

3,4-Methylenedioxymethamphetamine (MDMA or “Ecstasy”) is a psychoactive and hallucinogenic drug of abuse. MDMA has been shown to produce neurotoxicity both in animals and humans. MDMA and other amphetamines induce serotonergic and dopaminergic terminal neurotoxicity and also neurodegeneration in areas including the cortex, hippocampus, striatum and thalamus. Herein, we investigated the mechanisms involved in MDMA-induced neurotoxicity to neuronal serum free cultures from rat cortex. The hyperthermic effect produced by MDMA has been shown to be a clinically relevant aspect for the neurotoxic events. Thus, MDMA-induced toxicity to cortical neurons was evaluated both under normothermic (36.5°C) and hyperthermic (40°C) conditions. Our findings showed that MDMA produced neuronal apoptosis, accompanied by activation of caspase 3, in a concentration dependent manner. MDMA neurotoxicity was completely prevented by pre-treatment with a 5-HT\textsubscript{2A}-receptor antibody, which acted as an “irreversible non-competitive antagonist” of this receptor. Furthermore, MDMA depleted intracellular glutathione (GSH) levels in a concentration dependent manner, an effect that was attenuated by Ketanserin, a competitive 5-HT\textsubscript{2A}-receptor antagonist. Accordingly, N-acetylcysteine, an antioxidant and GSH precursor, also reduced MDMA-induced toxicity. Specific inhibitors of the inducible and neuronal nitric oxide synthase (NOS) partially prevented MDMA neurotoxicity, ascertaining the involvement of reactive nitrogen species, in the toxic effect. In conclusion, direct MDMA 5-HT\textsubscript{2A}-receptor stimulation produces intracellular oxidative stress that leads to neuronal apoptosis accompanied by caspase 3 activation.

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1. Introduction

It is widely acknowledged that the consumption of 3,4-methylenedioxymethamphetamine (MDMA; “Ecstasy”, “Adam”, “X”, “e”) may result in neurotoxic effects. MDMA-induced damage on serotonergic nerve endings in the forebrain, which lasts for months in rats and years in primates, has been demonstrated both biochemically and histologically (Ali et al., 1993; Bowyer et al., 2003; Green et al., 2003; Hatzidimitriou et al., 1999; Schmidt, 1987). Also, in human Ecstasy users a global and local reduction of serotonin transporters binding was found, in comparison to a control group and the decrease was positively correlated with the cumulative lifetime intake of Ecstasy (McCann et al., 1998). These studies indicate that MDMA-induced neurodegeneration may also occur in human users.

MDMA-induced neurotoxicity is, though, not only limited to serotonergic and dopaminergic neurons, since a broader neuronal cell death occurs in the brains of MDMA treated animals. Studies, that analysed the localization of MDMA-induced neuronal degeneration throughout the entire rat brain, have reported neuronal degeneration in different brain areas.
such as: the parietal cortex, the insular/perirhinal cortex, the ventromedial/ventrolateral thalamus, and the tenia tecta (Armstrong and Noguchi, 2004; Commins et al., 1987; Schmued, 2003). Accordingly, studies in vitro had shown that MDMA, and related amphetamines, induce neuronal apoptosis in cortical and cerebellar granule neurons (Capela et al., 2006a,b; Jimenez et al., 2004; Stumm et al., 1999). These studies demonstrated that MDMA-induced apoptotic neuronal death, was accompanied by activation of caspase 3, occurred in a concentration dependent manner and was characterized by endonucleosomal DNA cleavage, with differential expression of antiapoptotic and proapoptotic proteins (bcl-XL,b variants).

One important and considerable aspect of MDMA neurotoxicity, both in animals and humans, is the hyperthermia induced by MDMA. This represents a clinically relevant aspect in MDMA abusers, since body temperatures may reach up to 43 °C (Henry, 1992). Misuse of MDMA in crowded conditions with a high ambient temperature, physical activity and dehydration, i.e. under the conditions that MDMA is used at parties, may all contribute to increase the hyperthermic response induced by MDMA and thereby promote toxic events (Green et al., 2003). In agreement, we have previously demonstrated that MDMA and MDMA-metabolites induced apoptotic neuronal death in cortical neurons is potentiated under hyperthermic conditions (Capela et al., 2006a,b).

