Title:


Authors:

João Paulo Capela, Andreas Meisel, Artur Reis Abreu, Paula Séro Branco, Luísa Maria Ferreira, Ana Maria Lobo, Fernando Remião, Maria Lurdes Bastos and Félix Carvalho

Affiliation:

REQUIMTE, Toxicology Dept., Faculty of Pharmacy, Univ. Porto, Porto, Portugal (J.P.C., F.R., M.L.B., F.C.)
Neurology Dept., Charité Hospital, Humboldt Univ., Berlin, Germany (A.M.)
Running title: Neurotoxicity of Ecstasy Metabolites

*Corresponding Author: João Paulo Capela

REQUIMTE, Toxicology Department
Faculty of Pharmacy University of Porto
Rua Aníbal Cunha, 164
4099-030 Porto, Portugal
Fax: 00351 222003977
E-mail: joaocapela@ff.up.pt

Number of text pages: 27 (ex. References)
Number of words
Abstract: 213
Introduction: 608
Discussion: 1499
Number of figures: 9
Number of tables: 0
Number of references: 38

Abbreviations: MDMA, 3,4-Methylenedioxymethamphetamine ("Ecstasy"); α-MeDA, α-Methyldopamine; N-Me-α-MeDA, N-Methyl-α-methyldopamine; 5-(GSH)-α-MeDA, 5-(Glutathion-S-yl)-α-methyldopamine; MDA, 3,4-Methylenedioxyamphetamine; GSH, Glutathione; NAC, N-Acetylcysteine; ROS, reactive oxygen species; RNS, reactive nitrogen species.

Recommended section for assignment: Toxicology
Abstract:

3,4-Methylenedioxymethamphetamine (MDMA or “Ecstasy”), is a widely abused, psychoactive recreational drug. There are growing evidences that MDMA neurotoxic profile may be highly dependent on both its hepatic metabolism and body temperature. Metabolism of MDMA involves N-demethylation to 3,4-methylenedioxyamphetamine (MDA), which is also a drug of abuse. MDMA and MDA are O-demethylenated to N-methyl-α-methyldopamine (N-Me-α-MeDA) and α-methyldopamine (α-MeDA), respectively, both of which are catechols that can undergo oxidation to the corresponding ortho-quinones. In the presence of glutathione (GSH), ortho-quinones may be conjugated with GSH to form glutathionyl adducts. In this study we evaluated the neurotoxicity of MDMA and of three of its metabolites, obtained by synthesis, N-Me-α-MeDA, α-MeDA and 5-(GSH)-α-MeDA (5-(Glutathion-S-yl)-α-methyldopamine) in rat cortical neuronal serum free cultures under normal (36.5ºC) and hyperthermic (40ºC) conditions. Cell viability was assessed and the mechanism of cell death was also evaluated. Our study shows that these metabolites are more neurotoxic (5-(GSH)-α-MeDA being the most toxic) than the parent compound MDMA. The neurotoxicity of MDMA metabolites was partially prevented by the antioxidants N-Acetylcystein (NAC) and also, in a minor extent, by α-phenyl-N-tert-butyl nitrone (PBN). All the tested compounds induced apoptotic cell death in cortical neurons and their neurotoxic effect was potentiated under hyperthermic conditions. These data suggests that MDMA metabolites, especially under hyperthermic conditions, contribute to MDMA-induced neurotoxicity.
Introduction:

3,4-Methylenedioxymethamphetamine (MDMA or “Ecstasy”), is a widely abused, psychoactive recreational drug often ingested at dance clubs (Parrot, 2002). It is widely acknowledged that MDMA produces neurotoxic damage of 5-HT (serotonin) nerve endings, while the serotonergic cell bodies do not appear to be destroyed by the drug (Green et al., 2003). Moreover studies also report MDMA-induced neuronal degeneration in the parietal cortex, insular/perirhinal cortex, ventromedial/ventrolateral thalamus, and in the tenia tecta (Commins et al., 1987; Schmued, 2003). Evidence for the occurrence of MDMA-induced neurotoxic damage in human users remains a matter of debate, especially due to polydrug abuse among consumers (Green et al., 2003).

Several studies failed to prove serotonergic neurotoxicity when MDMA and MDA were injected directly into the brain (Esteban et al., 2001; Paris et al., 1992). Since they could not reproduce the serotonergic neurotoxicity seen after the peripheral administration of the drugs, it was postulated that systemic metabolism is needed for the occurrence of neurotoxic events (Monks et al., 2004).

Metabolism of MDMA involves N-demethylation to MDA. MDMA and MDA are O-demethylenated to N-methyl-α-methyldopamine (N-Me-α-MeDA) and α-methyldopamine (α-MeDA), respectively (Lim et al., 1988; Kumagai et al., 1991), both of which are catechols that can undergo oxidation to the corresponding o-quinones. These quinones are highly redox active molecules that can undergo redox cycling, which originates semiquinone radicals and leads to the generation of ROS and RNS (Bolton et al., 2000; Remião et al.,...
2002). The catecholamine oxidation process, can be catalyzed under physiological conditions by oxidative enzymes, such as xanthine oxidase, peroxidase, lipoxygenase, several copper-containing catechol oxidases, or in the presence of metal ions such as Cu\textsuperscript{2+}, Mn\textsuperscript{2+}, Fe\textsuperscript{3+}, and several copper and ferric chelates (Bindoli et al., 1992). Alternatively, as the reactive \(\alpha\)-quinone intermediates are Michael acceptors, cellular damage can occur through alkylation of crucial cellular proteins and/or DNA. In the presence of GSH, \(\alpha\)-quinone may be conjugated with GSH to form a glutathionyl adduct 5-(Glutathion-S-yl)-\(\alpha\)-methyldopamine (5-GSH-\(\alpha\)-MeDA) (Hiramatsu 1990; Carvalho et al., 2004b). This GSH conjugate remains redox active being readily oxidized to the quinone-thioether, which, after the reductive addition of a second molecule of GSH, yields a 2,5-bis-glutathionyl conjugate (for detailed insights on MDMA metabolism see Fig. 1). Taken all together, MDMA metabolism leading to the formation of reactive intermediates, ROS and/or toxic oxidation products may represent the triggering factors responsible for the toxicity exerted by this amphetamine (Carvalho et al., 2004b; Carvalho et al., 2004c).

A major feature of clinical cases related to MDMA toxicity is hyperthermia, with core body temperatures up to 43\(^\circ\)C having been reported (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 “rave” parties, may all contribute to increase the hyperthermic response induced by MDMA (Green et al., 2003).

The underlying mechanisms of MDMA-induced neurotoxicity, namely concerning the possible role of its metabolite(s), remain to be completely elucidated. Therefore, it was the aim of this study to evaluate and characterize
the neurotoxicity in rat cortical neuronal serum free cultures of MDMA and 3 of its main metabolites: N-Me-α-MeDA, α-MeDA and 5-(GSH)-α-MeDA. We have previously shown that exposition to hyperthermia (40°C) leads to a potentiation of the MDMA-induced neurotoxic effect (Capela JP, Ruscher K, Lautenschlager M, Freyer D, Durnagi U, Gaio AR, Bastos ML, Meisel A and Carvalho F (2005) Ecstasy-induced cell death in cortical neuronal cultures is 5-HT2A-receptor-dependent and potentiated under hyperthermia. Submitted elsewhere). So, in this study experiments were conducted in order to confirm the higher neuronal susceptibility towards MDMA metabolites at hyperthermic temperatures. Our results show that the 3 metabolites are more neurotoxic than MDMA, 5-(GSH)-α-MeDA being the most toxic.
Methods:

Materials

Materials for cell cultures were obtained from the following sources: neurobasal medium and supplement B27 from Gibco® (U.K.); modified Eagle’s medium, phosphate buffered saline (PBS), HEPES buffer, trypsin/EDTA, penicillin/streptomycin, L-glutamine, collagen-G and poly-L-lys in from Biochrom®, Berlin, Germany; multiwell plates from NUNC®; MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), MDMA (3,4-Methylenedioxymethamphetamine), PBN (α-phenyl-N-tert-butyl nit rone), NAC (N-Acetylcystein), reduced GSH, mushroom tyrosinase (4,400 units/mg) were obtained from Sigma-Aldrich®. The metabolites N-methyl-α-methyldopamine (N-Me-α-MeDA), α-methyldopamine (α-MeDA) and 5-(Glutathion-S-yl)-α-methyldopamine [5-(GSH)-α-MeDA] were synthesized by REQUIMTE/CQFB, Chemistry Dept, FCT, Univ. Nova de Lisboa. All other chemicals were purchased from Sigma-Aldrich® of the highest grade commercially available.

Synthesis of N-methyl-α-methyldopamine (N-Me-α-MeDA), α-methyldopamine (α-MeDA) and 5-(Glutathion-S-yl)-α-methyldopamine [5-(GSH)-α-MeDA]

Solvents were dried by standard methods and distilled before use. Analytical thin-layer chromatography was conducted on Merck Kieselgel 60, F254 silica gel 0.2 mm thick plates; column chromatography was performed on
Merck Kieselgel 60 (240-400mm) silica gel or reverse-phase RP-18 modified silica. Melting points were recorded on a Reichert-Thermovar hot stage apparatus and are reported uncorrected. Infrared (IR) spectra were recorded on Perkin Elmer Spectrum 1000 as potassium bromide (KBr) pellets or as film over sodium chloride (NaCl) windows. Proton and carbon nuclear magnetic resonance spectra (\(^1\)H and \(^13\)C NMR) were recorded on Bruker ARX 400 spectrometer, at 400 and 100.62 MHz respectively. Chemical shifts are expressed in ppm, downfield from TMS (\(\delta = 0\)) as an internal standard; J-Values are given in Hz. The exact attribution of NMR signals was performed using two dimensional NMR experiments. Mass spectra were acquired with a Micromass GC-TOF (GCT) mass spectrometer. HPLC was conducted on a Merck Hitachi system consisting of an L-7100 pump, a Rheodyne type injector, a D-7000 interface, and an L7450A diode array spectrometric detector.