Several studies have involved the 5-HT2A-receptor in the mechanism of MDMA-induced toxicity. In vivo, 5-HT2A-receptor antagonists, ketanserin, MDL 11,939 or ritanserin showed efficacy preventing neuronal damage in rats, although this effect was not dissociated from the hypothermic effect elicited by the antagonist alone (Johnson et al., 1993; Malberg et al., 1996; Schmidt et al., 1990). Moreover, in vitro we previously reported that the 5-HT2A-receptor antagonists Ketanserin and R-96544 protected cortical neurons against MDMA toxicity, in a temperature-independent manner (Capela et al., 2006b).

MDMA-induced neurotoxicity has also been shown to be associated with oxidative stress with the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Colado et al., 1997; Colado et al., 2001; Yeh, 1999). Our studies in cortical neuronal cultures have proven that MDMA-induced toxicity could be attenuated by pre-treatment with α-phenyl-N-tet-butyl nitrite (PBN), a free radical scavenger, Nω-nitro-arginine (L-NNA) and a non-specific nitric oxide synthase (NOS) inhibitor (Capela et al., 2006b).

The mechanisms involving MDMA-induced neuronal death, namely in the cortical brain area, are not fully understood. In the track for those mechanisms, in the present study we tested and proved, using cortical neuronal serum free cultures, the following hypothesis: (1) MDMA induces apoptosis accompanied by activation of caspase 3 in cortical neurons in a concentration dependent manner; (2) 5-HT2A-receptor antagonists prevent MDMA-induced cortical neurotoxicity, in a temperature independent manner; (3) MDMA produces oxidative stress, an event prevented by antioxidants and by specific inducible nitric oxide synthase (iNOS) and neuronal nitric oxide synthase (nNOS) inhibitors.

2. Materials and methods

2.1. Materials

Materials for cell cultures were obtained from the following sources: Neurobasal Medium and supplement B27 from Invitrogen (Paisley, UK); Modified Eagle’s medium, phosphate buffered saline (PBS), HEPES buffer, trypsin/EDTA, penicillin/streptomycin, L-glutamine, collagen-G and poly-L-lysine from Biochrom (Berlin, Germany). 3-(4,5-Dimethylthiazolyl-2yl)-2,5-diphenyl tetrazolium bromide (MTT), enzyme-standard for kinetic lactate dehydrogenase (LDH)-test, reduced (GSH) and oxidised glutathione (GSSG), glutathione redutase (GR) (EC 1.6.4.2), 2-vinylpyridine, β-nicotinamide adenine dinucleotide phosphate reduced form (β-NADPH), β-nicotinamide adenine dinucleotide reduced form (β-NADH), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), Ketanserin, NAC (N-acetylcysteine) and the peptide substrate for the caspase 3 assay acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) from Sigma–Aldrich (St. Louis, MO, USA). The drugs 1400W dihydrochloride and Nω-propyl-L-arginine were purchased from Tocris (Bristol, UK), MDMA (HCl salt) was extracted and purified from high purity MDMA tablets that were kindly provided by the Portuguese Criminal Police Department. The obtained salt was pure and fully characterized by NMR and mass spectrometry methodologies. Goat polyclonal antibody raised against the 5-HT2A-receptor (No. SC-15073) was purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were purchased from Sigma–Aldrich of the highest grade commercially available.