N-Me-\(\alpha\)-MeDA and \(\alpha\)-MeDA were prepared following the procedure of Borgman (Borgman, 1974) starting from the corresponding benzaldehyde and nitroethane. 5-(GSH)-\(\alpha\)-MeDA was prepared according to previously published methods (Hiramatsu et al., 1990, Miller et al., 1995) with modifications as reported below.

5-(GSH)-\(\alpha\)-MeDA: To a solution of \(\alpha\)-methyldopamine (0.020 g, 8.06x10\(^{-5}\) mol) in sodium phosphate buffer (80 mL, pH 7.4, 50 mM) at 25°C it was added mushroom tyrosinase (8000 units). The solution became red, indicating the formation of o-quinone. GSH (124 mg, 4.03x10\(^{-4}\) mol) was added and the solution red color changed with time to yellow (1 hour). The solution was carefully concentrated by rotary evaporation without heating and dissolved in 1 mL of water. The product purification was performed by reverse-phase RP-18
modified silica column chromatography first with water (150 mL) and the product separated using 10 x 7.5 mL of formic acid/water/methanol (1:49:50). Each fraction was checked for the presence of adduct using a UV-Vis detector. Fractions containing maxima at 232, 264 and 294 nm were separated and lyophilized to dryness. The product 5-(GSH)-α-MeDA (27 mg) was obtained as oil in 60% yield. The compound purity was checked by HPLC using a LiChrospher 100 RP-18 column, with two mobile phase solvents. Solvent A was prepared by adding concentrated trifluoroacetic acid (TFA) to deionized water until pH was 2.15. Solvent B was prepared by adding TFA to a 1:1 mixture of methyl cyanide (MeCN) and deionized water until pH was 2.15. The following mobile phase gradient was used: 0-10 min, 0-2% of solvent B; 10-15 min, 2-100% of solvent B; 15-20 min, 100-0% of solvent B. The compound eluted in 3 min. The peaks were monitored at 290 nm. \(^1\)H RMN (400 MHz, D\(_2\)O, \(\delta\): 6.72 (1H, s, H\(_{2/6}\)), 6.64 (1H, s, H\(_{2/6}\)), 4.27 (1H, m, Cys-α), 3.66 (1H, m, Glu-α), 3.58 (2H, m, Gly-α), 3.41 (1H, m, CH), 3.24 (1H, m, Cys-β), 3.06 (1H, m, Cys-β), 2.64 (d, J=6.6 Hz, 2H, CH\(_2\)), 2.35 (t, J=7.1 Hz, 2H, Glu-γ), 1.98 (2H, m, Glu-β), 1.15 (d, J=6.2 Hz, 3H, CH\(_3\)) ; \(^1\)C RMN (100.62 Hz, D\(_2\)O, \(\delta\)): 174.8 (CO Gly), 174.6 (CO Glu\(_{\varepsilon}\)), 173.9 (CO Glu), 172.1 (CO Cys), 144.6 (C\(_{Ar3}\)), 144.2 (C\(_{Ar4}\)), 128.8 (C\(_{Ar1}\)), 126.3 (C\(_{Ar2/6}\)), 119.4 (C\(_{Ar5}\)), 117.4 (C\(_{Ar2/6}\)), 54.1 (C Glu-α), 53.2 (C Cys-α), 49.1 (CH), 42.3 (C Gly-α), 39.3 (CH\(_2\)), 34.9 (C Cys-β), 31.4 (C Glu-γ), 26.2 (C Glu-β), 17.5 (CH\(_3\)); MS(FD) m/z 473 (MH\(^+\)).

**Cell Culture**
Primary neuronal cultures of cerebral cortex were obtained from embryos (E-18) of Wistar rats. Cultures were prepared according to Lautenschlager et al. (2000): 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 in dissociation medium, pelleted by centrifugation (210 g for 2 min), redissociated in starter medium (Neurobasal medium with supplemental B27, 100 U penicillin+streptomycin/ml, 0.5 mM L-glutamine, 25 µM glutamate), and plated in 48-well plates in a density of 1.5x10^5 cells/Well. 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 hr 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. In fact, our cultures only reach about 1% of microglia in respect to the total population at the 28th DIV, so only as a late stage event in old primary serum free cultures as described before (Lautenschlager et al., 2000). Therefore, microglia in this culture model is not likely to interfere with the mechanism of neurotoxicity.
Experimental Protocol

Previous experiments established 40°C as the temperature for the MDMA/hyperthermia experiments (Capela et al., submitted elsewhere). Cultures were treated after the 8th DIV with MDMA and metabolites (concentration range 100-800µM, single application without feeding for the following 24h or 48h) and were incubated under normal temperature (36.5°C). For hyperthermic experiments cells were placed under normal or hyperthermic temperature (40°C) for 24h and incubated with MDMA and the MDMA-metabolites N-Me-α-MeDA and α-MeDA. In experiments using protective agents, the free radical scavenger α-phenyl-N-tert-butyl nitrone (PBN) (100µM) and N-Acetylcysteine (NAC) (1mM) were applied to the culture 1 hour before the metabolites. The concentrations of NAC and PBN were chosen after screening experiments. Although higher doses of PBN were tested, the degree of protection was similar until 1mM. Since the protection was similar we used the 100µM concentration for PBN (data not shown). For NAC the highest degree of protection was attained with 1mM (data not shown). Protection experiments for 5-(GSH)-α-MeDA were performed at the 24h time-period. For N-Me-α-MeDA and α-MeDA it was used the 48h time-point for the protection experiments. Drugs were diluted in medium or purified water. Controls received an equivalent amount of vehicle. Cultured cells were assessed morphologically by phase contrast microscopy and viability by life-death assay at 2 different time points 24h and 48h.
Life–death assay

Cell damage was assessed quantitatively using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, which measures cellular metabolic activity. After adding 500 µg MTT/ml to each well, and incubating at 36.5°C for 35 min, the reaction was stopped by adding 10% sodium dodecyl sulfate (SDS) in 0.01 M hydrochloric acid (HCl), followed by over-night incubation at 36.5°C and photometric detection of formazan at 550 nm.

Ethidium bromide and acridine orange staining

The fluorescent DNA-binding dyes ethidium bromide and acridine orange were used to distinguish necrotic from apoptotic cells. The ethidium homodimer cannot penetrate intact cellular membranes and therefore stains the nucleus of cells red only when the membranes are disrupted, whereas acridine orange is membrane-permeable and stains living cells green. Primary cortical neurons, after 24h treatment with MDMA and its metabolites, were incubated with 2µg/ml acridine orange and 2µg/ml ethidium bromide for 5 min before imaging, using a fluorescence microscope with a standard fluorescein excitation filter (Leica, Heerbrueg, Switzerland).

Statistical analysis
Results are presented as mean ± SEM. To avoid possible variations of the cell cultures depending on the quality of dissection and seeding procedures, data were pooled from three representative experiments. The means for different treatment groups were compared using the Kruskal–Wallis test (one-way ANOVA on ranks), since normality conditions were not always satisfied, followed by the Student-Newman-Keuls post-hoc test once a significant \( p \) had been obtained. Details of the statistic analyses are described in each figure legend. Significance was accepted at \( p \) less than 0.05.
Results:

MDMA- and MDMA metabolites-induced neurotoxicity in cortical neuronal cultures is concentration-, temperature- and time-dependent. MDMA metabolites are more neurotoxic than the parent compound.

Cell viability assessed by the MTT test at the end of 24h incubation period revealed MDMA-, N-Me-α-MeDA- and α-MeDA-induced toxicity both at normothermic and hyperthermic conditions, as shown in Figure 2. At this time-point there was a concentration-dependent induced toxicity at both normothermia and hyperthermia for these compounds. Meanwhile, the hyperthermic condition did not change cell viability in control cells comparatively to the normothermic condition the observed toxicity was potentiated at 40ºC for all the studied compounds. Figure 2 shows the marked increase of the MDMA- and metabolites-induced neuronal toxicity under hyperthermia. Under normothermic conditions, after 24h incubation-period the toxicity elicited by the MDMA metabolites: N-Me-α-MeDA (Fig. 2B), α-MeDA (Fig. 2C) and 5-(GSH)-α-MeDA (Fig. 3) were shown to be more potent neurotoxins than MDMA itself. 5-(GSH)-α-MeDA proved to be the most toxic.

Cultures incubation with MDMA, N-Me-α-MeDA and α-MeDA for a longer time-period (48h) showed an increase in the neurotoxic effect (Fig. 4). Once more, N-Me-α-MeDA and α-MeDA showed to be more toxic than MDMA for the 48h time-period. As a result of its high toxicity after 24h incubation, 5-(GSH)-α-MeDA was not tested at the 48h time-period.
Taken all together MDMA, N-Me-α-MeDA and α-MeDA were shown to be neurotoxic in a concentration-, temperature- and time-dependent manner. These metabolites are more neurotoxic than the parent compound MDMA, 5-GSH-α-MeDA being the most toxic.

**Protective effect of NAC (1mM) and PBN (100µM) on the neurotoxicity induced by MDMA metabolites**

The addition of NAC (1mM), a precursor of GSH, and PBN (100µM), a free radicals scavenger one hour prior cultures stimulation with 5-(GSH)-α-MeDA provided protection against the metabolite-induced neurotoxicity in cortical neurons for the 24h, as revealed by the MTT test (Fig. 5). The stronger degree of protection afforded by NAC against the neurotoxicity of 5-(GSH)-α-MeDA was obtained for the 200µM concentration, where about 60% of cell viability was preserved. The same protective effect of NAC and PBN could also be observed against the N-Me-α-MeDA (Fig. 6) and α-MeDA (Fig. 7) neurotoxic effects after 48h incubation. The stronger degree of protection afforded by NAC against the neurotoxicity of α-MeDA and N-Me-α-MeDA was obtained for the 400µM concentration, where about 35% of cell viability was preserved.