2.2. Cell culture

Primary neuronal cultures of cerebral cortex were obtained from embryos (E-18) of Wistar rats. Cultures were prepared as previously described (Capela et al., 2006a,b). Briefly, cerebral cortex was dissected, meninges were removed and tissue was incubated for 15 min in trypsin/EDTA (0.05/0.02%, w/v, in PBS) at 37 °C; the cultures were rinsed twice with PBS and once with dissociation medium (modified Eagle’s medium with 10% fetal calf serum, 10 mM HEPES, 44 mM glucose, 100 U penicillin plus streptomycin/ml, 2 mM L-glutamine, 100 IE insulin/l), dissociated by Pasteur pipette again in dissociation medium, pelleted by centrifugation (210 x g for 2 min), re-dissociated in starter medium (Neurobasal medium with supplemental B27, 100 U penicillin + streptomycin/ml, 0.5 mM L-glutamine, 25 μM glutamate), and seeded out in 48-well or 6-well plates in a density of 1.5 x 10⁵ cells/cm². Wells were pre-treated by incubation with poly-L-lysine (0.25%, w/v, in PBS) over-night at 4 °C and then rinsed with PBS, followed by incubation with coating medium (dissociation medium with 0.03 w/v collagen G) for 1 h at 37 °C; then they were rinsed twice with PBS before the cells were seeded in starter medium. Cultures were kept at 36.5 °C and 5% CO2 and fed at the 4th day in vitro (DIV) with cultivating medium (starter medium without glutamate) by replacing one-half of the medium. The cultures were used for experiments after the 8th
DIV, containing ≤10% astroglial cells. Since these neuronal cultures are serum free, microglia are virtually absent in the cultures at the day of the experiments.

2.3. Experimental protocol

Previous published works established 40 °C as an adequate temperature for the hyperthermia experiments (Capela et al., 2006b). At the 8th DIV, neurons were stimulated by a single application of MDMA (100–800 μM). MDMA concentrations were selected according to the ones found in the rat brain following MDMA administration (Chu et al., 1996) and also to their ability to produce toxicity to cortical neurons (Capela et al., 2006b). Within the following 48 h after MDMA stimulation, cells were kept at 36.5 °C (normothermic conditions) or 40 °C (hyperthermic conditions) without feeding. The competitive 5-HT₂A-receptor antagonist Ketanserin (1 μM), the antioxidant and GSH precursor N-Acetylcysteine (NAC) (1 mM), the specific iNOS inhibitor 1400W (0.1 μM) and the specific nNOS inhibitor Nω-propyl-l-arginine (0.1 μM) were applied to the culture 1 h before MDMA. These concentrations were selected according to the drug selectivity for the target, and also, after screening experiments, their ability to provide protection, in accordance with their ability to produce toxicity to cortical neurons (Capela et al., 2006b). Within the following 48 h after MDMA stimulation, cells were kept at 36.5 °C. The 5-HT₂A-receptor antibody (1 and 2 μg/ml final concentration) was used as a “non-competitive irreversible” receptor blocker and was applied to the culture 1 h before MDMA. Experiments showed that the protection afforded by these agents was seen for tested MDMA concentrations. Herein, most of the data using these antagonists is shown for the 400 μM MDMA concentration, as representative of that protection. Drugs were diluted in medium or purified water. Administration of equivalent volumes of vehicle served as controls. Cultured neurons were assessed at two different time points (24 and 48 h).

2.4. Life-death assays

Cultured neurons were assessed morphologically by phase contrast microscopy at two different time points (24 and 48 h). Cell damage was also assessed quantitatively by the measurement of lactate dehydrogenase (LDH) release into the medium (as a measure of cell membrane integrity) by means of a kinetic photometric assay, as described previously (Capela et al., 2006b). Delta increase in LDH release into the medium was calculated by subtraction of the respective controls in U/ml.

2.5. Caspase 3 assay

Caspase 3 assay was based on a colorimetric assay previously described (Jimenez et al., 2004). The hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by caspase 3, results in the release of the p-nitroaniline (pNA) moiety, which has a high absorbance at 405 nm. Approximately 3 × 10⁶ cells were lysed in lysis buffer [50 mM HEPES, 1 mM DTT, 0.1 mM EDTA, 0.1% CHAPS (pH 7.4)] for 10 min on ice (no protease inhibitors). Cell lysate was added to assay buffer [100 mM NaCl, 50 mM HEPES, 10 mM DTT, 1 mM EDTA, 10% Glycerol, 0.1% CHAPS (pH 7.5)] containing 16 μM of the colorimetric caspase 3 substrate. After incubation at 37 °C for 2 h, absorbance was measured in a microplate reader at 405 nm. Protein concentrations were determined using the Bradford assay.

2.6. Glutathione measurement

Measurements of neuronal intracellular glutathione (GSH) levels were carried out according to a previously published method (Carvalho et al., 2004). Briefly, approximately 1.5 × 10⁶ cultured cells were scraped and proteins precipitated with perchloric acid (final concentration 5%), for 15 min at 4 °C, centrifuged, and the supernatant was neutralized with an equimolar solution of KHCO₃. GSH contents were measured by the rate of colorimetric change of 600 μM 5,5’-dithiobis(nitrobenzoic acid) at 412 nm in the presence of 1.4 U of glutathione reductase and 0.22 mM β-NADPH, using a microplate reader. Although oxidized glutathione (GSSG) was also evaluated, using 2-vinylpiridine to block free SH groups, intracellular GSSG levels were always found to be below the limit of quantification of the method, which values lie between 0 and 1 nmol/mg protein. Thus, GSH results reported in Fig. 3 include both reduced and negligible amounts of oxidized GSH levels. The perchloric acid pellet was suspended in 1 M NaOH and the protein measured by the Lowry method, using bovine serum albumin as standard. GSH contents were normalized to the total protein.

2.7. Statistical analysis

Results are presented as mean ± S.E.M. To avoid possible variations of the cell cultures depending on the quality of dissection and seeding procedures, data were pooled from three independent experiments. Non-parametric tests were used. Kruskal–Wallis test (one-way ANOVA on ranks) was used to compare means of different treatment groups, followed by the Student–Newman–Keuls post hoc test, if a significant p-value had been obtained. Details of the statistical analysis are described in each figure legend. Statistical significance was accepted at p-value less than 0.05.

3. Results

3.1. MDMA-induced apoptosis is accompanied by activation of caspase 3 in cortical neurons

Phase-contrast microscopy demonstrated that the predominant type of cell death following MDMA-stimulation was apoptosis. Phase-contrast microscopy revealed a delayed type of neuronal death, with typical features of apoptosis. Neurons exposed to increasing MDMA concentrations either under normothermic or hyperthermic conditions (48 h incubation) showed signs of neurite disintegration, cytoplasmic shrinkage, loss of membrane integrity and neuritic processes.
To assess whether neuronal apoptosis was accompanied by caspase activation, we measured caspase 3 activity. Caspase 3 was activated in a concentration dependent manner 48 h after the incubation of cortical neurons with MDMA, under normothermic conditions (Fig. 1).

3.2. MDMA-induced neurotoxicity is mediated by stimulation of the 5-HT2A-receptor

To test whether MDMA-induced neurotoxicity is mediated by agonism at the 5-HT2A-receptor we pre-incubated cultured cortical neurons with the 5-HT2A-receptor antibody, under hyperthermic conditions. The antibody acted as an “irreversible non-competitive antagonist” of the receptor. As shown in Fig. 2A, cultures pre-treatment with the antibody 1 h prior to the 48 h stimulation period with 400 µM MDMA, prevented the neurotoxic effects of MDMA. The 2 µg/ml antibody concentration was more effective than 1 µg/ml in protecting cortical neurons. Thus, the protective effect of the antibody was dependent on its concentration. The typical features of MDMA-induced apoptotic neurodegeneration, in cortical neurons under hyperthermic conditions, could be prevented by the antibody pre-treatment (Fig. 2B).