Cellular viability was preserved with 1mM NAC after 24h incubation with 800µM N-Me-α-MeDA and α-MeDA, as can be observed in the microphotographs included in figure 8. The metabolites-induced apoptosis was also reduced with NAC, as the morphology of cells depicted.
Phase-contrast microscopy showed that the mechanism of cell death induced by MDMA metabolites was typical of apoptotic cell death. Microphotographs included in figure 8 are an example for the apoptotic type of cell death induced by N-Me-α-MeDA and α-MeDA. Neurons exposed to increasing MDMA metabolites concentrations during 24h show progressive signs of neurite disintegration, chromatin condensation, membrane blebbing, cytoplasmic shrinkage, nuclear fragmentation, membrane integrity loss and of neuritic processes. In the ethidium bromide/ acridine orange staining (Fig. 9) living cells appear as cells with a regular-sized green fluorescent nucleus, while early apoptotic cells have a green fluorescent condensed, shrunken, or fragmented nucleus and late apoptotic cells have a red fluorescent condensed, shrunken, or fragmented nucleus. Necrotic cells exhibit a red fluorescent regular-sized or increased nucleus. The pictures in figure 9 suggest a higher degree of apoptosis at higher concentrations in a concentration-dependent manner for N-Me-α-MeDA and α-MeDA.
Discussion:

The key findings of our study were: (1) MDMA and its tested metabolites N-Me-\(\alpha\)-MeDA, \(\alpha\)-MeDA and 5-(GSH)-\(\alpha\)-MeDA induced cortical neurotoxicity in serum free cultures of cortical neurons, (2) neuronal death followed an apoptotic pattern. (3) The observed neurotoxicity was temperature-, time- and dose-dependent. (4) The tested metabolites were more neurotoxic than MDMA, (5) 5-(GSH)-\(\alpha\)-MeDA proved to be the most neurotoxic. (6) NAC and PBN partially protected against MDMA metabolites-induced neurotoxicity.

In vivo studies performed in rats demonstrated that the deleterious effects of MDMA are diverse, including cell death in several brain regions, as the cortex, striatum and thalamus (Commins et al., 1987; Schmued, 2003). Recent studies showed that MDMA-induced cell death in cultures of rat cortical neurons is accompanied by activation of neuronal apoptotic pathways (Stumm et al., 1999; Capela et al., submitted elsewhere). In the present study we showed that N-Me-\(\alpha\)-MeDA-, \(\alpha\)-MeDA- and 5-(GSH)-\(\alpha\)-MeDA-induced neurotoxicity is typical of apoptotical cell death. Herein, we also showed that MDMA itself is neurotoxic. In previous experiments, we have proven that MDMA-induced neurotoxicity in these cultures is dependent on direct MDMA 5-HT\(_{2A}\)-receptor-activation (Capela et al., submitted elsewhere). To our knowledge, this is the first study using neuronal cultures to evaluate the neurotoxicity elicited by MDMA metabolites.

MDA, N-Me-\(\alpha\)-MeDA, and \(\alpha\)-MeDA are major hepatic metabolites of MDMA (Monks et al., 2004). Carvalho and colleagues (2004a; 2004b) have proven in vitro that the concentration of N-Me-\(\alpha\)-MeDA and \(\alpha\)-MeDA decreases over time in biological media, due to their oxidation to the correspondent
aminochromes and conjugation with GSH. The reactive intermediates produced during the oxidation of these catecholamines into reactive ortho-quinones and/or aminochromes, can be conjugated with GSH to form the corresponding glutathionyl adducts. Aminochromes can also undergo further oxidation, leading to the formation of a of melanin type polymer formation (Zhang and Dryhurst, 1994). In fact, a dark brown/black turbidity, characteristic of these polymers, appeared in the neuronal medium, not only after incubation with N-Me-α-MeDA and α-MeDA, but also after incubation with 5-(GSH)-α-MeDA, as a late stage event (hours latter). In accordance, it was previously shown that GSH depletion is one of the early toxic events observed in rat cardiomyocytes exposed to N-Me-α-MeDA and α-MeDA (Carvalho et al., 2004b). The importance of the redox status in the conjugated or non-conjugated MDMA metabolites neurotoxicity was underlined in the present study, since protection was observed when NAC (1mM), a GSH synthesis precursor in the cells, was added to the neuronal cultures. NAC also attenuated the apoptosis induced by MDMA metabolites. Similar results were reported, using freshly isolated rat hepatocytes, with NAC (1mM) providing protection against N-Me-α-MeDA induced toxicity (Carvalho et al., 2004c).