3.3. MDMA concentration-dependently depleted intracellular GSH levels and induced oxidative stress in cortical neurons

Concerning the redox status, cortical neurons exposed to growing concentrations of MDMA during 48 h under normothermic conditions, suffered depletion intracellular GSH levels in a concentration-dependent manner (Fig. 3). The GSH depletion was not accompanied by a parallel increase in the levels of oxidized glutathione (GSSG). In fact, the levels of GSSG in the treated cells remained below the quantification levels of the method (data not shown). Importantly, pre-treatment with the competitive 5-HT2A-receptor antagonist ketanserin (1 µM) protected against the MDMA-induced depletion of the intracellular GSH levels (Fig. 3).

Also, N-acetylcysteine (1 mM), an antioxidant and GSH precursor, provided protection against MDMA-induced cortical neurotoxicity, as measured by LDH release (Fig. 4). Protection occurred at various MDMA concentrations under normothermic conditions.

3.4. Selective iNOS and nNOS inhibitors attenuated MDMA-induced neurotoxicity

To test whether the production of RNS is involved in MDMA-induced neurotoxicity we pre-incubated cultured cortical neurons with a specific iNOS inhibitor 1400W dihydrochloride (0.1 µM) and a specific nNOS inhibitor...
Intracellular GSH levels were depleted in a concentration-dependent manner, in neuronal cultures 48 h after exposure to MDMA under normothermic conditions. Neuronal cultures pretreated with Ketanserin (1 μM) were protected against MDMA-induced GSH depletion. Total intracellular GSH contents are presented in nmol/mg protein (n = 9 per condition out of three independent experiments). Multiple comparisons by the Student–Newman–Keuls post hoc test were performed after the Kruskal–Wallis test (**p < 0.01 MDMA vs. control; **p < 0.01 higher MDMA concentration vs. lower MDMA concentration; #p < 0.01 MDMA vs. MDMA + Ketanserin).

Data are presented as increase of LDH release relatively to respective controls (n = 16 per condition out of three different experiments). Multiple comparisons by the Student–Newman–Keuls post hoc test were performed after the Kruskal–Wallis test (**p < 0.01 MDMA vs. MDMA + NAC 1 mM).

Specific iNOS and nNOS inhibitors attenuated MDMA-induced neurotoxicity. Cortical cultures were exposed to 400 μM MDMA for 48 h under hyperthermic conditions. Absolute LDH activity (U/ml) values in the range of 1 to 10 μM (Battaglia et al., 1988). An agonist role has been confirmed by the demonstration that MDMA induces phosphatidylinositol (PI) turnover in cells expressing 5-HT<sub>2A</sub>- and 5HT<sub>2C</sub>-receptors (Nash et al., 1994). Serum free primary neuronal cultures lack serotonergic input because of the absence of cells producing 5-HT and serotonin. In the present neuronal culture model MDMA will act as an agonist at the 5-HT<sub>2A</sub>-receptor of pyramidal neurons.

The present study highlights that MDMA-induced cortical neurotoxic effects in cultured neuronal cultures are fully mediated by the 5-HT<sub>2A</sub>-receptor. We have previously shown that cultured primary cortical neurons express the 5-HT<sub>2A</sub>-receptor antagonist Ketanserin or R-96544 (Capela et al., 2006b). In accordance with MDMA-induced cortical neurotoxicity, caspase-like protease activity was found to be increased in rats’ frontal cortex 3 h after MDMA injection (O’Shea et al., 2005).

Specific iNOS and nNOS inhibitors provided protection against MDMA-induced cortical neurotoxicity. There is a general consensus that the 5-HT<sub>2A</sub>-receptor is a key site for hallucinogens action (Nichols, 2004). In rat brain, the 5-HT<sub>2A</sub>-receptor is found in the somata and dendrites of pyramidal cortical neurons (Xu and Pandey, 2000). The cortex brain area is known to be targeted by serotonergic neurons coming from the raphe nucleus and therefore is rich in this receptor. In accordance with MDMA-induced cortical neurotoxicity, caspase-1-like protease activity was found to be increased in rats’ frontal cortex 3 h after MDMA injection (O’Shea et al., 2005).  

MDMA binding affinity for the 5-HT<sub>2A</sub>-receptor has Ki values in the range of 1 to 10 μM (Battaglia et al., 1988). An agonist role has been confirmed by the demonstration that MDMA induces phosphatidylinositol (PI) turnover in cells expressing 5-HT<sub>2A</sub>- and 5HT<sub>2C</sub>-receptors (Nash et al., 1994). Serum free primary neuronal cultures lack serotonergic input because of the absence of cells producing 5-HT and serotonin. In the present neuronal culture model MDMA will act as an agonist at the 5-HT<sub>2A</sub>-receptor of pyramidal neurons.
higher than the competitive 5-HT2A-receptor antagonists, which may explain their non-fully protective effect (about 30% protection). However, we clearly demonstrated a full protection by the 5-HT2A-receptor antibody against MDMA-induced neurotoxicity in cortical neurons. The antibody served as an “irreversible non-competitive” 5-HT2A-receptor blocker and demonstrated that MDMA-induced neurotoxicity is, indeed, fully mediated by 5-HT2A-receptor stimulation.

Neuronal apoptosis mediated by 5-HT2A-receptor stimulation was accompanied by caspase 3 activation. Caspase 3 activity increased in a concentration dependent manner in cortical neuronal cultures exposed to MDMA. 5-HT2A-receptor activation is known to lead to the stimulation of the PI-specific phospholipase C (PLC) and also of the phospholipase A2 (PLA2) pathway. Clearly, specific hallucinogen ligands interact with the 5-HT2A-receptor to activate the PLC and PLA2 pathways to different extents (Nichols, 2004). There is evidence for a role of PLA2 in neuronal death after ischemic injury (Arai et al., 2001). Therefore, it seems likely that 5-HT2A-receptor stimulation by MDMA could lead to the activation of the PLA2 apoptosis pathway. The 5-HT2A-receptor is also known to promote neuronal firing by enhancing sensitivity to glutamatergic AMPA-receptors and increasing intracellular calcium ([Ca2+]i) levels (Azmitia, 2001). These actions may destabilize the internal cytoskeleton, which may be a major factor in the activation of cell apoptosis by 5-HT2A-agonism (Azmitia, 2001). Taking all these data into account, the present study provides new data to support the possible mechanism of neuronal apoptosis via 5-HT2A-receptor activation.

Studies support that hyperthermia is an important factor in MDMA-induced neuronal death. We have previously reported that cortical neurons incubated with MDMA (100–800 µM) for 48 h resulted in delayed neuronal death at all concentrations both under normothermic and hyperthermic conditions (Capela et al., 2006b). Overall, we could verify that there was a time-dependent and delayed MDMA-induced neuronal death and, also, hyperthermic conditions significantly potentiate MDMA-induced neurotoxicity. The long term neurotoxic effects, that follow MDMA administration have been shown to be related to the acute hyperthermia produced by the drug (Broening et al., 1995; Malberg and Seiden, 1998). Nonetheless, neurodegeneration does occur in normothermic animals (Broening et al., 1995; Farfel and Seiden, 1995). In accordance, prevention of MDMA-induced hyperthermia decreases the neurotoxicity and many drugs that protect against MDMA-induced neurotoxicity also decrease the animals body temperature (Farfel and Seiden, 1995; Malberg et al., 1996). In animal studies, researchers are faced with several uncontrollable factors affecting body temperature. We simulated the hyperthermia that follows MDMA administration by placing cortical neurons in an incubator at an environmental temperature of 40 °C (Capela et al., 2006b). A protective effect of selective 5-HT2A-receptor antagonists against MDMA-induced neuronal death in cortical cultures is presently shown, and was also previously proven, to be temperature independent (Capela et al., 2006b). Using neuronal cultures, we concluded that these blockers afforded protection directly, rather than indirectly via hypothemic effects which may be elicited by the drug in vivo (Johnson et al., 1993; Malberg et al., 1996; Schmidt et al., 1990). In accordance, in rats the activation of 5-HT2A-receptors by MDMA, a direct agonist, raises the body temperature (Blessing and Seaman, 2003).