Conjugation of electrophiles with GSH usually results in detoxification and their subsequent elimination as mercapturic acids (Monks et al., 2004). However, several examples exist where conjugation of GSH with electrophiles results in preservation or enhancement of biologic (re)activity. Ortho-Quinones, aminochromes, and GSH conjugates are known to cause irreversible inhibition of enzymes that possess either a GSH binding site and/or cysteine residues critical for enzyme function (Monks et al., 2004). Likewise, inhibition of
glutathione reductase and glutathione-S-transferase by quinines; glutathione reductase, selenium-dependent glutathione peroxidase, and glutathione-S-transferase by aminochromes, has been reported (Remião et al., 2002). Quinone thioethers have the ability to interfere with redox cycle, produce ROS and to arylate tissue macromolecules (Kleiner et al., 1998). Therefore all accounts for the potential role of MDMA thioether metabolites in MDMA-induced neurotoxicity (Monks et al., 2004). In this study the GSH conjugated derivative: 5-(GSH)-α-MeDA showed a potent neurotoxic effect in rat cortical neurons.

5-(GSH)-α-MeDA is metabolized via the mercapturic acid pathway within the central nervous system (CNS), forming 5-(cystein-S-yl)-α-MeDA and 5-(N-acetylcystein-S-yl)-α-MeDA (Miller et al., 1995). 5-(GSH)-α-MeDA is also readily oxidized to the corresponding quinone-GSH conjugate and undergoes addition of a second GSH molecule to form 2,5-bis-(glutathion-S-yl)-α-MeDA. Intracerebroventricular (ICV) injections of 5-(N-acetylcystein-S-yl)-α-MeDA and 5-(GSH)-α-MeDA into rats produced neurobehavioral changes characteristic of peripheral administration of MDMA/MDA as well as acute increases in brain 5-HT and DA concentrations (Miller et al., 1996). 2,5-bis-( glutathion-S-yl)-α-MeDA also proved to be a serotonergic neurotoxicant (Miller et al., 1997). In addition to the effects observed after ICV administration of 5-(N-acetylcystein-S-yl)-α-MeDA and 5-(GSH)-α-MeDA, their direct injection into the striatum, cortex, and hippocampus produced prolonged depletions in 5-HT and neurobehavioral changes similar to those obtained after in vivo administration of MDA and MDMA (Bai et al., 1999). 5-(N-acetylcystein-S-yl)-α-MeDA was also shown to be an extremely potent serotonergic toxicant (Bai et al., 1999). The existence of transporters capable of transferring GSH and systemic formed GSH conjugates
into the brain across the blood-brain barrier was previously suggested (Kannan et al., 1990). Moreover, recent experiments using in vivo microdialysis have provided direct evidence for the presence of GSH and N-acetylcysteine conjugates of MDMA metabolites in the brain, being the latter metabolite toxic to serotonergic neurons, after in vivo s.c. administration of MDMA (Jones et al., 2005). Therefore, the present study corroborates the important role of the GSH conjugated metabolites in MDMA-induced toxicity.

Colado et al. (1997) provided direct evidence that MDMA administration increased free radical formation in the rat brain. Sprague and Nichols (1995) also showed that MDMA administration to rats increased brain lipid peroxidation, a marker of free radical-induced damage. In accordance, herein we showed that PBN, a free radical scavenger, partially protected against the metabolites-induced neurotoxicity. The protective effect of PBN against MDMA-induced neurotoxicity in rats was previously reported by Yeh (1999). Hyperthermia has also been shown to increase free radical formation, an effect that may lead to neurotoxicity (Halliwell, 1992). Many in vivo studies support that hyperthermia plays a major role in MDMA-induced neuronal death. MDMA was shown to cause acute dose-dependent hyperthermia in rats, and in humans MDMA-induced hyperthermia can be fatal (Henry, 1992 and Green et al., 2003). Taking into account that MDMA metabolites are pro-oxidant compounds, the hyperthermia-induced oxidative stress, will probably potentiate their toxicity. In animal studies researchers are faced with several factors affecting body temperature. In this study the high body temperature was simulated by placing the cortical neurons in an incubator at an environment temperature of 40°C after addition of MDMA and MDMA metabolites. This
procedure has the advantage of a tight temperature control throughout the whole experiment. Herein we showed that the neurotoxicity of MDMA and MDMA metabolites was potentiated by hyperthermia.