Production of ROS/RNS has been demonstrated to contribute to the long-term neurotoxic effects produced by MDMA (Green et al., 2003). Specifically, peroxynitrite (ONOO−) formed by the interaction of nitric oxide (NO) with superoxide radicals is a major neurotoxin (Beckman et al., 1990). Also, hyperthermia prevents free radical formation, which is otherwise increased in rats undergoing hyperthermic brain temperature (Globus et al., 1995; Kil et al., 1996) as occurs in MDMA-administered animals. We have previously shown that the free radical scavenger PBN and the non-selective NOS inhibitor L-NNA provided protection against MDMA-induced neurotoxicity to cortical neurons, in a temperature independent manner (Capela et al., 2006b). Accordingly, PBN attenuated MDMA-induced neuronal damage in vivo, at a dose that did not modify hyperthermia, but was sufficient to scavenge the produced hydroxyl radicals (Yeh, 1999). MDMA was also shown to increase the formation of NO and nitrotyrosine in the rat striatum (Darvesh et al., 2005). Additionally, it was found that rat brain NOS activities in the cortex regions were significantly elevated at 6 h after MDMA administration (Zheng and Laverty, 1998). NO in the brain is synthesised by NO synthase, which is classified into 3 major isoforms, neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) (Pannu and Singh, 2006). Non-selective NOS inhibitors were shown to attenuate MDMA-induced neurotoxicity in rats (Darvesh et al., 2005; Zheng and Laverty, 1998). Furthermore, the specific nNOS inhibitor AR-R17477AR was reported to prevent the MDMA-induced rise of free radical formation in vivo, without affecting body temperature (Colado et al., 2001). Herein, we confirm and extend the findings of previous studies by showing that specific iNOS and nNOS inhibitors protect against MDMA-induced cortical neurodegeneration under hyperthermic conditions. It seems reasonable to believe that both iNOS and nNOS activities contribute to the formation of RNS by MDMA. In particular, NO mediated neurotoxicity may be dependent on the production of superoxide radical, which combines with NO to form the tissue-damaging ONOO−.

Incubation of cortical neurons with MDMA depleted intracellular GSH in a concentration-dependent manner. The decrease in the intracellular GSH levels is a marker of oxidative stress and leads to an increased vulnerability to further oxidative injury by MDMA. Pre-treatment with Ketanserin, an antagonist of the 5-HT2A-receptor, attenuated GSH depletion in neurons, thereby providing protection against MDMA neurotoxicity. Accordingly, N-acetylcysteine, an antioxidant and GSH precursor, provided protection against MDMA-induced cortical neurotoxicity. One might postulate that the activation of the 5-HT2A-receptors by MDMA could lead to a slow, but sustained, increase in the production of damaging ROS/RNS that promote oxidative stress thereby conducing to neuronal apoptosis, accompanied by caspase 3 activation.
The present study highlights the importance of the findings by Reneman et al. (2002) in human MDMA users. These authors evaluated the acute and chronic effects of MDMA on cortical 5-HT2A-receptors in rats and human brains. In rats, they observed a decrease followed by a time-dependent recovery of cortical 5-HT2A-receptor densities. In recent MDMA human users, post-synaptic 5-HT2A-receptor densities were significantly lower in all cortical areas studied (Reneman et al., 2002). These findings may represent neuro-adaptations by the over stimulation of the 5-HT2A-receptor leading to a down-regulation. On the other hand, they may also be viewed as the result of neurotoxic actions on the pyramidal cells of the cortex.

In conclusion, direct MDMA 5-HT2A-receptor stimulation produced intracellular oxidative stress that leads to neuronal apoptosis, accompanied by caspase 3 activation. The mechanism involved in the neuronal apoptosis mediated by 5-HT2A-receptor stimulation is worthy of further investigation.

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