The neurotoxicological evaluation of MDMA and/or its metabolites needs to address concentrations that simulate those obtained during chronic drug abuse. On the other hand, it is important to evaluate the mechanistic interactions of MDMA and its metabolites with cellular components, which is only possible using worst case approach concentrations. The MDMA concentrations used in this study were in accordance with other reports (Simantov and Trauber 1997; Stumm et al., 1999) that showed MDMA-induced apoptotical cell death in cultured cells. More importantly, Chu and colleagues (1996) have shown that s.c. administration of MDMA to rats at doses of 20 and 40 mg/kg results in brain concentrations of approximately $206 \mu$M (1h after) and $466 \mu$M (1.5h after) respectively, falling squarely in the range of those used in this study. Ricaurte et al. (2000) using an adjustment for body mass/surface area and drug clearance calculated that the equivalent dose in humans of a 20 mg/kg rat dose (an accepted neurotoxic dose to this species) to be 1.28 mg/kg or approximately 96 mg in a 75-kg individual. Human MDMA users typically use MDMA single dosages of 75–125 mg. The fact that some individuals report using up to 10-25 individual dosages (tablets) of MDMA per occasion (Parrott, 2005), further suggests that there is little or no margin of safety between the recreationally used and neurotoxic dosages of MDMA. Moreover, and although all human studies on MDMA brain concentration are post-mortem analyses, it was found that brain concentrations of MDMA and its metabolites are substantially higher (up to 30 times) than blood concentrations (García-Repetto...
et al., 2003). Most of the reported cases of serious toxicity or fatality have involved MDMA blood levels ranging from 0.5-10mg/l approximately 2-44µM (García-Repetto et al., 2003). Notably, it was already reported that in humans, N-Me-α-MeDA, a major toxic metabolite in the present study, reaches plasma concentrations similar to those of MDMA (Segura et al., 2001). N-Me-α-MeDA was previously detected in the brain of male SD rats after the s.c administration of a single high dose (40 mg/kg) of ecstasy (Chu et al, 1996). Jones and colleagues (2005) measured brain concentration of 5-(GSH)-α-MeDA to be 40–50µM after a single s.c. MDMA dose of 20mg/Kg. In fact, a multiple dosage regimen is often used in animal studies (Green et al., 2003), to match the pattern of MDMA use by abusers, suggesting that these metabolites may accumulate in the brain following multiple drug administration. It seems then reasonable to believe that the metabolites used in this study may achieve neurotoxic concentrations in vivo.

In conclusion, the present findings further support for the contribution of MDMA-metabolism and hyperthermia in the neurotoxicity exerted by “Ecstasy”. More studies are fundamental to determine the exact mechanisms by which MDMA metabolites access the brain and produce neurotoxicity.
REFERENCES


Carvalho M, Remião F, Milhazes N, Borges F, Fernandes E, Carvalho F and Bastos ML (2004c) The toxicity of N-methyl-α-methyldopamine to freshly isolated rat hepatocytes is prevented by ascorbic acid and N-acetylcysteine. Toxicology 200:193-203.


Footnotes

This work was supported by the Hermann & Lilly Schilling Foundation and “Fundação Calouste Gulbenkian” (Ref. FCG 10/04). J.P.C. was the recipient of a PhD grant from “Fundação para a Ciência e Tecnologia” (Ref. SFRD/BD/10908/2002).

Reprint requests should be sent to the correspondent Author.

João Paulo Capela

REQUIMTE, Toxicology Department

Faculty of Pharmacy University of Porto

Rua Aníbal Cunha, 164

4099-030 Porto, Portugal

Fax: 00351 222003977

E-mail: joaocapela@ff.up.pt
Figure Legends

Figure 1. Proposed pathway for MDMA metabolism. MDMA can undergo N-demethylation to MDA. Cytochrome P450 (CYP) enzymes, also mediate demethylation of MDMA and MDA to N-Me-α-MeDA and α-MeDA respectively. The catechols are readily oxidized to the corresponding o-quinones, which can enter redox cycles with their semiquinone radicals, leading to formation of ROS and RNS. On cyclization, ortho-quinones give rise to the formation of aminochromes and related compounds, such as 5,6 dihydroxyindoles, which can undergo further oxidation and polymerization to form brown or black insoluble pigments of melanin type. Alternatively, ortho-quinones can react readily with GSH to form the corresponding GSH conjugates like 5-GSH-α-MeDA.

Figure 2. The neurotoxicity induced by N-Me-α-MeDA and α-MeDA was stronger than that of MDMA and was potentiated under hyperthermic conditions. Cortical cultures were exposed at the 8th DIV to various MDMA (A), N-Me-α-MeDA (B) and α-MeDA (C) concentrations (100, 200, 400 and 800µM) during 24H under normal (36.5°C) and Hyperthermic temperature (40°C). Evaluation of the viability was performed by the MTT test and data is presented as percentage of the respective control cultures. The cellular viability of control cultures exposed 24h at 40°C was 99±1% relative to cultures at 36.5°C (n= 20 for A and n= 24 for B and C of 3 different experiments, Kruskal–Wallis Test: A=177.1, p<0.001; B= 196.6, p<0.001; C=220.9, p<0.001 followed by Student-Newman-Keuls post-hoc test). In each graphic all concentrations were different.
to the respective controls $p<0.01$. In the graphics $**p<0.01$ represent differences between cultures exposed at normal and hyperthermic temperature.

**Figure 3.** 5-GSH-α-MeDA was the most neurotoxic metabolite among the 3 herein tested. Cortical cultures were exposed at the 8th DIV to various 5-GSH-α-MeDA concentrations (100, 200, 400 and 800µM) during 24H under normothermia (36.5ºC). Evaluation of the viability was performed by the MTT test and data is presented as percentage of control cultures (n= 24 of 3 different experiments; Kruskal–Wallis Test=94.7, $p<0.001$ followed by Student-Newman-Keuls post-hoc test, were $p<0.01$ for all possible multiple comparisons except for 200 vs 800µM and 400 vs 800µM). In the graphic $**p<0.01$ represent differences to controls.

**Figure 4.** The neurotoxicity induced by N-Me-α-MeDA and α-MeDA was stronger than that of MDMA after 48h exposure. Cortical cultures were exposed at the 8th DIV to various MDMA (A), N-Me-α-MeDA (B) and α-MeDA (C) concentrations (100, 200, 400 and 800µM) during 48H under normal temperature (36.5ºC). Evaluation of the viability was performed by the MTT test and data is presented as percentage of control cultures (n= 24 for A, B and C of 3 different experiments, Kruskal–Wallis Test: A=100.2, $p<0.001$; B= 103.2, $p<0.001$; C=106.4, $p<0.001$ followed by Student-Newman-Keuls post-hoc test were $p<0.01$ for all possible multiple comparisons in each of the 3 graphics). In the graphics $**p<0.01$ represent differences to controls.

**Figure 5.** The protective effect of NAC (1mM) (A) and PBN (100µM) (B) against 5-GSH-α-MeDA-induced neurotoxicity. The graphics refer to the 24h incubation-
time under normothermia. Evaluation of the viability was performed by the MTT test and data is presented as percentage of control cultures. The MTT test measurements in arbitrary units were in the control 345±12, in NAC treated cells 327±7 and in PBN treated cells 335±13 (n= 24 per condition of 3 different experiments, Kruskal–Wallis Test: A=215.3, \( p<0.001 \) and B=190.4, \( p<0.001 \) followed by Student-Newman-Keuls post-hoc test **\( p<0.01 \)).

**Figure 6.** The protective effect of NAC (1mM) (A) and PBN (100µM) (B) against N-Me-α-MeDA-induced neurotoxicity. The graphics refer to the 48h incubation-time under normothermia. Evaluation of the viability was performed by the MTT test and data is presented as percentage of control cultures. The MTT test measurements in arbitrary units were in the control 617±13, in NAC treated cells 605±28 and in PBN treated cells 620±19 (n= 24 per condition of 3 different experiments, Kruskal–Wallis Test: A=112.9, \( p<0.001 \) and B=138.7, \( p<0.001 \) followed by Student-Newman-Keuls post-hoc test *\( p<0.05 \), **\( p<0.01 \)).

**Figure 7.** The protective effect of NAC (1mM) (A) and PBN (100µM) (B) against α-MeDA-induced neurotoxicity. The graphics refer to the 48h incubation-time under normothermia. Evaluation of the viability was performed by the MTT test and data is presented as percentage of control cultures. The MTT test measurements in arbitrary units were in the control 617±13, in NAC treated cells 605±28 and in PBN treated cells 620±19 (n= 24 per condition of 3 different experiments, Kruskal–Wallis Test: A=175.1, \( p<0.001 \) and B=215.3, \( p<0.001 \) followed by Student-Newman-Keuls post-hoc test *\( p<0.05 \), **\( p<0.01 \)).
**Figure 8.** Phase-contrast photos from primary cortical neuronal cultures at the 24h time-point incubated at normothermia: (A) control, (B) NAC 1mM treated, (C) exposed to 800µM of α-MeDA, (D) exposed to 800µM of α-MeDA and NAC 1mM, (E) exposed to 800µM of N-Me-α-MeDA, (F) exposed to 800µM of N-Me-α-MeDA and NAC 1mM. N-Me-α-MeDA and α-MeDA promote progressive loss of the dendrites and axons with typical apoptotical cell death morphology (magnification 400x). It is possible to notice the protection and the reduction of apoptotical features when NAC 1mM was added to the MDMA metabolites treated cells.

**Figure 9.** Fluorescence microscope photographs from ethidium bromide/acridine orange staining of primary cortical neuronal cultures at the 24h time-point incubated at normothermia: (A) control, (B) exposed to 200µM of α-MeDA, (C) exposed to 400µM of α-MeDA, (D) exposed to 200µM of 5-GSH-α-MeDA, (E) exposed to 400µM of 5-GSH-α-MeDA. Increasing concentrations of MDMA metabolites promote the augment of apoptotic cell death (magnification 400x).
Figure 1

Alkylation of crucial cellular proteins and/or DNA

$\alpha$-MeDA-α-quinone (R=NH$_2$)

N-Me-$\alpha$-MeDA-α-quinone (R=NHCH$_3$)

O$_2$.-$\alpha$-MeDopaminochrome (R=H)

$\alpha$-MeDA-α-semiquinone (R=NH$_2$)

N-Me-$\alpha$-MeDA-α-semiquinone (R=NHCH$_3$)

5,6-Dihydroxyindole

Autoxidation to melanin type polymer

ROS and RNS

2,5-bis(glutathion-S-yl)-α-MeDA (R=NH$_2$)

2,5-bis(glutathion-S-yl)-N-Me-$\alpha$-MeDA (R=NHCH$_3$)
**Figure 3**

Bar graph showing the concentration of 5-(GSH)-α-MeDA in mM, with % of control on the y-axis and Conc. mM on the x-axis. The graph includes bars for 0, 100, 200, 400, and 800 mM concentrations, with significant differences indicated by **. The graph indicates a significant decrease in % of control with increasing concentration of 5-(GSH)-α-MeDA.